CITRIC ACID PRODUCTION BY THE YEASTS *CANDIDA GUILLIERMONDII* AND *YARROWIA LIPOLYTICA*

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ABSTRACT

The aim of this thesis was to investigate the relationships, for a citric acid-producing strain of yeast, among the growth rate, sugar uptake rate and the citric acid production rate, and to investigate the hypothesis that citric acid production occurs when the growth rate slows, but the sugar uptake rate is maintained. As previous experimental work in the Department of Process and Environmental Technology (formerly Biotechnology Department) of Massey University had been performed in shake flask cultures only, it was desired to scale-up the culture into a 2l laboratory scale batch culture, and then into a chemostat culture. The first yeast investigated, *Yarrowia lipolytica* IMK2, failed to successfully scale-up, so further investigations were performed using the yeast *Candida guilliermondii* IMK1.

Experiments were performed in shake flask culture to investigate the effect of using mixed carbon sources to adjust the carbon uptake rate, and hence the citric acid production rate, but no effect was noticed with the mixtures tested.

Batch fermenter experiments were performed to investigate the effect of the culture pH, and the aeration rate, on citric acid production. The aeration rate was not observed to have an effect on the culture in the range tested (0.06 - 0.333 vvm), but the culture pH was observed to have an effect, with the maximum production occurring at pH 4.3, and no citric acid production occurring below pH 3.5.

Chemostat culture experiments were performed to investigate the effect of culture pH and the specific growth rate on citric acid production. The specific

growth rate was observed to have a significant effect, with the specific citric acid production rate increasing as the growth rate decreased. The effect of the culture pH was found to vary with the growth rate, with the maximum production rate and yield occurring at pH 3.8, and a growth rate of 0.02 h^{-1}. From cultures where the glucose was exhausted from the medium, and therefore glucose was a limiting nutrient, the specific citric acid production rate was observed to decrease as the glucose uptake rate decreased. Thus, it could be concluded that the specific citric acid production rate increased as the growth rate decreased, provided that the sugar uptake rate remained high.

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ABBREVIATIONS

ABBREVIATIONS OF UNITS

°C	degrees Celsius
cm	centimetre
g	gram
h	hour
kg	kilogram
kPa	kilopascal
l	litre
M	mole
mg	milligram
min	minute
ml	millilitre
mM	millimole
nm	nanometre
ppm	parts per million
rpm	revolutions per minute
µm	micrometre
v/v	volume per volume
vvm	volume per volume per minute
w/v	weight per volume

OTHER ABBREVIATIONS

AMP Adenosine monophosphate

ATP Adenosine triphosphate

bio biomass (dry weight)

D Dilution rate

DO Dissolved oxygen

EDTA Ethylenediaminetetraacetic acid

HPLC High Performance Liquid Chromatography

N Nitrogen

NAD Nicotinamide Adenine Dinucleotide

q specific growth rate

TCA Tricarboxylic acid

μ Specific growth rate

YNB Yeast nitrogen base

CHAPTER 1

INTRODUCTION

Citric acid is an organic acid produced naturally by most living organisms. Its low toxicity, palatability and ease of assimilation mean that it has many uses, particularly in the food and pharmaceutical industries. It is produced commercially by fermentation of glucose or molasses syrups by strains of the fungus *Aspergillus niger*, or by various yeasts.

Yeast fermentation has some advantages over the fungal fermentation: yeasts are easier to handle in a fermenter as they do not grow on probes or block ports; the form of the growth is usually as a homogenous suspension, rather than in the form of pellets or large aggregates; and they do not require a metal ion deficiency, thus eliminating an expensive medium pre-treatment step. Unfortunately, a side-effect of the yeast fermentation is the occasional by-production of isocitric acid.

Strains of yeast have been developed that can produce citric acid in a nitrogen limited medium containing an appropriate carbon source. The work described in this thesis was undertaken to investigate the relationship between growth rate, sugar uptake rate and citric acid production rate for a strain of yeast grown on glucose, and to test the hypothesis that citric acid production occurs when growth rate slows but the sugar uptake rate is maintained.

CHAPTER 2

LITERATURE REVIEW

2.1 Citric Acid

Citric acid ($CH_2COOHCOHCOOHCH_2COOH$) is a tricarboxylic acid, which was first isolated from lemon juice and crystallized as a solid by Scheele in 1784. Citric acid is widespread in living systems, as it forms an intermediate in the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle, through which carbohydrates are oxidized to carbon dioxide. The acid naturally occurs in high concentrations in citrus fruits, pineapples, pears, peaches and figs (6-9 % w/v of the juice). This widespread occurrence is an assurance of its non-toxic nature. Until the early part of this century, citric acid was produced commercially mainly by extraction from lemon juice. Some is still manufactured this way and is known as "natural citric acid". Today, however, most of the citric acid used in industry is produced by microbial fermentation, mainly using the fungus *Aspergillus niger*, but also using some strains of yeast. (Abou-Zeid and Ashy, 1984; Milsom and Meers, 1985; Rohr *et al*, 1983)

2.2 Uses of Citric Acid

Worldwide sales of citric acid are divided amongst three main fields approximately as follows: (Milsom and Meers, 1985)

Food, Confectionery and Beverages	75%
Pharmaceutical	10%
Industrial	15%

Food uses include acting as an antioxidant and / or an emulsifier for dairy products, as a stabiliser of fats and oils, as a food acidulant and flavour enhancer, and in frozen foods to inactivate trace metals, neutralise residual alkali and prevent changes in colour and flavour by lowering pH and inactivating enzymes. It is also used as a stabiliser of fats and oils, to aid emulsification in the manufacture of food, and as a food acidulant and flavour enhancer. (Kapoor *et al*, 1982; Milsom and Meers, 1985)

Confectionery uses include enhancement of flavour, inversion of sucrose, prevention of oxidation, adjustment of pH and production of darker colours in hard candies, jams and jellies. (Kapoor *et al*, 1982)

Beverage uses are as a flavour enhancer, a preservative and to eliminate haze caused by the presence of trace metals. In wine, citric acid prevents turbidity, inhibits oxidation and adjusts pH. Soft drinks manufacturers use citric acid to give a cool taste and help maintain carbonation. (Kapoor *et al*, 1982)

Pharmaceutical uses include acting as a preservative for blood (preventing clotting), in antacid and soluble aspirin preparation (as it produces effervescence when combined with bicarbonate, and as a flavouring agent) and as a buffering agent. (Abou-Zeid and Ashy, 1984; Kapoor *et al*, 1982; Milsom and Meers, 1985)

Industrially, citric acid is used for treatment of boiler water, in metal plating, for the replacement of phosphates in detergents, in cosmetics as an antioxidant

and synergist, in tanning and textiles, printing inks, to produce many useful esters with alcohols, and as a scrubbing agent for the removal of sulphur dioxide from flue gases. (Abou-Zeid and Ashy, 1984; Kapoor *et al*, 1982; Milsom and Meers, 1985)

Table 2.1 shows the value of citric acid imports into New Zealand over recent years.

Table 2.1 Citric acid imports into New Zealand from 1982 to 1992 (New Zealand Department of Statistics).

Year	Quantity (kg)	Value ($NZ)
1982 - 1983	717,000	1,483,000
1983 - 1984	939,000	1,705,000
1984 - 1985	939,000	1,705,000
1985 - 1986	1,036,000	2,521,000
1986 - 1987	1,021,000	2,425,000
1987 - 1988	1,100,000	2,475,000
1988 - 1989	922,000	2,132,000
1989 - 1990	1,244,000	2,663,000
1990 - 1991	1,003,000	1,836,000
1991 - 1992 (est)	860,000	1,763,000

2.3 Production of Citric Acid

The largest producer of citric acid is the United States , with the two main companies involved:- Pfizer Inc. (New York, New York), producing about 112,500 tonnes / annum and Miles Laboratories Inc. (Elkhart, Indiana), producing about 54,000 tonnes / annum (Kapoor *et al*, 1982)

Other major citric acid producing countries and companies include (Kapoor *et al*, 1982) :

West Germany	: Joh. A. Benckshiser, Ludwigshafen/Rhein
	: C.H. Boehringer Sohn, Ingelheim/Rhein
Belgium	: Citrique Belge, Tienen
France	: Rhone-Poulenc S.A., Paris
Taiwan	: San Fu Chemical Co. Ltd., Taipei
	: Tai Nan Fermentation Industrial Co. Ltd., Taipei
England	: John and E. Sturge, Ltd., Birmingham
Russia	
Canada	
Czechoslovakia	
Poland	
Israel	
Netherlands	
Austria	
Ireland	
Developing countries	

2.3.1 History of Production

Citric acid was first produced commercially in 1826 by John and Edmund Sturge from calcium citrate which was derived from lemon juice. Between thirty and forty tons of lemons were required to produce one ton of citric acid. By the turn of the twentieth century, about 10,000 tonnes per year of citric acid were produced from citrus fruits. In 1880 citric acid had been synthesised from glycerol by Grimoux and Adam. Wehmer, in 1893, discovered the possibility of a fermentation route for citric acid production when he noticed that certain species of *Penicillium* were able to accumulate significant quantities of citric acid when grown on solutions containing sugar. In 1917, Currie found that a strain of *Aspergillus niger* was able to produce higher citric acid yields. Currie joined with Chas. Pfizer and Co. Inc. and a citric acid plant using the new method opened in the US in 1923. (Abou-Zeid and Ashy, 1984; Milsom and Meers, 1985; Rohr *et al*, 1983)

This, and subsequent fermentation plants built around the world, all used the surface fermentation process, growing *A. niger* on media prepared from cane sucrose and inorganic salts. Soon processes based on cheaper beet molasses were introduced. Amelung (1930) made the earliest attempt to produce citric acid by a submerged fermentation technique. This process was first introduced commercially in 1952 in the US. Submerged fermentation processes using *A. niger* growing on media that were based on purified glucose syrups, beet or cane molasses have since been developed. (Abou-Zeid and Ashy, 1984; Milsom and Meers, 1985)

2.3.2 Production using Filamentous Fungi

In the surface fermentation process, broth, inoculated with *A. niger* spores, is placed in shallow pans. Humidified air is blown over the surface of the broth for five to six days, after which dry air is used. The spores germinate within 24 hours and mycelium covers the surface of the broth. The sugar concentration of 200 - 250 g/l is reduced to 10 - 30 g/l eight to ten days after inoculation. Very little citric acid is produced during growth. The initial pH is 5 - 6 and, upon germination of the spores, it rapidly approaches pH 1.5 - 2. The final yield of citric acid is in the range of 80 - 85% of the weight of the initial carbohydrate. (Atkinson and Mavituna, 1983)

In the submerged fermentation process, the inoculum usually consists of spores of a strain of *A. niger* grown on solid nutrient media. Initially the pH is about 4, and it falls to pH 1.5 - 2.0 during rapid uptake of ammonium ions. Little citric acid is formed in young cultures before a pH of 2.0 is reached. Air is continuously sparged at a rate of 0.5 - 1.5 vvm. In both fermentation processes, the amount of copper and iron ions added to the medium must be restricted, so steel fermenters cannot be used without glass or plastic coatings. (Atkinson and Mavituna, 1983)

Product recovery is by filtering the culture solution to remove the mycelium and adding calcium hydroxide to cause the precipitation of calcium citrate. The calcium citrate is then recovered by filtration and treated with sulphuric acid to precipitate calcium sulphate. The dilute filtrate containing the citric acid is purified by passing over activated carbon before being demineralized by ion exchange.

Next, the purified solution is evaporated, yielding crystals of citric acid, which are recovered by centrifugation. Citric acid is then marketed as an anhydrous crystalline chemical, as the crystalline monohydrate or as the crystalline sodium salt. (Atkinson and Mavituna, 1983)

2.3.3 Production using Yeasts

From 1965 onwards there has been progress towards using yeasts to produce citric acid from carbohydrates and alkanes. At the time that the processes using hydrocarbons as a substrate were being developed, petroleum products were still cheap and the production of citric acid from this substrate was economical. Since then, however, the price of petroleum products has risen significantly and the economics are now in favour of carbohydrates. Unlike hydrocarbons, which are becoming increasingly expensive and eventually will be in short supply, carbohydrates are renewable resources and can be used not only in pure form, but as impure residues from the processing of foodstuffs. (Abou-Zeid and Ashy, 1984; Milsom and Meers, 1985)

The range of yeasts reported to produce citric acid from various sources are species of *Candida, Hansenula, Pichia, Debaryomyces, Torulopsis, Kloeckera, Trichosporon, Torula, Rhodotorula, Sporobolomyces, Endomyces, Nematospora, Saccharomyces and Zygosaccharomyces.* Nowadays, those yeasts which accumulate high concentrations of citric acid are generally classified as *Yarrowia* e.g. *Y. lipolytica* or *Candida* e.g. *C. guilliermondii.* (Kapoor *et al*, 1982)

Candida genus is an imperfect yeast, with short-ovoid cells, (2 - 4.5) x (2.5 - 7) μm, multilateral budding, and blastospore and pseudomycelium production.

Yarrowia (also known as *Saccharomycopsis*) genus is an imperfect yeast, with short-ovoid to elongate cells, (2 - 4.5) x (4 - 22) μm, and multilateral budding on a narrow or broad base. Pseudomycelium, true mycelium, and arthrospores may be present (Meyer *et al*, 1984).

In the Department of Process and Environmental Technology (formerly Biotechnology Department) at Massey University, New Zealand, there is continuing interest in the use of whey permeate, a by-product of the dairy industry, as a substrate for production of citric acid. Previous studies with *A. niger* have demonstrated that lactose is a poor substrate for the fermentation as the galactose moiety of the sugar is not converted to citric acid and its presence has been shown to reduce the production of citric acid from the glucose moiety of the sugar. Thus investigations have begun into citric acid production using yeasts. (Gutierrez *et al*, 1992; McKay *et al*, 1990)

Yeasts have an advantage over *A. niger* for the formation of citric acid in that they do not require a metal ion deficiency, thus eliminating an expensive medium pre-treatment step. Yeasts are also easier to handle in a fermenter as they do not grow on probes or block ports, and the form of the growth is usually as a homogenous suspension, rather than in the form of pellets or large aggregates. (McKay *et al*, 1990; Rohr *et al*, 1983; Ward, 1989)

The major disadvantage of yeasts is the significant formation of isocitric acid as a by-product. This can amount for as much as 50% of the total acid, but is generally much lower. Also, the culture often requires pH control to achieve

high yields of citric acid (cf. *A niger*, where production is optimal at pH 2) and the higher pH required for production makes the culture more susceptible to contamination. (Rohr *et al*, 1983; Ward, 1989)

2.4 Biochemistry of Citric Acid Production by Yeasts

Citric acid is produced as an intermediate of the tricarboxylic acid cycle. Production of citric acid by yeasts requires a suitable carbon source, such as glucose, glycerol, sucrose, *n*-paraffin, ethanol, fatty acids and several natural oils. (Hattori *et al*, 1974; Ikeno *et al*, 1975). Also, the restriction of cell growth by limitation of a nutrient such as nitrogen, sulphur, phosphorus or magnesium is required. (Briffaud and Engasser, 1979a; Finogenova *et al*, 1986; Klasson *et al*, 1989; McKay *et al*, 1990; McKay *et al*, unpublished)

The reason why citric acid accumulates is not yet completely clear, but it has been proposed, when using a nitrogen-limited medium, that the low availability of nitrogen following its exhaustion causes an immediate decrease in AMP concentration in the cells. This causes inactivation of the NAD^+-isocitrate dehydrogenase within the mitochondria, as this enzyme has an absolute requirement for AMP. This results in an accumulation of isocitric acid, but the equilibrium constant of aconitase (which strongly favours citric acid) causes it to be citric acid which accumulates in high concentrations. (Evans and Ratledge, 1985; Mitsushima *et al*, 1978; Marchal *et al*, 1977b)

This model does not explain citric acid accumulation under condition of limitation of sulphur, phosphorus or magnesium. McKay *et al* (1990; unpublished)

have proposed that the slowing of growth rate, due to the exhaustion from the medium of these nutrients, causes changes in the cell and initiates production of citric acid by the yeast. McKay *et al* have shown that NAD^+-isocitrate dehydrogenase not only has a requirement for AMP, but also is inhibited by ATP. Hence, these authors concluded that it is the ratio of ATP : AMP which is critical to the regulation of NAD^+-isocitrate dehydrogenase. Essential to this condition being met is a high rate of carbon substrate catabolism causing a continued production of energy-rich metabolites, in combination with a restriction on energy utilization. Nutrient limitations such as nitrogen, sulphur and phosphorus meet these criteria.

Slowing of the growth rate can also lead to the production of a small quantity of 2-oxoglutarate by *Y. lipolytica* IMK2, both preceding and during citrate accumulation. If citrate production results from complete inactivation of isocitrate dehydrogenase, as proposed in the first model (Evans and Ratledge, 1985; Marchal *et al*, 1977a; Mitsushima, *et al* 1978) then accumulation of 2-oxoglutarate is surprising. Thus, deactivation of 2-oxoglutarate dehydrogenase may be the initial step of citric acid production, as is suggested for production by *A. niger*. (McKay *et al*, 1990; McKay *et al*, unpublished; Rohr *et al*, 1983)

2.5 Factors Affecting Citric Acid Production by Yeasts

2.5.1 Carbon Source

Production of citric acid during growth of yeasts on *n*-alkane was first

reported in the patent literature in 1967, using a species of *Candida* on a culture medium containing at least one of the normal alkanes with from nine to twenty carbon atoms. Starting from a highly reduced form of carbon such as *n*-alkanes, very high weight yields are possible. The theoretical yield is about 250%.

$$C_{16}H_{34} + 12.5\ O_2 \rightarrow 2.66\ C_6H_8O_7.H_2O + 4.66\ H_2O \qquad \text{[Eqn 2.1]}$$

The theoretical maximum yield for citric acid from glucose would be about 117%

$$C_6H_{12}O_6 + 1.5\ O_2 \rightarrow C_6H_8O_7.H_2O + H_2O \qquad \text{[Eqn 2.2]}$$

Allowing for production of biomass, carbon dioxide and maintenance energy, a reasonable target yield would be 175% on hydrocarbons and 80% on glucose. (Marchal *et al*, 1977a; Milsom and Meers, 1985)

Investigation into the productivity of citric acid, by strains of *Candida zeylanoides, C. citrica*, and *Saccharomycopsis lipolytica*, from individual *n*-alkanes of carbon numbers ten to twenty, showed that the highest production was obtained from the *n*-alkanes C_{13} to C_{18}. Effect of carbon numbers on yeast growth had a similar tendency to that on the productivity of citric acid. (Furukawa *et al*, 1977; Furukawa *et al*, 1982; Nakanishi *et al*, 1972)

A problem with hydrocarbon fermentations is the insolubility of the carbon source in an aqueous solution. It is essential to maintain a fine dispersion of the hydrocarbon in the aqueous phase, thus maximising the area for the cells to come into contact with the carbon source, and allowing good uptake. (Gutierrez

and Erickson, 1978; Milsom and Meers, 1985)

Glucose cultures have the advantage that the substrate is soluble in aqueous solutions and easily assimilated by the cells. Also, the oxygen requirement for the conversion of the substrate is much less. Furthermore, the citric acid is more suitable to food use as there is no possibility of traces of potentially carcinogenic hydrocarbons being carried over. The oil crisis of 1973/74 also gave processes using glucose an economic advantage over hydrocarbon processes. (Abou-Zeid and Ashy, 1984; Rehm and Reed, 1983)

Table 2.2 summarizes some of the final concentrations of citric acid observed during production from hydrocarbons and glucose.

Two phases are distinguishable during citric acid production in batch fermentation by yeasts growing on hydrocarbons or glucose in a nitrogen-limited medium.

1. Growth phase : lasts about 20 hours, after which the biomass concentration remains approximately constant.

2. Production phase : starts at the end of the growth phase. Citric acid is produced at a constant rate up to approximately 90 hours, after which the production rate declines, probably due to a decrease of physiological activity of the yeast. After about 160 hours, physiological activity usually ceases. (Briffaud and Engasser, 1979a; Enzminger and Asenjo, 1986; Maddox and Kingston, 1983; Hattori *et al*, 1974; Ikeno *et al*, 1975; Marchal *et al*, 1977a; Marchal *et al*, 1977b; Nakanishi *et al*, 1972; Treton *et al*, 1978)

Table 2.2 Final citric acid concentrations obtained during growth of yeasts on hydrocarbons and glucose

Reported By	Fermentation Time (h)	Initial Substrate Concentration (g/l)	Final Citric Acid Concentration (g/l)
Hydrocarbons			
Hattori (1974b)	80	78	120
Nakanishi (1972)	144	100	130
Treton (1978)	140	210 est	141
Ikeno (1975)	96	100	174
Furukawa (1977)	115	60	84
Furukawa (1982b)	168	45	76
Glucose			
Hattori (1974b)	144	78	42
Briffaud (1979a)	175	150 est	105
Treton (1978)	170	240	117
Ikeno (1975)	122	211	157
Klasson (1989)	120	130	30

Other carbon sources that have been investigated for citric acid production by yeasts include glycerol, some natural oils and fatty acids, sucrose, sorbitol, ethanol, cellulose, galactose, kraft black liquor, date seeds and cheese whey. Table 2.3 summarizes the citric acid yields obtained from some of these carbon sources.

During production from ethanol, the concentration of the substrate in the medium is very important as when the ethanol concentration rises above 1 g/l, citric acid synthesis is inhibited and mainly isocitric acid is accumulated. Above 20 g/l ethanol, both citric acid and isocitric acid production are inhibited, and acetic acid begins to accumulate. This can be overcome by a low initial concentration of ethanol followed by subsequent regular addition. (Finogenova *et al*, 1991)

Sugars present in the hemicellulose fraction of wood hydrolysate include xylose, arabinose, mannose and galactose. Improved yields would be obtained if the yeast were able to convert all of these sugars into citric acid. Thus, although a strain of *S. lipolytica* was able to ferment mannose as readily as glucose, with a yield of 0.41 g/g, it was unable to ferment the other sugars, even when present in combination with glucose. When glucose was present, no mannose was used by the yeast. (Maddox *et al*, 1985)

Production of citric acid from cellulose requires prior hydrolysis of the cellulose into sugars able to be used by the yeasts. During enzymatic hydrolysis, end-product inhibition by cellobiose and glucose can greatly reduce the rates. However, a simultaneous saccharification of the cellulose and fermentation of the sugars into citric acid would overcome this inhibition. A strain of *C. guilliermondii*

Table 2.3 Yields of citric acid obtained from various carbon sources

Carbon Source	Reported By	Max. Yield (%)
n-paraffin	Furukawa (1977)	151
	Furukawa (1982b)	150
	Nakanishi (1972)	150
	Hattori (1974b)	137
	Ikeno (1975)	161
Glucose	Ikeno (1975)	82
	Hattori (1974b)	54
	Treton (1978)	49
	Finogenova (1986)	45
	Gutierrez (1992)	38
	Maddox (1985)	30
	Maddox (1983)	51
	Briffaud (1979a)	75
Glycerol	Ikeno (1975)	59
	Hattori (1974b)	55
	Treton (1978)	57
	Finogenova (1986)	38
Sucrose	Hattori (1974b)	36
Galactose	Gutierrez (1992)	37
Mannose	Maddox (1985)	41

Sorbitol	Hattori (1974b)	26
Hexadecane	Finogenova (1986)	114
Ethanol	Finogenova (1986)	26
	Ikeno (1975)	34
Acetic acid	Finogenova (1986)	22
	Hattori (1974b)	32
	Potvin (1988)	47
Soybean oil	Hattori (1974b)	127
	Ikeno (1975)	115
Rapeseed oil	Ikeno (1975)	49
	Good (1985)	142
Coconut oil	Ikeno (1975)	100
Palm oil	Ikeno (1975)	155
Palm kernel oil	Ikeno (1975)	117
Olive oil	Ikeno (1975)	119
Linseed oil	Ikeno (1975)	97
Kraft black liquor	Potvin (1988)	47
Oleic acid	Hattori (1974b)	74
	Ikeno (1975)	107
Stearic acid	Ikeno (1975)	105
Palmitic acid	Ikeno (1975)	96
Myristic acid	Ikeno (1975)	89
Other fatty acids	Ikeno (1975)	insignificant

cultivated in a mixed enzyme-microbe system with a combination of fungal cellulases was able to convert cellulose into citric acid, but with only a low yield (0.1 g/g). The best results were obtained by conducting the process in two stages, the first to optimize the conditions for saccharification and cell growth, and the second to optimize saccharification and citric acid accumulation conditions. (Asenjo *et al*, 1982; Asenjo and Jew, 1983)

A strain of *C. tropicalis* was used by Potvin *et al* (1988) to ferment kraft black liquor, which typically contains significant amounts of acetic, formic, lactic and other carboxylic acids. When compared to fermentation on a synthetic acetate medium, the yeast showed good production on the liquor up to substrate concentrations of 15% (v/v). Above this, very little citric acid was produced. The acetate medium was able to produce citric acid even up to 35% (v/v) concentration.

Abou-Zeid *et al* (1983) investigated a mixture of date seed hydrolysate and whey using a strain of *C. lipolytica.* Maximum citric acid production occurred when a combination of 10 g/l of date seed hydrolysate and 15 g/l whey was used. The addition of 25 g/l of glucose to the whey and date seed hydrolysate increased the yield by 60%. Maltose addition (30 g/l) gave a 45% increase in yield, and sucrose (25 g/l) a 30% increase in yield. However, it is not clear how much, if any, of the sugars present in the date seed hydrolysate and whey were used during the process.

The carbon source can have a significant effect on the relative amounts of citric and isocitric acids accumulated extracelluarly by strains of yeast. Table 2.4 summarizes the main effects.

Table 2.4 Affect of carbon source on the relative amounts of citric and isocitric acid accumulated.

Carbon Source	Reported By	Citric Acid (%)	Isocitric Acid (%)
n-paraffin	Nakanishi (1972)	50 - 90	10 - 50
	Hattori (1974b)	53	47
	Treton (1978)	67	33
Glucose	Hattori (1974b)	88	12
	Treton (1978)	91	9
	Finogenova (1986)	84	16
Glycerol	Hattori (1974b)	91	9
	Treton (1978)	92	8
	Finogenova (1986)	88	12
Sucrose	Hattori (1974b)	81	19
Sorbitol	Hattori (1974b)	84	16
Hexadecane	Finogenova (1986)	43	57
Ethanol	Finogenova (1986)	23	77
Acetic Acid	Hattori (1974b)	52	48
	Finogenova (1986)	57	43
Soybean oil	Hattori (1974b)	52	48
Oleic Acid	Hattori (1974b)	55	45

Glucose and glycerol tend to be better carbon sources than hydrocarbons, oils or organic acids to minimise isocitrate production. However, careful selection of an appropriate strain can assist to minimise the isocitric acid production on any particular substrate.

Examination of the intracellular concentrations of citric and isocitric acids by Treton *et al* (1978), for a strain of *S. lipolytica*, showed 5% isocitric acid and 95% citric acid at late exponential phase when grown on glucose. When grown on *n*-paraffin the intracellular isocitric acid concentration was 10% during the exponential phase, falling to 7% in the late exponential phase. In the surrounding medium, however, isocitric acid was about 33% of the total citric acid. This observation indicates that permeability barriers may be important in the excretion and accumulation of isocitric acid. Further investigation showed that in the presence of glucose or glycerol, neither citric nor isocitric acid was consumed provided that the major carbon source concentration did not fall below a certain threshold (a level low enough to prevent reconsumption of the acids during a normal fermentation). In the presence of *n*-paraffin, however, citric and isocitric acids were consumed, but the consumption of isocitric acid occurred after a significant lag period and was slower than that of citric acid. In the presence of citric acid there was no consumption of isocitric acid. These observations indicate that during a fermentation conducted on *n*-paraffin both citric and isocitric acids are excreted and citric acid alone is reconsumed, albeit at a slower rate than that of production, thus causing the higher accumulation of isocitric acid during cultivation on *n*-paraffin.

Reasons for the nonconsumption of excreted isocitric acid in the presence of citric acid include:

1. If the same permease mediates the uptake of both acids, competitive inhibition may occur to the detriment of isocitric acid uptake. In the experiments performed there was always an excess of citric acid over isocitric acid.

2. If the carriers are different, citric acid could act as an inhibitor of the isocitric acid-specific transport system.

2.5.2 Oxygen

Citric acid fermentation is aerobic, with a considerable oxygen requirement, especially for highly reduced substrates, such as *n*-alkanes. As equation 2.1 in section 2.5.1 shows, production of citric acid from a C_{16} *n*-alkane requires 12.5 moles of oxygen for every mole of alkane. Glucose, as shown by equation 2.2 (section 2.5.1), requires 1.5 moles of oxygen per mole of glucose. Thus, provision of oxygen is very important to the fermentation.

