A STUDY OF CITRIC ACID PRODUCTION BY SUBMERGED AEROBIC FERMENTATION USING THE FUNGUS *Aspergillus niger*

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ABSTRACT

The aim of this work was to obtain detailed information on the production of citric acid during submerged fermentation by *Aspergillus niger*, in an attempt to understand and optimize the process. Initial experiments were performed to determine the effect of interruptions to aeration on citric acid production. Unless the value of the Dissolved Oxygen Tension (DOT) of the culture fell below the DOT_{crit} (20% of saturation), no gross effect was observed. When the DOT value fell to zero, citric acid production ceased. Production however, recovered after recommencement of aeration, albeit after a delay.

Experiments were performed in batch fermentation using various non-carbohydrate medium components as the growth-limiting nutrient. Nitrogen-, phosphate- or sulphate-limited cultures resulted in strong citric acid production. The most significant observation during these fermentations was that the maximum citric acid production rate occurred prior to the exhaustion of the limiting nutrient, i.e. when the organism was at a positive growth rate.

Chemostat experiments were performed in order to determine the effect of the growth rate and the culture DOT on citric acid production. Maximum citric acid production rates and yields were achieved at low growth rate (μ = 0.017 h^{-1}) and high DOT (90% of saturation) values. The specific citric acid production rate was twice the maximum observed in batch fermentation, and the product yield was increased

by 23%.

The information regarding growth rate and DOT gained from the chemostat experiments was applied to a continuous fed-batch fermentation using nitrogen as the growth-limiting nutrient. The overall fermenter productivity attained was double that of the batch fermentation, resulting in a halving of the fermentation period. This is the first reported use of the continuous fed-batch technique for citric acid production.

In all three fermentation modes (batch, chemostat and fed-batch), nitrogen limitation was superior to phosphate limitation in terms of citric acid production rates and yields. A double nitrogen/phosphate limitation gave results almost identical to a nitrogen limitation. The evidence suggests that the nitrogen nutrient exerts a form of catabolite repression on citric acid accumulation.

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ABBREVIATIONS

ABBREVIATIONS OF UNITS

^{o}C	degrees Celcius
d	day
g	gram
h	hour
l	litre
m	meter
mg	milligram
min	minute
ml	millilitre
mm	millimeter
mM	millimole
nm	nanometer
rpm	revolutions per minute
ul	microlitre
vvm	volume per volume per minute

OTHER ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
D	Dilution rate
DOT	Dissolved Oxygen Tension
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic acid
HPLC	High Performance Liquid Chromatography
ID	Internal Diameter
N	Nitrogen
NAD	Nicotinamide Adenine Dinucleotide

NADH	Reduced Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
PO_4^{3-}	Phosphate
TCA	Tricarboxylic Acid
u	Specific Growth Rate

CHAPTER 1

INTRODUCTION

Citric acid is an organic acid with many industrial uses, particularly in the food and pharmaceutical industries due to its low toxicity, ease of assimilation and palatability. It is produced commercially by fermentation of sugar solutions, generally molasses or glucose syrups. Details of commercial processes remain secret, but in general, citric acid production is by strains of the fungus *Aspergillus niger* using the submerged aerobic fermentation process. This process has been reviewed extensively (Kapoor *et al.*, 1982; Kristiansen *et al.*, 1982; Rohr *et al.*, 1983). However, the precise details of a typical batch process have not been satisfactorily reported, nor has the biochemical mechanism of citric acid accumulation been adequately explained. Answers to some of these mysteries of the citric acid fermentation may already be known to various industrial concerns, but in view of the need for commercial secrecy, have not been published in the scientific literature.

The work described in this thesis was undertaken to provide detailed information on the parameters controlling the course of a typical batch fermentation process, and to attempt to understand the biochemical mechanism of citric acid accumulation. Inevitably, the simple description of a process led to the desire for optimization, and thus, studies were performed subsequently in chemostat culture, a technique which is also valuable in biochemical studies. From this work, it was possible to develop a fed-batch

culture technique for citric acid production.

CHAPTER 2

PRODUCTION OF CITRIC ACID BY FERMENTATION

2.1 INTRODUCTION

Citric acid is a tricarboxylic acid, which was first isolated from lemon juice and crystallized as a solid by Scheele in 1784. It is a natural constituent of citrus fruits, other fruits such as pineapples, pears and figs and animal tissues. Until the early part of this century, commercial citric acid production was from lemon juice. At present, most of the citric acid used in industry is produced by fungal fermentations, using sugar cane or beet molasses as the carbohydrate source. The estimated production in the major citric acid-producing nations is shown in Table 2.1 (Kapoor *et al.*, 1982).

The United States is the largest producer of citric acid, with two major companies involved:- Miles Laboratories Inc., (Elkhart, Indiana) and Pfizer Inc. (New York, New York). Other important producers include:

- England: John and E. Sturge, Ltd, Birmingham.
- West Germany: Joh. A. Benckshiser, Gmbtt, Ludwigshafen/Rhein.
- Belgium: Citrique Belge, Tienen.
- France: Rhone-Poulenc S.A., Paris.
- Republic of China: San Fu Chemical Company, Ltd, Taipei.

Citric acid is used for a variety of purposes. The

Table 2.1 Estimated production of citric acid by various countries (Kapoor *et al.*, 1982).

Countries	Estimated Production (Tons/year)
Western European countries, United Kingdom, France, Netherlands, Belgium, Austria, West Germany and Ireland	100,000
U.S.A.*	128,000
U.S.S.R.	20,000
Canada	10,000
Japan	7,000
Czechoslovakia	4,000
Australia	3,000
Poland	2,250
Developing countries	12,000
Israel	4,000
Others	16,000

* Hossain (1983).

food and beverage industries use about 70%, the pharmaceutical industry about 12% and other industries about 18% of the total produced (Atticus, 1975). In the food industry, citric acid is used primarily as an acidulant. It is also used as a flavour enhancer and preservative in carbonated beverages. The pharmaceutical industry uses citric acid as an acidulant, while the chemical and cosmetic industries use it for its sequestering and plasticizing properties (Meyrath, 1967). Other industrial applications include the treatment of boiler water, metal plating, detergent formulation, tanning and textiles. The most recently available figures for importation of citric acid into New Zealand are shown in Table 2.2.

There is an extensive body of literature on citric acid production by fermentation, including more than 450 reports published world-wide in the last 20 years. Amongst these reports are some comprehensive reviews (e.g. Loesecke, 1945; Foster, 1949; Perlman and Sih, 1960; Lockwood and Schweiger, 1967; Smith *et al.*, 1974; Lockwood, 1975; Berry *et al.*, 1977; Miall, 1978; Kapoor *et al*,. 1982; Kristiansen *et al*,. 1982; Rohr *et al*,. 1983). These authors have adequately covered the work performed over the last 50 years, consequently no attempt will be made here to give a detailed review of the literature. Instead, this chapter will highlight those factors shown to be important in the fermentation process and which relate to this study.

Table 2.2 Citric acid imports into New Zealand from 1976 to 1984 (New Zealand Department of Statistics).

Year	Quantity (kg)	Value ($NZ)
1976 - 1977	1,100,000	1,400,000
1977 - 1978	735,000	1,300,000
1978 - 1979	1,115,039	1,314,886
1979 - 1980	890,805	1,287,566
1980 - 1981	653,391	1,201,367
1981 - 1982	999,076	1,799,679
1982 - 1983	717,368	1,483,246
1983 - 1984	82,000	465,000

2.2 HISTORY

The history of citric acid production by fungi has been extensively reviewed by Foster (1949) and Miall (1975). The development of a fermentation process for the production of citric acid can be conveniently divided into three phases (Perlman and Sih, 1960). Wehmer (1893) began the first phase, when he reported the use of *Penicillium lacteum* and *Mucor piriformis* for the production of citric acid, which occurred when the organisms were grown on the surface of media containing carbohydrate and inorganic salts. An attempt was made to use this process for the commercial production of citric acid in a factory in Thann, Alsace, in 1893. It was abandoned in 1903 because of many difficulties, among them degeneration of the organism, contamination, long fermentation times and high costs. The groundwork, from which all microbial citric acid production processes later developed, is attributed to Wehmer.

The second phase, the result of Currie's research, reported in 1917 (Perlman and Sih, 1960), was surface fermentation using *Aspergillus niger*. Currie was the first to report the importance of the use of pure reagents in the fermentation medium to obtain increased yields. He joined Chas. Pfizer and Co. Inc., Brooklyn, New York, and with this company was partly responsible for the development of a citric acid process, which was first operated on a commercial scale in 1923.

The third phase, which is continuing now, is the submerged fermentation process. This began in 1938 with the publication of Perquin's thesis (Perlman and Sih, 1960), and

resulted in a shift in emphasis from surface culture to large-scale submerged fermentation. Surface culture methods continued in use for some years, but have now largely been replaced by the more efficient submerged culture methods. Details of the surface culture method can be found in Prescott and Dunn's (1959) review, while the review by Smith *et al.* (1974) covers the submerged culture methods.

2.3 CITRIC ACID-ACCUMULATING ORGANISMS

Many different groups of fungi have been found to accumulate citric acid, particularly the genera *Aspergillus* and *Penicillium*. Many strains of these two genera have been used by investigators studying citric acid production. They have been reviewed by Loesecke (1945), Foster (1949) and Perlman and Sih (1960). However, it is obvious from the literature that only selected strains of *Aspergillus niger*, mainly mutants, are used in the commercial production of citric acid.

Various yeasts, particularly of the genera *Candida* and *Saccharomycopsis*, have been shown to accumulate citric acid. Kapoor *et al.* (1982) have published a comprehensive review of this subject, and in the same review, mention is made of the fact that bacteria such as *Bacillus licheniformis*, *Bacillus subtilis* and *Brevibacterium flavum* have been shown to accumulate citric acid.

2.4 INDUSTRIAL PROCESSES

There are three basic processes used for commercial production of citric acid (Lockwood, 1975; Rohr *et al.*, 1983):-

1. The Koji fermentation process
2. The liquid culture shallow pan process
3. The submerged fermentation process

The precise technical details of these processes remain secret, but examination of the relevant patents issued to the companies concerned has given some information as to the processes used (Lockwood,1975).

The Koji Fermentation Process

This is a simple fermentation process, developed in Japan. Cooked, solid vegetable residues, usually sweet potato or wheat bran, are spread in trays and inoculated with a selected strain of *A. niger*. During incubation, the amylase produced by *A. niger* saccharifies the starch and much of the hydrolysed sugar is then converted to citric acid. The temperature of the solid mass is maintained at 28 °C and the pH drops to 1.8 to 2.0 as citric acid accumulates. The mass is extracted with water in percolators after 5 to 8 days incubation and the citric acid is purified. The estimated annual production by this method is only 2500 tons (Lockwood, 1975; Rohr *et al.*, 1983).

The Liquid Culture Shallow Pan Process

More commonly referred to as the surface culture method, this is the oldest method in use in the U.S.A. and

Europe. It it estimated that in a large citric acid production plant, approximately 30 acres of shallow pans are required. To prevent metal ion contamination of the substrate, the pans are manufactured from high-purity stainless steel or aluminium. The most common substrate is beet molasses, but raw sugar or glucose syrups can also be used. Traces of iron are removed by treating the molasses with ferrocyanide and filtering off the resulting precipitate. The medium is then adjusted to pH 2.5 to 4.0 using sulphuric acid. Inoculation is with spores of *A. niger* blown over the sterile solution in the pans. The spores rapidly germinate and cover the solution with a thin white pellicle of mycelium. The temperature is maintained at 30°C and sterile humidified air is blown slowly over the surface of the solution for about 5 to 6 days. After 8 to 10 days of incubation, the sugar content has been reduced from 200 g/l to 10 g/l and the maximum citric acid concentration is achieved. The yield of citric acid from this process is approximately 85% on the basis of sugar used.

The Submerged Fermentation Process

A schematic diagram of this process is shown in Figure 2.1 (Lockwood, 1975). The raw materials used are generally beet molasses, glucose syrups and high-test cane syrup. A pelletal form of a selected strain of *A. niger* is used to inoculate the fermenter. The culture is agitated and aerated throughout the entire fermentation. The duration of the fermentation depends on the initial sugar concentration,

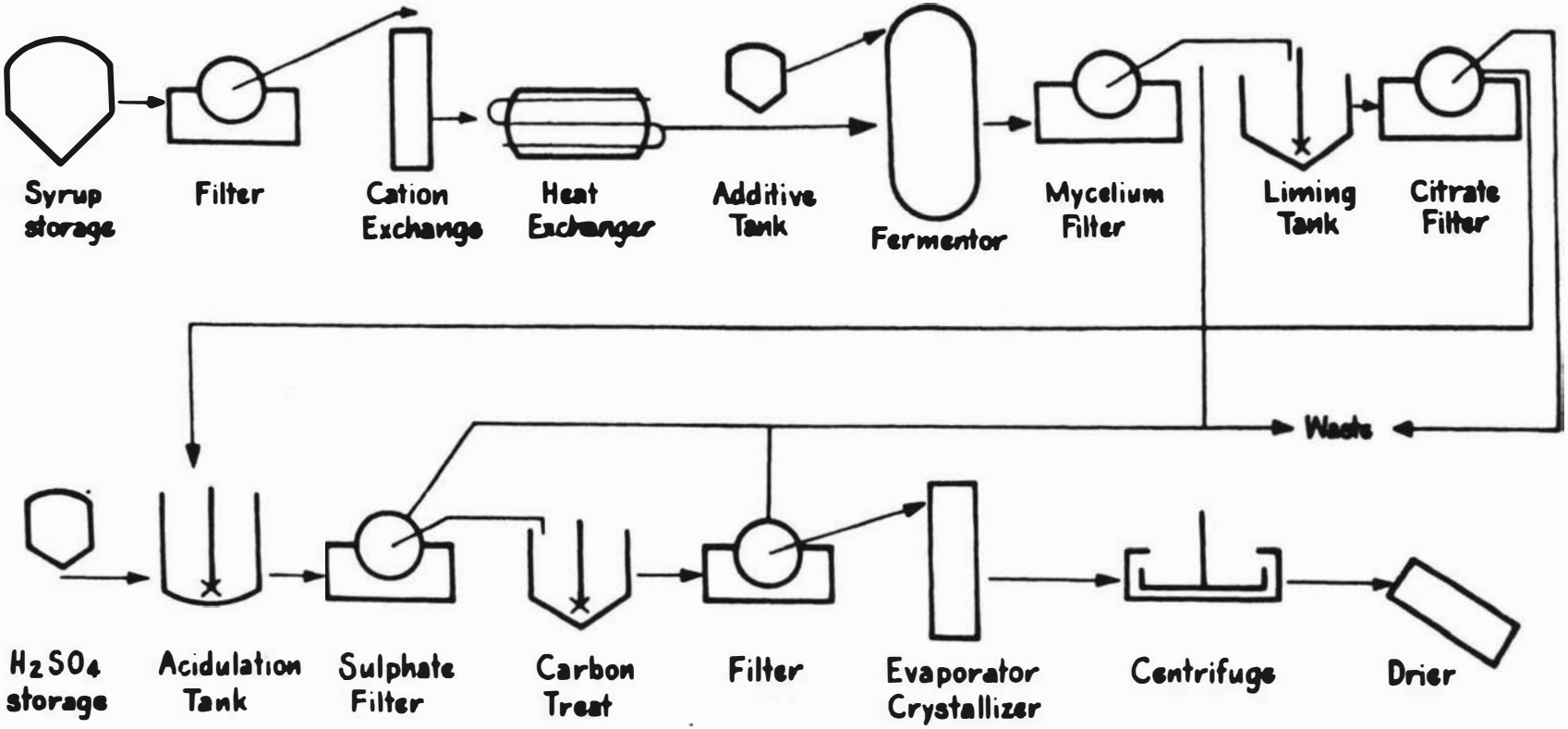


Figure 2.1 Flow diagram for submerged citric acid manufacture (Lockwood, 1975)

subsequent sugar additions and the amount of growth. It usually ranges from 6 to 14 days, but for a given set of conditions, it is a constant period. The citric acid yield from this process is about 95% on the basis of sugar utilisation (Lockwood and Batti, 1965). The submerged fermentation process, because of the following advantages:

- higher yield of citric acid based on sugar used
- improved process control
- reduced fermentation period
- reduced requirement for floor space
- reduced manual handling
- lower investment cost

is the preferred process for current commercial citric acid production (Sodeck *et al.*, 1982).

In summary, approximately 80% of the citric acid required in the western world is produced by the submerged fermentation process, using molasses as the substrate (Sodeck *et al.*, 1982).

2.5 BIOCHEMISTRY OF CITRIC ACID ACCUMULATION BY *A. NIGER*

Many theories have been put forward to explain the accumulation of citric acid by *A. niger* (e.g. Perlman and Sih, 1960; Meyrath, 1967; Kubicek *et al.*, 1980; Hossain *et al.*, 1984). The situation, however, is still that no single hypothesis fully explains the optimum physiological conditions required to obtain high yields. It is generally accepted that the final step in the synthesis of citric acid is the condensation of acetyl CoA and oxaloacetate (Figure 2.2), and that this condensation is the major route of

citric acid synthesis (Kapoor *et al.*, 1982). Two problems, then, need to be addressed. First, the reason why citric acid is accumulated rather than metabolised. Second, the source of oxaloacetic acid, since accumulation of citric acid implies some disturbance in the normal operation of the TCA-cycle, which consequently prevents the production of oxaloacetic acid by this route.

The mechanism of citric acid production by *A. niger* was studied by Shu *et al.* (1954), using a medium containing glucose-1-C^{14} as the sole carbon source. Following mathematical analysis of their data, they concluded that 37 to 40% of the total citric acid was formed from C_4 - dicarboxylic acid, *which had been produced via the TCA-cycle*. In contrast, Bomstein and Johnson (1952) and Cleland and Johnson (1954) demonstrated that very little C_4 - dicarboxylic acid was produced via the TCA cycle when the fermentation conditions were such to give 50 to 70% yields of citric acid. Using the radio labelled glucose as substrate, Cleland and Johnson (1954) concluded that glucose was first split into two C_3 -fragments (pyruvic acid) followed by the formation of a C_2 -fragment (acetyl CoA) by decarboxylation and a C_4 -fragment (oxaloacetic acid) by carboxylation. These two fragments then condensed to form citric acid.

Since then, the enzymes phosphoenolpyruvate carboxykinase and pyruvate carboxylase have been demonstrated to be active during citric acid production (Woronick and Johnson, 1960; Bloom and Johnson, 1962). It has also been reported that pyruvate carboxylase, isocitrate

lyase and oxaloacetate hydrolase are active in *A. niger* during oxalic acid and citric acid production (Verhoff and Spradlin, 1976). Hence, there is evidence that a major source of oxaloacetic acid during citric acid accumulation is *via* carboxylation of a C_3-fragment.

There a number of reports in the literature concerning the involvement of some TCA-cycle enzymes in citric acid accumulation. Three key enzymes that have been examined are aconitase and both NAD-linked and NADP-linked isocitrate dehydrogenases. Ramkrishnan *et al.* (1955) reported that citric acid accumulated due to the disappearance of aconitase and isocitrate dehydrogenase at the end of the initial growth phase. It was not stated , however, which isocitrate dehydrogenase was examined. They further demonstrated that during citric acid accumulation, the activity of the condensing enzyme increased tenfold and that the accumulated citric acid inhibited the activity of isocitrate dehydrogenase. However, the presence of aconitase and both NAD-linked and NADP-linked isocitrate dehydrogenases during citric acid accumulation was demonstrated by La Nauze (1966), although in lower activity during the citric acid production phase than during the initial growth phase of the fungus. There is a major defect in the report of Ramkrishnan *et al.* (1955), in that they failed to give details of the method used for the preparation of the cell-free extract, and more importantly, failed to give details of the precautionary measures taken to avoid enzyme inactivation during this preparation

procedure. Thus, the observed disappearance of these enzymes may have been the result of inactivation during the preparation procedure, particularly since many subsequent investigators have demonstrated these enzymes to be active during citric acid accumulation. Ahmed *et al.* (1972) re-examined the role of the TCA-cycle during citric acid accumulation, in particular studying mitochondrial function, TCA-cycle enzymes and intermediates of the TCA-cycle. They demonstrated that certain TCA-cycle enzyme activities, e.g. the citric acid condensing enzyme, aconitase and both NAD-linked and NADP-linked isocitrate dehydrogenases, were as high during the production phase as during the initial growth phase. The presence of TCA-cycle intermediates, as demonstrated by Ahmed (1970), gives no support to the concept of a complete blockage of the TCA-cycle during citric acid accumulation.

Szczodrak (1981) studied the activities of aconitase, both NAD-linked and NADP-linked isocitrate dehydrogenases and citrate synthase (condensing enzyme) in cell-free extracts of *A. niger* during citric acid production. He reported that during the production phase, the activities of aconitase and both NAD-linked and NADP-linked isocitrate dehydrogenases decreased significantly compared with their activities during the growth phase (but did not disappear completely) and that citrate synthase activity was maintained at a constant level throughout the entire fermentation. Hossain *et al.* (1984) calculated the specific production rate of citric acid over the entire fermentation period and showed a relationship between this and the

activities of aconitase and both NAD-linked and NADP-linked isocitrate dehydrogenases. The maximum specific citric acid production rate coincided with the maximum observed activities of these three enzymes. Thus, although the pattern of enzyme activity shown by Hossain *et al.* (1984) agreed with that of Szczodrak (1981), the latter author failed to report citric acid production rates, and did not provide sufficient data for such calculations. Hence, whereas Szczodrak (1981) postulated that citric acid accumulation followed a decrease in activity of these enzymes, Hossain *et al.* (1984) postulated that the enzyme activities were high in response to large losses of citric acid from the cell at a time when the organism was still growing and needed to "scavenge" this intermediary metabolite. Then, as the specific growth rate decreased, so did the level of enzyme activity. Consequently, it was postulated that these enzyme activities are influenced by the citrate concentration, rather than the citrate concentration being influenced by the enzyme activities. Unfortunately, Hossain *et al.* (1984) did not provide any data regarding growth rate.

There are some reports in the literature which indicate a blockage of the TCA-cycle at the step of 2-oxoglutarate dehydrogenase. Kubicek and Rohr (1977, 1978) were unable to detect this enzyme in cell-free extracts of *A. niger* during citric acid production in a sucrose-based synthetic medium. Hossain *et al.* (1984) were also unable to detect this enzyme when using a synthetic medium with sucrose, glucose or

fructose as the sole carbon source. It was, however, detected when either lactose or galactose was the sole carbon source, both of which are poor substrates for citric acid production. Both groups postulated a blockage in the TCA-cycle at the step of 2-oxoglutarate dehydrogenase as being responsible, at least in part, for citric acid accumulation. Hossain *et al.* (1984) further postulated that the blockage was caused by the action of glucose and fructose in repressing this enzyme, but that galactose and lactose did not cause such repression. In contrast, Meixner-Monori *et al.* (1985) were able to detect and measure 2-oxoglutarate dehydrogenase activity, when, during the enzyme assay, the reaction was initiated by the addition of coenzyme-A rather than by addition of 2-oxoglutarate. Thus, the activity of 2-oxoglutarate dehydrogenase was measurable at all sampling times during citric acid accumulation. They suggested that the inability of other workers to detect the activity of this enzyme was the result of attempting to initiate the reaction by the addition of substrate, as opposed to addition of coenzyme-A. The former method will show activity only if the enzyme is active at normal biosynthetic levels, and will show an apparent complete inhibition of activity where the enzyme may be present as a partially inactivated enzyme complex. Thus, very low levels of enzyme activity, rather than no activity, would probably have been observed by Kubicek and Rohr (1977, 1978) and Hossain *et al.* (1984) using the latter method. On this basis, the hypothesis of Hossain *et al.* (1984) of complete blockage of the TCA-cycle at the step of 2-oxoglutarate

dehydrogenase, if modified to read "significant inhibition of" remains valid and no longer contradicts the results of Ahmed (1970).

After an extensive study of the physiological aspects of the citric acid fermentation, Kubicek and Rohr (1981) suggested that the following metabolic events are responsible for citric acid accumulation by *A. niger*:

(a) excessive catabolism of glucose *via* the hexose monophosphate pathway due to poor regulation at the phosphofructokinase and phosphoenolpyruvate carboxykinase steps

(b) uninfluenced rate of citric acid formation by poorly regulated citrate synthase

(c) incomplete operation of the TCA-cycle due to inhibition of 2-oxoglutarate dehydrogenase by glucose and NH_4^+ ions, and inhibition of isocitrate dehydrogenase and succinic dehydrogenase by several metabolites

(d) anaplerotic formation of oxaloacetate by constitutive, weakly-regulated pyruvate carboxylase.

Since then, reports from the same laboratory have attempted to explain some of these metabolic events. Habison *et al.* (1983) examined the role of phosphofructokinase, pyruvate carboxylase and citrate synthase. They concluded that the key regulatory enzyme was phosphofructokinase, in that feedback inhibition of this enzyme by citrate was impaired. They reported that this situation arose when the intracellular NH_4^+ level was increased. Kubicek *et al.* (1979) and Ma *et al.* (1985) reported that increased protein

degradation and consequent increase in intracellular NH_4^+ levels, occurred during Mn^{2+} deficient conditions during the production phase. Rohr *et al.* (1983) showed phosphofructokinase activity to be insensitive to citrate repression in the presence of physiological concentrations of NH_4^+ *in vitro*. They postulated that an explanation for citric acid accumulation was reduced feedback inhibition of phosphofructokinase due to the release of NH_4^+ ions resulting from protein turnover.

Rohr *et al.* (1983) suggested a mechanism for citric acid accumulation in which the TCA-cycle, due to the impaired activity of 2-oxoglutarate dehydrogenase, operates a mechanism resembling the "horseshoe-cycle" which is known to operate in facultative anaerobic bacteria, and in which the other dicarboxylic acids are formed by the reduction of oxaloacetate. Thus, pyruvate carboxylase is formed by the fungus to ensure a sufficient supply of oxaloacetate.

By the measurement of key enzymes, Kubicek and Rohr (1977), showed the ratio of glycolytic to pentose phosphate pathway metabolism of glucose to be 2:1 under unlimited growth conditions. However, during citric acid accumulation the involvement of glycolysis increases to a ratio of 4:1. Consequently, reoxidation of glycolytic NADH, produced during ATP formation, is necessary for citric acid accumulation. Kubicek *et al.* (1980) suggested that *A. niger* contains a standard respiratory chain sensitive to antimycin and azide, and an alternative branch sensitive to salicylhydroxamic acid (SHAM) (Figure 2.3). They showed that citric acid accumulation was strongly inhibited by SHAM, but

growth was insensitive, and that both growth and citric acid accumulation were sensitive to antimycin and azide. They also suggested that a high culture Dissolved Oxygen Tension (DOT) is necessary to maintain the activity of this alternative branch, hence the reason for the requirement for a high culture DOT for significant citric acid accumulation to occur. In other microorganisms, this branch of the pathway has been reported to facilitate the reoxidation of cytoplasmic NADH in the absence of oxidative phosphorylation so that glycolytic ATP formation can proceed. Thus, the increased respiratory activity (which in part is not coupled to ATP synthesis), and the deregulation of phosphofructokinase from citrate inhibition by increased intracellular NH_4^+ levels, stimulate a metabolic flux through glycolysis without significant metabolic control. This situation, given the anaplerotic nature of the pyruvate carboxylase enzyme and the unusual operation of the TCA-cycle (in that oxoaloacetate is metabolised to both malic acid and citric acid due to the low level of activity of 2-oxoglutarate dehydrogenase), results in a rise in the citrate concentration.

There are little data published on the role of isocitrate lyase in citric acid production. Two groups investigated isocitrate lyase activity during citric acid production by different strains of *A. niger* (Ahmed *et al.*, 1972; Ng *et al.*, 1973). Both reported that the enzyme was active throughout the fermentation. From mass balance work, Verhoff and Spradlin (1976) postulated a scheme of citric

Figure 2.2 The Tricarboxylic acid cycle with Glyoxylic acid cycle and carbohydrate input.

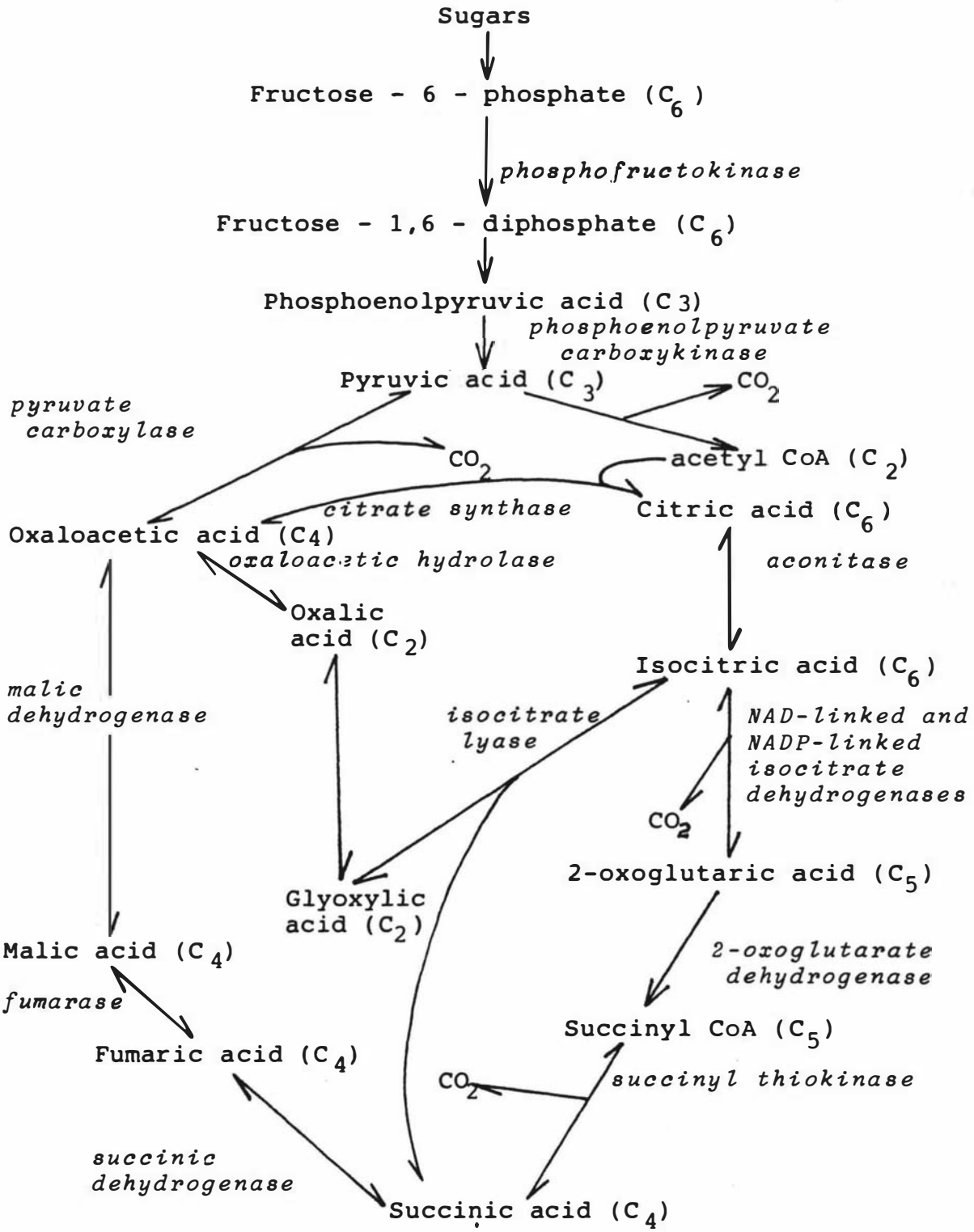
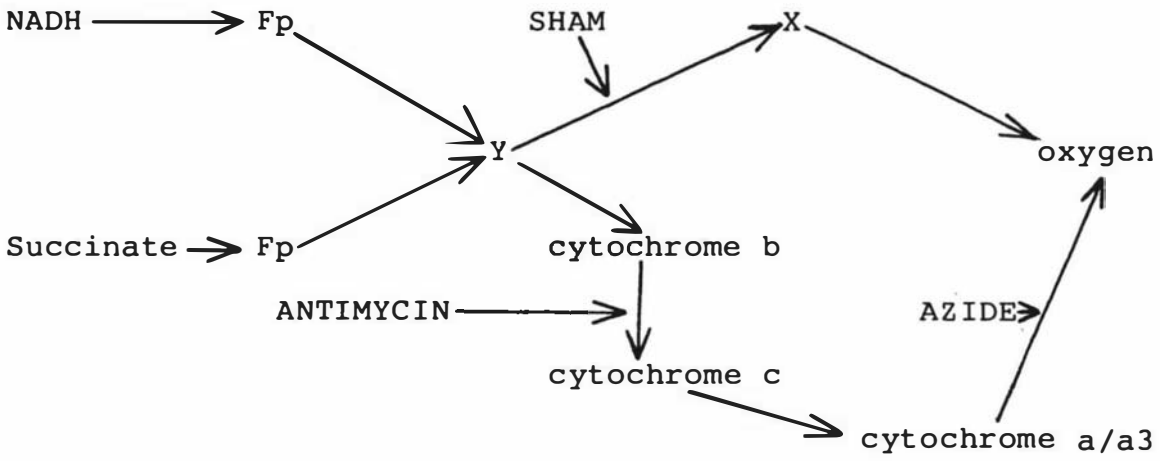


Figure 2.3 Alternative pathway for the non-phosphorylating reoxidation of reduced adenine nucleotides



Fp = Flavoproteins

X and Y are as yet unidentified

(Kubicek *et al.*, 1980).

acid accumulation involving isocitrate lyase. It involved the accumulation of lipids by the organism, which caused the glyoxylate cycle to operate in an attempt to convert the lipids back to sugars. Because an excess of sugar is still present, oxalic acid accumulates. With the conversion of oxalic acid to glyoxylate (Figure 2.2), the subsequent reaction glyoxylate to isocitrate (the enzyme isocitrate lyase) is the reverse of that normally operating when the glyoxylate cycle is in operation, so isocitrate is formed. However, this hypothesis was based on mass balance equations derived from data obtained from batch fermentations, in which they started from a premise that citric acid production occurred only after active growth had ceased. However, Hossain *et al.* (1984) have shown this not to be the case. Therefore, while this hypothesis cannot be dismissed, it must be treated with some caution.

In summary, a great deal of information with regard to the operation of the TCA-cycle, and more recently some with regard to the glyoxylate cycle during citric acid accumulation has been published. However, much of it is conflicting, and the situation, although slightly clearer than in 1980, remains uncertain.

2.6 ENVIRONMENTAL FACTORS AFFECTING CITRIC ACID ACCUMULATION

Much contradiction exists in the literature regarding the effects of different environmental factors on citric acid production. Possibly, this can be explained because different workers have used (1) different strains of

organism, (2) different media and (3) different sources of nutrient chemicals, possibly contaminated with different levels of trace elements. Also, it must be borne in mind that the various environmental factors can interact with each other. Hence, the following sections summarise those factors which are known to be important.

2.6.1 Form of Growth

When filamentous fungi are grown in submerged culture the type of growth varies from the "pellet" form, consisting of a compact discrete spherical mass of hyphae, to the "filamentous" form in which the hyphae form a homogenous suspension dispersed throughout the medium. The pellet mode of growth is generally believed to be desirable in the citric acid fermentation and its formation depends upon the fungal species, the size of the inoculum, the growth medium and the physical environment within the culture vessel e.g. pH, aeration and agitation (Whitaker and Long, 1973).

Schweiger and Snell (1949) developed a medium in which *A. niger* grew in the form of small pellets, averaging 0.1 mm diameter, which were composed of short stubby, forked, bulbous mycelia. They reported that the slime-forming tendencies of the fungus were eliminated and aeration was more easily achieved. Carilli *et al.* (1961) observed that the filamentous form of *A. niger* reduced the oxygen level in the medium to zero after 15 hours of fermentation. When the fungus was induced to form pellets, the viscosity of the suspension was considerably lower and the dissolved oxygen level considerably higher. They concluded that the pellet

form of growth was essential to maintain an excess of oxygen in the culture medium and hence achieve higher yields of citric acid. Clark *et al.* (1966) reported that filamentous growth of *A. niger* has little capacity to produce citric acid and its occurrence has always resulted in poor yields. They further demonstrated that Mn^{2+} ions induced filamentous rather than pelletal growth during submerged fermentation.

Heinrich and Rehm (1982) studied citric acid production from *A. niger* in both shake-flask culture and in a stirred fermenter. They obtained higher citric acid yields in the shake-flask than in the stirred fermenter culture. The reason given for the difference was the different modes of growth, i.e. pellets in shake-flask and filaments in the stirred fermenter. They concluded that the filamentous growth in the fermenter was due to Mn^{2+} ion contamination of the medium from impurities in the stainless steel parts.

Whitaker and Long (1973) published a review in which the importance of the pelletal form of growth was extensively discussed. However, they did not present evidence to demonstrate clearly whether it is the pelletal form or the fermentation conditions (which coincidentally encourage pellet formation) which is important in citric acid production. The efficiency of aeration of the fermentation liquor is much greater in a pelletal culture than in a filamentous culture. However, there is at present no experimental data published regarding the aeration efficiency *within* pellets. Also, such factors as Mn^{2+} ions, the presence of which is known to be detrimental to citric acid production, were postulated to act through their effect

on fungal morphology, in which the filamentous form of growth is encouraged. The work of Habison *et al.* (1983) (Section 2.5) indicates that the Mn^{2+} ion effect is biochemical, and related to the intracellular NH_4^+ ion concentration.

2.6.2 pH

The maintenance of proper acidity of the fermentation medium is important for successful production of citric acid. A low pH favours citric acid accumulation (and coincidentaly minimizes the danger of microbial contamination), while a high pH favours the production of oxalic acid (Prescott and Dunn, 1959). In general, the best citric acid-producing strains possess the greatest tolerance of acidic conditions, but the most favourable pH will depend largely on the fungal strain used (Loesecke, 1945). Berry *et al.* (1977) have suggested that the most favourable pH value is between 2.0 and 3.0. A higher pH is favoured during the initial stages of the fermentation since this facilitates mycelial growth; thereafter the pH is permitted to drop during the fermentation as the citric acid accumulates in the medium.

Shu and Johnson (1948b) demonstrated that the initial pH value of a sucrose-based synthetic medium influenced the rate of citric acid production in submerged culture. They observed that at initial pH 1.7 both growth and citric acid production were greatly retarded. They obtained the highest yield at initial pH 3.7 to 4.2. Banik (1975) adjusted the pH of a sucrose-based synthetic medium to 2.0, 2.5, 3.5,

4.0, 4.5 and 5.0 and found that the optimum initial pH for the production of citric acid was 3.5. However, it has been suggested that the optimum initial pH varies depending on the nature of the substrate; a pH value of 2.5 to 4.0 is optimum for defined media while an initial pH of 6.0 to 7.5 is required in molasses medium (Berry *et al.*, 1977). Hossain *et al.* (1983) observed that an initial pH of 4.5 was optimal for citric acid production from whey permeate.

Kristiansen and Charley (1981) conducted experiments in a continuous culture chemostat at a growth rate of 0.075 h^{-1} and pH values ranging from 1.5 to 3.5. They calculated the specific citric acid production rates and concluded that the optimum pH value for citric acid production was pH 1.75. They also demonstrated that at a growth rate of 0.075 h^{-1}, the overall citric acid productivity and the biomass concentration were independent of the pH within the range 1.6 to 3.9.

