CITRIC ACID PRODUCTION BY IMMOBILIZED CELLS OF THE YEAST *CANDIDA GUILLIERMONDII*.

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ABSTRACT

The feasibility of using cells of *Candida guilliermondii* immobilized onto sawdust particles for production of citric acid was investigated.

C. guilliermondii IMK1 from a stock culture (Department of Process and Environmental Technology, Massey University, Palmerston North, New Zealand) was reisolated for further study including strain improvement work by induced mutation using UV light. A mutant strain DT2 was isolated which produced a citric acid concentration of 9.2 g/l (yield 25 % (w/w)) in shake flask culture, using a defined medium containing 36 (g/l) glucose, compared with 4.9 (g/l) citric acid produced (yield 14 % (w/w)) by the parent strain. Experiments in a laboratory scale batch fermenter, in which a higher concentration of citric acid (11.7 g/l) was achieved, proved that citric acid production using the mutant strain *C. guilliermondii* DT2, could be scaled up successfully from shake flask to a 2 l fermenter. This mutant was used throughout subsequent experiments.

Sawdust was selected, as the most appropriate support material to immobilize the mutant strain *C. guilliermondii* DT2 via the adsorption method.

Experiments using different concentrations of nitrogen nutrient in defined medium using cells of *C. guilliermondii* DT2 immobilized onto sawdust particles, in repeated batch shake flask culture, demonstrated a marked effect of the nitrogen concentration on citric acid production. Thus, an overall

productivity of 0.11 (g/l.h) was obtained using a defined medium containing 0.53 (g/l) ammonium chloride, compared to overall productivities of 0.04 (g/l.h) and 0.01 (g/l.h) using defined media containing 0.1 (g/l) and no ammonium chloride, respectively. No significant effect of nitrogen concentration on citric acid yield was observed in this investigation. In contrast, similar experiments, in repeated batch shake flask culture, for the effect of phosphate concentration on citric acid production showed no effect of phosphate on either the production rate or yield of citric acid.

In bubble column culture experiments, using cells of *C. guilliermondii* DT2 immobilized onto sawdust, the importance of pH control in citric acid production was demonstrated. In addition, it was demonstrated that the activity of immobilized cells which have lost the ability to produce citric acid can be revived by supplying medium containing sufficient concentrations of nitrogen and phosphate. Reduction of the nitrogen concentration in the medium from 0.53 (g/l) to 0.05 (g/l), provided that the reactor was well established, showed no significant influence on citric acid productivity, but significantly improved the citric acid yield. The highest productivity of around 0.21 - 0.24 (g/l.h) at a dilution rate of 0.21 h^{-1}, accompanied by a citric acid yield of about 10 - 11 % (w/w), was reached and maintained for more than 140 hours of stable operation.

Overall, it was concluded that cells of *C. guilliermondii* were succesfully immobilized onto sawdust particles, and the immobilized cell reactor produced

citric acid at a higher rate compared to a free cell system. In particular, a high rate of citric acid production in a bubble column reactor, operated in continuous mode, was achieved.

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CHAPTER 1

INTRODUCTION

Citric acid is produced commercially through a fermentation process, and selected strains of *Aspergillus niger* are usually applied in this process. However, several species of yeasts, especially from the genus *Candida*, have proved their potential as citric acid producers.

Yeasts have several advantages compared to fungal species, including ease of growth and ease of handling in a fermenter since they do not block ports or grow on probes, and they grow as a homogeneous suspension rather than as pellets or large aggregates. Moreover, yeasts can be grown on various kinds of carbon source including n-alkanes and sugars. Due to their ease of assimilation and lower formation of the by-product, isocitric acid, sugars , particularly glucose, are auspicious carbon sources for the production of citric acid (Rohr *et al*, 1983; McKay *et al*, 1990).

Recently, with respect to the optimization of the process, mutation of strains (Furukawa *et al*, 1977, 1982; McKay *et al*, 1990; Gutierrez and Maddox, 1993) and immobilization of cells (Maddox and Kingston, 1983; Kautola *et al*, 1991; Rymowicz *et al*, 1993; Forster *et al*, 1994) have become of central interest to workers to improve reactor productivity. Hence, it seemed appropriate to investigate the use of immobilized cells of *C. guilliermondii* for

the production of citric acid.

The present work aimed to examine the performance of immobilized cells of *C. guilliermondii* for citric acid production from glucose in repeated batch shake-flask culture and in a continuous bubble column reactor. Attempts to accelerate the process by means of manipulation of limiting nutrient concentrations, i.e. nitrogen and phosphate, were also examined in this work.

CHAPTER 2

LITERATURE REVIEW

2.1 Citric Acid

Citric acid or 1,2,3-propane tricarboxylic acid-2 hydroxy is a useful organic acid, which has a high water solubility and a mildly acidic taste. The natural occurrence of citric acid in the plant and animal kingdom indicates that this compound is non-toxic, and is able to be applied safely in the food and drink industries which are the main consumers of this acid.

Until the early twentieth century citric acid was produced mostly from extraction of citrus fruits, though in 1893 Wehmer discovered that citric acid can be produced by fermentation from sugar using certain moulds of the genera *Penicillium* and *Mucor*.

Today, citric acid is manufactured mostly through a fermentation process, with the fungal species *Aspergillus niger* dominating the choice of micro-organism for this purpose for nearly 80 years, until several yeasts within the genus of *Candida* were taken into consideration by many workers (Rohr *et al*, 1983; Abou-Zeid and Ashy, 1984; Milsom and Meers, 1985).

2.2. Uses of Citric Acid

Generally citric acid is marketed either in the anhydrous form or as the monohydrate. The demand for this compound from various industries is

increasing considerably throughout the world.

Table 2.1 shows the application of citric acid in various industries. Based on the characteristics : ease of assimilation, pleasant sour taste, low toxicity and enhancing properties to an existing flavour, citric acid is used mostly in the food and soft drink industries (75%). Other industries which consume significant amounts of citric acid are the pharmaceuticals and cosmetics industries (10%). The application of citric acid in the pharmaceutical field is referred to as the effervescent effect, which is produced when mixed with carbonate and bicarbonates, for instance in the preparation of soluble aspirin and antacid (Rohr *et al*, 1983; Milsom and Meers, 1985; Marison, 1988).

Today, due to the chelating property of this compound, citric acid is being used more and more in the detergent industry, and has attracted many workers to do research related to this purpose. Indeed, some countries do not allow the sale of detergents containing polyphosphate, so citric acid is indicated to replace the position of polyphosphate in the detergents completely (Crueger and Crueger, 1989).

Table 2.1 : Uses of citric acid

Industry	Area	Uses
Beverage	General	Flavour enhancer Preservative Haze eliminator Prevents deterioration
Beverage	Wine	Prevents turbidity
Beverage	Soft drinks	"Cool" taste Aids carbonation
Food	Confectionery	Flavour enhancer
Food	Frozen food	Anti-oxidant
Food	Dairy products	Colour enhancer pH regulator Inactivates trace metals
Pharmaceutical		Solvent and flavouring Effervescent with H_2CO_3
Cosmetics		Anti-oxidant and synergist
Other		In detergents Tanning Textiles

From : Marison (1988)

2.3. Production of Citric Acid

Citric acid is produced commercially in many countries including United States, Germany, Belgium, France, Taiwan, England, Russia, Canada, Czechoslovakia, Poland, Israel, Netherlands, Austria, Ireland and some developing countries. United States with two main companies : Pfizer Inc.

(New York, New York), produces about 112,500 tonnes/annum and Miles Laboratories Inc. (Elkhart, Indiana), produces about 54,000 tonnes/annum, making the U.S.A the largest producer of citric acid (Robert-Thomson, 1993).

2.3.1. History of Production

In the early stage of the citric acid history, production was only through a chemical process involving the extraction of citrus fruits which contain around 7 to 9% of citric acid. Grimaux and Adam, in 1880, had synthesized citric acid from glycerol, and this encouraged other workers to explore other raw materials for citric acid production. The study of the microbial process was started in 1893, when Wehmer discovered that certain species which belong to *Penicillium* (termed by Wehmer as *Cytromyces*) were able to produce large amounts of citric acid when inoculated into media containing sugar. However, Wehmer had unsatisfactory results when he attempted to produce citric acid using *Penicillium lactum* and *Mucor pyriformis* on a commercial scale. This was suggested to be due to the problem of contamination and the long duration of the fermentation (Rohr *et al*, 1983; Milsom and Meers, 1985).

Aspergillus niger has an important role in the development of the citric acid fermentation process. It has dominated the choice of microorganism for this purpose for a long period of time. It was started by the important finding of Thom and Currie in 1916, that *Aspergillus niger* could grow well at extremely low pH values, and that, fortunately, pH values around 2.0 to 3.5 are

favourable for the production of citric acid. In addition, operation at this low pH value reduces the risk of contamination. Moreover, Currie, in 1917, found that a high sugar concentration provided propitious conditions for optimal production, and it was observed that the highest yields of citric acid can be achieved when the development of mycelium is restricted (Rohr *et al*, 1983; Abou-Zeid and Ashy,1984).

In 1923, a plant for citric acid fermentation was opened in New York which applied the fermentation technique developed by Thom and Currie in 1916. In this process, *A. niger* was grown in surface culture on sugar media. In 1930, Amelung made the earliest attempts to produce citric acid by the submerged-growth technique, and this process was introduced commercially in the United States in 1952 and in Mexico in 1959 (Abou-Zeid and Ashy, 1984).

2.3.2. Production Using Aspergillus niger

Aspergillus niger has now been used for many years, on both a laboratory and industrial scale, for the production of citric acid by fermentation process. A major advantage of *A. niger* is its ability to grow under highly acidic conditions, and, providentially, the optimum yield of citric acid from the fermentation is generally achieved at pH values less than 3. Operation at higher pH values can lead to the accumulation of oxalic and gluconic acid as by products (Rohr *et al*, 1983).

The earliest processes for production of citric acid were carried out in

surface culture, where sterile medium was placed in stainless steel or aluminium trays, inoculated with spores of *A.niger*, and incubated at a temperature of 28 - 30°C and relative humidity of 40 - 60% for 8 - 12 days. The fungal mycelium grows and spreads over the surface, and the accumulation of acid causes a decrease of medium pH value (Marison, 1988).

The starting pH of the medium is adjusted according to the substrate being used in the process. When using media based on sucrose, e.g. molasses, the pH should be adjusted to around 5 to 7, because *A. niger* will not germinate at higher hydrogen ion concentrations. This has been suggested to be due to the presence of acetic acid as a constituent of molasses (Milsom and Meers, 1985).

In later years, the submerged fermentation process was introduced, and it proved to be a significant improvement over the surface culture procedure, due to a shorter fermentation period of only 3 - 5 days to run a fermentation. In both surface and submerged fermentation processes, at the end of the process, the mycelium may be re-used by adding fresh medium, after the liquor has been drained off (Marison, 1988).

2.3.3. Production Using Yeasts

In contrast to the utilization of *Aspergillus niger* for citric acid production, yeasts require a shorter culture period, and usually grow as a homogeneous suspension rather than as pellets or as large aggregates. In addition, yeasts can be used at very high initial sugar concentrations.

Combination of these characteristics offers a high yield per period of time, and ease of handling (Milsom and Meers, 1985; McKay *et al*, 1990; Tani *et al*, 1990).

Several species within the genus *Candida* are the most frequently used on a wide variety of substrates, with the emphasis being on the use of glucose or n-alkanes as the carbon source. When either glucose or n-alkanes are used as substrate, the accumulation of acid occurs after the completion of the growth phase and it is indicated by the exhaustion of the nitrogen source from the medium (Milsom and Meers, 1985; Marison, 1988).

Generally, the fermentation using yeasts is carried out using submerged culture, with high aeration, a temperature around 22 - 30°C, pH 4.5 and fermentation period of 3 - 6 days. The main drawback of using yeasts is the formation of isocitric acid as a by-product, which might reach 50% of the total acid yield. Methods to control the accumulation of isocitric acid include inhibition of the activity of aconitase, and appropriate selection of the species and substrate for the process (Rohr *et al*, 1983; Marison, 1988).

2.4. The Yeast

Since they possess no chlorophyll, yeasts are true fungi, and form one of the important subgroups of fungi, which belong to the subdivision of the *Thallophytes*. Yeasts are grouped together in three families, known as the *Saccharomycetaceae, Sporobolomycetaceae*, and *Cryptococcaceae*. They lie

between bacteria and higher fungi in respect to the average size of the single cell. The group is distinguished by a form of vegetative reproduction. As with bacteria yeasts are widespread in nature. Generally, yeasts can be found in areas where carbohydrates are present, such as in the soil of vineyards and orchards; from the surface of grapes, apples, and most sweet fruits; from citrus fruits; and from the leaves and other parts of plants (Prescott and Dunn, 1959; Bailey and Ollis, 1986).

Yeast reproduction occurs through various ways, including sexual and asexual paths. Sexual reproduction occurs through the formation of zygotes or by sporulation. Zygotes are obtained by conjugation of two haploid cells to form a diploid cell. In addition to being a sexual form of reproduction, where it is important for producing new hybrids, sporulation maintains the viability of the species during hostile changes in the environment. Certain characteristics must be fulfilled to provide suitable conditions for sporulation : the yeast cells must be young and vigorous, they must be supplied with adequate air and water, the medium should be adjusted to a particular pH value, and it must be free from inhibitory substances. Each variety of yeast has a particular range of temperature for sporulation. The temperature optimum for most of the yeasts lies between 25 and 30°C (Prescott and Dunn, 1959; Bailey and Ollis, 1986).

Asexual reproduction occurs through budding and fission. In budding, a small offspring cell begins to grow on the side of the original cell. Fission occurs by division of the cell into two new cells (Bailey and Ollis, 1986).

2.5. Factors Affecting Citric Acid Production by Yeasts.

Since high yielding strains and appropriate media have an important influence on the fermentation efficiency, these two elements have received serious consideration and attention. Many workers have tried to select, and to develop, reasonably good strains as well as to optimize the medium composition.

2.5.1. Strain Selection and Improvement.

Many species within the genus *Candida* are capable of producing citric acid, but most of them also accumulate d-isocitric acid as a by-product in significant amounts. Nakanishi *et al* (1972) found that *Candida guilliermondii*, when grown on n-paraffin, could produce citric acid accompanied by only small quantities of isocitric acid, although the total acid production was not as high as when using *Candida zeylanoides*. Furukawa *et al* (1977; 1982) reported that mutagenesis with N-methyl-N'-nitro-N-Nitrosoguanidine (NTG) could create new strains with higher productivity and which produced a higher ratio of citric acid to total acid, when compared to their parent strains. McKay *et al* (1990) reported that ultra-violet light mutagenesis, followed by subsequent selection, increased the production of citric acid from glucose by *Yarrowia (Syn. Candida) lipolytica* IFO 1658 by two fold, and by *Candida guilliermondii* NRRL Y-448, from galactose, six fold. In agreement with the result of McKay *et al* (1990), Gutierrez and Maddox (1993) reported that the

mutant strain of *C. guilliermondii*, known as IMK1, produced citric acid at a concentration almost seven times higher than that of the parent strain NRRL Y-448, although the mutant grew considerably slower than the parent, with correspondingly lower biomass yield.

2.5.2. Growth Conditions

The success of fermentation processes is absolutely dependent upon the composition of the medium and the environmental conditions. The main factors affecting the process are the carbon source, nitrogen, oxygen, phosphate, metal ions, pH and temperature.

2.5.2.1. Carbon Sources.

Carbon is the major constituent required by microorganisms, so carbon normally is present as the main component in the growth media.

Since first reported in the patent literature by a Japanese company, a number of studies on the formation of citric acid from n-alkanes by yeasts within the genus *Candida* have appeared during the 1970's (Milsom and Meers, 1985).

An investigation into the effect of various individual n-alkanes on the production of citric acid by *C. zeylanoides* showed that between carbon numbers 10 to 20, higher production of citric acid was obtained from n-alkanes of C_{13} to C_{18}, especially C_{16} to C_{18} (Nakanishi *et al*, 1972). Using *C.citrica* on

various n-alkanes with carbon numbers from 11 to 17, plus NP-mixture (mixture of n-paraffin of C_{16} and n-paraffin-SHR 13002 of Mitsui Texaco) (Table 2.2), it was shown that the highest citric acid yields were attained from n-paraffins of C_{16} and the NP-mixture (Furukawa *et al*, 1977).

Table 2.2 : Citric acid and isocitric acid production from various n-paraffins using *C. citrica.*

n-Paraffin	Growth (OD)	Citric acid Conc'n (g/l)	Citric acid Yield %(w/w)	Isocitric acid Conc'n (g/l)	Isocitric acid Yield % (w/w)
C 11	0.065	36.2	60.3	3.3	5.5
C 12	0.097	44.1	73.5	5.6	9.3
C 14	0.143	49.1	81.8	2.6	4.3
C 15	0.138	43.5	72.5	2.1	3.5
C 16	0.147	54.4	90.7	3.8	6.3
C 17	0.210	50.7	84.5	4.1	6.8
P-4*	0.160	46.2	77.0	3.2	5.5
NP-SHR 13002*	0.129	51.7	86.2	3.5	5.8
P-7*	0.112	46.4	77.3	2.7	4.5

Cultured at 30°C for 7 days; * = NP mixture

From : Furukawa *et al* (1977).

Examination of the effect of n-paraffin concentration on citric acid

production and cell growth using a mutant strain of *Saccharomycopsis (Syn. Candida) lipolytica* in the concentration range of 45 - 90 (g/l) showed that increased concentrations provided increased production of citric acid as well as of isocitric acid and biomass concentration (Furukawa and Ogino, 1982).

Since sugars are metabolised rapidly by microorganisms such as fungi or yeasts, they are excellent carbon sources for many fermentation processes, including that for citric acid production. Hattori *et al* (1974) observed the effect of various carbon sources on the ratio of citric acid to total acid production, using *C. zeylanoides*, and the results, of six days fermentation at temperature 30°C and at pH value of 5.5, are summarized in Table 2.3.

Table 2.3 : Effect of carbon sources on citric acid fermentation using *C. zeylanoides*.

Carbon source	Biomass conc'n mg/ml	Citric acid (A) mg/ml	d-iso citric (B) mg/ml	A+B	A/A+B
Glucose	11.6	37.0	4.8	41.8	0.883
Glycerol	12.4	39.0	4.1	43.1	0.905
Sucrose	12.0	23.0	5.4	28.4	0.810
Sorbitol	10.0	17.0	3.2	20.2	0.842
Acetic acid	8.9	13.0	12.0	25.0	0.520
Oleic acid	8.6	32.0	26.0	58.0	0.552
Soybean oil	12.2	51.0	48.0	99.0	0.516
n-paraffin	14.0	56.2	50.5	106	0.527

From : Hattori *et al*, 1974

Rohr *et al* (1983) mentioned that the advantage of using glucose as the carbon source was that considerably less isocitric acid was formed as by-product. However, Briffaud and Engasser (1979) found that the specific rate of acid poduction from glucose was rather lower than that obtained by Marchal *et al* (1977) who used n-paraffin as the carbon source. However, with glucose only small quantities of isocitric acid were formed, 10% instead of 30%, and the specific oxygen consumption rate was almost threefold lower than with n-paraffin.