The production of citric acid from *n*-paraffin by yeasts can be considerably affected by aeration and agitation conditions. Using a strain of *C. zeylanoides*, an increase in the production rate of just over 20% was achieved by increasing the agitation from 500 rpm to 700 rpm and aeration from 0.5 vvm to 2.0 vvm. The ratio of citric to isocitric acid was unaffected. A strain of *C. citrica* also showed an increase of about 50% in the rate of citric acid production by increasing the aeration rate from 0.3 vvm to 1.0 vvm. Raising the agitation speed from 250 rpm to 600 rpm further increased the citric acid production by about

70%. A strain of *S. lipolytica* showed no significant change in citric acid production rate following an increase in the aeration rate to above 0.2 vvm, even when air enriched with 42% O_2 was used. Aeration at 0.2 vvm gave a dissolved oxygen concentration of about 40% of saturation. Another strain of *S. lipolytica* showed no significant change in production of citric acid when the aeration rate was varied from 0.2 to 0.8 vvm, but an increase in the agitation rate from 350 rpm to 500 rpm gave a 35% increase in citric acid production and a 60% decrease in isocitric acid production. In small jar fermenters, such as the ones used in these experiments, the agitation rate can have a significant affect on the dissolved oxygen (DO) concentration. This is because an increase in agitation causes a decrease in gas bubble size, and thus a greater surface area from the same volume of gas. As diffusion of oxygen from the gas phase into the culture medium occurs only at the surface of the bubbles, an increase in the surface area causes an increase in the overall rate of oxygen transfer, and thus an increase in the DO concentration. (Furukawa *et al*, 1977; Furukawa and Ogino, 1982; Hattori *et al*, 1974b; Marchal *et al*, 1977a)

During growth on glucose, the specific oxygen consumption rate is lower by about threefold than during growth on *n*-paraffins. For a strain of *S. lipolytica*, an increase in concentration of gas oxygen of about ninefold resulted in a 100% increase in the specific rate of citric acid production. Using a strain of *C. tropicalis* it was discovered that the accumulation of citric acid increased with dissolved oxygen concentration up to about 60 ppm, then decreased rapidly above this. Isocitric acid accumulation decreased with increasing DO concentration and became negligible around 50 ppm. The total citric and isocitric

acid concentration remained nearly constant, independent of the DO concentration. With a supply of air at normal pressure the DO concentration is about 6 ppm. Pure oxygen at about 170 kPa is needed to obtain a DO concentration of 60 ppm. (Briffaud and Engasser, 1979a, 1979b; Okoshi *et al*, 1986)

Finogenova *et al* (1991) found that, during growth on ethanol, a strain of *C. lipolytica* showed inhibition of yeast growth at an oxygen concentration of about 30% of saturation, but not at 60 - 65% or 90 - 95% saturation. At 90 - 95% saturation, citric and isocitric acids were excreted in approximately equal quantities. Decreasing the oxygen concentration to 60 - 65% saturation resulted in only slightly decreased isocitric acid production, but citric acid production was only 40% of previous. At about 30% saturation, citric acid production stopped.

In summary, oxygen is very important in the production of citric acid. An increase in the culture DO concentration, caused by an increase in the aeration rate, agitation rate, or the use of oxygen enriched gas, usually causes an increase in the rate of citric acid production.

2.5.3 pH

Control of the culture pH is important during the cultivation of yeasts. Unlike the fungus *A. niger*, which can thrive at very acidic pH levels, and where production of citric acid is optimal at pH 2, yeasts cultures require a higher pH level for optimum production, and culture pH cannot be allowed to fall naturally.

For a strain of *C. zeylanoides* grown on *n*-paraffin, total citric and isocitric

acid production was found to be maximum at pH 6.5, with a slight decrease (5%) at pH 5.5, and a larger decrease (25%) at pH 4.5. Similarly , a strain of *S. lipolytica* showed maximum production at pH 6.5, with a 8% increase in production over that at pH 5.5. At pH 7.0, production decreased by 15%. For another strain of *S. lipolytica*, the specific rate of citric acid production was found to increase steadily from pH 3.0 to pH 4.5, followed by a slight increase up to pH 5.0, before decreasing slightly at pH 6.0. Raising the pH from 3.0 to 5.0 gave a 350% increase in the rate of citric acid production. In the experiments with this *S. lipolytica* strain, the yeast cells were first grown at pH 5.0, and then the pH was adjusted to the value to be investigated. Thus, the affect of pH on rate of citric acid production was measured for the production phase only. (Furukawa and Ogino, 1982; Marchal *et al*, 1977a; Nakanishi *et al*, 1972)

Production of citric acid from ethanol using a strain of *C. lipolytica* was investigated by Finogenova *et al* (1991). No difference in total citric and isocitric acid production between pH 4.5 and pH 6.0 was observed, but the ratio of the two acids changed significantly. At pH 4.5, the citric acid concentration was nearly double that of isocitric acid, but at pH 6.0 the reverse was true. At pH 7.0, citric and isocitric acid production ceased.

Hattori and Suzuki (1974a) investigated the effect of pH on polyol production by citric acid-accumulating yeasts. Production of polyols such as mannitol, arabitol and erythritol has been observed from citric acid-producing yeasts growing on *n*-alkanes at low pH, such as pH 3.5. Decreasing the medium pH from 5.5 to 3.5 caused the intracellular pH to drop from 6.5 - 6.7 to 5.5 - 5.7, and polyol production was found to occur between intracellular pH values of 5.3 -

5.7. Studies of the enzymes involved in polyol production showed that their activity was stimulated at around pH 5.5, causing polyol production when the culture pH drops to around pH 3.5. Citrate synthase activity was significantly depressed at pH 5.5. For a non-polyol producing yeast strain, a drop in culture pH to 3.5 resulted in an intracellular pH of about 4.8, which caused inhibition of most enzymes, including those involved in polyol production.

To summarize, culture pH has a significant affect on citric acid production. Maximum citric acid production usually occurs around pH 6.5 when culture growth and citric acid production are conducted at the same pH value. However, when the culture pH is adjusted after growth, the maximum citric acid production rate occurs around pH 5. Production of polyols has been observed for some citric acid-accumulating yeasts, and their production is stimulated by a drop in culture pH to 3.5.

2.5.4 Temperature

Investigation using strains of *C. zeylanoides*, *C. citrica* and *S. lipolytica* grown on *n*-paraffins have all shown good citric acid excretion rates between 25° to 33°C, with optimum production at 30°C. Above 35°C, production of citric acid decreases sharply. (Furukawa *et al*, 1977; Hattori *et al*, 1974b; Marchal *et al*, 1977a; Nakanishi *et al*, 1972)

2.5.5 Nutrients

2.5.5.1 Nutrient Limitation

Citric acid production by yeasts requires the restriction of cell growth to stimulate production. Most fermentations have been performed using a limitation of nitrogen for this purpose. During growth on *n*-paraffin, by strains of *S. lipolytica* and *C. zeylanoides*, cell mass increased with increased concentration of the limiting nutrient, nitrogen, but the specific rate of citric acid production tended to decrease with increasing cell biomass. Thus a compromise must be reached between a high specific rate of production and sufficient biomass to obtain the optimum for total citric acid production. Over a range of ammonium salt concentrations from 1 to 50 g/l, the optimum concentration for citric acid production was 4 g/l. Since the biomass increased steadily with increasing ammonium salt concentration, it was clear that nitrogen was the limiting nutrient. (Hattori *et al*, 1974b; Marchal *et al*, 1977; Nakanishi *et al*, 1972) During growth on glucose, Klasson *et al* (1989) found no change in the specific rate of citric acid production over the initial ammonia concentration range of 0.11 - 0.44 g/l.

Citric acid production has also been observed for limiting nutrients other than nitrogen, such as sulphur, magnesium, phosphate and potassium. For a *Yarrowia lipolytica* strain, cells limited by the sulphate concentration, in the presence of an excess of nitrogen, had an identical rate of citric acid production to that of nitrogen-limited cells. In a phosphate-limited (0.15 - 0.30 mM) medium cells showed an increasing specific rate of citric acid production with increasing

limiting nutrient, but the maximum specific rate was only 43% of that observed during nitrogen limitation. The specific rate fell very quickly between 0.15 - 0.23 mM phosphate. For magnesium-limited cells, with a limiting concentration of 15 μM, very low quantities of citric acid were produced. Raising the concentration to 50 μM resulted in a specific rate of production only 45% of that for nitrogen limitation. Thus significant citric acid production occurs under magnesium or phosphate limitation only when a threshold concentration of the nutrients is exceeded. Potassium-limited cells produced only minor accumulations of citric acid at the concentrations tested (up to 0.10 mM). (McKay *et al*, 1990; McKay *et al*, unpublished)

2.5.5.2 Nitrogen

The form of the nitrogen used in the fermentation is important, especially as nitrogen is commonly used as the limiting nutrient. Investigations into various inorganic nitrogen sources have shown ammonium salts to be the most easily assimilated by citric acid producing yeasts. A strain of the yeast *C. citrica* was able to use all of the ammonium salts tested (ammonium sulphate, ammonium chloride, ammonium nitrate and ammonium phosphate) with fairly similar production of biomass and citric acid. When the only form of nitrogen was sodium nitrate, however, both the biomass and citric acid production decreased greatly. A strain of *C. lipolytica* growing on a medium which already contained some organic nitrogen, from date seed hydrolysate and whey, also benefitted most from a nitrogen supplementation in the form of ammonium salts rather than

nitrate. (Abou-Zeid *et al*, 1983; Furukawa *et al*, 1977)

For a *C. zeylanoides* strain growing on *n*-paraffin, the ratio of citric acid to isocitric acid increased with increasing ammonium ion concentration. Increasing the ammonium chloride concentration from 4 g/l to 50 g/l caused isocitric acid production to decrease to 14% of previous, while citric acid decreased to only 61%. Studies with other ammonium salts revealed similar decreases in isocitric acid production. This effect also occurred with strains of *C. lipolytica, C. tropicalis* and another strain of *C. zeylanoides.* However, since the citric acid concentration also decreases with high ammonium ion concentration, albeit more slowly, this is not a particularly practical method for reducing isocitric acid production. (Hattori *et al*, 1974b)

2.5.5.3 Phosphate

The phosphate concentration has a significant effect on citric acid production, but not on the ratio of citric acid to isocitric acid. Hattori *et al* (1974b) found that for a strain of *C. zeylanoides*, grown on *n*-paraffin, the optimum concentration of potassium dihydrogen phosphate was 0.3 g/l.

2.5.5.4 Metal Ions

For a strain of *C. zeylanoides* grown on a synthetic medium containing *n*-paraffin as the substrate, iron salt ($FeSO_4.7H_2O$) was observed to have a significant effect on citric acid production. The total amount of citric acid

production was greatly affected by the iron concentration, with maximum production over a range from 0 - 20 mg/l being at 0.1 mg/l of the iron salt. A *C. citrica* strain, grown on *n*-paraffin, in a medium containing trace iron from the addition of 0.3 g/l of yeast extract, showed a decrease in citric acid production by addition of 1 mg/l iron salt to the medium, and further decrease by the addition of 5 mg/l of the iron salt. With a strain of *S. lipolytica* grown on a synthetic medium, with *n*-paraffins as the substrate, an increase in iron salt concentration from 10 to 400 mg/l resulted in only a slight decrease in the specific production rate of citric acid. Reduction of the iron concentration to below 10 mg/l by the addition of the iron complexing agent quinaldinic acid, however, had a large affect on the fermentation. 10 mg/l of quinaldinic acid resulted in a 20% increase in the citric acid production rate, and 100 mg/l of the quinaldinic acid resulted in a 40% increase. These results indicate that while a small amount (e.g. 0.1 mg/l) of iron salt is necessary for the fermentation, addition of extra iron salt, up to 20 mg/l, causes a large decrease in the production of citric acid. Beyond 20 mg/l, further addition of iron salt does not appear to have a large effect on the fermentation. In all cases, an increase in the iron salt concentration caused an increase in isocitric acid production. (Furukawa *et al*, 1977; Hattori *et al*, 1974b; Marchal *et al*, 1977a)

Manganese salt ($MnSO_4.4H_2O$) addition to yeasts grown on *n*-paraffins had no effect on the ratio of citric to isocitric acid. Increasing the manganese salt concentration from 0.2 to 2.0 mg/l caused a 40% increase in citric acid production. Further increase to 20 mg/l resulted in a 60% drop in production. There was only a slight increase in the citric acid production between 0.5 and 1.0

mg/l of the manganese salt. (Furukawa *et al*, 1977; Hattori *et al*, 1974b)

Addition of zinc ions (as $ZnSO_4.7H_2O$) to a strain of *C. zeylanoides* grown on *n*-paraffin, resulted in an 80% increase in citric acid production between 0.2 and 2.0 mg/l addition. Further addition up to 20 mg/l had no significant affect. For a strain of *C. citrica*, grown in a medium containing trace zinc ions from the addition of 0.3 g/l of yeast extract, the addition of 0 to 5 mg/l of the zinc salt caused only a slight increase in citric acid production. These results indicate that the addition of a small amount of zinc ions (up to 2.0 mg/l) can improve citric acid production. Further addition of zinc does not seem to have any affect on performance. (Furukawa *et al*, 1977; Hattori *et al*, 1974b)

Cupric sulphate ($CuSO_4.5H_2O$) addition has been reported to have a large affect on citric acid production. For a *C. zeylanoides* strain growing on *n*-paraffin, an increase in the cupric salt from 0.05 to 0.1 mg/l caused citric acid production to decrease to 40% of previous. However, further addition up to 1.0 mg/l had no significant effect. For a strain of *S. lipolytica*, grown on a medium containing 0.3 g/l of yeast extract, addition of 0.1 mg/l of the cupric salt produced a 50% increase in citric acid production and a 60% decrease in isocitric acid production compared to production without any addition. Further addition of the cupric salt caused a slow decrease in citric acid accumulation. A strain of *C. citrica* also showed an optimum concentration of 0.1 mg/l cupric sulphate. (Furukawa *et al*, 1977; Furukawa *et al*, 1982b; Hattori *et al*, 1974b)

Boron addition, in the form of sodium borate ($Na_2B_4O_7.10H_2O$), to the fermentation on *n*-paraffins, gave an small increase in citric acid at its optimum addition concentration of 0.1 mg/l, and caused isocitric acid to decrease by

around a third. Cobalt ions ($CoCl_2.6H_2O$) gave a similar result at 0.01 mg/l. Molybdenum ion ($(NH_4)_6Mo_7O_{24}.4H_2O$), at 0.05 mg/l, however, decreased citric acid yield by around 20%. (Furukawa *et al*, 1977; Furukawa *et al*, 1982b)

No reports were found in the literature about the effect of metal ion concentrations on citric acid production by yeasts growing on glucose.

Table 2.5 summarizes the optimum concentrations of metal ions reported for citric acid production by yeasts.

2.5.5.5 Yeast Extract

Furukawa *et al* (1977) used a strain of *C. citrica*, grown on *n*-paraffins, to examine the effects of yeast extract and casamino acids on the production of citric acid. Varying the yeast extract concentration from 0.1 to 0.6 g/l had negligible effect, but casamino acids gave a maximum of a 16% increase in production after a change from 0 to 0.3 g/l. An increase to 0.7 g/l casamino acids gave a 8% decrease in production.

2.5.5.6 Others

As isocitric acid production is a major side reaction for yeasts producing citric acid, especially when growing on hydrocarbons, methods of reducing isocitric acid production may be important to the economic viability of the fermentation process. Aconitase inhibitors, such as monofluoroacetate (100 mg/l) or α,α'-dipyridyl, have been reported, by Nakanishi *et al* (1972), to increase citric

Table 2.5 Optimum metal salt concentrations for citric acid production by yeast strains grown on *n*-paraffin.

Metal Salt	Optimum Concentration (mg/l)
$FeSo_4.7H_2O$	0.1
$MnSO_4.4H_2O$	2.0
$ZnSO_4.7H_2O$	2.0
$CuSO_4.5H_2O$	0.1
$Na_2B_4O_7.10H_2O$	0.1
$CoCl_2.6H_2O$	0.01
$(NH_4)_6Mo_7O_{24}.4H_2O$	0

acid production by as much as 180% while reducing isocitric acid production to only 18% of previous. However, such inhibitors may be too costly to justify their use.

2.6 Chemostat Fermentations

Continuous fermentation for the production of citric acid could be advantageous commercially, as a continuous system can give a more consistent product and provide a steady stream of crude product for further processing. Continuous systems also require a lower capital investment and less labour, as they have a higher volumetric efficiency and less down-time. As a nutrient limitation is required to stimulate citric acid production by yeasts, continuous fermentation can be achieved by chemostat culturing.

Hattori *et al* (1974b) investigated a nitrogen limited chemostat culture, growing on *n*-paraffin, using a strain of *C. lipolytica*. The specific production rate (g/g.h) of citric acid increased as the dilution rate increased from 0.012 h^{-1} to 0.025 h^{-1}. In the dilution rate range 0.025 h^{-1} to 0.05 h^{-1} no clear trend was apparent. A linear relationship was observed between the dilution rate and the ratio of citric acid to isocitric acid, with an increase in the dilution rate from 0.012 h^{-1} to 0.05 h^{-1} causing citric acid to decrease from 92% to 63% of the total acid. Biomass concentration increased slightly as the dilution rate decreased, which may be due to the accumulation of storage compounds at the lower dilution rates.

Aiba and Matsuoka (1978) investigated another *C. lipolytica* strain, growing on *n*-alkanes in a nitrogen limited chemostat culture. Over a dilution rate range

of 0.0128 to 0.0730 h^{-1}, no significant difference in the ratio of citric to isocitric acid was observed. The citric acid specific production rate reached a maximum at a dilution rate of 0.0412 h^{-1}, approximately 3-fold greater than the rates at the maximum and minimum dilution rates examined. Biomass concentration generally decreased with increased dilution rate.

Aiba and Matsuoka (1979) also investigated a *C. lipolytica* strain in a nitrogen limited chemostat culture, growing on glucose. Over dilution rates of 0.0122 h^{-1} to 0.0769 h^{-1}, the specific rate of citric acid production was maximum at 0.0300 h^{-1}. Biomass remained fairly constant over the range of dilution rates tested. The ratio of citric to isocitric acid also remained fairly constant, but with a slight decrease at the higher dilution rates.

Klasson *et al* (1989) found that a *C. lipolytica* strain, in a nitrogen limited chemostat, grown on glucose, could be run in the chemostat culture for over 1000 hours with no problems. Biomass decreased with increased dilution rate, and increased with higher concentrations of nitrogen in the feed media. Over a dilution rate range of 0.03 to 0.13 h^{-1}, the maximum specific rate of citric acid production occurred at a dilution rate of about 0.06 h^{-1}. No trend with the nitrogen content of the feed media was observed.

In a chemostat culture, dilution rate appears to be an important factor, as the specific production rate of citric acid changes significantly with dilution rate. The maximum specific production rates occur at moderate dilution rates, between about 0.03 and 0.06 h^{-1}, with the rate decreasing at higher or lower dilutions. Biomass tends to decrease as the dilution rate increases, probably due to the production of storage compounds at the lower dilution rates.

2.7 Cell Recycle Fermentations

Cell growth, in chemostat fermentations, consumes a significant fraction of the substrate at the expense of the product. Fermentations using cell recycle would overcome much of this loss of substrate for the production of cell biomass. Cell recycle systems could also be used to prolong the effective production phase and shorten the lag phase before fermentation.

With a strain of *C. lipolytica* producing citric acid from growth on hydrocarbons, Gledhill *et al* (1973) investigated a non-sterile, semicontinuous, cell recycle system. Cell recycle was achieved by removing a portion of the broth from the fermenter after inoculation, growth and citric acid production under aseptic conditions, then separating out the yeasts under nonaseptic conditions using a centrifuge. The yeast cells were diluted to the original volume in fresh medium and returned to the fermenter. Using this technique, the citric acid fermentation could be extended for several days, with a gradual decrease in production rate over repeated cell recycles. The production rate during the semicontinuous cell recycle phase was also greatly affected by the stage of the fermentation that the process was initiated at. A higher concentration of citric acid in the media resulted in a lower production rate.

A similar fermentation system was used with a strain of *S. lipolytica* by Furukawa and Ogino (1982a). The overall productivity of the citric acid was maintained at a nearly 60% greater rate than for a batch culture, although the maximum productivity rate was slightly lower. Productivity during the effective phase decreased gradually over the course of the fermentation.

Enzminger and Asenjo (1986) used a continuous stirred tank bioreactor with cell recycle, to produce citric acid from glucose by a strain of *S. lipolytica.* The cells were collected by filtration from the culture exit stream and pumped back to the fermenter. In a batch culture, after cell mass levelled off, the citric acid production rate was constant for over 350 hours, until the citric acid concentration reached 105 g/l. Centrifuging and washing the cells allowed another 250 hours of acid production at an increased specific production rate (although total production rate was slightly lower due to a decrease in biomass). Using the cell recycle system, cell mass steadied off after 30 - 40 hours of recycle, and steady state levels of citric acid were achieved for over 200 hours of operation. Specific rate of total acid production, yield on glucose, and volumetric productivity were all similar to or higher than batch values.

Kim *et al* (1987) used a continuous stirred tank membrane reactor with a strain of *S. lipolytica.* Cell recycle was by a 0.45 μm membrane filter to prevent yeast cell exit, with a periodic reverse flow to wash cells back into the fermenter. If a suitable media and environment to support long term yeast cell activity was provided, constant activity without visible sign of decline could be obtained for over a month.

Cell recycle fermentations have been reported to successfully improve the overall productivity of the citric acid fermentation over that achieved by batch cultures. The fermentation could be maintained at a high production rate for much longer than a batch fermentation. Continuous recycle systems that collected the cells by filtration from the exit stream for return into the fermenter appeared more stable than the semicontinuous systems that removed batches

of culture and centrifuged to separate the cells for return to the fermenter.

2.8 Immobilized Cells

As with cell recycle systems, immobilized cell cultures would overcome the loss of substrate for cell growth and allow long production phases.

Briffaud and Engasser (1979a, 1979b) produced citric acid from glucose in a trickle flow fermenter using a strain of *S. lipolytica*, immobilized on cylindrical wood chips. Less than 5% of the yeasts were unbound in the liquid medium, indicating that wood chips were very efficient for yeast adsorption. The growth phase was linear, compared with exponential for an unbound batch culture. Citric acid production began at the end of the growth phase and continued at a constant rate for about 80 hours. The specific rate of citric acid production was 60% of the rate for an unbound batch culture. The culture could be regenerated by the addition of ammonia, which induced a new growth and excretion phase. The change in growth pattern and reduction in acid production were caused by oxygen diffusional limitations, mainly in the biomass film, and alterations in the bound cell metabolism. The importance of oxygen diffusional limitation was demonstrated by a decrease in the specific production rate when the biomass was in a thicker film.

Immobilization in a polyacrylamide gel was performed for a strain of *S. lipolytica* producing citric acid on glucose by Maddox and Kingston (1883). By comparison with cells that had been harvested, washed, and suspended in the glucose solution without being immobilized, it was seen that the immobilization

caused no loss of activity. In comparison to free cells in a fermenter, the immobilized cells had a low citric acid production rate, about 15% of the free cell rate. As the immobilization procedure had caused no activity loss, the physical/chemical environment may be responsible for the difference, such as dissolved oxygen concentration. Production rates for the immobilized cells were maintained for 45 days, and the cells could be stored for 14 days at 4°C without loss of activity.

A *C. tropicalis* strain immobilized in a carrageenin gel by Potvin *et al* (1988) was able to produce citric acid on kraft black liquor. Unlike a free cell culture, the immobilized cells were not inhibited by a concentration of 25% of the liquor. On a synthetic acetate media, immobilized cells had a specific production rate of only about half of that for free cells. The improvement in performance on the kraft black liquor may be due to the support acting as a microfilter, keeping out lignin fragments while allowing acetate to diffuse readily.

Several immobilization techniques were compared by Kautola *et al* (1991) using a strain of *Y. lipolytica*. The citric acid productivity was measured in repeated batch fermentations, each of 6 days duration. The highest productivity was obtained during the first batch, with small 2-3 mm calcium alginate beads, polyurethane gel cubes, and free cells all achieving the same productivity. The larger 5-6 mm calcium alginate beads and 5-8 mm carrageenin beads had about 65% of this productivity. The productivities of the *S. lipolytica* strain immobilized in polyacrylamide gel by Maddox and Kingston (1983) were under one third of the highest productivity achieved in these experiments. After the third batch, the free cell productivity decreased markedly, but the alginate bead immobilized yeast

continued fairly steadily. The other immobilized yeasts decreased in productivity fairly steadily. The bead size had a strong effect. A decrease in bead diameter to one half increased the productivity by about threefold after the second batch. Passive immobilization using hydrophobic carriers was not as effective. During the first batch the productivities were only 20% of those of the free cells, and decreased close to zero during the second and third batch. In an airlift fermenter, 5-8 mm carrageenin beads performed about 25% better than 5-6 mm calcium alginate beads, which had a productivity very close to the first batch fermentation.

Citric acid producing yeast cells have been reported to be successfully immobilized. Production by the immobilized cells can be maintained for longer periods of time than free batch cells. Generally, however, the immobilized cells have a lower specific production rate than free cells. Table 2.6 summarizes some of the production rates achieved.

Table 2.6 Maximum specific citric acid production rates reported for batch, chemostat, cell recycle and immobilized cell fermentations by yeasts growing on glucose.

Reported By	Specific Production Rate (g/g.h)
Batch Culture	
Klasson (1989)	0.053
Maddox (1983)	0.070
Briffaud (1979a)	0.041
Enzminger (1986)	0.040
Chemostat Culture	
Klasson (1989)	0.110
Aiba (1979)	0.037
Cell Recycle	
Enzminger (1986)	0.045
Immobilized Cells	
Maddox (1983)	0.010
Briffaud (1979b)	0.025
Kautola (1991)	0.021

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microbiological Media

Yeast Nitrogen Base (YNB) without amino acids or ammonium sulphate (Difco) was used as the basal medium. Sterile stock solutions of glucose, or other carbon source, ammonium chloride, antibiotics and the YNB were added to a solution of potassium dihydrogen phosphate in distilled water, which had been adjusted to pH 5 by the addition of 5 M sodium hydroxide, and which had been sterilized by autoclaving at 121°C for 20 minutes. Tables 3.1 to 3.4 detail the media used in different aspects of the work.

3.1.2 Gases

Oxygen-free nitrogen was supplied by New Zealand Industrial Gases Limited, Palmerston North, New Zealand.

Table 3.1 Medium for Batch Culture and Inoculum Preparation

Component	Concentration (g/l)[1]
Glucose	72
Yeast Nitrogen Base (w/o amino acids and ammonium sulphate)	3.4
Streptomycin	0.050
Kanamycin	0.010
Potassium Dihydrogen Phosphate	2.7
Ammonium Chloride	0.535

[1] Medium made to volume with distilled water

Table 3.2 Medium for Chemostat Fermentation

Component	Concentration (g/l)[1]
Glucose	10 or 20[2]
Yeast Nitrogen Base	3.4
(w/o amino acids and ammonium sulphate)	
Streptomycin	0.050
Kanamycin	0.010
Potassium Dihydrogen Phosphate	2.7
Ammonium Chloride	0.535

[1] Medium made to volume with distilled water

[2] 10 g/l glucose used for high and medium dilution rates.

20 g/l glucose used for low dilution rate.

Table 3.3 Medium for Shake Flask Cultures

Component	Concentration (g/l)[1]
Glucose	36.0
[Alternative Carbon Source	
Fructose	36.0
Glycerol	18.4
Succinic Acid]	23.6
Yeast Nitrogen Base	3.4
(w/o amino acids and ammonium sulphate)	
Streptomycin	0.050
Kanamycin	0.010
Potassium Dihydrogen Phosphate	2.7
Ammonium Chloride	0.535

[1] Medium made to volume with distilled water

Table 3.4 Medium for agar plates used in inoculum preparation

(for *Yarrowia lipolytica* IMK2)

Component	Concentration (g/l)[1]
Glucose	36
Yeast Nitrogen Base	3.4
(w/o amino acids and ammonium chloride)	
Streptomycin	0.050
Kanamycin	0.010
Ammonium Chloride	0.535
Calcium Carbonate	20
Agar	15

[1] Medium made to volume with distilled water

3.1.3 Chemicals

Chemicals used for analytical work and fermentations were all of analytical grade. Chemical suppliers were:

-BDH Chemicals Ltd (Palmerston North, New Zealand).

ammonium chloride; buffer solutions (pH 4.0 and 7.0); calcium carbonate; glucose; glycerol; methanol; potassium dihydrogen phosphate; sodium hydroxide.

-Ajax Chemicals (Sydney, Australia)

agar.

-Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)

calcium EDTA; citric acid; kanamycin; streptomycin.

-Bevaloid Chemicals Ltd. (Levin, New Zealand)

bevaloid 6009D antifoam

-May and Baker (Lower Hutt, Wellington, New Zealand)

orthophosphoric acid

-US Biochemical Corp. (Cleveland, Ohio, U.S.A.)

fructose

-Yellow Springs Instrument Co. Inc. (Yellow Springs, Ohio, U.S.A.)

YSI buffer for glucose

-Difco Laboratories (Detroit, Michigan, U.S.A.)

yeast nitrogen base w/o amino acids and ammonium chloride; potato dextrose agar

-Scientific Supplies Ltd. (Auckland, New Zealand)

succinic acid

-Polychem (Auckland, New Zealand)

ethanol

3.1.4 Organisms

The organisms used were *Yarrowia lipolytica* IMK2 and *Candida guilliermondii* IMK1.