2.6.3 Temperature

The importance of incubation temperature in determining the yield of citric acid has been emphasised by many investigators (e.g. Kitos *et al.*, 1953; Martin, 1957; Kristiansen and Charley, 1981). The temperature used will depend in part on the organism and the fermentation conditions. The optimum temperature range of 28 to 30 °C for *A. niger* has been proposed for high yields and rapid rates of accumulation (Prescott and Dunn, 1959). Doelger and Prescott (1934) found that increasing the temperature above 30 °C decreased the citric acid yield and increased

oxalic acid accumulation.

2.6.4 Aeration

Citric acid is an oxidation product of a hexose sugar, and therefore, during its production under submerged culture conditions, it is necessary to ensure a supply of oxygen exceeding that required for growth alone.

Karow and Waksman (1947) obtained maximum yields of citric acid when pure oxygen instead of air was supplied to the cultures. They concluded that oxygen is a limiting factor in the production of citric acid in submerged fermentation. They did not however, report the oxygen uptake rates.

Using a synthetic sucrose-based medium in 50-gallon tanks, Buelow and Johnson (1952) found that by increasing the airflow rate from 0.9 to 3.5 mmol oxygen/l.min and increasing the agitation speed, the fermentation time was decreased by approximately 40 hours, and the yield increased from 55 to 80% (based on sugar utilised). No culture DOT values, oxygen uptake or carbon dioxide production rates were reported and so no comparisons of these rates with those of other workers is possible.

Kovats and Gackowska (1976) reported that an interruption to aeration during the fermentation adversely affected citric acid production. Kubicek *et al*. (1980) reported that a short interruption to aeration (20 minutes) resulted in a complete and irreversible loss of ability to produce citric acid, but had no effect on the viability of the organism. The industrial importance of this finding, is

that if a mechanical breakdown occurs and lasts longer than 20 minutes, the fermentation will have to be abandoned. Fundamentally, it indicates that the citric acid producing capability of the organism is fragile, in that it will not survive adverse conditions for more than a few minutes, whereas the viability of the organism is unaffected. The authors did not attempt to explain this effect. However, it is unlikely that it is a genetic effect, such as plasmid curing, due to the rapidity of the loss of the property (Scott, pers. comm., 1986). A possibility is that induction of a biochemical pathway, not normally operating under conditions suitable for citric acid accumulation, occurs. Once induced, this pathway continues operation even after recommencement of aeration, with citric acid as a substrate. However, it must be emphasised that this is speculation. Kubicek *et al.* (1980) reported critical DOT values for *A. niger* of 18 to 21 mbar (10 to 12% of air saturation) during the growth phase, and 23 to 26 mbar (12 to 15% of air saturation) during the production phase. The minimum DOT value for citric acid production was 25 mbar (15% of air saturation) and production increased steadily between 40 and 150 mbar (25 and 75% of air saturation). They reported specific oxygen uptake rates of 0.12 mmol/gDW.h and specific carbon dioxide production rates of 0.06 mmol/gDW.h. These figures are significantly lower than those reported by Siebert and Schulz (1979) which were in the order of 1 to 10 mmol/gDW.h for the specific oxygen uptake rate, and 0.1 to 2.0 mmol/gDW.h for the specific

carbon dioxide production rate. Measurements of the specific oxygen uptake rate of fungi in other aerobic fermentations, such as penicillin production (Varder and Lilly, 1982), are of the same order as those reported by Siebert and Schulz (1979), so the data of Kubicek *et al.* (1980) must be treated with some caution. Overall, few data are available on the oxygen uptake and carbon dioxide production rates during the citric acid fermentation.

2.6.5 Nutritional Status of the Growth Medium

It is generally accepted that in batch fermentation microoganisms will grow at their maximum specific growth rate, until at least one nutrient in the local environment becomes growth-limiting. Unless the growth-limiting nutrient is replenished, it will eventually be exhausted from the local environment and this is termed "nutrient exhaustion". This phenomenon is used in fermentation processes to allow the organism to multiply to an optimum biomass concentration by the use of a non-carbohydrate growth-limiting substrate such as nitrogen. Once the growth-limiting nutrient is exhausted from the medium, the organism converts the remaining carbohydrate to the desired product rather than to biomass.

2.6.5.1 Carbohydrate

Pure sugars such as glucose or sucrose were used as the carbon source in much of the early work on citric acid production, but in present commercial practice the raw material is generally cane or beet molasses. Early workers established that a relatively high sugar concentration of

100 to 200 g/l was required to give high yields of citric acid (based on sugar utilised) and that in weaker sugar solutions, a large proportion of the sugar was required as an energy source for growth; consequently less acid could be produced (Currie, 1917; Porges, 1932; Doelger and Prescott, 1934). In current commercial practice, using submerged fermentation, the concentration of sugar in the medium is adjusted to 160 g/l (Berry *et al.*, 1977).

Various sugars have been investigated as carbohydrate sources for citric acid production, including glucose, sucrose, arabinose, fructose, lactose, galactose and mannose. Very little citric acid was obtained from galactose in comparison with other sugars (Amelung, 1927; Noguchi, 1962). Hossain *et al.* (1984) investigated citric acid production from different sugars including sucrose, glucose, fructose, lactose and galactose. They showed that the maximum rate of citric acid production using sucrose was at least 2.5 times higher than that obtained using any of the other sugars. No citric acid was produced when galactose was the sugar source and when a mixture of glucose and galactose was used as the sugar source, Hossain *et al.* (1985) showed galactose to have an inhibitory effect on citric acid production from glucose.

2.6.5.2 Nitrogen

Nitrogen is generally used as the growth-limiting nutrient during citric acid production in batch fermentation, so that the fermentation operates under nitrogen exhaustion during the production phase. However,

there are no published data showing the exact relationship between exhaustion of nutrient nitrogen and the initiation of citric acid production. Loesecke (1945) reported that a low nitrogen concentration favours citric acid production, while a high nitrogen concentration favours abundant growth of mycelium, with low citric acid production. This effect of the high concentration probably occurs for two reasons; firstly, by the time the nitrogen is exhausted from the medium less sugar remains for conversion to citric acid since it has already been converted to biomass and carbon dioxide; and secondly, the higher the biomass concentration, the more difficult it is to maintain a sufficiently high DOT for citric acid production to occur. Thus, the need to limit biomass is emphasized.

The nitrogen source is usually ammonium sulphate, ammonium nitrate, sodium nitrate, potassium nitrate or ammonia. No single source is superior to any others and the advantages occasionally observed are a measure of the purity of the compound used (Perlman and Sih, 1960).

2.6.5.3 Phosphate

Generally, potassium dihydrogen orthophosphate is used when phosphate needs to be added to commercial fermentation substrates. It is also the source in most synthetic media. A patent by Szucs in 1944 (Shu and Johnson, 1948a) claimed that phosphate limitation was required for citric acid production in submerged culture, but it was later established by the latter authors that this work had been performed using an insufficiently purified phosphate source,

which still contained some trace metal ions, and that while the phosphate concentration should be low, it need not be limiting to allow citric acid accumulation. Except for the more recent work by Kristiansen *et al.* (1982) in continuous culture chemostat operated with a phosphate-limited feed medium, very little information exists on the effect of phosphate limitation on citric acid production. Kristiansen *et al.* (1982) showed that less citric acid was produced during phosphate-limited steady states than during those of nitrogen limitation. However, they concluded that the amount of excess nitrogen in the medium was the important factor rather than the lack of phosphate, in that the higher the excess of nitrogen, the less citric acid was produced.

2.6.5.4 Trace Elements

Many variations have been reported in the requirements for some essential trace elements, such as Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+}, in the citric acid fermentation, reflecting the different strains of *A. niger* used, as well as the different sources of nutrient chemicals. It is well established that the trace metal requirements must be investigated whenever a new strain or substrate is used (Berry *et al.*, 1977).

Clark *et al.* (1966) studied the effect of various trace metals on citric acid production and concluded that very low levels of Fe^{2+}, Cu^{2+} and Zn^{2+}, and the total absence of Mn^{2+} were required for high yields of citric acid.

No data are available on trace metal uptake rates during the citric acid fermentation. The only trace metal to have been studied in any detail is Mn^{2+}. Kubicek and

Rohr (1977) demonstrated that Mn^{2+} in the fermentation medium decreased the specific activity of citrate synthase, while increasing the specific activities of aconitase and both NAD-linked and NADP-linked isocitrate dehydrogenases. Habison *et al.* (1983) reported increased intracellular NH_4^+ levels when Mn^{2+} was absent from the fermentation medium, possibly due to increased protein degradation. The high level of NH_4^+ deregulates the citrate inhibition of phosphofructokinase, thus facilitating the accumulation of citric acid. However, in Mn^{2+}-sufficient media, the intracellular NH_4^+ levels were lower, as was the citric acid yield (Habison *et al.*, 1983).

The presence of Mn^{2+} has also been reported to encourage filamentous as opposed to pelletal morphology (Kisser *et al.*, 1980; Heinrich and Rehm, 1982).

The trace metals Fe^{2+}, Cu^{2+} and Zn^{2+} have been reported to have a critical influence on citric acid production. Several reports indicate that some or all of these metals should be absent from the medium or present in very low concentration. Shu and Johnson (1947, 1948b), for example, reported that Fe^{2+} must be present in low concentrations, yet La Nauze (1966) implies that it should be absent for high yields of citric acid. Cu^{2+} has been reported to be an antagonist to Fe^{2+} (Schweiger, 1961), which, if this is the case, suggests that a lower concentration of Fe^{2+} will negate the requirement for Cu^{2+}. It has been stated that onset of Zn^{2+} deficiency during growth of the fungus signals the transition from the growth phase to the production phase

(Kapoor *et al.*, 1982). However, it is generally accepted that this transition occurs at the point of nitrogen exhaustion.

2.6.6 Presence of Methanol and Other Compounds

A major advance in the technology of the citric acid fermentation was the observation by Moyer (1953a, b, 1954) that the addition of alcohols or esters to the fermentation medium resulted in increased citric acid production. He demonstrated that the addition of methanol (final concentration 2 to 4% (v/v)) at inoculation greatly enhanced citric acid production by *A. niger* from crude carbohydrate sources such as gelatinised cornstarch, blackstrap molasses and beet molasses. Hossain *et al.* (1984) reported that no citric acid was produced when galactose was the substrate. However, the addition of 3% (v/v) methanol on day 3 of the fermentation caused citric acid to be produced from galactose. The exact role of methanol was not stated by either author, but it was not assimilated during the fermentation. Several other workers have reported that other compounds are also beneficial in increasing the citric acid yield, for example glycerol (Leopold, 1971) and fatty acids (Millis *et al.*, 1963).

2.7 RELATIONSHIP BETWEEN GROWTH RATE AND CITRIC ACID PRODUCTION

Gaden (1955) proposed a classification scheme of fermentations in which there were three types. Type I fermentations convert the energy source directly to product

as a result of metabolism, and as such are growth-associated. For Type II fermentations, the product formation exhibits some relationship with growth rate, but it is not directly growth-associated. In Type III fermentations, growth-associated metabolism of the energy source has passed its maximum and has almost ceased before the period of maximum product formation. The classical examples of the three types are ethanol, citric acid and penicillin for Type I, Type II and Type III, respectively.

Many reports have been published on the submerged batch fermentation of citric acid but few data are presented on growth rates, as opposed to mycelial dry weights. It is possible to calculate approximate specific growth rates at particular times during the fermentation from the data of some authors (e.g. Siebert and Schulz, 1979; Hossain *et al.*, 1983, 1984) due to their frequency of sampling. However, most authors have not sampled frequently enough. Hossain *et al.* (1984) demonstrated that the maximum specific rate of citric acid production occurred prior to the exhaustion from the medium of nutrient nitrogen, the growth-limiting substrate, and not later in the fermentation, as has been generally accepted in the literature. This implies that the maximum production occurs at a positive growth rate. Because of the well-established variation of the specific growth rate during a batch fermentation, the culture will be at the optimum growth rate for citric acid production for only a short period of time. This was recognised by Kristiansen and Sinclair (1979), and Kristiansen and Charley (1981), both of whom used chemostat continuous culture to

investigate, amongst other parameters, the effect of growth rate on citric acid production. The former authors used *Aspergillus foetidus*, whilst the latter used *A. niger*. Both authors reported an optimum specific growth rate of approximately 0.075 h^{-1} for citric acid production. However, neither group recorded the DOT of the culture at steady state, nor did they attempt to control it. Hence, although they maintained aeration and agitation conditions constant for all steady states, it is clear that different DOT values must have existed since the oxygen uptake rate would have varied with the growth rate. The reported optimum growth rates must therefore be called into doubt, as at least two parameters (DOT and growth rate) changed for each steady state, when it was assumed that only the growth rate changed. Only the *value* of the optimum growth rate is questioned, however, not its existance.

2.8 CONTINUOUS CULTURE CHEMOSTAT PRODUCTION OF CITRIC ACID

Most published work on citric acid production using the submerged fermentation process has been from laboratory or pilot scale batch fermentation. This may contribute to the varied observations and consequent lack of a single unanimously supported hypothesis for the mechanism of citric acid accumulation by *A. niger*.

Lesniak and Stawicki (1979) used a "semi-continuous" system of removal of a given volume of fermentation broth, containing a representative amount of biomass and replacement with fresh medium at daily intervals. This was

in effect a repeated fed-batch culture. They did not attempt a chemostat study, citing technical problems with maintaining a constant volume. This problem has also been experienced by other workers attempting chemostat studies with filamentous organisms (Kristiansen and Charley, 1981; Clark, 1982; Clark *et al*., 1983). The main problem involves rapid blockage of the overflow port if a standard weir tube is employed. However, methods have been devised to control the outlet flow, usually involving application of a vacuum on an electrically controlled time signal (Clark *et al*., 1983).

A major use of the continuous culture chemostat is as a research tool. Thus, steady state conditions may be achieved, in contrast to the transient conditions in traditional batch fermentation. In turn, this allows control, and hence investigation, of various environmental factors. However, it must be emphasised that to interpret correctly data from a chemostat, only the one parameter under investigation can be permitted to change, unless, in a designed experiment provision is made for the study of two or more parameters. In this situation, a factorial experimental design must be used.

The commercial advantage of using a continuous culture chemostat is greater productivity than from a batch process, since the conditions required for the maximum productivity can be maintained for long periods. In contrast, during a batch process these conditions may occur only for a very short period of time. Kristiansen and Charley (1981) reported a 3-fold increase in productivity over published

literature values for batch stirred tanks and semi-continuous tower processes. They stated, however, that they used a sucrose-based synthetic medium, and comparison was with values reported for both synthetic media and molasses.

Studies on citric acid production in chemostat culture have been described by Kristiansen and Sinclair (1979) and Kristiansen and Charley (1981), using both nitrogen- and phosphate-limited conditions. Their studies pertained mainly to the effects of growth rate (see Section 2.7) and pH value (see Section 2.6.2) on citric acid production.

2.9 CONTINUOUS FED-BATCH CULTURE

The term fed-batch culture was defined by Yoshida *et al.* (1973) as a batch culture continuously fed with a growth-limiting substrate. There is no continuous removal of culture so there is a constantly increasing volume. Thus, it is distinguished from the constant volume continuous culture chemostat.

Pirt (1974) developed mathematical expressions for the continous fed-batch system, while Dunn and Mor (1975) developed Pirt's work further. Both of these authors used Monod's equation as the basis of their theoretical studies. A comprehensive review of fed-batch techniques has recently been published (Yamane and Shimizu, 1984), but no mention is made of their application to citric acid production.

Continuous fed-batch systems are used industrially for penicillin production and waste treatment (Pirt, 1974), bakers yeast production (Woehrer and Roehr, 1981) and the

production of some amino acids and extracellular enzymes (Yamane and Shimizu, 1984). Kristiansen and Charley (1981) stated that citric acid was produced commercially using fed-batch fermentation. However, they gave no details of the process and failed to provide any patent or literature reference.

In the continuous fed-batch fermentation, a quasi-steady state is obtained when the growth-limiting substrate is exhausted from the batch fermentation broth and growth-limiting amounts are then added continuously. The fed-batch quasi-steady state and chemostat steady state are comparable, in that in both cases the growth rate equals the dilution rate. However, the difference is that in the chemostat, dilution rate (and hence, growth rate) is constant as a consequence of the constant volume, whereas with the fed-batch system, the dilution rate decreases with time due to the increase in volume. The dilution rate in fed-batch fermentation can be maintained constant for some time by increasing the flow rate in proportion to the increase in volume (Pirt, 1974; Dunn and Mor, 1975). Thus, the growth rate can be controlled in fed-batch fermentations.

Industrially, the use of the fed-batch fermentation allows the application of data accumulated from chemostat studies, and reduces the "down time" of expensive capital equipment when compared with the batch process. The fed-batch system is technically easier to operate than the chemostat, because there is no requirement for exact volume control and therefore there are no overflow port blockage

problems, which is particularly important when using filamentous fungi.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Microbiological Media

The Sucrose-Beef Extract medium adopted for the sporulation of *A. niger* MH 15-15 was that described by Sanchez-Marroquin *et al* (1970) (Table 3.1). The liquid media described by Kristiansen and Charley (1981), or modifications thereof, were used in the fermentation experiments (Tables 3.2 and 3.3).

Table 3.1 Sucrose-Beef Extract Medium (Sanchez-Marroquin *et al.*, 1970).

Component	Concentration (g/l)*
Sucrose	2.5
Beef Extract	10.0
Sodium Chloride	5.0
Agar	15.0

* The medium was made to volume with distilled water.

Table 3.2 Medium for Batch Fermentation (Kristiansen and Charley, 1981).

Component	Concentration (g/l)*
Sucrose	140.0
$(NH_4)_2SO_4$	2.0
KH_2PO_4	2.0
$MgSO_4.7H_2O$	0.5
Fe^{2+} a	0.1×10^{-3}
Zn^{2+} b	0.1×10^{-3}
Cu^{2+} c	60.0×10^{-6}

Table 3.3 Feed medium for Chemostat Fermentation (Kristiansen and Charley, 1981).

Component	Concentration (g/l)*
Sucrose	50.0
$(NH_4)_2SO_4$	1.0
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.1
Fe^{2+} a	0.1×10^{-3}
Zn^{2+} b	0.1×10^{-3}
Cu^{2+} c	60.0×10^{-6}

* Medium made to volume with distilled water and adjusted to pH 6.5 using 1.0 M NaOH

a as $(NH_4)_2SO_4Fe_2(SO_4)_2.24H_2O$; b as $ZnSO_4.7H_2O$

c as $CuSO_4.5H_2O$

3.1.2 Gases

The following gases were supplied by New Zealand Industrial Gases Limited, Palmerston North, New Zealand: oxygen-free nitrogen; dry air; carbon dioxide; 4% carbon dioxide in oxygen-free nitrogen; 16% oxygen in oxygen-free nitrogen.

3.1.3 Chemicals

Chemicals used for fermentation and analytical work were all of analytical grade. Their sources were:

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

ammonium ferrous sulphate; ammonium sulphate; buffer tablets, pH 4.0 and 7.0; citric acid; coomassie brilliant blue G250; copper sulphate; ethylene diaminetetracetic acid; fructose; hydrazine sulphate; hydrochloric acid; magnesium chloride; magnesium sulphate; orthophosphoric acid; di-potassium hydrogen phosphate; potassium dihydrogen phosphate; potassium cyanide; sodium molybdate; sulphuric acid; tris(hydroxymethyl) methylamine.

-Ajax Chemicals (Sydney, Australia).

acetonitrile (HPLC grade), methanol (HPLC grade).

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

adenosine 5-monophosphoric acid (AMP, muscle adenylic acid); adenosine 5-triphosphoric acid (ATP, sodium salt); beef extract; bovine serum albumin; *B*-nicotinamide adenine dinucleotide (NAD); *B*-nicotinamide adenine dinucleotide, reduced form (NADH, disodium salt); *B*-nicotinamide adenine dinucleotide phosphate (NADP, monosodium salt); *B*-

nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, tetrasodium salt); cis-aconitic acid; co-carboxylase (aneurine pyrophosphate: thiamine pyrophosphate chloride); co-enzyme A (sodium salt); DL-isocitric acid (trisodium salt); L-cysteine hydrochloride; glucose; malic dehydrogenase; pyruvic acid (sodium salt); sucrose.

-Swift Consolidated (NZ) Ltd, (Wellington, New Zealand).

Dow-Corning antifoam A.F. emulsion (Food Grade).

3.1.4 Organism

The organism used was *Aspergillus niger* MH 15-15, isolated by Hossain *et al.* (1983) as a mutant strain of *A. niger* IMI 41874. Cultures of *A. niger* MH 15-15 were grown on slopes of Sucrose-Beef Extract Agar (Table 3.1), where they sporulated profusely.

Spores of *A. niger* MH 15-15 were preserved using the technique described by Hossain (1983) and Hossain *et al.* (1983), which was as follows:

a) The organism was grown on slopes of Sucrose-Beef Extract Agar for 8-9 days.

b) The spores were harvested in sterile distilled water and the spore suspension was shaken for one hour to break the spore chains and clumps. The suspension was then filtered through sterile glass wool to remove any remaining clumps, and the concentration was adjusted to 1-2 x 10^8 spores/ml using distilled water.

c) The spore suspension (2 ml) was dispensed

aseptically into 3 ml of nutrient broth, containing 30% (v/v) glycerol, contained in a 10 ml capacity screw cap bottle (15 such bottles were prepared from a single slope culture). The inoculated bottles were then stored at -20°C. When spores were required for subculturing, a loopful from a thawed bottle was transferred to a slope of Sucrose-Beef Extract Agar, and incubated at 30°C for 6-8 days. A further subculture from this initial slope, onto a fresh agar slope, was carried out to obtain improved sporulation. This second slope was used for up to 3 months as the stock slope culture for inoculum preparation.

3.2 MEDIA STERILISATION

All microbiological media were sterilised by autoclaving at 121°C for 15 minutes. All fermentation media were sterilised at 121°C for 20 minutes.

3.3 CLEANING OF GLASSWARE

All glassware was washed in hot Pyroneg(R) solution, rinsed in tap water, then in distilled water, and hot air dried. Glassware used in enzyme assays, trace metal assays, and storage of HPLC solvents was treated with chromic acid after the detergent wash, then rinsed thoroughly with deionised water. It was then treated with 2.0 M NaOH solution, and again thoroughly rinsed with deionised water. Finally, it was treated with a 50% (v/v) solution of hydrochloric acid in deionised water, and thoroughly rinsed in Milli-Q deionised water.

3.4 ANALYTICAL METHODS

3.4.1 pH Measurement

pH measurements were performed routinely using a Metrohm pH meter E520 (Metrohm A.G., Herisau, Switzerland).

3.4.2 Determination of Mycelial Dry Weight

A specific volume of fermenter culture (about 25 ml) or the entire contents of a shake-flask culture (about 100 ml) were filtered through a Buchner funnel, using a pre-weighed Whatman No. 54 filter paper which had been dried at 105°C ± 2°C and stored in a desiccator. The fungal mass was washed three times with deionised water (about 400 ml) and dried to constant weight at 105°C ± 2°C.

3.4.3 Analysis of Sugars

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

A *u*-Bondapak family Carbohydrate Analysis Column (3.9 mm ID x 300 mm, part number 84308, Waters Associates) was used for the analysis.

The detector was a Model 401 Differential Refractometer (Waters Associates). The response was recorded on a CR600 twin-pen, flat-bed chart recorder (J.J. Lloyd Instruments Ltd, Southhampton, England).

The analyses were conducted at ambient temperature. The solvent system was a mixture of acetonitrile and water

(ratio 80:20) (Hossain *et al.*, 1984). The solvent flow rate was 1.5 to 2.0 ml/min, depending on the resolution required. Between 25 and 100 *u*l of sample was injected into the chromatograph, the exact amount depending on the sugar concentration in the sample.

The quantitation of sugars was performed by measuring the peak height of the sugar in the sample, and comparing this with a standard curve of peak height. Standard curves were linear up to 20 g/l concentration in a 50 *u*l injected sample.

3.4.4 Determination of Citric Acid

Citric acid was determined by an HPLC method. The equipment used was as described in Section 3.4.3., with the exception of the column. For this analysis, a *u*-Bondapak C18 reverse-phase column (4.0 mm ID x 250 mm, Bio-sil ODS-10, Bio-Rad Laboratories, Richmond, California, U.S.A.) was used. The solvent system was 2% (w/v) potassium dihydrogen orthophosphate prepared using Milli-Q deionised water and adjusted to pH 2.45 with orthophosphoric acid (Coppola *et al.*, 1978). The solvent flow rate was 1.5 ml/min. Samples of between 25 and 100 *u*l were injected into the chromatograph. The amount of citric acid in the samples was calculated by measuring the peak height of acid with reference to peak height of the standard curve. The standard curve was linear up to 30 g/l concentration for a 50 *u*l injected sample.

Thorough cleaning of this column was necessary following the use of the phosphate-based solvent. This was

achieved using freshly degassed Milli-Q deionised water at a flow rate of 1.5 ml/min for 1 hour. The flow rate was then reduced to 0.1 ml/min overnight. This was then followed with a methanol/water mixture (ratio 50:50) and pure methanol at flow rates of 0.5 ml/min for 30 minutes and 1.0 ml/min for 20 minutes, respectively.

3.4.5 Determination of Total Nitrogen

This was performed using the micro-kjeldahl method (N.Z.S. 2246, 1969).

A known volume of the fermentation liquor, or a known weight of oven-dried mycelia (20 to 40 mg N) was transferred to a Kjeldahl digestion flask. Sodium sulphate (2.0 g), mercuric sulphate solution (5 ml; of 150 g red mercuric oxide dissolved in a solution of 180 ml concentrated sulphuric acid plus 1320 ml water) and concentrated sulphuric acid (20 ml) were added to the Kjeldahl flasks. The contents of the flasks were brought to the boil and heated until the solution was clear. Heating was continued for a further 1 hour. The cooled contents were transferred quantitatively to a 100 ml volumetric flask. Aliquots (10 ml) of the digested samples were used to determine the ammonia concentration in the samples. This was done by steam distillation in a Markham still apparatus following the addition of aqueous sodium hypophosphite (5 ml; 15% (w/v)) and aqueous sodium hydroxide (15 ml; 60% (w/v)). The distillate containing ammonia was collected in aqueous boric acid solution (10 ml; 2% (w/v)) containing 2 drops of screened methyl red indicator (2 g methyl red and

1 g methylene blue dissolved in 1000 ml 96% (v/v) ethanol), and titrated with 0.002 M HCl to grey-green end point. At this concentration, 1 ml acid = 0.028 g nitrogen. All determinations were carried out in duplicate.

3.4.6 Determination of Inorganic Phosphate

This was performed by measuring the blue colour intensity formed by the reduction of molybdophosphoric acid to molybdenum blue, of uncertain composition, in the presence of hydrazine sulphate (Vogel, 1961). The intensity of the blue colour is proportional to the phosphate concentration in the sample. Sample and standard solutions containing up to 0.1 mg of phosphorous as the orthophosphate in 25 ml, were mixed with molybdate solution (5 ml; 12.5 g sodium molybdate in 5.0 M sulphuric acid , and diluted to 500 ml with 5.0 M sulphuric acid), and hydrazine sulphate (2 ml; 1.5 g hydrazine sulphate in 1.0 litre Milli-Q deionised water), diluted to the mark with deionised water in a 50 ml volumetric flask, and well mixed. The flasks were then immersed in a boiling water bath for 10 minutes. They were removed and cooled rapidly in iced water. When cool, the contents were again mixed, and the volume readjusted to 50 ml with Milli-Q deionised water. The absorbance of the samples was measured at 830 nm against deionised water. The standard curve was linear in the range 0 to 4.0 mg/l phosphorous.

3.4.7 Determination of Sulphate

This was performed by the Chemistry Department, Massey University, Palmerston North, New Zealand, using the method of Michalk and Manz (1980). The method is based on the precipitation of sulphate by the addition of barium chloride in excess, and determination of unconsumed barium ions in solution by atomic absorption spectrophotometry.

An amount of 0.5 to 1 ml of sample or standard solution was mixed with uranyl acetate solution (2ml; 8g uranyl acetate in 1.0 litre of Milli-Q deionised water) to remove protein and phosphate. The mixture was centrifuged at 5,000 x *g* for 20 minutes. A 1 ml amount of supernatant liquid was then mixed with barium chloride solution (1 ml; 120 mg in 1.0 litres of Milli-Q deionised water). After 16 to 24 hours, the mixture was centrifuged, and 1 ml of supernatant liquid was mixed with caesium chloride solution (9 ml; 200 mg in 1.0 litres Milli-Q deionised water). The barium ion concentration was then determined by atomic absorption spectrophotometry at 553.4 nm using a nitrous oxide-acetylene flame.

3.4.8 Determination of Mg^{2+} and Trace Metals

Determinations of Cu^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+} were performed by the New Zealand Dairy Research Institute (Palmerston North, New Zealand). For Cu^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+}, no pretreatment of samples was necessary. For Mg^{2+}, 0.05 g of liquid sample was weighed into a 100 ml volumetric flask, to which 40 ml of Milli-Q deionised water was added. To this was added 2 ml of a 5% Sr^{2+} (w/v) solution, and the

sample was diluted to the mark using Milli-Q deionised water and well mixed. All five metal ions were measured using a GBC 903 Atomic Absorption Spectrophotometer (GBC, Dandenong, Victoria, Australia). The following wavelengths were used: Cu^{2+} at 324.8 nm; Fe^{2+} at 248.3 nm; Zn^{2+} at 213.9 nm; Mn^{2+} at 279.5 nm; Mg^{2+} at 285.2 nm.

3.5 CULTURE CONDITIONS

3.5.1 Preparation of Spore Suspension

A spore suspension was prepared in sterile distilled water from the spores scraped from 10-day old culture grown on slopes of Sucrose-Beef Extract Agar as described in Section 3.1.4.

3.5.2 Shake-Flask Culture

Experiments were conducted in 250 ml Erlenmeyer flasks containing 100 ml of medium. The flasks were treated with Sigmacote (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) which coated the inner surface of the flasks with a microscopically thin film of silicone, thus preventing wall growth of mycelium. After coating, the flasks were dried in hot air and then washed in Milli-Q deionised water and hot-air dried before use.

Spores (approximately 1×10^8) from a standard spore suspension (Section 3.1.4) were used to inoculate 100 ml of fermentation medium which was then incubated at $30^{o}C$ on an Environ-Shaker Model 3597 at an operating speed of 180 rpm. For sampling, the entire contents of a flask were taken.

3.5.3 Batch Fermenter Culture

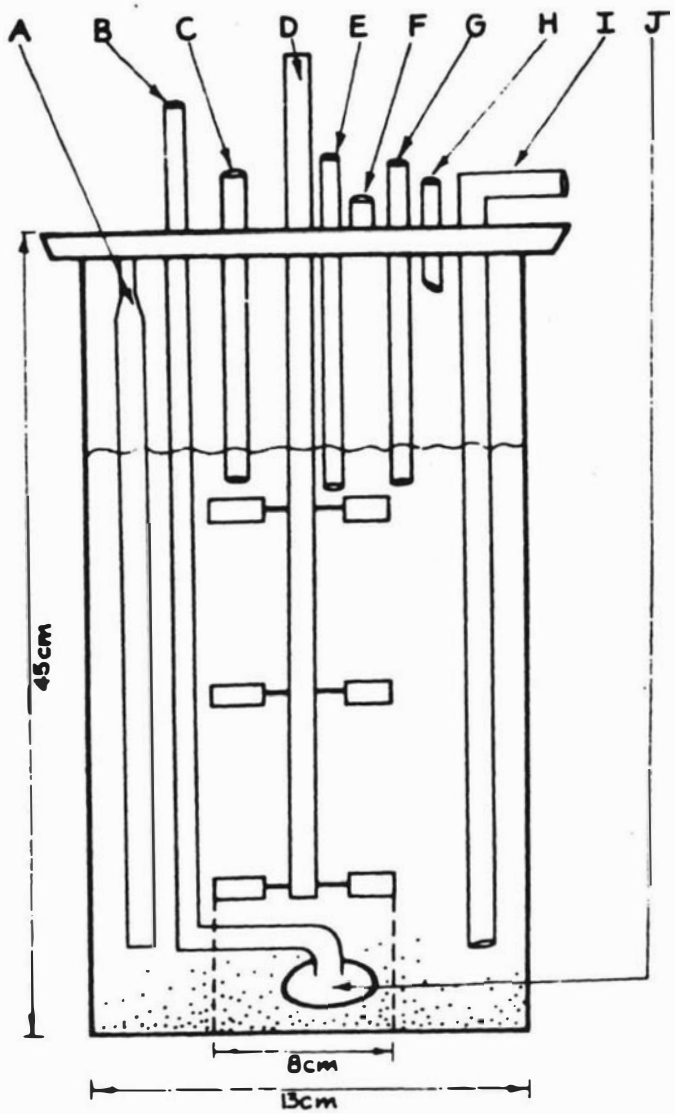
The fermentation apparatus used was constructed in the Biotechnology Department, Massey University. Figure 3.1 shows a schematic diagram of the fermenter vessel, while Figure 3.2 shows a schematic diagram of the fermenter unit and its ancillary equipment. The fermenter vessel used was a seven litre capacity glass jar (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.), with a working volume of five litres, and was provided with a stainless steel head containing ports for the insertion of probes and the other facilities required. The dimensions of the fermenter vessel and impellers are shown in Figure 3.1. The fermenter vessel was provided with four baffles each of 2 cm width, which extended from the head to within 5 cm of the vessel base.

Agitation was provided by an assembly of three, 4-bladed impellers mounted vertically at intervals of 7, 18 and 28 cm above the base of the vessel on the central impeller shaft. This was driven by a D.C. 1/4 H.P. variable speed motor from the top of the fermenter vessel. Variable speed was obtained using an electronic controller. Impeller speeds from 0 to 1000 rpm were attainable.

The fermenter temperature was maintained at 30 $\pm$ 0.2 ^{o}C by means of hot or cold water flowing through hollow baffles and was controlled by an electronic thermostat. The temperature was continuously recorded using a Honeywell Varsaprint Multipoint chart recorder (Amiens, France).

Air was supplied to the fermenter vessel from the

Figure 3.1 A schematic diagram of the fermenter vessel, showing various facilities and probes in the vessel head



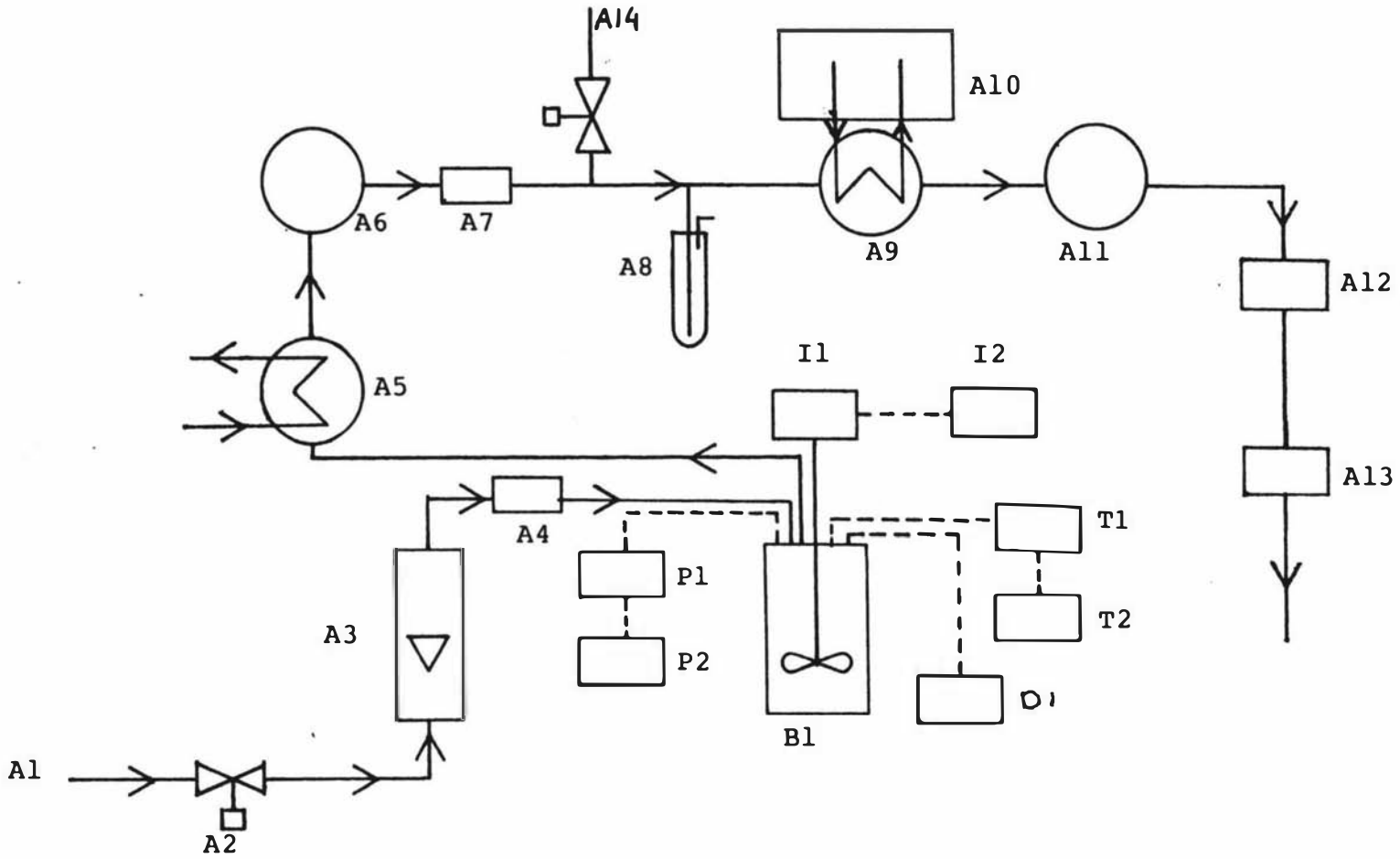
A Baffle
B Air inlet
C pH probe
D Impeller drive shaft
E DOT probe
F Inoculation port
G Reference probe (pH)
H Air outlet
I Sample tube
J Sparger

University compressed air line, through a cleaner/manostat assembly (Manostat SPNZ 102MOA, MacEwans Machinery Ltd, Palmerston North, New Zealand) to a flowstat (type MN, B.R. Hamersham Ltd, Lower Hutt, New Zealand) to regulate the flow to the rotameters. The rotameter (Series 1100, tube H-G-300, Fisher Controls Ltd, Croyden, England) controlled the airflow to the fermenter over the range 0.6 to 5.0 litre/min. The measured air was passed through a sterile glass wool-packed filter, then a sterile millipore filter (0.22 μm, Millipore Corporation, Bedford, Massachusetts, U.S.A.) before entering the culture vessel through a 4 cm stainless steel sintered metal plate.

Exhaust air was vented through a water cooled condenser to prevent the loss of fermentation liquid by evaporation. The condenser was connected to a water trap, to retain any small amounts of water vapour carried past the condenser. From the water trap, the gas passed through a second sterile millipore filter. The filter was connected to a distilled water lute, causing a back-pressure of 60 cm water (equivalent to 53 mm Hg) on the fermenter vessel. The water lute was connected to a second water cooled condenser, through which water from a resevoir held at 1.0 $\pm$ 0.2^{o}C was pumped, which was in turn connected to a second water trap. From this second water trap, the exhaust air entered the carbon dioxide gas analyzer (Type 2H/34 Infra-Red gas analyzer, Analytical Development Company, Herts, England), thence to an oxygen analyzer (Servomex$^{(R)}$ Oxygen Analyzer 540A, Sybron Taylor, Taylor Instrument Ltd, Sussex, England), so that the carbon dioxide production and the

A1 Compressed air line
A2 Manostat
A3 Rotameter
A4 Air filters
A5 Condenser
A6 Water trap
A7 Air filter
A8 Distilled water lute
A9 Condenser
A10 Resevoir (held at 1 ^{o}C)
A11 Water trap
A12 Carbon dioxide analyser
A13 Oxygen analyser
A14 Calibration gas line
B1 Fermenter vessel
D1 DOT recorder
I1 Impeller drive
I2 Agitation controller
P1 pH controller
P2 pH recorder
T1 Temperature controller
T2 Temperature recorder

Figure 3.2 A schematic diagram of the batch fermenter and its ancillary equipment



oxygen uptake rates of the fermentation could be monitored. Both gas analysers were connected to a twin pen flat bed recorder (J.J. Lloyd Instruments Ltd, Southampton, England) and continuously monitored (Figure 3.2) (Brooks *et al.*, 1982).