2.5.2.2. Oxygen

The oxygen supply is critically important for aerobic fermentation processes like citric acid production, and highly reduced substrates require a higher oxygen supply, as shown in equations 2.1 and 2.2 below :

$C_{16}H_{34} + 12.5\ O_2 \rightarrow 2.66\ C_6H_8O_7H_2O + 4.66\ H_2O$ (2.1)

$C_6H_{12}O_6 + 1.5\ O_2 \rightarrow C_6H_8O_7H_2O + H_2O$ (2.2)

Production of 2.66 moles of citric acid monohydrate from a C_{16} n-alkane requires 12.5 moles of oxygen for every mole of alkane, whereas production from glucose requires only 1.5 moles of oxygen per mole of glucose (Roberts-Thomson 1993).

Okoshi *et al* (1987), discovered that the accumulation of citrate using *C. tropicalis* was affected by the concentration of dissolved oxygen. The accumulation of citrate improved with an increased dissolved oxygen

concentration up to 60 ppm, and decreased sharply above a concentration of 60 ppm. In contrast, the concentration of dissolved oxygen had an opposite influence on the accumulation of isocitric acid, i.e. the increase of DO concentration resulted in a lower concentration of isocitric acid.

Maximum production of total acid during growth of *C. zeylanoides* can be reached at a moderate supply of oxygen (0.2 vvm), and an increase in the aeration rate had only a limited effect on the productivity. A strain of *Saccharomycopsis (Syn. Candida) lipolytica* showed no significant change in citric acid production rate following an increase in the aeration rate above 0.2 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 (Furukawa *et al*, 1977; Marchal *et al*, 1977; Furukawa and Ogino, 1982). Roberts-Thomson (1993), using *C. guilliermondii*, reported that aeration in the range 0.04 - 0.2 vvm had very little effect on either the citric acid production or glucose utilisation. However, this author did not measure the dissolved oxygen concentration of the media.

2.5.2.3. Nitrogen

The fermentation process for citric acid production by yeasts is biphasic, where citric and isocitric acid accumulation occurs after the end of the cellular growth phase. Therefore, higher concentrations of the limiting nutrient, such as nitrogen and phosphate, cause a higher growth rate, but tend to decrease the

citric acid formation rate. This phenomenon suggests that restriction of the cells' growth will stimulate citric acid accumulation by diverting the carbon source from biomass production to citric acid production. Thus, a compromise must be reached between a high specific rate of production and sufficient biomass to obtain the maximum total citric acid production (Nakanishi *et al*, 1972; Marchal *et al*, 1977; Roberts-Thomson, 1993).

Table 2.4 : Effects of inorganic nitrogen sources on growth and citric acid production using *C. citrica*.

Nitrogen source	Growth (OD)	Citric acid Conc'n (g/l)	Citric acid Yield % (w/w)	Isocitric acid Conc'n (g/l)	Isocitric acid Yield % (w/w)
$(NH_4)_2SO_4$	0.264	61.4	102.4	5.1	8.5
NH_4Cl	0.219	58.6	97.7	3.9	6.5
NH_4NO_3	0.343	68.9	114.8	9.0	15.0
$NH_4H_2PO_4$	0.314	58.6	97.7	7.6	12.6
$NaNO_3$	0.037	22.6	37.7	16.1	26.8

Cultured at 30°C for 7 days, Nitrogen sources = 4 g/l.

From : Furukawa *et al* (1977).

Conventionally, nitrogen is supplied in the form of ammonium sulfate or nitrate. Using *C. citrica*, ammonium nitrate gave slightly higher citric acid production compared to other ammonium salts, as shown in Table 2.4 (Furukawa *et al*, 1977).

2.5.2.4. Phosphate

Citric acid production is considerably influenced by the initial concentration of phosphate. Hattori *et al* (1974), as summarized in Table 2.5, reported that for citric acid production using *C. zeylanoides* growing

Table 2.5 : Effect of phosphate concentration on the ratio of citric acid to total acid.

KH_2PO_4 conc'n (g/l)	Biomass conc'n (g/l)	Citric acid conc'n (g/l)	Isocitric acid conc'n (g/l)	Ratio of citric acid to total acid
0.0	4.0	7.8	6.9	0.530
0.10	8.0	28.9	27.6	0.513
0.30	23.6	60.1	60.0	0.500
0.50	28.0	53.8	48.2	0.527
1.0	39.2	39.3	35.9	0.523
3.0	72.8	10.9	9.8	0.527

From : Hattori *et al* (1974)

on n-paraffin, an increase in phosphate concentration up to 0.3 g/l was followed by an increase in citric acid production. However, at a phosphate concentration above 0.3 g/l production decreased, although growth of the organism increased steadily up to 3.0 g/l of phosphate concentration. This result confirms the importance of the concentration of the limiting nutrient in citric acid production. Meanwhile, the ratio between citric acid and total acid was not

affected by the various concentrations of phosphate.

2.5.2.5. Metal Ions

Maddox *et al* (1994), reported that the accumulation of citric acid by a strain of *C. guilliermondii*, in contrast to *Aspergillus niger*, was not affected by the presence of certain metal ions which are commonly found in raw materials, such as, copper, iron, manganese and Zinc.

2.5.2.6. pH

Each yeast strain has a particular pH range which is favourable for its growth, so control of the culture pH is important during the cultivation. Unlike the fungus *A. niger*, which can grow at a very acidic level, and where production of citric acid is maximal at pH 2, yeast cultures require a higher pH level for maximum production. Lowering of the pH value may lead to the formation of polyhydroxy compounds (e.g. erythritol and arabitol). Suitable pH values for citric acid production are in the range 5.5 to 6.5, and *S. lipolytica* has been reported to give maximum production at pH 6.5 (Nakanishi *et al*, 1972; Furukawa and Ogino, 1982; Rohr *et al*, 1983).

Roberts-Thomson (1993) reported that the highest yield and rate of citric acid production using *C. guilliermondii* occured at pH 4.3, while the highest glucose consumption rate occured at pH 3.5. However, no citric acid was produced at the latter pH value.

2.5.2.7. Temperature

Observations into the effect of temperature on citric acid and isocitric acid production have been carried out by Nakanishi *et al* (1972), Furukawa *et al* (1977) and Marchal *et al* (1977). All showed that temperatures between 25°C to 33°C are favourable for grown of *C. zeylanoides, C. citrica and S. lipolytica* on n-paraffin. Optimum acid production was obtained at a temperature of 30°C, and temperatures above 35°C led to a reduction of productivity.

2.5.3. Cell Immobilization Methods

Immobilization of cells can be defined as restriction of cell movement by means of an insoluble support material without altering their metabolic activity. In general, immobilization systems provide several advantages : the reaction rate is accelerated due to the increased cell density per unit reactor; wash-out can be avoided; and the growth phase of the cells can be maintained. High cell activity and long term stability of immobilized cells can be achieved through suitable choice of support and immobilization method (Blanch, 1984; Scragg, 1988). According to Scragg (1988), several techniques are available for immobilization of cells, and they can be categorized into two basic groups : attachment and entrapment (Table 2.6).

Table 2.6 : Methods of immobilizing cells

Attachment	
without support	Aggregation or floc formation, crosslinking, covalent bonding.
with support	Adsorption to ion-exchange or to inorganic support, Biofilm formation.
Entrapment	Organic polymers, Inorganic polymer, Use of a semi-permeable membrane.

From : Scragg (1988)

2.5.3.1. Immobilization Without a Support

This technique was developed based on the properties of the cell itself, such as the tendency of some microorganisms like yeast to form aggregates or floc particles in suspension cultures. However, the link will not be adequate if it relies only on that phenomenon, so the technique usually needs the addition of chemical compounds to stimulate the aggregation or floculation.

Another approach to this method is cross linking between free amino and/or carboxyl goups which are present in the cell walls of the organism. However, as with aggregation or floculation, it also needs chemical reagents to stabilize the linkages. Usually coupling reagents like glutaraldehyde can

improve the strength of the linkage. However, this technique has never been employed widely, due to toxic effects of the chemical reagents on the microorganisms (Scragg, 1988).

2.5.3.2. Covalent binding

The principle of this method is to form a covalent bond between cells and support material. The bond is normally formed between a functional group of the support and amino or carboxyl groups on the cell wall. To stimulate the linkages, functional groups of the support are usually activated by coupling agents, such as aminosilane, isocyanate, carbodiimide, or glutaraldehyde. The coupling agents will place a specific group on the support surface, and these groups are later reacted with reactive groups on the cell surface.

This method is more suitable for enzyme immobilization rather than cell immobilization, due to the toxicity of the coupling reagents which can cause severe damage to the cell (Kolot, 1981; Blanch, 1984; Scragg, 1988).

2.5.3.3. Adsorption

The adsorption of cells onto an inert support material is the most economical procedure of immobilization. The link formed is dependent upon the carrier surface properties and the chemistry of the support, besides the characteristics of the cells themselves. Many microorganisms have a natural tendency to adhere to solid surfaces, and microbial cells can behave as cations

or anions depending on the pH of the medium. Based on these two characteristics, adsorption of cells onto inert supports can be manipulated. In addition, many microorganisms produce extracellular polysaccharides which can help to anchor the cells to the support, giving, in some cases, a very strong attachment.

Table 2.7 shows a variety of carriers for cell immobilization by adsorption methods. However, strict control of pH and ionic strength is often critically important for this procedure, because the charges on the cells and the support are reliant on them. Alteration of the pH will lead to the reduction of the adsorption level, and hence to elution of the immobilized cells (Blanch, 1984; Scragg, 1988).

Table 2.7 : Materials used for cell immobilization by adsorption.

Support	Cell type	Product
Cellex-E	*Azotobacter vinelandii*	-
Ion exchanger	*Saccharomyces cerevisiae*	Ethanol
Wood chips	*Saccharomyces cerevisiae*	Ethanol
Ceramics	*Acetobacter*	Acetic acid
Porous glass	*Saccharomyces carlsbergensis*	Beer
Controlled pore glass	Mixed culture	Methane
Fritted glass	*E. coli*	Biomass
Glass fibre pads	*Zymomonas mobilis*	Ethanol
Anthracite	*Pseudomonas sp*	Phenol degradation

From : Scragg (1988)

2.5.3.4. Entrapment

Entrapment is the most commonly used method of immobilizing both viable and non-viable cells. Immobilization by entrapment differs from the other methods, in that cells are free and it is possible to maintain their growth phase. However, they are restricted in movement by the lattice structure of a gel or by a semi-permeable membrane. Entrapment of cells can be done by either placing a cross-linked polymeric network around the cells, or by placing the cells inside polymeric materials followed by crosslinking of the polymer chains.

The porosity of the gel lattice or semi-permeable membrane is the most important aspect for determining the activity of the immobilized cells. The structure should be tight enough to prevent leakage of the cells, and at the same time should be able to facilitate free movement of the substrate to penetrate into the system and allow the products to come out from the barrier. It can be achieved by appropriate adjustment of the concentration of the polymers.

The advantages of this method are that the cells can multiply inside the support, and the system has a high stability, so wash out is less likely to occur even at a high dilution rate. However, the specific activity of the immobilized cells is usually lower than that of free cells (Blanch, 1984; Scragg, 1988).

2.5.4. Immobilized Cell Bioreactors

2.5.4.1. General

The main function of a properly designed bioreactor is to provide a controlled environment in order to achieve the optimal desired product formation by the particular fermentation culture employed (Scragg, 1988). In fermentation processes, the stirred vessel is the most frequently employed type of reactor. However, such reactors are not particularly suitable for the application of immobilized cells, due to the high shear rate which may lead to disruption of the immobilized bioparticles (Fonseca *et al*, 1986).

Bubble column, air-lift and fluidized-bed reactors are the most common examples of immobilized cell reactors. Aeration and mixing in these fermenters are provided by use of gas to circulate the culture within the reactor. The gas (usually air) is distributed through the reactor base, where the size of bubbles is controlled by means of a sparger and the air flow rate being used (Dieter Deckwer, 1985).

2.5.4.2. Citric Acid Production

The use of immobilized cell reactor for citric acid production has been investigated by several workers. Maddox and Kingston (1983) claimed that entrapment of *Saccharomycopsis (Syn. Candida) lipolytica* IFO 1658 in polyacrylamide gel did not reduce the cell activity, although the rate of citric acid production was only 1/7 of that of the free cells. However, the

immobilized cell activity was maintained for 45 days. Subsequently, Kautola *et al* (1991), using *Yarrowia (Syn. Candida) lipolytica* A-101, studied different carriers for immobilization by entrapment. In this work it was shown that the bead size had a strong influence on the productivity. A decrease in the Ca-alginate immobilized yeast bead diameter to one half increased the volumetric productivity by about three fold. The highest citric acid productivity, in repeated-batch shake flask experiments, was achieved using small 2 - 3 mm Ca-alginate beads. However, experiments in an air-lift bioreactor showed that K-carrageenan was slightly better as carrier than alginate. In addition, the influence of bead porosity, which is related directly to the pre-polymer concentration, has been studied by Rymowicz *et al* (1993), using *Y. lipolytica* A-101. They observed that increasing the alginate concentration reduced the citric acid yield. In this work, it was also observed that the column height - to - diameter ratio of an air-lift bioreactor had a significant influence on the citric acid productivity. Using continuous production in an air-lift bioreactor, the citric acid productivity was improved about 2.5 fold, compared to the maximum achievement in repeated-batch shake flask. The highest productivity in the air-lift bioreactor was maintained for 13 days at a dilution rate of 0.023 h^{-1}. However, increasing the dilution rate to 0.045 h^{-1} caused a significant reduction of the productivity. Meanwhile, Forster *et al* (1994), using *Y. lipolytica* EH 59 and *Y. lipolytica* H 181, reported that encapsulation of cells in sodium cellulose sulfate (CS) and poly (dimethyldiallylammonium chloride)

resulted in lower citric acid productivity and yield compared to the result using free cells.

Table 2.8 : Summary of published works of citric acid production using immobilized cell of *Candida lipolytica.*

Authors (year)	Reactor	Productivity (g/l.h)	Yield % (w/w)
Maddox &Kingston (1983)	Shake-flask	0.05	70
Kautola *et al* (1991)	Shake-flask	0.155	-
Kautola *et al* (1991)	Air-lift	0.120	-
Rymowicz *et al* (1993)	Air-lift	0.350	-
Forster *et al* (1994)	Fluidized-bed	0.125	25

Table 2.8 shows the comparison of citric acid productivity and yield obtained in various bioreactors, by a number of authors. It is shown that the highest citric acid productivity (0.35 g/l.h) was achieved with *S. lipolytica* entrapped in Ca-alginate, during fermentation in an air-lift bioreactor.

2.6. Objective of Present Work

The objective of the present work was to improve the performance of a strain of *C. guilliermondii* for citric acid production by means of immobilizing the cells by adsorption onto an inert support material.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Microbiological Media

Glucose was used as the carbon source while Yeast Nitrogen Base (YNB) without amino acids and ammonium sulphate (Difco laboratories, Detroit, Michigan, USA) was used as the basal medium. Sterile stock solutions of ammonium chloride, phosphate buffer, amino acids, YNB and antibiotics were added to a solution of glucose in distilled water, which had been sterilized by autoclaving at 121°C for 15 min. Tables 3.1 to 3.3 show the details of 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 agar plates used in strain isolation

Component	Concentration (g/l)
Glucose	36
Yeast Nitrogen Base (w/o amino acids and ammonium sulphate)	1.7
Ammonium chloride	0.53
Potassium di-hydrogen orthophosphate	1.3
di-potassium hydrogen orthophosphate	0.95
Histidine	0.01
Methionine	0.02
Tryptophan	0.02
Calcium carbonate	20
Agar	15

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 solution (pH 4 and 7); glucose; methanol; potassium dihydrogen orthophosphate; di-potassium hydrogen orthophosphate; calcium carbonate; formaldehyde solution.

-Ajax chemicals (Sydney, Australia)

agar

-Sigma chemical Co. (St Louis, Missouri, USA)

citric acid; kanamycin; streptomycin; DEAE-Sepharose.

Table 3.2 : Medium for inoculum preparation, shake flask culture and initial medium for bubble column culture.

Component	Concentration (g/l)
Glucose	36 or 50
Yeast Nitrogen Base	1.7
Ammonium chloride	0.53
Potassium di-hydrogen orthophosphate	1.3
di-potassium hydrogen orthophosphate	0.95
Histidine	0.01
Methionine	0.02
Tryptophan	0.02
Streptomycin	0.1
Kanamycin	0.02

Table 3.3 : Medium for batch fermentation

Component	Concentration (g/l)
Glucose	50 or 70
Yeast Nitrogen Base	1.7
Ammonium chloride	0.53
Potassium di-hydrogen orthophosphate	1.3
di-potassium hydrogen orthophosphate	0.95
Histidine	0.01
Methionine	0.02
Tryptophan	0.02

-Bevaloid chemicals Ltd (Levin, New Zealand)

bevaloid 6009 D antifoam

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

orthophosphoric acid

-Yellow Springs Instrument Co. Inc. (Yellow Springs, Ohio, USA)

YSI buffer for glucose analysis

-Difco Laboratories (Detroit, Michigan, USA)

Yeast Nitrogen Base without amino acid and ammonium chloride; potato dextrose agar (PDA).

-Polychem (Auckland, New Zealand)

ethanol.

3.1.4 Organism

The organisms used were *Candida guilliermondii* strain IMK1, re-isolated from a stock culture (Department of Process and Environmental Technology, Massey University, Palmerston North, New Zealand); and strain DT2 a mutant derivative of IMK1, which was isolated on a defined medium containing glucose (36 g/l) and calcium carbonate (20 g/l), (Table 3.1), following UV mutagenesis (see sections 3.5 and 3.6).

Stock cultures were maintained on slopes of potato dextrose agar supplemented with glucose (7 g/l), and stored in the cold room (4°C). Fresh cultures were prepared every 2 months for use in inoculum preparation.

3.1.5 Support Materials for Immobilization

Materials used were ; sawdust, from a cherry tree; glass beads of 4.5 - 5.5 mm diameter (BDH Ltd., Poole, England); and DEAE Sepharose Cl-6B (Sigma Chemical Co., St. Louis, Missouri, USA).

3.2. Media Sterilization

Yeast Nitrogen Base (without amino acids and ammonium sulfate), streptomycin, kanamycin, histidine, methionine and tryptophan were sterilized by filtration through a 0.45 µm membrane (Millipore Corporation, Bedford, USA). Ammonium chloride and phosphate buffer solution were sterilized by autoclaving at 121°C for 15 min. Glucose was sterilized in the fermenter vessel by autoclaving as for ammonium chloride and phosphate buffer.

Antifoam (10 g/l, bevaloid 6009 D) and 10 M KOH were sterilized by autoclaving at 121°C for 15 min.

3.3. Cleaning of Glassware

All glassware was washed in hot pyroneg solution, rinsed in tap water, then in distilled water, followed by hot air drying.