Yarrowia lipolytica IMK2, a derivative of IFO 1658, was selected for increased citric acid production following UV mutagenesis and isolation in the presence of 0.8M potassium citrate pH 5.5). (Ian M^{c}Kay, personal communication)

Candida guilliermondii IMK1, a derivative of NRRL Y-448, was isolated on a defined medium containing glucose (72 g/l) and citrate (0.4 M) following UV mutagenesis (McKay *et al*, 1990).

Stock cultures were maintained on slopes of Potato Dextrose Agar at 4°C. Fresh stocks were subcultured every 6 months for use in inoculum preparation.

3.2 Media Sterilization

Glucose, fructose, glycerol and succinate stock solutions were sterilized by autoclaving at 121°C for 15 minutes.

Yeast nitrogen base, ammonium chloride, streptomycin and kanamycin stocks were filter sterilized (0.45 μm).

Potassium phosphate solution was sterilized in the fermentation equipment by autoclaving at 121°C for 20 minutes.

Antifoam (10 g/l bevaloid 6009D) and 5M NaOH were sterilized by autoclaving at 121°C for 15 minutes.

3.3 Cleaning of Glassware

All glassware was washed in hot Pyroneg$^{(R)}$ solution, rinsed in tap water, then in distilled water, followed by hot air drying.

3.4 Analytical Methods

3.4.1 pH Measurement

pH measurement in the shake flask experiments was performed using a Orion Research Digital Ionalyzer Model 701A (Watson Victor Ltd., New Zealand)

pH measurements in batch and chemostat experiments were performed using a Horizon pH controller model 5997-20 (Horizon Ecology Co., Chicago, Illinois, U.S.A.)

The electrodes used were combination pH electrodes (Broadley James Corporation, Santa Anna, California, U.S.A)

3.4.2 Determination of Cell Biomass

For shake flask and batch fermentation experiments, biomass was determined by reading the absorbance at 600 nm, following dilution in water to ensure that the absorbance stayed within the instrument range, and converting to dry weight using a calibration curve. The calibration curve was prepared in a similar manner to that described for biomass determination for chemostat experiments.

For chemostat experiments, biomass was determined by centrifuging a 25 ml sample, in duplicate, for 10 minutes at 4000 rpm using a BGH Hermle Z320 centrifuge (Berthold Hermle Gmbtl & Co., Gosheim, Germany) followed by resuspension in distilled water and re-centrifugation prior to drying overnight at 105°C. Absorbance of cell biomass at 600 nm was used to determine if steady state condition had been achieved.

3.4.3 Citric Acid Determination

Citric acid analysis was carried out using High Performance Liquid Chromatography (HPLC), (Waters Associates Model ALC/GPC 244 liquid chromatograph with a model 6000A solvent delivery system and a U6K septumless injector (Waters Associates Inc., Millipore Corporation, Milford, Massachusetts, U.S.A.)).

A C-18 reverse phase column, 4.6mm x 220mm, (Brownlee, San Francisco, U.S.A.) was used for the analysis.

The detector was a Model 401 Differential Refractometer (Waters Associates), while the response was recorded on a Waters 740 Data Module recorder.

The analyses were carried out at ambient temperature. The solvent system was 20 g/l potassium dihydrogen phosphate prepared using Milli-Q deionised water and adjusted to pH 2.5 using orthophosphoric acid. The solvent flowrate was 2.0 ml/min. Samples of 50 µl were injected into the chromatograph. The integrator was calibrated using standard solutions of 1 g/l and 10 g/l citric acid before assaying samples. Calculation was by comparison of peak area.

3.4.4 Carbon Sources Determination

Glucose : Analysis was carried out using a YSI fixed enzyme sugar analyzer (Yellow Springs Instrument Co.)

Succinate : Analysis was carried out using HPLC, as for citric acid determination (described in section 3.4.3).

Glycerol : Analysis was by an HPLC method. The equipment used was as described for citric acid (Section 3.4.3), with the exception of the column. A Sugar-PAK 1 column, 6.5mm x 300 mm (Waters Chromatography Division, Millipore Corporation) was used for the analysis. The solvent system was 0.050 g/l CaEDTA in Milli-Q deionized water. Solvent flowrate was 0.5 ml/min and the column was heated to 90°C using a Waters Model CX4-2 heating unit. A sample size of 50 µl was injected into the chromatograph. A 5 g/l standard was used to compare peak area with the sample.

Fructose : Analysis was by an HPLC method, as for glycerol determination.

3.4.5 Polyol Determination

An HPLC method was used to detect the presence of glycerol, mannitol, erythritol and arabitol. The method used was the same as described for glycerol determination in section 3.4.4.

3.4.6 Determination of Nitrogen Limitation

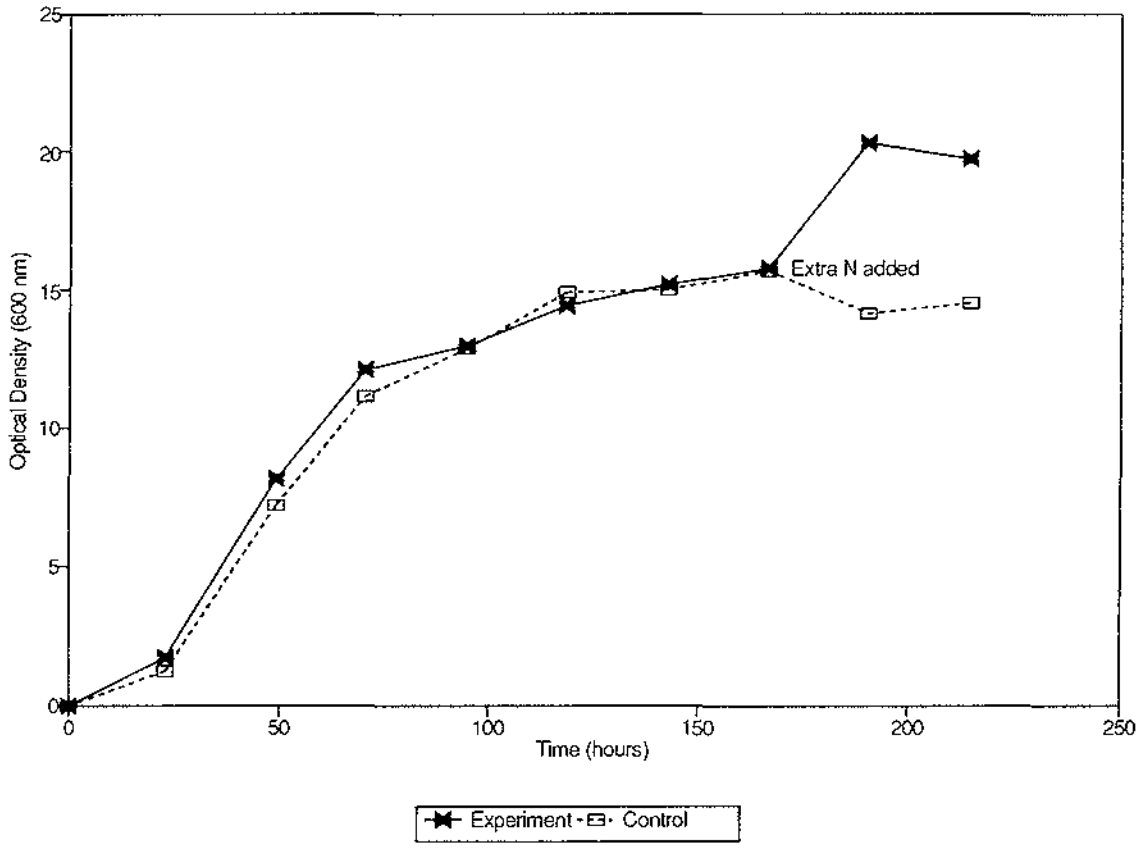
Nitrogen limitation was proved by the addition of extra nitrogen to a shake flask culture after the stationary growth phase had been achieved, and observing additional growth. Figure 3.1 shows the resulting growth curve.

3.5 Preparation of Samples

3.5.1 Sample Preparation for HPLC Analysis

Cells were removed by centrifugation for 5 minutes at 5000 rpm using a Clandon T52 centrifuge (Clandon Scientific Ltd., Hants, GU 12 5QR, U.K.). The supernatant liquid was filtered through a membrane (0.45 μm). For citric acid analysis the sample was acidified by adding 10% (v/v) orthophosphoric acid.

Figure 3.1 Proof that nitrogen is the growth limiting nutrient



3.5.2 Sample Preparation for Glucose Analysis

Cells were removed by centrifugation as for HPLC preparation. The supernatant liquid was diluted as required with distilled water to remain in the YSI instrument range.

3.6 Culture Conditions

3.6.1 Inoculum Preparation

Yarrowia lipolytica IMK2 :

The organism was taken from the stock culture using a sterile loop and streaked out by the 16-streak method onto an agar plate (Table 3.4). After incubation at 30°C for 48 to 72 hours the inoculum was prepared by selecting a colony which produced large amounts of citric acid, as demonstrated by the zone of clearing in the calcium carbonate, and suspending the yeast cells in 10 ml of potassium dihydrogen phosphate solution (20 mM).

Candida guilliermondii IMK1 :

The organism was taken from the stock culture using a sterile loop and inoculated into a 250 ml conical flask containing 50 ml of medium (Table 3.1). The culture was incubated at 30°C on a gyratory shaker at 200 rpm (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.) until late exponential phase (generally 48 to 72 hours).

3.6.2 Shake-Flask Culture

Experiments were conducted in 250 ml Erlenmeyer flasks containing 50 ml of medium (Table 3.3). 1 ml of inoculum was added to each flask. Flasks were incubated at 30°C on a gyratory shaker (New Brunswick Scientific Co.) at an operating speed of 200 rpm. Culture pH was adjusted twice daily to pH 5.2, if necessary, by addition of 10M NaOH. Samples were taken either daily or twice daily as required.

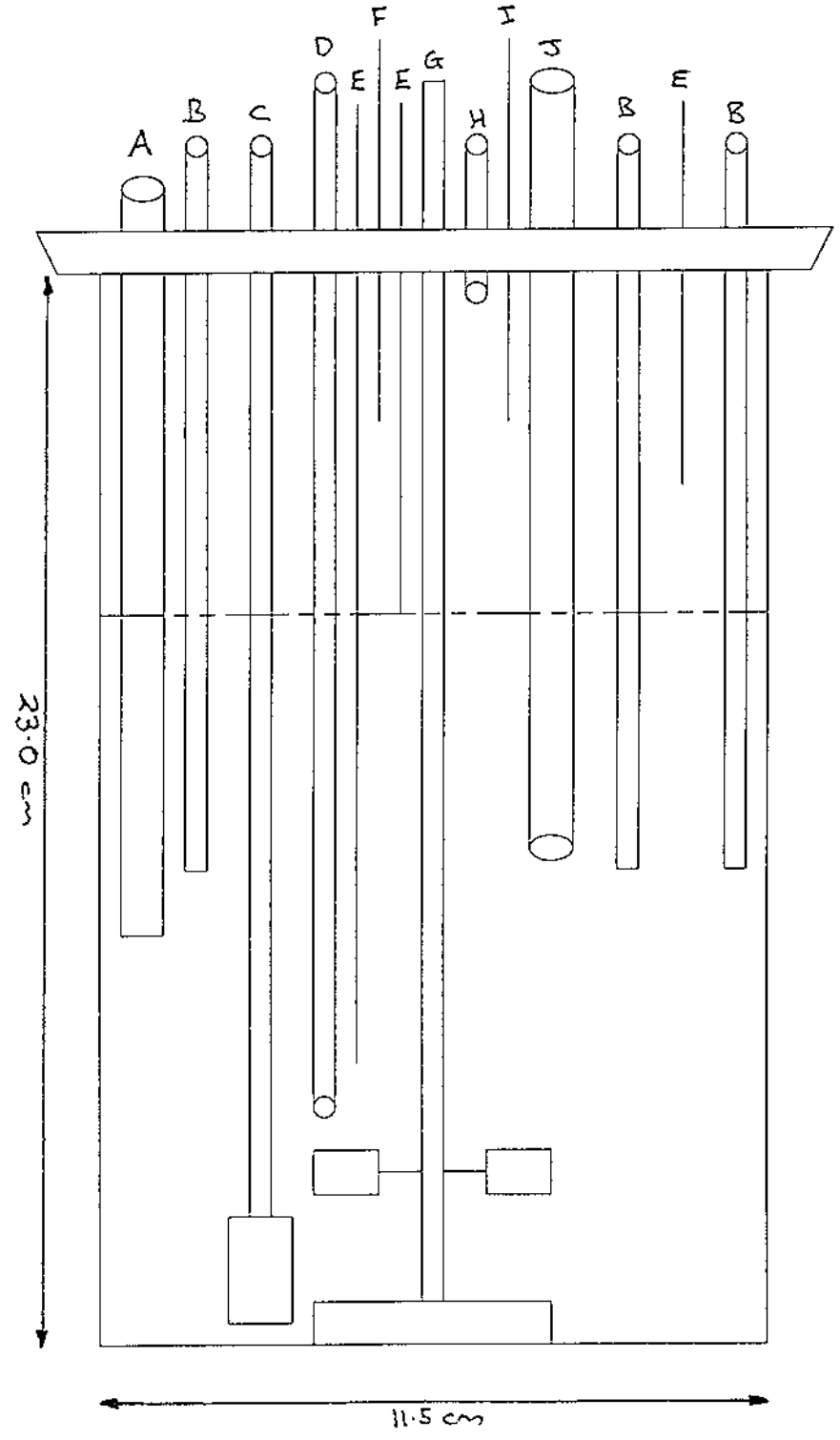
3.6.3 Batch Fermenter Culture

Figure 3.2 shows a diagram of the fermenter vessel, while Figure 3.3 shows a diagram of the fermenter head. The fermentation apparatus used was a Biogen benchtop culture apparatus. A 2-litre capacity glass jar (New Brunswick Scientific Co.) was used as the fermenter vessel with a working volume of 1.5 litres. The vessel was provided with a polyethylene-polypropylene head containing holes for the insertion of probes and the other facilities required.

Agitation was provided by a 6-bladed disc-turbine impeller mounted 3 cm above the base of the vessel on the central impeller shaft. This was driven from the base of the fermenter using indirect magnetic coupling to turn the impeller. Variable speed was obtained using an electronic controller. Agitation speed could vary from 0 to 1000 rpm.

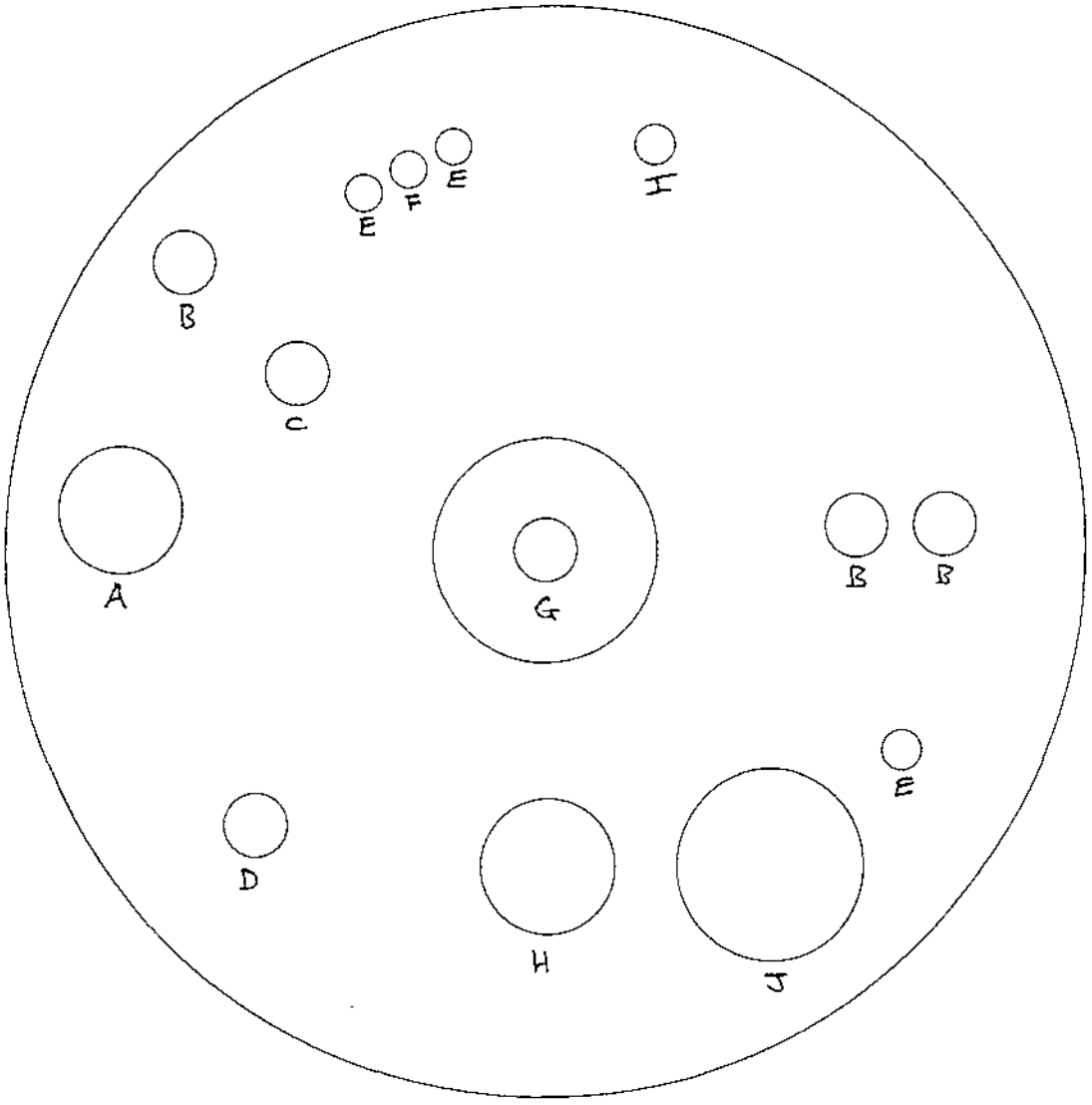
The fermenter temperature was maintained at 30°C by means of a thermocouple inserted into a close-ended metal tube inserted into the fermenter

Figure 3.2 The batch fermenter



A Heating element
B Thermocouple port
C Air diffuser
D Sample Port
E Foam Detector
F Antifoam addition port
G Impeller
H Air outlet
I NaOH addition port
J pH probe

Figure 3.3 The batch fermenter head



A Heating element
B Thermocouple port
C Air diffuser
D Sample Port
E Foam Detector
F Antifoam addition port
G Impeller
H Air outlet
I NaOH addition port
J pH probe

head, connected to an electronic thermostat which controlled a heating element inserted into another closed metal tube in the head. Temperature was continuously recorded using a Honeywell Varsaprint Multipoint chart recorder (Amiens, France).

Air was supplied to the fermenter from the Massey University compressed air line, through a pressure controller (Smiths, U.K.) where pressure was maintained at 163 kPa to a gap meter (Gap Basingstoke, England) to regulate the flowrate. The gap meter controlled the airflow to the fermenter over the range of 0 to 0.6 l/min. The air then passed through a sterile millipore filter (Millex-FG_{50}, 0.2 µm, Millipore Corporation, Molsheim, France) and entered the vessel either through the central agitation shaft or through a diffuser. Exhaust air passed through a sterile cotton wool filter.

Culture pH was measured using a combination pH electrode (Broadley James Corporation) connected to a Horizon pH Controller Model 5997-20 (Ecology Co.). The pH controller automatically controlled the culture pH by connection to a Masterflex fixed speed (1 rpm) peristaltic pump, size 13 pump head (Cole Parmer Instrument Co., Chicago, Illinois, U.S.A.), which would dose 10M NaOH when the culture pH fell below the set point. The pH controller was also connected to the Honeywell Varsaprint multipoint chart recorder and culture pH was continuously recorded. The pH value of the samples was regularly checked by an independent pH meter and any discrepancies were corrected.

Foam was detected by conductivity between two probes, one submerged in the culture and one 3 cm above the culture fluid. A foam controller (electronics workshop, Process and Environmental Technology Department, Massey

University, New Zealand) connected to a Masterflex peristaltic pump dosed sterile 10 g/l Bevaloid 6009D antifoam to control foaming problems. A mechanical foam breaker, consisting of two plastic ties, attached to the central agitation shaft about 2 cm above the surface of the culture fluid also helped to maintain low foam levels.

Culture samples were taken by temporarily blocking the air exit line and opening the sample port. The build up of pressure in the vessel forced some culture out the submerged sample line. Sample sizes of 15 ml were taken.

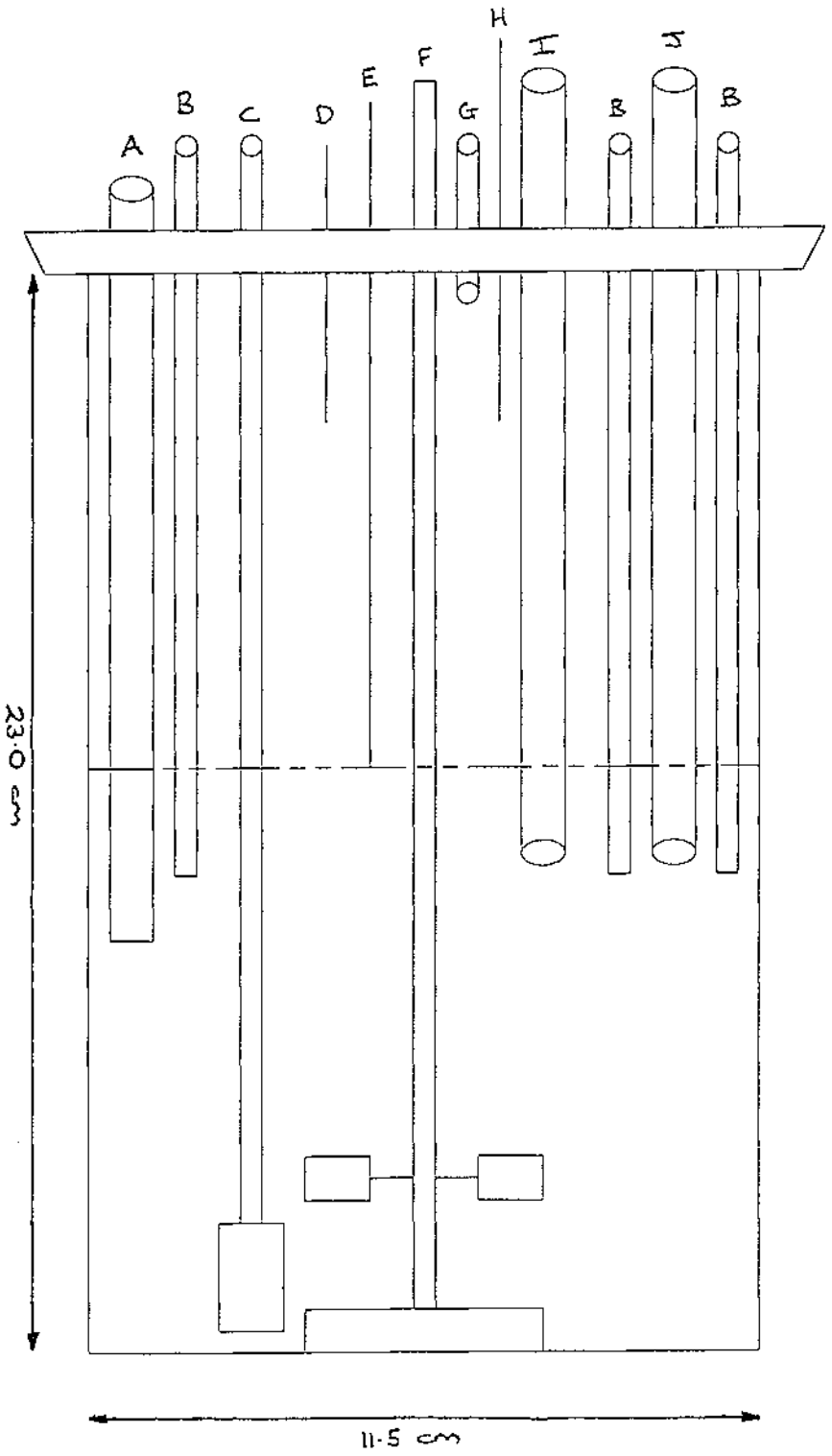
3.6.4 Chemostat Continuous Culture

The equipment described for batch culture (Section 3.6.3) was used for chemostat culture with a few minor variations (described below). A working volume of 1 litre was used. Figures 3.4 and 3.5 show diagrams of the fermenter vessel and fermenter head respectively.

Air was supplied to the fermenter as described for the batch culture, but the air entered the vessel via a diffuser, rather than by the central agitation shaft. Exhaust air was vented, through a water cooled condenser to reduce any loss of fermentation liquid by evaporation, into a water trap.

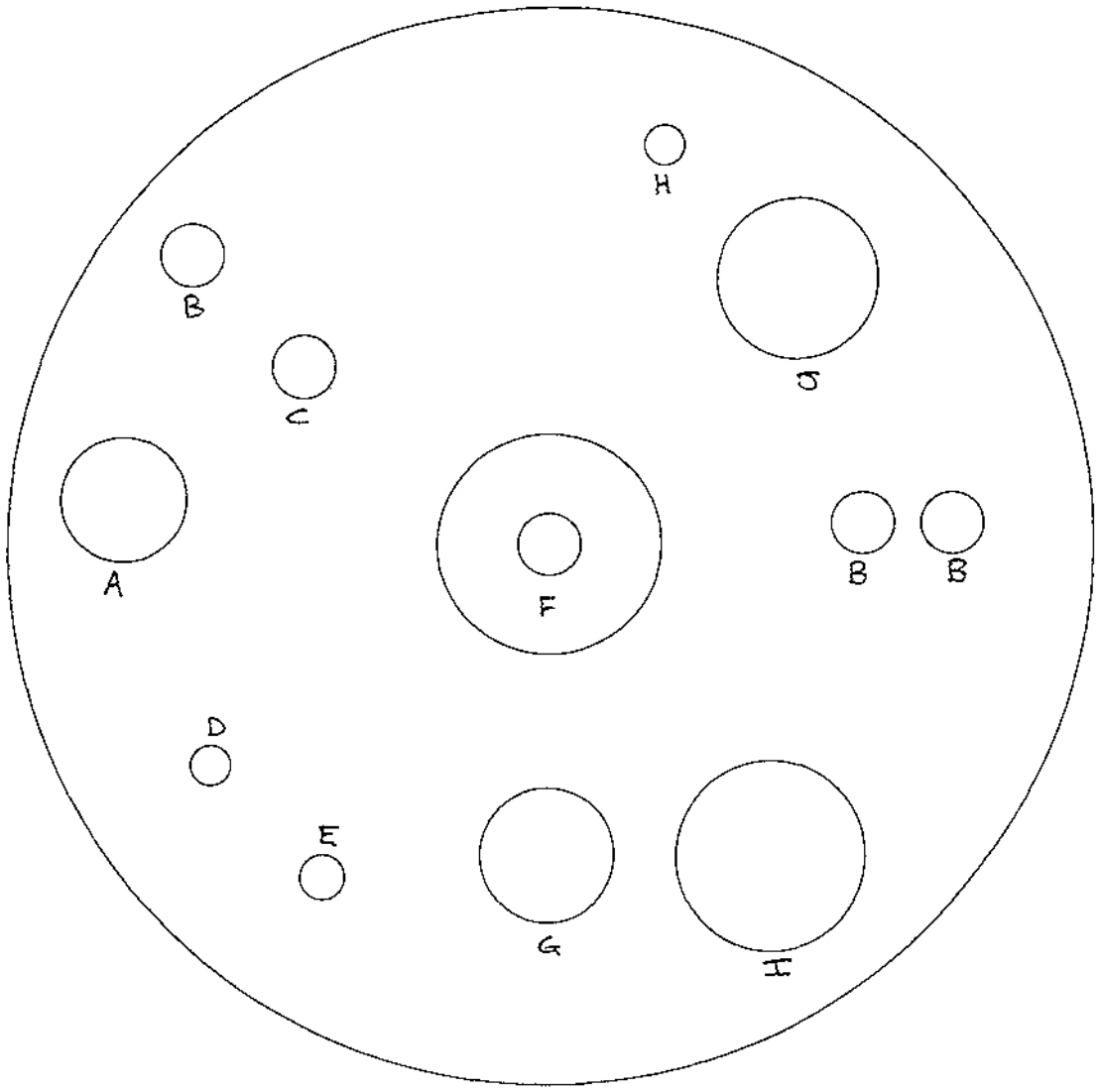
Culture dissolved oxygen tension was measured using a series 900 glass galvanic dissolved oxygen probe, type M1016-5050 (New Brunswick Scientific Co.). The probe was connected to a Honeywell Varsaprint Multipoint chart recorder through a Hewlett-Packard 2470B amplifier, allowing continuous recording of the culture DOT. Prior to inoculation of the fermenter the probe was

Figure 3.4 The chemostat fermenter



A Heating element
B Thermocouple port
C Air diffuser
D Influent port
E Effluent port
F Impeller
G Air outlet
H NaOH addition port
I pH probe
J DO probe

Figure 3.5 The chemostat fermenter head



A Heating element
B Thermocouple port
C Air diffuser
D Influent port
E Effluent port
F Impeller
G Air outlet
H NaOH addition port
I pH probe
J DO probe

calibrated *in situ* by sparging oxygen-free nitrogen gas through the air supply system to obtain zero saturation conditions, and then the medium was aerated vigorously to give conditions of 100% saturation of oxygen.