The culture pH was measured using an E.I.L 33 1070 030 toughened glass electrode and an E.I.L 33 1320 210 laboratory sealed reference electrode (Electronic Instruments Ltd, Richmond, Surrey, England) connected to a Horizon pH Controller Model 5997-20 (Ecology Co., Oak Park Avenue, Chicago, Illinois, U.S.A.). The pH controller was connected to the Honeywell Varsaprint multipoint chart recorder. The culture pH was continuously recorded, but not controlled. The pH value of each fermentation sample was measured independently to check the accuracy of the fermenter pH measurement, and any discrepancies were corrected.

Dissolved oxygen tension was measured using a series 900 galvanic dissolved oxygen probe (Type M 1016-5002, New Brunswick Scientific Co., Inc., New Jersey, U.S.A.), and continously recorded using the multipoint chart recorder. The probe was calibrated *in situ*, prior to the inoculation of the fermenter, by sparging the contents of the fermenter with oxygen-free nitrogen gas through the air supply system to obtain zero saturation conditions. The contents of the fermenter vessel were then aerated vigorously to give 100% of saturation. The saturation condition was held for 12 hours to ensure stable dissolved oxygen probe operation and

the chart recorder was set at 95% full scale. After the completion of the fermentation, the probe calibration was checked to ensure that no significant deterioration of the probe had occurred during the fermentation.

3.5.4 Continuous Fed-Batch Fermentation

The equipment described in Section 3.5.3, was used for fed-batch fermentation, with the same ancillary equipment except that pH was not continuously monitored. The feed medium was added through port C (Figure 3.1), delivered by a peristaltic pump (Model 1612, Instrumentation Specialties, Lincoln, Nebraska, U.S.A.). Port G (Figure 3.1) was stoppered in this series of experiments. It was considered that the frequency of sampling, and the independent measure of pH was sufficient to monitor adequately the pH of the fermentation.

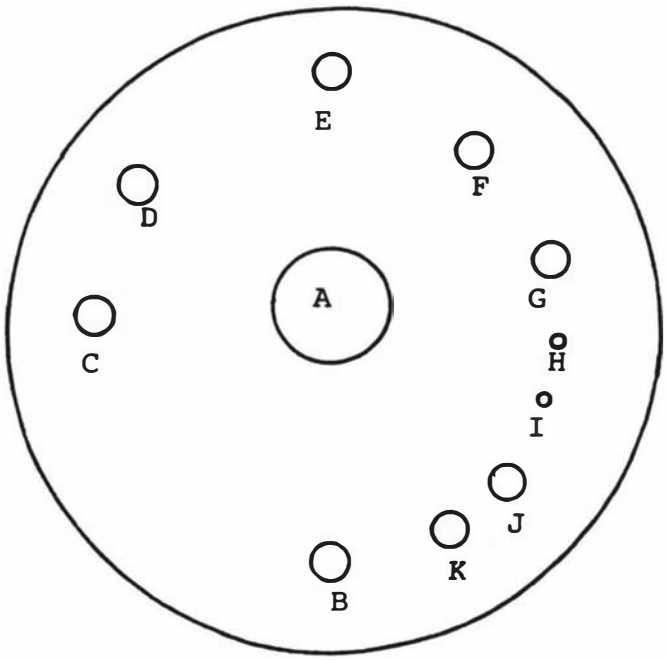
3.5.5 Chemostat Continuous Culture

The fermenter used was a Multigen F2000 Benchtop culture apparatus (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.A.). Figure 3.3 shows a diagram of the fermenter head, and Figure 3.4 shows a schematic diagram of the fermenter plus its ancillary equipment.

The fermenter vessel was a 2 litre capacity glass jar of working volume 1.6 litre, with a polyethylene-polypropylene head containing holes for the insertion of probes and other sensors. The vessel was unbaffled, the various probes and tubes providing sufficient baffling.

Agitation was provided by an impeller assembly of three, 6-bladed disc-turbine impellers mounted at equally

Figure 3.3 The chemostat fermenter head



A Air inlet/Agitator shaft
B DOT probe
C Sample port
D Medium-feed inlet
E pH probe
F Thermocouple/Temperature control
G Inoculation port
H Acid inlet
I Alkali inlet
J Air exit
K Overflow port

spaced intervals from 5 cm above the vessel base to 5 cm below the culture surface. This was driven by indirect magnetic coupling through the base of the vessel. Agitation speeds from 0 to 1000 rpm were attainable.

Temperature control was by means of a heating tape wrapped around the outside of the fermenter vessel. With the thermocouple wire inserted into a glass tube of water, inserted in the fermenter head, the temperature of the vessel contents was maintained at 30 $\pm$ 0.5°C. Temperature was continuously monitored and recorded using a Honeywell Varsaprint Multipoint chart recorder (Amiens, France).

Air supply and exhaust air measurements were performed as described in Section 3.5.3, except that the rotameter was a Fisher Controls Model E-300, with an operating range of 100 to 1200 ml/min.

Culture pH was monitored during the batch phase, and controlled, if necessary, during the continuous culture phase using a Horizon pH Controller Model 5997-20 (Ecology Co., North Oak Park Avenue, Chicago, Illinois, U.S.A.). The pH probe used was a combination Orion Model 90-14 (Orion Research, Cambridge, Massachusetts, U.S.A.). In all experiments it was desired to maintain the culture at pH 2.0 $\pm$ 0.1 during the continuous phase. In some experiments this value was attained and held without any control. However, provision was made for automatic control *via* addition of 0.1 M HCl or 0.1 M NaOH by Masterflex peristaltic pumps (Cole-Palmer Instrument Co., Chicago, Illinois, U.S.A.). The culture pH was continuously recorded using the multipoint

chart recorder. Independent pH measurements of samples were carried out as described in Section 3.5.3.

A glass galvanic dissolved oxygen probe, type M1016-0770 (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.A.) was used to measure the culture dissolved oxygen tension. The probe was connected to a model DO-40 Dissolved Oxygen Analyser (New Brunswick Scientific Co. Inc.) which used a 10 mv output for chart recorder operation. The probe was calibrated *in situ*, prior to inoculation of the fermenter, by the method described in Section 3.5.3. Culture DOT was continuously recorded using the multipoint chart recorder. The culture DOT was automatically controlled using an electronic analogue circuit, designed in such a way as to increase (if below the DOT set-point) or decrease (if above the DOT set-point) the agitation speed. When at the set-point the agitation speed would remain constant. The system, a modification of that reported by Clark *et al.* (1985), allowed control of the DOT to $\pm$ 2% of saturation and rapidly returned the DOT to the set-point following disturbances (Figure 3.5).

The overflow method used was that described by Clark (1982). A weir tube was found to be unsatisfactory for mycelial organisms, because the tube rapidly became partially blocked and the overflow was not a homogeneous mixture of fermenter contents. Thus biomass build-up occurred. The system used (Figure 3.6) was periodical evacuation of the overflow resevoir, thus withdrawing culture through a vertically positioned overflow tube. The vacuum was applied for 15 seconds every 15 minutes. Culture

A1	Compressed air line
A2	Manostat
A3	Rotameter
A4	Air filters
A5	Condenser
A6	Water trap
A7	Solenoid valve
A8	Air filters
A9	Distilled water lute
A10	Condenser
A11	Resevoir (held at 1^{o}C)
A12	Water trap
A13	Carbon dioxide analyser
A14	Oxygen analyser
B1	Fermenter vessel
C1	Cam-timer
D1	DOTrecorder
D2	DOT analyser
D3	DOT controller
D4	Magnetic drive motor
F1	Feed-medium resevoir
F2	Feed-medium pump
O1	Overflow resevoir
O2	Solenoid valve
O3	Vacuum pump
P1	pH controller
P2	pH recorder
P3	Acid pump
P4	Alkali pump
P5	Acid resevoir
P6	Alkali resevoir

Figure 3.4 A schematic diagram of the chemostat continuous culture fermenter and its ancillary equipment

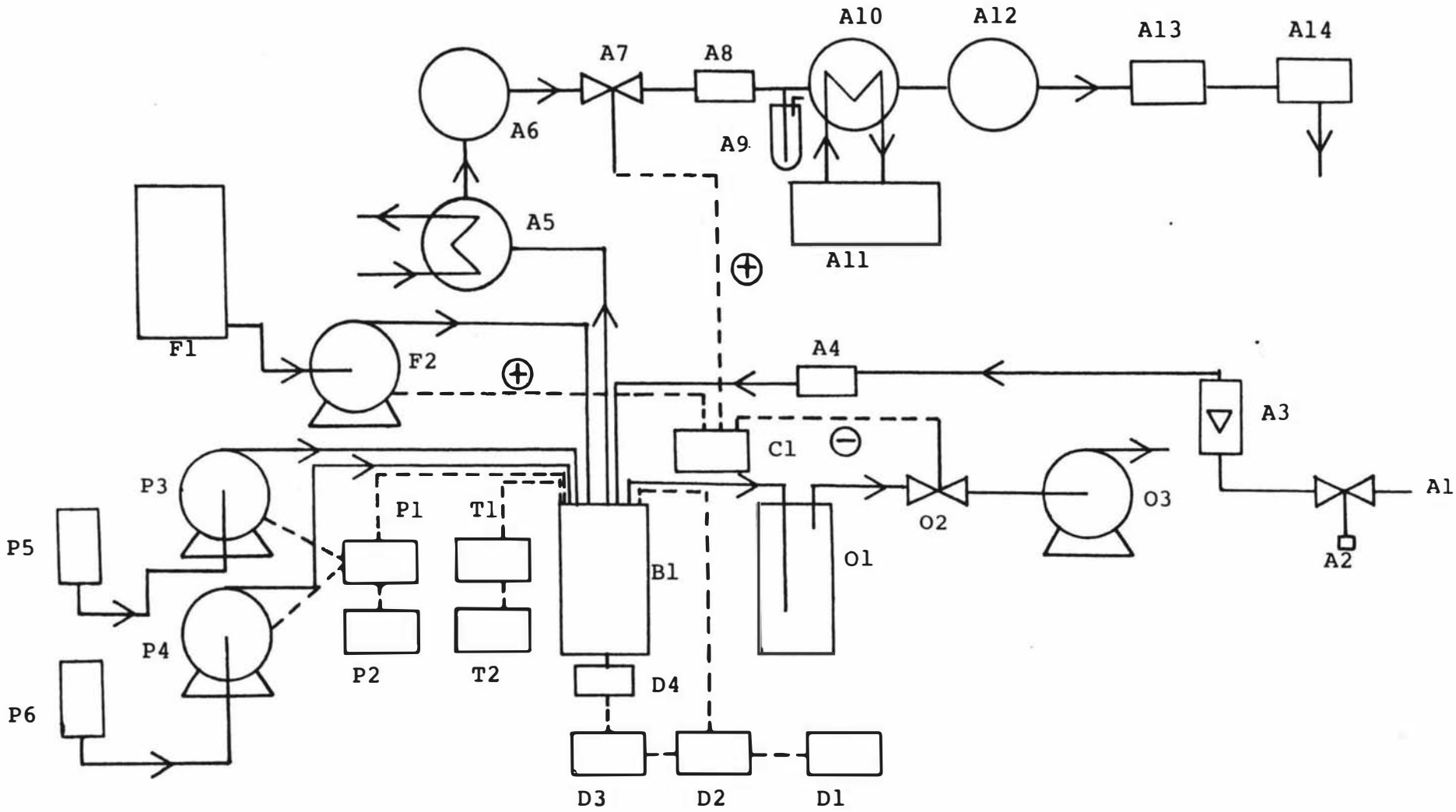


Figure 3.5 legend

A Air supply
B Electric motor
C DOT probe
D PI controller

Figure 3.6 legend

A1 Exit air line
A2 Solenoid valve
B1 Fermenter vessel
C1 Relay switch
C2 Timer
C3 Cam-timer container
F1 Feed-medium resevoir
F2 Feed-medium pump
O1 Overflow resevoir
O2 Solenoid valve
O3 Vacuum pump

Figure 3.5 A schematic diagram of the DOT control system which used proportional-integral control of agitation speed

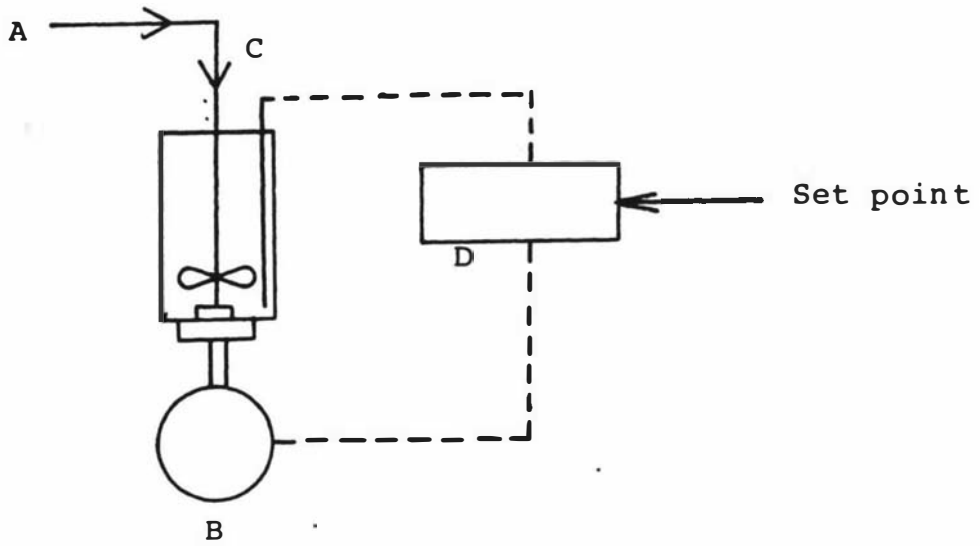
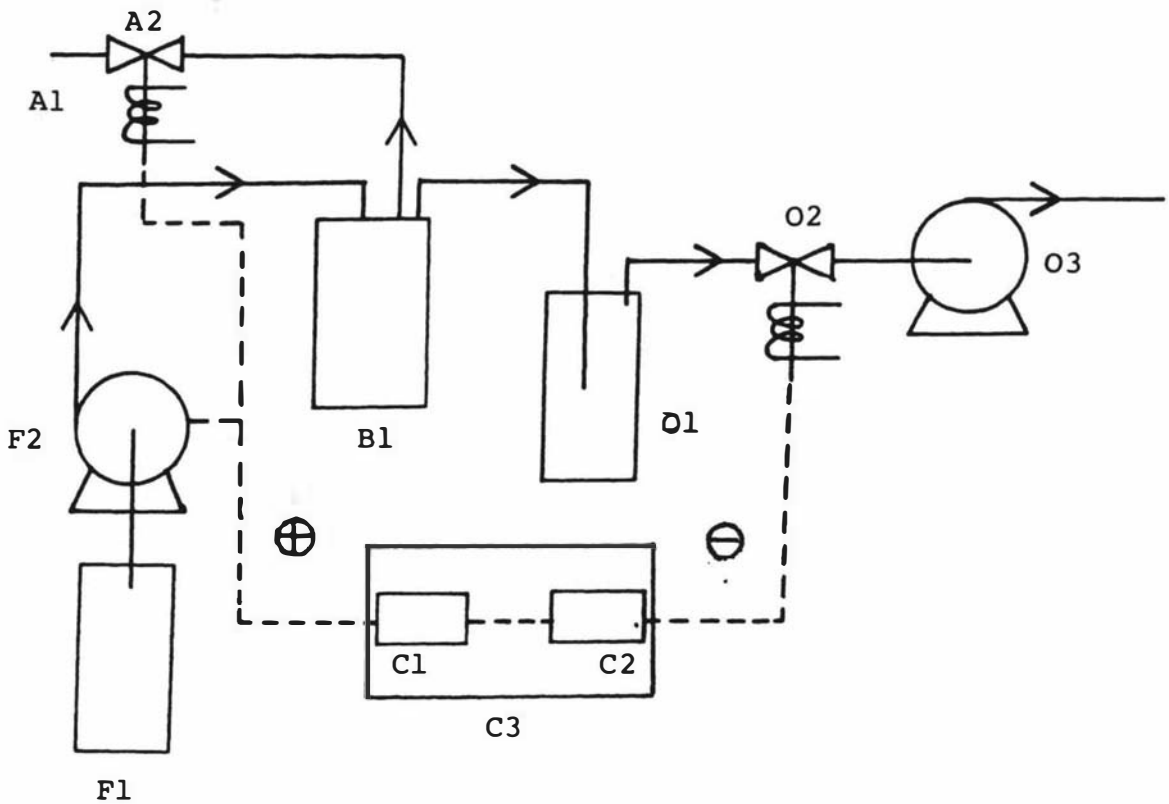


Figure 3.6 A schematic diagram of the chemostat continuous culture overflow control system



volume, and hence, dilution rate varied in a cyclic manner, by approximately 3%, using this procedure. This was considered acceptable. In order to prevent the free flow through of feed medium into the vessel, and reversed flow of effluent gas through the oxygen and carbon dioxide analysers, while the vacuum was being applied, a solenoid valve was positioned in the effluent gas line between the exit filter and the water lute, and was connected to a cam-timing device. The feed pump was also connected to the cam-timer. The normal valve position was open, with the feed pump on. When the cam-timer opened the solenoid valve on the vacuum line, it opened the relay switch, closing the solenoid valve on the effluent gas line and turning off the feed pump (Figures 3.4 and 3.6). The pump used on the medium feed line was a Hughes Micro Metering pump series II (F.A. Hughes and Co., Ltd, Surrey, England).

3.5.6 Sterilization

The fermenter vessel containing the medium and all systems in the fermenter head except the pH probes, were sterilized in an autoclave for 20 minutes at 121^{o}C, as were the feed medium in the resevoirs for Sections 3.5.4 and 3.5.5.

The pH probes were sterilized by immersing in 2% (v/v) formaldehyde solution for 30 minutes and washed thoroughly with sterile distilled water before insertion into the fermenter vessel. The glass apparatus, pipettes and glass wool-packed air filters were sterilized by dry heat at 160^{o}C for 2 hours.

3.5.7 Preparation of Inoculum for Fermenter Experiments

Spores (approximately 1×10^8) (Section 3.1.4) were used to inoculate 100 ml of medium in a 250 ml Erlenmeyer flask. The inoculated medium was then incubated at 30 °C in shake-culture for 36 to 40 hours for the batch and fed-batch experiments, and 24 hours for the chemostat experiments. The contents of the flask (containing small pellets of about 0.2 to 0.3 mm diameter) were used for the inoculation of the fermenters (10% (v/v) inoculum).

3.5.8 Sampling of Fermenters

The routine withdrawal of samples (about 30 ml volume) for analysis was performed through the sample withdrawal system of the vessels. Prior to sampling, the withdrawal system was flushed with 10 to 15 ml of the culture fluid to remove any "dead" volume. The volumes of flush and samples withdrawn from the fermenter were recorded and taken into account during the volumetric analysis of fermentation components.

3.5.9 Avoidance of Wall Growth in Fermenter Culture

Mycelial growth around the vessel walls was periodically dislodged with a teflon-covered bar magnet inside the fermenter vessel which was secured and moved with a horse shoe magnet from outside the vessel.

3.6 PREPARATION OF SAMPLES FOR HPLC ANALYSIS

Mycelium was removed by filtration and the samples were centrifuged at about 2500 x *g* using a Wifug Chemico Centrifuge (Stockholm, Sweden). The supernatant liquid was then filtered through a membrane (pore size 0.45 *u*m) using a Swinney Filter Kit (Millipore Corporation, Bedford, Massachusetts, U.S.A.).

3.7 ENZYME ASSAYS

3.7.1 Preparation of Cell-Free Extract

Fermentation samples (about 50 ml) or whole shake-flask contents (about 100 ml) were filtered through a Buchner funnel using Whatman No. 54 filter paper. The mycelium was washed twice with cold Milli-Q deionised water (about 4 ^{o}C), followed by washing with sufficient 0.1 M potassium phosphate buffer (pH 7.4) to bring the pH to neutrality. The mycelium was then rewashed with cold deionised water.

The washed mycelium was suspended in cold 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA (10 ml extraction solution/g wet weight mycelium). The suspension was placed into a pre-cooled homogenizer container (a stainless steel bottle of 75 ml capacity) which contained the required amount of ballotini beads (about 5 g/g wet weight mycelium) of 0.5 mm diameter (Glasperlen, Kt. Nr. 54180, B. Braun Melsungen AG., Germany). The stainless steel container was placed inside the holding chamber of a rotary cell homogenizer (Cell Homogenizer MSK, Type 853034, B. Braun Melsungen AG, Germany) which operated at either 2000 or 4000 rpm. The machine and the material were cooled

for about 1 minute by passage of liquid carbon dioxide through the system. This coolant flow was continued throughout homogenization. Shaking was continued for 1 minute at 4000 rpm at an operating temperature of about 3 to 4 °C. The homogenate was then separated from the ballotini beads by filtering through a glass sinter. The filtered liquid was then centrifuged at 25000 x *g* for 30 minutes at 0 °C using a refrigerated centrifuge (Sorvall Superspeed RC 2-B Automatic Refrigerated Centrifuge). The creamy supernatant liquid was maintained at 3 to 4°C and assayed for enzyme activity as soon as possible. Where necessary, the extract was diluted using 0.1 M potassium phosphate buffer, pH 7.4.

All assays were carried out at room temperature (about 23°C) using a Shimadzu UV-120-02 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Assays were conducted in triplicate and the average result was recorded.

The specific activity of the enzymes was expressed as *u*moles of product/min/mg of protein. The amount of product formed during the reaction time was calculated from the standard curve of that product.

3.7.2 Protein Estimation

The protein content of cell-free extracts was estimated using the method of Bradford (1976).

Diluted cell-free extract (0.1 ml, containing 10 to 100 *u*g protein) was pipetted into a test tube. To this was added 5 ml of protein reagent (Coomassie Brilliant Blue G250, 100 mg dissolved in 50 ml 95% ethanol, to which was

added 100 ml of 85% (w/v) orthophosphoric acid, and the mixture was diluted with Milli-Q deionised water to a final volume of 1.0 litres, and filtered before use) and mixed gently. The blue colour obtained after 2 minutes is stable for up to 1 hour. The absorbance was measured at 595 nm against a reagent blank, and compared to a standard curve constructed using Bovine Serum Albumin (BSA). A reduction factor of 2.1 equivalent protein was required, due to the use of BSA as the standard solution, when calculating the absolute protein concentration in the sample (Chong, Pers Comm, 1984).

3.7.3 Aconitase (E.C. 4.2.1.3)

The activity of this enzyme was determined by measuring the rate of appearance of cis-aconitate from isocitrate according to the method of La Nauze (1966).

The reaction system contained: 0.1 M DL-isocitrate in 0.5 M potassium phosphate buffer (pH 7.4), 0.4 ml; diluted enzyme extract, 0.1 ml; and 0.05 M phosphate buffer (pH 7.4), 2.5 ml. The blank contained (in 3 ml) all components except the substrate. The rate was obtained by monitoring the increase in absorbance at 240 nm over a period of 5 minutes.

3.7.4 NAD-Linked Isocitrate Dehydrogenase (E.C. 1.1.1.41)

The activity of this enzyme was determined by measuring the rate of formation of NADH from NAD according to the method of La Nauze (1966).

The reaction system contained: 0.5 M potassium

phosphate buffer (pH 7.4), 0.2 ml; 0.005 M NAD, 0.3 ml; 0.0025 M AMP, 0.3 ml; 0.1 M $MgCl_2$, 0.1 ml; 0.3 M KCN (freshly neutralized), 0.1 ml; 0.1 M DL-isocitrate, 0.1 ml; diluted enzyme extract, 0.1 ml; Milli-Q deionised water, 1.8 ml. The blank contained (in 3 ml) all components other than the substrate. The rate was obtained by measuring the increase in absorbance at 340 nm over a period of 5 minutes.

3.7.5 NADP-Linked Isocitrate Dehydrogenase (E.C. 1.1.1.42)

The activity of this enzyme was determined by measuring the rate of formation of NADPH from NADP according to the method of La Nauze (1966).

The reaction system contained: 0.5 M potassium phosphate buffer (pH 7.4), 0.2 ml; 0.0025 M NADP, 0.3 ml; 0.1 M $MgCl_2$, 0.1 ml; 0.3 M KCN (freshly neutralized), 0.1 ml; 0.1 M DL-isocitrate, 0.1 ml; diluted enzyme extract, 0.1 ml; Milli-Q deionised water, 2.1 ml. The blank contained (in 3 ml) all the components other than the substrate. The rate was obtained by monitoring the increase in absorbance at 340 nm over a period of 5 minutes.

3.7.6 2-Oxoglutarate Dehydrogenase (E.C. 1.2.4.2)

The activity of this enzyme was determined by measuring the rate of formation of NADH from NAD according to the method of Reed and Mukherjee (1969).

The reaction system contained: 0.5 M potassium phosphate buffer (pH 8.0), 0.3 ml; 10 mM $MgCl_2$, 0.3 ml; 0.01 M NAD, 0.3 ml; 30 mM cysteine hydrochloride (neutralized before use), 0.3 ml; 20 mM thiamine pyrophosphate, 0.03 ml; 3 mM Coenzyme A (prepared freshly before use), 0.06 ml; 0.1

M 2-oxoglutarate, 0.03 ml; diluted enzyme extract, 0.1 ml; Milli-Q deionised water, 1.58 ml. The blank contained (in 3 ml) all components other than the substrate. The rate was obtained by monitoring the increase in absorbance at 340 nm over a period of 5 minutes.

3.7.7 Pyruvate Carboxylase (E.C. 6.4.1.1)

The activity of this enzyme was determined by measuring the rate of oxidation of NADH to NAD according to the method of Feir and Suzuki (1969).

The reaction system contained: 50 mM tris hydrochloride buffer (pH 7.9), 1.5 ml; solution containing 5 mM sodium pyruvate, 5 mM sodium bicarbonate, 3 mM $MgCl_2$, 1.2 mM ATP, and 33 mM KCl, 1 ml; 0.1 mM NADH, 0.3 ml; malic dehydrogenase, 1 unit (0.1 ml); diluted enzyme extract, 0.1 ml. The blank contained (in 3 ml) all components other than the substrate. The rate was obtained by monitoring the decrease in absorbance at 340 nm over a period of 5 minutes.

3.7.8 Isocitrate Lyase (E.C. 4.1.3.1)

The activity of this enzyme was determined by measuring the rate of formation of glyoxylic acid phenylhydrazone according to the method of Dixon and Kornberg (1959).

The reaction system contained: 0.5 M potassium phosphate buffer (pH 6.85), 0.4 ml; 0.1 M DL-isocitrate in 0.05 M potassium phosphate buffer (pH 6.85), 0.5 ml; 0.1 M $MgCl_2$, 0.1 ml; 0.1 M phenylhydrazine, 0.02 ml; diluted enzyme extract, 0.1 ml; Milli-Q deionised water, 1.78 ml. The blank contained (in 3 ml) all components other than the

substrate. The rate was obtained by monitoring the increase in absorbance at 324 nm over a period of 5 minutes, and calculated using the molar extinction coefficient for glyoxylic acid phenylhydrazone of 1.7×10^{-4}.

3.8 CALCULATIONS

3.8.1 Statistical Analyses

From the data generated during chemostat continuous culture experiments at various DOT and specific growth rate values, regression equations were obtained. Lack of fit tests were performed and equations showing no significant lack of fit were used to predict response surfaces as 3-dimensional contour diagrams by means of a program developed at Massey University for an IBM XT personal computer (Boag, pers comm., 1984). From these response surfaces, optimum conditions for citric acid production were predicted, and where there was no significant lack of fit, the apparent optimum condition was tested experimentally. Correlation coefficients for the entire correlation matrix were obtained.

3.8.2 Carbon Balance

The carbon balance was calculated to determine the fate of the carbohydrate source carbon. The following assumptions were made:-

a) Protein content of biomass was expressed as a percentage nitrogen of the mycelial dry weight x 6.25

b) Protein carbon content is 36% based on an average amino acid of molecular weight 100

c) Biomass carbohydrate carbon content is 44%

3.8.3 Gas Balance

The gas mass balance equations used were those described by Brooks (1978) and Brooks *et al.*(1982).

Oxygen uptake and carbon dioxide production were calculated thus:

(Oxygen in) - (Oxygen out) = Oxygen uptake

(Carbon Dioxide out) - (Carbon Dioxide in) = Carbon Dioxide Production

The true flow rate of the air was calculated using the equation:

$$F_2 = F_1 \times \frac{293}{760} \times \frac{P_1}{(273 + T_1)}$$

where F_1 = observed flow rate (ml/min)

P_1 = observed pressure of volumetric standard (mm Hg)

T_1 = observed temperature of volumetric standard (^{o}C)

F_2 = flow rate corrected to standard conditions, ml/min at 760 mm Hg, 20 ^{o}C.

When actual flow measurement was made, the true flow rate was found by measuring the temperature and pressure of the gas and substituting in equation (1):

$$F_3 = F_2 \times \left(\frac{P_2}{P_c}\right)^a \times \left(\frac{T_c}{T_2}\right)^b$$

where F_3 = true flow rate at 760 mm Hg and 20°C for any scale reading

P_c = calibration pressure of variable area flowmeter (gauge + BP) (mm Hg absolute)

P_2 = observed pressure of operation of variable area flowmeter (gauge + BP) (mm Hg absolute)

T_c = calibration temperature of variable area flowmeter (°K)

T_2 = observed operating temperature of variable area flowmeter (°K)

a = pressure correction index (Table 3.4)

b = temperature correction index (Table 3.4)

Using this method, the accuracy of the flow measurement can be $\pm$ 2.0% of the actual flow rate.

Table 3.4 Variation of pressure and temperature correction indices with flow rate in variable area flowmeters (Brooks, 1978).

Flow Rate (ml/min)	a	b
0 - 20	0.75	2.0
20 - 150	0.75	1.5
150 - 1000	0.75	1.0
1000 and over	0.5	0.75

3.9 DISCUSSION OF METHODS

3.9.1 Organism

The organism used in this project, *A. niger* MH15-15, was a mutant isolated for its ability to produce citric acid from whey permeate (Hossain *et al.*, 1983). This must be considered when comparisons are made between data obtained in this project and data published in the literature, which are generally obtained from *A. niger* strains selected for their ability to produce citric acid from the substrate used in the reported work. In this project, the substrate used was sucrose. This sugar was used because it is a more favourable substrate than lactose for strong citric acid production.

3.9.2 Chemostat Continuous Culture

Some problems were experienced with the chemostat continuous culture system. In an initial series of experiments, it was found that steady state conditions were achieved after 3 residence volumes had passed through the fermentation vessel. Previous culture history had no effect on the steady state, once achieved.

Subsequent to this series of experiments, a period of some 8 months occurred during which no steady states were able to be achieved. This was due to washout, which apparently commenced after approximately 1.75 to 2 residence volumes had passed through the fermentation vessel. This occurred at all dilution rates tested, from 0.045 to 0.12 h^{-1}. When the continuous feed was turned off, and the culture batched, washout ceased, a slight increase in

biomass concentration (measured as mycelial dry weight) occurred and citric acid production commenced. Once the continuous feeding was resumed, washout again resulted. During this period, the same culture of *A. niger* MH15-15 produced citric acid in batch fermentations at the same production rates as those observed prior to the onset of this problem, giving similar final citric acid concentrations and yields. In an attempt to solve the problem, fresh batches of all chemicals used in the medium were obtained, and all metal tubes, probes and other metal components, except the agitator, were removed from the fermentation vessel, and replaced by glass. However, no improvement occurred. Eventually, by subculturing from the frozen stocks, a stock culture was found that did not wash out, and gave results similar to those obtained in chemostat continuous culture prior to the onset of this problem. Further frozen stocks of this culture were made (Section 3.1.4) and used for subsequent work. No further problems were experienced.

The reason for the abnormal behaviour of the culture is not known. However, a possible explanation was suggested by Lawrence (pers. comm., 1985) following his attendance at a symposium in East Germany in 1984 with the theme of "Problems of Phage in Biotechnology". Six of the groups that attended this symposium reported washout in chemostat continuous culture in pilot plant or commercial scale fermentations. In all 6 reports, bacteria were the fermenting organisms, and bacteriophages were found to be responsible for the washout of the culture (Hoppe *et al.*,

1984; Kirchubel, 1984; Krivisky, 1984; Mach, 1984; Prauser, 1984; Wunsche 1984). Lysis occurs during active growth of an organism. If the culture is actively growing as in a chemostat, lysis could occur. If however, active growth has ceased, as occurs in batch fermentation subsequent to the exhaustion of the growth-limiting nutrient (nitrogen), the phage cannot lyse the host cell, as the culture is not "growing", merely increasing the mycelial dry weight by means of storage carbohydrate production. There are no reports of fungal phage in the literature, though virus-like particles have been observed in yeasts (Bruenn, 1980). Consequently, the possibility of a phage-like contamination of the culture in the present study, cannot be discounted.

Another possibility is that due to the fact that the culture was growing continuously in chemostat, a chemical or combination of chemicals, was produced under these conditions that caused the cells to lyse. No search was undertaken to detect and identify this chemical, if in fact it existed.

Without further investigation, the former explanation involving virus/plasmid contamination of the culture appears to be the more likely of the two possibilities.

CHAPTER 4

THE EFFECT OF INTERRUPTIONS TO THE AIR SUPPLY DURING CITRIC ACID PRODUCTION

4.1 INTRODUCTION

It is generally accepted that aeration during the production of citric acid by *A. niger* in submerged batch fermentation is critical if high yields are to be obtained. Kovats and Gackowska (1976) reported that an interruption to aeration during batch fermentation adversely affects citric acid production. Kubicek *et al.* (1980) found that a 20 minute interruption to aeration during idiophase did not reduce the viability of the organism, but irreversibly destroyed its ability to produce citric acid. Rohr *et al.* (1983) noted that the ability to produce citric acid was irreversibly retarded, but not totally destroyed after an interruption of 20 minutes. They also reported a slight stimulatory effect on biomass production.

Kubicek *et al.* (1980) reported critical DOT values of 9 to 10% of saturation and 12 to 13% of saturation for trophophase and idiophase, respectively. They also showed that citric acid production steadily increased between DOT values of 25 and 75% of saturation.

On the basis that interruptions to aeration are not uncommon in practice during fermentation processes, it was decided to re-examine these reports concerning the effect

of interruption of aeration on citric acid production.

4.2 RESULTS

The medium used was that described in section 3.1 (Table 3.2), with the exception that the $(NH_4)_2SO_4$ concentration was reduced to 1.0 g/l in order to restrict the final biomass concentration and thereby allow the maintenance of a higher DOT value. The experiments were carried out in three situations: entirely in shake-flask culture; a combination of fermenter and shake-flask culture; and entirely in fermenter culture. In all three situations, interruptions to aeration were performed by flushing the cultures with oxygen-free nitrogen gas.