3.4 Analytical Methods

3.4.1 pH Measurement

pH measurements during the shakeflask and bubble column culture experiments were performed using a pH M 82 Standard pH meter (Radiometer, Copenhagen, Denmark). pH measurements during batch fermenter experiments were performed in situ using a Horizon pH controller model 5997-20 (Horizon Ecology Co., Chicago, Illinois, USA). The electrodes used were combination pH electrodes (Broadley James Corporation, Santa Anna, California, USA).

3.4.2 Determination of Cell Biomass

For all experiments, free cell biomass was determined by reading the absorbance of the culture at 600 nm using a PU 8625 UV/VIS spectrophotometer (Philips Analytical, York Street, Cambridge, Great Britain) following dilution in water to ensure that the absorbance stayed within the instrument range. The value was converted to biomass dry weight using a calibration curve. The calibration curve was prepared by centrifugation of 50 ml of defined sample, in duplicate, for 10 minutes at 4200 rpm using a BGH Hermle Z 320 centrifuge (Berthold Hermle GmBH and Co., Gosheim, Germany) followed by resuspending twice in distilled water and re-centrifugation, prior to drying overnight at 105°C.

Immobilized cell biomass from bubble column cultures was determined by counting the colony number of cells stripped off from the support material

followed by spreading on PDA plates and incubation at 30°C for 5 days. The colony count was converted to dry weight using a conversion factor. The factor was estimated by spreading a sample of known cell concentration on PDA plates and counting the colony number after incubation.

3.4.3 Citric Acid Determination

Determination of citric acid concentration was carried out by High performance liquid chromatography (HPLC) using a model ALC/GPC 244 liquid chromatograph with a model 6000 A solvent delivery system and a U6K septumless injector (Waters Associates Inc., Millipore Corporation, Milford, Massachusetts, USA).

A C-18 reverse phase column,4.6 mm X 220 mm, (Brownlee, San Fransisco, USA), was used for the analysis. The detector was a model 401 differential refractometer, (Waters Associates), and 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 orthophosphate, prepared using Milli-Q deionized water and adjusted to pH 2.45 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 the samples. Calculation of citric acid concentration was by comparison of peak area.

3.4.4 Glucose Determination

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

3.4.5 Microscopic Observation

Microscopic observation was carried out using an olympus CH microscope, Olympus optical Co., Japan.

3.5 Mutation Procedure

3.5.1 Preparation of Cells

Cells for mutation were prepared by growing strain IMK1, (which had previously been reisolated on plates of a defined medium (Table 3.1)), in a 250 ml conical flask containing 30 ml of synthetic medium (Table 3.2). After incubation at 30°C for 24 hours at a shaker speed of 200 rpm (see section 3.9.2), the cells were collected by centrifugation at 5000 rpm using a Clandon T 52 centrifuge (Clandon Scientific Ltd., Hants, UK.), followed by rinsing twice with sterile saline solution (7 g/l NaCl). The cells were then resuspended in 10 ml sterile saline solution and placed in a sterile petri dish.

3.5.2 Mutation

Mutation was carried out by exposing the cell suspension to UV light at 254 nm (Gelman Sciences Pty Ltd, Lanecove NSW, Australia). The cell

suspension was placed 30 cm below the UV light for 5 min, followed by placing in the dark for 20 min. The culture was then diluted and spread onto plates of defined medium supplemented with calcium carbonate (20 g/l) (Table 3.1).

The exposure time of five minutes was the time needed to reduce the cell number by 2-3 log cycles, and was determined previously from a death curve.

3.6. Isolation of Mutant

Mutants were isolated following observation of the colonies on a defined medium (Table 3.1). Since the desired activity was acid production, a clear zone was formed around colonies which produced citric acid, due to dissolution of calcium carbonate by the acid. The biggest and the fastest clear zone-producers were isolated and maintained as working cultures.

3.7 Preparation of Samples

3.7.1 Sample Preparation for HPLC Analysis

Cells were removed from cultures by centrifugation for 5 min at 4200 rpm using a BHG, Hermle Z 320 centrifuge (Berthold Hermle GmbH and Co, D-7209 Gosheim, Germany). The supernatant liquid was filtered through a 0.45 µm membrane. For citric acid analysis the sample was acidified by adding 10% (V/V) orthophosphoric acid.

3.7.2 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 linear operation range (0 - 5 g/l).

3.7.3 Sample Preparation for Count of Immobilized Cells.

Wet sawdust from bubble column cultures was collected aseptically following separation from the liquid, and gently rinsed with sterile water to remove free cells. Three grams of wet sawdust were added to 30 ml sterile peptone water, and blended in a stomacher (Lab-blender 400, Seward Laboratory, UAC House, Black friars Road, London) for 1 min. The cell suspension was then shaken well and diluted prior to spreading on PDA plates for a colony count.

The moisture content of the sawdust was determined by drying 10 g of wet sawdust at 50°C for 24 h (to constant weight).

3.8. Cell Immobilization

3.8.1 Preparation of Support Materials for Immobilization

A. Glass beads

Five grams of glass beads were washed twice with distilled water followed by autoclaving at 121°C for 15 min, prior to addition into shakeflask culture.

B. DEAE Sepharose

DEAE sepharose (5 g) was treated in an identical manner to glass beads before being added into shakeflask culture.

C. Sawdust

The sawdust was sieved to a size range of 420 - 600 µm (Endecott Test sieve shaker, Endecotts Ltd, London, England). The particles were then held in water at 60°C for an hour, followed by washing three times with distilled water, to remove water-soluble compounds from the sawdust which might inhibit the growth of yeast.

Following autoclaving at 121°C for 15 min, the sawdust was added to shakeflask culture or packed into the bubble column reactor.

3.8.2. Cell Immobilization for Shakeflask Culture

Cell immobilization for shakeflask culture was performed by pouring sterilized support material into late exponential phase cultures. After incubation for 24 h, the liquid containing free cells was removed by decanting, and the support materials were washed twice with sterile distilled water prior to addition of fresh medium.

3.8.3 Cell Immobilization for Bubble Column Cultures

The sterilized column reactor was packed with sterile sawdust, and a 50 ml inoculum was pumped into the column followed by about 50 ml of fresh

medium. The reactor was then allowed to stand, as a batch culture, with aeration, for about 20 hours, to allow the cells to attach to the sawdust particles.

3.9 Culture Conditions

3.9.1 Inoculum Preparation

A 250-ml shakeflask containing 50 ml of synthetic medium (Table 3.2) was inoculated with one loopfull of cells taken from the stock culture. The culture was then incubated at 30°C on a Gallenkamp orbital incubator, (Watson Victor, Ltd, New Zealand), until late exponential phase (generally 40 to 48 hours).

3.9.2 Shakeflask Culture Using Freely Suspended Cells

Experiments were conducted in 250-ml erlenmeyer flasks containing 50 ml of medium (Table 3.2). One loop-full of inoculum from late exponential phase (section 3.9.1) was added to each flask. Flasks were incubated at 30°C in a Gallenkamp orbital incubator at an operating speed of 200 rpm. Culture pH was adjusted twice daily to pH 5.2 for the first two days, then maintained around pH 4.2, by addition of 10 M KOH. Samples were taken twice daily.

3.9.3 Batch Fermenter Culture Using Freely Suspended Cells

Batch fermentation experiments were carried out using a Multigen benchtop culture apparatus (Figure 3.1). A 2-litre capacity glass jar (New

Brunswick Scientific Co, New Brunswick, New Jersey, USA.) 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 could be obtained using an electronic controller.

The fermenter temperature was maintained at 30°C by means of a thermocouple inserted into a close-ended metal tube inserted into the fermenter head, connected to an electronic thermostat which controlled a heating element inserted into another closed metal tube in the head. Temperature was continously recorded using a Honeywell versaprint multipoint chart recorder (Amiens, France).

Air supply to the fermenter was taken from the Massey University compressed air line, through a pressure controller (Norgren, Martonair (NZ), Ltd, Auckland), where pressure was maintained at 150 KPa, to a gap meter (Gap, Basingstoke, England) to regulate the flowrate. The gap meter maintained the air flow to the fermenter at 0.6 l/min. The air then passed through a sterile packed cotton wool filter and entered the vessel through a diffuser. Exhaust air passed through a sterile cotton wool filter.

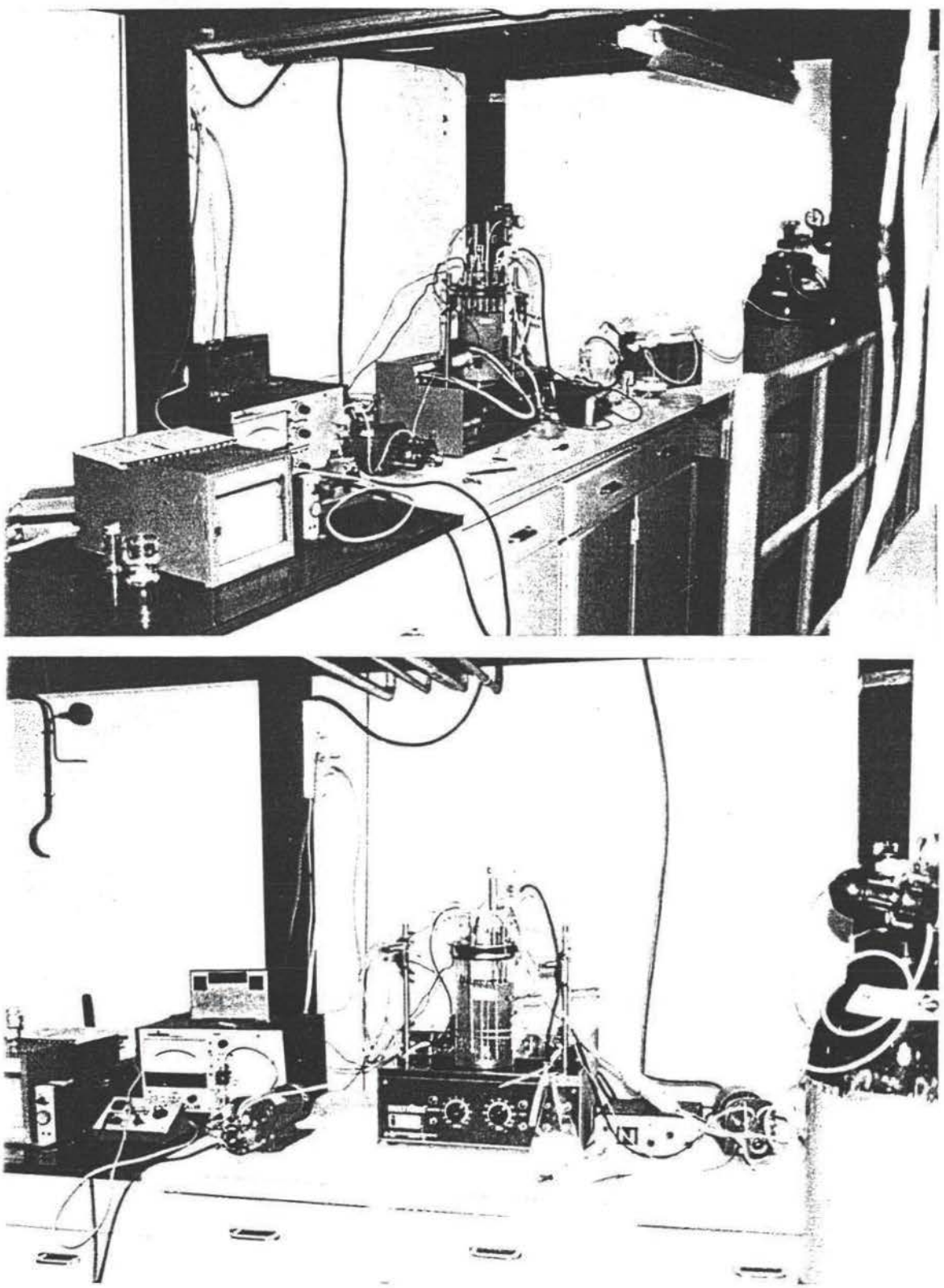
Culture pH was measured using a combination pH electrode, connected to

a pH controller. The pH controller automatically controlled the culture pH by connection to a masterflex peristaltic pump, size 13 pump head (Cole Parmer Instrument Co, Chicago, Illinois, USA) which would dose 10 M NaOH when the culture pH fell below the set point (pH 5.3 for the first 48 h, then changed to pH 4.3 for the rest of fermentation). The pH controller was also connected to the Honeywell versaprint multipoint chart recorder and culture pH was continously recorded. The pH value of the samples was regularly checked by an independent pH meter and any disrepancies 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 6009 D antifoam to control any foaming problems.

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 of the submerged sample line. A sample size of 10 ml was taken.

Figure 3.1 : Two photographs of the batch fermenter, and its ancillary equipment.



3.9.4 Repeated Batch Culture Using Immobilized Cells

These experiments were carried out in a similar manner to shakeflask experiments using free cells, but the cells were immobilized onto support materials. The medium was changed by decanting the liquid followed by rinsing the support particles twice with sterile water to remove any unattached cells. Fresh medium was then added, and the cultures were reincubated as before.

3.9.5 Bubble Column Culture Using Immobilized Cells

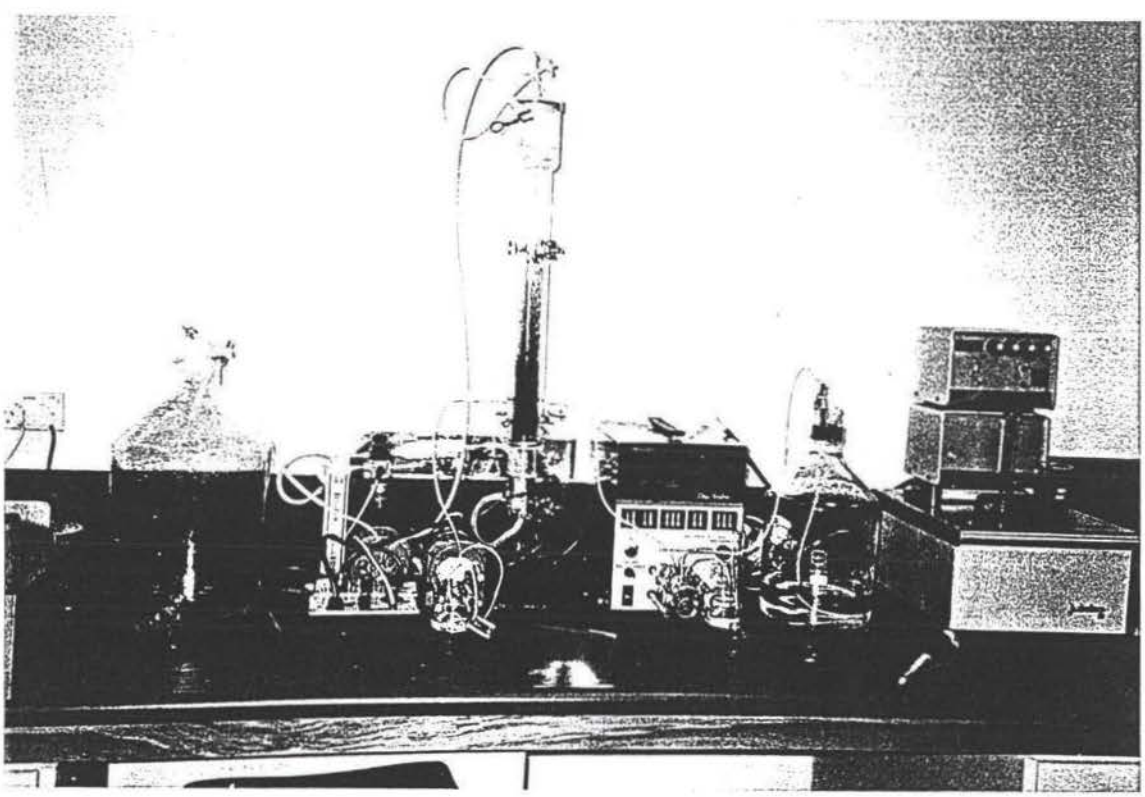
The experiments were carried out using an acrylic column reactor, with a total volume of 250 ml (Figure 3.2), (Mechanical workshop, Process and Environmental Technology Department, Massey University, New Zealand). Medium was pumped continously into the column by a masterflex digi-staltic pump (Masterflex, Cole Parmer Instrument Co., Chicago, Illinois, USA) with a size 13 pump head. A double pump head was used to check the dilution rate, by means of one head being used for the medium supply line and another for measuring the pump flow rate.

The air supply was controlled and filtered as for batch fermenter culture (section 3.9.3), and fed into the bottom of a central draught tube through a sparger at a flow rate of 0.6 l/min. Exhaust gas escaped through the top of the column. Aeration provided both the agitation to bring about the mixing of the culture and dispersal of the oxygen into the broth.

A 250-ml working volume was maintained by setting the outlet tube at certain height, while the outlet pump flowrate was always faster then the medium supply rate. A metal filter was used to prevent the escape of support material from the reactor.

The culture temperature was maintained at 30°C using a water jacket where the water was circulated by a Julabo HP water circulator, and the water temperature was controlled by Julabo HTC temperature controller (Julabo

Figure 3.2 : A photograph of the bubble column reactor, and its ancillary equipment.



Labortechnik GmBH, D-7633 Seelbach, Germany).

Samples were taken twice daily by redirecting the effluent line to the sampling line.

3.9.6. Sterilization of Fermentation Equipment

The fermenter vessel for batch experiments, containing glucose and all the ancillary systems except the pH probe and the condenser, was sterilized by autoclaving at 121°C for 15 min.

The pH probe and condenser were sterilized by immersing in 50% (v/v) 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 min. Cotton wool filters were sterilized at 160°C for 20 h.

The column reactor for bubble column cultures was sterilized by passing a 2% formaldehyde solution through the system for 5 h, followed by thorough rinsing with sterile distilled water.

CHAPTER 4

STRAIN IMPROVEMENT STUDIES USING UV LIGHT MUTATION

4.1. Introduction

There is a general agreement that the effectiveness of a fermentation process depends mainly on the strain being used and the fermentation system itself. It was desired in this thesis to develop a citric acid production process using an improved strain, and to use the improved strain as an immobilized cell culture in a continuous fermentation system. The first step in the study was to mutagenise *Candida guilliermondii* using UV light, and to evaluate the resulting mutants for citric acid production in shake flask and batch fermenter culture.

4.2. Reisolation and Mutation of *Candida guilliermondii* IMK1

Since storage might have caused a degeneration of the cells' metabolic activity, and based on the hypothesis that not all of the cells present in the culture possess the same metabolic activity, reisolation of the yeast strain *C. guilliermondii* IMK1 from a stock culture was performed to select a single colony which produced citric acid in the highest concentration and in the shortest time. On the basis of formation of clear zones on plates of a defined medium supplemented with calcium carbonate (Table 3.1), a single colony of

C. guilliermondii was reisolated after 7 days of incubation at 30°C.

In an attempt to improve the citric acid productivity, UV light irradiation was applied to the reisolated cells, followed by an identical isolation procedure as for reisolation of *C. guilliermondii* IMK1. Two mutant strains, named DT1 and DT2, were isolated after 6 days of incubation at 30°C, and were maintained as working cultures on slopes of PDA at 4°C.