Foam control was not necessary during chemostat culture.

Feed medium was added to the culture using a Masterflex peristaltic pump (5-100 rpm, model 7546-10, Cole-Parmer Instrument Co). Dilution rate was controlled by a solid state Masterflex controller attached to the pump. Culture volume was controlled by setting the height of the exit tube.

The culture was initially grown under batch fermentation conditions at pH 5.2 until late exponential phase before switching on the feed medium and adjusting the pH if required.

Samples were taken from the exit line.

3.6.5 Sterilization

The fermenter vessel containing the potassium dihydrogen phosphate buffer and all the systems in the fermenter head, except the pH probe and the condenser, was sterilized by autoclaving at 121°C for 20 minutes, as was the buffer for the feed medium in the reservoirs for the chemostat culture.

The pH probe and condenser were sterilized by immersing in 50% ethanol for 12-18 hours, followed by thorough rinsing in sterile distilled water before insertion into the fermenter vessel.

All pump tubes and feed lines were sterilized by autoclaving at 121°C for 15 minutes.

3.6.6 Avoidance of Biomass Build-up on Vessel Walls

Yeast growth and foam residue on the walls of the vessel was periodically dislodged using a teflon covered bar magnet inside the fermenter vessel which was secured and moved by a horse shoe magnet from outside the vessel.

3.7 Discussion of Methods

3.7.1 Foaming

Both of the yeasts studied in this work showed a natural tendency to form a foam of cells due to the aeration method of bubbling fine gas bubbles through the culture. This resulted in a clarified broth with a thick layer of foam containing the yeast cells in the head space. Mechanical foam breakers alone, attached to the agitation shaft, proved unable to control the foam levels, especially as foam became trapped between probes where the foam breaker did not reach. Manual addition of antifoam when foam levels rose above acceptable was unsatisfactory as major problems arose overnight. Hence an automatic antifoam addition system based on detection of conductivity between two probes, causing drip feed addition of an antifoam agent, was added to the fermenter. This proved satisfactory along with the mechanical foam breakers in controlling the foam levels and maintaining the bulk of the yeast cells in the culture medium.

3.7.2 Aeration

Initially air was supplied to the culture through the central agitation shaft and out of a series of small holes just below the impellers. However, problems occurred due to wearing of the plastic head-piece of the agitation shaft, thus allowing the air to short-circuit through the head space instead of through the culture medium. Attempts to fix the problem by adding an improved o-ring seal to the head piece proved unsatisfactory, so an alternative air distribution system through a diffuser separate from the agitation shaft was finally used.

CHAPTER 4

STUDIES USING *YARROWIA LIPOLYTICA* IMK2

4.1 Introduction

Initial experiments were performed using a high citric acid-producing mutant strain of the yeast *Yarrowia lipolytica* IMK2. Previous experimental work with this mutant had been carried out in the Process and Environmental Technology (formerly Biotechnology) and Food Technology Departments of Massey University, but in shake flask culture only. It was desired in this thesis to scale-up the fermentation to batch fermenter culture and then to use chemostat culture to investigate the kinetics of the fermentation and the hypothesis that citric acid production occurs when the growth rate slows but the sugar uptake rate is maintained. An investigation into the effect of an altered sugar uptake rate on citric acid production was attempted by the use of mixed carbon sources in shake flask cultures.

4.2 Effect of Mixed Carbon Sources

Shake flask experiments were performed to investigate the use of mixed substrates as a means of controlling the carbon uptake rate and thus, possibly, the citric acid production rate. The ability to control the carbon uptake rate by the use of a mixed carbon source would allow a useful method of investigating this parameter under otherwise identical batch or chemostat fermentation conditions.

The citric acid production rate may be limited by the rate of uptake of substrate into the cell. Uptake sites in the cell membrane vary for different types of substrates. By providing two carbon sources that enter the cell by different uptake sites, an increase in the total carbon uptake rate may be achieved, thus increasing the rate of citric acid production. If the substrates compete for the same uptake site, however, no increase in uptake rate or citric acid production rate would occur. Thus, this experiment was designed to investigate if a change in the specific production rate of citric acid occurred by using glucose, fructose, succinate and mixtures thereof, as carbon sources, and to study the relationship between total carbon uptake rate and citric acid production rate.

The total substrate concentration was 2M in all experiments. Glucose and fructose were tested alone and in a 1 : 1 mixture with each other. Succinate was tested only in a 1 : 1 ratio with glucose. Figures 4.1 and 4.2 show citric acid production during growth of *Y. lipolytica* on different carbon sources. From a plot of the citric acid concentration against the integral of the biomass of the culture as a function of time, the specific rate can be calculated as the gradient of the curve at each point. When the specific rates are constant, such a curve will yield a straight line. The integral of the biomass of the culture as a function of time can be approximated by measuring the area under the growth curve (Hollander and Stouthamer, 1979). The growth curves for the different cultures were almost identical, as shown in Figure 4.3, with the cultures growing on the glucose and succinate mixture producing a slightly higher final biomass concentration. The specific citric acid production rate for each carbon source tested is shown in Table 4.1.

Figure 4.1 Citric acid production on glucose, fructose, and 1 : 1 glucose : fructose carbon sources

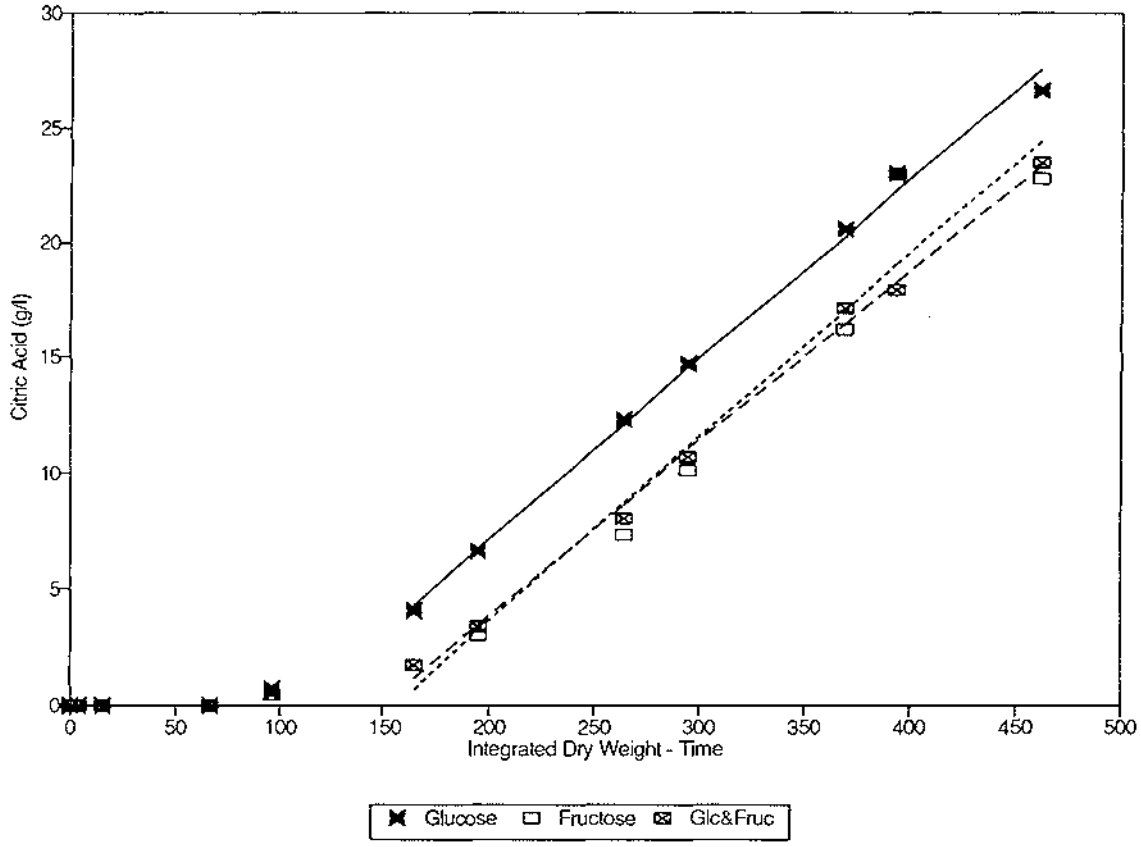


Figure 4.2 Citric acid production on glucose and 1 : 1 glucose : succinate carbon sources

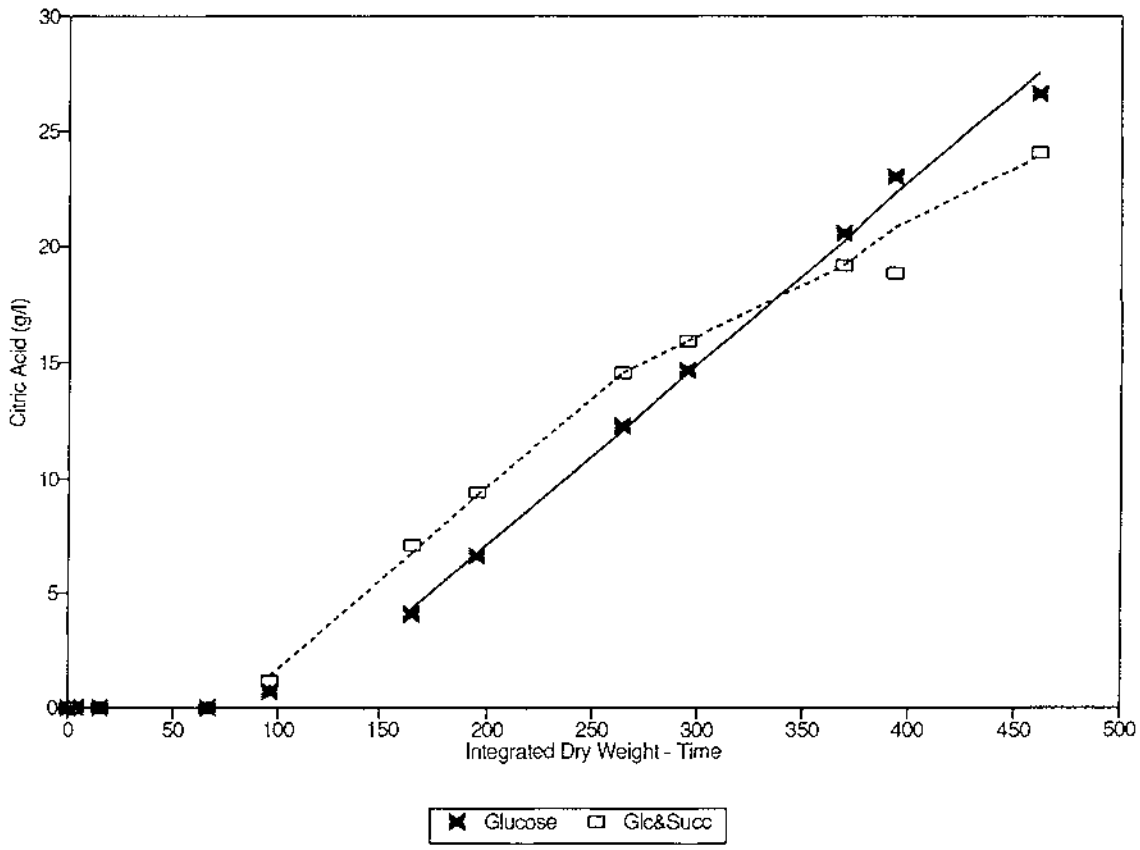


Figure 4.3 Growth curve of *Y. lipolytica* on mixed carbon sources

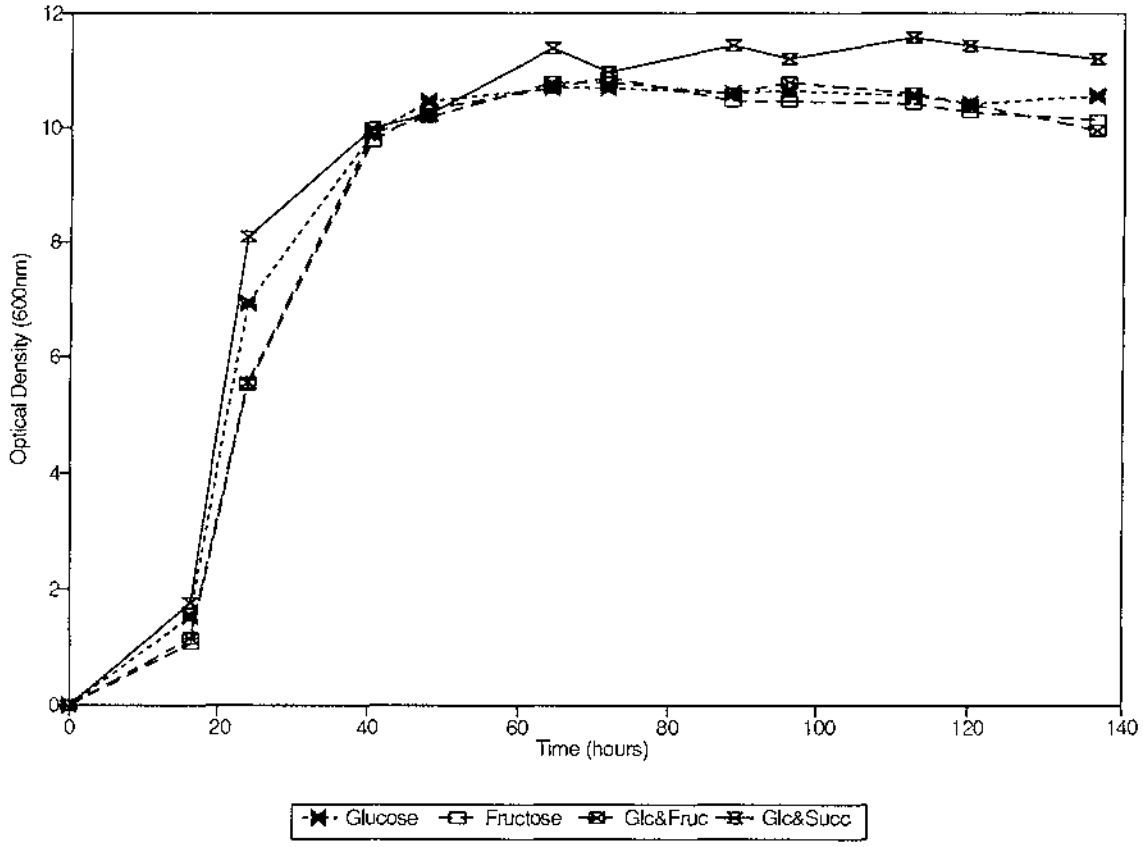


Table 4.1 Results of the mixed carbon sources shake-flask experiments for *Yarrowia lipolytica* IMK2.

Carbon Source		Specific Rate of Citric Acid Production (g/g.h)
Glucose		0.078
Fructose		0.080
Glucose and Fructose		0.075
Glucose and Succinate	(initial)	0.078
	(final)	0.045

The production rates were basically identical during growth on glucose, fructose and the glucose-fructose mixture, although the fructose-containing cultures showed a slight delay in starting citric acid production. With the glucose-succinate substrate mixture, the initial production rate was the same as for glucose alone, but it slowed down approximately halfway through the fermentation to only 58% of the initial rate. This decrease in rate was probably due to exhaustion of the glucose from the medium, following which the yeast produced citric acid from succinate alone. No substrate concentration analyses were done, nor any further investigations, as problems with the batch fermentation, described in Section 4.4, resulted in abandonment of studies with this yeast.

4.3 Effect of Aeration

Preliminary investigations within the Food Technology and Biotechnology Departments of Massey University had suggested that for the yeast strain *Candida guilliermondii* IMK1, shake flask fermentations under low oxygen conditions, although proceeding very slowly, showed a higher specific rate of citric acid production than fermentations conducted under high aeration conditions (Cambourn, personal communication). Thus, an experiment was performed using the yeast *Y. lipolytica* IMK2 to see if a similar phenomenon occurred. The fermentation was carried out in 250 ml conical flasks containing 200 ml of standard medium, as described in Section 3.1.1., with a fermentation trap to

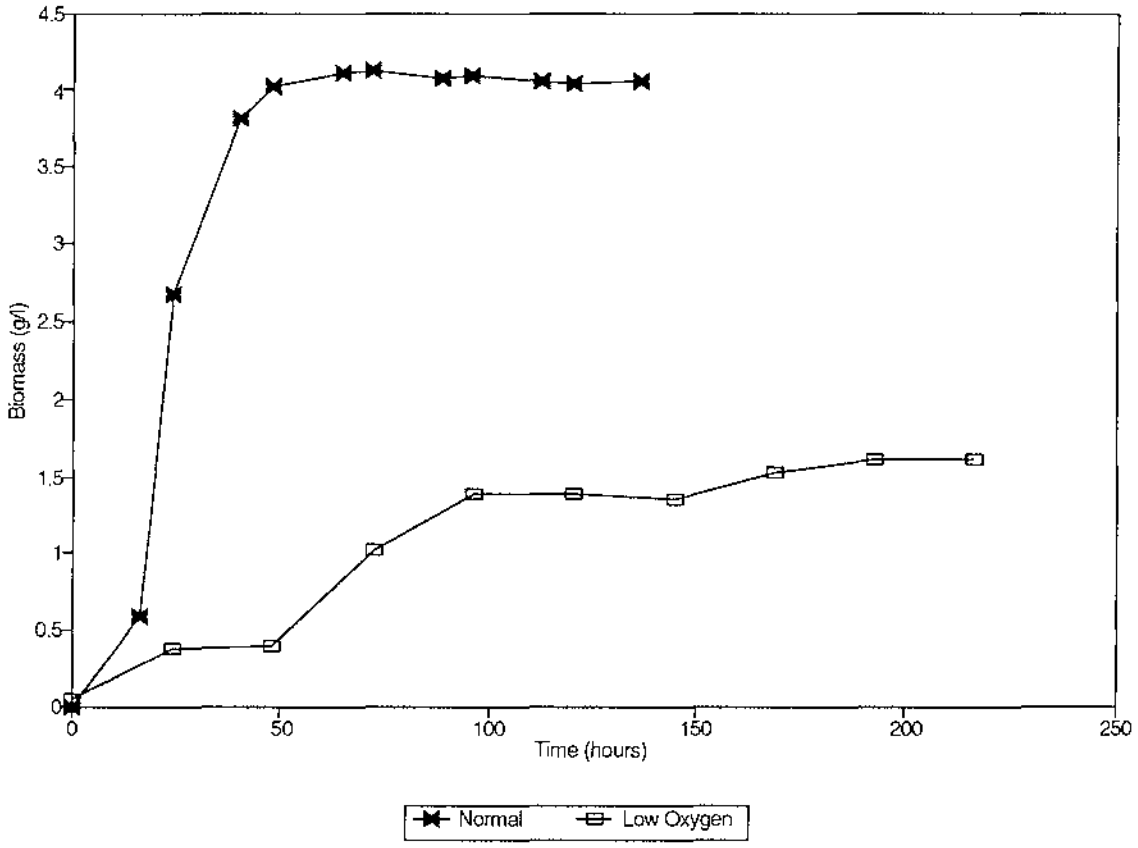
reduce diffusion of oxygen from the atmosphere into the flask. The flasks were incubated on a shaker at 50 rpm. Some oxygen would be expected to enter the culture during sampling and pH control, but the oxygen concentration should be very low. Cultivation was otherwise as described for a normal shake flask culture, as described in Section 3.6.

Growth of the culture proceeded much slower than that observed during a normal fermentation, i.e. a shaker speed of 250 rpm and no fermentation trap. Figure 4.4 shows a comparison of the growth curves. Even after 215 hours of cultivation, however, there was virtually no citric acid produced, with one of the replicate flasks containing less than 0.2 g/l of citric acid, and the other none. The normal shake flask cultures produced up to 25 g/l of citric acid after 136 hours of cultivation. These results indicate that for the yeast *Y. lipolytica* IMK2, a low oxygen concentration does not promote a high specific citric acid production rate.

4.4 Studies in Fermenter Culture

The purpose of the initial experiments was to gather data on the normal behaviour of the yeast during growth in a fermenter. Problems occurred, however, as the yeast consistently failed to produce citric acid in significant quantities. The highest value obtained was a citric acid concentration of 4.6 g/l from 17.7 g/l of glucose after 138 hours of fermentation, giving a yield of only 0.26 g/g. This compared poorly to the typical performance in shake flask culture of up to 25 g/l citric acid after 112 hours and a yield of 0.47 g/g.

Figure 4.4 Growth curve of *Y. lipolytica* during normal (high) and low oxygen shake flask experiments



The medium used was as described in Section 3.1.1, except that in the initial experiments the ammonium chloride concentration was 0.27 g/l and the glucose concentration was 36 g/l. The culture conditions were as described in Section 3.6. Table 4.2 summarizes the results of the experiments and some of the problems that occurred during this work.

The low biomass concentrations in the early experiments, runs 1 - 6, may have been due to the high aeration rate of 0.6 vvm. The air bubbles caused the yeast cells to form a foam in the head-space of the fermenter vessel, leaving a clarified broth. Manual addition of antifoam helped to control the foam, but the problem still occurred overnight. In addition, the aeration, by pumping gas through the culture, may have caused some loss of ammonium ions as ammonium gas, contributing to the lower biomass in the fermenter compared to the shake flask cultures. Hence, the aeration rate was reduced to 0.066 vvm to try to reduce these effects.

Attempts to improve the culture performance by increasing the glucose and nitrogen concentrations to 72 and 0.535 g/l respectively, adjusting the culture pH from 5.3 to 4.3, increasing the aeration rate from 0.066 vvm to 0.333 vvm, and installing an automatic antifoam addition system, all failed to produce a significant improvement. A fresh stock culture was also tested to see if the strain had altered due to frequent subculturing, but no change in the citric acid production was observed, and the old stock culture performed typically in shake flask experiments.

Table 4.2 Batch fermentations of *Yarrowia lipolytica* IMK2. (In chronological order)

Expt. No.	Final Biomass (g/l)	Citric Acid Produced (g/l)	Duration of fermen-tation (h)	Conditions: pH aeration NH_4Cl	Comments
1	5.57	0	113.5	pH 5.3 0.6 vvm 0.27 g/l	pH control trouble, no foam, atypical growth, contami-nation suspected
2	0.10	-[1]	40	pH 5.3 0.6 vvm 0.27 g/l	aeration lost, foaming, not growing properly
3	0.12	-	40	pH 5.3 0.6 vvm 0.27 g/l	aeration lost, foaming, not growing properly
4	0.83	-	72	pH 5.3 0.6 vvm 0.27 g/l	aeration lost, manual A/F[2] addition, biomass too low
5	1.46	-	97	pH 5.3 0.6 vvm 0.27 g/l	aeration lost, pH increase to 6.7 at 97 hours
6	1.14	-	65	pH 5.3 0.6 vvm 0.27 g/l	manual A/F addition, pH increase to 6.5 at 41 hours

Expt. No.	Final Biomass (g/l)	Citric Acid Produced (g/l)	Duration of fermen-tation (h)	Conditions: pH aeration NH_4Cl	Comments
7	0.39	0.5	65	pH 5.3 0.066 vvm 0.27 g/l	manual A/F addition, biomass loss at 65 hours
8	0.85	-	64	pH 4.3 0.066 vvm 0.27 g/l	manual A/F addition, pH not dropping, biomass too low
9	1.20	2.0	120.5	pH 5.3 0.066 vvm 0.535 g/l	manual A/F addition, fresh stock culture
10	3.46	1.0	113	pH 5.3 0.066 vvm 0.535 g/l	fungi contami-nation noticed at 113 hours
11	1.90	0.4	160	pH 4.3 0.066 vvm 0.535 g/l	manual A/F addition, pH not dropping
12	3.14	4.6	138	pH 5.3 0.333 vvm 0.535 g/l	automatic A/F addition
Shake Flask	4.06	22.2	112	0.535 g/l NH_4Cl	

[1] not tested due to poor growth

[2] A/F = antifoam

To investigate if the presence of iron ions from the steel in the fermenter, or the antifoam being added, were causes of the poor fermenter performance, these factors were tested in shake flask culture. Flasks were set up that contained steel ball-bearings to simulate the metallic steel in the fermenter, while in other flasks, drops of antifoam were added twice daily during sampling and pH control to mimic the antifoam addition in a batch fermenter. Table 4.3 shows the results. The growth of the cultures was identical under all conditions.

No significant differences in citric acid production were observed in the shake flasks. Although the type of steel tested was probably not quite the same, as ball bearings were used for the shake flask experiment, it is unlikely that the failure of the batch culture is due to either antifoam addition or the presence of steel in the fermenter.

4.5 Discussion

The aim of the first part of this work was to achieve some control over the substrate uptake rate, and thus to be able to test the hypothesis that the citrate production rate is related to the substrate uptake rate. Growth on the two sugars, glucose and fructose, individually, resulted in similar citrate production rates. When the two sugars were present in a mixture, the production rate showed no change. Hence, although the sugar analyses were not performed, this result shows that this mixture of carbon sources would not be useful in the verification of the above hypothesis. It is possible that the two sugars are taken up at the same transport site, and that they compete with each other.

Table 4.3 Citric acid production in shake-flask cultures investigating the effect of metallic steel and antifoam addition on *Yarrowia lipolytica* IMK2.

Growth Condition		Final Citric Acid Concentration (g/l)
Control	(I)	26.7
	(II)	25.5
Steel	(I)	25.8
	(II)	24.0
Antifoam	(I)	23.8
	(II)	25.7

When using a mixture of glucose and succinate as the carbon source, the citrate production rate exhibited a diauxic pattern. Thus, the initial rate was identical to that observed during production from glucose alone, but it subsequently decreased sharply to a lower value. Possibly, this shift coincided with exhaustion of glucose, implying that glucose and succinate are taken up consecutively rather than simultaneously, but no analyses were performed to verify this. Further, an experiment using succinate, alone, as the carbon source was not performed. Nevertheless, this result demonstrates that the concept of manipulating the substrate uptake rate by using different substrates or mixtures thereof, and observing the effect on the citrate production rate, remains valid. In the present thesis, however, this was not explored further because of the difficulty of culturing *Y. lipolytica* IMK2 in a fermenter.

The report of Cambourn (personal communication) that a strain of *C. guilliermondii* grown under extreme oxygen limitation showed an increased specific citrate production rate, was of interest because of its possible application to a two-stage fermentation process, i.e. initial yeast growth under strong aeration, followed by citrate accumulation under oxygen limitation. Thus, this report was investigated using *Y. lipolytica* IMK2.

Under conditions of low oxygen supply, the growth of the yeast was inhibited, showing that oxygen is required for biomass production. This limitation of growth, however, did not promote citric acid production. A possible reason for this is that citric acid production also requires oxygen, with 1.5 moles of oxygen required to produce 1 mole of citric acid from 1 mole of glucose (Eqn. 2.2, Section 2.5.1), and all the available oxygen was being used for biomass

production.

During studies in a laboratory-scale batch fermenter, the process did not produce citric acid at a rate or yield comparable to that of the shake-flask cultures. Problems with foaming and low biomass were mostly overcome by decreasing the aeration rate and the addition of antifoam. However, a significant improvement in fermentation performance was not achieved by increasing the aeration rate, lowering the pH, or increasing the nitrogen and glucose concentrations. Iron has been observed to reduce the production of citric acid by yeasts (Furukawa *et al*, 1977; Hattori *et al*, 1974b; Marchal *et al*, 1977a), but the presence of metallic steel, or the addition of antifoam, were indicated by shake flask cultures to be unlikely as causes for this scale-up failure. The reason for failure to scale-up successfully remains unknown, and further experimentation with this yeast was not undertaken.

4.6 Conclusions

The citric acid production rate can be varied by the use of glucose and succinate as carbon sources. Growth on glucose and fructose gave identical citrate production rates, probably because the two sugars use the same uptake site. Possibly, when present in a mixture, succinate was not used for production of citric acid until glucose was exhausted from the medium.

Cultivation of *Y. lipolytica* IMK2 under low oxygen conditions gives a much slower rate of growth and final biomass concentration, and does not promote a high specific rate of citric acid production.

It proved impossible to successfully scale-up cultivation of *Y. lipolytica* IMK2 from a shake flask culture to a 1.5 l batch fermentation. The reason for the failure remains unknown, but it is not due to contaminating iron or antifoam.

CHAPTER 5

BATCH CULTURE STUDIES USING *CANDIDA GUILLIERMONDII* IMK1

5.1 Introduction

Following the failure to successfully scale-up the cultivation of *Y. lipolytica* RB82 from a shake flask culture to a laboratory-scale batch fermenter, work commenced with the yeast *Candida guilliermondii* IMK1. As with the *Y. lipolytica* strain, previous experimental work had been carried out in the Process and Environmental Technology Department of Massey University with *C. guilliermondii* IMK1 in shake flask culture only. In this thesis it was desired to scale-up the fermentation to batch fermenter culture, and then to use chemostat culture to investigate the fermentation kinetics and the hypothesis that citric acid production occurs when the growth rate slows but the sugar uptake rate is maintained. The effect of the pH of the culture, and the aeration rate, were also investigated, as means of adjusting the sugar uptake rate and citric acid production rate. An investigation into the effect of mixed carbon sources on the substrate uptake rate, and hence on the citric acid production rate, was also investigated in shake flask culture.