4.2.1 Shake-Flask Culture

Three sets of cultures were set up, one as a control, one which was flushed with oxygen-free nitrogen gas for 20 minutes on both the first and third days after inoculation, and one which was flushed with oxygen-free nitrogen gas for 120 minutes on both the first and third days after inoculation. During the flushing, the flasks were removed from the shaker and the nitrogen gas was delivered at a flow rate of 100 ml/min *via* a sterile glass tube with the tip just below the surface of the culture. The control flasks remained on the shaker at all times. Citric acid concentration in the flasks was measured daily and the results are shown in Figure 4.1. Interruptions to aeration, particularly for 120 minutes, retarded citric acid production, but this effect was not permanent. By the fifth

Figure 4.1 Effect of interruptions to aeration on citric acid production in shake-flask culture

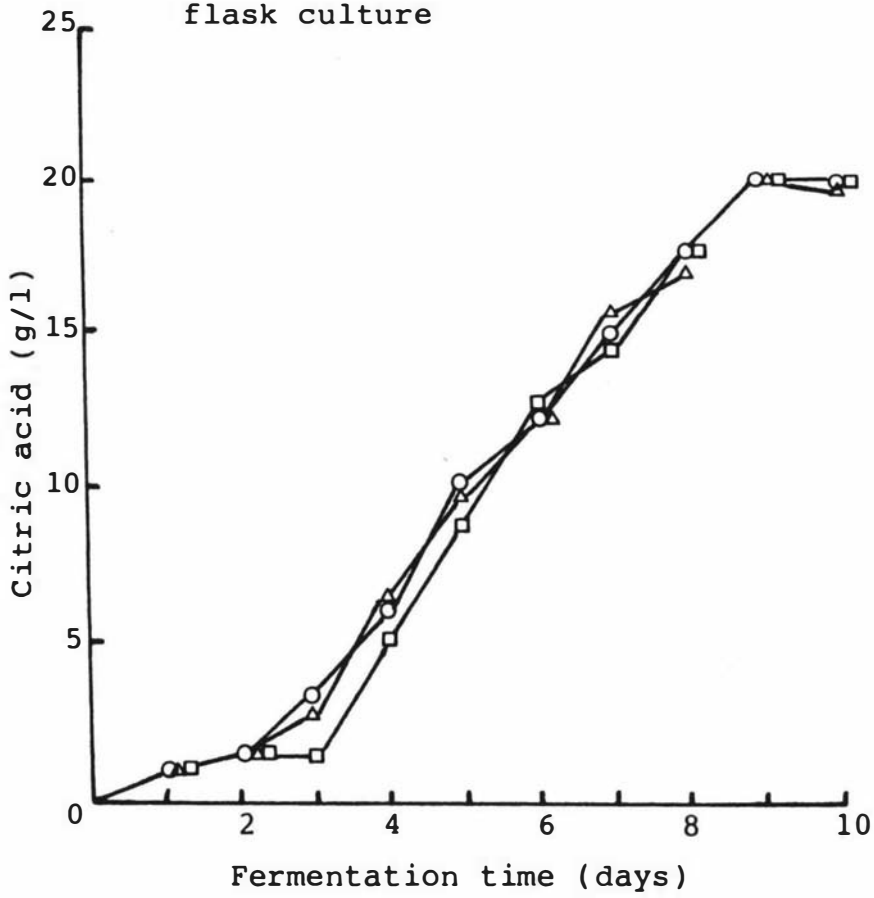
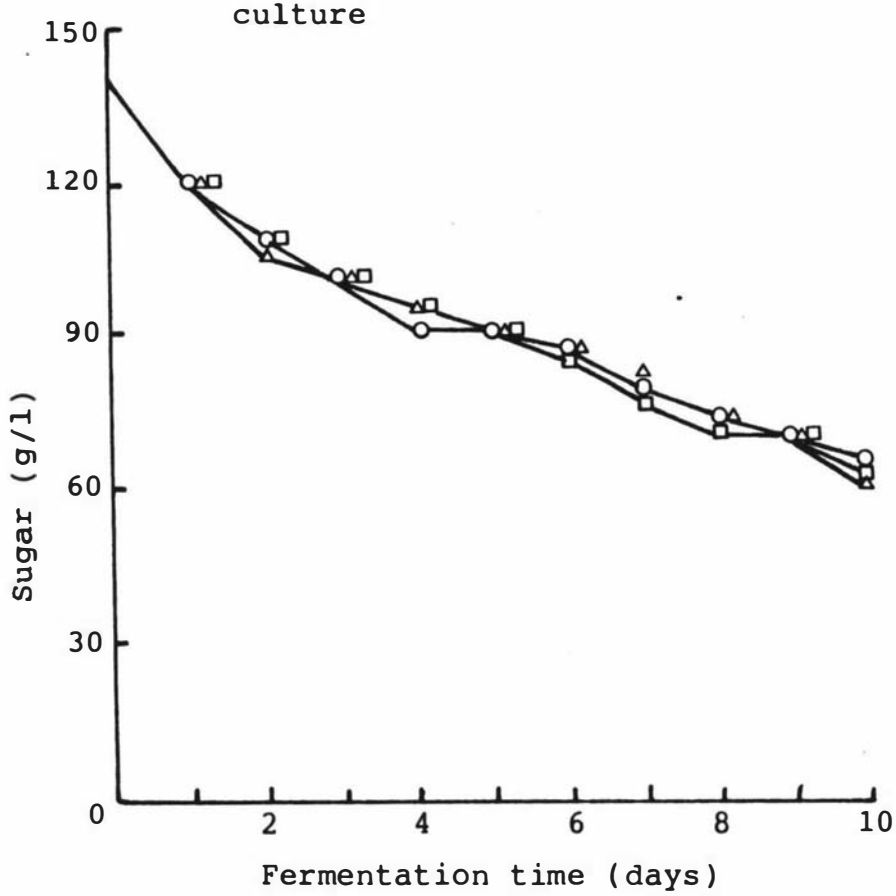
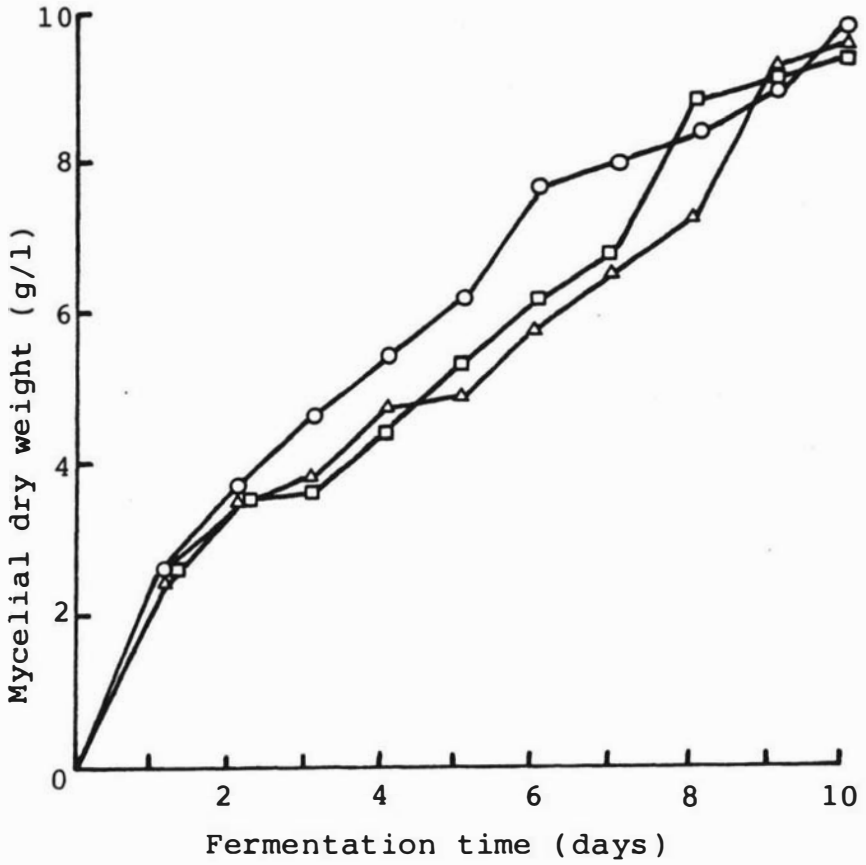


Figure 4.2 Effect of interruptions to aeration on sugar utilisation in shake-flask culture



(O,control, no interruptions; Δ,nitrogen gas flushed for 20 min on days 1 and 3; □ ,nitrogen gas flushed for 120 min on days 1 and 3)

Figure 4.3 Effect of interruptions to aeration on biomass production in shake-flask culture



(Symbols as for Figure 4.1)

day of fermentation, the citric acid concentrations under all conditions were approximately equal. Sugar utilisation (Figure 4.2) was not affected by the interruptions, but biomass production (Figure 4.3) appeared to be delayed. However, the final biomass concentrations were not significantly different.

4.2.2 Combination of Fermenter and Shake-Flask Culture

The experiment was performed in a combination of fermenter culture, as described in section 3.5.3 except that the effluent gas was not analysed, and in shake-flask culture. The aeration and agitation rates for the fermenter were 0.5 vvm and 200 rpm, respectively. The fermenter was inoculated and citric acid production was monitored over a 10 day period. During the fermentation, samples (3 x 50 ml) were withdrawn daily into sterile 250 ml flasks. One flask was immediately (within 1 minute) placed on the shaker and used as a control. The other two flasks were flushed with oxygen-free nitrogen gas as described in section 4.2.1, one for 20 minutes, the other for 120 minutes, prior to being placed on the shaker. Samples (5 ml) were withdrawn from the flasks on each of the following three days and analysed for citric acid and sugar. The results in Table 4.1 show the effect of the interruptions to aeration on citric acid production in the flasks. Results from samples withdrawn from the fermenter on days 1, 2 and 3 suggest that the interruption to aeration caused a slight initial inhibition of citric acid

Table 4.1 The effect of interruption to aeration on citric acid production in samples withdrawn from a fermenter

Day from fermenter	Day in shake-flask	Citric Acid Concentration (g/l)		
		Control	Interruptions	
			20 min	120 min
0	0	0	0	0
	1	1.0	1.4	1.4
	2	2.1	2.2	1.9
	3	3.9	4.8	4.5
1	0	1.6	1.6	1.6
	1	1.9	1.5	1.5
	2	4.1	4.6	4.4
	3	7.1	7.3	7.1
2	0	3.7	3.7	3.7
	1	6.8	6.2	6.6
	2	9.5	11.2	10.2
	3	13.3	16.4	14.7
3	0	8.3	8.3	8.3
	1	8.8	8.5	8.8
	2	12.9	12.2	12.1
	3	14.4	14.9	14.7
4	0	9.3	9.3	9.3
	1	10.2	10.7	10.4
	2	13.9	15.5	14.0
	3	17.1	17.8	17.2

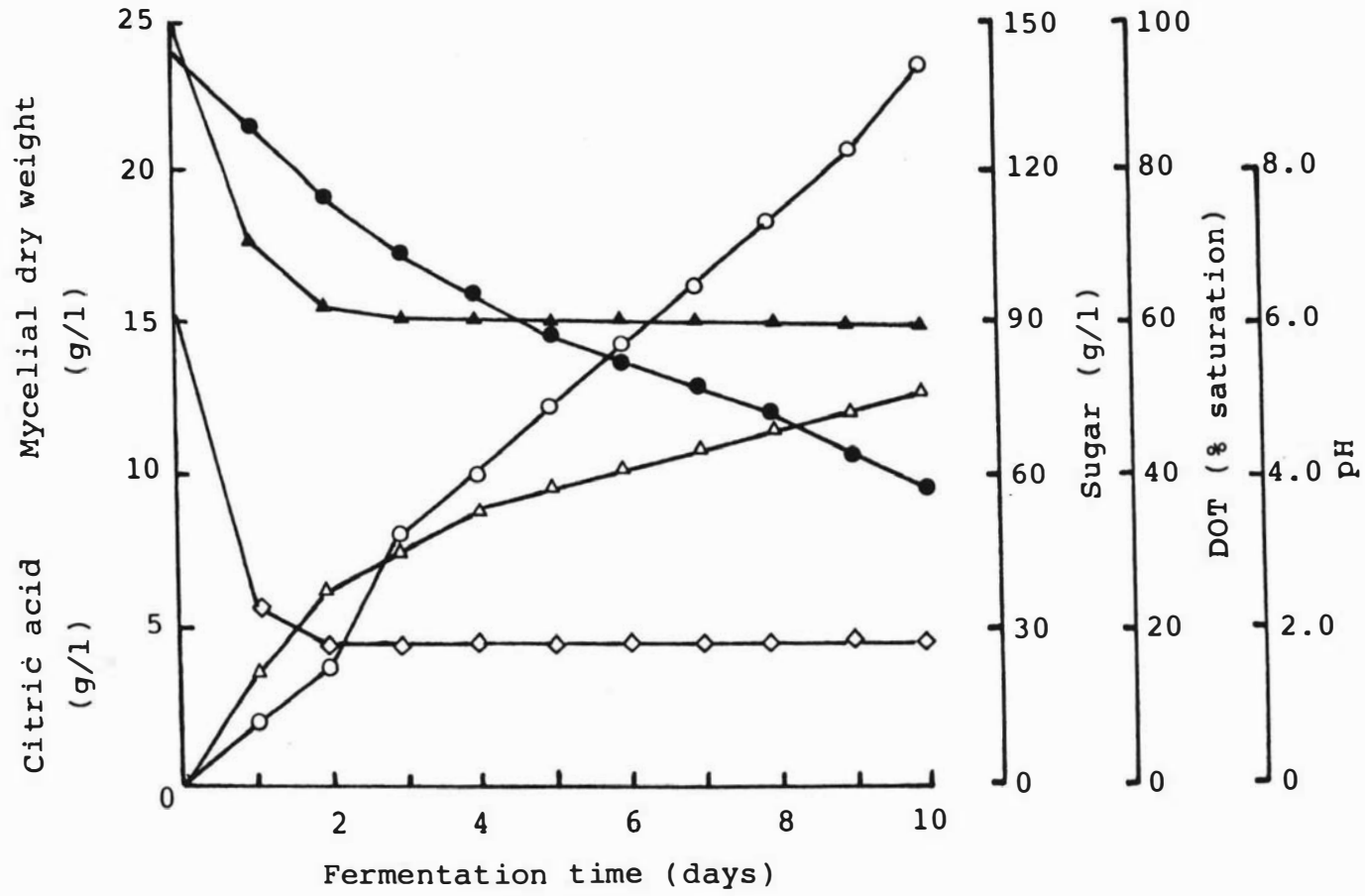
Day from fermenter	Day in shake-flask	Citric Acid Concentration (g/l)		
		Control	Interruptions	
			20 min	120 min
5	0	12.0	12.0	12.0
	1	12.4	12.3	12.1
	2	16.4	17.3	17.0
	3	22.3	23.6	22.7
6	0	15.2	15.2	15.2
	1	15.4	15.4	15.5
	2	17.7	17.7	17.3
	3	20.9	20.5	20.0
7	0	16.1	16.1	16.1
	1	16.9	16.2	16.1
	2	21.9	22.0	21.9
	3	25.4	25.7	25.3
8	0	18.6	18.6	18.6
	1	21.0	19.5	20.0
	2	25.0	24.0	25.4
9	0	21.8	21.8	21.8
	1	23.8	22.3	23.0
10	0	24.8	24.8	24.8
	1	25.7	25.0	25.2
	2	27.1	28.5	27.9

production, but this was quickly overcome. Samples withdrawn from the fermenter after day 3 behaved similarly to each other, showing that the interruptions had no lasting effect on the citric acid production of the cultures. The interruptions to aeration had little effect on sugar utilisation (data not shown).

4.2.3 **Fermenter Culture**

The experiments were performed in fermenter culture as described in section 3.5.3, except that no analysis of effluent gas was performed. Aeration and agitation rates were 1.0 vvm and 200 rpm, respectively. A control fermentation was performed with no interruption to aeration (Figure 4.4). Fermentations were then performed in which oxygen-free nitrogen gas was sparged at a flow rate of 1.0 vvm for either 20 or 120 minutes on the first and third days of the fermentation. Agitation was continued throughout the interruption period. A further experiment was performed where, instead of replacing the air with nitrogen gas, the aeration and agitation were discontinued for a 20 minute period on the first and third days of the fermentation. The citric acid production under the various conditions is plotted in Figure 4.5, and shows that under the conditions of two 20 minute interruptions, production was similar to that of the control. However, when the interruptions were for 120 minutes, citric acid production was severely retarded, although the rate of production finally attained was equal to, that of the control. After the first 120 minute interruption (on day 1) mycelial growth was severely

Figure 4.4 Time course profile of the control fermenter culture (no interruptions)



(O,citric acid; ●,sugar; △,mycelial dry weight; ▲,DOT; ◇,pH)

retarded, and did not recover until the sixth day of fermentation (Figure 4.6). The biomass concentration at the end of the fermentation was not too dissimilar to that of the control. The rate of sugar utilisation (Figure 4.7) was unaffected by the first 120 minute interruption, but was retarded after the second interruption. Recovery was observed at the same time as mycelial growth recommenced.

The product yields (based on sugar utilised) after 10 days of fermentation were 45% for the control fermentation, with similar values obtained for both of the fermentations with 20 minute interruptions. However, the product yield for the fermentation interrupted for periods of 120 minutes, was only 21%.

The DOT profiles for the experiments in the fermenter are shown in Figure 4.8. When the air supply was interrupted for 120 minutes, the DOT decreased to zero over a period of 35 minutes. In contrast, when the interruptions were for 20 minutes, the DOT did not decrease to zero, the lowest value reached being 20% of air saturation. During nitrogen sparging of uninoculated growth medium, the recorded DOT decreased to zero within 10 minutes. This indicates that the mycelial pellets retain dissolved oxygen for some time after the interruption to the air supply, resulting in a slower rate of degassing. In all experiments, after recommencement of the air supply the DOT rose to a level higher than that immediately prior to the interruption and then gradually decreased, suggesting that the rate of metabolism is temporarily depressed during oxygen starvation.

Figure 4.5 Effect of interruptions to aeration on citric acid production in fermenter culture

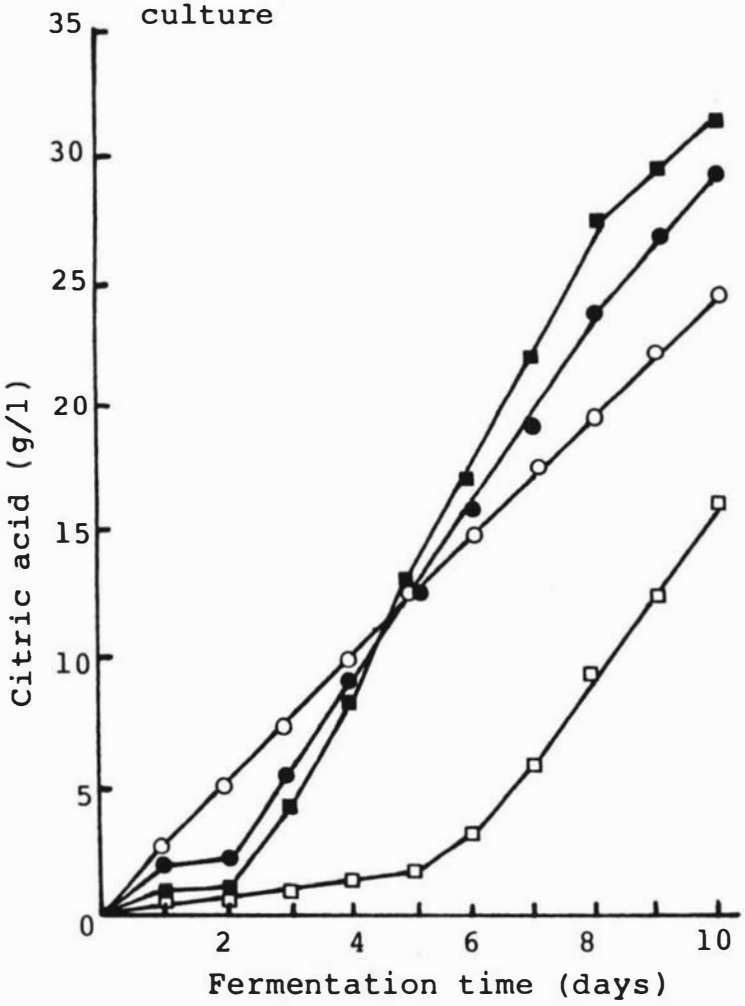
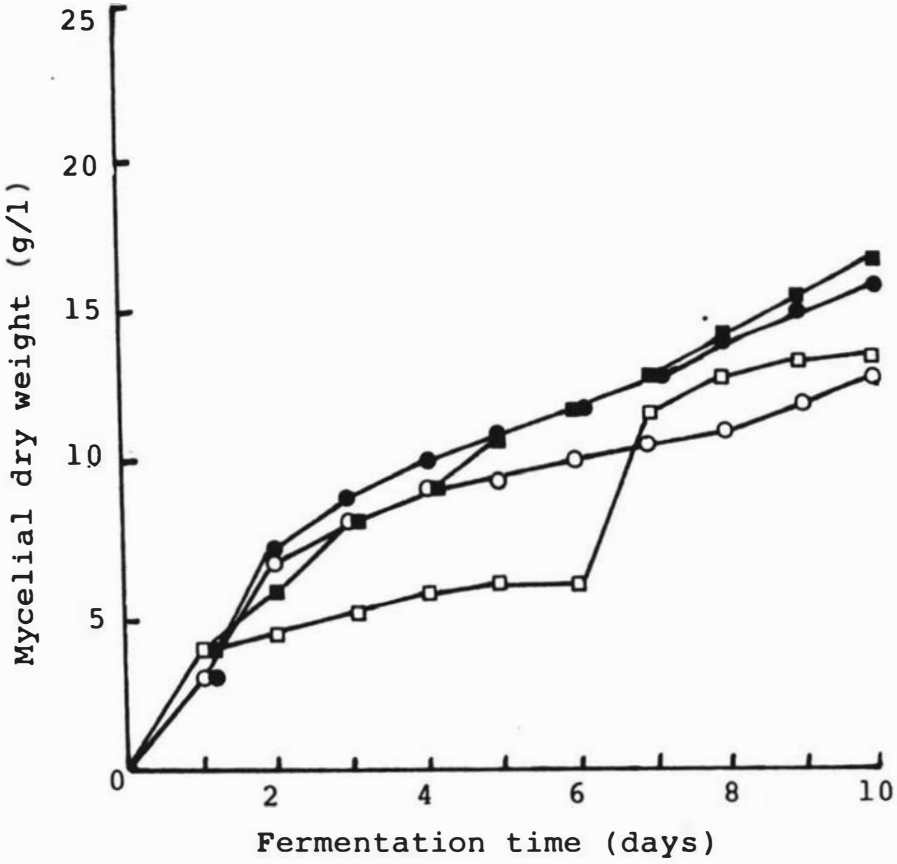
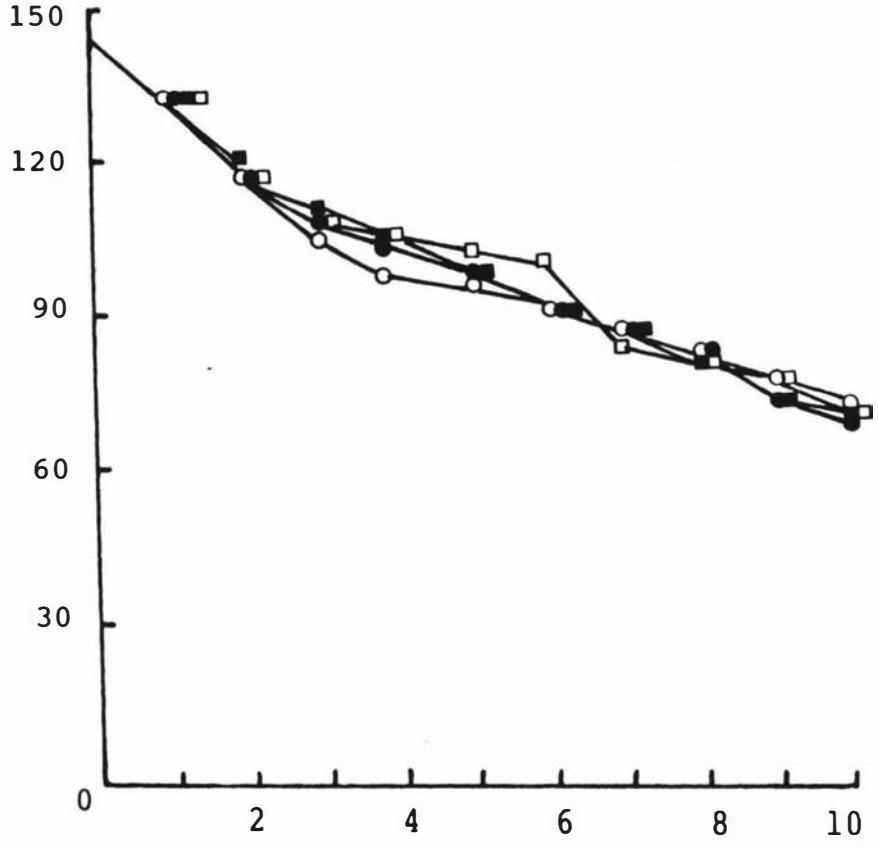


Figure 4.6 Effect of interruptions to aeration on biomass production in fermenter culture



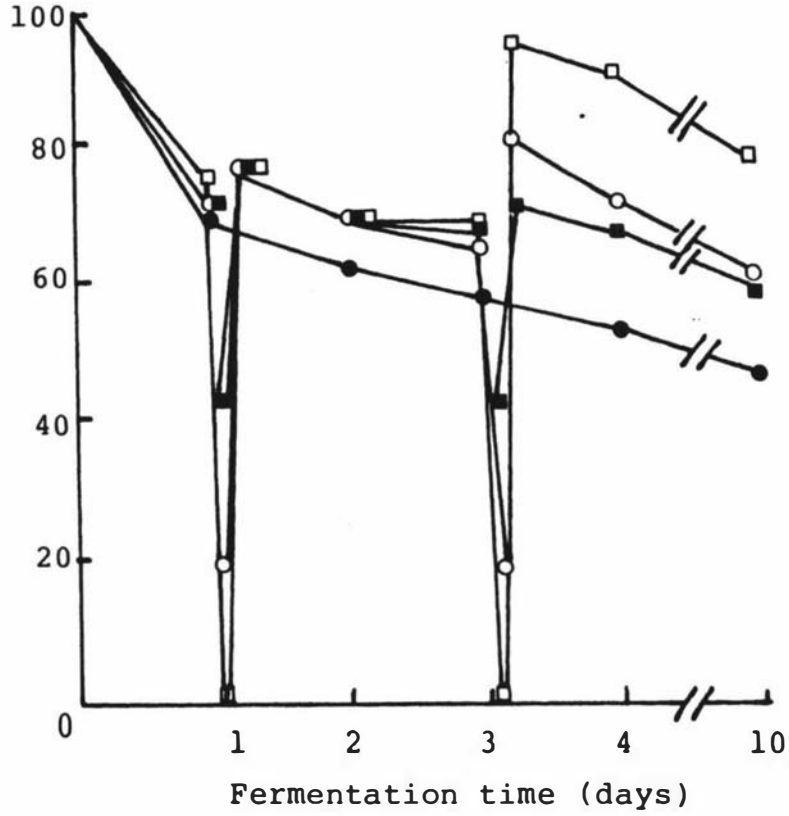
(●,control, no interruptions; ○,nitrogen gas sparged into the culture for 20 min on days 1 and 3; ■,agitation and aeration of the culture suspended for 20 min on days 1 and 3; □,nitrogen gas sparged into the culture for 120 minutes on days 1 and 3)

Figure 4.7 Effect of interruptions to aeration on sugar utilisation in fermenter culture



(Symbols as for Figure 4.5)

Figure 4.8 Effect of interruptions to aeration on the DOT of the cultures in the fermenter



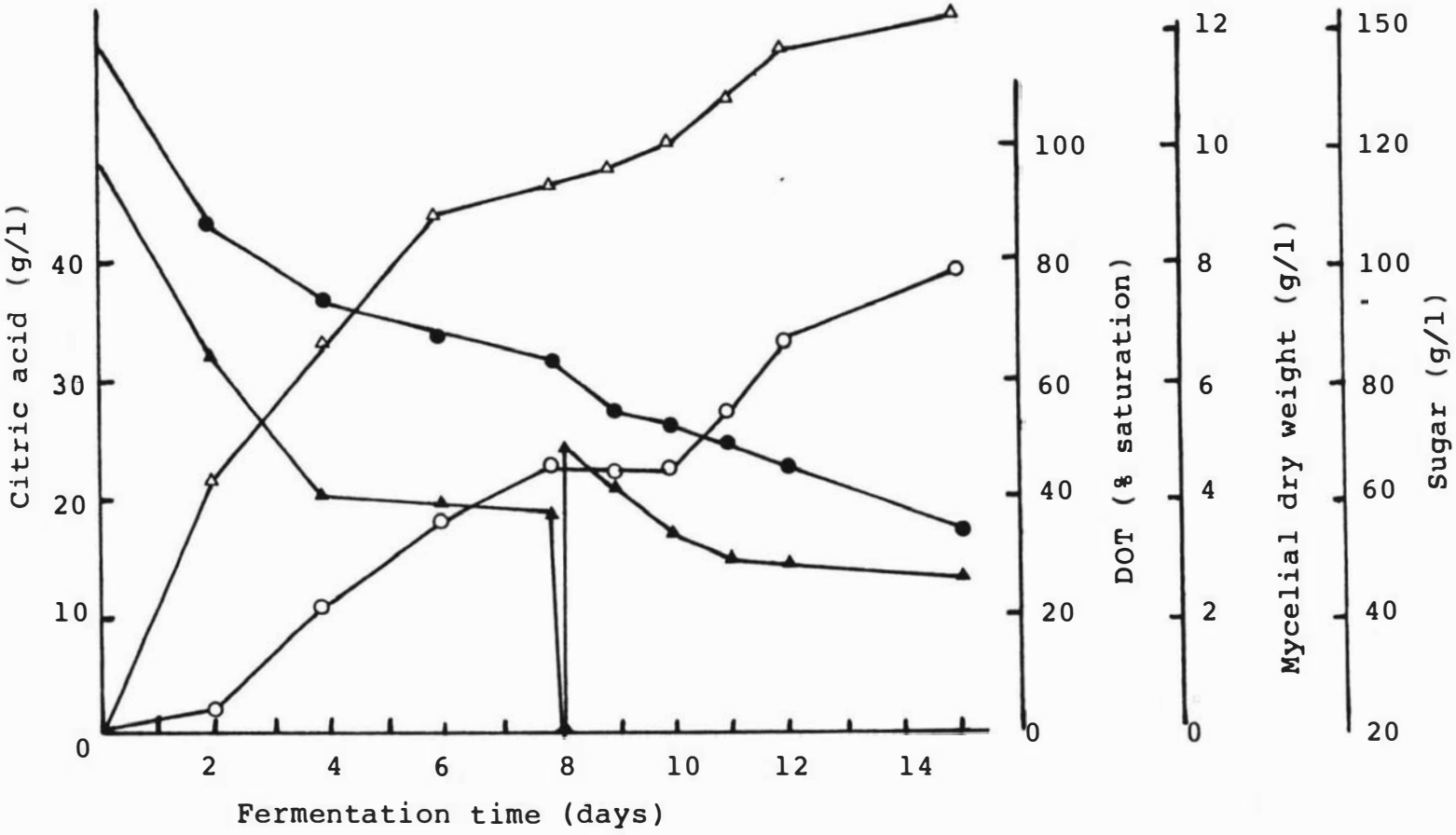
In all of these experiments, the interruptions to the aeration had been made while the organism was actively growing. Consequently, a further experiment was performed in which the interruption was delayed until active growth had ceased, as measured by the exhaustion of the nutrient nitrogen source from the growth medium. This occurred by the fourth day after inoculation. Then, on the eighth day, the air supply was replaced with nitrogen gas for a period of 120 minutes. The results are shown in Figure 4.9. On sparging with the nitrogen gas, the DOT of the culture decreased to zero over a 35 minute period and remained at this level for 85 minutes before air was reintroduced. Citric acid production ceased for 2 days, but then resumed at a rate similar to that observed prior to the interruption. Sugar utilisation was hardly affected. The increase in biomass which was observed following the exhaustion of nutrient nitrogen was probably due to the accumulation of carbohydrate reserves by the organism.

4.3 DISCUSSION

The results from the experiments carried out entirely in shake-flask culture indicated a slight inhibitory effect on citric acid production of interruptions to aeration on the first and third days after inoculation, although this effect was reversible. These results are in agreement with those of Kubicek *et al.* (1980), who reported that trophophase cultures were able to recover from a temporary interruption to aeration.

In the second series of experiments using a combination

Figure 4.9 Effect of interruption to aeration in a 2.0 litre fermenter. Aeration was interrupted on day 8 by replacing the air supply with nitrogen gas for 120 min



(O,citric acid ; Δ,mycelial dry weight; ●,sugar; ▲,DOT)

of fermenter and shake-flask culture, the effects of interruptions during both trophophase and idiophase were investigated. Slight inhibitory effects on citric acid production occurred during trophophase, but were quickly overcome. However, during idiophase, no effect was observed. These results appear to contradict those of Kubicek *et al.* (1980), who reported that idiophase cultures failed to recover from temporary interruptions to the air supply. Since interruptions to the air supply directly affect the DOT of the culture, experiments were performed entirely in fermenter culture, so that the DOT could be monitored continuously. In the experiments where the interruption periods were of 20 minutes duration, the DOT never fell below 20% of saturation. This is above the critical DOT value of 12 to 13% of saturation reported by Kubicek *et al.* (1980), so the lack of any gross effect of the interruptions on citric acid production can be explained on this basis. Similar results have been reported for penicillin production (Varder and Lilly, 1982). In contrast, when the air supply to the fermentation was interrupted for 120 minutes, the DOT value decreased to zero and was maintained at that level for 85 minutes. In this case, mycelial growth and citric acid production, but not sugar utilisation, ceased. This was not a permanent effect and both biomass production and citric acid production rates eventually recovered to equal that of the control culture, albeit with a reduced product yield. Thus, both trophophase and idiophase cultures can recover after a period of 85 minutes at a DOT value of zero

saturation. For the experiment involving a combination of shake-flask and fermenter culture, it must be assumed, that the critical DOT value was not reached. This was probably a reflection of the methodology used in that the dissolved oxygen was not completely removed from the mycelial pellets. Hence, a temporary interruption to the air supply *per se* is not as important as the value to which the DOT decreases, and the rate of decrease will depend on the oxygen demand of the culture. Thus, in the event of a temporary interruption to the air supply during a fermentation, attention to the DOT value will indicate whether any inhibitory effect is likely to occur. If the DOT remains above the critical value, no real effect will be apparent. However, below this value, production will be inhibited for some considerable time, although subsequent recovery can occur. Since sugar utilisation continues during the interruption, reduced product yields can be expected. The fate of the sugar during this time is unknown.

4.4 CONCLUSIONS

The main conclusion that can be drawn from this series of experiments is that if the air supply to a citric acid-producing culture is interrupted so that the DOT value falls to zero, inhibition of citric acid production will occur. However, both trophophase and idiophase cultures can recover even after a period of 85 minutes at a DOT value of zero. Nonetheless, there will be a significant lag period between reconnection of the air supply and the observed recovery.

Furthermore, product yields will be depressed due to continued sugar utilisation during the interruption.

Interruptions to aeration that do not reduce the DOT value to less than the critical DOT value (12 to 13% of saturation) for citric acid production, will have no gross effect on the culture. Thus, during an equipment breakdown, which is the cause of most interruptions to aeration in practice, attention to the DOT value will indicate whether any inhibitory effect should be expected.

CHAPTER 5

THE EFFECT OF DIFFERENT NUTRIENT LIMITATIONS ON CITRIC ACID PRODUCTION IN BATCH CULTURE

5.1 INTRODUCTION

It is generally recognised that to achieve high citric acid production rates and yields, a nutrient other than the sugar source should be exhausted from the medium after the attainment of sufficient biomass, so that the remaining sugar is then converted to citric acid. Most literature reports refer to the nutrient nitrogen as the limiting substrate and very few data have been published on limiting biomass growth with any other nutrients. Reports exist using phosphate as the limiting nutrient (Shu and Johnson, 1948; Berry *et al.*, 1977; Kristiansen *et al*,. 1982; Jernejc *et al*,. 1982) but no reports have been published in which any other nutrient was deliberately examined as the limiting factor. The purpose of this section was to examine citric acid production and associated parameters, under the following nutrient limitations: nitrogen, phosphate, sulphate, magnesium, iron, copper and zinc.

5.2 LIMITATION OF TRACE METALS

These experiments were performed in shake-flask culture using the medium of Kristiansen and Charley (1981) (Table 3.2) modified as follows. Nitrogen-limited cultures using $(NH_4)_2SO_4$ (1.0 g/l) as the nitrogen source were employed as controls. For the trace metal limitations the $(NH_4)_2SO_4$ concentration was also 1.0 g/l, while two concentrations of

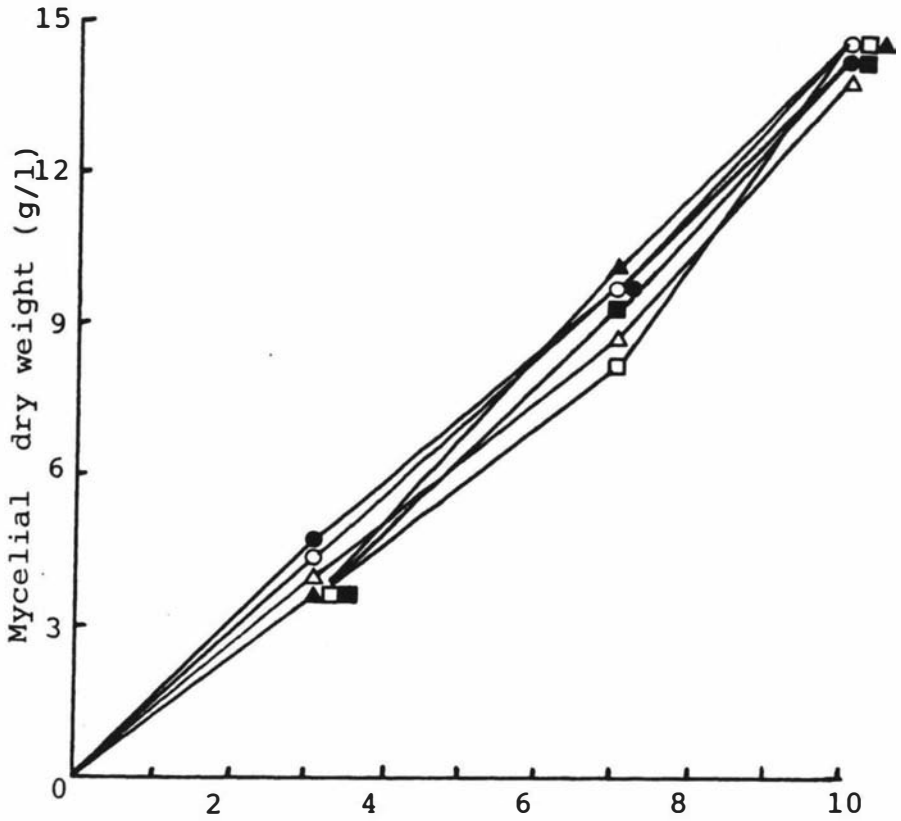
the limiting metal ion were used, 10% of the amount shown in Table 3.2, and its complete absence.

The results for biomass production (Figure 5.1) under the various limitations show very little difference from that of the control (Figure 4.3) (approximately 15 g biomass/l after 10 days), suggesting that in no case was a trace metal limitation actually achieved. Hence it must be assumed that sufficient quantities of these trace metals were present in the spore inoculum or as contaminants in the other medium components. No analyses were performed on the media for metal ion composition.

The citric acid production under the various limitations is presented in Figure 5.2. These results indicate that after a 10 day fermentation, very little citric acid was produced under any condition when compared with the control (Figure 4.1) (approximately 20 g/l). Thus it is clear that reduction of the trace metal content of this growth medium has an adverse effect on citric acid production. The sugar utilisation under the various limitations is shown in Figure 5.3. For the control culture (Figure 4.2), a sugar utilisation of approximately 70 g/l was observed after 10 days of fermentation. Thus, reduction of the trace metal content of the growth medium has an adverse effect on this parameter.

The results of these experiments show, firstly, that a true limitation of the metal ions was not achieved. However it can be concluded that any reduction in the trace metal content of this medium has an adverse effect on sugar

Figure 5.1 Effect of metal ion limitations on biomass production in shake-flask culture



(○ , no Zn^{2+}; ● , 0.01 mg/l Zn^{2+}; △ , no Fe^{2+}; ▲, 0.01 mg/l Fe^{2+}; □ , no Cu^{2+}; ■ , 0.006 mg/l Cu^{2+})

Figure 5.2 Effect of metal ion limitations on citric acid production in shake-flask culture

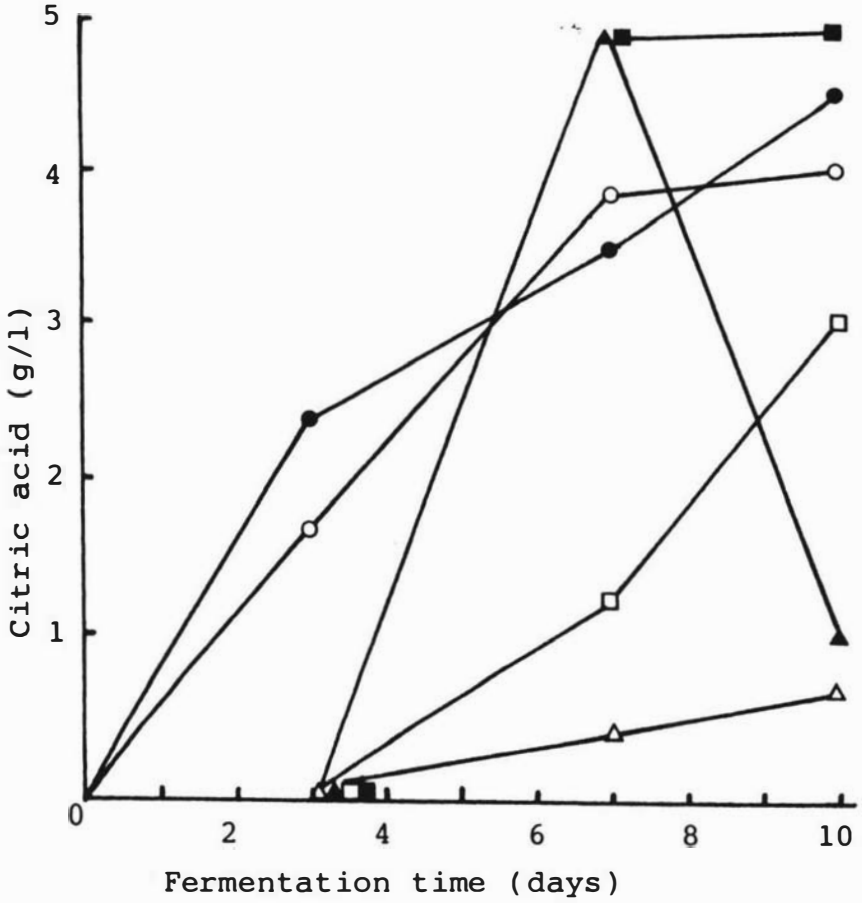
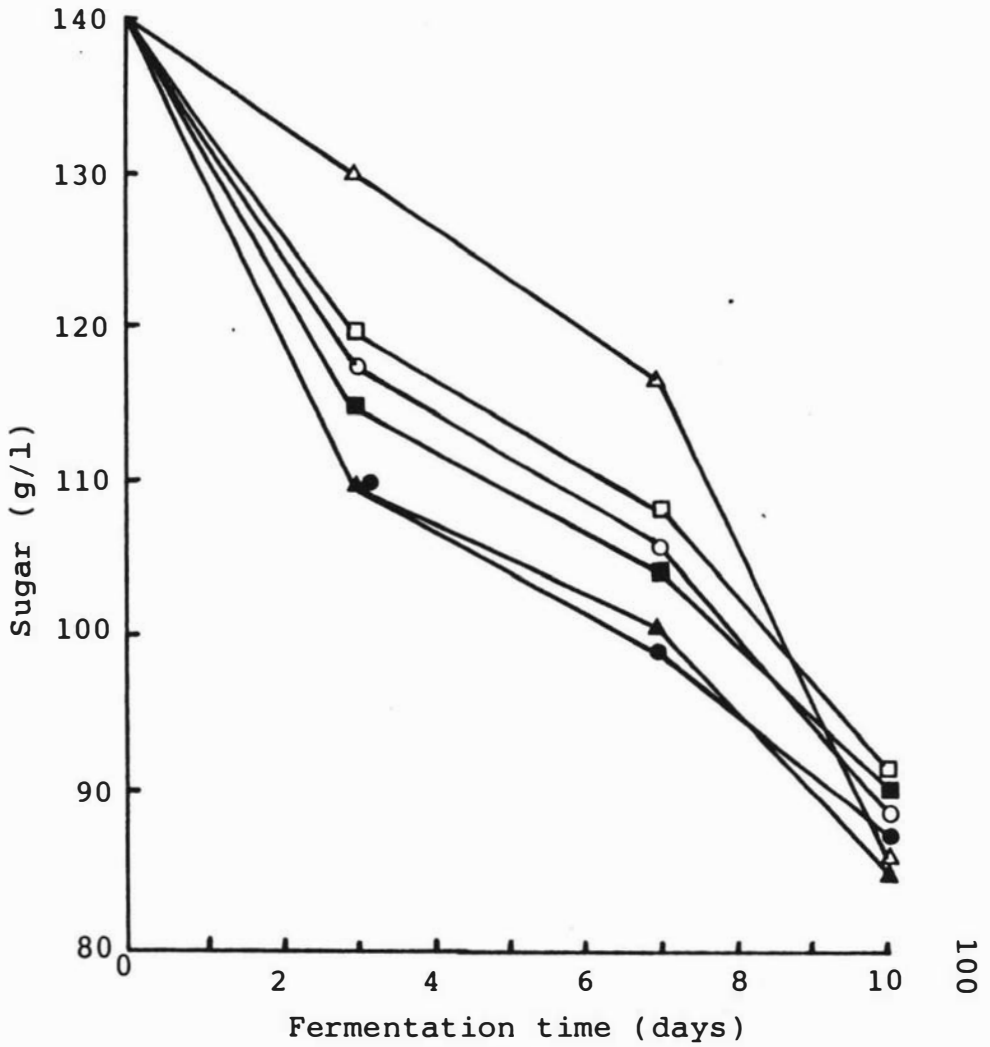


Figure 5.3 Effect of metal ion limitations on sugar utilisation in shake-flask culture



(Symbols as for Figure 5.1)

utilisation and citric acid production. On this basis therefore, it was not considered worthwhile to conduct further experiments to achieve true limitation.