4.3. Studies of Mutant in Shake flask Fermentation

A comparative study of the two selected mutants DT1 and DT2 with the parent strain IMK1 was conducted in shakeflask culture with freely suspended cells using the medium described in Table 3.2 containing 36 (g/l) glucose. Figure 4.1 demonstrates that the parent strain, IMK1, and the two mutants, DT1 and DT2, grew in an almost identical manner , and produced similar final biomass concentrations (3.88 g/l, 3.37 g/l and 3.45 g/l for IMK1, DT1 and DT2 respectively). The maximum specific growth rates were also about the same (0.12 h^{-1}) for the three strains.

The two mutants were superior to the parent in respect of their abilities to produce citric acid (Figure 4.2). The highest citric acid concentration (9.2 g/l) was obtained using strain DT2, after 133 h of cultivation, while DT1 produced 7.1 (g/l) citric acid after the same fermentation time. The lowest citric acid concentration (4.9 g/l) was achieved

Figure 4.1 : Growth curves of IMK1, DT1 and DT2 in shake flask cultures

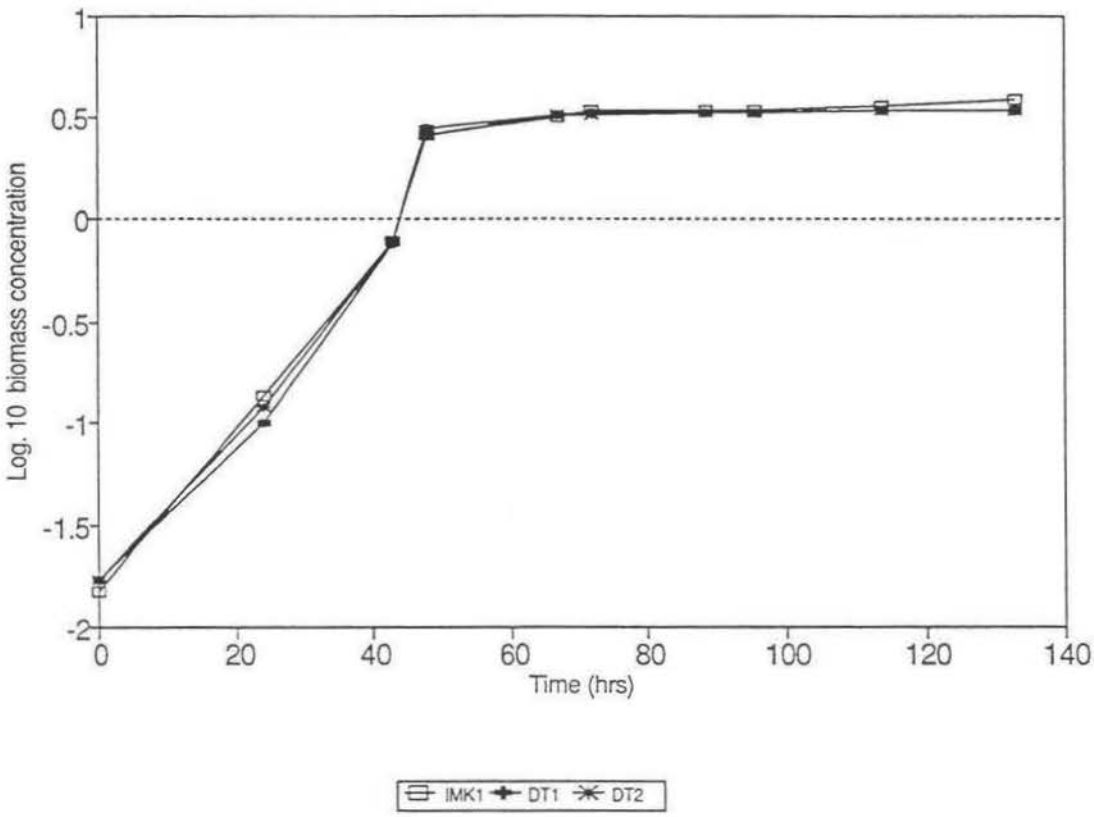


Figure 4.2 : Comparison of citric acid production in shake flask culture

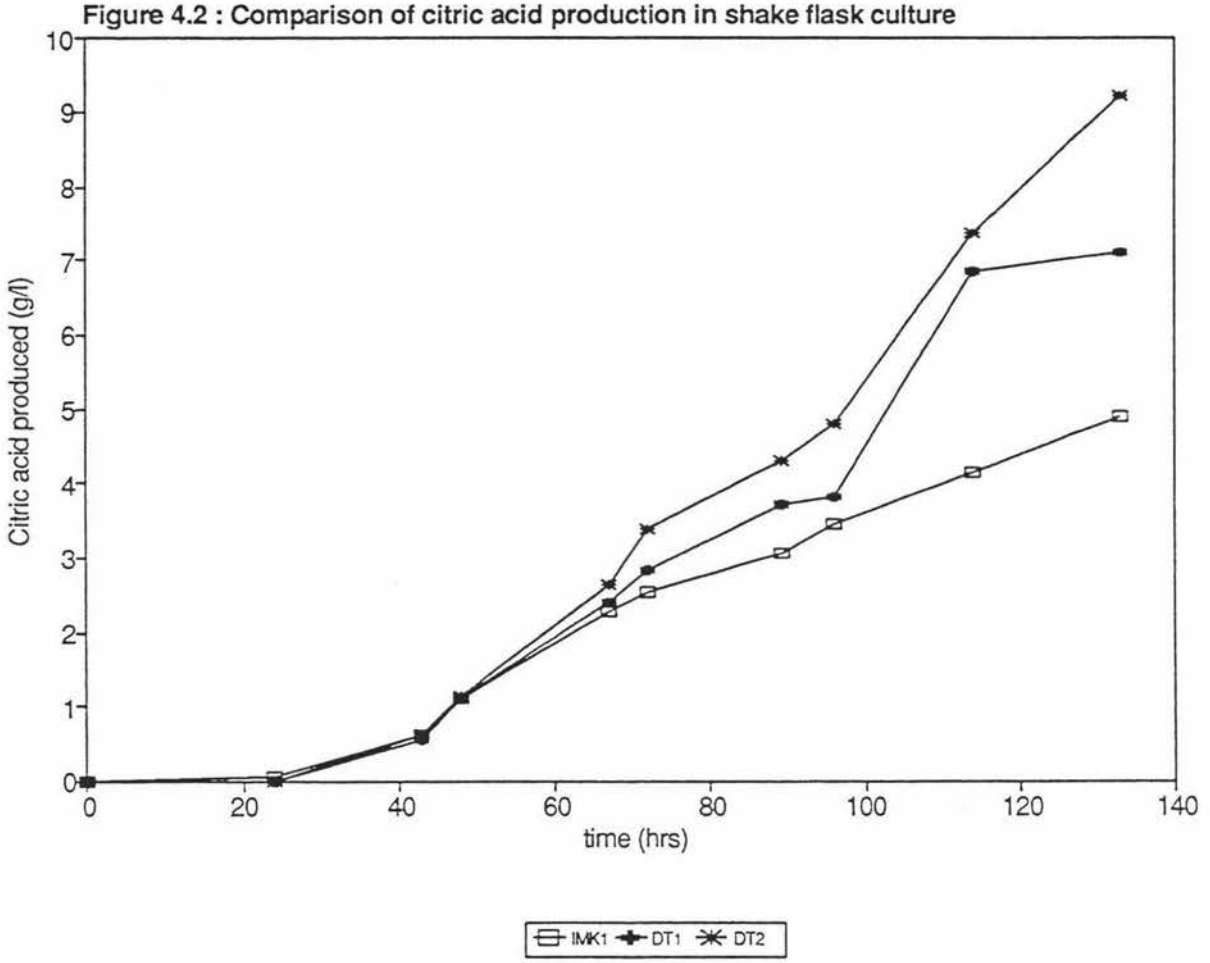
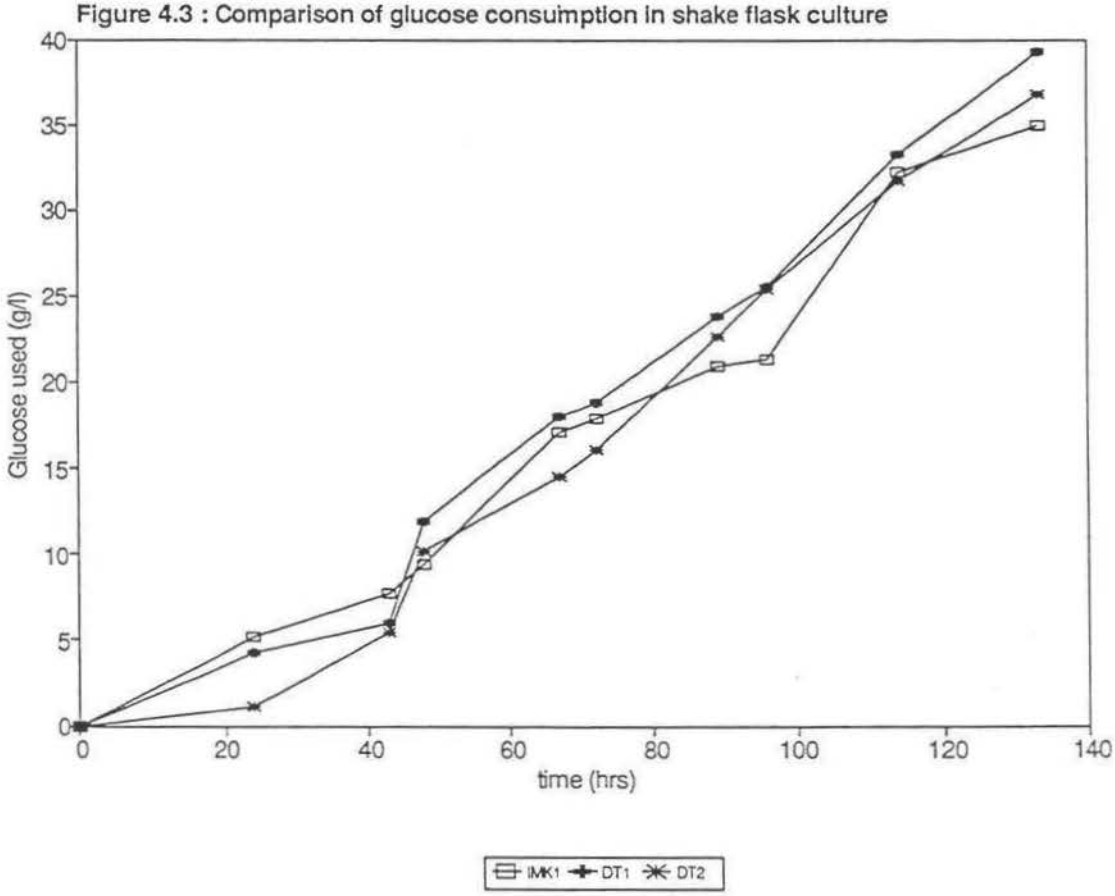


Figure 4.3 : Comparison of glucose consumption in shake flask culture



by the parent strain, IMK1. However, the citric acid production patterns were basically similar for the three strains, with the bulk of the production occurring after the exponential growth phase.

The glucose analysis result (Figure 4.3) illustrates that the glucose was essentially exhausted from the media after 133 h of fermentation, and the overall glucose consumption rates were 0.263 (g/l.h), 0.295 (g/l.h) and 0.277 (g/l.h) for IMK1, DT1 and DT2, respectively. Citric acid yields, based on sugar utilized, were 14 % (w/w), 18 % (w/w) and 25 % (w/w) for IMK1, DT1 and DT2 respectively.

The mutant strain DT2 showed the highest overall citric acid productivity value of 0.07 (g/l.h), compared with 0.05 (g/l.h) for DT1 and 0.04 (g/l.h) for IMK1. Hence, this mutant was selected for use in further studies.

4.4. Studies of Mutant in Batch Fermenter

The previous sub-chapter (4.3) reported the study of an improved strain of *C. guilliermondii* in shakeflask culture. This strain was now studied in batch fermenter culture in an attempt to provide better conditions for production of citric acid.

In order to observe the effect of glucose concentration and to provide a comparison with shakeflask culture, two runs of batch fermentation were carried out under otherwise identical conditions. The first run was performed using a normal medium with 50 (g/l) glucose (Table 3.3), while for the second

Figure 4.4 : Growth curve of strain DT2 in batch culture (glucose 50 g/l)

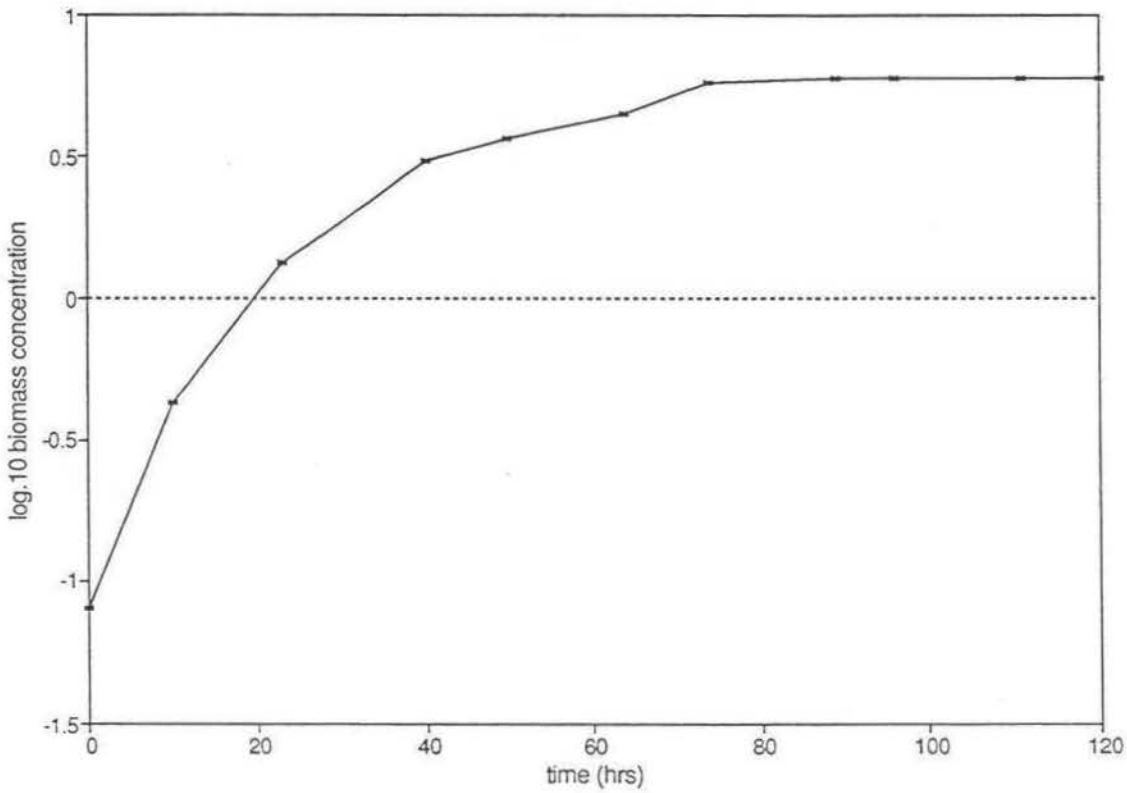


Figure 4.5 : Growth curve of strain DT2 in batch culture (glucose 70 g/l)

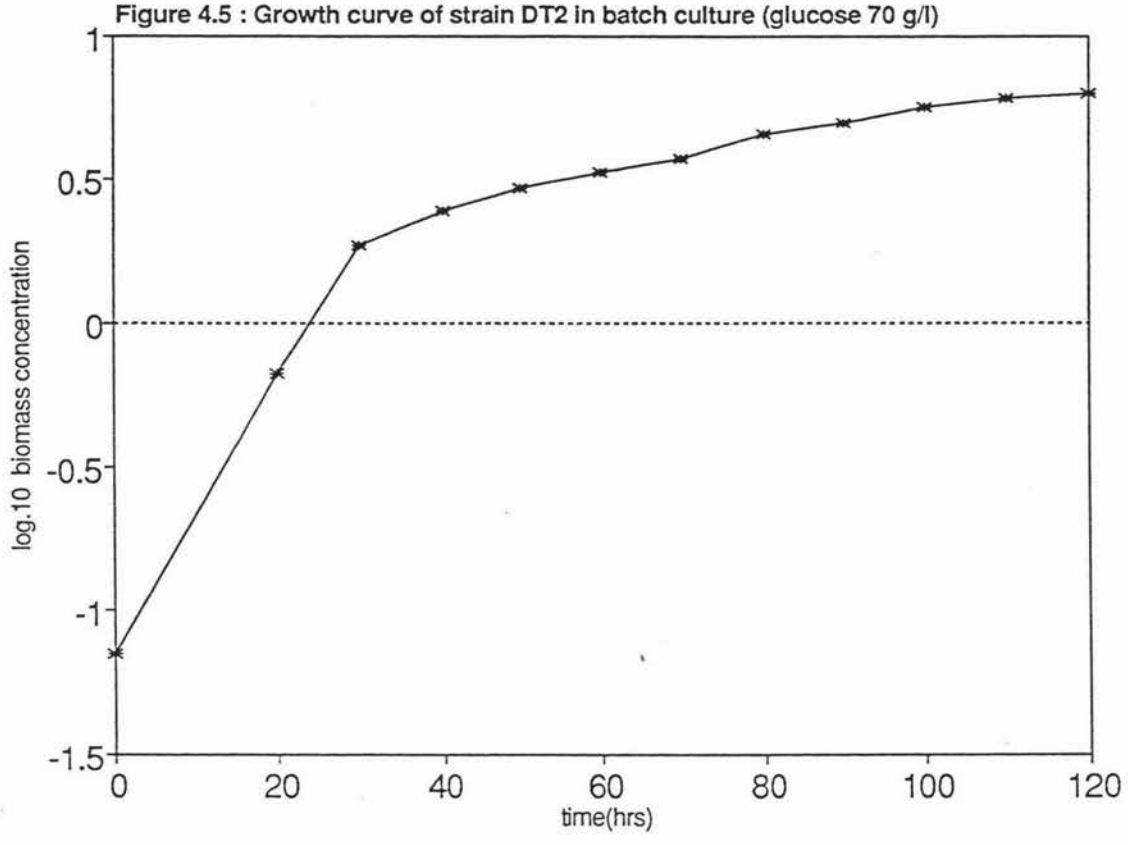


Figure 4.6 : Citric acid production and glucose utilization by strain DT2 in batch culture with an initial glucose concentration of 50 g/l