5.2 Effect of Mixed Carbon Sources

Experiments were performed in shake flask culture with *C. guilliermondii* IMK1, to investigate whether, by using different mixtures of substrates, the

substrate uptake rate could be manipulated, and to observe the effect on the citric acid production rate. Work with the *Y. lipolytica* strain (Section 4.2) had suggested that the concept of manipulating the rates by the use of different carbon sources was valid, and it was desired to determine if a similar result would be observed for the *C. guilliermondii* strain.

The total substrate concentration was 2M in all experiments. Glucose was tested alone, while fructose and glycerol were tested in 1 : 1 mixtures with glucose. Figures 5.1 to 5.3 show the citric acid production and substrate utilization during growth of *C. guilliermondii* IMK1 on the different carbon sources. Figure 5.4 shows a comparison of the citric acid production for the carbon sources tested. The results for the different carbon sources are summarized in Table 5.1.

The specific citric acid production rates were basically identical in all cases. The specific glucose consumption rates were nearly identical for the pure glucose and glucose-glycerol mixture, but slightly higher for the glucose-fructose mixture. Some fructose was used simultaneously with the glucose, but only at 10% of the rate of the glucose consumption. Glycerol was used in only one of the duplicate flasks, and at a rate of only 3% of that of the glucose. Despite running the experiments for nearly 193 hours, glucose was not exhausted from the medium in any of the cultures.

The growth curves for the cultures are shown in Figure 5.5. The cultures containing glucose and fructose as the carbon sources were slower to commence growth than the others, and achieved a slightly lower final biomass than the cultures growing on glucose alone. The cultures growing on the glucose and

Figure 5.1 Citric acid production and substrate utilization of *C. guilliermondii* during growth on glucose (2M)

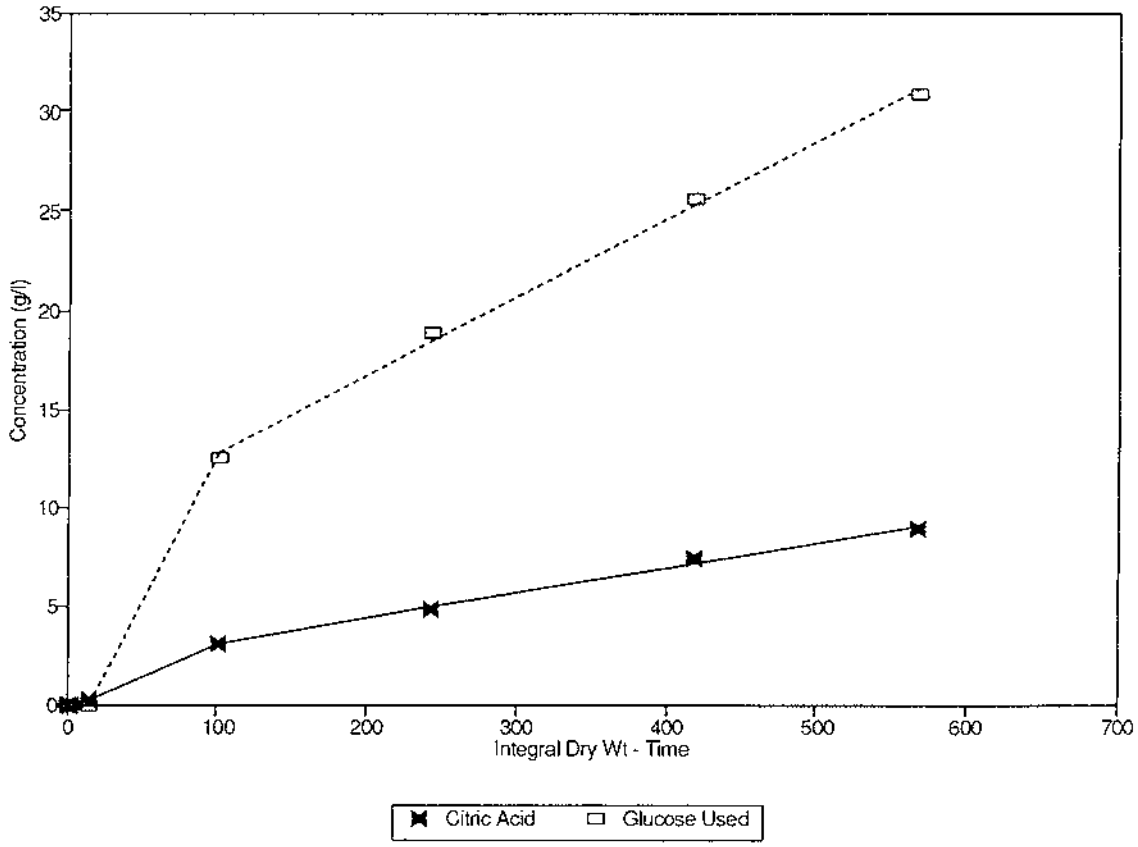


Figure 5.2 Citric acid production and substrate utilization of *C. guilliermondii* during growth on a 1 : 1 glucose : fructose mixture

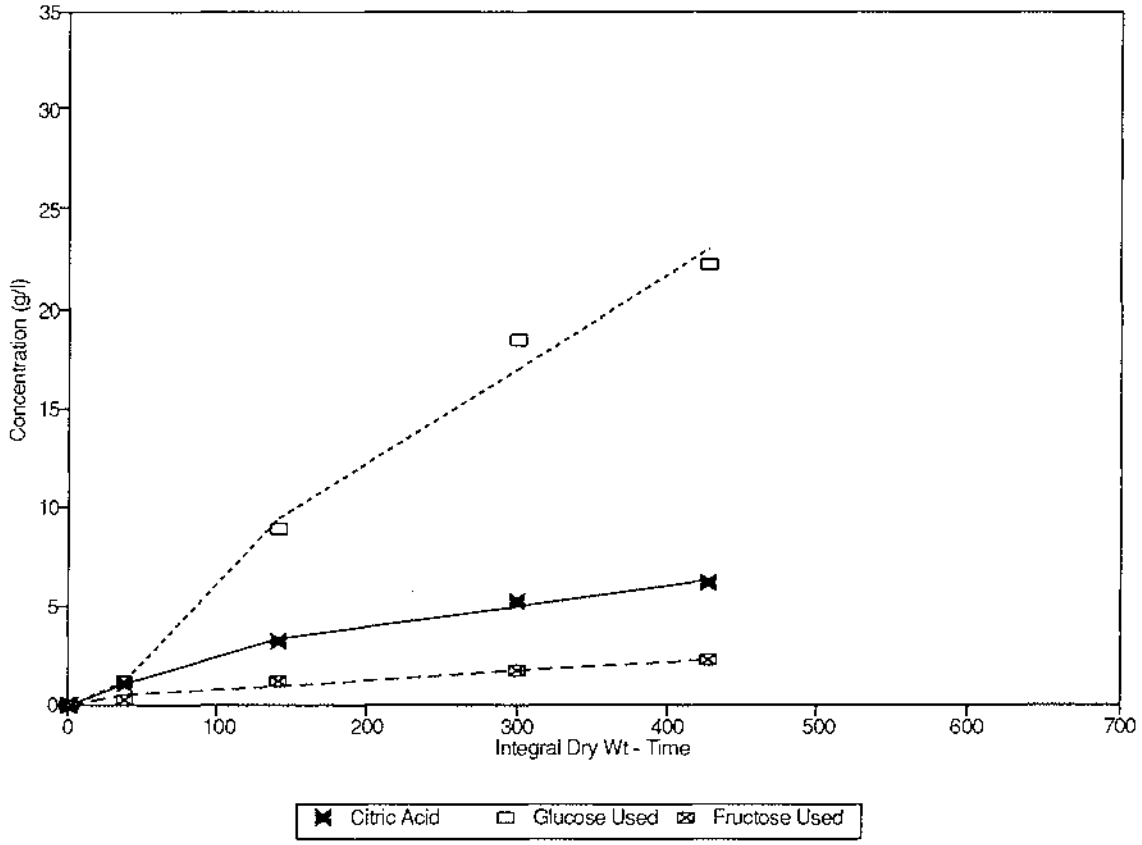


Figure 5.3 Citric acid production and substrate utilization of *C. guilliermondii* during growth on a 1 : 1 glucose : glycerol mixture

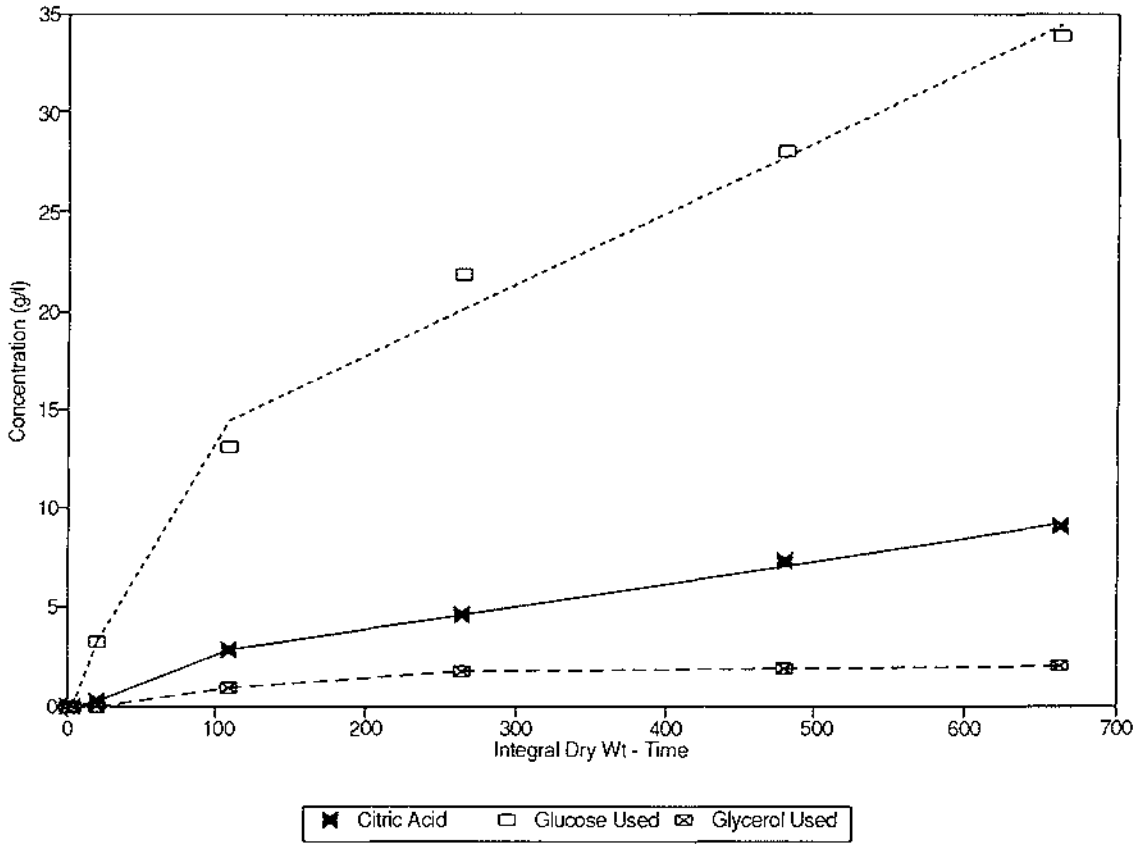


Figure 5.4 Comparison of citric acid production during growth of *C. guilliermondii* in glucose, 1 : 1 glucose : fructose and 1 : 1 glucose : glycerol

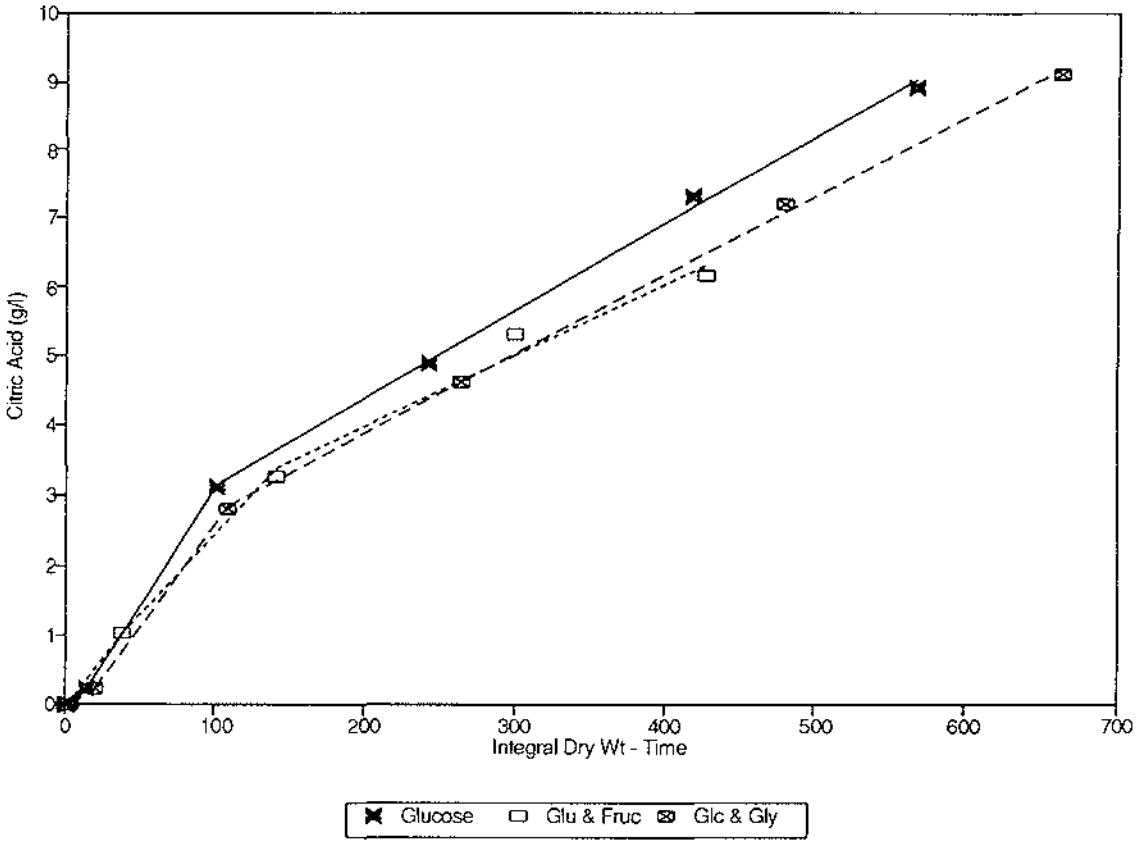


Figure 5.5 Growth curves of *C. guilliermondii* during growth on mixed carbon sources

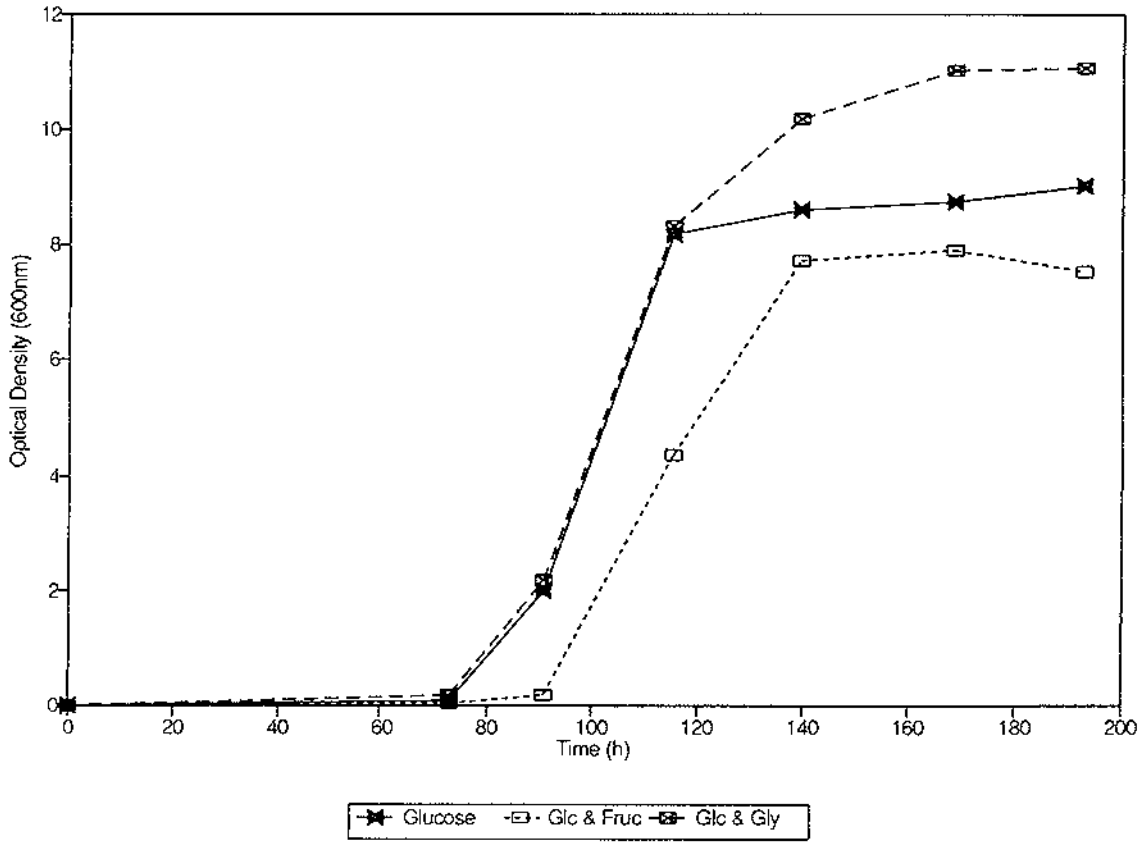


Table 5.1 Results of the mixed carbon source shake-flask experiments for *Candida guilliermondii* IMK1.

Carbon Source	Citric Acid Specific Production Rate (g/g.h)	Glucose Specific Consumption Rate (g/g.h)	Other Carbon Source Specific Consumption Rate (g/g.h)	Citric Acid Yield (g/g)
Glucose	0.013	0.039	-	0.29
Glucose and Fructose	0.010	0.047	0.005	0.25
Glucose and Glycerol	0.011	0.036	0.001[1]	0.24[2] 0.27[3]

[1] Glycerol used in only one of the duplicate flasks.

[2] Glycerol used converted to moles, then back to equivalent weight of glucose, for direct comparison with other results.

[3] Result for duplicate flask that did not use any glycerol.

glycerol mixture obtained a higher final biomass than the pure glucose flask. These differences are probably not significant.

5.3 Effect of pH

Experiments were performed in a laboratory-scale batch fermenter to investigate the effect of the pH of the culture on the citric acid production rate and glucose consumption rate. The ability to vary these rates by adjusting the pH would allow a method of investigating these parameters. The cultures were all grown initially at pH 5.3. In one experiment, the culture was controlled at this value throughout the subsequent production phase. To investigate the other pH values (pH 3.3 and pH 4.3), towards the end of the growth phase the pH was allowed to decrease naturally to the desired value, at which point it was controlled. This allowed the effect of the pH to be determined during the production phase only. Figure 5.6 shows that the growth curves were nearly identical for the experiments.

Figures 5.7 and 5.8 show the citric acid production and glucose consumption for the experiments, and the results are summarized in Table 5.2.

The highest yield and rate of citric acid production occurred at pH 4.3, with the rate being 37% higher than that at pH 5.3. Some difficulty occurred in the experiment where it was attempted to hold the culture at pH 3.3. After late exponential growth, the pH controller was adjusted to the pH 3.3 set-point, and the culture pH was allowed to fall naturally. However, the culture pH did not fall below pH 3.5. After reaching pH 3.5, citric acid production ceased, and the pH

Figure 5.6 Growth curves of *C. guilliermondii* during cultivation for the pH experiments

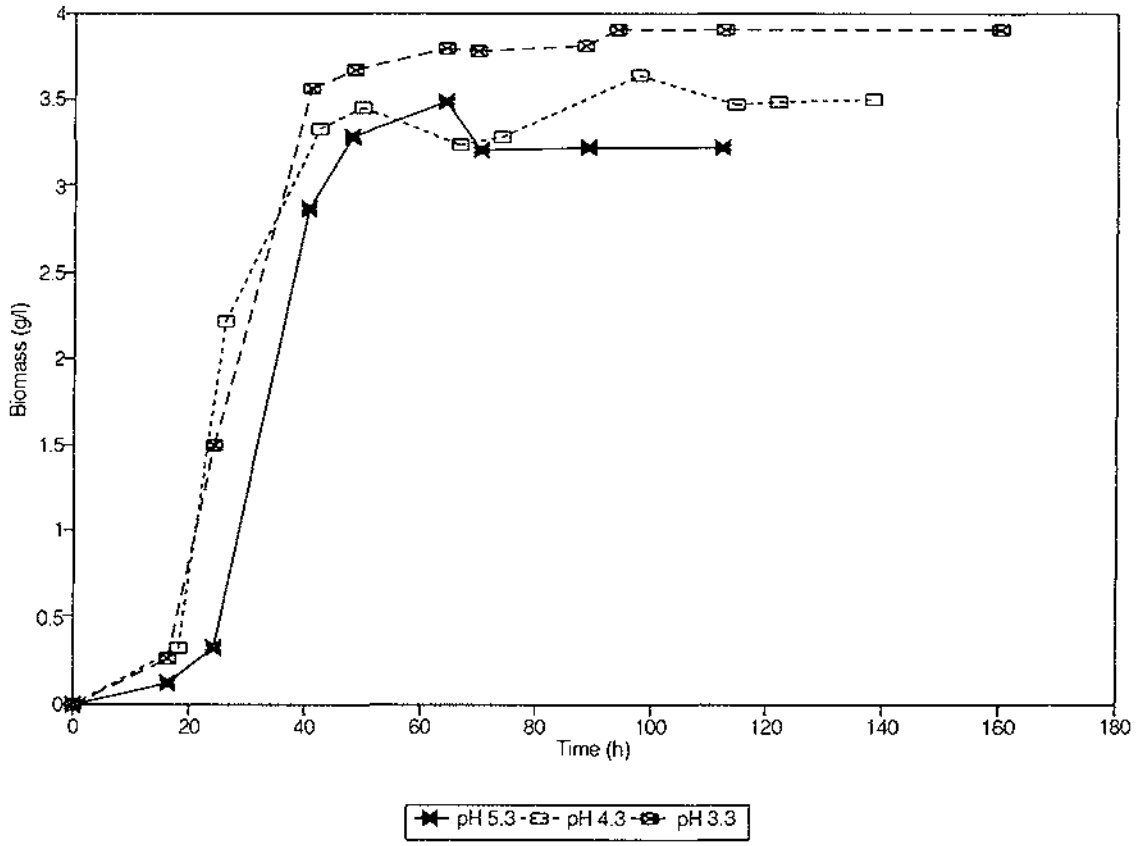


Figure 5.7 Citric acid production by *C. guilliermondii* as a function of the pH value of the culture

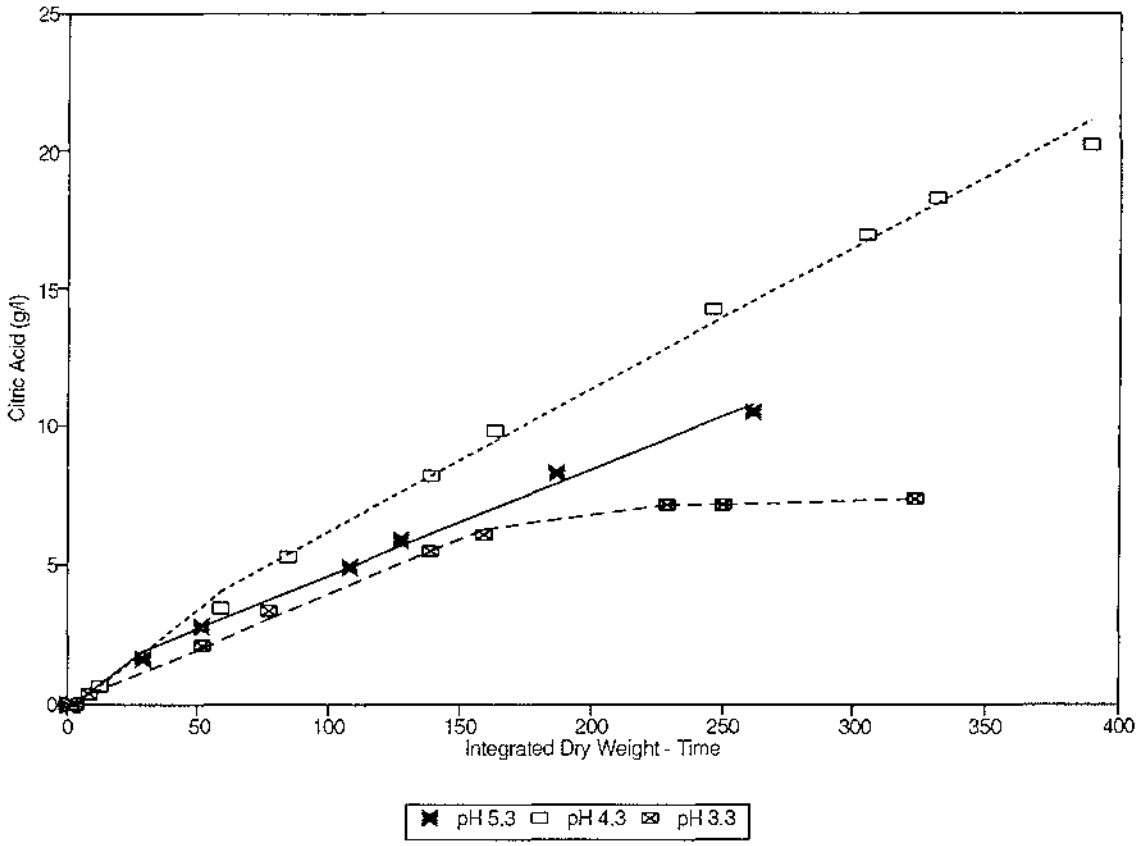


Figure 5.8 Glucose consumption by *C. guilliermondii* as a function of the pH value of the culture

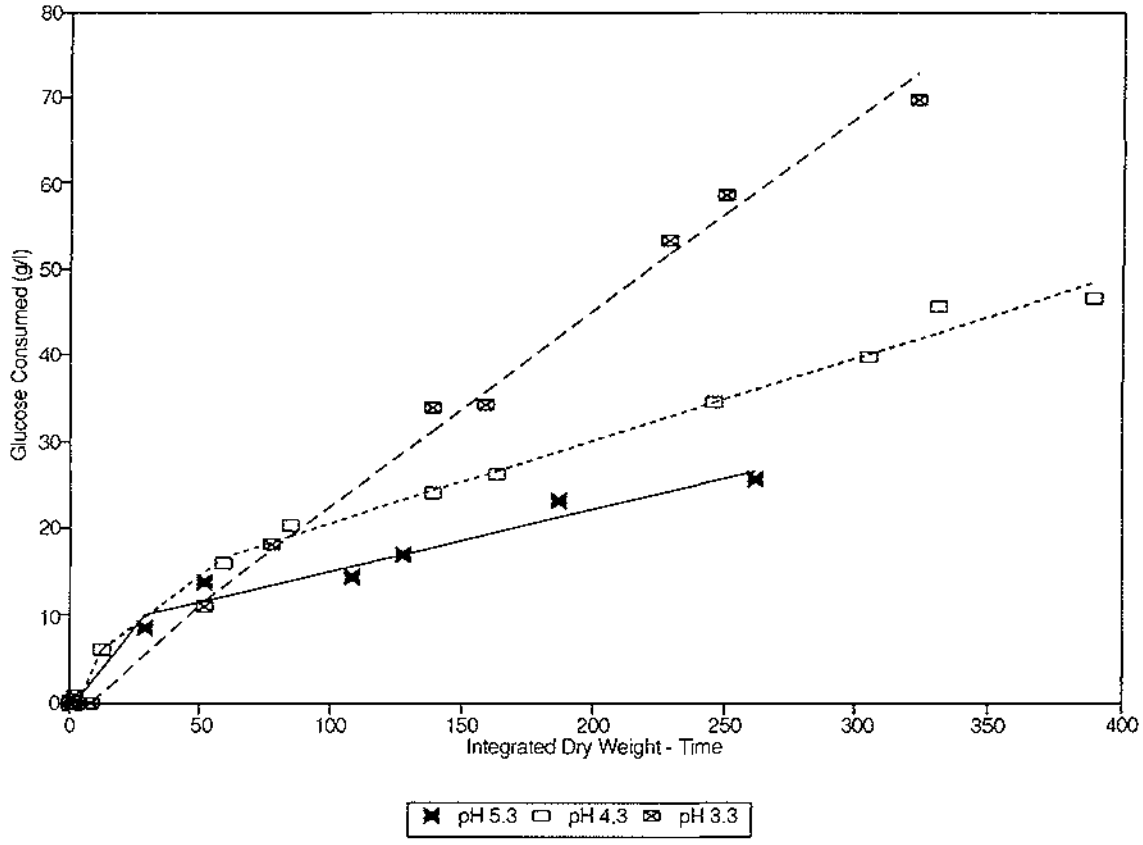


Table 5.2 Results of pH experiments.