5.3 LIMITATION OF NUTRIENT NITROGEN, PHOSPHATE, SULPHATE AND MAGNESIUM AT LOW LEVELS OF BIOMASS

These experiments were performed in fermenter culture as described in section 3.5.3. The concentration of the limiting nutrient was set so that a biomass concentration of approximately 15 g/l was achieved in each experiment, allowing the maintenance of a high DOT value, while the agitation rate was kept at 200 rpm to minimise the effect of shear forces. The aeration rate was sufficiently low (0.5 vvm) so as to minimise the error involved in effluent gas analyses (Brooks *et al*,. 1982). For the various limitations, the medium described in Table 3.2 was modified as shown in Table 5.1. The limiting nutrient concentrations were nitrogen (0.21 g/l), phosphorous (0.070 g/l expressed as phosphate), sulphur (0.073 g/l expressed as sulphate), and magnesium (0.002 g/l).

5.3.1 Time Course of Fermentations

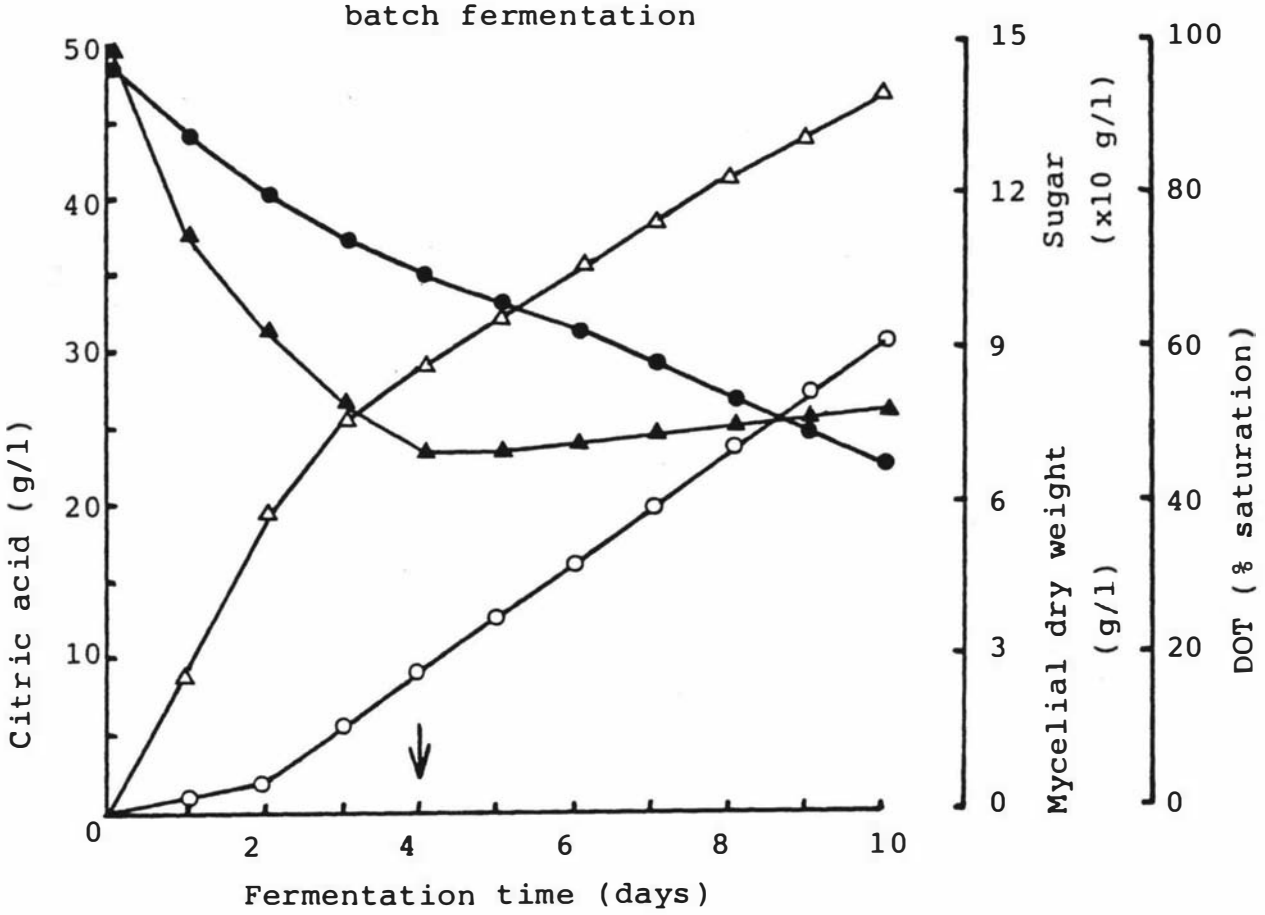
Figure 5.4 depicts the fermentation under nitrogen-limited conditions, and shows that nitrogen was exhausted by day 4. The phosphate concentration at this time was 970 mg/l and no further uptake occurred during the remainder of the fermentation. The very low concentrations of SO_4^{2-} and Mg^{2+} required for these two ions to be growth limiting nutrients (see below) were such that their utilisation could

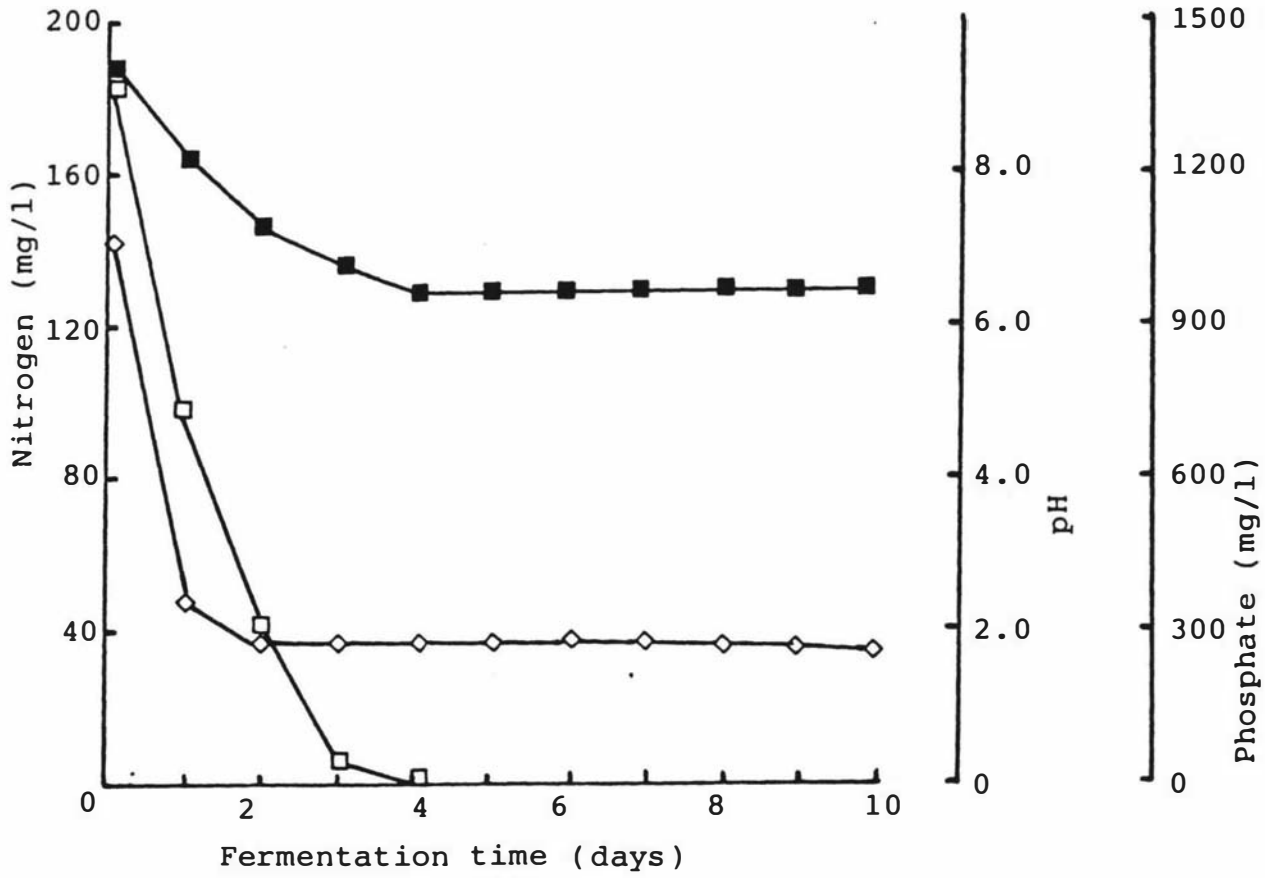
Table 5.1 Limiting nutrient concentrations to obtain 15 g/l biomass

Constituent (g/l)	Limitation			
	N	PO_4^{3-}	SO_4^{2-}	Mg^{2+}
$(NH_4)_2SO_4$	1.0	1.1	0.1	1.1
NH_4Cl	-	-	0.9	-
KH_2PO_4	2.0	0.1	2.0	2.0
$MgSO_4.7H_2O$	0.5	0.5	-	0.02
$MgCl_2$	-	-	0.5	-

All other constituents were as in Table 3.2.

Figure 5.4 The time course of a nitrogen-limited batch fermentation





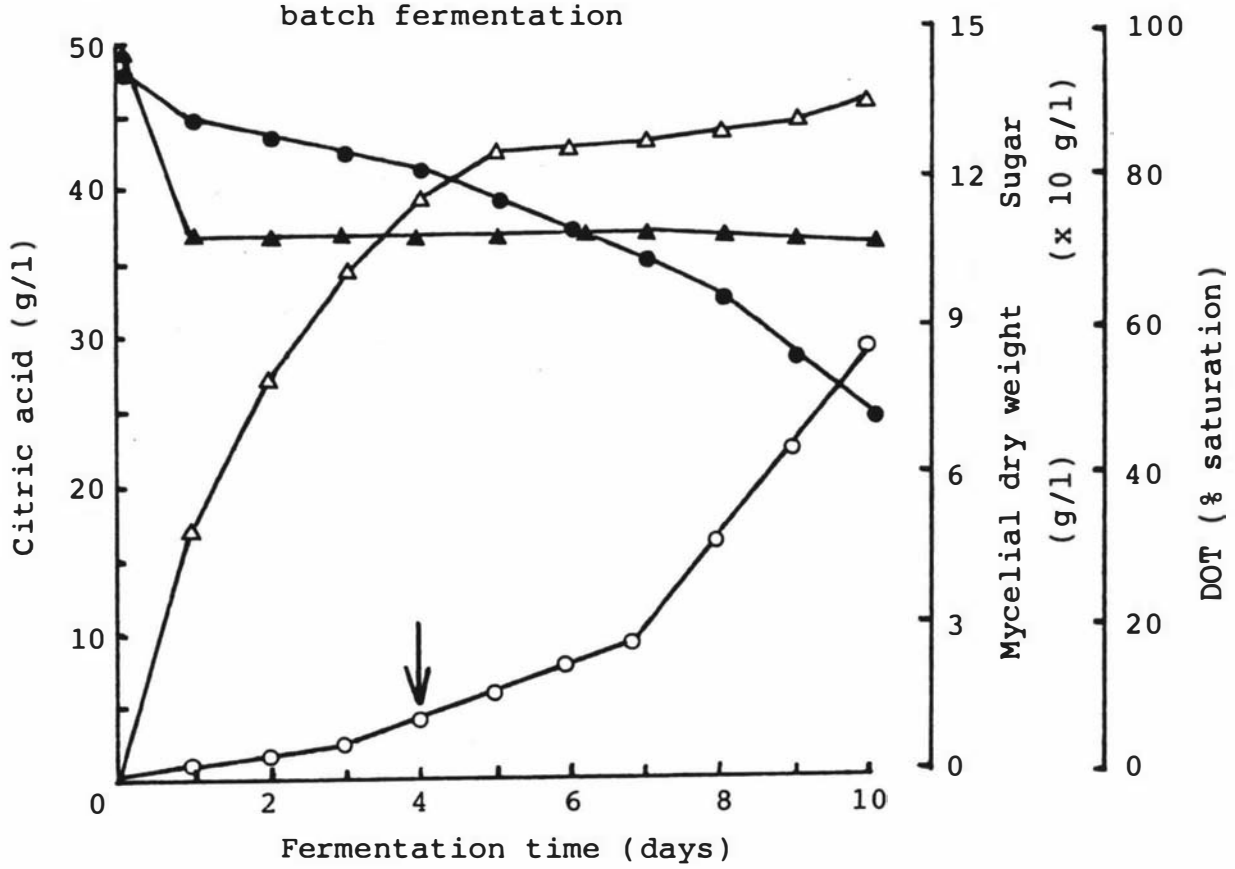
(○, citric acid; ●, sugar; △, mycelial dry weight; ▲ DOT; □, nitrogen; ■, phosphate; ◇, pH; ↓, limiting nutrient exhausted)

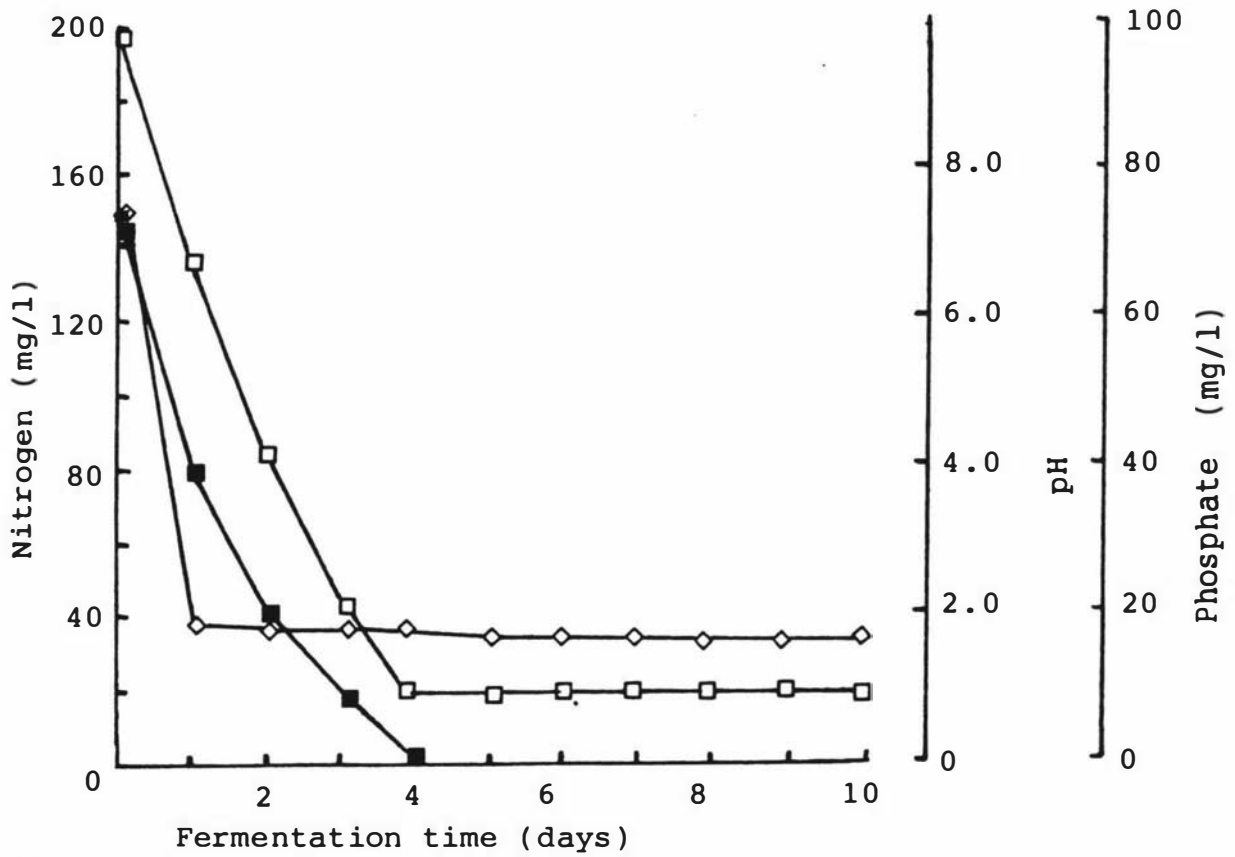
not be determined accurately. However, it is unlikely that they were growth limiting in this fermentation. Biomass continued to increase after nitrogen exhaustion and can probably be attributed to the accumulation of storage carbohydrate. Citric acid production commenced before day 1, i.e. prior to nitrogen exhaustion, and a concentration of 32 g/l (representing a yield of 46% based on sugar utilised), was achieved at day 10 (Table 5.2).

The phosphate-limited fermentation (Figure 5.5) shows that the limiting nutrient was exhausted on day 4. The biomass continued to increase after this time despite there being no further uptake of nitrogen after phosphate exhaustion. This was probably due to the accumulation of storage carbohydrate. The excess nitrogen concentration was 20 mg/l. Citric acid production commenced before day 1, prior to phosphate exhaustion. However, a noticeable increase in production rate occurred after day 7. A final citric acid concentration of 28 g/l was obtained, representing a yield of 43% based on sugar utilised (Table 5.2). The DOT during the fermentation was approximately 1.5 times higher than that observed during the nitrogen-limited fermentation.

The course of the sulphate-limited fermentation is shown in Figure 5.6. Sulphate was exhausted from the medium on day 4, and no further uptake of nitrogen or phosphate was observed after this time. The increase in biomass subsequent to the sulphate exhaustion from the medium was probably caused by the production of storage carbohydrate. Citric

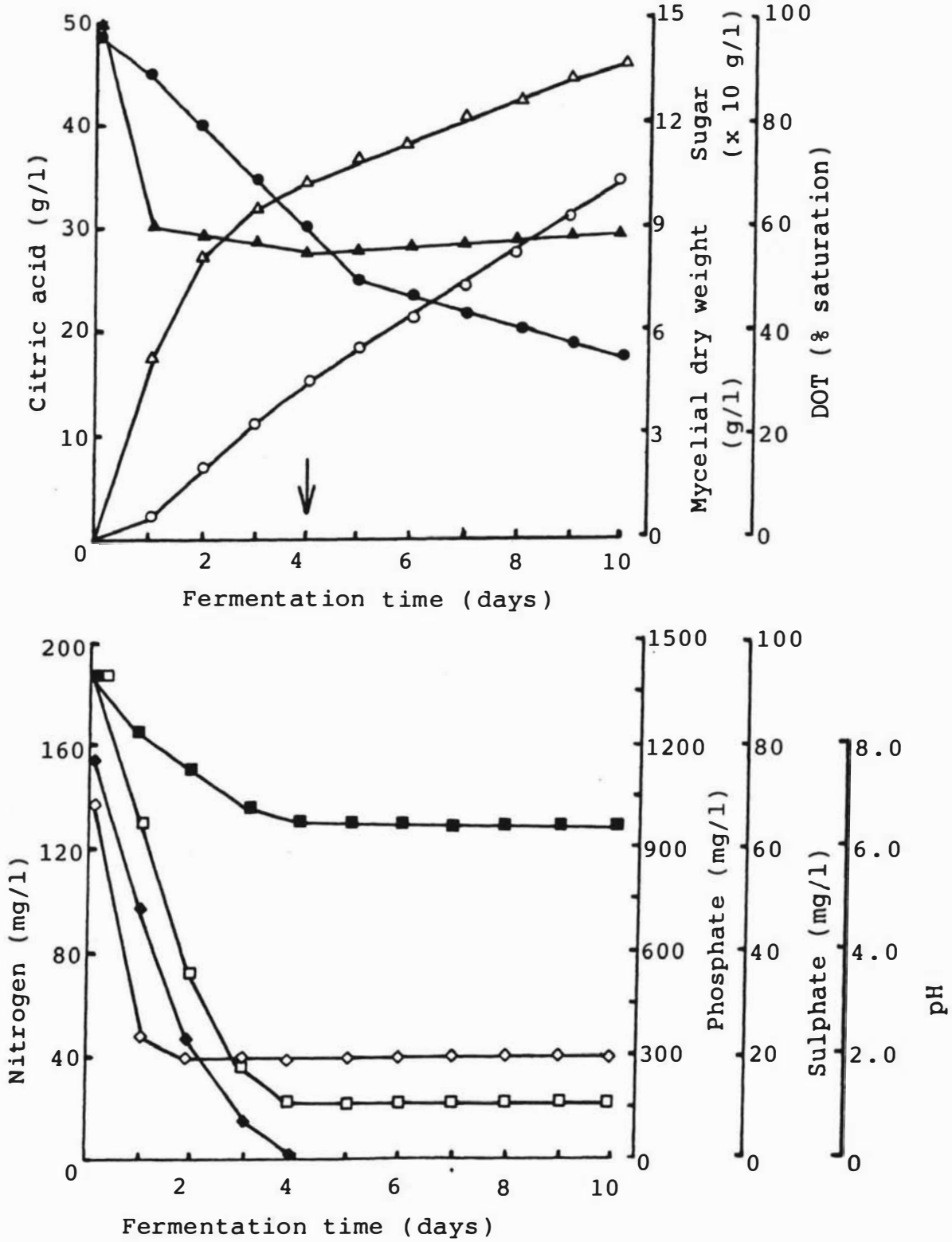
Figure 5.5 The time course of a phosphate-limited batch fermentation





(○, citric acid; ●, sugar; △, mycelial dry weight; ▲ DOT; □, nitrogen; ■, phosphate; ◇, pH; ↓, limiting nutrient exhausted)

Figure 5.6 The time course of a sulphate-limited batch fermentation



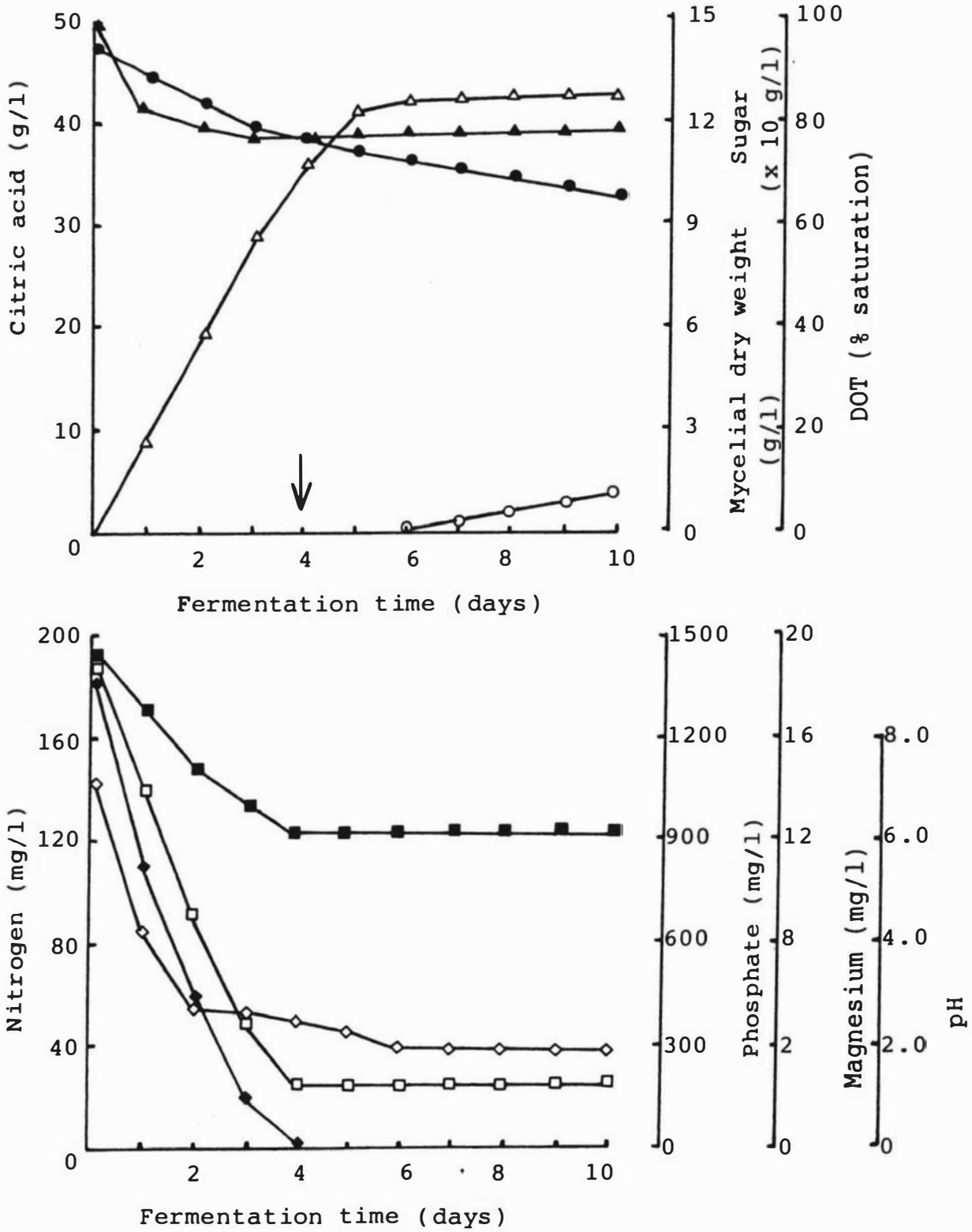
(○, citric acid; ●, sugar; △, mycelial dry weight; ▲ DOT; □, nitrogen; ■, phosphate; ◇, pH; ⇓, limiting nutrient exhausted ◆, sulphate;)

acid production commenced after day 1, before sulphate exhaustion. The final concentration on day 10 of 35 g/l represented a yield of 34% based on sugar utilised (Table 5.2).

The course of the magnesium-limited fermentation is shown in Figure 5.7. Magnesium was exhausted on day 4 and subsequent to its exhaustion from the medium, no further uptake of nitrogen or phosphate was observed. The increase in biomass after magnesium exhaustion can again be attributed to storage carbohydrate production. Citric acid production did not commence until after day 6 and a final concentration at day 10 of 4 g/l represented a yield of 10% based on sugar utilised (Table 5.2). The DOT remained high (approximately 80% of saturation), during the fermentation, reflecting a reduced requirement for oxygen. The pH profile was different from the other limitations in that it had decreased to pH 4.0 on day 1 and by day 5 had reached only pH 2.5. A trace of 2-oxoglutaric acid was detected in the fermentation medium at this time. After citric acid production had commenced, the pH decreased to pH 2.0.

From these results it can be seen that the highest citric acid yields were obtained from nitrogen- and phosphate-limited fermentations. For these two limitations the citric acid production and sugar utilisation were also similar. In the case of sulphate limitation, although more citric acid was produced compared with nitrogen- or phosphate-limited fermentations, more sugar was used and consequently the citric acid yield was lower. The magnesium limitation adversely affected sugar utilisation, which had

Figure 5.7 The time course of a magnesium-limited batch fermentation



(○, citric acid; ●, sugar; △, mycelial dry weight; ▲ DOT; □, nitrogen; ■, phosphate; ◇, pH; ↓, limiting nutrient exhausted ◆, magnesium;)

Table 5.2 Summary of different nutrient-limited fermentations at low biomass levels

Limiting nutrient	Biomass (g/l)	Sugar used (g/l)	Citric Acid (g/l)	Citric Acid Yield (%)
N	14	70	32	46
PO_4^{3-}	14	65	28	43
SO_4^{2-}	14	106	35	34
Mg^{2+}	14	38	4	10

Results are expressed after 10 days of fermentation.

There was no residual limiting nutrient remaining in the fermentation liquor after day 4.

consequent effects on citric acid production and yield. The biomass production was similar under all limitations. At this stage however, it must be cautioned that a true comparison cannot yet be made of the different nutrient limitations except for that with magnesium. This is because the fermentations differred in their DOT values, a parameter known to affect citric acid production (Section 2.6.4).

On the basis that a similar biomass production occurred under the different nutrient limitations, it can be proposed that a minimal medium for the growth of *A. niger* would include N, PO_4^{3-}, SO_4^{3-}, and Mg^{2+} present in the ratio 210:70:73:2. A slight increase in any three of these nutrients would render the fourth the limiting factor.

5.3.2 **Rates of Growth, Product Formation and Nutrient Uptake**

From the preceding data, it was possible to calculate rates of citric acid and carbon dioxide production, and uptake rates of sugar, nitrogen, phosphate, oxygen, sulphate and magnesium (these last two only when they were used as the limiting nutrient).

The rate data are shown in Figure 5.8 for a nitrogen-limited fermentation. The maximum volumetric citric acid production rate (210 mg/l.h) was observed on day 4 and the maximum specific rate (25 mg/gDW.h) on day 3. One feature of the data is that at the point of the maximum specific citric acid production rate, nutrient nitrogen was still being consumed by the organism, implying that there was still a positive specific growth rate. The value of the latter, based on mycelial dry weight determination, was

0.005 h^{-1}. On examining the growth rate data, it is seen to be a positive value throughout the fermentation. However, this must be treated with some caution since the growth rate should be zero after the exhaustion of the limiting nutrient. The positive values recorded, therefore, are due to the accumulation of storage carbohydrate and reflect the method of biomass determination, i.e. mycelial dry weight. If the growth rate data were based on the mycelial nitrogen concentration (not determined in this experiment) then the growth rate would be zero after the exhaustion of nutrient nitrogen.

Figure 5.9 shows the rate data under phosphate limitation. The maximum citric acid production rate, both volumetric and specific, occurred on day 8, well after phosphate exhaustion on day 4. There was no nutrient uptake occurring at this time except for sugar, for which a peak was observed. Coincident with the maximum citric acid production rate, there was a sharp decrease in the specific carbon dioxide production rate. The maximum observed volumetric citric acid production rate of 275 mg/l.h was greater than that observed under nitrogen limitation, but the specific rate, at 20 mg/gDW.h, was less. In contrast to the nitrogen-limited fermentation, most of the citric acid production occurred during the final 3 days of the fermentation.

The rate data for the sulphate-limited fermentation are shown in Figure 5.10. The volumetric and specific rates were similar to those observed during the nitrogen-limited

fermentation, peaking on days 3 and 4, respectively. Also, the maximum observed citric acid production rate occurred while the sulphate uptake rate was at a positive value, implying that a positive growth rate existed at this time. The calculated specific growth rate was approximately 0.004 h^{-1}.

The rate data for magnesium limitation are shown in Figure 5.11. The citric acid production rates, both volumetric and specific, reached their maxima on day 8. No citric acid was produced before day 6. The oxygen uptake rate increased slightly and the carbon dioxide production rate decreased markedly from day 7 to 8 during the increased citric acid production rate. No other nutrient uptake was occurring at this time and the calculated specific growth rate was approximately 0.001 h^{-1}.

One significant aspect of comparison between the nitrogen- and phosphate-limited fermentations is the large difference in oxygen requirements. The ratio of citric acid produced to oxygen used during the course of the fermentation is shown in Table 5.3. At the peak of the specific citric acid production rate under nitrogen limitation on day 3, the ratio was twice that of the phosphate-limited fermentation at that time. However, late in the phosphate-limited fermentation, when the citric acid production rate was highest, the ratio was at least 30 times larger than that under nitrogen limitation during the same period. This difference occurred only after phosphate exhaustion from the medium. Thus, under conditions of phosphate exhaustion, only small amounts of oxygen are

required for citric acid accumulation to occur.

All of the specific rate data described above were calculated using mycelial dry weight as a measurement of biomass. The data described in Figures 5.4 to 5.11 all show that once the limiting nutrient was exhausted, uptake of only sugar and oxygen continued, i.e. all other nutrients ceased to be consumed. Therefore, although the biomass continued to increase, this was probably due to storage carbohydrate production rather than active growth. A more true set of specific rate data would be obtained if the limiting nutrient content of the biomass were used to calculate the specific rates. However, this would not significantly alter any of the conclusions drawn so far.

5.3.3 Analysis of Selected Enzymes

Selected enzymes in mycelial cell-free extracts were investigated to determine their activities at various times during the nitrogen-limited fermentation. Figure 5.12 shows the activities of the following enzymes: aconitase, NAD-linked isocitrate dehydrogenase, NADP-linked isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, isocitrate lyase and pyruvate carboxylase. The results confirm the report of Hossain *et al.* (1984), that the maximum observed specific citric acid production rate coincides with the maximum observed activities of aconitase and NAD-linked and NADP-linked isocitrate dehydrogenases. In addition, isocitrate lyase showed a maximum at this time. Further there was no measurable pyruvate carboxylase activity until after growth had ceased. In contrast to Hossain *et al.*

For rate data shown in Figures 5.8, 5.9, 5.10 and 5.11, the following symbols were used:

- ○ , volumetric citric acid production rate expressed as mg/l.h
- ● , specific citric acid production rate expressed as mg/gDW.h
- △ , specific sugar uptake rate expressed as mg/gDW.h
- ▲ , specific nitrogen uptake rate expressed as mg/gDW.h
- □ , specific phosphate uptake rate expressed as mg/gDW.h
- ✕, specific sulphate uptake rate (Figure 5.10 only) expressed as mg/gDW.h
- ✕ , specific magnesium uptake rate (Figure 5.11 only) expressed as mg/gDW.h
- ■ , specific oxygen uptake rate expressed as mmol/gDW.h
- ◇ , specific carbon dioxide production rate expressed as mmol/gDW.h
- ◆ , specific growth rate expressed as h^{-1}

All axes, unless otherwise specified, represent the specific product formation or nutrient uptake rates.