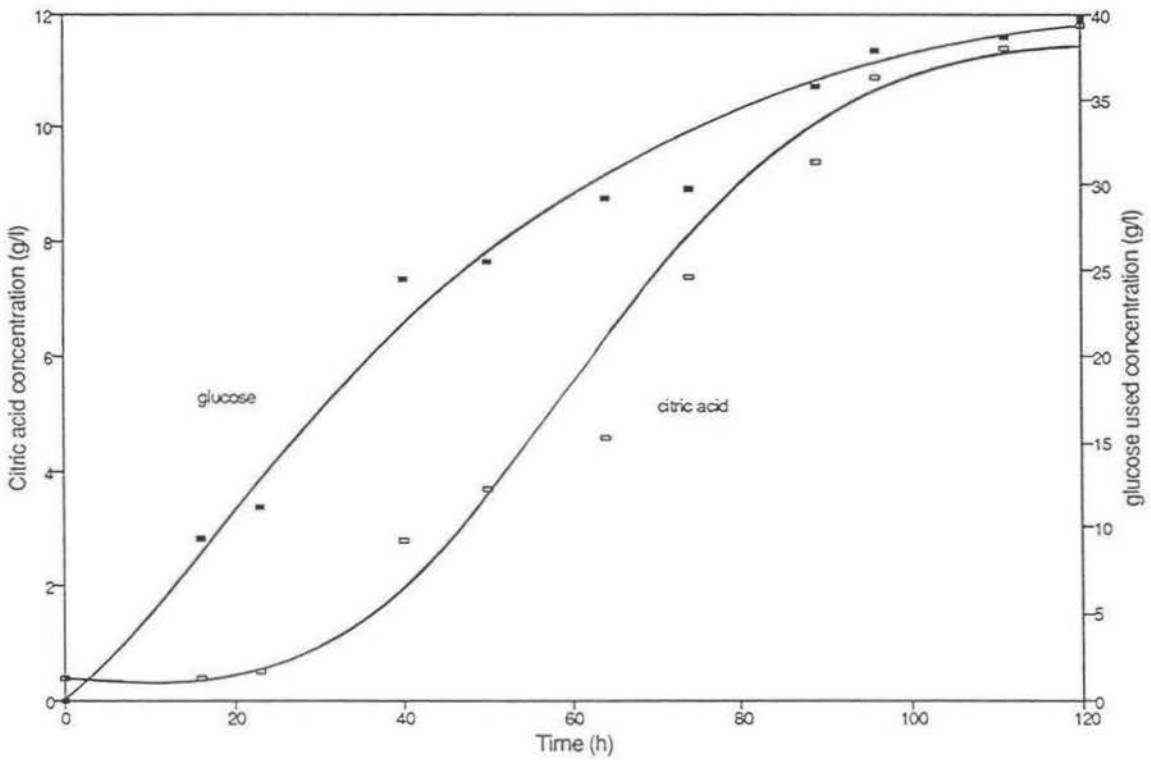
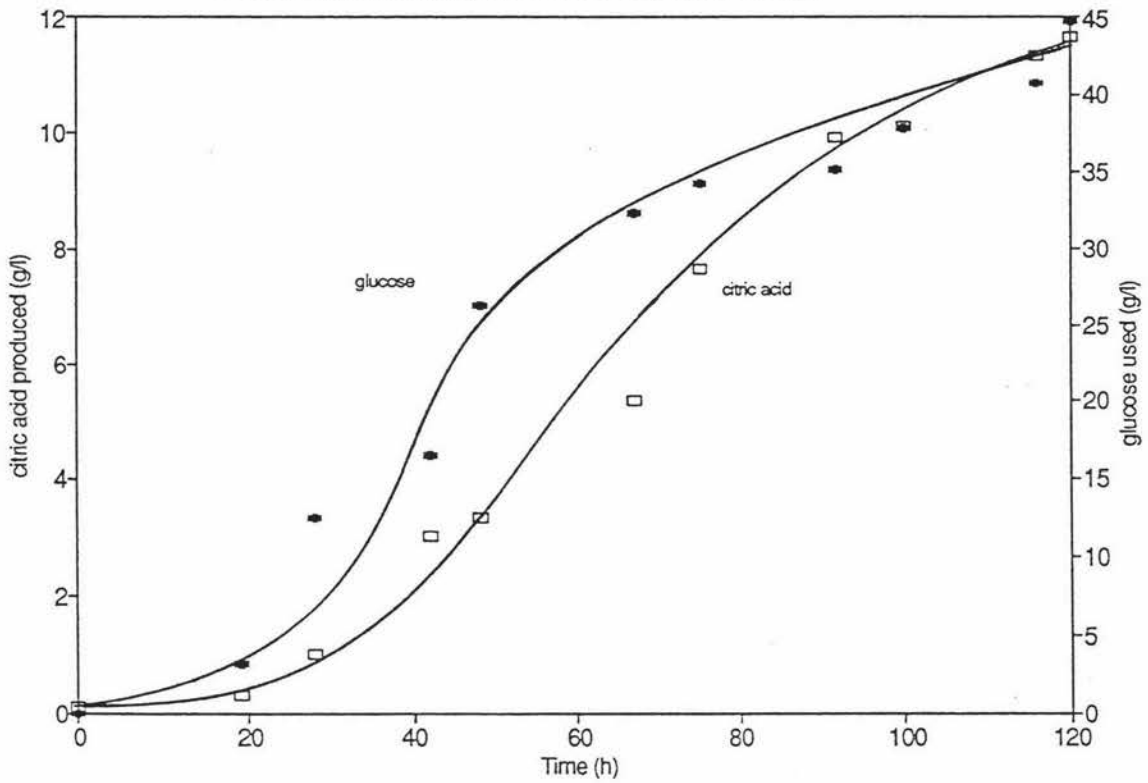


Figure 4.7: Citric acid production and glucose utilization by strain DT2 in batch culture with an initial glucose concentration of 70 g/l



run the glucose concentration was increased to 70 (g/l). The experimental results (Figures 4.4 to 4.7) demonstrate that the increased glucose concentration had no significant influence either on the biomass growth rate or product formation rate. In both experiments the maximum specific growth rate of strain DT2 was the same as for shakeflask culture, i.e. 0.12 h^{-1}, with a maximum biomass concentration of 6.1 (g/l) and 6.3 (g/l) for the first and second runs respectively.

Since the batch fermenter cultures provided a better environmental control, such as culture pH value, aeration rate and mixing, it allowed the culture to perform better than in shakeflask . The reactor productivities in batch fermenter culture were significantly higher than in shake flask. Thus, the overall citric acid productivities in batch fermenter culture were 0.098 (g/l.h) and 0.097 (g/l.h) in media with 50 (g/l) glucose and 70 (g/l) glucose, respectively. In contrast, the reactor productivity in shakeflask culture was 0.07 (g/l.h), with an initial glucose concentration of 36 (g/l).

Figures 4.8 and 4.9 demonstrate that the citric acid production rate during the two batch fermentation experiments followed similar patterns, where the highest instantaneous rates, 0.18 (g/l.h) and 0.17 (g/l.h) representing specific production rates of 0.05 and 0.04 (g citrate/g biomass.h), with initial glucose concentrations of 50 g/l and 70 g/l respectively, occured around the middle of the fermentation period.

Citric acid yields, based on the weight of product formed per sugar used,

Figure 4.8 : Citric acid production rate during batch fermentation with an initial glucose concentration of 50 g/l

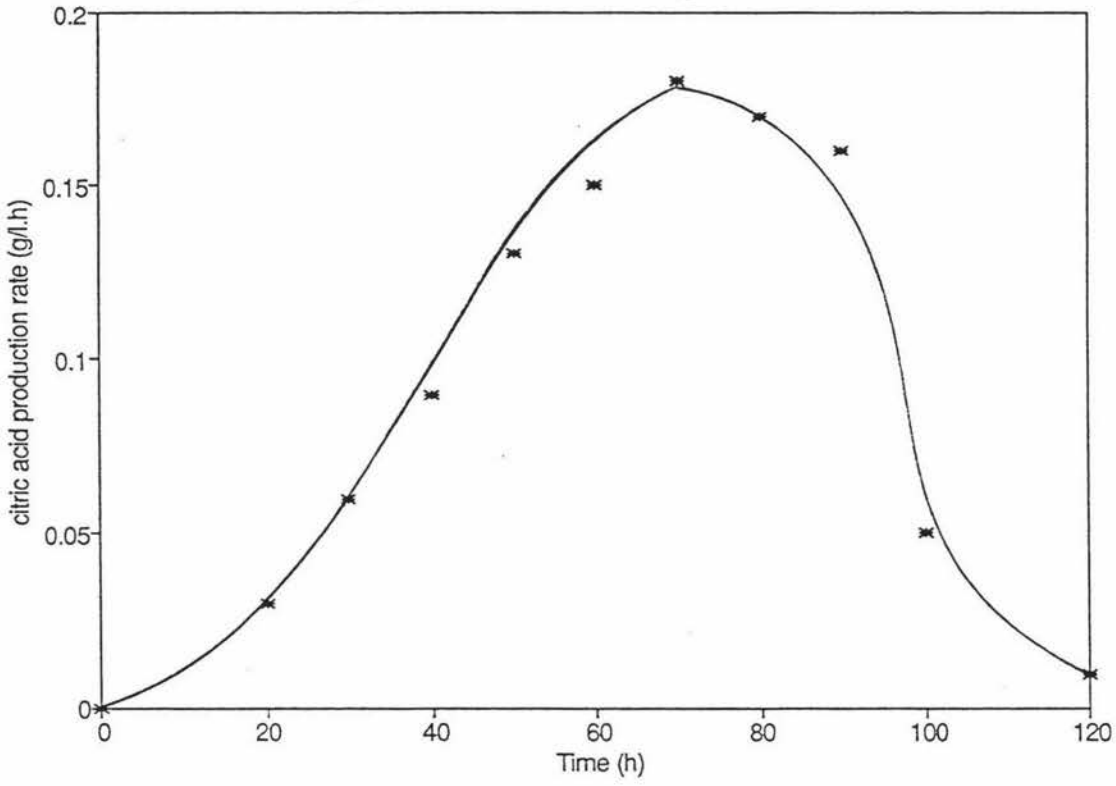
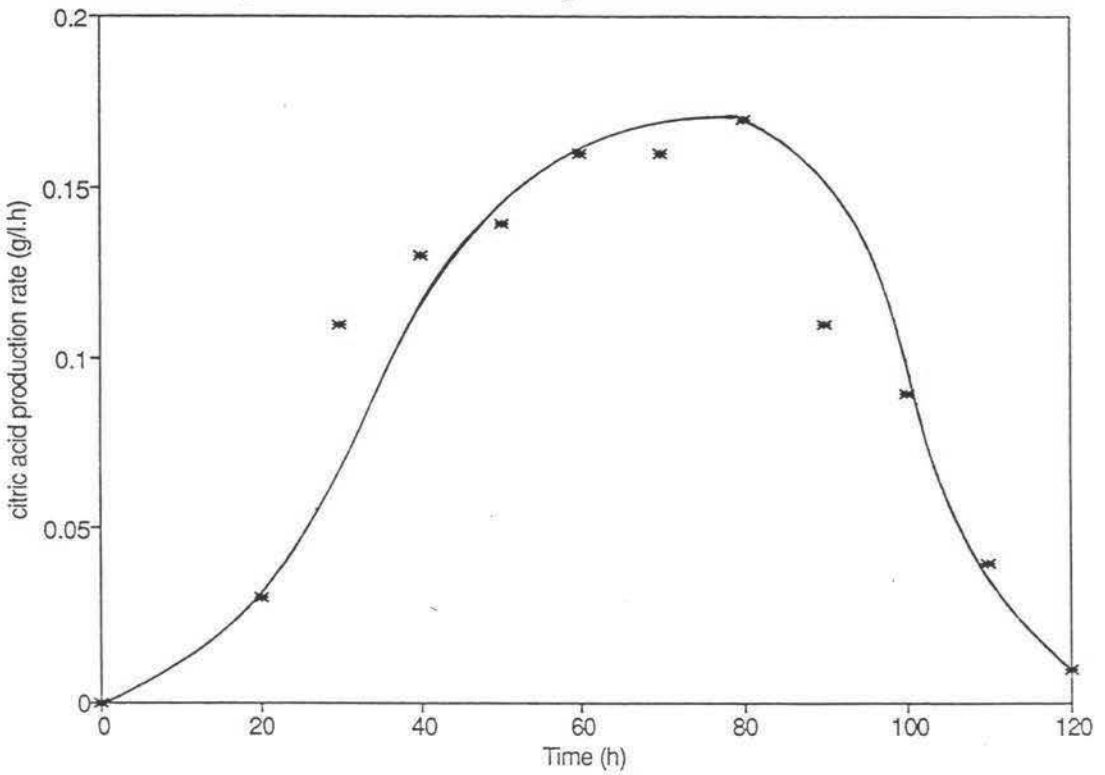


Figure 4.9 : Citric acid production rate during batch culture with an anitial glucose concentration of 70 g/l



were 33 % (w/w) and 28 % (w/w) for the batch fermenter cultures starting with 50 (g/l) glucose and with 70 (g/l) glucose respectively. In comparison, the yield in shakeflask was 25 % (w/w).

Table 4.1 shows a summary of the comparison between shakeflask and batch fermentation cultures for strain DT2.

From the results of these experiments, it was apparent that the shake flask experiment with a mutant strain DT2 could be scaled-up to a laboratory scale batch fermenter. Moreover, the bigger scale and better control allowed higher reactor productivities to be achieved.

Table 4.1 : Comparison between shakeflask and batch fermenter cultures.

Fermentation system	Citric acid Yield % (w/w)	Overall citric acid productivity (g/l.h)	μ max (h^{-1})
Shake flask	25	0.069	0.12
Batch culture (w/ 50 g/l glucose)	33	0.098	0.12
Batch culture (w/ 70 g/l glucose)	28	0.097	0.12

4.5. Discussion

The aim of the work described in this chapter was to isolate a mutant strain of *C. guilliermondii* which has a better ability than the parent, in terms of citric acid production, and then to test this mutant in a laboratory-scale batch fermenter, in order to obtain "baseline" data prior to the development of an immobilized cell culture system.

The UV light mutation gave a significant improvement for the *C. guilliermondii* strain, though no appropriate explanation can be given for this phenomenon. Nevertheless, this result demonstrates that the concept of manipulating the strain by using UV light mutagenesis remains valid. The selected mutant strain was tested in a laboratory-scale batch fermenter to study its performance under suitable environmental conditions. These conditions were selected based on recent work performed in this laboratory with strain IMK1 (Roberts-Thomson, 1993). While it is possible that the optimum conditions for strain DT2 may be different to those for strain IMK1, further studies in batch fermenter culture were considered inappropriate.

The reactor yields and productivities in batch fermenter culture experiments were higher than those observed in shakeflask culture. Fluctuations in the culture pH during fermentation in shake flask, due to insufficient adjustment during the cultivation period, may also have been a factor.

4.6. Conclusion

The ability of the yeast strain *C. guilliermondii*, in term of citric acid production, can be improved by the use of a UV light mutation method. In respect of citric acid production, the mutant strains DT1 and DT2 were superior to the parent strain IMK1, though in general they behaved similarly during their growth on a defined medium.

Two experiments in batch fermenter culture have proved that it is

possible to succesfully scale-up the cultivation of *C. guilliermondii DT2* from a shakeflask culture to 1.5 l batch fermentation, and the kinetic data obtained will provide a reference point by which to judge the development of an immobilized cell reactor system.

CHAPTER 5

USE OF IMMOBILIZED CELLS OF STRAIN DT2 IN SHAKE FLASK CULTURES

5.1. Introduction

Following the success of improving the catalytic capability of the yeast strain *C. guilliermondii* using UV light mutagenesis, work continued with cell immobilization of the mutant strain DT2.

The majority of the published work on citric acid production using immobilized yeast refers to the entrapment or encapsulation of *C. (Yarrowia) lipolytica* using a variety of polymers (Maddox & Kingston, 1983; Kautola *et al* ., 1991; Rymowicz *et al*., 1993; Forster *et al*, 1994). There has been no work reported so far on the study of immobilized *C. guilliermondii* for citric acid production and little on the use of adsorption as the immobilization technique. Therefore, an attempt was made to investigate the effect of immobilization on citric acid production using the mutant strain *C. guilliermondii* DT2.

Since adsorption is the simplest technique of whole cell immobilization, it was desired to immobilize the cells via adsorption onto a cheap and readily available solid material. Then the effects of nitrogen (N) and phosphate (P) concentrations on citric acid production would be investigated, as a possible means of stimulating the production of citric acid, and also to control

contamination by limiting these two components in the medium.

5.2. Selection of Support Material

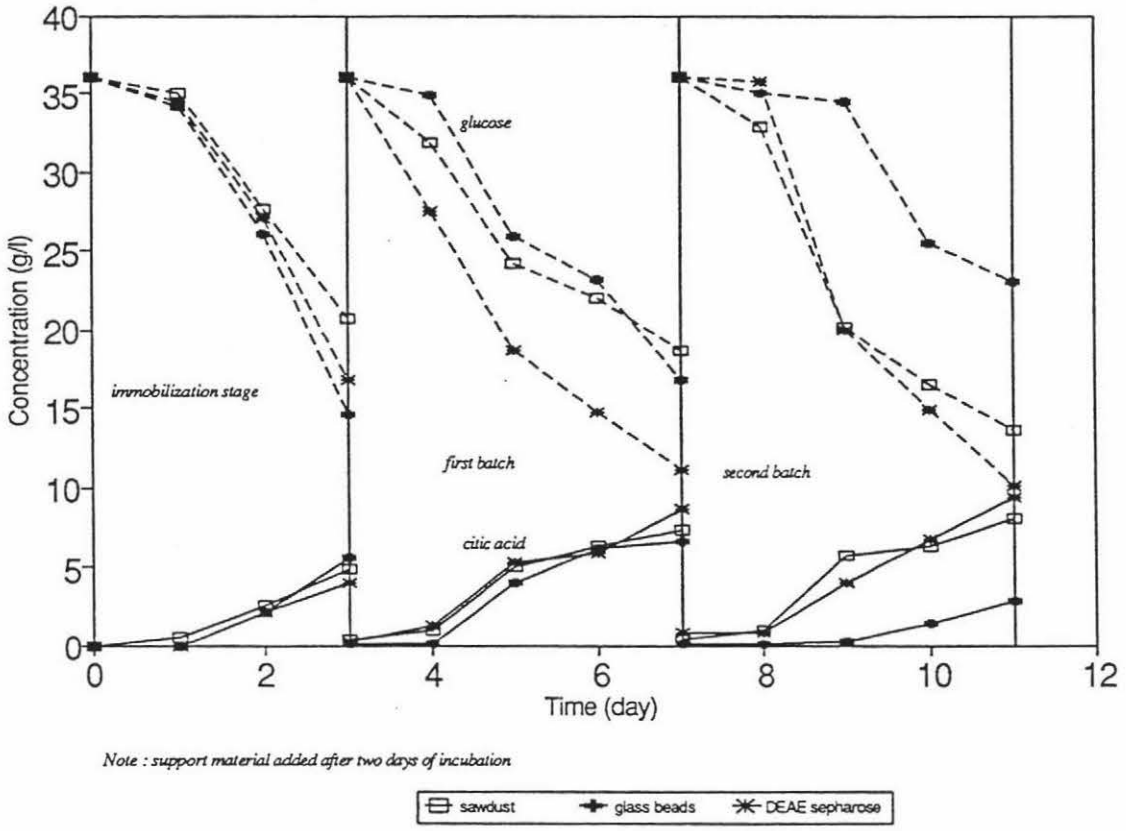
The most common reason given for not using the adsorption method to immobilize cells, is that it often lacks a linkage between the cells and the support. However, with an adequate selection of the support material, it should be possible to obtain satisfactory stability of an immobilized cell system. Thus three alternative support materials were studied, i.e. sawdust, glass beads and DEAE-sepharose, in repeated-batch shake flask culture. In order to determine the stability and efficiency of the bioparticles obtained by immobilizing the yeast strain *C. guilliermondii* DT2 via adsorption, citric acid production was used as the barometer. Two different concentrations of ammonium chloride in the media were used to provide a comparison, and also to estimate the influence of freely suspended cells on the system, since at lower level of nitrogen cell growth, and thus release of freely suspended cells, should be restricted.