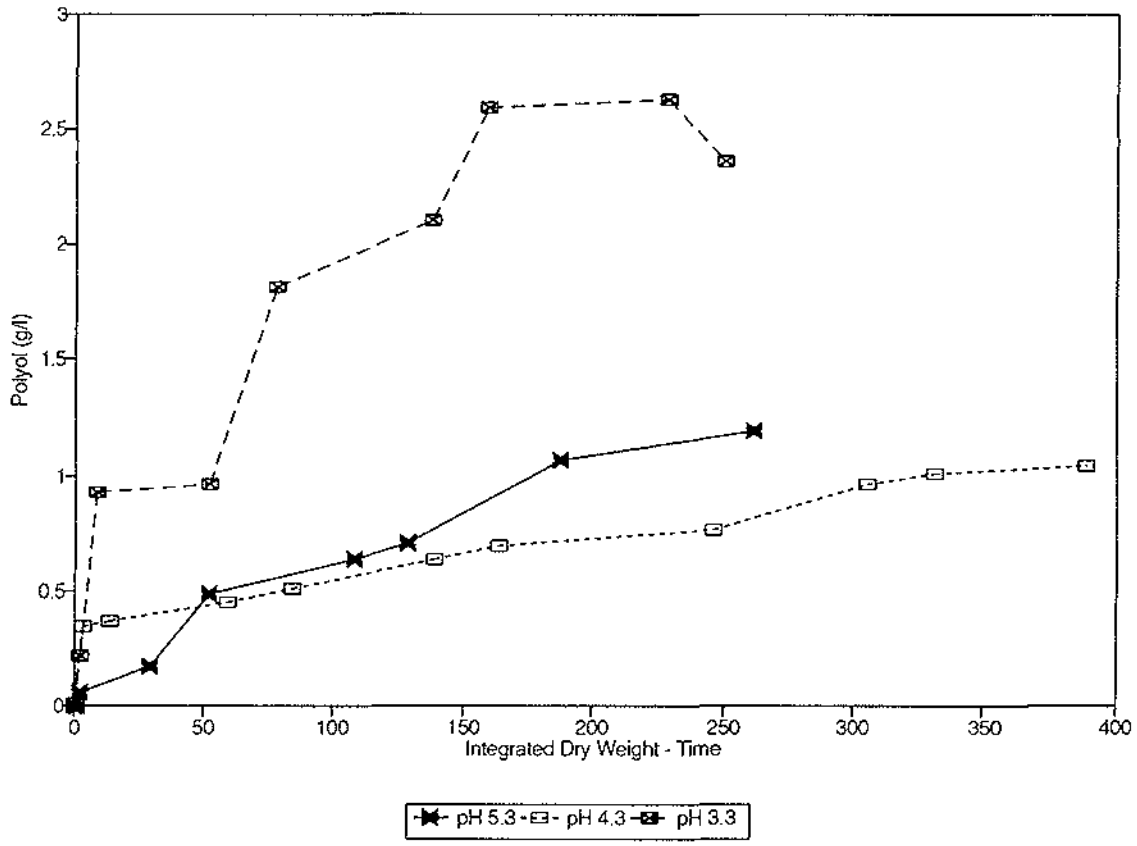
pH	Citric Acid Specific Production Rate (g/g.h)	Glucose Consumption Specific Rate (g/g.h)	Citric Acid Yield (g/g)
5.3	0.038	0.071	0.41
4.3	0.052	0.096	0.44
3.3	0.040 initial 0 final	0.226	0.10

would not drop further. Thus some citric acid was produced initially, at a rate similar to that observed in the experiment where the culture was controlled at pH 5.3. Despite the lack of citric acid production at pH 3.5, the specific rate of glucose consumption was very high. As the production of polyols has been reported in the literature at low pH values (Section 2.5.3), the cultures were tested for the production of the polyols glycerol, mannitol, erythritol and arabitol. Figure 5.9 shows the total polyol production results. Although the culture at pH 3.5 produced about twice as much polyols than the others, there was still only 2.6 g/l produced over the period of the culture.

5.4 Effect of Aeration

Reports in the literature (Section 2.5.2) indicate that an increase in the dissolved oxygen concentration in the culture generally results in an increase in the rate of citric acid production. Thus, altering the aeration rate may allow a means of varying the citric acid production rate in otherwise identical batch or chemostat conditions. The dissolved oxygen tension can be increased in a small jar fermenter by either increasing the aeration rate or the agitation rate. The agitation rate can have a significant affect on the DO concentration, as an increase in agitation causes a decrease in gas bubble size, and thus a greater surface area from the same volume of gas. As diffusion of oxygen from the gas phase into the culture medium occurs only at the surface of the bubbles, an increase in the surface area causes an increase in the overall rate of oxygen transfer, and thus an increase in the DO concentration. Increasing the aeration

Figure 5.9 Polyol production by *C. guilliermondii* during cultivation at different pH values



rate causes an increase in the number of gas bubbles, so for a given agitation rate, an increase in aeration also causes an increase in the surface area of the gas-liquid interface, and hence an increase in the dissolved oxygen tension. Due to technical problems with the equipment, the actual dissolved oxygen tension could not be measured during these experiments.

All the cultures were grown at pH 5.3 until the late exponential phase, and then the pH was allowed to fall naturally to pH 4.3, at which point it was controlled. Figures 5.10 to 5.16 show the citric acid production and glucose consumption for the various aeration conditions investigated, and for shake flask cultures as a comparison. Figure 5.17 displays the effect of the aeration rate on the specific rates of citric acid production and glucose utilization, while Figure 5.18 shows the growth curves for the experiments. The results are summarized in Table 5.3, and show that the aeration rate had little effect on the citric acid production rate.

As shown in Figure 5.18, and observed from the maximum growth rates given in Table 5.3, the experiments conducted at an aeration rate of 0.133 vvm, and both 500 and 800 rpm agitation speeds, did not display a typical growth pattern, with slower growth occurring. The reason for this atypical growth is unknown, but the difference in the growth may be partially responsible for the low yields. The other experiments all displayed similar growth patterns.

Figure 5.10 Citric acid production and glucose consumption by *C. guilliermondii* when grown at an aeration rate of 0.333 vvm, and an agitation rate of 500 rpm

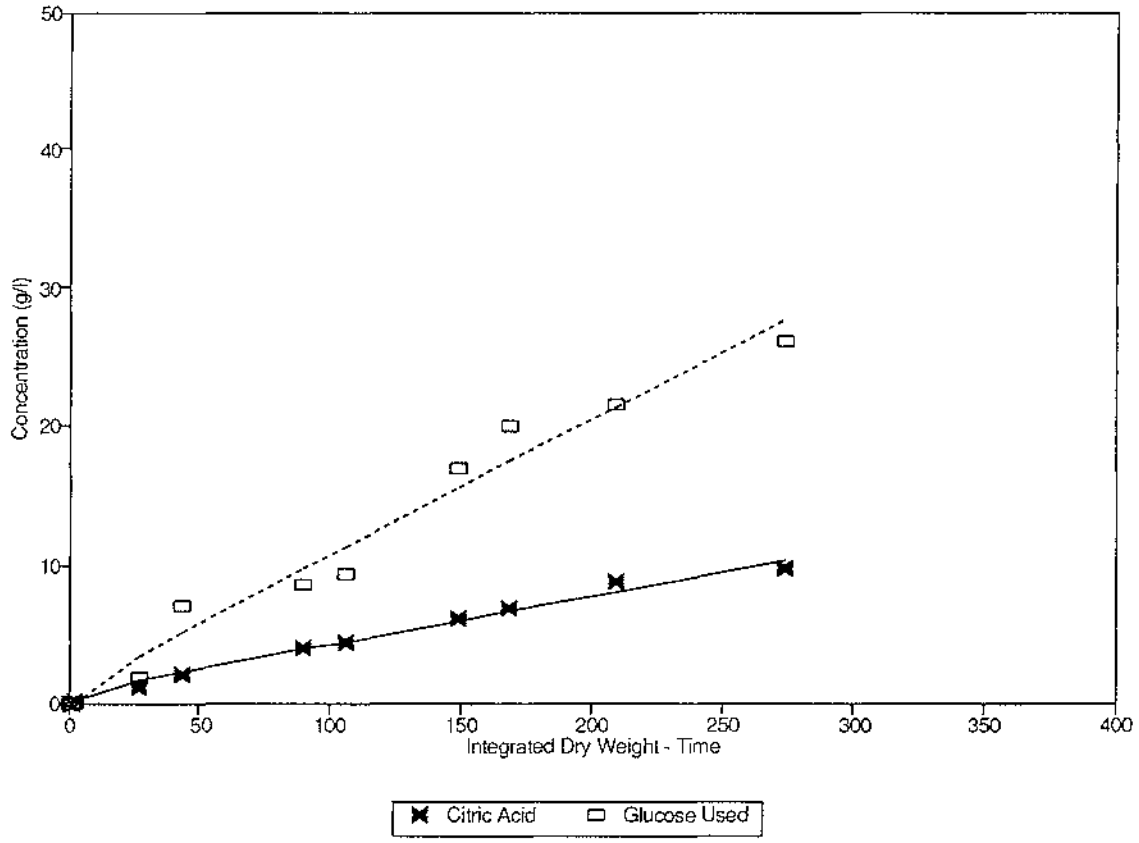


Figure 5.11 Citric acid production and glucose consumption by *C. guilliermondii* when grown at an aeration rate of 0.200 vvm, and an agitation rate of 500 rpm

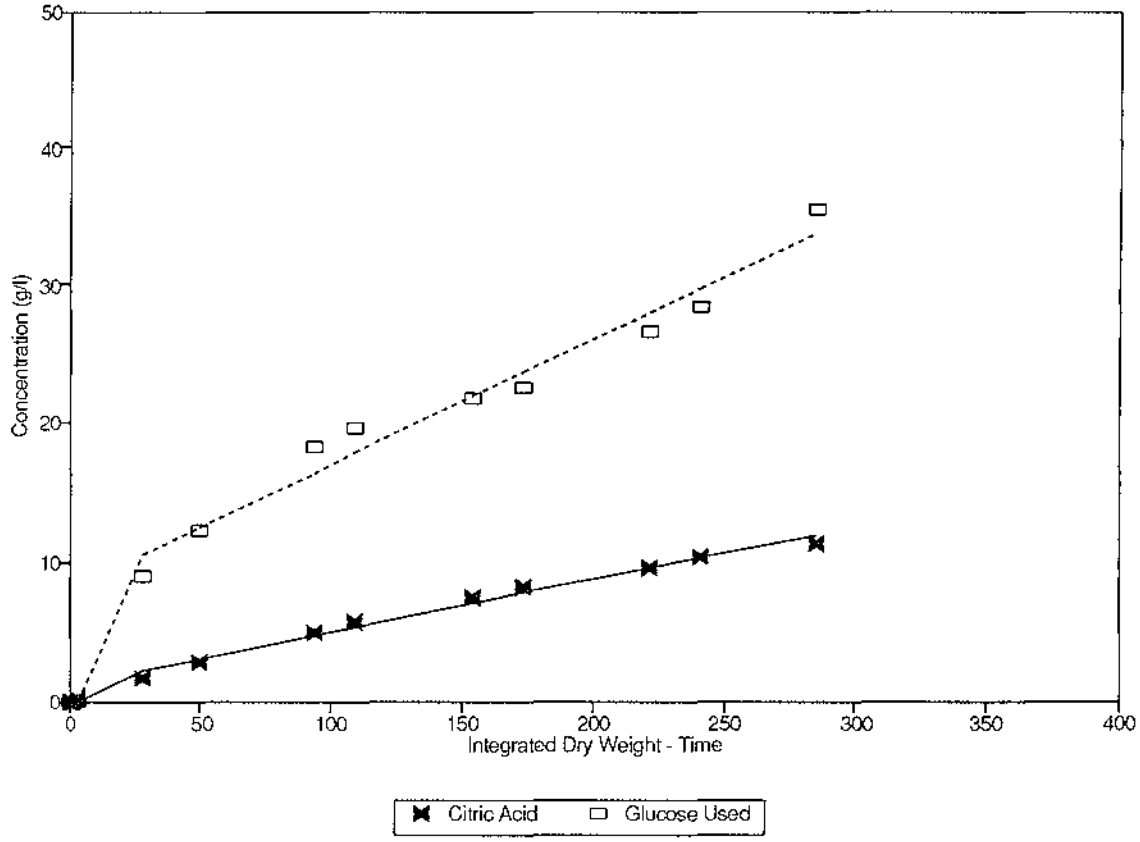


Figure 5.12 Citric acid production and glucose consumption by *C. guilliermondii* when grown at an aeration rate of 0.133 vvm, and an agitation rate of 500 rpm

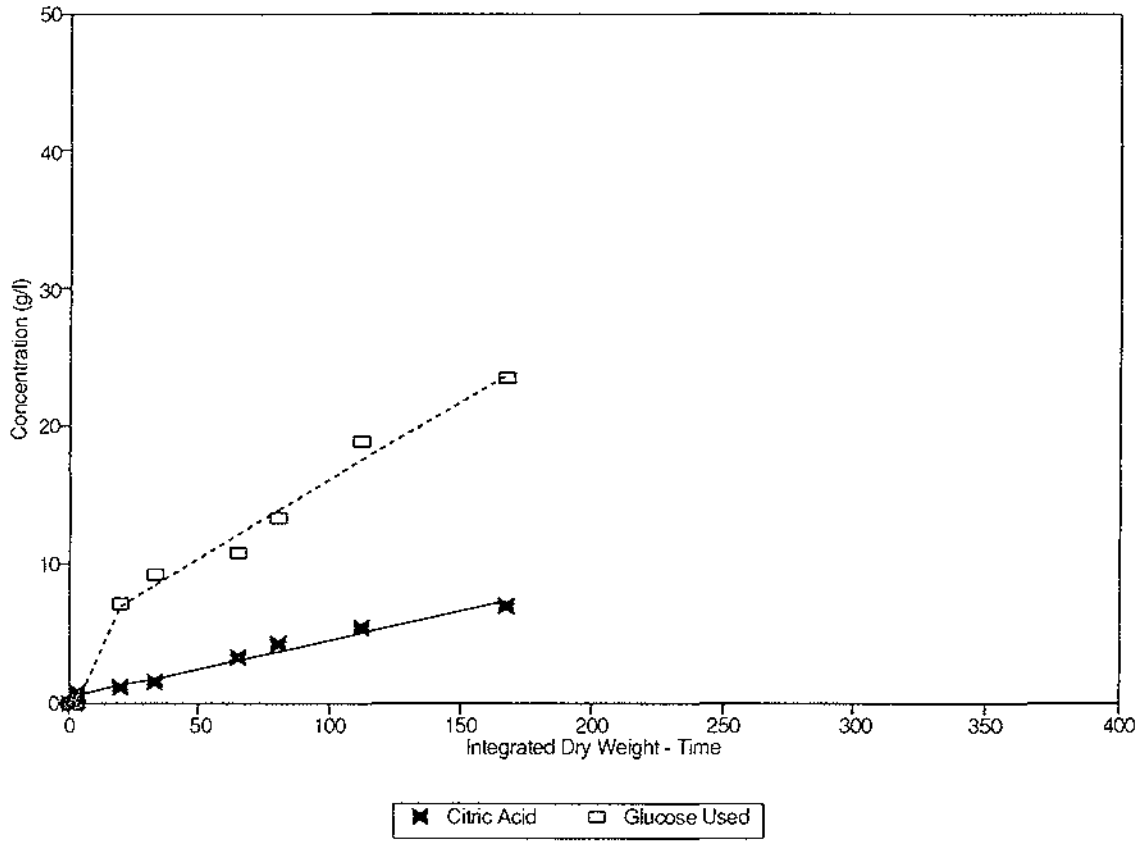


Figure 5.13 Citric acid production and glucose consumption by *C. guilliermondii* when grown at an aeration rate of 0.067 vvm, and an agitation rate of 500 rpm

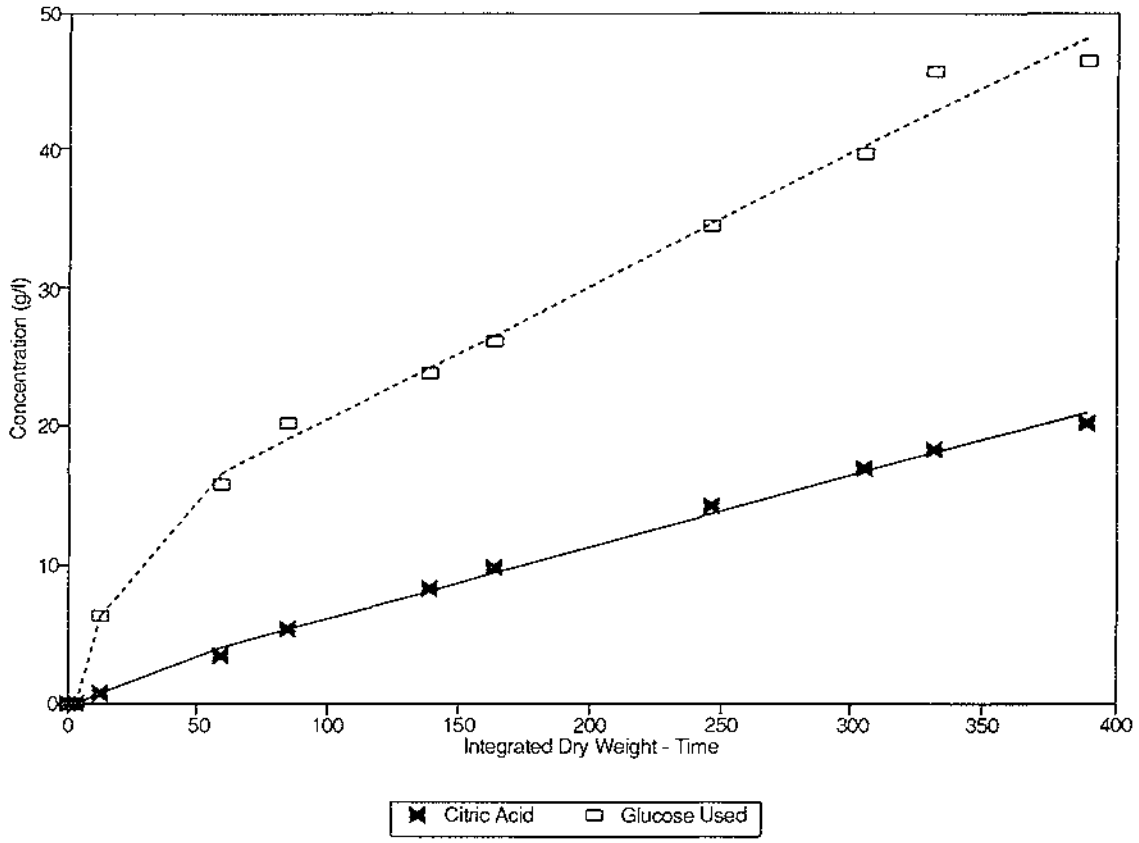


Figure 5.14 Citric acid production and glucose consumption by *C. guilliermondii* when grown at an aeration rate of 0.040 vvm, and an agitation rate of 500 rpm

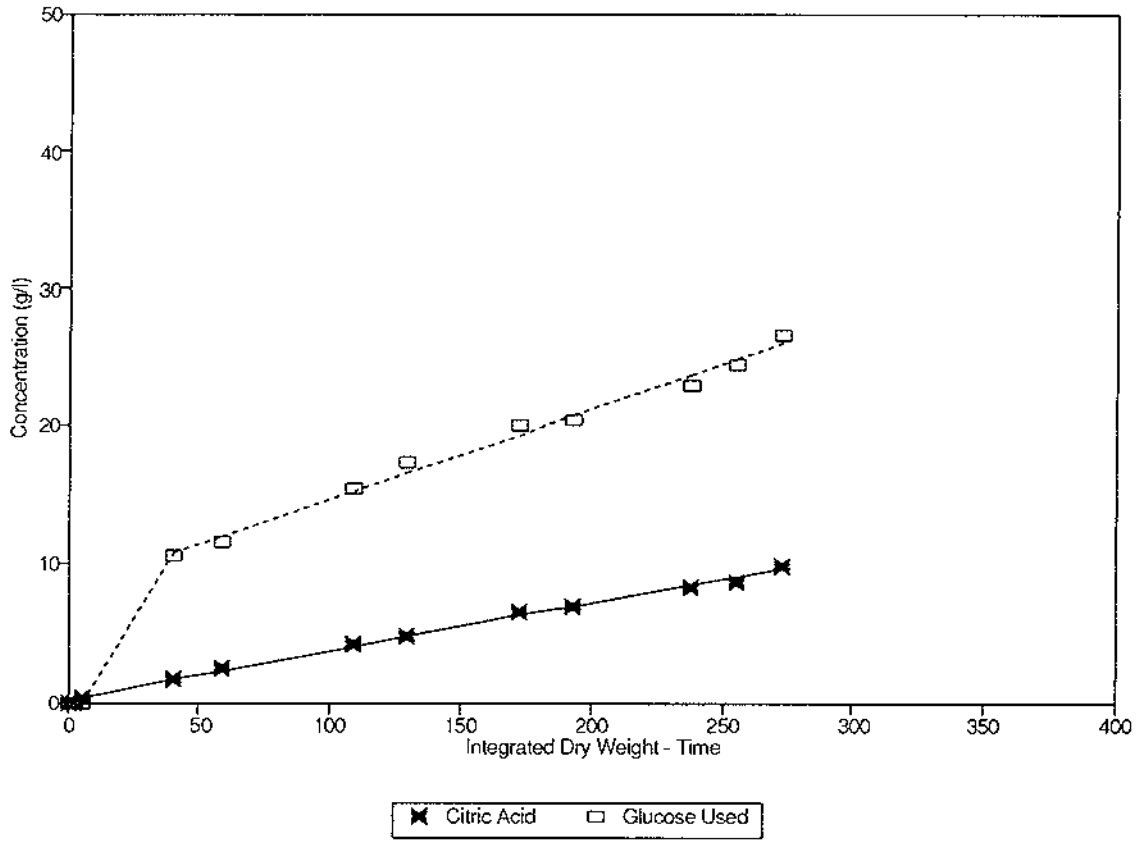


Figure 5.15 Citric acid production and glucose consumption by *C. guilliermondii* when grown at an aeration rate of 0.133 vvm, and an agitation rate of 800 rpm

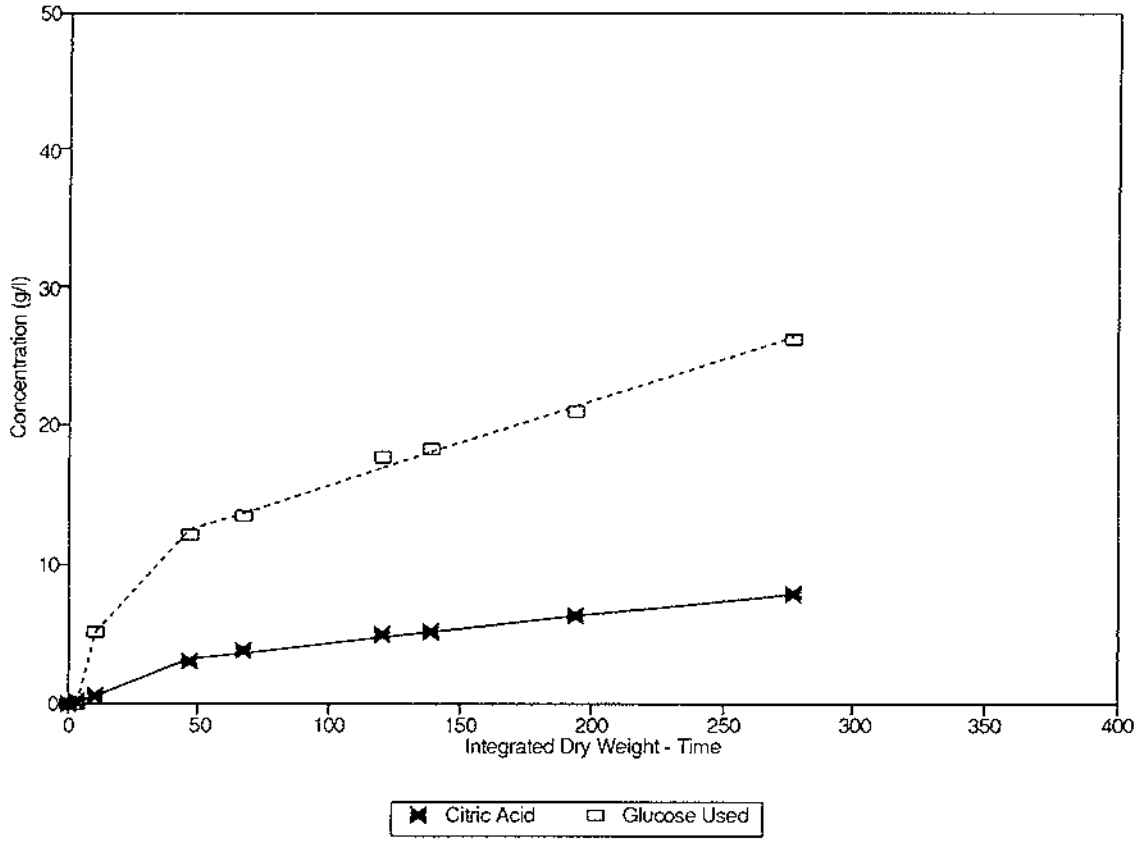


Figure 5.16 Citric acid production and glucose consumption by *C. guilliermondii* when grown in shake flask culture

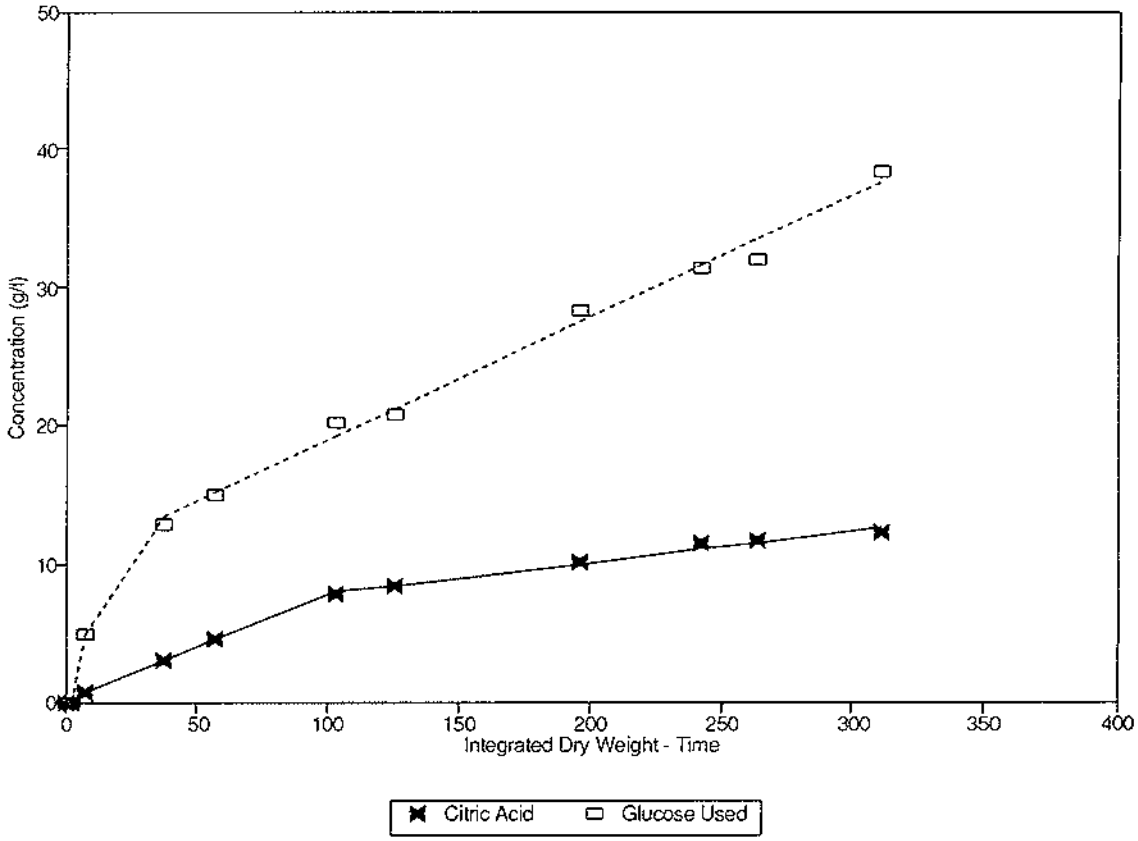


Figure 5.17 The effect of aeration on citric acid production and glucose consumption rates during growth of *C. guilliermondii* at pH 4.3, and an agitation rate of 500 rpm