Figure 5.8 Growth rate, product formation rates and nutrient uptake rates under nitrogen limitation

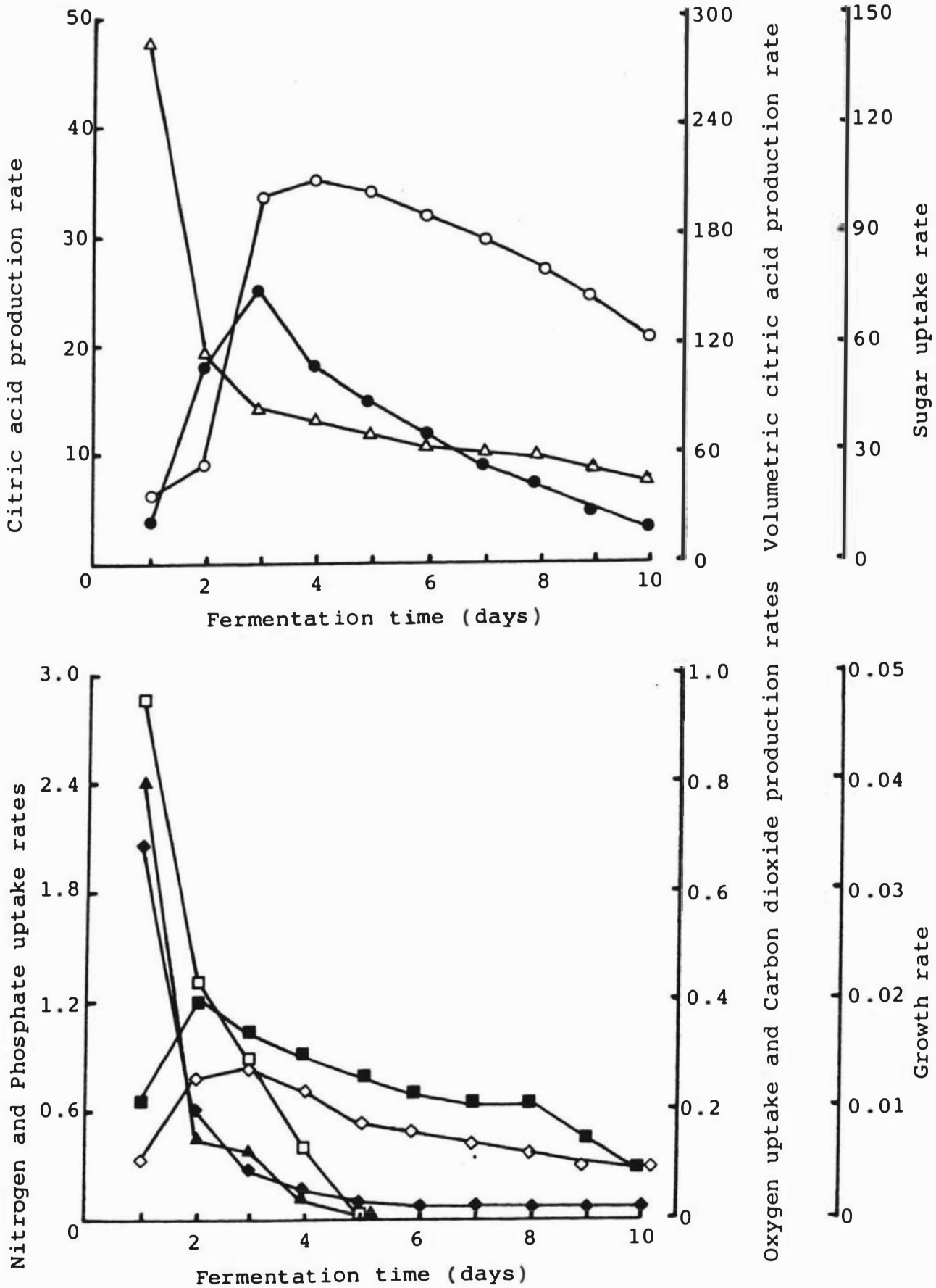
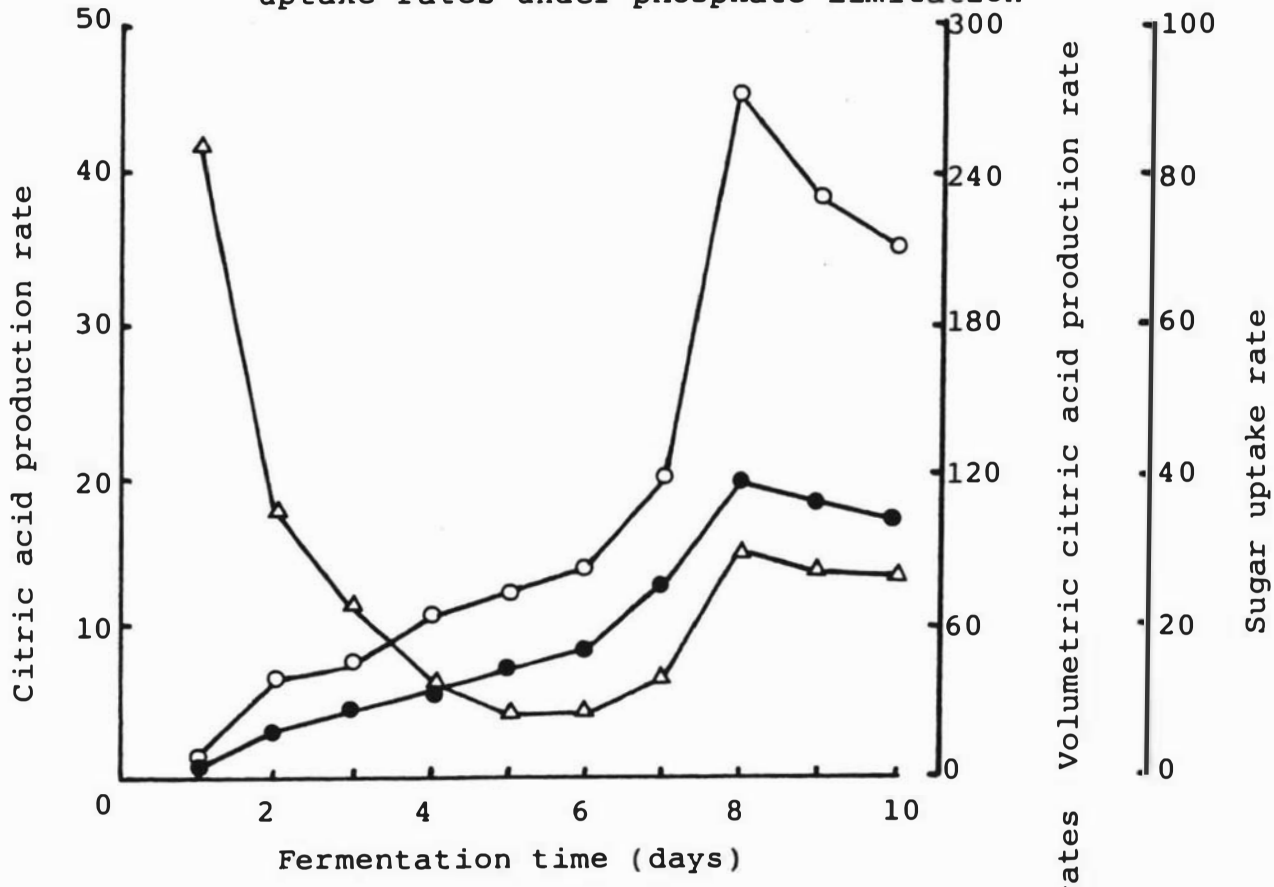


Figure 5.9 Growth rate, product formation rates and nutrient uptake rates under phosphate limitation



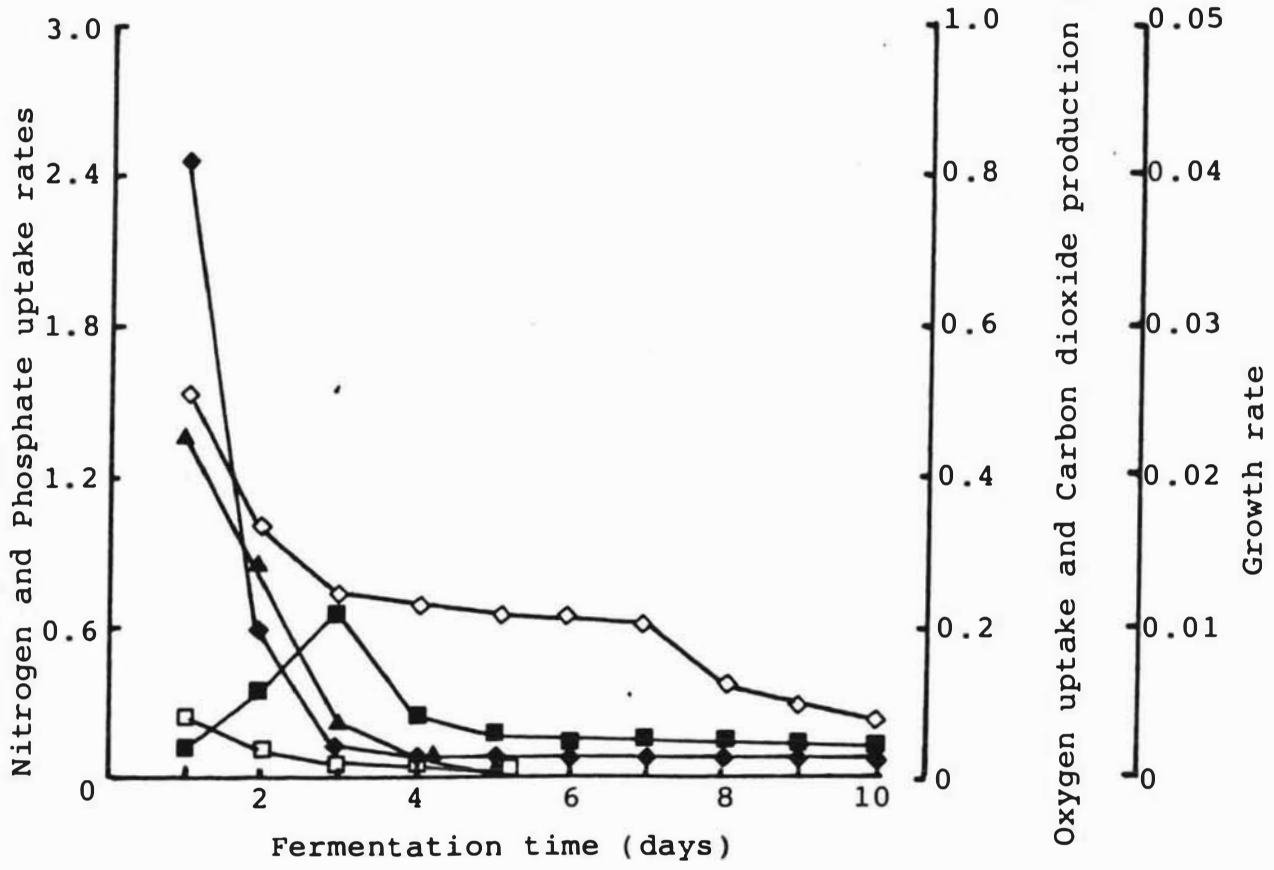


Figure 5.10 Growth rate, product formation rates and nutrient uptake rates under sulphate limitation

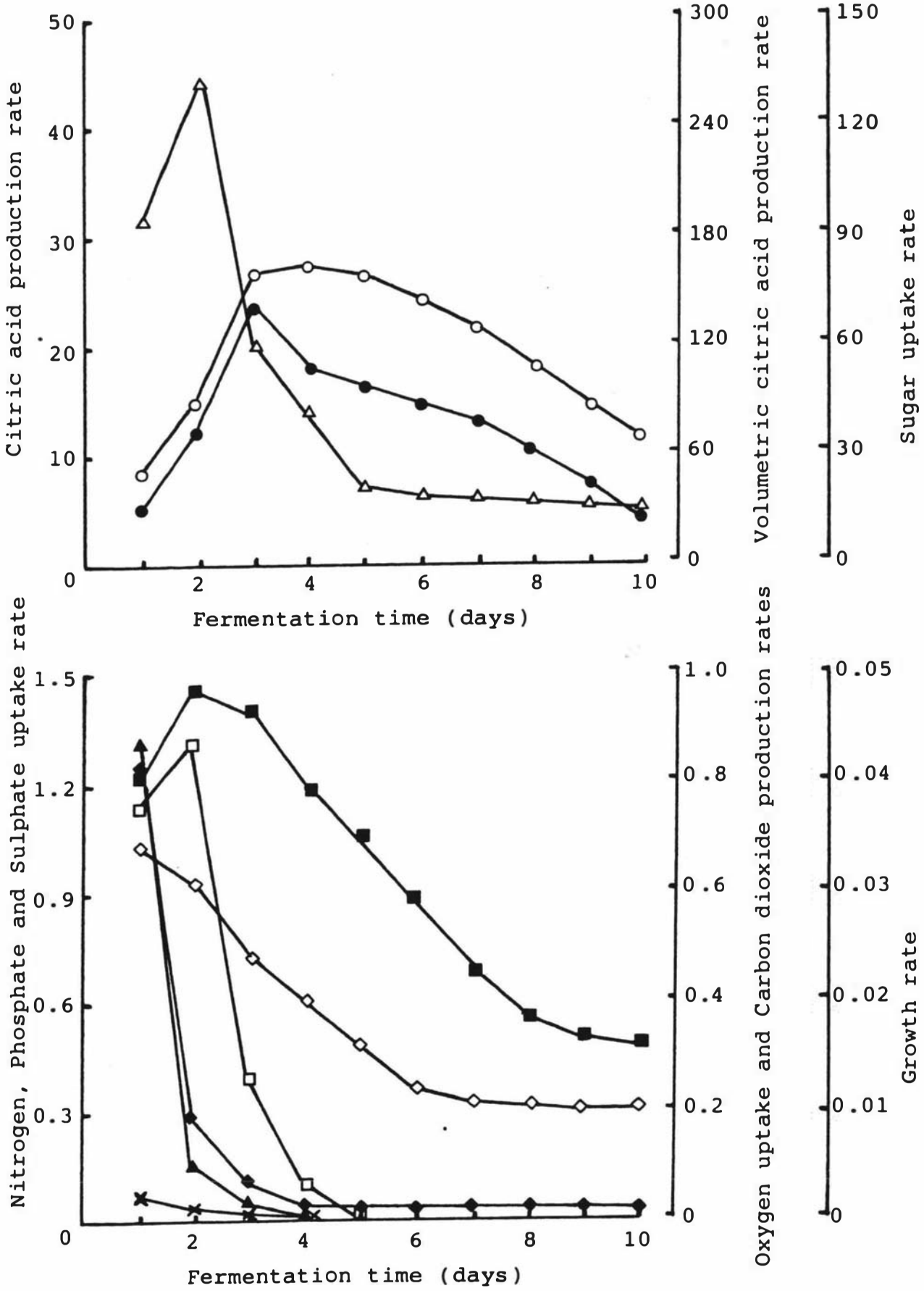
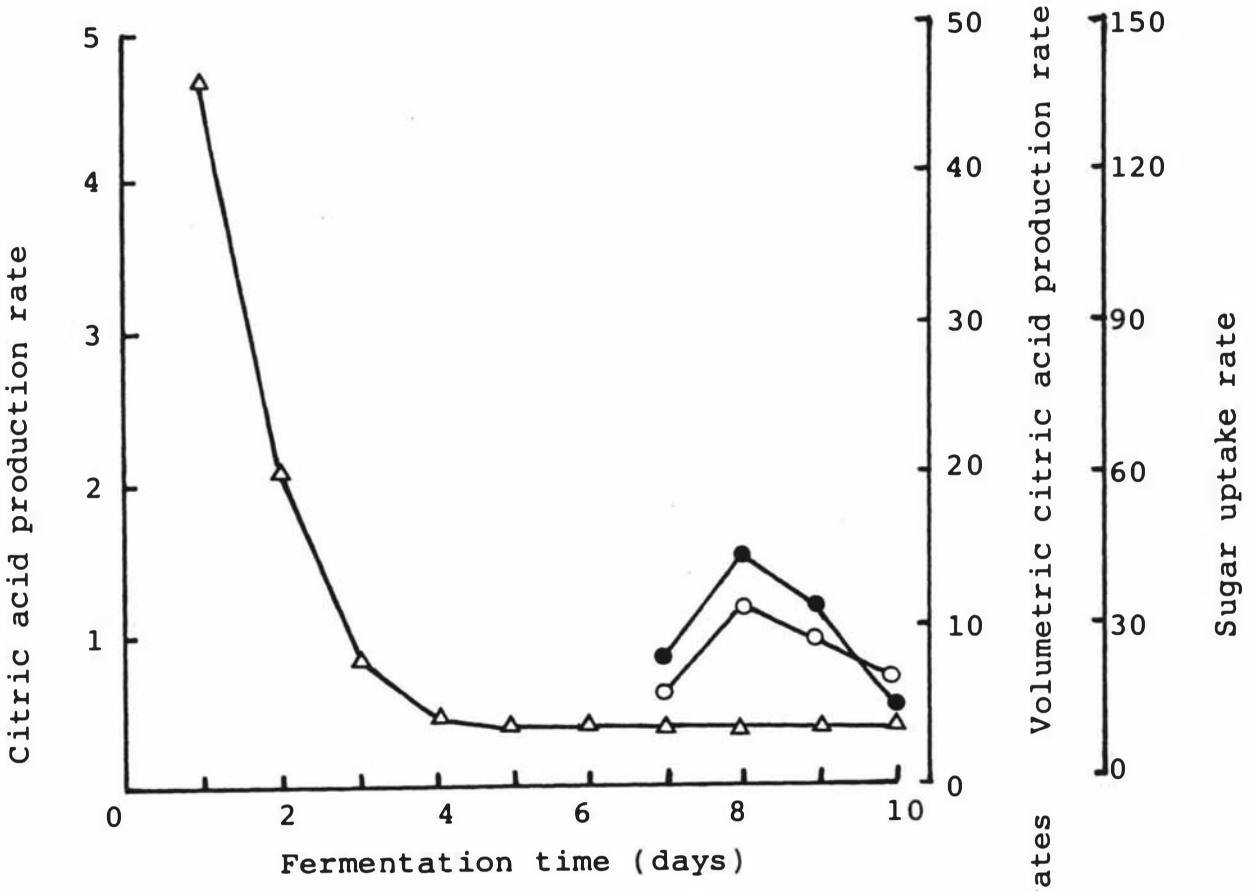


Figure 5.11 Growth rate, product formation rates and nutrient uptake rates under magnesium limitation



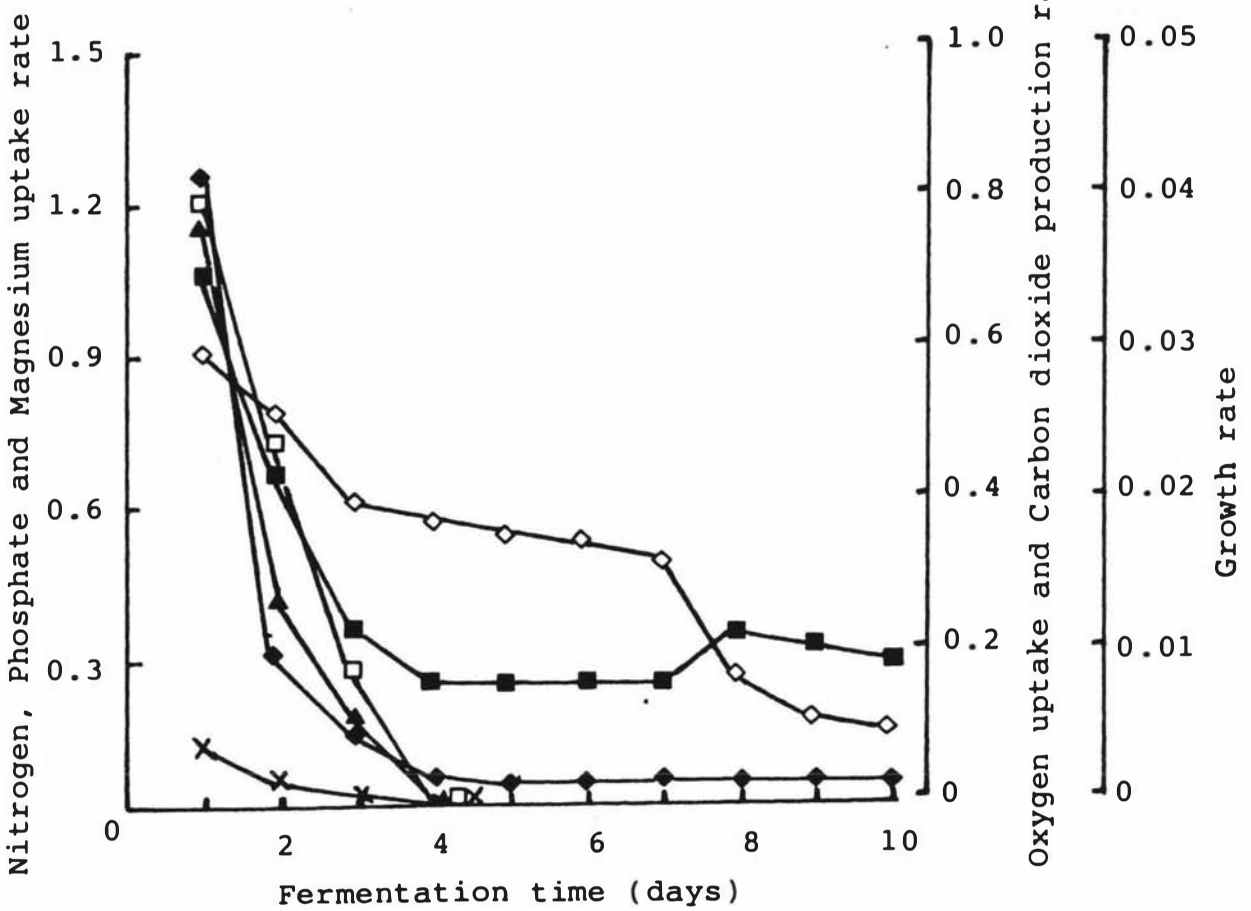
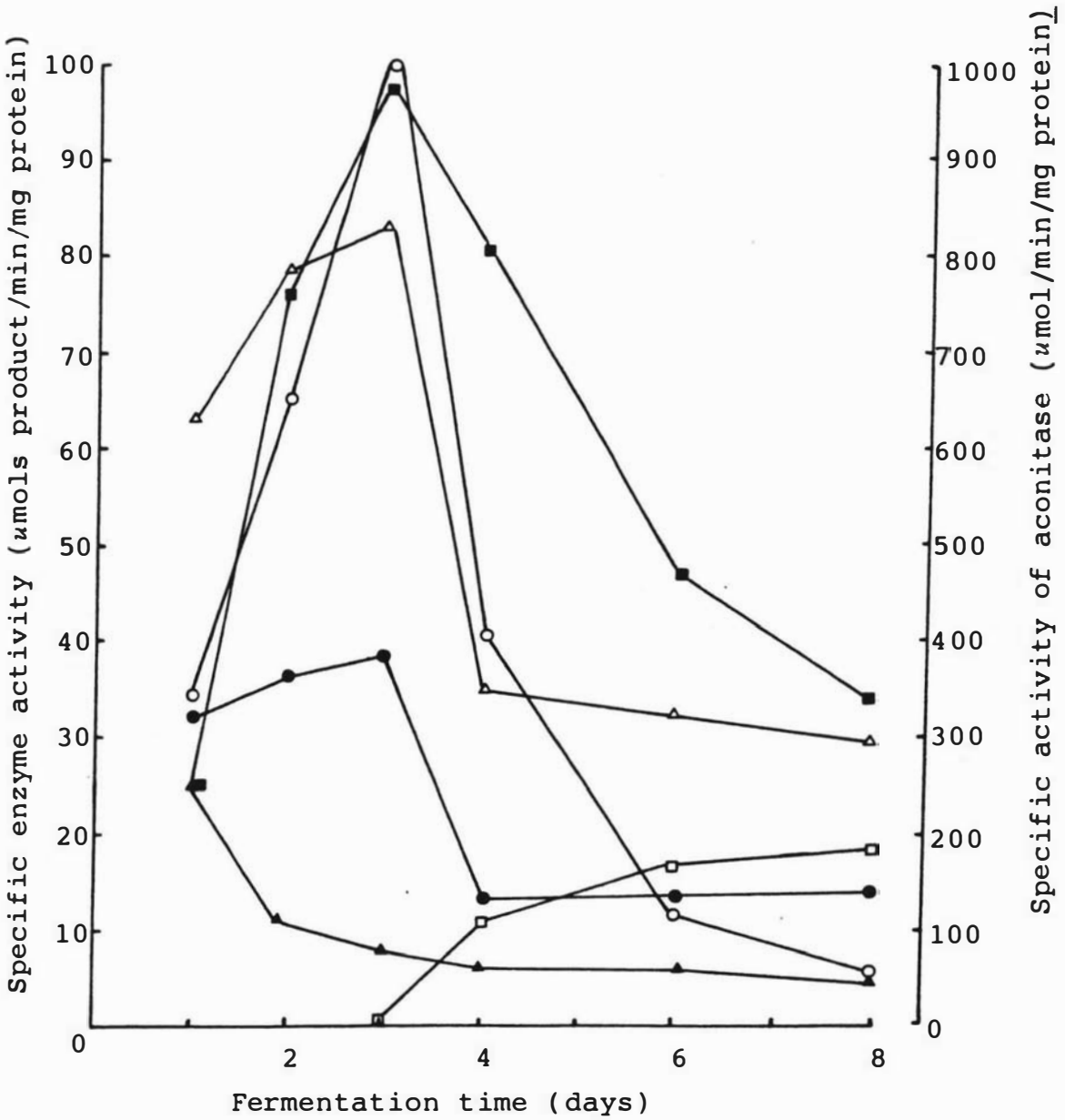


Table 5.3 The molar ratio of citric acid produced to oxygen used as calculated from the respective specific rates

Day of Fermentation	Ratio under nitrogen limitation	Ratio under phosphate limitation
1	9.1	51.8
2	0.9	0.1
3	9.4	4.6
4	3.8	49.1
5	4.6	61.9
6	7.1	94.3
7	7.6	16.9
8	4.0	76.1
9	6.8	250.0
10	7.9	460.0

Figure 5.12 The specific activities of some enzymes in a nitrogen-limited batch fermentation



(○, aconitase; ●, NAD-isocitrate dehydrogenase; △, NADP-isocitrate dehydrogenase; ▲, 2-oxoglutarate dehydrogenase; □, pyruvate carboxylase; ■, isocitrate lyase)

(1984), 2-oxoglutarate dehydrogenase activity was observed throughout the fermentation. However, this contradiction can be explained by the method of enzyme analysis used. Thus Hossain *et al.* (1984) initiated the reaction by the addition of enzyme, whereas in the present study, the reaction was initiated by the addition of the substrate.

During the other nutrient-limited fermentations, the enzyme assays were performed only on days 2 and 8. However, no useful information could be obtained from the results.

5.3.4 Discussion

Examination of the time course data (Figures 5.4 to 5.7) for the various nutrient limitations indicates that the major proportion of the citric acid produced was formed after exhaustion of the limiting nutrient. This is the prevalant interpretation in such recent reviews as those by Kapoor *et al.* (1982), Kristiansen *et al.* (1982) and Rohr *et al.* (1983). However, while this general interpretation was confirmed, it is evident that during the nitrogen- and sulphate-limited fermentations, the maximum specific citric acid production rate occurred prior to the exhaustion of the limiting nutrient, i.e. when active growth was still occurring, albeit slowly. It is not possible to compare directly these results with those of other authors, since so few rate data have been presented at, or around, the time of nutrient exhaustion. However the implication from the present data is that an optimum specific growth rate exists for citric acid production. Unfortunately, some deficiencies are apparent in the above experiments. In particular, the

volumetric citric acid production rates were rather low, reflecting the low level of biomass. Also, the fermentation did not proceed to completion. Thus, further experiments were performed in which higher levels of biomass were used (Section 5.4).

It was considered that the magnesium limitation was not worth pursuing further, since it was not conducive to citric acid production. It was also decided that because of the similarity of results between nitrogen- and sulphate-limited fermentations, the latter would not be further investigated. This decision was made on the basis that the citric acid yield under sulphate limitation was lower than that under nitrogen limitation. Also, both limitations both restrict protein synthesis and nitrogen contents of both biomass and fermentation liquors are markedly easier to monitor than the sulphate contents. It is also more likely that in the commercial situation, nitrogen would be the limiting nutrient rather than sulphate.

With regard to the enzyme analyses performed during the nitrogen-limited fermentation the results support the work of Hossain *et al.* (1984), with the exception of the 2-oxoglutarate dehydrogenase activity. Thus the concept of repression of this enzyme being responsible, at least in part, for citric acid accumulation must be modified to "partial repression" or "partial inhibition" as suggested by Meixner-Monori *et al.* (1985). Hossain *et al.* (1984) did not analyse isocitrate lyase during their experiments, but data have been reported by Ahmed *et al.* (1972). The present

results show that the enzyme is most active at the same time as aconitase and the isocitrate dehydrogenases, and thus may be the major means of providing oxaloacetate during rapid production and loss of citric acid from the cell. Pyruvate carboxylase appears to fulfill this function only after active mycelial growth has ceased. Overall, the present results do not contradict the view of Hossain *et al.* (1984) that the observed maximum activities of aconitase and the isocitrate dehydrogenases at the time of maximum specific citric acid production rate are the response of the cell to diminished intracellular levels of citric acid. Indeed, isocitrate lyase may be added to this list of enzymes.

An interesting feature of the phosphate-limited fermentation compared to the nitrogen- or sulphate-limited fermentations is the lowered oxygen demand of the former during citric acid production. If this effect can be verified under conditions of higher biomass (Section 5.4) it may have important implications for the power requirements of the fermentation. The biochemical explanation of the observed result is not clear, but it may be connected to the alternative mechanism of the non-phosphorylating re-oxidation of adenine nucleotides as suggested by Kubicek *et al.* (1980) (Section 2.5). Under conditions of phosphate deficiency, oxidative phosphorylation is restricted and the alternative mechanism, which requires a high DOT but uses substrate-level phosphorylation, is used for nucleotide reoxidation. However, this remains speculative.

5.4 LIMITATION OF NUTRIENT NITROGEN, PHOSPHATE AND DOUBLE NITROGEN/PHOSPHATE AT HIGH LEVELS OF BIOMASS

In the previous section, the effects of various nutrient limitations on citric acid production were investigated. However, the experiments were performed at relatively low levels of biomass with the result that the volumetric citric acid production rates were rather low and the fermentation did not go to completion. The intention now was to repeat the experiments involving nitrogen and phosphate limitation, using higher levels of biomass to correct these defficiencies.

Kristiansen *et al.* (1982) reported that in a phosphate-limited fermentation, the amount of excess nitrogen in the medium should be as low as possible, but no data were provided. To investigate this claim, experiments were performed to determine the effect on citric acid production of various levels of excess nitrogen in a phosphate-limited medium. The fermentations were performed in shake-flask, using the medium of Kristiansen and Charley (1981) (Table 3.2), modified as follows: KH_2PO_4 (0.1 g/l) was used, to give a PO_4^{3-} concentration of 0.07 g/l , while the nitrogen concentrations investigated were 0.25 g/l, 0.315 g/l 0.42g/l, 0.525 g/l and 0.63 g/l obtained from $(NH_4)_2SO_4$ concentrations of 1.2 g/l, 1.5 g/l, 2.0 g/l, 2.5 g/l and 3.0 g/l, respectively.

The results are shown in Figures 5.13, 5.14 and 5.15 for biomass, citric acid production and yield, and sugar utilisation, respectively. No major effect on biomass production was observed. However, there was a significant

effect on citric acid production and yield, in that the lower the nitrogen excess, the higher the citric acid production and yield. For the three lowest levels of nitrogen excess, the sugar utilisations were similar, but with the two largest excess nitrogen levels, markedly more sugar was used and was probably converted to carbon dioxide. Extrapolating from the data, it appears that the best situation, both for improvement of citric acid production and on purely economic grounds, would be a double nitrogen/phosphate limitation. Figure 5.16 shows the residual nitrogen concentrations in the fermentation liquor.

Experiments were now performed in fermenter culture to investigate citric acid production under conditions of nitrogen, phosphate and double nitrogen/phosphate limitation. The operating conditions were as described in Section 3.5.3, with the agitation and aeration rates set at 250 rpm and 0.8 vvm, respectively. The media used were based on that of Kristiansen and Charley (1981) (Table 3.2) modified as follows: for nitrogen limitation, no modification; for phosphate limitation, KH_2PO_4 at 0.2 g/l, giving 146 mg/l as PO_4^{3-} and $(NH_4)_2SO_4$ at 2.2 g/l giving 460 mg/l as N to allow an excess of 10% of the initial nitrogen concentration; and for the double nitrogen/phosphate limitation KH_2PO_4 at 0.2 g/l (146 mg/l as PO_4^{3-}) and $(NH_4)_2SO_4$ at 2.0 g/l (420 mg/l as N) were used. All other medium constituents were as in Table 3.2. The fermentation period was extended to 14 days to allow the process to go to completion.

Figure 5.13 The effect on biomass of various levels of excess nutrient nitrogen in phosphate-limited culture

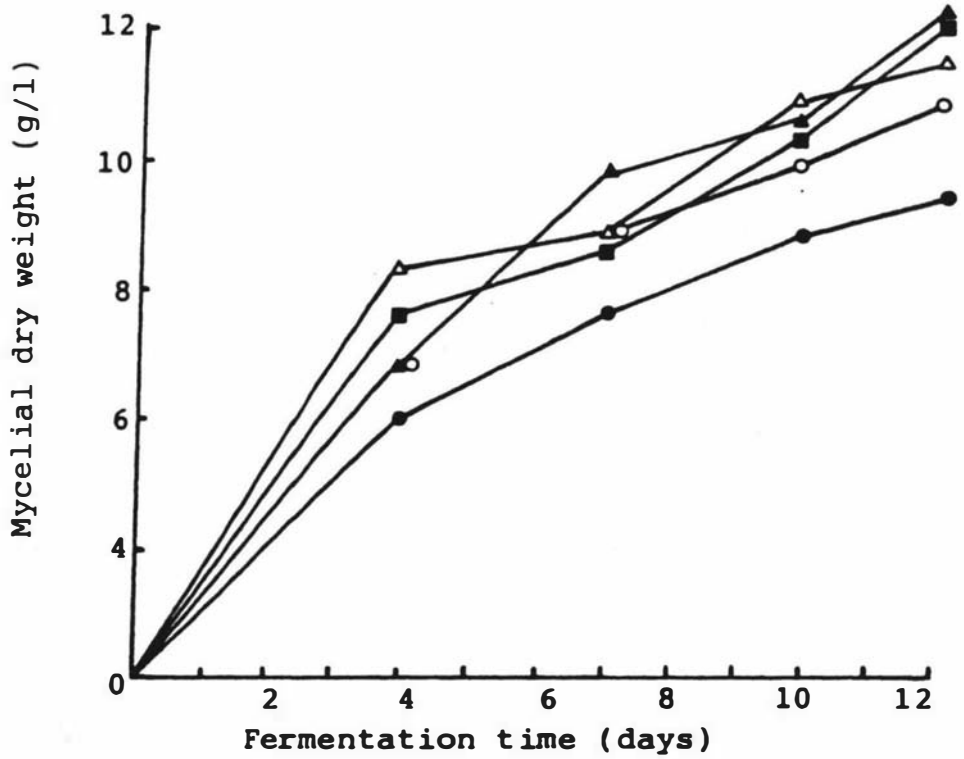
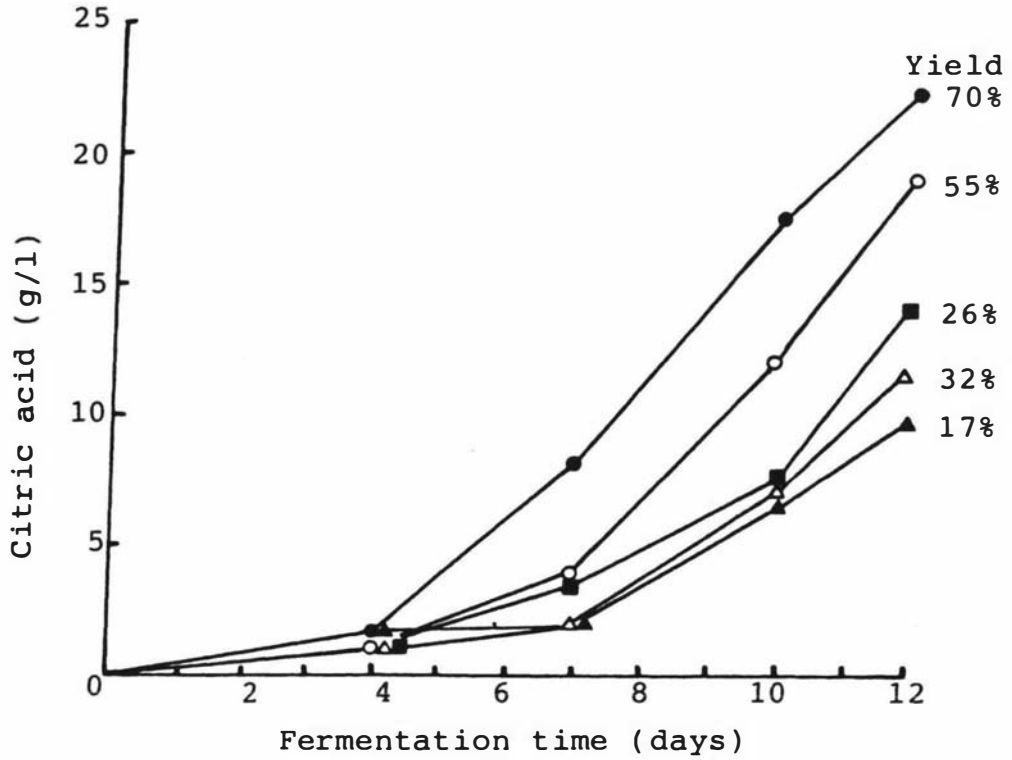


Figure 5.14 The effect on citric acid production and yield of various levels of excess nutrient nitrogen in phosphate-limited culture



(In g/l $(NH_2)_4SO_4$: ●, 1.2; ○, 1.5; ▲, 2.0; △, 2.5; ■, 3.0)

Figure 5.15 The effect on sugar utilisation of various levels of excess nutrient nitrogen in phosphate-limited culture

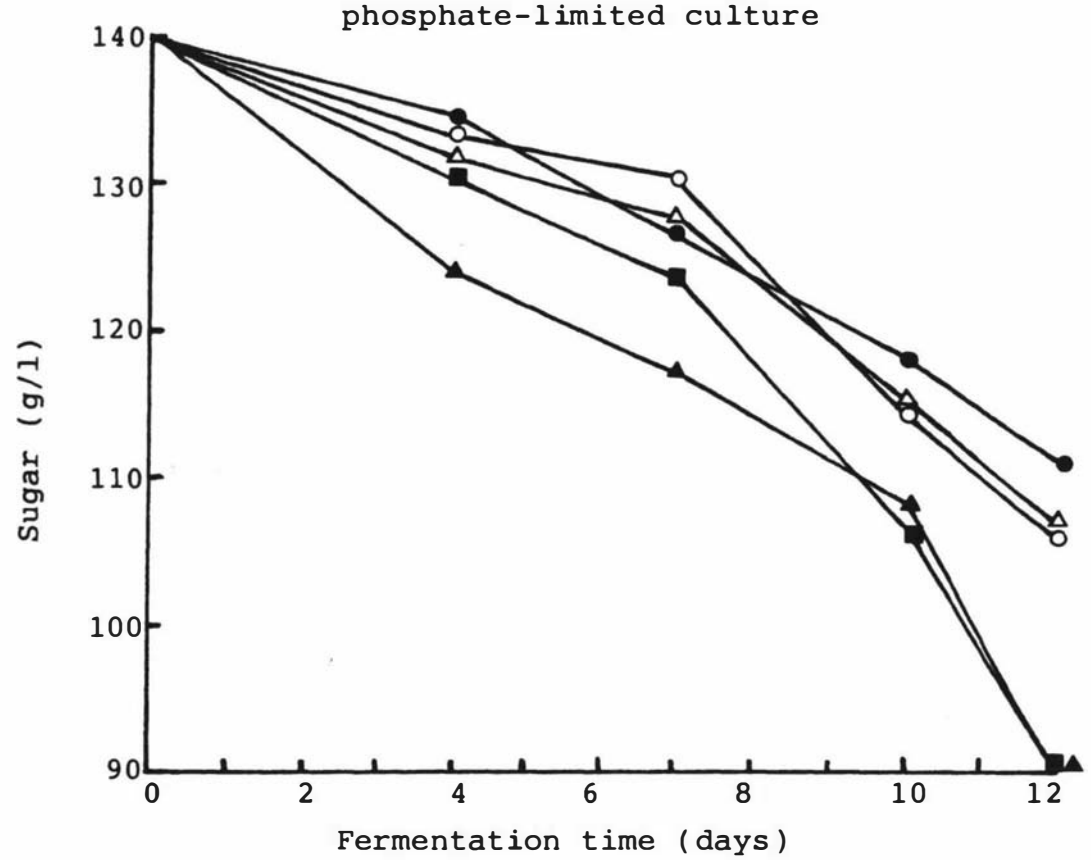
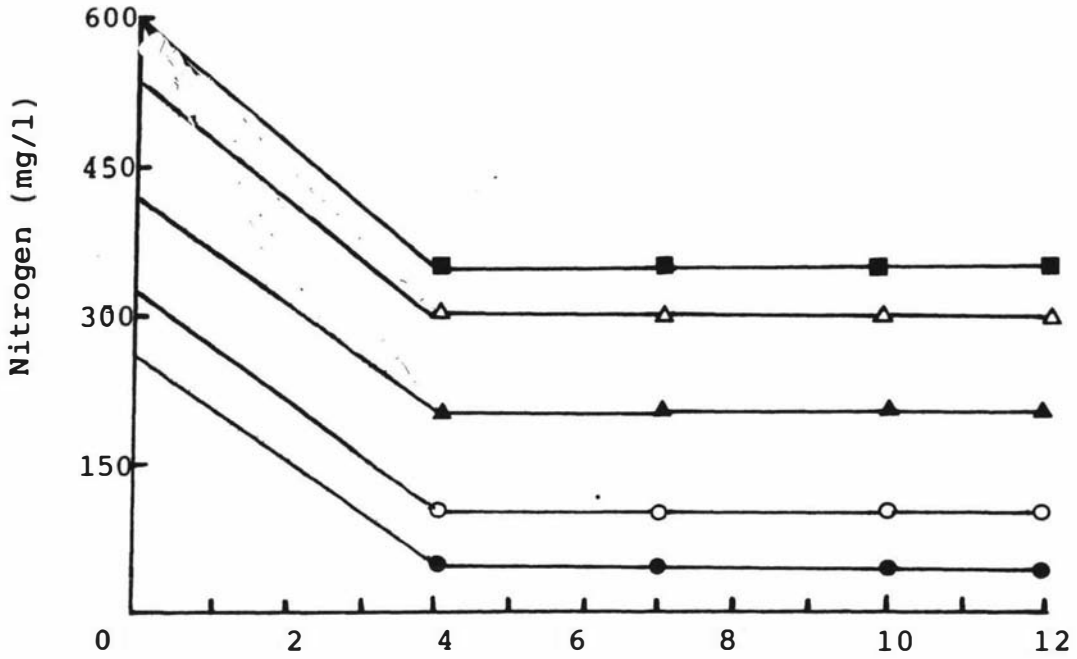


Figure 5.16 The levels of excess nutrient nitrogen in phosphate-limited culture



(Symbols as for Figure 5.13)

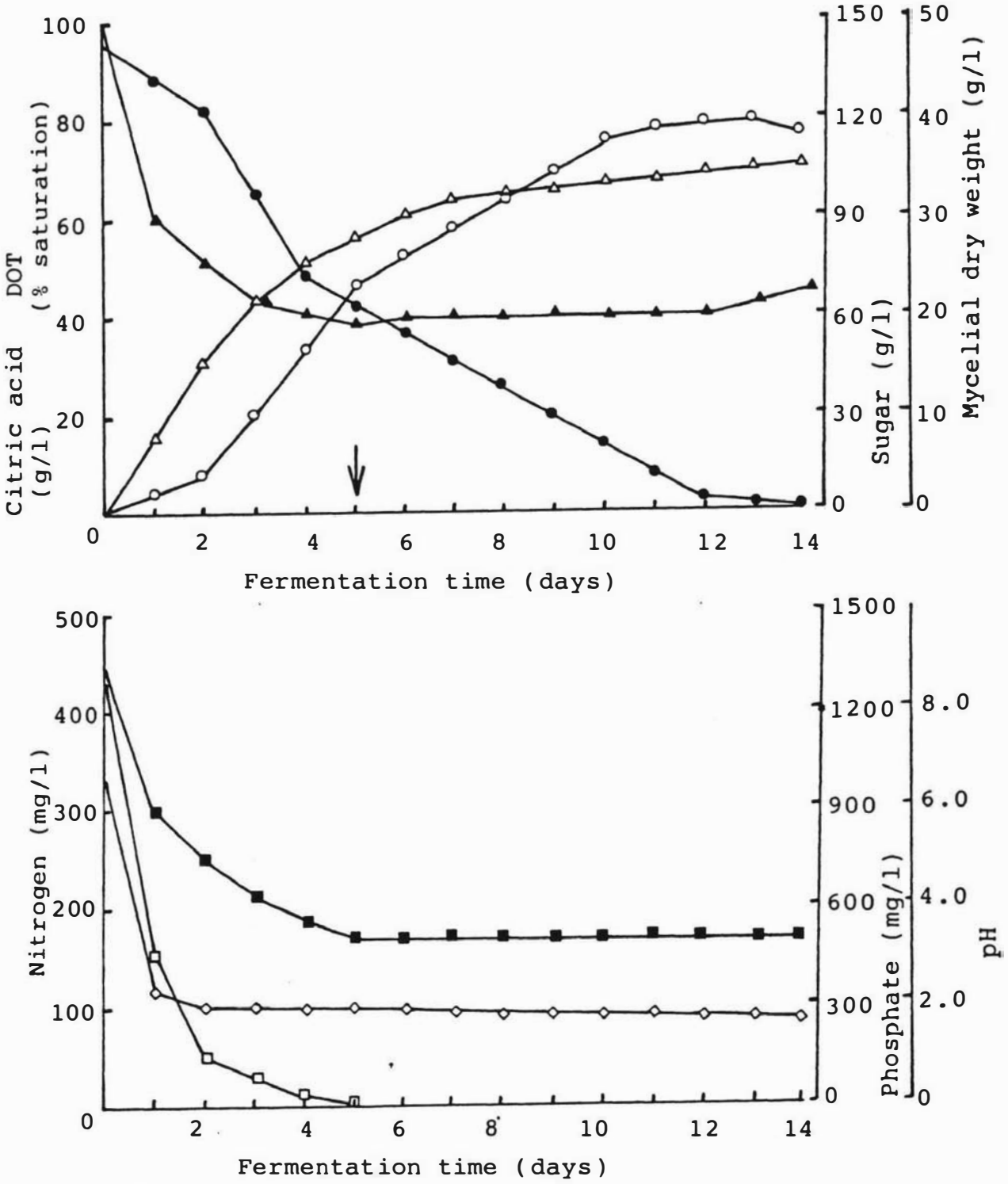
5.4.1 Time Course of Fermentations

The time course of a nitrogen-limited fermentation is shown in Figure 5.17. Nitrogen was exhausted by day 5 and subsequent to this exhaustion, no further phosphate uptake occurred. The phosphate concentration in the liquor at this time was 550 mg/l. Citric acid production commenced after day 1, prior to nitrogen exhaustion. The DOT was 40% of saturation for most of the fermentation period. The sugar was exhausted by day 14, showing that the fermentation went to completion. At this time there was 35 g/l biomass and a final citric acid concentration of 79 g/l, representing a yield of 56% based on sugar used (Table 5.4).