The amount of support material used in each flask was 2.5 g for sawdust and 5 g for glassbeads and DEAE-sepharose. Initial experiments were performed using the medium described in Table 3.2 with a glucose concentration of 36 g/l and an ammonium chloride concentration of 0.53 g/l. After inoculation of the flasks, the support materials were added after 2 days of incubation, and the medium was changed after 3 and 7 days of incubation.

Figure 5.1 shows the accumulation with time of citric acid and the utilization with time of glucose. Using glass beads as the support material, both citric acid production and glucose utilization decreased markedly after the second medium change. Thus the citric acid concentration after 4 days in the first batch was 6.6 g/l while it was only 2.8 g/l after 4 days in the second batch. Similarly, glucose utilization dropped from 19.3 g/l in the first batch to 13 g/l in the second. Meanwhile the product formed and the sugar utilized during four days cultivation in the first and second batches using cells immobilized onto sawdust and DEAE-sepharose showed no significant differences. This indicated that the cells were attached in sufficient strength onto the particles of sawdust and DEAE-sepharose, whereas the decline of sugar utilization during the cultivation of immobilized cells on glassbeads indicated that the cell concentration was reduced, probably due to wash-out.

Table 5.1 shows that the highest yields (based on gram citric acid produced per gram of glucose used) in the two batches were achieved using cells immobilized onto sawdust, whereas the highest productivity (0.09 g/l.h) was reached using cells immobilized onto DEAE-sepharose.

Figure 5.1: Evolution of citric acid and glucose concentrations during repeated batch shake flask cultures using defined medium containing 0.53 (g/l) nitrogen.



Note : support material added after two days of incubation

Table 5.1 : Comparison of the productivities and yields of cells of DT2 immobilized onto various support materials, with an initial ammonium chloride concentration of 0.53 (g/l) (based on Figure 5.1).

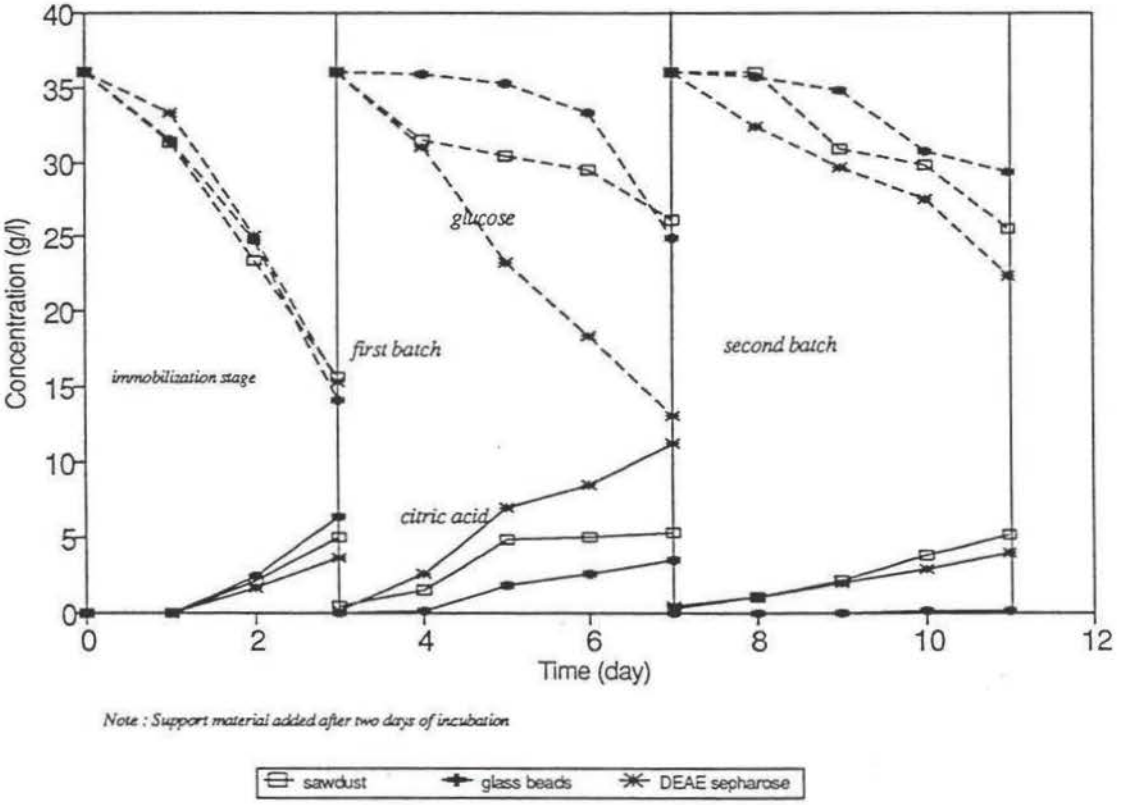
Support material	Yield % (w/w), 1st	Yield % (w/w), 2nd	Overall productivity (g/l.h), 1st	Overall productivity (g/l.h), 2nd
Glass bead	34	22	0.07	0.03
Sawdust	43	36	0.08	0.08
DEAE-sepharose	35	34	0.09	0.09

1st = first batch; 2nd = second batch.

A further study of the support material selection was conducted using similar conditions to the above, but now the nitrogen concentration in the medium was reduced to 1/5 of that used previously. This was to limit the growth of the immobilized cells, and to restrict any contribution from freely suspended cells in the flasks. The results (Figure 5.2) showed that only the cells immobilized onto sawdust accumulated the same citric acid concentration during four days cultivation in the first and second batches. Overall, the acid production was lower in all aspects compared to the results using the higher nitrogen concentration (Fig. 5.1).

Interestingly, citric acid production by the cells immobilized onto glassbeads and DEAE-sepharose declined dramatically from the first batch to the second batch. In the first batch, immobilized DT2 on DEAE-sepharose accumulated 11.2 (g/l) citric acid, but fell to only 3.8 (g/l) citric acid in

Figure 5.2 : Concentration of citric acid and glucose during repeated-batch shake flask cultivation using lower N medium (0.1 g/l).



Note : Support material added after two days of incubation

the second batch. Similarly, the cells immobilized on glassbeads produced 3.5 (g/l) citric acid in the first batch, and only 0.1 (g/l) citric acid in the second batch. A possible reason for this result is that the cells were not attached firmly to the solid particles. Thus, freely suspended cells dominated the process in the first batch and were washed out when the culture was transferred to the second batch.

Table 5.2 demonstrates that only the cells immobilized onto sawdust showed a similar yield and citric acid productivity during the first and second batches.

Table 5.2 : Comparison of the productivities and yields of cells of DT2 immobilized onto various support materials, with an initial ammonium chloride concentration of 0.1 (g/l) (based on Figure 5.2).

Support material	Yield % (w/w), 1st	Yield % (w/w) 1st	Overall productivity (g/l.h), 1st	Overall productivity (g/l.h), 2nd
Glass bead	31	3	0.04	0.002
Sawdust	49	50	0.05	0.05
DEAE-sepharose	49	28	0.12	0.04

1st = first batch; 2nd = second batch

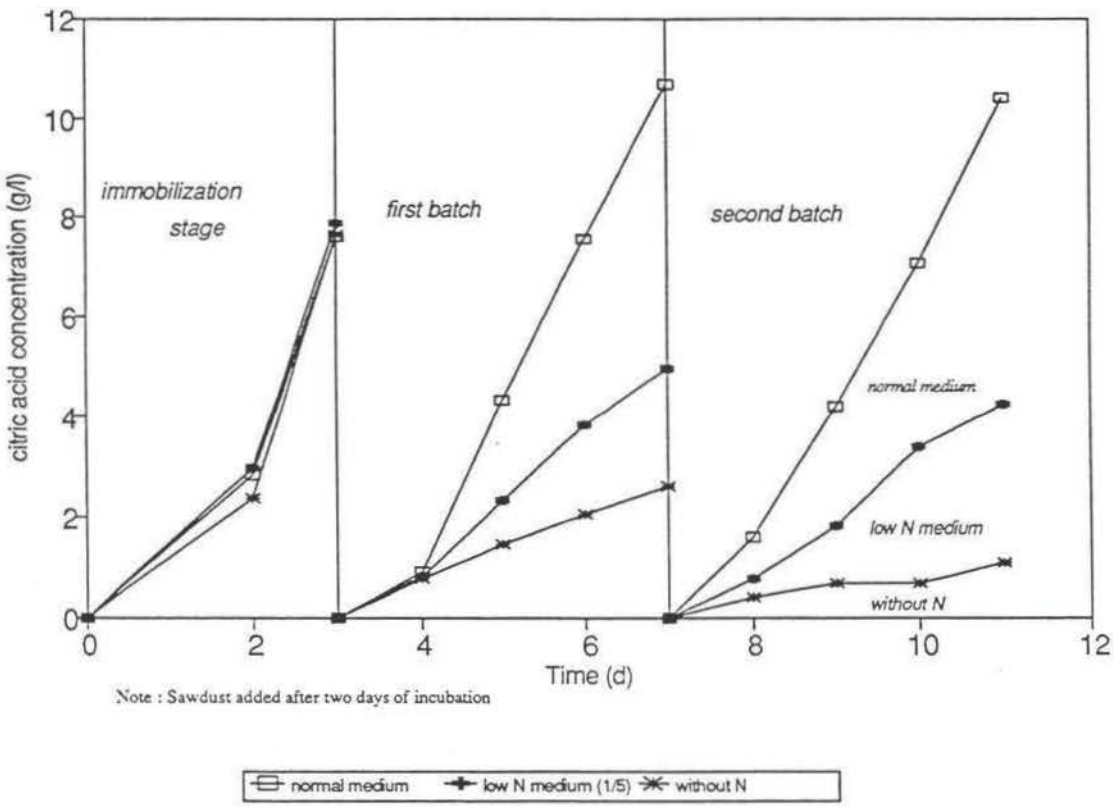
Based on these results, and the fact that it is cheap and easy to handle, sawdust was selected for further studies.

5.3. Effect of Nitrogen Concentration

Experiments were performed in the four-day repeated-batch shake flask cultures, as described above, to investigate whether, by varying the nitrogen concentration, the citric acid production could be manipulated . The defined medium containing 36 (g/l) glucose (Table 3.2) with three different ammonium chloride concentrations was used, i.e. 0.53 (g/l), 0.1 (g/l) and absence of ammonium chloride. The results (Figure 5.3) demonstrated that the production of citric acid by immobilized *C. guilliermondii* DT2 had a strong relationship with the nitrogen concentration in the medium. In the medium containing ammonium chloride at 0.53 (g/l) (Table 3.2), the immobilized cells produced 10.7 (g/l) and 10.4 (g/l) citric acid in the first and second batches, respectively. In contrast, when the nitrogen was reduced to 0.1 (g/l) the cultures accumulated only 4.9 (g/l) and 4.3 (g/l) during cultivation in the first and second batches, respectively. Then, when nitrogen was eliminated completely from the medium, the citric acid production was even lower, at only 2.6 (g/l) and 1.1 (g/l) citric acid during fermentation in the first and second batches, respectively.

Table 5.3 demonstrates that lowering of the nitrogen concentration of the medium resulted in decreased productivities. In contrast, the yield values during the first batch were approximately equal, though there was a decrease in the second batch when ammonium chloride was absent from the medium.

Figure 5.3 : Comparison of citric acid production in repeated-batch shake flask using different N concentrations.



Note : Sawdust added after two days of incubation

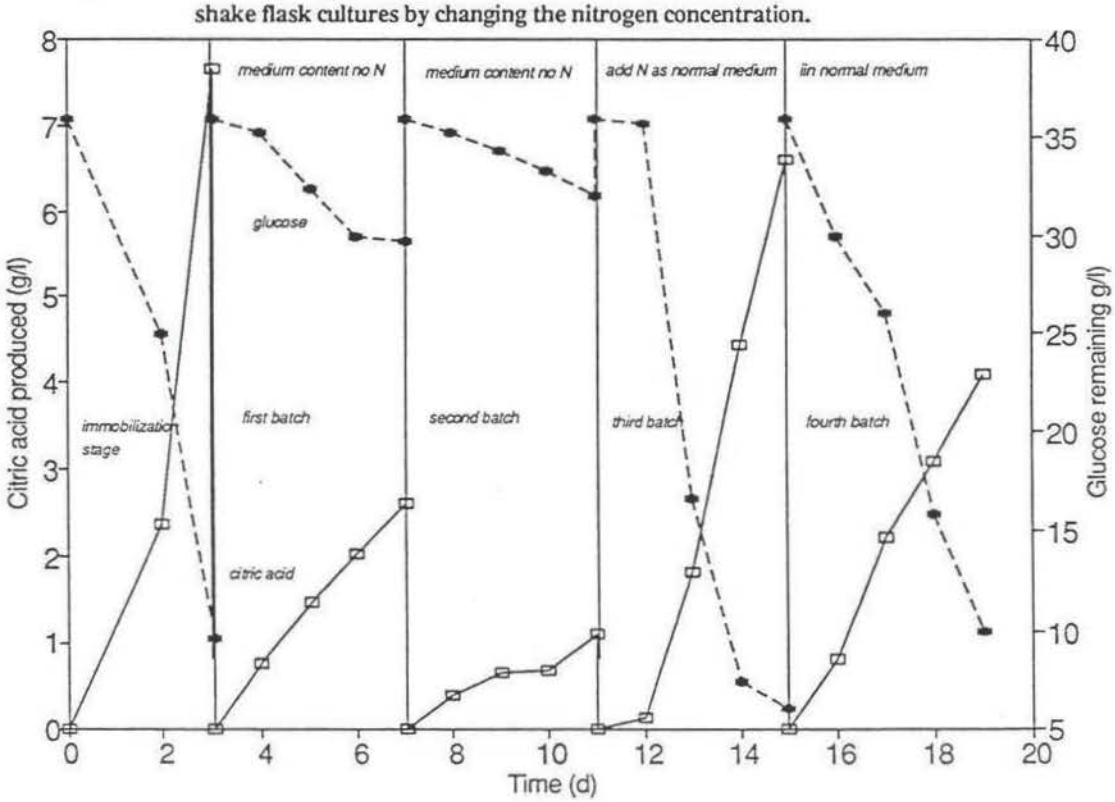
Table 5.3 : Comparison of the productivities and yields of immobilized cells of DT2 in media with various N concentrations (based on Figure 5.3).

NH_4Cl conc'n (g/l)	Yield % (w/w), 1st	Yield % (w/w), 2nd	Overall productivity (g/l.h), 1st	Overall productivity (g/l.h), 2nd
0.53	38	38	0.11	0.11
0.10	39	37	0.05	0.04
nil	41	27	0.03	0.01

1st = first batch; 2nd = second batch

The effect of the nitrogen concentration was further investigated using four-day repeated-batch shake flask culture using a different experimental design. The experiment was now conducted in four batches, where the medium used was the defined medium containing no nitrogen for the first two batches, and then 0.53 (g/l) ammonium chloride was added for the last two batches. The results (Figure 5.4) demonstrated that citric acid production, as well as glucose utilization, were dramatically increased in the third batch compared to the first two. However, in the fourth batch the citric acid production declined. The citric acid yield decreased gradually from batch to batch, starting with 41 % (w/w) in the first batch, where the medium contained no N, to 16 % (w/w) in the fourth batch, where 0.53 (g/l) ammonium chloride was present in the medium. A possible reason for this finding was contamination, where the high glucose consumption was caused by interference from a contaminant.

Figure 5.4 : Evolution of glucose and citric acid concentrations in repeated-batch shake flask cultures by changing the nitrogen concentration.



Note : Sawdust added after two days of incubation

Nevertheless, the experiments have indicated that immobilized cells which are performing poorly can be revived by the provision of nitrogen nutrient.

5.4. Effect of Phosphate Concentration

Conceptually, the experiments were performed in a similar manner to previous work (section 5.3), but now the nitrogen concentration was the same for all experiments (0.53 g/l) and the concentration of phosphate in the medium was varied. Thus the phosphate concentrations used (in the form of KH_2PO_4 and K_2HPO_4) were 2.3 (g/l), 0.2 (g/l) and absence of phosphate. Figure 5.5 shows that the highest citric acid formation in the first and second batches (13.2 g/l and 12.8 g/l respectively) was achieved in the medium where phosphate was reduced to 1/10 of the normal concentration in the defined medium (Table 3.2). However, production in the medium containing the normal phosphate concentration (2.3 g/l) still exceeded 10 g/l. When no phosphate was added to the medium, the citric acid production was only slightly lower, suggesting that the phosphate content of the medium does not have a significant influence on the citric acid productivity. However, Table 5.4 shows that total elimination of phosphate from the medium does cause an increase in the citric acid yield.