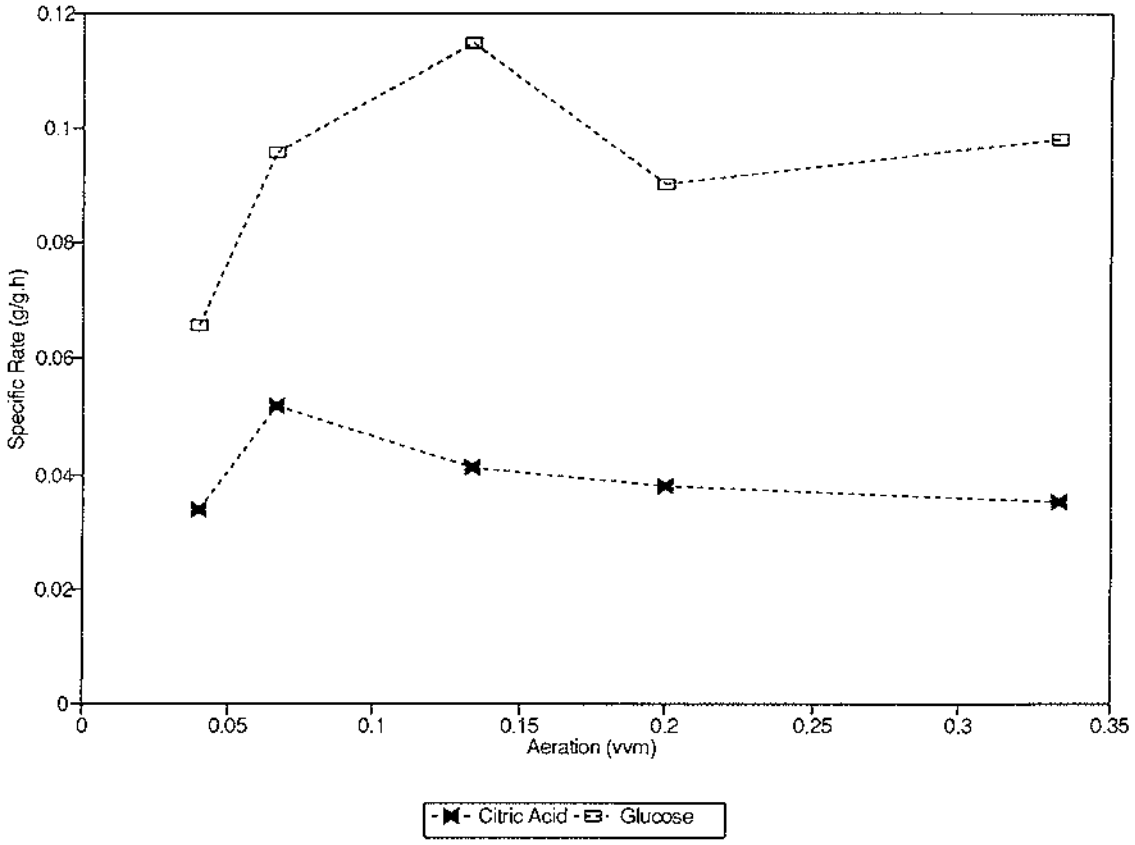


Figure 5.18 Growth curves of *C. guilliermondii* during cultivation at different aeration rates

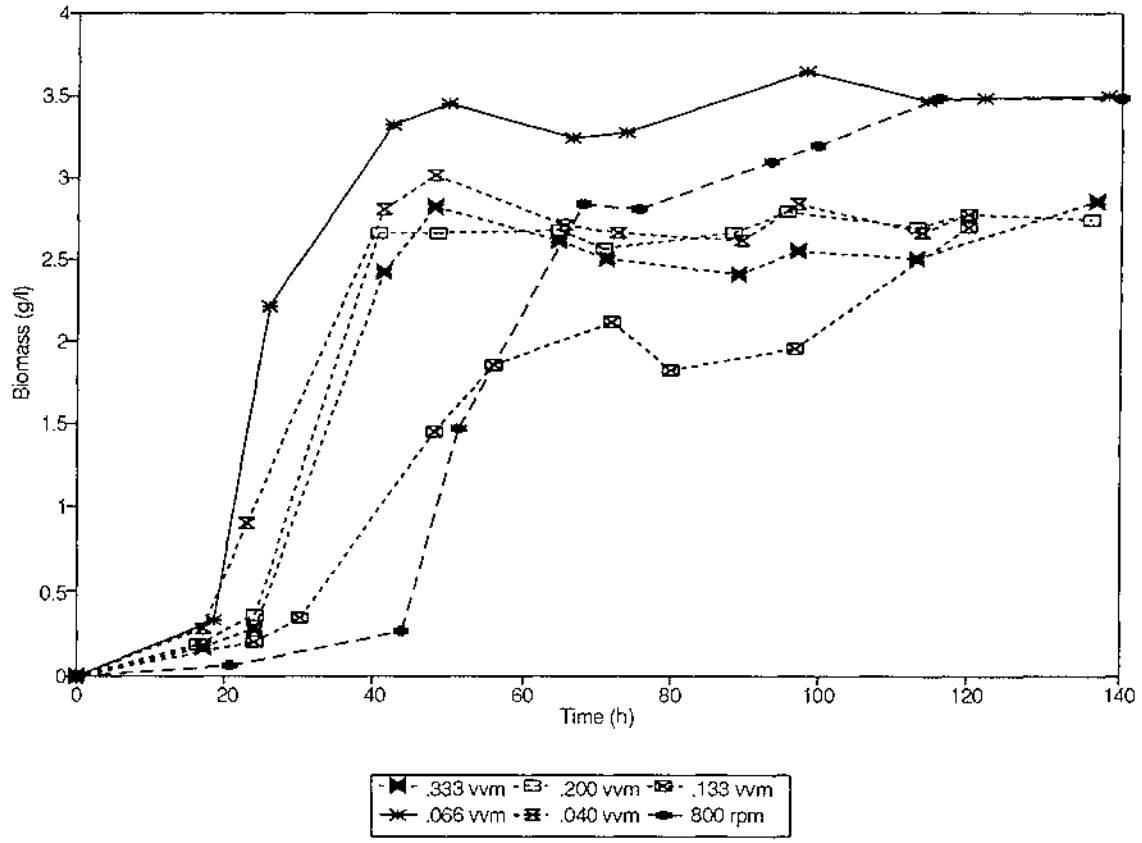


Table 5.3 Results of aeration experiments.

Aeration / Agitation Conditions	Maximum Growth Rate (h^{-1})	Citric Acid Specific Production Rate (g/g.h)	Glucose Specific Consumption Rate (g/g.h)	Citric Acid Yield (g/g)
0.040 vvm 500 rpm	0.13	0.034	0.066	0.37
0.067 vvm 500 rpm	0.12	0.052	0.096	0.44
0.133 vvm 500 rpm	0.09	0.041	0.115	0.31
0.133 vvm 800 rpm	0.07	0.020	0.060	0.30
0.200 vvm 500 rpm	0.12	0.038	0.090	0.36
0.333 vvm 500 rpm	0.12	0.035	0.098	0.37
Shake Flask	0.12	0.022	0.088	0.34

5.5 Discussion

The aim of the use of mixed carbon sources was to achieve some control over the substrate uptake rate, and thus be able to test the hypothesis that the citrate production rate is related to substrate uptake rate. However, similar citrate production rates were achieved with the pure glucose, and the glucose-fructose and glucose-glycerol mixtures. In all experiments the glucose was taken up preferentially, with fructose being simultaneously taken up, but at only 10% of the rate of glucose, and the glycerol being simultaneously taken up in only one of the duplicates, and at only 3% of the rate of the glucose. It is possible that the glucose and fructose were being taken up at the same transport site, and thus competing with each other, but that the glucose was preferred. The glycerol uptake, however, was probably subject to catabolite repression, and was not taken up in any significant quantity in the presence of glucose. The citric acid yields, specific glucose consumption rates and specific citric acid production rates in this experiment were lower than those observed in the batch fermenter experiments, and in another shake flask culture growing on glucose alone (Section 5.4). These experiments were performed last, so deterioration of the stock culture may have occurred. This could be combated by plating out and reisolating the organism to prepare a fresh stock culture. Fluctuations in the culture pH, due to insufficient adjustments during the cultivation period, may also have been a factor. As the glucose consumption rate was lower than expected, glucose was never exhausted from the medium during the culture time period investigated. For these reasons, the production rates during growth on glycerol

or fructose alone were not measured.

No variation of the citric acid production rate was obtained by the use of mixtures of glucose with fructose or glycerol. However, it is possible that the use of either fructose or glycerol as the carbon source in the absence of glucose may give some variation over the substrate uptake rate and the citric acid production rate. In this thesis, however, time restrictions resulted in no further investigation of the effect of mixed carbon sources.

From the batch fermentation experiments, the culture pH was demonstrated to have a major effect on the glucose consumption rate. As the pH decreased, the specific rate of glucose utilization increased, with a very high consumption rate at pH 3.5, despite the fact no citric acid was produced, nor any quantities of polyol significant enough to account for the 77 g/l of glucose that was used. The final biomass concentration of the pH 3.3 culture was only slightly higher than that of the other cultures, ruling out the production of large quantities of storage compounds as the reason for the high glucose utilization. Although it is possible that another undetermined product was produced from the glucose and excreted into the culture medium, the most likely end-product of the glucose was carbon dioxide, indicating a high energy requirement by the yeast when cultured at the low pH value. This energy may have been required for active transportation of hydrogen ions out of the cell to maintain a constant intracellular pH value.

The citric acid production rate, was also affected by the culture pH, with the maximum value occurring at pH 4.3. Thus, some control over the citric acid production rate and glucose utilization rate can be achieved after the bulk of the

growth has occurred, when the growth rate approaches zero due to exhaustion of the limiting nutrient, by altering the pH of the fermentation. However, if the pH falls to 3.5, citric acid production ceases. Culture pH has an effect on the rate of production as the extracellular pH influences the intracellular pH, and thus the activity of intracellular enzymes. Enzyme operation can be stimulated or inhibited by the intracellular pH, and it appears that for the yeast *C. guilliermondii* IMK1, the enzymes responsible for the production of citric acid operate best at an intracellular pH correlating to an extracellular pH of around 4.3.

A report in the literature for a strain of *S. lipolytica* indicates a similar effect, with the citric acid production rate increasing by 340% from pH 3.0 to pH 4.5, with a slight further increase between pH 4.5 and pH 5.0, and a slight decrease in the citric acid production rate between pH 5.0 and pH 6.0. In this report, the cultures were all first grown at the same pH value, and then adjusted after the growth phase to the pH under investigation, thus showing the effect of the pH on the specific rate of citric acid production in the production phase, after the biomass growth has finished (Marchal *et al*, 1977).

Aeration was found to have very little effect on the citric acid production or glucose utilization. A nearly 10-fold increase in the aeration rate from 0.040 to 0.333 vvm resulted in virtually no change in the specific rate of citric acid production. Increasing the agitation rate also did not appear to improve the citric acid production, although the atypical growth pattern may have affected these results. Thus, aeration was found to be of little use as a method of controlling the rates in the fermentation, at least in the range tested in these experiments. Under lower aeration conditions, an effect may have been observed. This was

unusual, as most of the reports in the literature indicated that an increase in the aeration rate resulted in an increase in the production of citric acid. However, one strain of *S. lipolytica* cultured on *n*-paraffins has been reported, by Marchal *et al* (1977), to show no significant change in the citric acid specific production rate at aeration rates above 0.2 vvm, even when air enriched with 42% O_2 was used.

It was desired in this thesis to observe the relationship between the growth rate, substrate uptake rate and the citric acid production rate. During the production phase of a batch culture, when the growth phase has finished, it may be possible to represent this relationship by equation 5.1, where the specific production rate of citric acid is directly proportional to the substrate uptake rate.

$$q_{\text{citric acid production}} = k.q_{\text{substrate utilization}} \qquad \text{[Eqn 5.1]}$$

Table 5.4 summarizes the values for the constant k calculated from equation 5.1 for the batch and shake flask cultures performed.

Thus, it can be seen that while there is some variation, overall the constant values are similar for similar culturing techniques, with a range from 0.20 to 0.32 in shake flask cultures, and a range from 0.35 to 0.54 for batch fermenter cultures, if the pH 3.3 experiment, where the citric acid production ceased, is ignored. The reason for the difference between the shake flask and batch fermenter culture results may be due to the improved control of conditions, such as pH, in the fermenter culture, resulting in better performance. Ignoring the fermenter cultures that had atypical growth patterns, the results display even

Table 5.4 Relationship between specific citric acid production and substrate utilization rates for all *C. guilliermondii* IMK1 experiments

Experimental Conditions	k
Mixed carbon sources experiment	
Glucose only	0.32
Glucose and Fructose	0.20
Glucose and Glycerol	0.32
pH experiment	
pH 5.3	0.54
[1] pH 4.3	0.54
pH 3.3	0.18
Aeration experiment	
0.040 vvm, 500 rpm	0.52
[1] 0.067 vvm, 500 rpm	0.54
[2] 0.133 vvm, 500 rpm	0.36
[2] 0.133 vvm, 800 rpm	0.34
0.200 vvm, 500 rpm	0.42
0.333 vvm, 500 rpm	0.35
Shake Flasks	0.25

[1] Data from the same culture used in both experiments.

[2] Atypical growth patterns.

more consistency for the relationship between substrate uptake rate and citric acid production rate, with three cultures giving a constant k value of about 0.54, one culture giving a slightly lower value of 0.42 and only one culture giving a lower value of 0.35. Therefore it can be concluded that there is a relationship between the citric acid production rate and the substrate uptake rate, as described by equation 5.1, and in batch culture, the value of the constant is typically 0.54. Reports in the literature for yeast strains growing on glucose give similar k values, of 0.64 and 0.57 for strains of *S. lipolytica* (Briffaud and Engasser, 1979a; Enzminger and Asenjo, 1986).

5.6 Conclusions

No control over the citric acid production rate was obtained by the use of mixtures of glucose with fructose or glycerol. In all the experiments the glucose was taken up preferentially, with fructose being simultaneously taken up, but at only 10% of the rate of glucose, and the glycerol being simultaneously taken up in only one of the duplicates, and at only 3% of the rate of the glucose.

The culture pH was demonstrated to have a major effect on the production phase, after the growth rate approximated zero. As the pH decreased, the specific rate of glucose utilization increased. The citric acid production rate also was affected by the pH, with the maximum production occurring at pH 4.3. Some control over the citric acid production rate and glucose utilization rate can be achieved in the production phase after growth has ceased, by altering the pH of the fermentation, but if the pH falls to 3.5, citric acid production ceases.

Aeration was found to have very little effect on the citric acid production or glucose utilization. A nearly 10-fold increase in the aeration rate from 0.040 to 0.333 vvm resulted in virtually no change in the specific rate of citric acid production. Thus, aeration was found to be of little use as a method of controlling the rates in the fermentation, at least in the range tested in this work.

There is a relationship between the citric acid production rate and the substrate uptake rate, during the production phase, and in batch fermenter culture, in the range of pH 4.3 to pH 5.3, the value of the constant is typically 0.54.

CHAPTER 6

CHEMOSTAT STUDIES USING *CANDIDA GUILLIERMONDII* IMK1

6.1 Introduction

Chemostat cultures maintain the organism in a constant metabolic state. The growth can be controlled at a constant rate by the dilution rate of the culture, thus enabling investigation into the hypothesis that citric acid production occurs when the growth rate slows but the substrate uptake rate is maintained. Experiments were performed in a nitrogen limited chemostat culture to investigate the effect of the specific growth rate on citric acid production by *Candida guilliermondii* IMK1. As the culture pH of was shown to have an effect on the specific citric acid production and glucose consumption rates after growth had finished, it was decided to also investigate the effect of pH on a growing culture in the chemostat.

6.2 Results of Chemostat Cultures

As the maximum specific growth rate observed in batch culture was 0.12 h^{-1}, the dilution rate had to remain below this to prevent culture wash-out. It was decided that the maximum dilution rate for the chemostat experiments would be two-thirds of the maximum growth rate, i.e. 0.08 h^{-1}. The minimum dilution rate chosen was 0.02 h^{-1}. Due to the failure of the batch culture to produce citric acid below pH 3.5, the minimum pH value was chosen to be above this, at pH 3.8.

pH 5.2 was chosen for the maximum pH value. Fermentations were conducted in pairs, with the first steady state being achieved from a fresh batch culture, and then the conditions were adjusted to allow the second steady state to be achieved. Steady state was assumed if, after three residence times from the change from batch to chemostat operation, or from the adjustment of the culture conditions, samples taken at least half a residence time apart showed no significant variation. It was of interest to determine whether the increased length of the culture time and change of conditions would affect the second steady state. A factorial experiment was set up to investigate these factors, and the experimental design is shown in Table 6.1, along with the coded variables allocated to assist with the statistical analysis of the data. Centre-points were added at pH 4.5 and dilution rate 0.05 h^{-1} to allow replicates for the data analysis. A randomly selected order was used to perform the experiments. The culture conditions were as described in Section 3.6.

A problem occurred in the low (0.02 h^{-1}) dilution rate experiments, when all of the glucose provided (10 g/l) was used by the culture, resulting in glucose limitation in experiments 6 and 9. Comparison of the duplicate centre-points and high (0.08 h^{-1}) dilution rate experiments showed that there was no significant effect on the specific citric acid production rate or the glucose consumption rate of the run order, i.e. first or second steady state position. An effect was noticed on the final biomass concentration, but from the duplicate centre-point results only. As only four data points could be used in each set to investigate the effect of the steady state order, there are very low degrees of freedom in this analysis, and thus a relatively high error influence. This could be the cause of the

Table 6.1 Experimental design of chemostat experiments, and allocation of coded variables to pH, specific growth rate (μ) and steady state order.

Run No.	pH uncoded	μ uncoded	Order uncoded	pH coded	μ coded	Order coded
11	3.8	0.02	First	-1	-1	-1
9	5.2	0.02	First	1	-1	-1
1	3.8	0.08	First	-1	1	-1
7	5.2	0.08	First	1	1	-1
6	3.8	0.02	Second	-1	-1	1
12	5.2	0.02	Second	1	-1	1
4	3.8	0.08	Second	-1	1	1
2	5.2	0.08	Second	1	1	1
5	4.5	0.05	First	0	0	-1
3	4.5	0.05	First	0	0	-1
8	4.5	0.05	Second	0	0	1
10	4.5	0.05	Second	0	0	1

observed contradiction between the results for the replicate centre-points and the replicate high dilution rates. Appendix I shows the analyses for the effect of steady state order on the culture. These results indicate that there would be very little error by ignoring the steady state order during analysis of the effect of pH and dilution rate on the specific rates in chemostat culture. Due to time limitations, and the fact that the order of the steady state was not considered to be significant, the glucose-limited experiments were not repeated, and the glucose concentration in the feed medium was increased to 20 g/l for the other two low dilution rate experiments to prevent a repeat of this glucose limitation. Table 6.2 gives the steady state concentrations, and Table 6.3 the calculated rate data for the chemostat experiments.

Chemostat culture theory predicts that the biomass concentration is dependent only on the growth-limiting nutrient concentration, in this case nitrogen. However, the biomass concentration, based on dry weight, was observed to vary at the different steady states. This is almost certainly due to the accumulation of storage compounds by the yeast. Therefore, some of the specific rate data were calculated based on the yeast nitrogen content, instead of on biomass.

Multivariate linear regression analysis, using the Minitab package (Copyright Pennsylvania State University), was used to develop regression equations for the experimental data. This gives an empirical model, in which the terms and coefficients are related to , but do not necessarily determine the value of, the experimental values. The general model used had the form:

$$\hat{Y} = B_0 + B_1A + B_2B + B_3A.B + B_4A^2$$

Table 6.2 Steady state concentrations during nitrogen-limited chemostat cultures.

Run No.	Citric Acid Produced (g/l)	Glucose Used (g/l)	Biomass (g/l)	% Nitrogen in Biomass	Citric Acid Yield (g/g)
11	8.8	16.0	2.28	3.1	0.55
9	3.9	9.9[1]	2.07	-	-
1	1.0	3.0	1.13	6.2	0.33
7	1.3	4.5	1.51	4.6	0.30
6	4.1	10.4[1]	2.12	-	-
12	6.3	13.9	2.17	3.2	0.45
4	1.3	4.1	1.22	5.7	0.30
2	1.5	4.9	1.46	4.8	0.31
5	1.6	5.2	1.54	4.5	0.31
3	1.8	5.5	1.47	4.8	0.33
8	2.2	6.1	1.80	3.9	0.36
10	2.7	7.1	1.73	4.0	0.38

[1] Glucose limiting.

Table 6.3 Specific rates of substrate uptake (q_{glc}) and product formation (q_{cit}) at steady states in nitrogen limited chemostat cultures.

Run No.	q_{cit} ($g/g_{bio}.h$)	q_{glc} ($g/g_{bio}.h$)	q_{cit} ($g/g_N.h$)	q_{glc} ($g/g_N.h$)
11	0.077	0.141	2.51	4.57
1	0.069	0.212	1.12	3.43
7	0.070	0.238	1.52	5.14
12	0.058	0.128	1.80	3.97
4	0.081	0.268	1.43	4.69
2	0.084	0.269	1.74	5.60
5	0.052	0.169	1.15	3.71
3	0.063	0.188	1.32	3.93
8	0.061	0.169	1.57	4.36
10	0.078	0.205	1.93	5.07

q_{cit} / q_{glc} ($g/g_{bio}.h$) = Specific rate of citric acid production / glucose consumption per gram of biomass in the culture

q_{cit} / q_{glc} ($g/g_N.h$) = Specific rate of citric acid production / glucose consumption per gram of nitrogen added in the feed medium

where: $\hat{Y}$ = the value of the variable being regressed

B = coefficients

A = coded variable for pH

B = coded variable for specific growth rate

B_0= constant or Y-intercept

Due to limitations in the experimental design, some of the effects are aliased with others. Thus the effect B_1A actually estimates the effects of A + A.B^2, B_2B estimates B + A^2.B and B_4A^2 estimates A^2 + B^2 + A^2.B^2. The effects of the interactions A.B^2, A^2.B and A^2.B^2 are probably negligible, but the effect of B^2 could be significant. It cannot be determined from this experiment whether any significant effect of B_4 is due to A^2, B^2, or a combination of both.

Table 6.4 shows the full regression of the models. The coefficients found to be statistically significant using the t-test are indicated.

The models that did not display a significant lack of fit were used to predict response surfaces, constructed as 3-dimensional shadow-contour diagrams, using the computer program Graftool (3-D Visions Corporation, California, U.S.A.).

Figure 6.1 shows the predicted citric acid production rate ($g/g_N.h$). As demonstrated by their significance in the equation, the most important factors are the growth rate and the growth rate - pH interaction, with the specific citric acid production rate ($g/g_N.h$) increasing as the growth rate decreases, and the effect of the pH varying with the growth rate. At a high pH value, the specific citric acid

Table 6.4 Full regression models for nitrogen-limited chemostat cultures.

$\hat{Y}$	B_0	B_1	B_2	B_3	B_4
q_{cit} $(g/g_{bio}.h)$*	0.0635	0.0044	0.0043	0.0052	0.0084
q_{cit} $(g/g_N.h)$	1.49	-0.089	-0.351[1]	0.266[1]	0.311[3]
q_{glc} $(g/g_{bio}.h)$	0.183	0.0004	0.0562[1]	0.0066	0.0078
q_{glc} $(g/g_N.h)$*	4.27	0.177	0.222	0.477[2]	0.225
Citric Acid Yield	0.345	-0.0275[1]	-0.0950[1]	0.0225[2]	0.060[1]
Biomass	1.63	0.049	-0.446[1]	0.103[2]	0.143[3]
% Nitrogen in Biomass	4.30	-0.287[2]	1.09[1]	-0.337[1]	-0.063

Statistical significance of coefficients indicated thus:
[1] = 2% level
[2] = 5% level
[3] = 10% level

* Models demonstrating a significant lack of fit

production rate ($g/g_N.h$) is less affected than at low pH, i.e. at pH 5.3 (coded = 1) decreasing the dilution rate from 0.08 h^{-1} (coded = 1) to 0.02 h^{-1} (coded = -1) caused the specific citric acid production rate to only increase from 1.63 to 1.80 $g/g_N.h$, while at the lower pH value of 3.8 (coded = -1) the same change in dilution rate caused the specific citric acid production rate to increase from 1.28 to 2.51 $g/g_N.h$. The maximum specific citric acid production rate ($g/g_N.h$) occurred at the low growth rate - low pH corner.

Figure 6.2 shows the predicted specific glucose consumption rate ($g/g_{bio}.h$). The most important factor was the growth rate, with the specific glucose consumption rate ($g/g_{bio}.h$) decreasing steadily as the growth rate decreases, as the requirement to consume glucose for growth decreases with the growth rate.

The citric acid yield is shown in Figure 6.3. As indicated by the equation in Table 6.4, the pH, growth rate, and the pH - growth rate interaction all have a significant affect on the citric acid yield. The maximum yield occurred at the low growth rate - low pH corner.

Figure 6.4 shows the predicted steady state biomass concentrations. The biomass concentration changes due to the production of storage compounds. The % of nitrogen in the biomass, shown in Figure 6.5, is also an indicator of storage compound production, as they consist of carbohydrates, not proteins, and thus do not contain any nitrogen. Therefore, a decrease in the % of nitrogen is due to an increase in storage compounds. As indicated by the equations for biomass and % nitrogen, and Figures 6.4 and 6.5, the most important factor for storage carbon production is the growth rate, although the pH and the pH - growth rate interaction also have an effect. i.e. at pH 5.2, decreasing the dilution

Figure 6.1 Predicted specific citric acid production rate ($g/g_N.h$) at steady state during nitrogen-limited chemostat culture

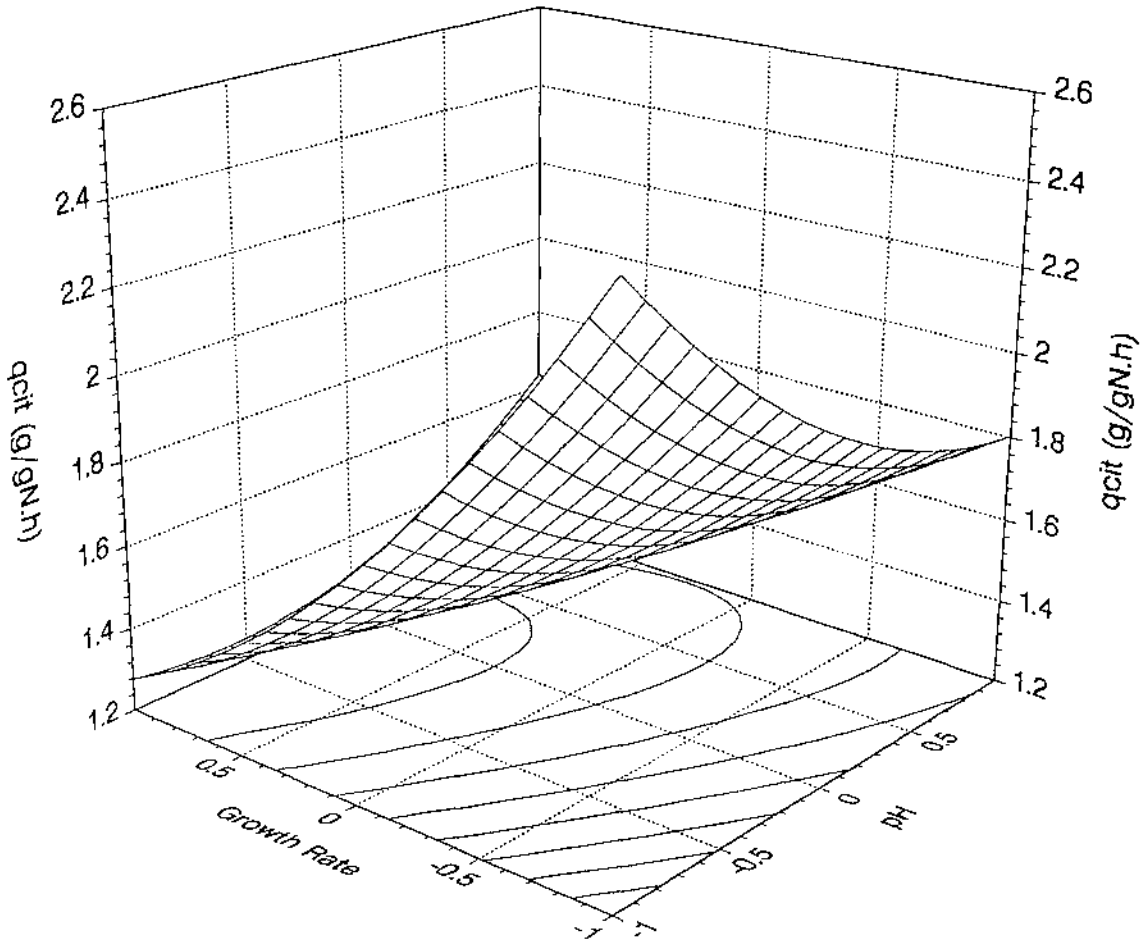


Figure 6.2 Predicted glucose consumption rate ($g/g_{bio}.h$) at steady state during nitrogen-limited chemostat culture