Figure 5.18 shows the time course of a phosphate-limited fermentation. The phosphate was exhausted by day 5, when the liquor nitrogen concentration was 50 mg/l. There was no further uptake of nitrogen after phosphate exhaustion. Citric acid production commenced on day 2, prior to phosphate exhaustion. The fermentation did not go to completion, with 50 g/l sugar remaining at day 14, i.e. approximately 33% of the initial sugar concentration. The final citric acid concentration of 40 g/l on day 14 represents a yield of 44% based on sugar used. The biomass concentration (24 g/l) was less than that observed in the nitrogen-limited fermentation (Table 5.4). The DOT was 50% of saturation or higher during the fermentation.

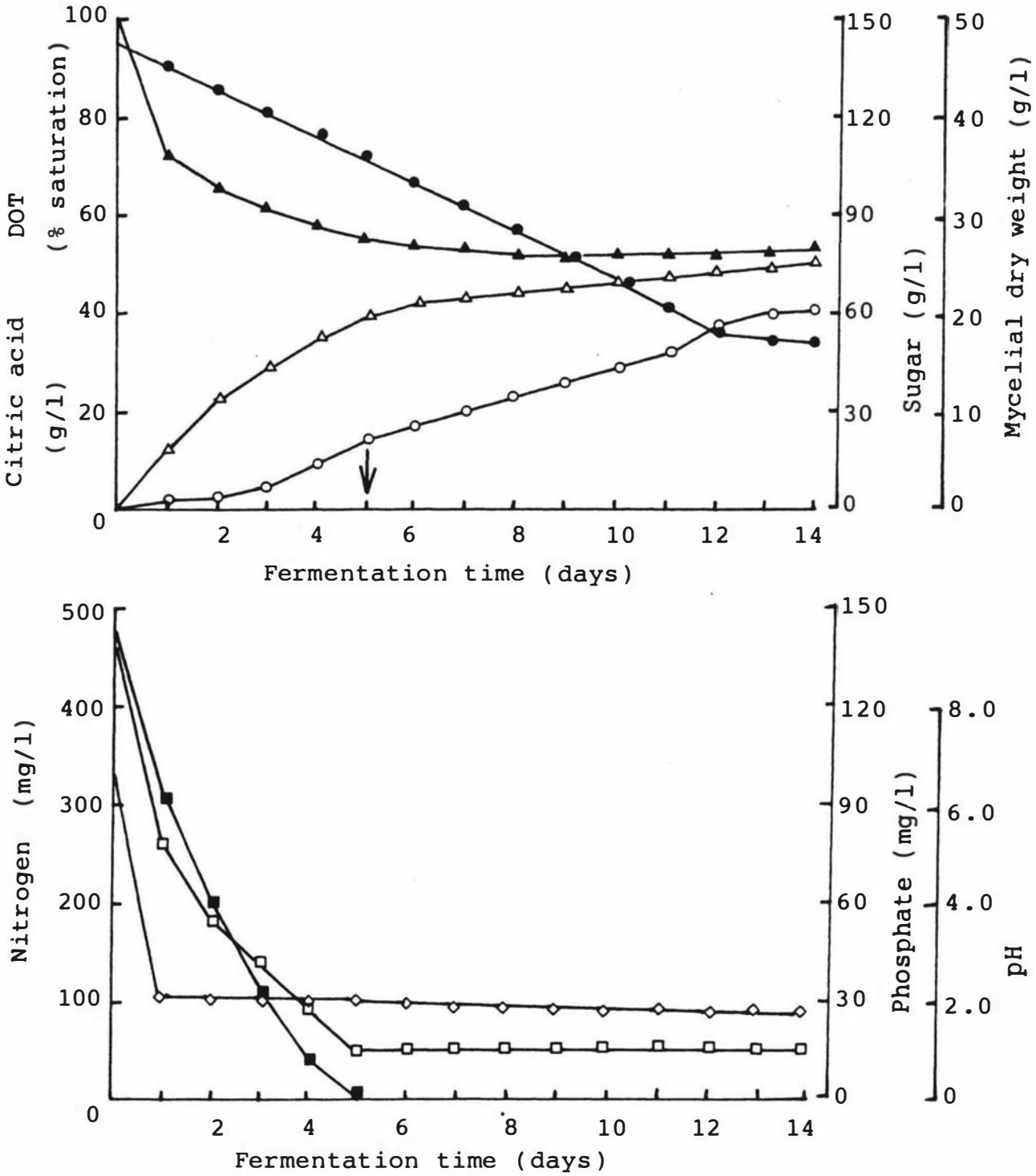
The double nitrogen/phosphate-limited fermentation was performed and the time course is shown in Figure 5.19. This fermentation closely resembled that described for the

Figure 5.17 Time course of a nitrogen-limited batch fermentation with high levels of biomass



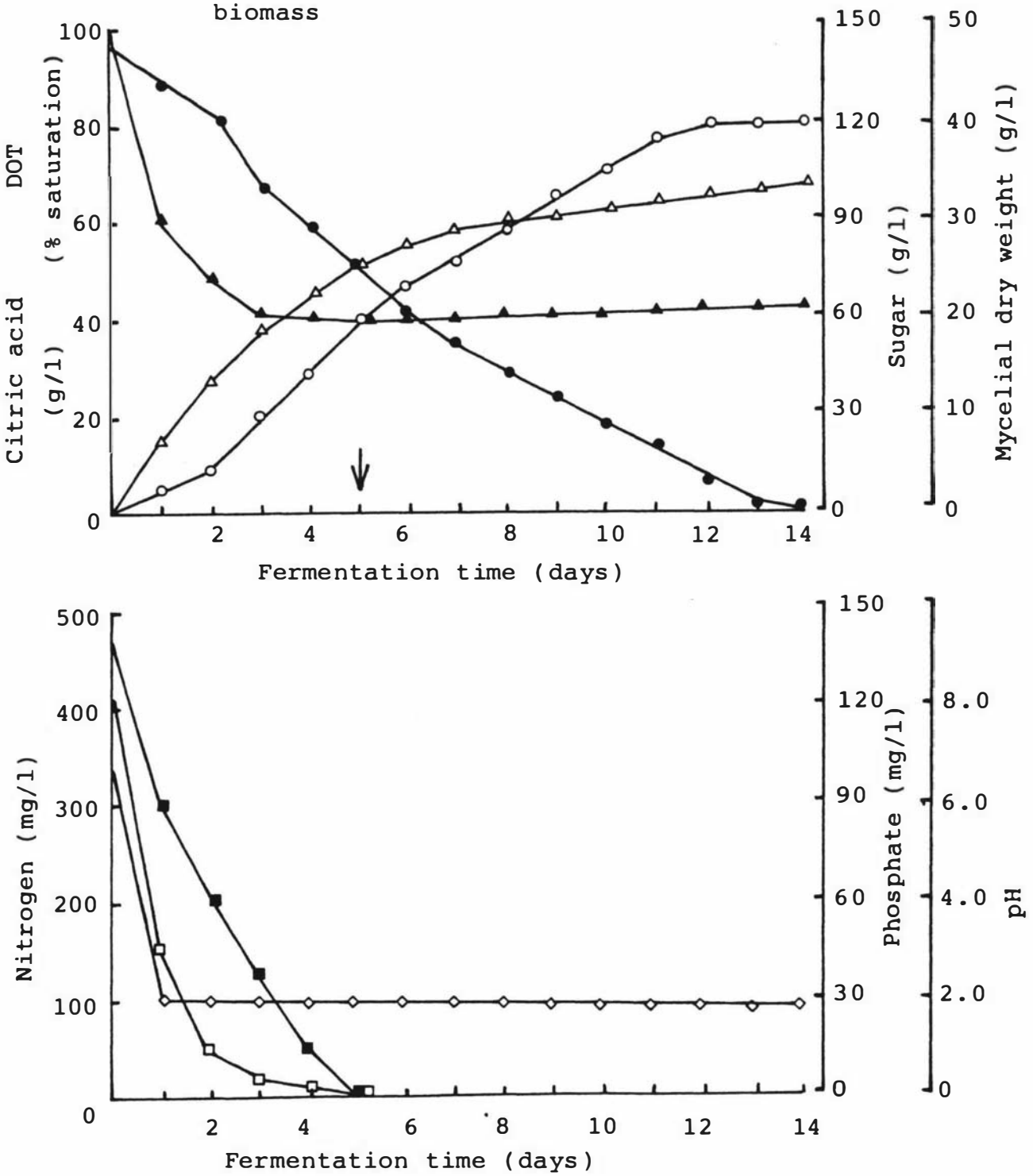
(○, citric acid; ●, sugar; △, mycelial dry weight; ▲, DOT; □, nitrogen; ■, phosphate; ◇, pH; ⇓, limiting nutrient exhausted)

Figure 5.18 The time course of a phosphate-limited batch fermentation with high levels of biomass



(○, citric acid; ●, sugar; △, mycelial dry weight; ▲, DOT; □, nitrogen; ■, phosphate; ◇, pH; ⇓, limiting nutrient exhausted)

Figure 5.19 The time course of a double nitrogen/phosphate-limited batch fermentation with high levels of biomass



(○, citric acid; ●, sugar; △, mycelial dry weight; ▲, DOT; □, nitrogen; ■, phosphate; ◇, pH; ⇓, limiting nutrient exhausted)

Table 5.4 Summary of different nutrient-limited fermentations at high biomass levels

Limiting nutrient	Biomass (g/l)	Sugar used (g/l)	Citric Acid (g/l)	Citric Acid Yield (%)
N	35	140	79	56
PO_4^{3-}	24	90	40	44
N and PO_4^{3-}	34	140	80	57

Results are expressed after 14 days of fermentation.

There was no residual limiting nutrient in the fermentation liquor after day 5.

nitrogen-limited fermentation (Figure 5.17), with the exception that both nitrogen and phosphate were exhausted by day 5. The final citric acid concentration of 80 g/l, representing a yield of 57% based on sugar used, and the biomass concentration of 34 g/l on day 14 (Table 5.4) were remarkably similar to the values for nitrogen limitation.

From these results, it is clear that the nitrogen-limited fermentation was superior to the phosphate-limited fermentation in terms of citric acid production and yields. The double nitrogen/phosphate limitation closely resembled the nitrogen-limited fermentation.

The increase in biomass observed after the exhaustion of the limiting nutrients was probably due to the production of storage carbohydrate by the organism.

5.4.2 Rates of Growth, Product Formation and Nutrient Uptake

From the data presented in Section 5.4.1, it is possible to calculate rates of citric acid and carbon dioxide production and the uptake rates of nitrogen, phosphate, sugar and oxygen. Figure 5.20 shows the rate data for the nitrogen-limited fermentation. The maximum citric acid production rates, both volumetric and specific, were observed on day 3, i.e. prior to nitrogen exhaustion on day 5, at values of 690 mg/1.h and 35 mg/gDW.h, respectively. As expected, the volumetric rates were higher than those observed under conditions of low biomass levels (Figure 5.4). The maximum specific sugar uptake rate coincided with the citric acid production rate maxima. The specific growth rate at this point was 0.012 h^{-1} and the specific nitrogen

uptake rate was 0.13 mg/gDW.h. The specific oxygen uptake rate and carbon dioxide production rates were 1.0 and 0.3 mM/gDW.h, respectively.

The rate data for the phosphate-limited fermentation are presented in Figure 5.21. The maximum citric acid production rates, both volumetric and specific, occurred on day 3, prior to phosphate exhaustion on day 5, at values of 150 mg/l.h and 25 mg/gDW.h, respectively. In addition, there was a second peak of citric acid production rates, on day 10. The maxima observed were less than those under nitrogen-limited conditions. There was no peak of specific sugar uptake rate. The specific growth rate on day 3 was 0.013 h^{-1}. During the latter part of the fermentation, the specific oxygen uptake rate was significantly lower than that observed for the nitrogen limitation (Figure 5.20).

The data presented in Figure 5.22 are those of the double nitrogen/phosphate limitation and are remarkably similar to those of the nitrogen limitation (Figure 5.20). This invites the suggestion that a double nitrogen/phosphate limitation is in effect a nitrogen limitation.

The difference in oxygen requirements between nitrogen- and phosphate-limited fermentation observed at low biomass levels was again observed at the high biomass level (Table 5.5). However, the difference was not so marked.

5.4.3 Discussion

The main feature of these results is that the maximum citric acid production rates, both volumetric and specific,

For rate data shown in Figures 5.20, 5.21 and 5.22, the following symbols were used:

- ○ , volumetric citric acid production rate expressed as mg/l.h
- ● , specific citric acid production rate expressed as mg/gDW.h
- △ , specific sugar uptake rate expressed as mg/gDW.h
- ▲ , specific nitrogen uptake rate expressed as mg/gDW.h
- □ , specific phosphate uptake rate expressed as mg/gDW.h
- ■ , specific oxygen uptake rate expressed as mmol/gDW.h
- ◇, specific carbon dioxide production rate expressed as mmol/gDW.h
- ◆, specific growth rate expressed as h^{-1}

All axes, unless otherwise specified, represent the specific product formation or nutrient uptake rates.

Figure 5.20 Growth rate, product formation rates and nutrient uptake rates during a nitrogen-limited batch fermentation at high biomass level

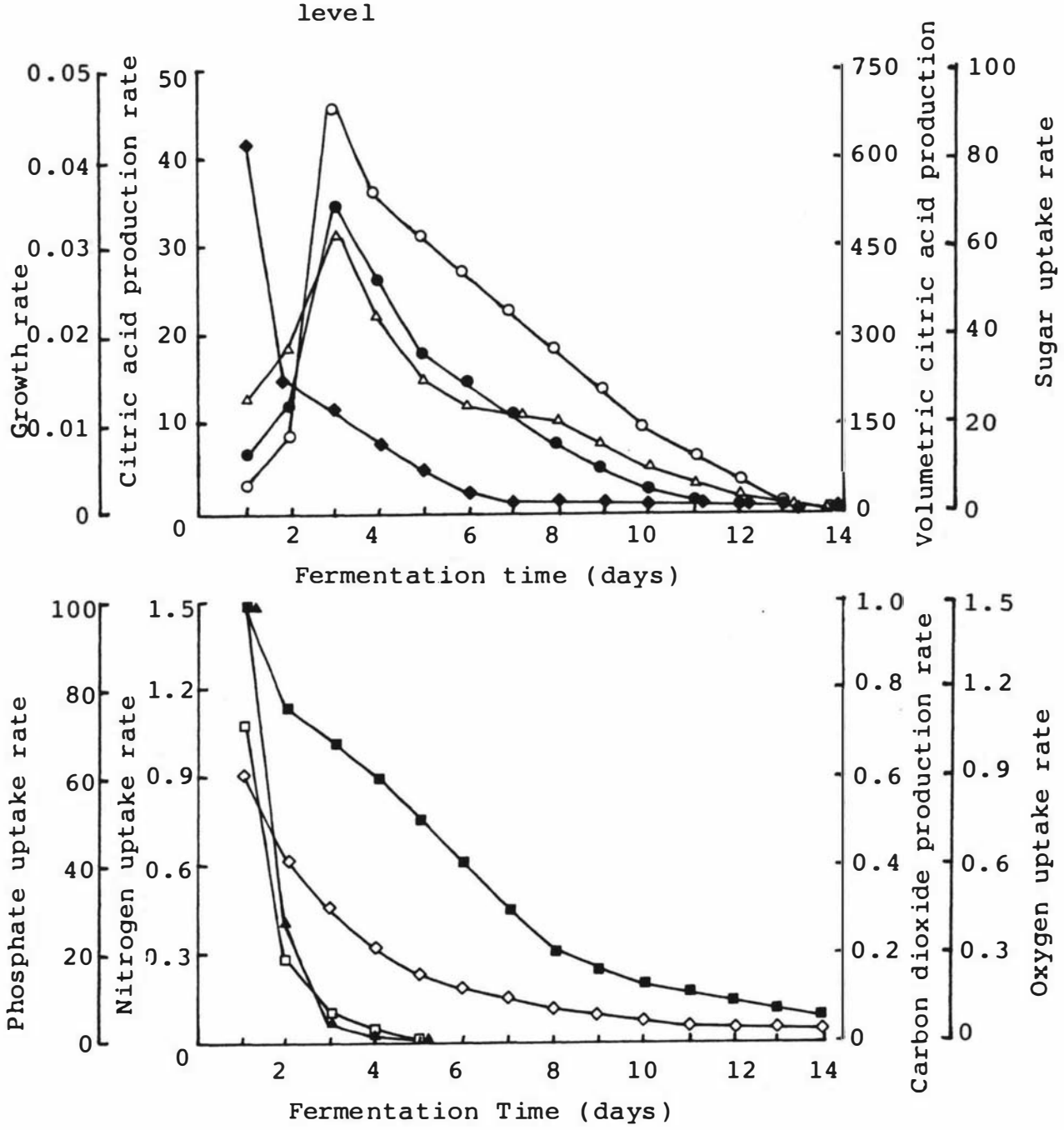


Figure 5.21 Growth rate, product formation rates and nutrient uptake rates during a phosphate-limited batch fermentation at high biomass level

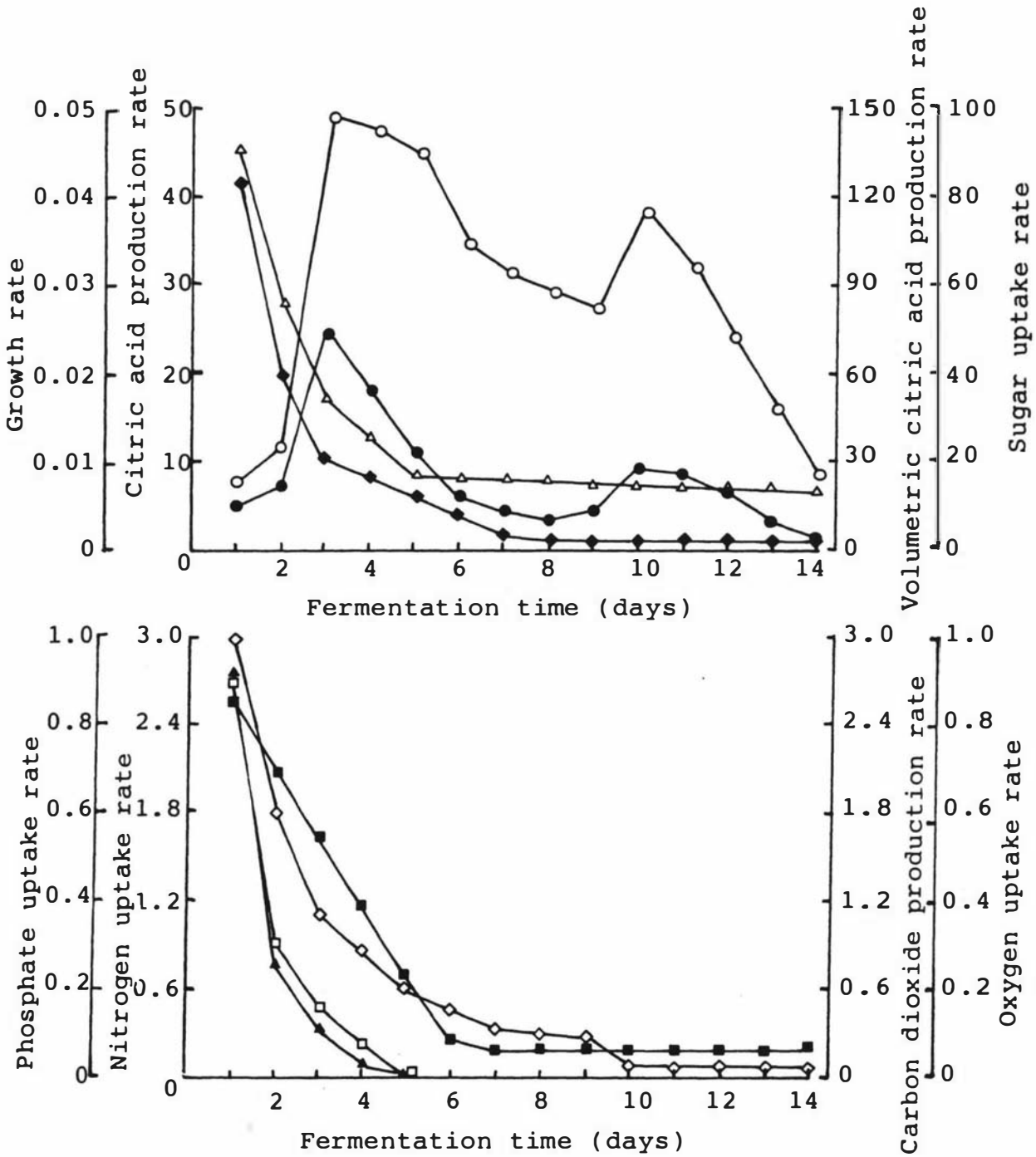


Figure 5.22 Growth rate, product formation rates and nutrient uptake rates during a double nitrogen/phosphate-limited batch fermentation at high biomass level

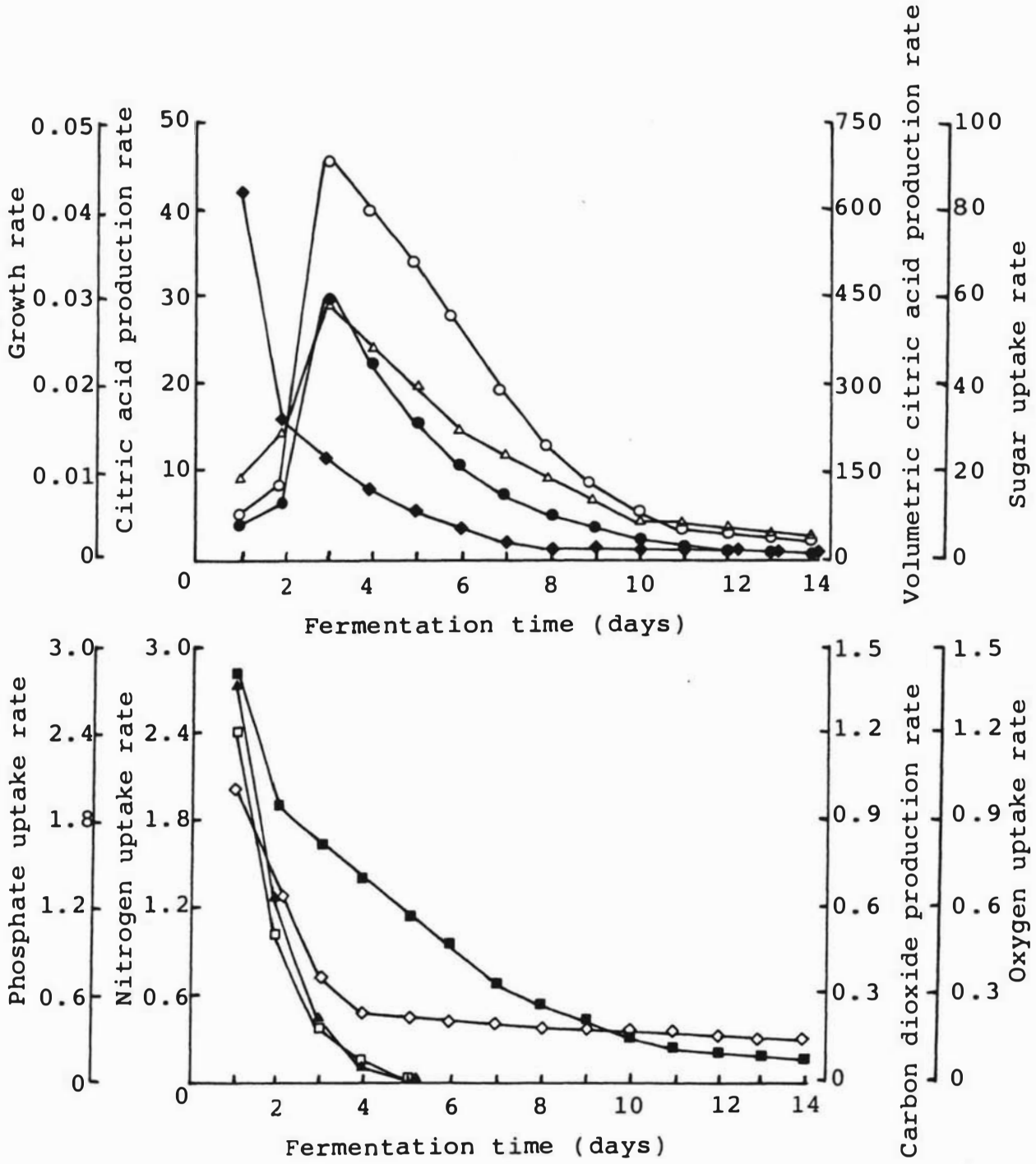


Table 5.5 The molar ratio of citric acid produced to oxygen consumed at high biomass levels, calculated using the respective specific rates

Day of fermentation	During N limitation	During PO_4^{3-} limitation	During dual limitation
1	1.5	3.4	1.4
2	3.1	2.2	3.0
3	12.0	11.8	11.2
4	10.1	8.5	9.4
5	7.7	13.0	7.0
6	9.3	12.5	8.0
7	7.3	17.0	6.5
8	13.1	13.0	11.0
9	11.0	25.0	9.0
10	10.5	45.2	9.0
11	10.5	41.4	6.0
12	2.5	29.3	2.0
13	2.5	5.4	1.0
14	1.0	5.4	0.8

occurred prior to the exhaustion of the limiting nutrient from the growth medium, i.e. when the specific growth rate was positive and not after cessation of active growth, as is often stated in the literature. This was particularly marked in the case of the nitrogen-limited fermentation.

The specific growth rate during a batch fermentation is generally calculated from the increase in biomass, usually measured as mycelial dry weight, over a given period of time. It is assumed that the increase in mycelial dry weight is due to the growth of the organism. However, if the growth-limiting nutrient is approaching exhaustion, or has been exhausted from the medium, any increase in mycelial dry weight may not be due to active growth. Further, because of the rapid decline in growth rate as the limiting nutrient approaches exhaustion, accurate measurements of the specific growth rate are not possible. For these reasons, it was decided that a thorough examination of the effect of specific growth rate on citric acid production was warranted and this required a study in chemostat culture. A continuous culture chemostat is a useful research tool as it allows a study of the effect of changing one parameter, e.g. growth rate, while all other parameters are maintained constant.

If, as seems probable at this stage, an optimum specific growth rate exists for citric acid production, then it should be possible to maintain the maximum citric acid production rate for extended time periods during a fermentation process. This can be achieved in both chemostat and continuous fed-batch culture, as in both techniques specific growth rates less than the maximum can be

maintained constant.

With regard to the phosphate-limited fermentation, at this stage there appears to be little advantage in limiting this nutrient when compared with the nitrogen-limited fermentation. The difference in oxygen uptake observed when the fermentations were performed at low biomass levels was not so apparent at the higher levels of biomass. Further, the results show that in a phosphate-limited fermentation the level of excess nitrogen remaining after phosphate exhaustion has an effect on citric acid production and yields. On economic grounds, it would be preferable to have no excess nutrients present after the required biomass concentration has been attained. The present results show that when nitrogen and phosphate are used together as limiting nutrients, the situation resembles nitrogen rather than phosphate limitation. To investigate these different nutrient limitations more fully, experiments in chemostat culture are required.

The reason why excess nitrogen in a phosphate-limited culture should have a detrimental effect on citric acid production is not clear. One possible explanation is that the nutrient NH_4^+ ion may exert a form of catabolite repression. From Figures 5.20, 5.21 and 5.22, it was observed that the nitrogen uptake rate at the time of maximum citric acid production rate was approximately 0.1 mg/gDW.h. It has been suggested above that an optimum specific growth rate exists for citric acid production. An optimum nitrogen uptake rate may also exist.

5.5 CONCLUSIONS

The major conclusion that can be drawn from this study in batch fermentation is that citric acid production can readily occur under conditions of nitrogen, phosphate or sulphate limitation. Further, the maximum citric acid production rate occurs prior to the exhaustion of the limiting nutrient from the medium, suggesting that an optimum specific growth rate exists for citric acid accumulation. However, due to the diffulties in measuring growth rates accurately in batch fermentations, studies in chemostat culture are required to verify this effect.

CHAPTER 6

THE PRODUCTION OF CITRIC ACID IN CHEMOSTAT CONTINUOUS CULTURE

6.1 INTRODUCTION

During a chemostat fermentation, the organism can be maintained in a constant metabolic state, thus allowing accurate determination of the effects of different environmental variables. The results described in the previous chapter indicated that there may be an optimum specific growth rate for citric acid production. Also in the previous chapter, it was stated that it was difficult to compare accurately the fermentations operated under different nutrient limitations because of the different DOT values that were maintained. Thus, it was decided to perform a study in chemostat culture to determine the effects of two variables, specific growth rate and DOT, on citric acid production, and if possible determine the optimum conditions for these two variables. The limiting nutrients used to obtain steady states were nitrogen and phosphate. Subsequently, a combined nitrogen/phosphate double limitation was performed.

6.2 NITROGEN-LIMITED FERMENTATIONS

The design for the experiment was a 3^2 factorial experiment using three values of the two variables, specific growth rate and DOT. The centre point chosen for the

specific growth rate was the optimum value reported by Kristiansen and Charley (1981) of 0.075 h^{-1}, while the other values used were 0.045 h^{-1} and 0.12 h^{-1}. Ideally the latter two values should be equidistant from the centre point, but the selected values were used because of the limitations of the equipment. The centre point chosen for the DOT was 50% of saturation, while the other two values were 25% and 75% of saturation. These values were used because Kubicek *et al.* (1980) reported a positive effect on citric acid production over this range. Three replicates of the centre point were performed to allow calculation of error. Use was made of coded variables (allocated as in Table 6.1) to assist in the statistical analysis of the data. These codes were used in the multivariate linear regression analysis, using the Minitab package (Copyright Pennsylvania State University) on the University main computer, to develop regression equations for the experimental data. The general model used had the form:

$$\hat{Y} = B_0 + B_1A + F_2B + B_3A.B + B_4A^2 + B_5B^2 + B_6A^2.B + B_7A.B^2 + B_8(A.B)^2$$

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

B = coefficients

A = coded variable for specific growth rate

B = coded variable for DOT

B_0 = constant or Y-intercept

It is important to note that multivariate linear regression gives an empirical model. The terms and coefficients are empirically related to, but do not necessarily determine the

Table 6.1 Allocation of coded variables to specific growth rate (u) and DOT

Run Order	u uncoded	DOT uncoded	u coded	DOT' coded
3	0.045	25	-1	-1
9	0.045	50	-1	0
2	0.045	75	-1	+1
6	0.075	25	0	-1
1, 5, 10	0.075	50	0	0
8	0.075	75	0	+1
4	0.12	25	+1	-1
11	0.12	50	+1	0
7	0.12	75	+1	+1

value of, the experimental variables.

For each steady state investigated, a new culture was established to avoid any problems associated with strain degeneration. The process was operated initially as a batch fermentation using the medium described in Table 3.2, adjusted to pH 6.5. After inoculation with a 24 hour-old shake-flask culture (10% (v/v) inoculum) (Section 3.5.7), the pH was allowed to drop naturally to pH 2.3 at which time the continuous feed was started using the medium described in Table 3.3, adjusted to pH 4.5. The aeration rate was adjusted to the 0.5 l/min (0.35 vvm) mark on the rotameter while the agitation speed range was controlled as described in Section 3.5.5 to maintain a constant DOT value. In most cases the pH of the culture remained naturally at pH 2.0, but the automatic control was available if required. The culture was assumed to be in steady state after three residence times, and thereafter successive samples taken at least half a residence time apart showed no significant variation.

The steady state concentrations recorded for the various operating conditions are shown in Table 6.2 and the calculated rate data in Table 6.3. The theory of the chemostat culture predicts that biomass concentration is dependent on only the concentration of the growth-limiting nutrient, in this case nitrogen. However, the observed biomass concentrations, based on mycelial dry weight, varied considerably at different steady states. This was almost certainly due to accumulation of storage carbohydrate by the

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

DOT (% Satn)	μ (h^{-1})	Citric Acid (g/l)	Biomass (gDW/l)	(gN/l)	(gPO_4^{3-}/l)	% N in biomass	% PO_4^{3-} in biomass	Sugar Uptake[1] (g/l)	Citric Acid Yield (%)	PO_4^{3-} uptake[2] (g/l)
25	0.045	2.2	2.5	0.21	0.025	8.4	1.0	5.4	40.7	0.025
25	0.075	1.4	5.7	0.22	0.060	3.9	1.1	5.5	24.5	0.060
25	0.12	2.8	7.3	0.20	0.062	2.7	0.8	13.0	21.5	0.062
50	0.045	4.2	6.0	0.22	0.187	5.2	4.5	8.9	47.0	0.187
50	0.075	6.5	5.6	0.21	0.070	3.8	1.3	21.8	29.8	0.070
50	0.075	6.1	6.0	0.21	0.062	3.5	1.0	21.7	28.1	0.062
50	0.075	5.9	6.5	0.20	0.056	3.1	0.9	22.6	26.1	0.056
50	0.12	0.4	5.2	0.21	0.140	4.0	2.7	6.0	6.7	0.140
75	0.045	7.2	3.7	0.21	0.070	5.7	1.9	12.0	60.0	0.070
75	0.075	1.7	4.8	0.20	0.041	4.2	0.9	14.5	11.7	0.041
75	0.12	NIL	2.6	0.22	0.130	8.5	5.0	4.3	NIL	0.130

1 initial concentration = 50 g/l

2 initial concentration = 0.353 g/l

There was no residual nitrogen present at steady state (initial concentration 0.21 g/l expressed as nitrogen)

Table 6.3 Volumetric and specific rates of nutrient uptake and product formation at steady states in nitrogen-limited chemostat experiments.