Figure 5.5 : Comparison of citric acid production in repeated batch shake flask using different P concentrations

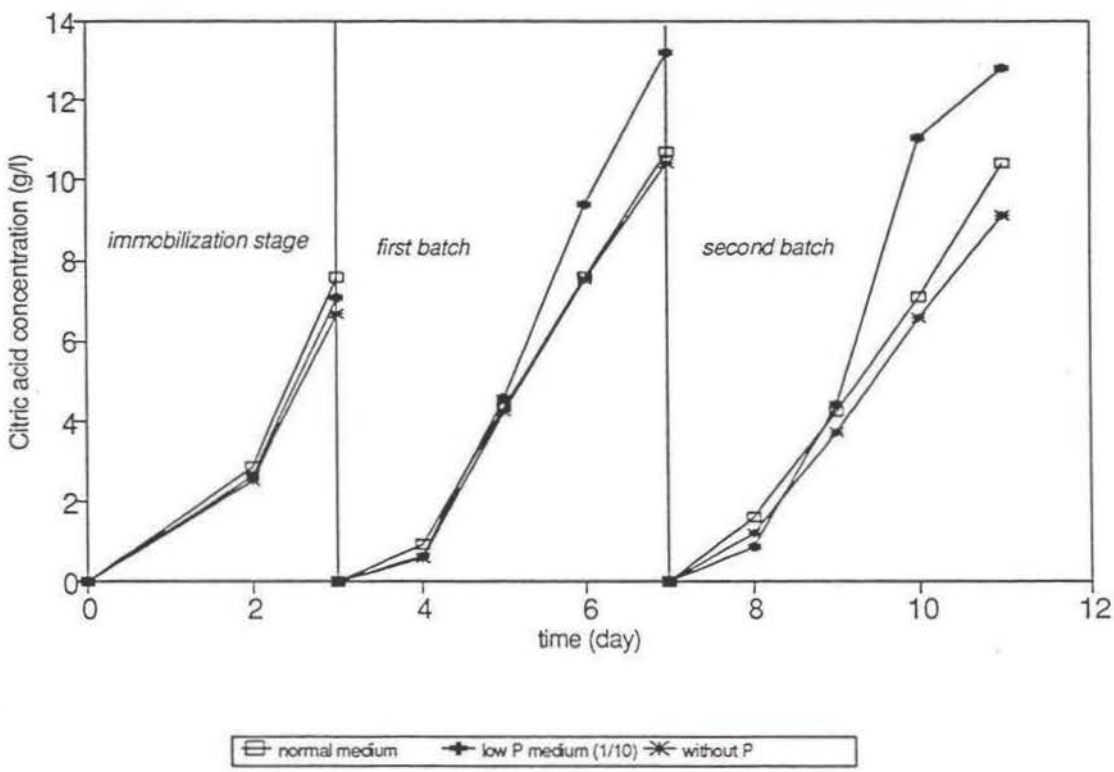


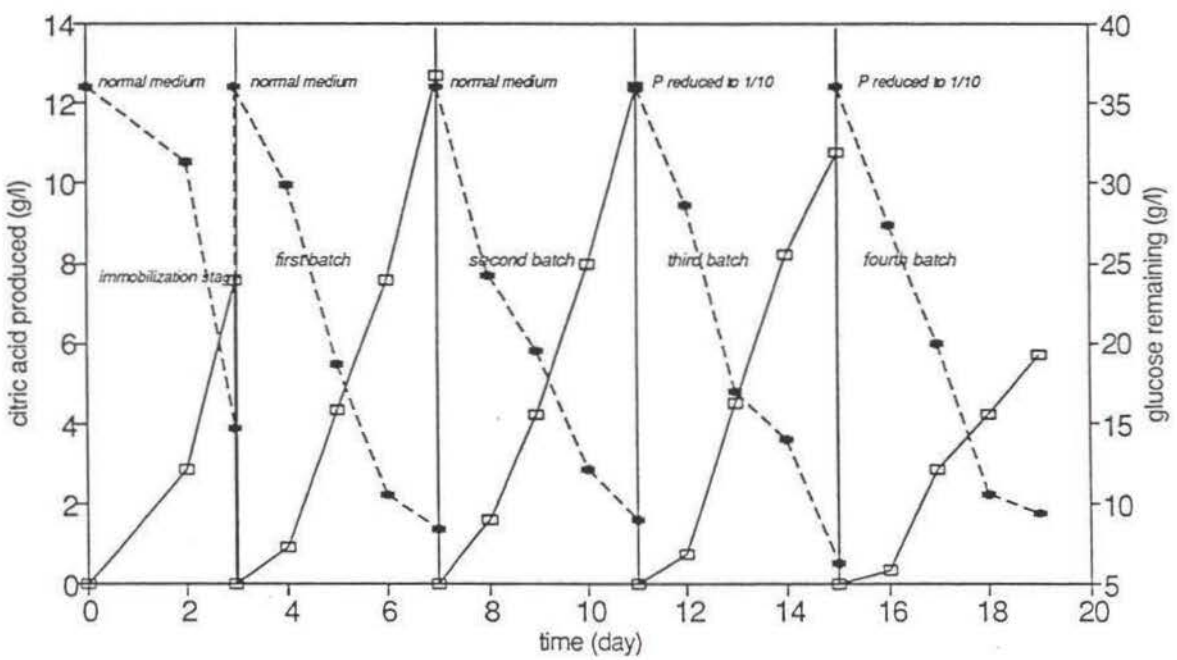
Table 5.4 : Comparison of the productivities and Yield of immobilized cells of DT2 in media with various P concentrations (based on Figure 5.5).

Phosphate conc'n (g/l)	Yield % (w/w), 1st	Yield % (w/w), 2nd	Overall productivity (g/l.h), 1st	Overall productivity (g/l.h), 2nd
2.3	39	38	0.11	0.11
0.2	46	40	0.14	0.10
nil	83	78	0.11	0.10

1st = first batch; 2nd = second batch; Phosphate = KH_2PO_4 and K_2HPO_4

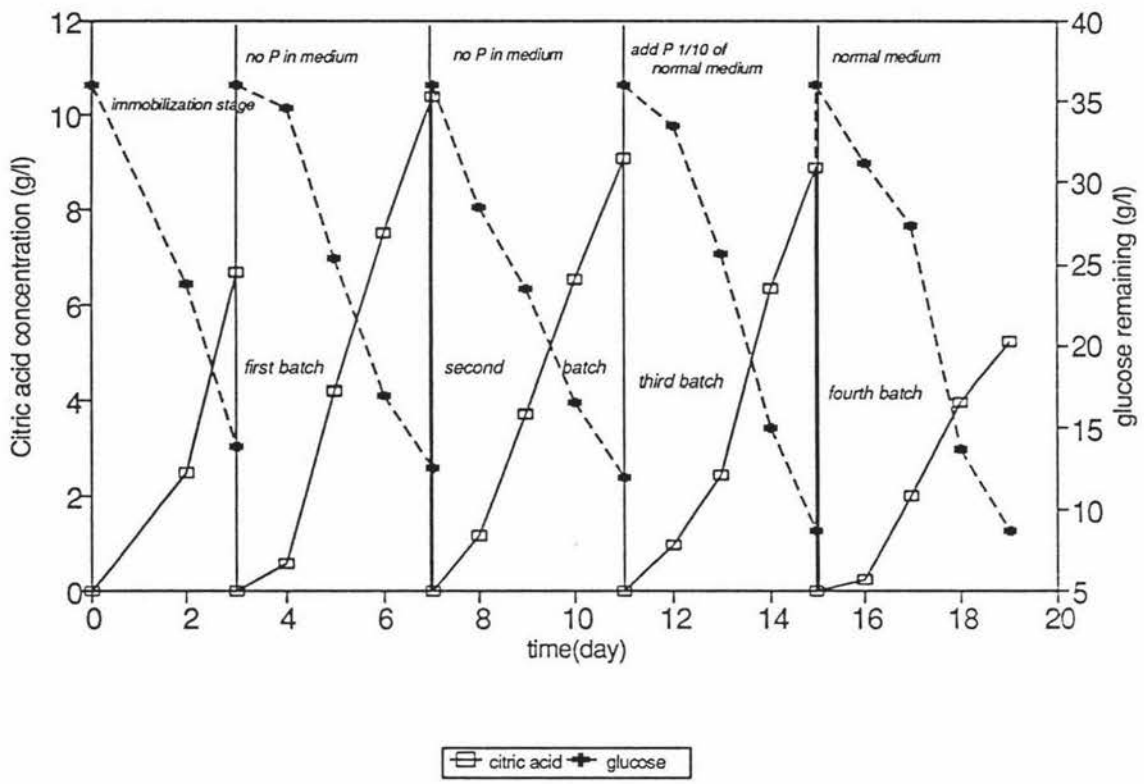
Using the same approach as with the nitrogen concentration (described above), supporting experiments were performed for the present study, using 4-day repeated-batch shake flask culture. For the first experiment (Figure 5.6), after the immobilization phase, the immobilized cells were cultivated in the defined medium containing 2.3 (g/l) phosphate, as described in Table 3.2, for the first two batches, after which the phosphate concentration was reduced to 1/10 for the last two batches. Citric acid production was slightly lower in the third batch than in the second, and went even lower in the last batch. However, the glucose utilization in all batches was similar. This result suggests that a minimum phosphate concentration is required to maintain the activity of the immobilized cells over long periods. In the second experiment (Figure 5.7), the process was commenced with the cultivation of the immobilized cells in the defined medium containing no phosphate for the first and second batches, after

Figure 5.6 : Evolution of citric acid and glucose concentrations in repeated-batch shake flask cultures



Note : Sawdust added after two days of incubation

Figure 5.7 : Evolution of the citric acid and glucose concentrations in repeated batch shake flask cultures by increasing the P concentration of the medium.



which it was increased to 1/10 of that in Table 3.2 in the third batch, and the normal concentration (2.3 g/l) for the fourth batch. The results indicated that citric acid production and citric acid yield progressively deteriorated with each batch, and is apparently contradictory to the result shown in Figure 5.6. The citric acid yields obtained in the first two batches, where the medium contained no phosphate, were 44 % (w/w) and 38 % (w/w) respectively for the first and second batches. These values were significantly lower than those obtained in the previous experiment using the same medium composition (Table 5.4). Since the result in the previous experiment (Table 5.4) was out of the line with all others, a possible explanation for the inconsistency is error of measuring the glucose concentration remaining in the culture during that experiment.

5.5. Discussion

The objective of the present work was to select an appropriate support material for immobilization of the mutant strain *C. guilliermondii* DT2, and to test whether the productivity of the yeast cells for citric acid production could be stimulated by limiting nitrogen and phosphate in the medium.

From the results summarized in Table 5.1, it seemed very difficult to decide between DEAE-sepharose and sawdust as the most appropriate material for the adsorption of *C. guilliermondii* cells, since DEAE-sepharose showed a higher productivity than sawdust. On the other hand, sawdust showed a better

result in terms of yield achievement. However, the result of the following experiment (Table 5.2), where the nitrogen concentration in the medium was reduced to 1/5 of normal, suggested that the cells immobilized onto sawdust had better stability, in respect of cell attachment.

Since the production of citric acid by yeast is biphasic, i.e. the accumulation of product starts at the end of the growth phase, it was attempted to improve the productivity by reducing the concentration of the limiting nutrient. However, the result (Table 5.3) demonstrated that lowering the nitrogen concentration obviously reduced the productivity. In contrast, the nitrogen concentration showed no significant influence on the citric acid yield, except when it was completely absent.

A similar effect of the nitrogen concentration was apparent in Figure 5.4, where productivity values were lower in a medium containing no nitrogen. However, addition of nitrogen allowed the ability of the cells to produce citric acid to be revived.

The study of the effect of reducing the phosphate concentration of the medium showed no significant influence on the productivity value (Figure 5.5).

The subsequent experimental results for the effect of phosphate concentration (Figures 5.6 and 5.7), were in agreement with the result summarized in Figure 5.5 , in terms of citric acid productivity. However, there was an inconsistency of the yield obtained when phosphate was absent from the medium, which created difficulty in seeing the effect of phosphate on citric

acid yield. Otherwise, the phosphate concentration appeared to have no major affect on the production of citric acid using immobilized cells of *C. guilliermondii*.

5.6. Conclusion

Sawdust was selected as the support material for further studies, due to the stability of the immobilized cells, especially when the experiments were performed at the lower nitrogen concentration. In addition sawdust is cheaper than DEAE-sepharose, and easier to handle when involved in the fermentation process.

The concentration of nitrogen in the medium was demonstrated to have a major effect in improving the productivity of citric acid production. In contrast, the phosphate concentration showed no significant influence on either the productivity or yield of citric acid. This finding may be useful to reduce the fermentation cost, by means of complete elimination of phosphate from the medium, and, also, the absence of phosphate might inhibit the growth of any contaminant microorganisms.

CHAPTER 6

USE OF IMMOBILIZED CELLS OF STRAIN DT2 IN A BUBBLE COLUMN REACTOR

6.1. Introduction

As the operation of an immobilized cell reactor in a batch mode is not likely to be economically attractive, so now further work in this thesis was performed in continuous mode operation using a bubble column reactor.

The aim of the present work was, of course, to study the performance of the immobilized cells of *C. guilliermondii* DT2 at a dilution rate value much higher than the maximum specific growth rate of the cells. This is because application of an immobilized cell system becomes advantageous only if it is operated at a dilution rate higher than normal washout conditions, under which a free cell system can not be operated (Black, 1986). The possibility of improving the citric acid production rate by means of adjusting the concentration of limiting nutrients, was also studied in the present work.

6.2. Effect of Phosphate Concentration

A bubble column reactor with a working volume of 250 ml was used for all experiments. The initial medium for cell growth and immobilization was a fully defined medium containing 50 (g/l) glucose, and had a pH value of 6.2, as described in Table 3.2.

Five grams of sawdust were packed to immobilize cells of *C. guilliermondii* DT2 in the reactor column. Continuous feeding of fresh medium, at an initial dilution rate of 0.1 h^{-1}, was commenced after allowing the column to stand in batch conditions for about 20 hours in order to facilitate an adequate contact time between cells and sawdust particles. The bioreactor performance during the fermentation period was monitored via measuring : pH of the culture, glucose concentration remaining, citric acid produced and microscopic observation.

Based on the experimental result in section 5.4, where a decreased phosphate concentration in the medium increased the citric acid yield, and supported by the view that less phosphate in the medium would minimize the risk of contamination, the first experiment was designed to manipulate the performance of the reactor by lowering the phosphate concentration. This could also allow the removal of the antibiotics from the culture medium.

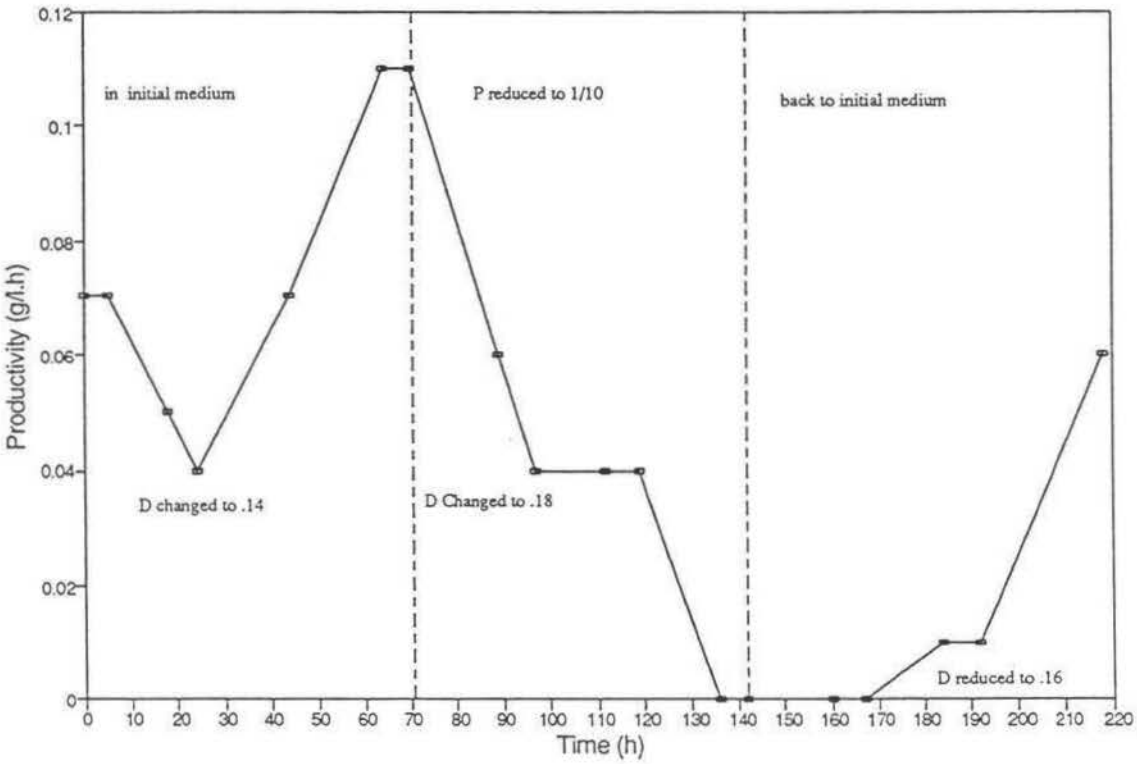
The results, summarized in Table 6.1, demonstrate that reduction of the phosphate content of the medium from 2.3 (g/l) to 0.2 (g/l) caused the culture pH value to decrease, and resulted in a lowered citric acid yield from 10.2 % to, eventually, 0 % (w/w).

Table 6.1 : Effect of dilution rate and phosphate content of the medium on citric acid yield.

Time (h)	D (h^{-1})	Phosphate (g/l)	pH	Yield % (w/w)
0	0.1	2.3	3.5	5.2
5	0.1	2.3	3.6	7
18	0.1	2.3	3.6	4
24	0.1	2.3	3.1	3.6
44	0.14	2.3	2.9	5.9
64	0.14	2.3	3.1	10.5
70	0.14	2.3	3.3	10.2
89	0.14	0.2	2.7	5.6
97	0.18	0.2	2.8	3.6
112	0.18	0.2	2.7	3.2
119	0.18	0.2	2.5	2.4
136	0.18	0.2	2.4	0
142	0.18	0.2	2.3	0
160	0.18	2.3	2.8	0
167	0.18	2.3	2.8	0
184	0.18	2.3	3.1	1.8
192	0.16	2.3	3.4	3.3
218	0.16	2.3	3.9	5.6

Since phosphate is supplied as a buffer as well as a nutrient, reduction of the phosphate concentration in the medium directly reduced the buffering capacity. Therefore, when the system was supplied with a low-phosphate, lowering of the culture pH was inevitable due to acid production. Eventually, when the pH value had fallen to pH 2.4, citric acid production ceased.

Figure 6.1 : Evolution of citric acid productivity during the first run of bubble column culture using immobilized cells of strain DT2.



However, some recovery of the activity occured as the phosphate concentration was restored after 160 h of operation.

Figure 6.1 shows that the highest citric acid productivity (0.11 g/l.h) was obtained after 64 hours of fermentation, in the initial medium, at a dilution rate of 0.14 h^{-1}. It was maintained for only 6 hours and dropped gradually as the medium was changed to the lower phosphate concentration. However, the productivity began to recover as the phosphate concentration was subsequently restored.

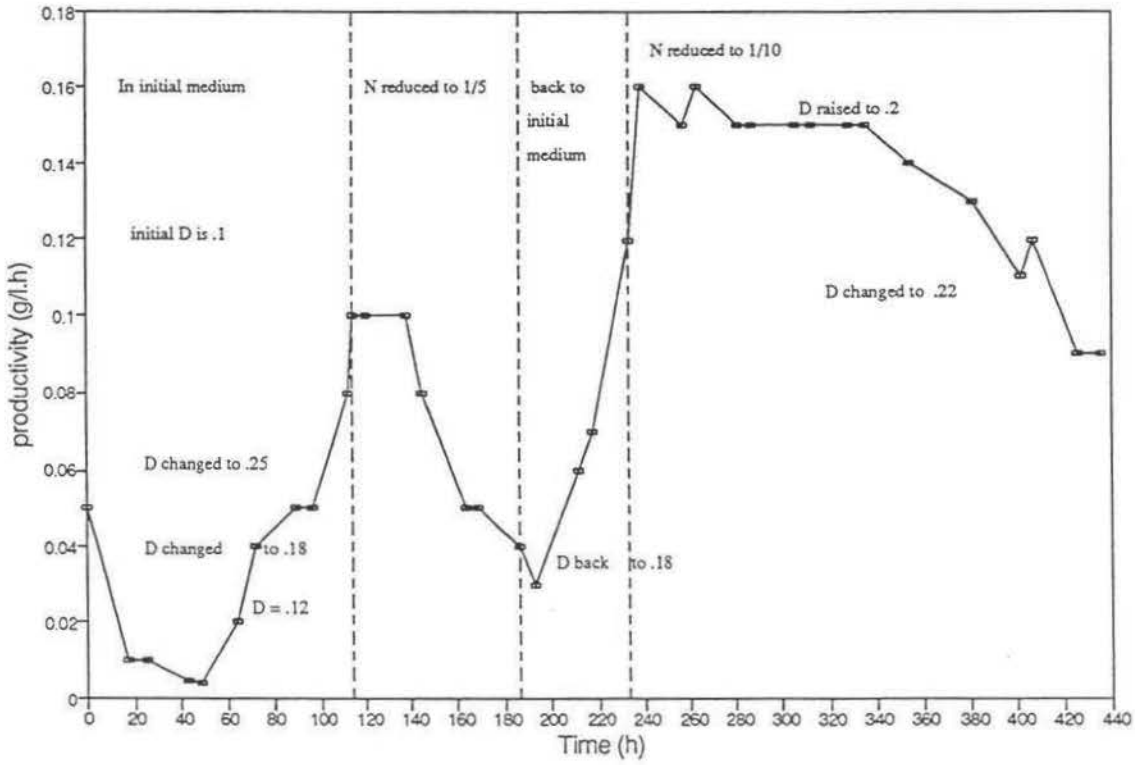
6.3. Effect of Nitrogen Concentration

Following the failure of improving the reactor performance by reducing the phosphate concentration in the medium, the second experiment of the bubble column reactor was designed to investigate the possibility of reducing the nitrogen concentration in the medium, and to study its effect on citric acid productivity.