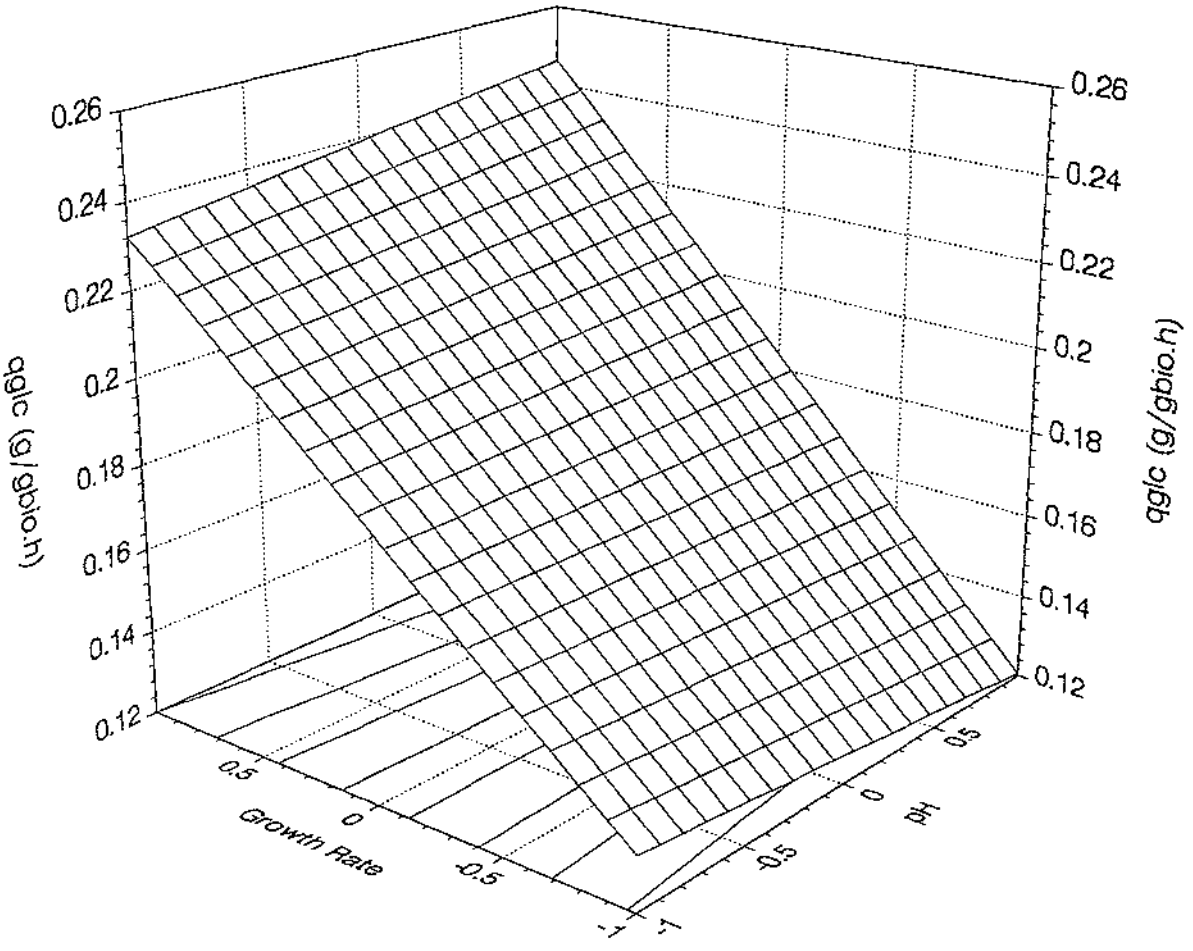


Figure 6.3 Predicted citric acid yield at steady state during nitrogen-limited chemostat culture

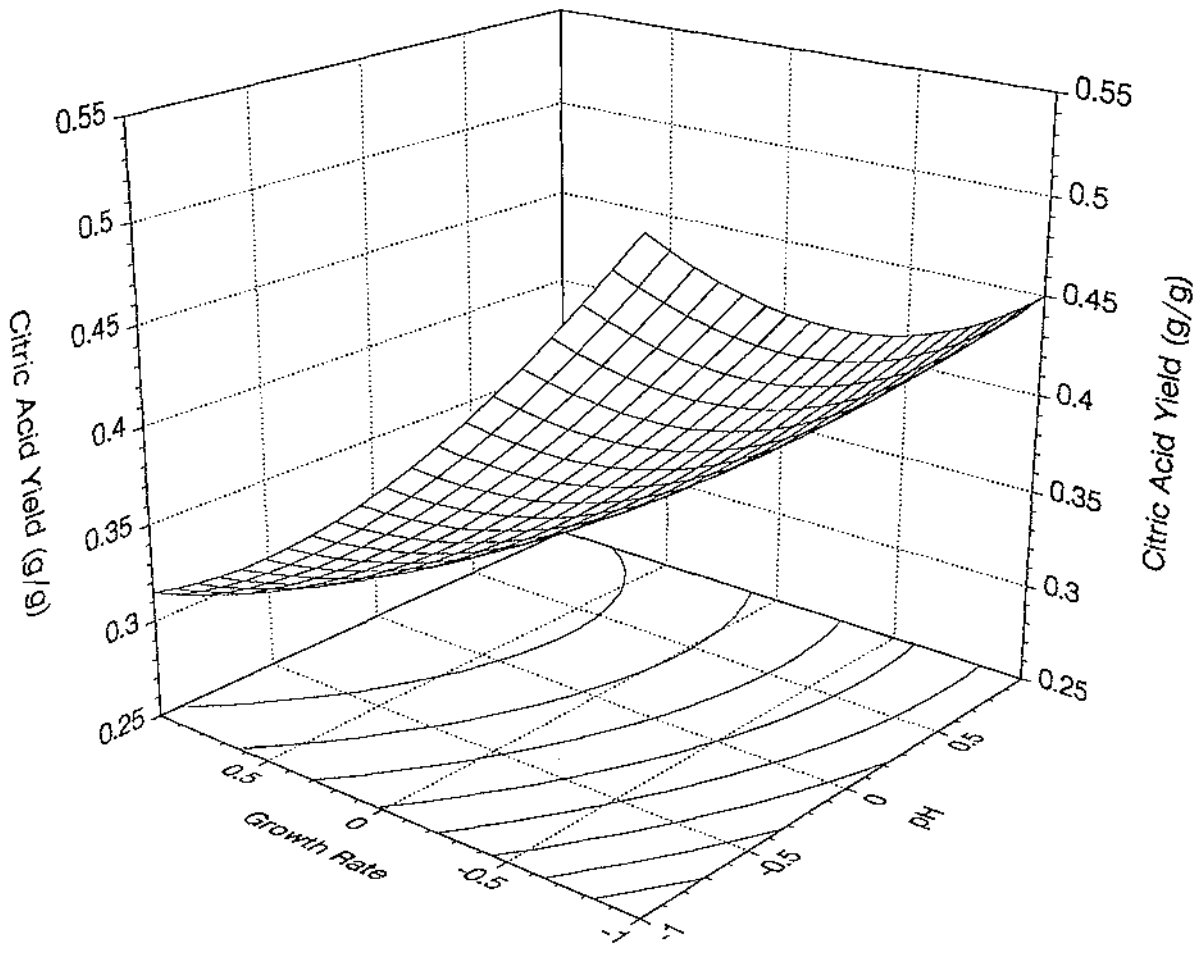


Figure 6.4 Predicted biomass concentration at steady state during nitrogen-limited chemostat culture

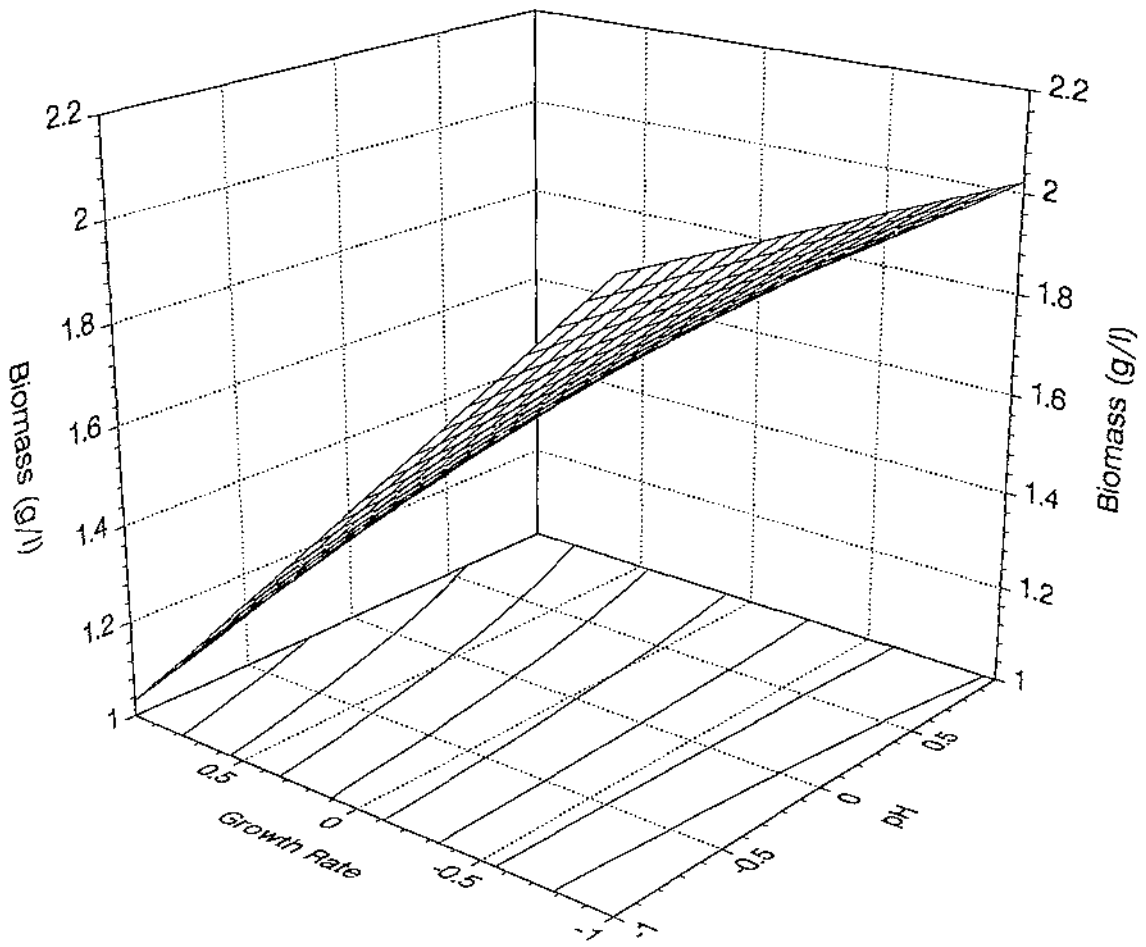
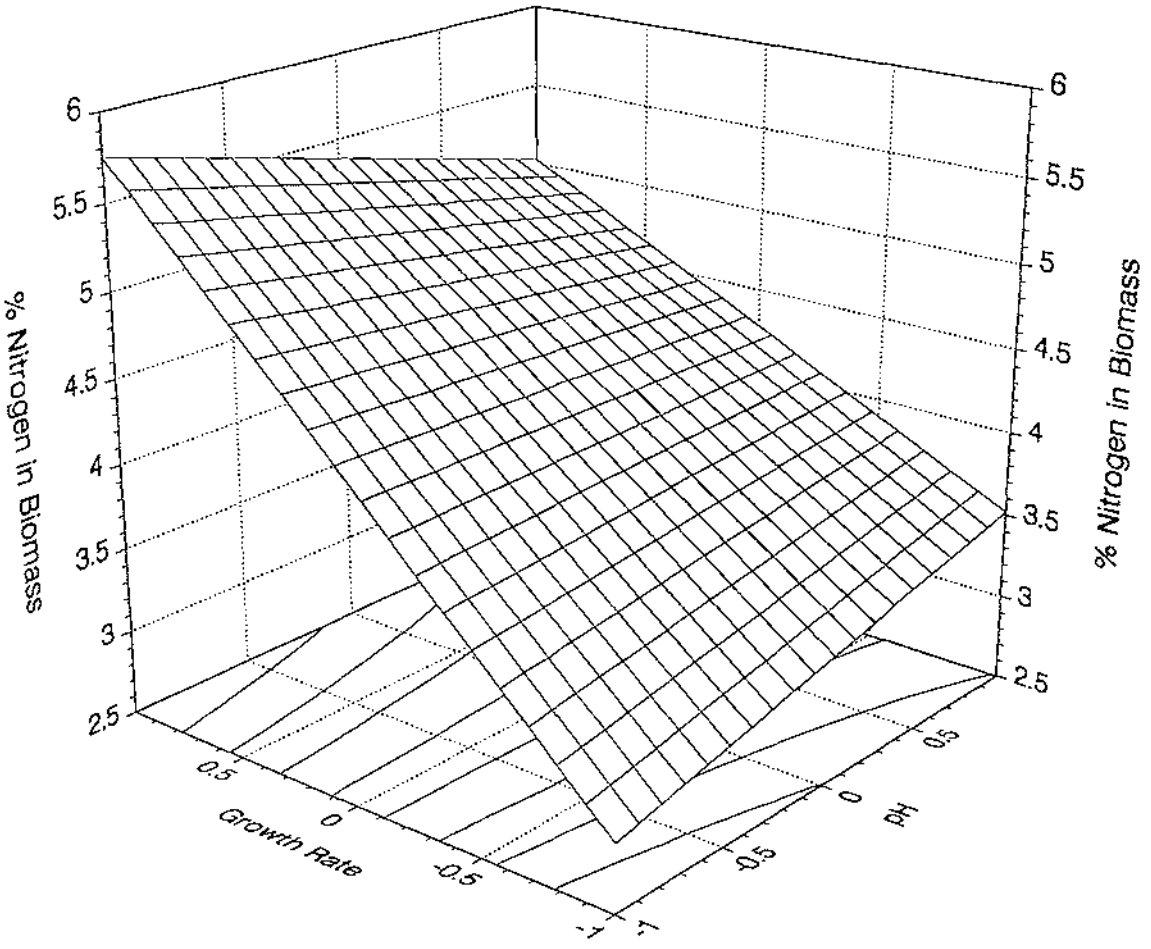


Figure 6.5 Predicted % of nitrogen in biomass at steady state during nitrogen-limited chemostat culture



rate from 0.08 h^{-1} to 0.02^{-1} causes the biomass to increase from 1.34 to 2.02 g/l and the % nitrogen to decrease from 3.52 to 5.03 %, whereas at pH 3.8, decreasing the dilution rate over the same range causes the biomass to increase from 1.03 to 2.13 g/l and the % nitrogen to decrease from 5.75 to 2.90 %.

Correlation coefficients were determined for all Y data with all other Y data. Table 6.5 shows the correlation coefficients found to be greater than 0.5, which was used as a cut-off value.

The data show relationships between the specific growth rate and the specific glucose uptake rate ($g/g_{bio}.h$), specific citric acid production rate ($g/g_N.h$), citric acid yield, biomass production, and the % of nitrogen in the biomass. No correlations were observed with the culture pH. The specific citric acid production rate ($g/g_{bio}.h$) had a correlation with the glucose uptake rate, and when calculated per gram of nitrogen added, the specific citric acid production rate ($g/g_N.h$) had a strong correlation with the citric acid yield. The citric acid yield had a strong correlation with the biomass production and a negative relationship with the specific glucose uptake rate ($g/g_{bio}.h$). Biomass production had negative correlations with the specific glucose uptake rate ($g/g_{bio}.h$) and the % of nitrogen in the biomass, and a positive correlation with the specific citric acid production rate ($g/g_N.h$). The specific glucose uptake rate ($g/g_{bio}.h$) had a strong correlation with the % of nitrogen in the biomass. These data suggest that to obtain a high citric acid production rate ($g/g_N.h$) and yield, the growth rate of the culture should be low.

Table 6.5 Correlation coefficients of data from a nitrogen-limited chemostat culture.

Data Set	Correlation Coefficient
Specific citric acid production rate ($g/g_{bio}.h$)	0.58
- Specific glucose consumption rate ($g/g_{bio}.h$)	
Specific citric acid production rate ($g/g_{bio}.h$)	0.74
- Specific glucose consumption rate ($g/g_N.h$)	
Specific citric acid production rate ($g/g_N.h$)	0.82
- Biomass	
Specific citric acid production rate ($g/g_N.h$)	0.88
- Citric acid yield	
Specific citric acid production rate ($g/g_N.h$)	-0.66
- Specific growth rate	
Specific glucose consumption rate ($g/g_{bio}.h$)	-0.77
- Citric acid yield	
Specific glucose consumption rate ($g/g_{bio}.h$)	-0.83
- Biomass	
Specific glucose consumption rate ($g/g_{bio}.h$)	0.79
- % Nitrogen in biomass	
Specific glucose consumption rate ($g/g_{bio}.h$)	0.54
- Specific glucose consumption rate ($g/g_N.h$)	
Specific glucose consumption rate ($g/g_{bio}.h$)	0.94

- Specific growth rate	
Citric acid yield - Biomass	0.91
Citric acid yield - Specific growth rate	-0.87
% Nitrogen in biomass - biomass	-0.98
% Nitrogen in biomass - Specific growth rate	0.90
Biomass - Specific growth rate	-0.93

6.3 Discussion

From the results for the nitrogen-limited chemostat cultures, the specific citric acid production rate ($g/g_{bio}.h$) showed no clear relationship with either the culture pH or the growth rate. This was probably due to the biomass concentration being affected by the growth rate. Chemostat theory states that the biomass concentration should be unaffected by the growth rate, but in practice the growth rate did affect biomass concentration, almost certainly due to the production of storage compounds. When the specific citric acid production rate was calculated based on the concentration of the limiting nutrient added to the culture, giving units of $g/g_N.h$, the specific citric acid production rate was observed to decrease with increased growth rate, and to be affected by the interaction between the pH and the growth rate. The maximum production rate and citric acid yield occurred at the low pH and dilution rates. Decreasing the growth rate from 0.08 to 0.02 h^{-1} increased the specific citric acid production rate ($g/g_N.h$) by 95 % and the yield by 75 % at pH 3.8, and by 10 % and 48%, respectively, at pH 5.2. This result was unexpected when compared to the batch culture, where an optimum pH for citric acid production was observed at pH 4.3. However, the batch culture experiments were performed to investigate the effect of the pH when the growth had ceased, and the chemostat results indicate that it is the interaction between the culture pH and the growth rate, not the culture pH alone, that has the more significant affect on the specific citric acid production rate ($g/g_N.h$). This interaction effect may be partially due to the growth pattern of *C. guilliermondii* IMK1. Unlike the yeasts reported in the literature, and *Y. lipolytica*

RB82, *C. guilliermondii* IMK1 does not produce citric acid only after growth has ceased, but also produces it while growing. This is demonstrated by Figure A1 in Appendix II, where citric acid production in batch culture is observed to commence at the start of exponential growth for *C. guilliermondii* IMK1, but does not commence until the end of exponential growth for *Y. lipolytica* RB82. Thus, unlike the effect in batch culture, where, after the growth had ceased, the optimum specific citric acid production rate occurred at pH 4.3, and citric acid production decreased with decreasing pH, in chemostat culture, where the culture is still growing, the effect of the culture pH changes with the growth rate, and the optimum pH for citric acid production varies accordingly, i.e. at a growth rate of 0.08 h^{-1}, the optimum pH was 5.2, and at a growth rate of 0.02 h^{-1} the optimum pH was 3.8. Reports in the literature (Aiba and Matsuoka, 1979; Klasson *et al*, 1989) indicate that the specific rate of citric acid production for yeasts cultured on glucose varies with the growth rate, and the optimum growth rates are low to moderate (0.03 - 0.06 h^{-1}). For the fungus *A. niger*, producing citric acid in a nitrogen-limited chemostat culture, the maximum specific citric acid production rate and yield was also observed to occur at a low dilution rate, 0.017 h^{-1}. (Dawson, 1986)

A major aim of the current investigation was to investigate the hypothesis that citric acid production occurs when the growth rate slows, but the sugar uptake rate is maintained. The chemostat culture results show that as the growth rate slows, so did the sugar uptake rate, probably as there was a reduction in the demand for sugar for biomass production. However, even at the minimum growth rate tested, the glucose consumption rate remained fairly high, approximately 2-

fold higher than for batch cultures. The specific citric acid production rate was approximately 50% higher than that achieved in batch cultures. Thus, although the glucose uptake rate was not maintained as the growth rate slowed, due to the decrease in demand for biomass production, it did remain comparatively high, and the specific citric acid production rate ($g/g_N.h$) was observed to increase as the growth rate decreased. Therefore, it can be stated that the specific citric acid production rate ($g/g_N.h$) increases when the growth rate decreases, and the sugar uptake rate remains high.

6.4 Conclusions

The specific rate of citric acid production ($g/g_{bio}.h$) was unaffected by either the growth rate or the pH, probably due to the variation in the production of storage compounds by the yeast at different growth rates. When calculated according to the limiting nutrient concentration however, the specific citric acid production rate was observed to increase as the growth rate decreased, and the optimum pH was affected by the growth rate. The maximum citric acid production rate ($g/g_N.h$) and yield occurred at pH 3.8, and a growth rate of 0.02 h^{-1}. The specific citric acid production rate ($g/g_N.h$) increased as the growth rate decreased, and the sugar uptake rate remained high.

CHAPTER 7

FINAL DISCUSSION AND CONCLUSIONS

The major aim of this thesis was to investigate the relationship between the growth rate, sugar uptake rate, and citric acid production rate, and to investigate the hypothesis that citric acid production occurs when the growth rate slows but the sugar uptake rate is maintained. Initially, the experiments were performed using the yeast *Yarrowia lipolytica* IMK2, which displays the typical production curve described in the literature, where the production of citric acid does not begin until the late exponential phase of growth, when the growth rate is declining towards zero. However, following the failure of this yeast to scale-up successfully into a batch fermenter, experiments proceeded using the yeast *Candida guilliermondii* IMK1. This yeast does not display the typical production curve, and citric acid production begins at the early exponential phase of growth. Thus, the relationship observed for this yeast between the growth rate and the citric acid production rate may not be the same as for other, more typical, citric acid producing yeasts.

The experiments investigating citric acid production in batch fermentation revealed that the pH of the culture had an affect on the production of citric acid, but that the aeration rate, between 0.040 and 0.333 vvm, did not. Thus, when experiments were performed in a chemostat culture to investigate the effect of the growth rate on citric acid production, the effect of the pH was also investigated. The results showed that the maximum specific citric acid production rate ($g/g_N.h$) and yield occurred at low growth rates and low pH values, and that the effect of

the pH varied according to the growth rate. The observation that the effect of the pH varied with the growth rate would not have been easy to observe if a factorial experimental design had not been used to set up the chemostat experiments. No reports have been discovered in the literature which describe the use of a factorial experimental design to investigate the production of citric acid by yeasts.

Reports in the literature suggest that an optimum growth rate exists for the production of citric acid. In this thesis, however, an optimum was not observed. It is possible that if the growth rate was decreased further, such an optimum may have been observed, but reducing the dilution rate further in the chemostat culture would have presented technical difficulties. However, the use of a continuous fed-batch culture to control the growth rate via the addition of the growth-limiting nutrient may be feasible. As the specific growth rate would be controlled by the continuous addition of growth limiting amounts of nitrogen, the volume of the culture would increase continuously. This would result in a continually decreasing growth rate, and the feed rate of the limiting nutrient would have to continuously increase. Such a fed-batch system has been reported for citric acid production by *A. niger*, although the feed rate of the limiting nutrient was increased stepwise, causing oscillation in the growth rate, and specific growth rates down to 0.003 h^{-1} could be obtained. (Dawson, 1986)

Glucose exhaustion occurred in two of the low dilution rate experiments. Appendix III shows the effect of this exhaustion on the biomass concentration and specific rates. The reduction in the specific glucose uptake rate due to the limitation in glucose availability resulted in a slight decrease in the biomass concentration, probably due to a slight reduction in the production of storage

compounds, but a major decrease in the specific citric acid production rate occurred. This demonstrates the effect of the glucose uptake rate on the citric acid production rate at a constant growth rate.

In conclusion, the specific citric acid production rate ($g/g_N.h$) was observed in chemostat culture to increase as the growth rate decreased, while the sugar uptake rate was still high. When the sugar uptake rate decreased, so did the citric acid production rate.

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APPENDIX I

For the high dilution rate duplicates, the data was collected at the same dilution rate so the only factors having an effect would be the pH, the steady state order, and any pH - order interaction. For the centre-point duplicates, the only factor having an effect would be the steady state order, as the pH and dilution rate are constant. The equation is:

$$\hat{Y} = B_0 + B_1\text{order} + B_2\text{pH} + B_3\text{pH.order}$$

As only 4 data points could be used in each set to investigate the effect of the steady state order, there are very little degrees of freedom in this analysis, and thus a higher error influence, which could be the cause of the contradiction between the results of the effect of steady state on the biomass production for the replicate centre-points and the replicate high dilution rates.

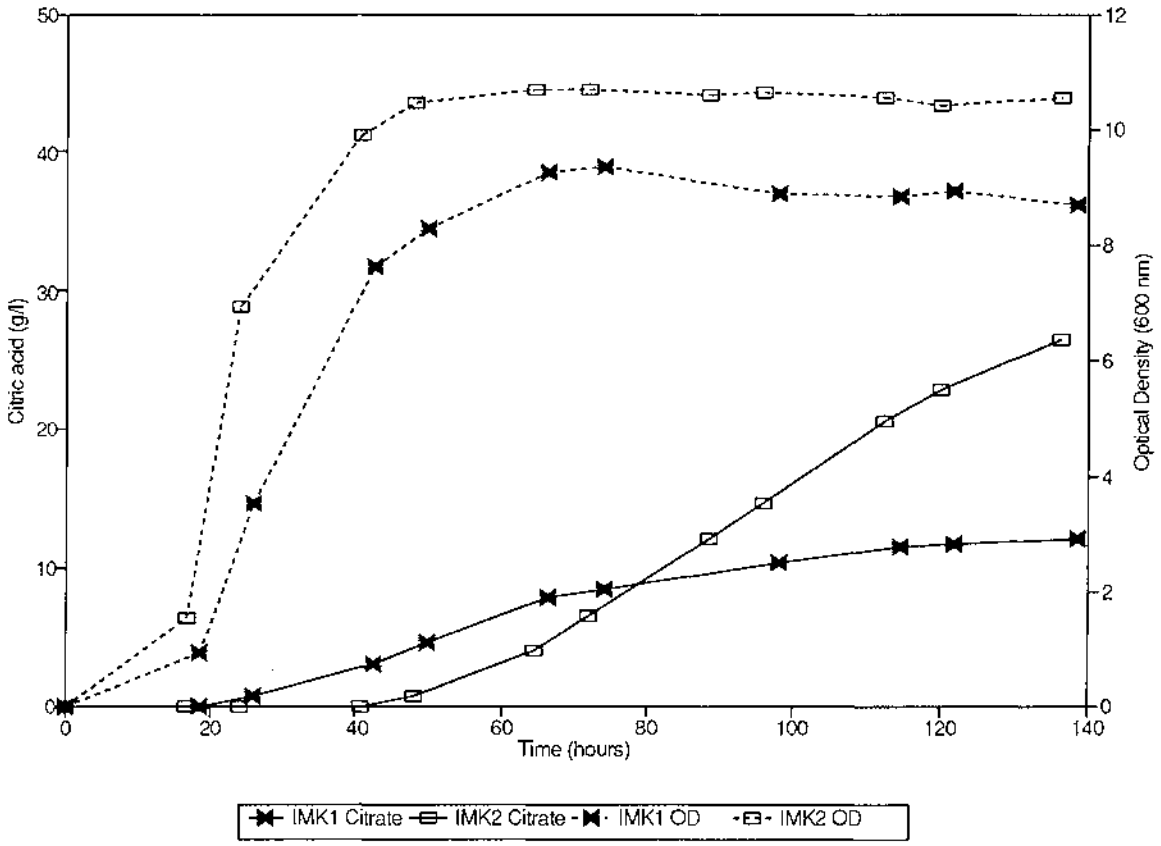
Table A1 Affect of steady state order on the nitrogen limited chemostat culture.

Chemostat Conditions	$\hat{Y}$	B_0	B_1	B_2	B_3
High Dilution Rate	q_{cit}	0.0703	0.00055	0.0067	0.0061
High Dilution Rate	q_{glc}	0.247	0.0219	0.0070	0.0063
High Dilution Rate	Biomass	1.33	0.0083	0.152[1]	-0.0367
Centre-point	q_{cit}	0.0635	0.0060	-	-
Centre-point	q_{glc}	0.1830	0.0046	-	-
Centre-point	Biomass	0.0635	0.130[1]	-	-

[1] significant at the 5% level

APPENDIX II

Figure A1 Comparison of typical growth and citric acid production by *Candida guilliermondii* IMK1 and *Yarrowia lipolytica* IMK2.



APPENDIX III

Table A2 Results of the low growth rate experiments and the experiments where glucose was exhausted from the medium.

Glucose Limiting	Biomass (g/l)	Specific Citric Acid Production Rate ($g/g_{bio}.h$)	Specific Glucose Consumption Rate ($g/g_{bio}.h$)
No	2.28	0.077	0.141
No	2.17	0.058	0.128
Yes	2.12	0.039	0.098
Yes	2.07	0.038	0.096