The basic fermentation conditions were as previously described (Section 6.2). The experiment commenced at an initial dilution rate of 0.1 h^{-1}, as for the first experiment. However, since it was observed during the first experiment that some sawdust remained static at the base of the column instead of fluidizing, the amount of sawdust used was reduced to only three grams (dry weight).

The results are summarized in Table 6.2 and Figure 6.2. By comparing Figure 6.2 and Figure 6.1, it is apparent that the citric acid productivities

Figure 6.2 : Evolution of citric acid productivity in the second run of bubble column culture using immobilized cells of strain DT2



during the first 70 hours of the second experiment were significantly lower than those obtained during the first experiment. Since the amount of sawdust packed was reduced from 5 to 3 grams, where the sawdust particles' capacity of adsorbing cells of *C. guilliermondii* is 1.3 X 10^8 colony-forming units per gram sawdust (dry weight), the low value of initial productivities is suggested to be due to a lower biomass density within the reactor. However, increasing the dilution rate to 0.25 h^{-1} was followed by an improving citric acid productivity as well as citric acid yield. The highest productivity obtained at this dilution rate was 0.1 (g/l.h) representing a yield of 8 % (w/w), based on glucose utilized. However, reduction of the nitrogen concentration in the medium to 0.1 (g/l) resulted in a sharp drop in productivity to 0.03 (g/l.h), representing a yield of 1.4 % (w/w).

Table 6.2 : Effect of dilution rate and nitrogen content in the medium,on pH and citric acid yield.

Time (h)	D (h^{-1})	NH_4Cl conc'n (g/l)	pH	Yield % (w/w)
0	0.1	0.53	3.9	5
17	0.1	0.53	5.9	1.5
25	0.1	0.53	5.6	1.8
43	0.1	0.53	5.7	1.2
49	0.1	0.53	4.8	0.8
65	0.1	0.53	3.5	2.5
72	0.12	0.53	3.3	4.4
89	0.18	0.53	3.5	4.5
96	0.18	0.53	4.1	4.6
112	0.25	0.53	4.2	5.4
114	0.25	0.53	4.2	8
120	0.25	0.1	4.0	8
137	0.25	0.1	4.7	6.3
144	0.25	0.1	4.7	5.4
163	0.25	0.1	4.8	3.1
168	0.25	0.1	4.8	3
186	0.25	0.1	4.8	2.3
193	0.25	0.1	4.9	1.4
212	0.18	0.53	3.2	4.9
218	0.18	0.53	3.3	5.9
233	0.18	0.53	3.1	7.8
238	0.18	0.05	2.9	9.6
257	0.18	0.05	3.5	12.5
263	0.18	0.05	3.5	14.2

Table 6.2 continue to next page

Table 6.2 : continue

Time (h)	D (h^{-1})	NH_4Cl conc'n (g/l)	pH	Yield % (w/w)
281	0.18	0.05	3.5	13.3
287	0.18	0.05	3.5	12.1
306	0.18	0.05	3.4	13.3
313	0.2	0.05	3.4	17.8
328	0.2	0.05	3.4	15.5
335	0.2	0.05	3.5	16.6
354	0.2	0.05	3.5	10.6
381	0.2	0.05	3.5	9.6
402	0.2	0.05	3.4	11.7
407	0.22	0.05	3.6	11.7
425	0.22	0.05	3.8	7.8
435	0.22	0.05	3.7	7.4

Restoration of the ammonium chloride concentration to 0.53 (g/l), accompanied by reduction of the dilution rate to 0.18 h^{-1}, resulted in an increased citric acid productivity to about 0.15 - 0.16 (g/l.h), representing specific production rates of about 0.025 - 0.027 (g citrate/g biomass.h), and a yield of about 12 to 16.6 % (w/w). This productivity value was maintained for about 100 hours despite a subsequent reduction in the nitrogen content of the medium, but then declined. Small increases in the dilution rate did not prevent this decline.

The highest productivity value (about 0.16 g/l.h) was maintained for 116 hours.

In order to improve further the citric acid productivity, and to facilitate a comparison study on the effect of cell density on the reactor performance, a third experiment with the bubble column culture was performed. Now the column was packed with 5 grams of sawdust, while the other initial experimental conditions were identical to the previous experiment (second run). However, in this case, the experiment commenced with a dilution rate of 0.13 h^{-1}, i.e. just above the maximum growth rate of the cells (0.12 h^{-1}).

Since the results of the first experiment had suggested that a low culture pH value during operation of the reactor might cause citric acid production to cease, the value was maintained above pH 3 by means of adjusting the dilution rate. After 91 hours of fermentation, the ammonium chloride concentration in the medium was reduced to 0.05 (g/l), and the antibiotics were omitted from the medium. This medium composition was maintained until the end of the experiment.

The results of the third experiment are presented in Table 6.3 and Figure 6.3. Figure 6.3 demonstrates that a progressive increase of the dilution rate up to 0.2 h^{-1} was accompanied by an increased productivity, whereas a reduction of the ammonium chloride concentration to 0.05 (g/l) had no significant influence on this parameter. Indeed, Table 6.3 shows that the yield, based on glucose used, improved somewhat as the ammonium chloride concentration was reduced.

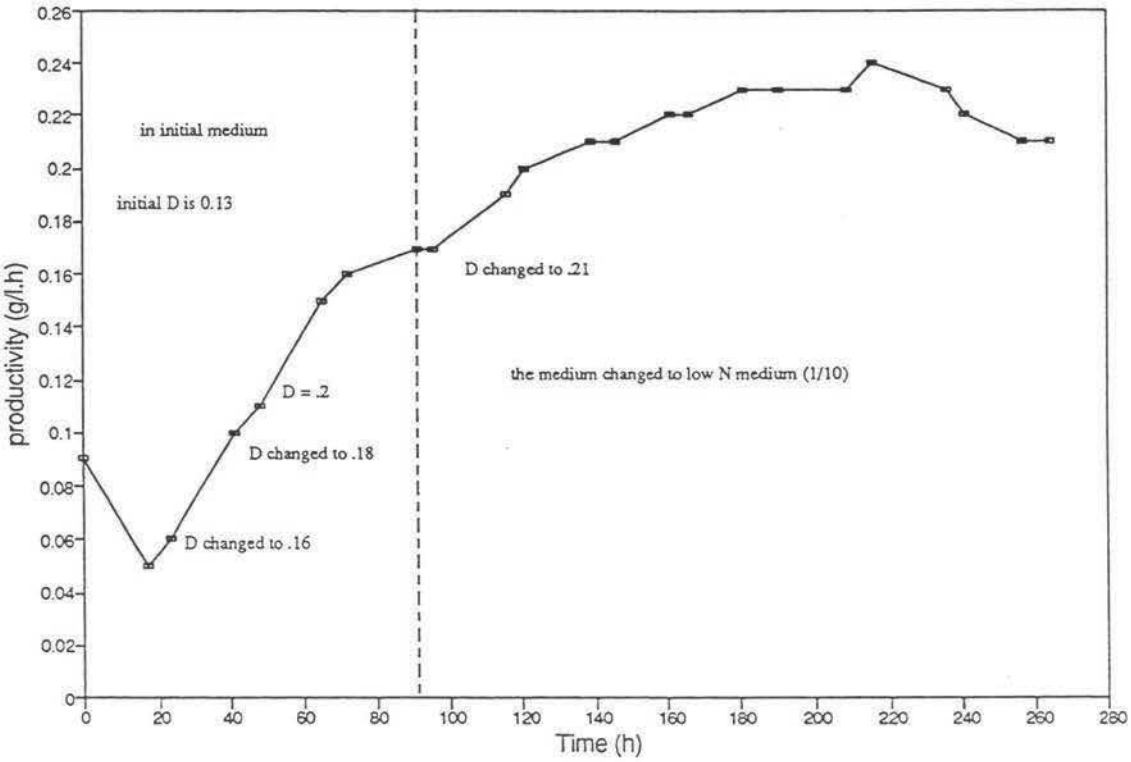
The highest citric acid poductivity obtained during the third experiment of

the bubble column culture was around 0.2 - 0.24 (g/l.h), representing specific production rate of about 0.02 - 0.024 (g citrate/g biomass.h), (Figure 6.3), which was maintained for 143 hours. The highest citric acid yield achieved was around 10 - 11 % (w/w), based on glucose utilized.

Table 6.3 : Effect of dilution rate and nitrogen content of the medium on pH and citric acid yield, during the third experiment of bubble column culture.

Time (h)	D (h^{-1})	NH_4Cl conc'n (g/l)	pH	Yield % (w/w)
0	0.13	0.53	3.0	6.7
17	0.13	0.53	3.0	4.3
23	0.13	0.53	3.0	5.3
41	0.16	0.53	2.7	4.5
48	0.18	0.53	2.8	4.5
65	0.2	0.53	3	7.3
72	0.2	0.53	3	7.7
91	0.2	0.53	3.2	7.4
96	0.2	0.05	3.1	9.3
116	0.21	0.05	3.2	9.8
121	0.21	0.05	3.2	9.6
139	0.21	0.05	3.1	10.3
146	0.21	0.05	3.2	8.5
161	0.21	0.05	3.2	10.5
166	0.21	0.05	3.2	10.7
181	0.21	0.05	3.2	10.5
191	0.21	0.05	3.1	10.4
209	0.21	0.05	3.2	10.4
216	0.21	0.05	3.3	11
236	0.21	0.05	3.6	10
241	0.21	0.05	3.5	10.6
257	0.21	0.05	3.6	11.1
264	0.21	0.05	3.6	11.4

Figure 6.3 : Evolution of citric acid productivity in the third run of bubble column culture using immobilized cells of strain DT2



6.4. Discussion

The result of the first experiment using the bubble column reactor, using a medium containing a low phosphate concentration, suggested that a minimum culture pH needs to be maintained to allow citrate production. Unfortunately, in these experiments, culture pH control was possible only by adjusting the dilution rate. This was in contrast to the experiments in repeated batch shake flask culture, where the culture pH was adjusted regularly by addition of 10 M KOH. However, it was found from the first experiment that the immobilized cell activity could be revived by restoring the medium to a higher content of phosphate concentration (2.3 g/l).

The amount of sawdust particles packed in the column was critical, since it is related to the capacity of cell attachment. The higher amount of sawdust used will provide a higher cell density in the reactor, but, a higher amount of sawdust will also mean more sawdust remaining static on the base of column, which could easily block the aeration line. So, consideration should be made between sufficient cell concentration to produce the highest reactor productivity and the amount of sawdust which will allow a reasonable fluidization characteristic.

Adjusment of the nitrogen content of the medium appeared to give contradictory results. In the second experiment (Figure 6.2), the first reduction of the concentration resulted in a sharp decline in citric acid productivity, but in the second reduction, and in the third experiment (Figure 6.3), no such effect

was observed. It is suggested that this apparent contradiction reflects the different stages of establishment of the reactors in the various stages of the two experiments. Thus, early in the second experiment the reactor was less well established than it was later, or in the third experiment, when the nitrogen supply was reduced, and so a productivity decline was noted. Once the system was well established, however, as it was late in the second experiment and in the third experiment, the nitrogen requirement is less and a reduction can be more easily tolerated. Under this condition, a reduced nitrogen supply can result in an increased citric acid yield, presumably because glucose is diverted from biomass production to citrate production.

By commencing the third experiment of the bubble column reactor at a dilution rate of 0.13 h^{-1}, followed by increasing the dilution rate to 0.21 h^{-1}, it was proved that the cells of *C. guilliermondii* were immobilized in sufficient strength onto the sawdust particles. It was supported by the fact that this immobilization system performed well under conditions of restricted growth, by means of using a low nitrogen content in the medium. However, the citric acid yield obtained using bubble column culture was only 1/4 of the average achievement using repeated batch shake flask, and 1/3 of that obtained using freely suspended cells in batch fermenter culture. A possible explanation for this result is that the sugar consumed during the experiment using bubble column culture was mainly used for growth of cells, since a continuous fermentation culture might allow the cells to maintain their growth phase,

particularly in the medium containing a high concentration of nitrogen.

The maximum specific citric acid production rate, based on productivity per biomass concentration, in bubble column culture was only about half of that obtained using freely suspended cells in batch fermenter culture. However, this specific production rate, in continuous operation as using bubble column culture, can be maintained for long period of process, while in batch fermenter culture the maximum specific production rate only occur at certain point within the period of process.

6.5. Conclusions

1. The mutant strain of *C. guilliermondii* DT2 was immobilized succesfully onto sawdust particles, and used in a bubble column reactor for citric acid production. A reactor productivity of 0.20 - 0.24 (g/l.h) was maintained for several days. This is higher than was obtained with free cells (Chapter 4).

2. The culture pH must be maintained at a minimum value to allow citric acid production to occur.

3. Once a reactor has been established, adjustment of the nitrogen supply can be made without any significant effect on the citric acid productivity. Indeed, a decreased nitrogen supply can result in an increased citric acid yield.

4. The citric acid yields observed in the bubble column reactor were generally lower than those observed in repeated batch culture.

CHAPTER 7

FINAL DISCUSSION AND CONCLUSIONS

The feasibility of using immobilized cells of *C. guilliermondii* for citric acid production, from glucose, was investigated in this thesis.

The starting point of the investigation was reisolation of a strain of *C. guilliermondii* IMK1 from a stock culture (Department of Process and Environmental Technology, Massey University, Palmerston North, New Zealand), which was followed by a strain improvement programme.

Strain improvement was performed by induced mutation using Ultra Violet light, followed by subsequent selection in petri dishes. Two mutants (*C. guilliermondii* DT1 and DT2) which produced more citric acid than the parent, were selected for further work.

A comparative study of the two selected mutants with the parent strain IMK1, which was carried out in shake flask culture, demonstrated the superiority of the mutants, particularly DT2, in producing citric acid. However, the specific growth rates of the three strains were about the same. Further examinations of the mutant (*C. guilliermondii* DT2) using a laboratory scale batch fermenter, proved that the mutant strain DT2 could be used succesfully in a fermenter culture.

The experiments in chapter 5 describe an investigation into the use of immobilized cells of strain DT2 in shake flask culture. The investigation was

initiated with the selection of a support material, which proved that among the three options chosen , i.e. sawdust, DEAE-sepharose and glass beads, sawdust was the most suitable support material for cell immobilization of the mutant strain *C. guilliermondii* DT2 via the adsorption method.

When investigating the effect of the nitrogen nutrient concentration, using defined medium in repeated batch shake flask culture, production of citric acid was obviously related to the amount of initial nitrogen provided in medium. A very low citric acid concentration was produced when using defined medium containing no nitrogen. This finding was suggested to be due to the low number of cells firmly attached onto the sawdust particles during the immobilization stage, and the growth of immobilized cells was restricted by limitation of nitrogen in the medium. The highest overall productivity (0.11 g/l.h), which was obtained using defined medium containing 0.53 (g/l) ammonium chloride (Table 5.3), was considerably higher than the overall productivity achieved using freely supended cells in the same medium (Table 4.1). This demonstrated the superiority of the immobilized cell system over the freely suspended cells.

A similar approach, in repeated batch shake flask culture, was used to investigate the effect of phosphate concentration in the medium. Using phosphate concentrations in the range of 0 - 2.3 (g/l), no significant changes in either citric acid production or citric acid yield were observed.

The effects of limiting nutrients, phosphate and nitrogen, were once again

investigated in chapter 6. Now the experiments were carried out in continuous mode operation using a bubble column reactor. However, the experiments for the effect of limitation of the phosphate concentration in the medium failed to produce sufficient data to support those that were obtained using repeated batch shake flask culture. This failure was suggested to be due to poor control of the culture pH value, which relied on the buffering capacity of the phosphate in the supplied medium and adjustment of the feed medium dilution rate. Nevertheless, the importance of adequate pH control was demonstrated. Reduction of the concentration of ammonium chloride in the medium, provided that the system was well established, caused an increase in the citric acid yield, although no significant influence was observed on citric acid production rate.

The advantages of using an immobilized cell reactor include achievement of a higher production rate by means of a higher biomass density within the reactor, and the possibility of operating at a dilution rate much higher than the specific growth rate of the cells. In this work it has been suggested that the biomass concentration in the reactor is directly related to the amount of support material packed. It has been clearly proved that the cells of mutant strain *C. guilliermondii* DT2 were well immobilized onto the sawdust particles, where the system was stable for more than 140 h of operation at a dilution rate of 0.21 h^{-1}.

In conclusion, the major aim of this work, which was to develop and evaluate an immobilized cell reactor for citric acid production using *C.*

guilliermondii, has been achieved. The results clearly prove that the productivity of the immobilized cell reactor is superior to that of free cells, although the citric acid yield and specific production rate obtained in bubble column culture were markedly lower than those obtained using freely suspended cells in batch fermenter culture. This superiority was emphasized by the performance of the immobilized cells in a bubble column culture, where the maximum productivity achieved (0.21 - 0.24 g/l.h) was much better than most published results. However, the results obtained in this work are rather lower than those obtained by Rymowicz *et al* (1993). Table 7.1 presents a comparison of the results obtained in this work and in the other published works.

Table 7.1 : Comparison of citric acid producivity achieved using immobilized yeast cell by several authors.

Authors (year)	Organism	Immobilization/carrier	Reactor	Productivity (g/l.h)	Yield % (w/w)
Maddox & Kingston (1983)	*S.lipolytica*	Entrapment/ polyacrylamide	Shake flask	0.05	70
Kautola *et al* (1991)	*S.lipolytica*	Entrapment/ alginate	Shake flask	0.155	
Kautola *et al* (1991)	*S.lipolytica*	Entrapment/ carrageenan	Air-lift	0.12	
Rymowicz *et al* (1993)	*S.lipolytica*	Entrapment/ alginate	Air-lift	0.35	
Forster *et al* (1994)	*S.lipolytica*	Encapsulation/CS	Fluidized bed	0.125	
This work	*C. guilliermondii*	Adsorption/ sawdust	Shake flask	0.11	40
This work	*C. guilliermondii*	Adsorption/ sawdust	Bubble column	0.21 - 0.24	11

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