DOT	u	Citric Acid				Sugar		
(% Satn)	(h^{-1})	(mg/l.h)	(mg/gDW.h)	(mg/gN.h)	(mg.gPO_4^{3-}.h)	(mg/1.h)	(mg/gDW.h)	(mg/gN.h)
25	0.045	99.0	40.0	471	3960	243	98	1160
25	0.075	105	18.4	477	1750	413	72	1880
25	0.12	336	46.0	1680	5400	1560	213	7800
50	0.045	190	31.5	860	1010	400	67	1820
50	0.075	488	87.1	2320	6960	1635	251	7800
50	0.075	458	76.3	2180	7630	1630	267	7750
50	0.075	443	71.4	2210	7900	1690	300	8070
50	0.12	48	9.2	229	343	270	52	1290
75	0.045	324	87.6	1540	4630	900	243	4290
75	0.075	128	26.6	640	3200	1090	227	5440
75	0.12	NIL	NIL	NIL	NIL	516	198	2440

DOT (% Satn)	u (h^{-1})	Nitrogen (mg/gDW.h)	Phosphate (mg/gDW.h)	Oxygen (mM/l.h)	Oxygen (mM/gDW.h)	Carbon Dioxide (mM/l.h)	Carbon Dioxide (mM/gDW.h)	Respiratory Quotient
25	0.045	3.8	0.45	5.5	2.2	1.6	0.6	0.3
25	0.075	2.9	0.8	1.3	0.8	1.3	0.2	0.8
25	0.12	3.3	1.0	10.5	1.4	ND	ND	ND
50	0.045	1.7	1.4	12.9	2.2	5.9	1.0	0.5
50	0.075	2.8	0.9	16.4	2.9	11.2	2.0	0.7
50	0.075	2.6	0.8	16.2	2.7	10.8	1.8	0.7
50	0.075	2.4	0.6	16.9	2.6	11.1	1.7	0.7
50	0.12	4.8	3.2	11.8	2.3	9.0	1.8	0.8
75	0.045	2.6	0.9	8.4	2.3	2.2	0.6	0.4
75	0.075	3.1	0.6	4.6	1.0	6.0	1.3	0.4
75	0.12	10.1	6.0	1.2	0.5	2.3	1.2	2.1

organism as indicated by the experimentally determined values for the biomass nitrogen content, which are equal to the uptake of nitrogen from the growth medium. Therefore, some of the specific rate data shown in Table 6.3 are based on mycelial nitrogen content, rather than on mycelial dry weight. Use of the mycelial phosphate content, estimated from the amount of phosphate consumed from the growth medium, is another means of expressing specific rate data.

It was not possible to generate data on trace metal uptake rates, because the concentrations present in the culture medium were higher than those in the feed medium. This was almost certainly caused by contamination from metal components within the fermenter.

Table 6.4 shows the full regression equations of models which fitted the data and which exhibited no significant lack of fit. Footnotes are used to indicate the coefficients found to be statistically significant using the T-test. Lack of fit tests on the models were performed using the Minitab routine. The regression equations which exhibited a significant lack of fit are shown in Appendix 3.

Using the correlation matrix facility of the Minitab Package, correlation coefficients were determined for all $\hat{Y}$ data with other $\hat{Y}$ data. Table 6.5 shows the correlation coefficients found to be greater than 0.5, which was used as a cut-off value. With the exception of sugar uptake rate, no specific nutrient uptake rates correlated with specific citric acid production rate, or citric acid yield, i.e. correlation coefficients were less than 0.5. However, the data clearly show a strong positive relationship between the

Table 6.4 Full Regression Models for Nitrogen-Limited Chemostat Cultures.

$\hat{Y}$	B_0	B_1	B_2	B_3	B_4	B_5	B_6	B_7	B_8
Volumetric citric acid production rate (mg/l.h)	475^a	-4.12	-61^c	-142^b	-359^a	-368^a	440^a	41.8	23.0
Specific citric acid production rate (mg/gDW.h)	73.7^a	-7.6	-9.6^c	-23.7^b	-51.4^a	-55.3^a	76.7^a	10.2^c	12.8^c
(mg/gN.h)	2347^a	28	-337	-970^c	-1720^b	-1826^a	2370^a	367^c	340
(mg/gPO_4^{3-}.h)	8450^a	2610^b	-286	-1212^c	-420	-7837^a	2894^b	558	2859^b
Citric acid yield	28.1^a	-1.2	-19.8^a	-12.5^b	-11.6^b	-0.9	12.4^b	2.3^c	-2.2^c
Volumetric sugar uptake rate (mg/l.h)	1703^a	86	138	-347^c	-880^b	-1205^a	1102^a	197^c	170
Specific sugar uptake rate (mg/gN.h)	7027^a	165	354	-2723^b	-2593^b	-4873^a	5056^a	1968^b	1160^c
Volumetric nitrogen uptake rate (mg/l.h)	18.3^a	0.1	10.6^a	3.1^c	3.0^c	1.05	-1.3	4.1^b	-1.0
Specific nitrogen uptake rate (mg/gDW.h)	2.8^a	0.6^c	1.4^b	2.9^a	-0.05	0.1	2.8^a	1.7^a	-0.5^c
Storage carbon yield based sugar utilised[1]	23.0^a	-9.5^b	19.8^a	-11.8^b	28.4^a	25.8^a	-54.5^a	-28.8^a	-7.4^c
Carbon dioxide yield based on sugar utilised	32.1^a	-4.9	-16^b	24.4^b	-14.1^b	17.1^b	-36.9^a	29.3^a	7.1^c

Statistical significance of coefficients are indicated thus: a = 2% level; b = 5% level; c = 10% level

1 assumed carbon content of biomass after nitrogen and phosphate contents were substracted from the total biomass

Table 6.5 Correlation coefficients of data from nitrogen-limited chemostat cultures

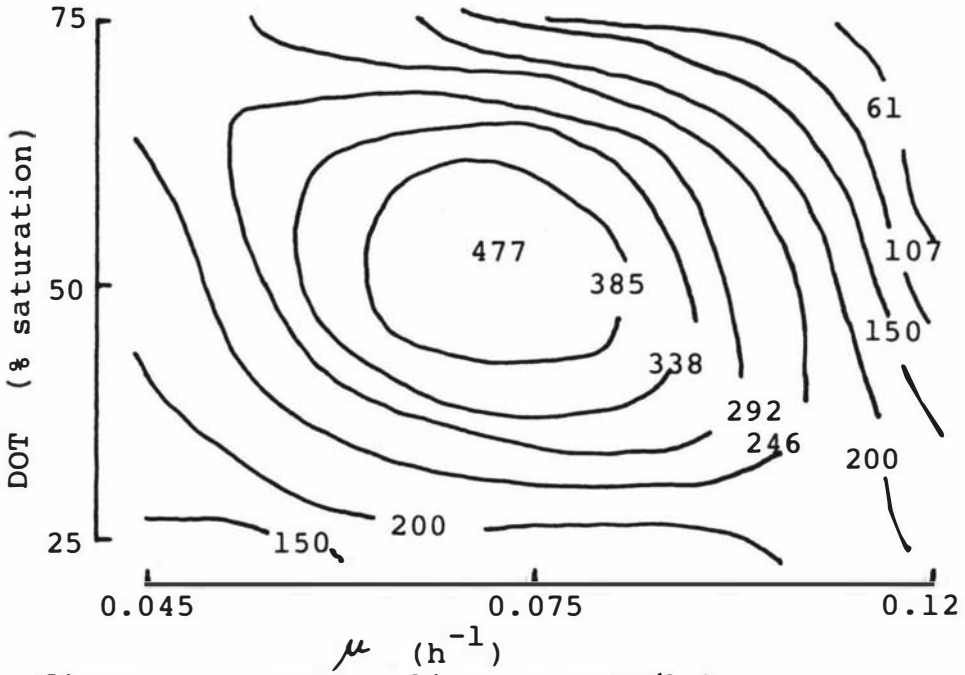
Data Set	Correlation Coefficient
Specific citric acid production rate (mg/gDW.h) - DOT	0.95
Citric acid yield based on sugar used - u	-0.83
Carbon dioxide yield based on sugar used - u	-0.52
Storage carbon yield based on sugar used - u	0.73
Specific citric acid production rate (mg/gDW.h) - Specific sugar uptake rate (mg/gDW.h)	0.64
Storage carbon yield based on sugar used - Specific sugar uptake rate (mg/gDW.h)	-0.57
Carbon dioxide yield based on sugar used - Specific sugar uptake rate (mg/gDW.h)	-0.59

specific citric acid production rate and the DOT value of the culture. A negative correlation of specific growth rate is observed with the citric acid yield and with the carbon dioxide yield. However, a positive correlation is observed with the storage carbon yield. In the case of the specific sugar uptake rate (which is dependent upon specific growth rate), a negative correlation is observed with both storage carbon yield and carbon dioxide yield. These data suggest that to obtain high citric acid production rates and yields, the DOT value of the culture should be high and the specific growth rate low.

In an attempt to determine the optimum specific growth rate and DOT values for maximum citric acid production rates and yields, the regression equations from Table 6.4 were used to predict response surfaces, constructed as 3-dimensional contour diagrams using a programmed IBM XT personal computer (Boag, pers. comm. 1985). Figure 6.1 shows the predicted volumetric citric acid production rate as an elongated dome with the apex at the centre point. Figure 6.2, which shows the predictions for the specific citric acid production rate based on total biomass (mycelial dry weight), also shows a maximum at the centre point, but exhibiting a ridge from DOT 75% of saturation/u = 0.045 h^{-1} to DOT 25% of saturation/u = 0.12 h^{-1}. If, however, the specific citric acid production rate is predicted based on mycelial nitrogen or phosphate contents, as shown in Figures 6.3 and 6.4 respectively, the overall shape of the contour surface does not alter greatly, but the maximum point is

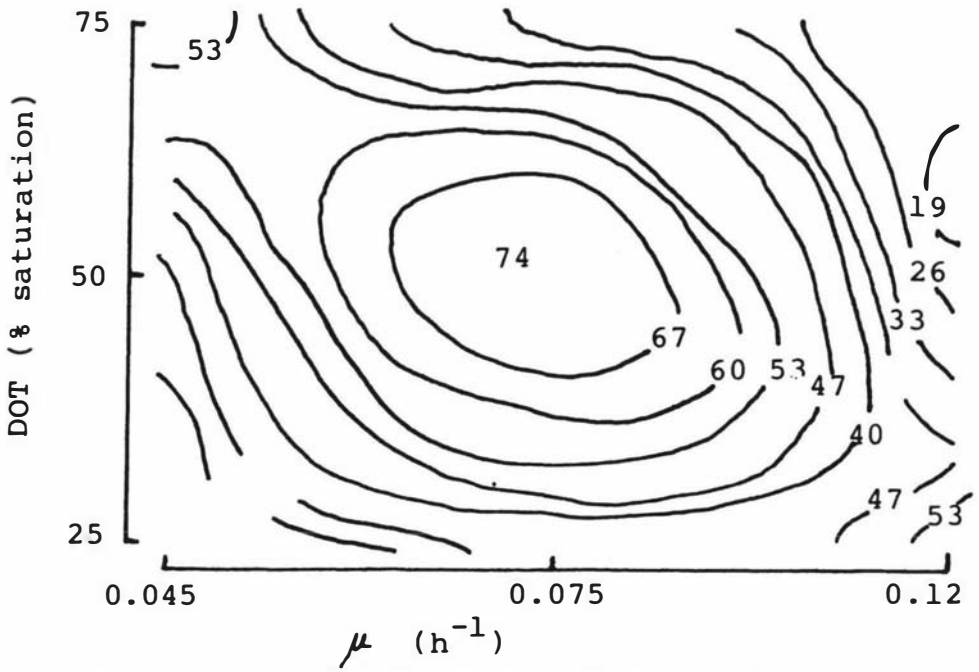
predicted to be at one of the corner points, i.e. DOT = 75% of saturation/u = 0.045 h^{-1}, rather than at the centre point. Figure 6.5 shows that the same corner point is predicted to give the greatest citric acid yield based on sugar used. In view of the apparent contradiction of the predicted optima in Figures 6.2, 6.3 and 6.4, the predictions were extrapolated outside the range of experimental data, using coded values of -1.5 and +1.5 for both variables. The predictions for specific citric acid production rates using total biomass (Figure 6.6) and biomass nitrogen content (Figure 6.7) were constructed using the same equations as for Figures 6.2 and 6.3, respectively. Figure 6.8 represents the extrapolated predictions of the citric acid yield based on sugar used. All three Figures, 6.6, 6.7 and 6.8, clearly show the maximum in each to be at the point of lowest possible specific growth rate and highest possible DOT. The maximum predicted specific citric acid production rates in Figures 6.2 and 6.3 are 74 mg/gDW.h and 2479 mg/gN.h, respectively. The predicted values at a DOT of 100% saturation and u = 0.010 h^{-1} are 286 mg/gDW.h and 10,220 mg/gN.h. The predicted citric acid yield at this point is 100%. In an attempt to test this prediction, a steady state was established at DOT = 90% of saturation and u = 0.017 h^{-1}. This DOT value was the highest maintainable level, and the value of u was used for technical reasons. The results of this steady state are presented in Table 6.6. When compared with the steady states shown in Table 6.3, the volumetric citric acid production rate was less than that observed at the centre point of the

Figure 6.1 Predicted volumetric citric acid production at steady states during nitrogen-limited chemostat continuous culture



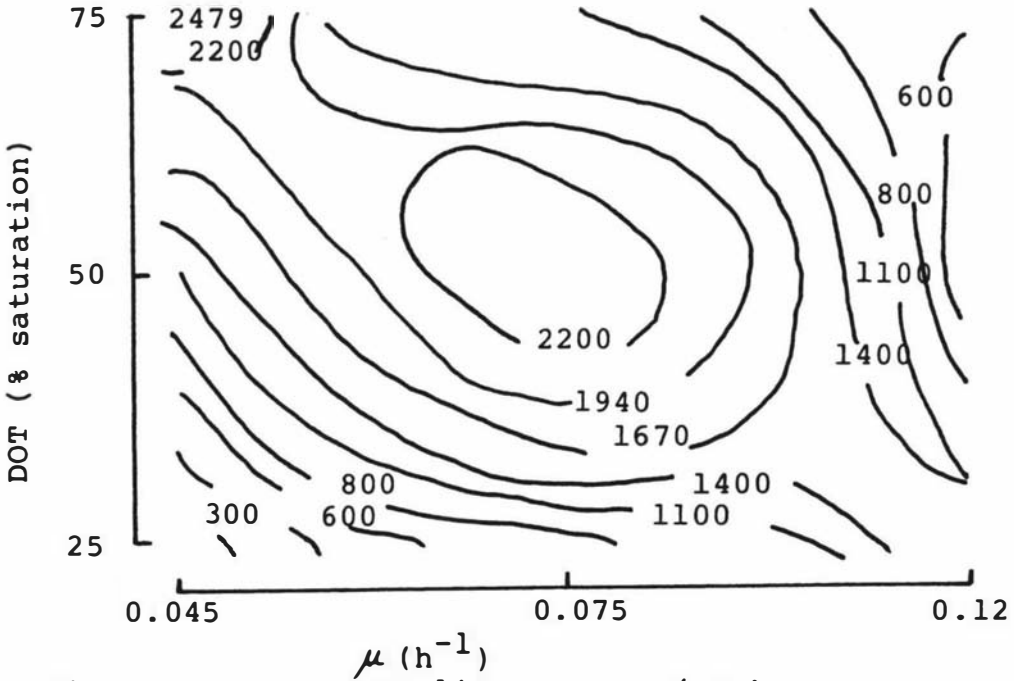
figures on contour lines are mg/l.h

Figure 6.2 Predicted specific citric acid production rate at steady states during nitrogen-limited chemostat continuous culture



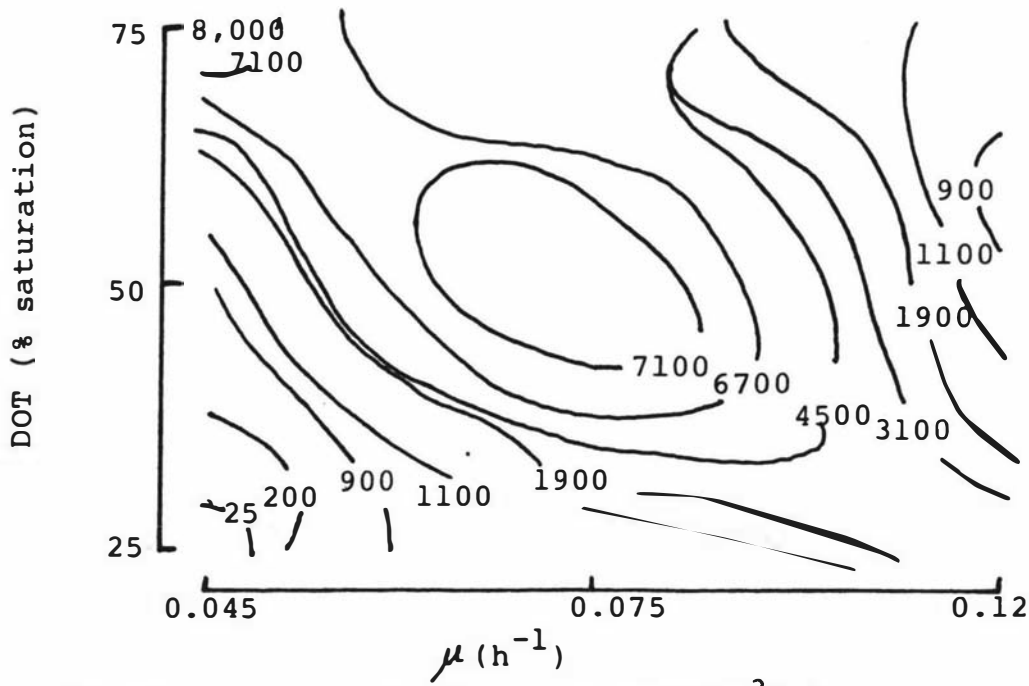
figures on contour lines are mg/gDW.h

Figure 6.3 Predicted specific citric acid production rate calculated using mycelial nitrogen content at steady states during nitrogen-limited chemostat continuous culture.



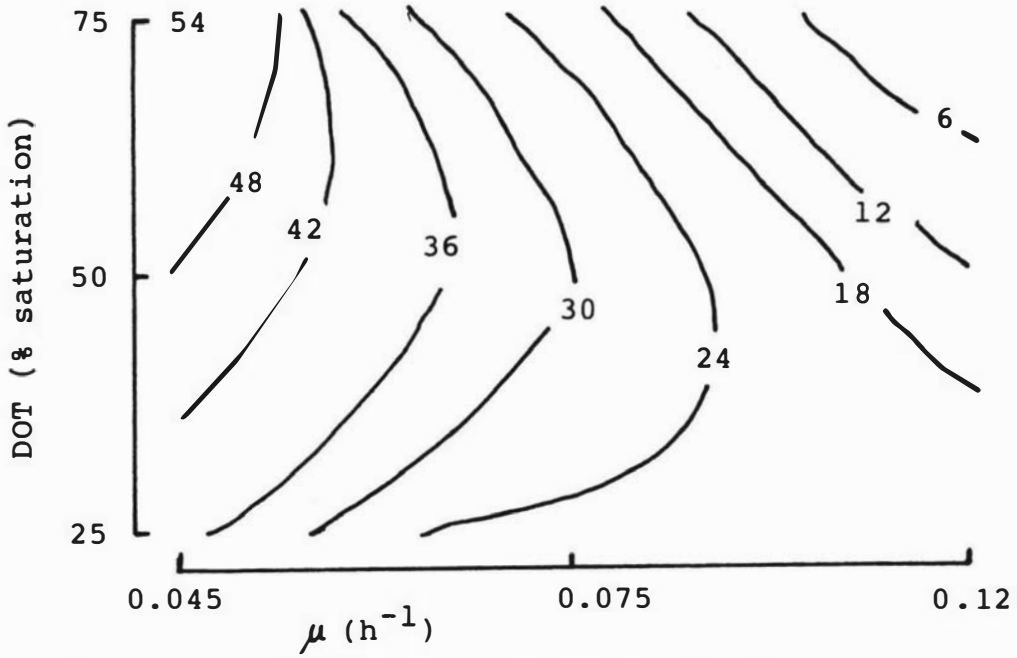
figures on contour lines are mg/gN.h

Figure 6.4 Predicted specific citric acid production rate calculated using mycelial phosphate content at steady state during nitrogen-limited chemostat continuous culture.



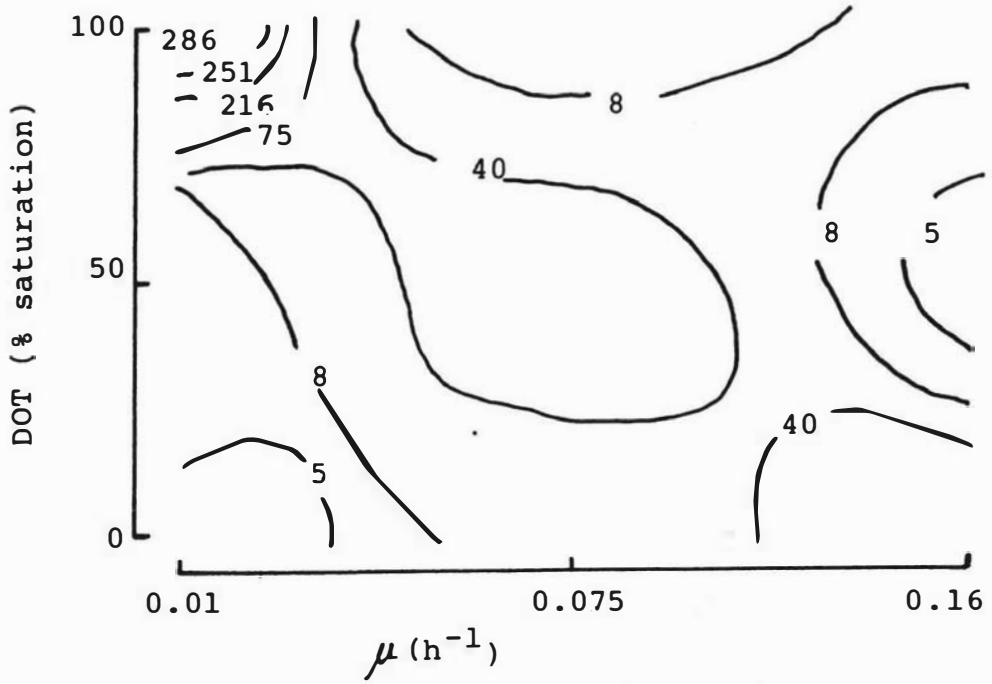
figures on contour lines are mg/gPO_4^{3-}.h

Figure 6.5 Predicted citric acid yield based on sugar utilised at steady states during nitrogen-limited chemostat continuous culture.



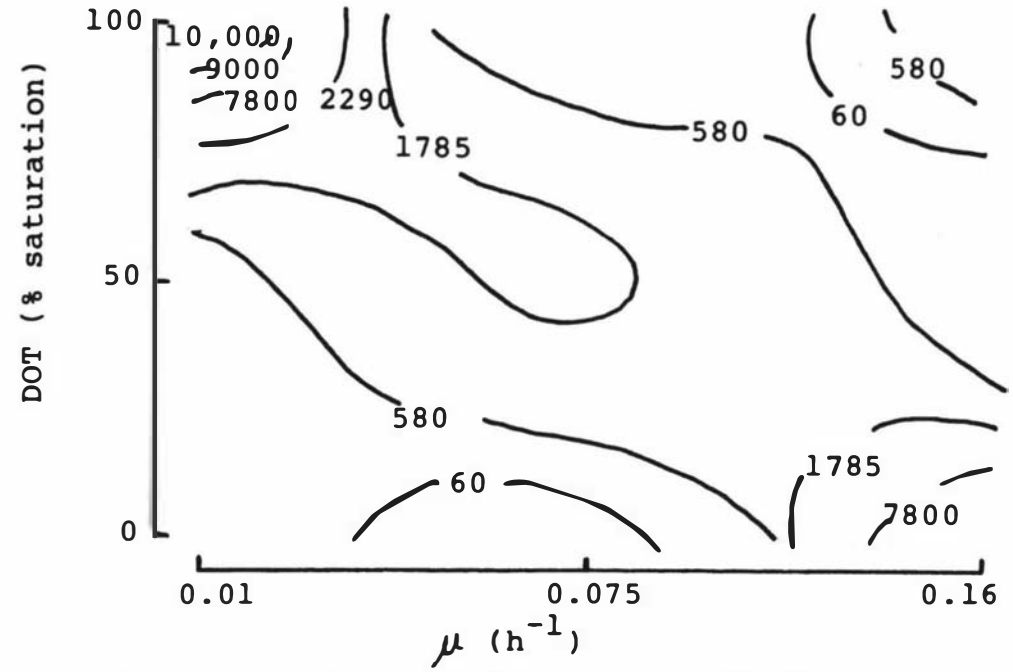
figures on contour lines are % yield

Figure 6.6 Extrapolated specific citric acid production rate at steady states during nitrogen-limited chemostat continuous culture.



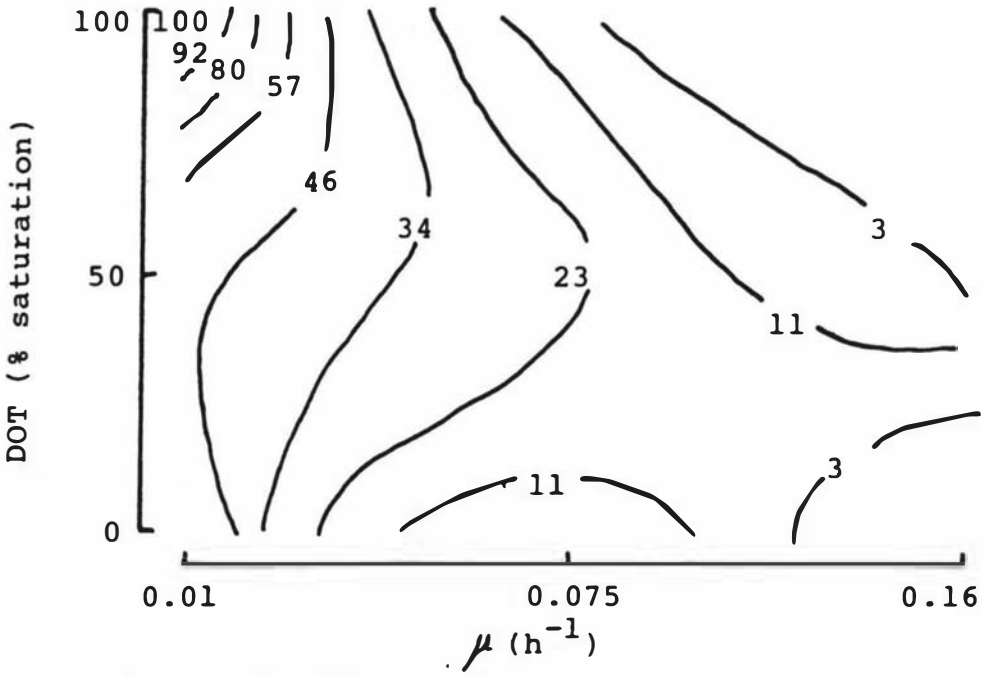
figures on contour lines are mg/gDW.h

Figure 6.7 Extrapolated specific citric acid production rate calculated using mycelial nitrogen content at steady states during nitrogen-limited chemostat continuous culture.



figures on contour lines are mg/gN.h

Figure 6.8 Extrapolated citric acid yield based on sugar utilised during nitrogen-limited chemostat continuous culture.



figures on contour lines are % yield

Table 6.6 Concentrations, and volumetric and specific rates of product formation and nutrient uptake at the steady state u = 0.017 h^{-1}; DOT = 90% of saturation in nitrogen-limited culture.

Citric Acid (g/l)	Biomass (gDW/l)	Biomass (gN/l)	Biomass (gPO_4^{3-}/l)	% N in biomass	% PO_4^{3-} in biomass	Sugar uptake[1] (g/l)	Citric Acid Yield (%)	PO_4^{3-} uptake[2] (g/l)
23.7	5.1	0.21	0.13	4.1	2.5	36.5	65	0.13

Volumetric citric acid (mg/l.h)	Specific citric acid (mg/gDW.h)	Specific citric acid (mg/gN.h)	Specific citric acid (mg/gPO_4^{3-}.h)	Sugar (mg/l.h)	Sugar (mg/gDW.h)	Nitrogen (mg/gDW.h)
403.	79.3	1920	3090	620	120	0.7

Phosphate (mg/gDW.h)	Oxygen (mM/l.h)	Oxygen (mM/gDW.h)	Carbon Dioxide (mM/l.h)	Carbon Dioxide (mM/gDW.h)	Respiratory Quotient
0.4	15.8	3.1	8.5	1.7	0.5

1 initial concentration 50 g/l; 2 initial concentration 0.353 g/l

There was no residual nitrogen present at steady state (initial concentration of 0.21 g/l expressed as nitrogen)

experimental design, but the specific citric acid production rate was slightly higher. The citric acid yield, however, was increased considerably. Thus, the steady state conditions shown in Table 6.6 result in a combination of high yield and high specific citric acid production rate. The specific citric acid production rate at this steady state condition was significantly higher (by a factor of 2.4) than the maximum observed in batch fermentation (Figure 5.19), but the volumetric production rate was less than 60% of the maximum observed.

In an attempt to increase the volumetric citric acid production rate, an experiment was performed at the steady state condition of DOT = 90% of saturation/u = 0.017 h^{-1} in which the biomass concentration was to be doubled. To obtain the higher level of biomass, the nitrogen concentration in the feed medium was increased to 420 mg/l (2g/l $(NH_4)_2SO_4$) (210 mg/l N in the medium previously). However, under these conditions it was found to be impossible to maintain a DOT value of greater than 60% of saturation. The results of this steady state are presented in Table 6.7. The experiment was successful in that the volumetric citric acid production rate was increased from 403 to 520 mg/l.h, although the specific citric acid production rate was reduced from 79 to 51 mg/gDW.h and the yield based on sugar used was reduced from 65 to 52%. This was probably due to the DOT being maintained at only 60% of saturation. Such a result was predicted by the correlation of specific citric acid production rate and DOT as shown in

Table 6.7 Concentrations, and volumetric and specific product formation and nutrient uptake rates, with increased biomass level, at the steady state u = 0.017 h^{-1}; DOT = 60% of saturation in nitrogen-limited culture.

Citric Acid (g/l)	Biomass (gDW/l)	(gN/l)	(gPO_4^{3-}/l)	% N in biomass	% PO_4^{3-} in biomass	Sugar uptake[1] (g/l)	Citric Acid Yield (%)	PO_4^{3-} Uptake[2] (g/l)
30.5	10.2	0.42	0.27	4.1	2.6	58.7	52	0.27

Volumetric citric acid (mg/l.h)	Specific citric acid (mg/gDW.h)	(mg/gN.h)	(mg/gPO_4^{3-}.h)	Sugar (mg/l.h)	(mg/gDW.h)	Nitrogen (mg/gDW.h)
520	51.0	1240	1930	998	97.8	0.7

Phosphate (mg/gDW.h)	Oxygen (mM/l.h)	(mM/gDW.h)	Carbon Dioxide (mM/l.h)	(mM/gDW.h)	Respiratory Quotient
0.45	24.4	2.4	6.2	0.6	0.25

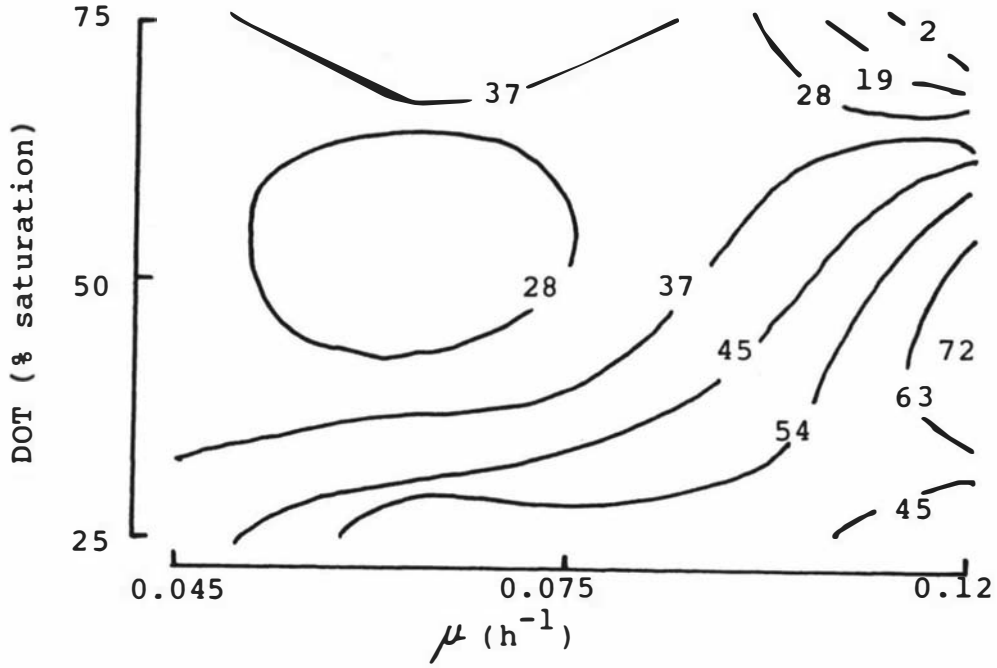
1 initial concentration 100 g/l; 2 initial concentration 0.353 g/l

There was no residual nitrogen present at steady state (initial concentration 0.42 g/l expressed as nitrogen)

Table 6.5. The volumetric rate of 520 mg/l.h was still, however, lower than the maximum observed in batch fermentation (Figure 5.19).

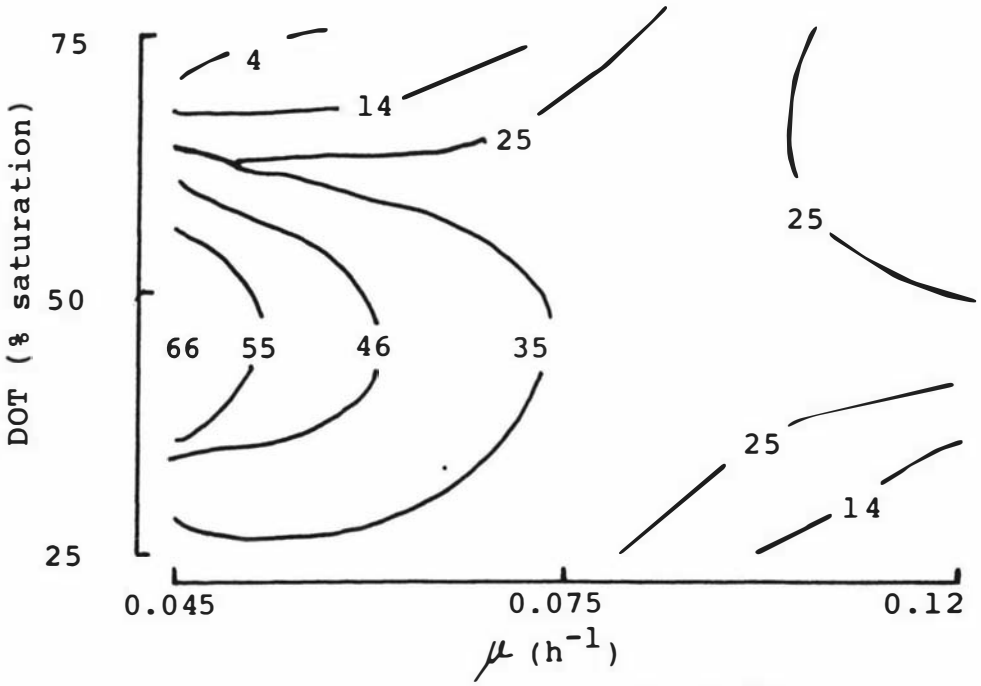
The carbon balances shown in Table 6.8 suggests that citric acid, carbon dioxide and biomass are the only three fates of the feed carbon. Only the fate of the carbon used is shown, the residual substrate carbon being ignored. The assumptions made in the calculation of the biomass content were as follows:- the percentage nitrogen in the biomass was multiplied by 6.25 to obtain the biomass crude protein content (including nucleic acids); carbon is 36% of the average amino acid of molecular weight 100; carbon is 44% of biomass carbohydrate using $C_6H_{10}O_5$. Thus, the addition of the three columns for each steady state do not all total 100%. They are however, sufficiently close to 100% to be reasonably certain that no other major product was formed during citric acid production, in the nitrogen-limited fermentation. It is recognized that the biomass composition is based on an assumption, rather than experimentally determined data. However, no other TCA-cycle acids, or oxalic acid or gluconic acid were observed as products of the fermentation. These compounds would have been detected by the HPLC analysis methods used, had they been present. The predicted response surfaces of citric acid yield, storage carbon yield and carbon dioxide yield, all based on sugar used are shown in Figures 6.5, 6.9 and 6.10, respectively. As expected, the fermentation conditions which maximise citric acid yield are quite different to those for storage carbon yield, the latter being favoured by a high

Figure 6.9 Storage carbon yield based on sugar utilised at steady states during nitrogen-limited chemostat continuous culture.



figures on contour lines are % yield

Figure 6.10 Carbon dioxide yield based on sugar utilised at steady states during nitrogen-limited chemostat continuous culture.



figures on contour lines are % yield

Table 6.8 Carbon balances as % carbon used in nitrogen-limited chemostat cultures

DOT (% Satn)	u (h^{-1})	Citric Acid	Carbon Dioxide	Biomass	Total
25	0.045	20.5	11.5	67.1	99.1
25	0.075	10.3	9.2	82.3	101.8
25	0.12	21.3	3.5	73.5	98.3
50	0.045	24.8	25.6	55.1	105.5
50	0.075	22.7	37.1	42.3	102.2
50	0.075	23.0	35.0	44.8	102.8
50	0.075	21.9	36.3	41.2	98.4
50	0.12	2.5	41.7	44.2	88.4
75	0.045	31.5	24.3	44.0	99.8
75	0.075	11.3	26.5	59.3	96.8
75	0.12	NIL	20.5	79.0	99.5

specific growth rate and a relatively low DOT. Carbon dioxide yield is favoured by a low specific growth rate and a relatively low DOT value.

6.3 PHOSPHATE-LIMITED FERMENTATION

The experimental design used was the same as that described in Section 6.2, with the coded variables allocated as in Table 6.1, and the model as described in Section 6.2. Fermentation conditions were as described in Section 6.2, with the exceptions that in the feed medium the PO_4^{3-} concentration was 0.07 g/l (0.1 g/l as KH_2PO_4) and that of nitrogen was 0.23 g/l (1.1 g/l as $(NH_4)_2SO_4$). The criteria for steady state were as described in Section 6.2.

The steady state concentrations recorded for the corner points and the centre point are shown in Table 6.9. Two features to note are, firstly, the low biomass concentrations, caused by the low concentrations of the growth-limiting nutrient, and, secondly, at a high specific growth rate no citric acid was produced at either the low or high DOT values. Table 6.10 shows the rate data. For reasons previously outlined (Section 6.2), some of the specific rate data are presented based on mycelial nitrogen and phosphate contents, in addition to mycelial dry weight. Trace metal analysis of the fermentation liquor again gave values higher than those in the feed medium, probably due to leaching from the fermenter parts.

The volumetric citric acid production rates are lower than those observed under nitrogen limitation (Table 6.3), due to lower biomass concentrations under phosphate

Table 6.9 Steady state concentrations during phosphate-limited chemostat experiments

DOT (% Satn)	u (h^{-1})	Citric Acid (g/l)	Biomass (gDW/l)	(gN/l)	(gPO_4^{3-}/l)	% N in biomass	% PO_4^{3-} in biomass	Sugar uptake[1] (g/l)	Citric Acid Yield (%)	N[2] Uptake (g/l)
25	0.045	1.0	3.0	0.124	0.07	4.1	2.0	9.0	11.1	0.124
75	0.045	1.2	8.7	0.095	0.07	1.1	0.8	7.5	16.0	0.095
50	0.075	1.0	1.3	0.085	0.07	6.5	4.6	12.6	9.3	0.085
50	0.075	0.8	1.3	0.084	0.07	6.5	4.6	11.1	8.0	0.084
50	0.075	0.9	1.2	0.084	0.07	7.0	5.0	11.6	9.0	0.084
75	0.12	NIL	0.5	0.040	0.07	8.0	12.0	2.1	NIL	0.040
25	0.12	NIL	2.0	0.170	0.07	8.5	3.0	11.5	NIL	0.170

1 initial concentration 50 g/l

2 initial concentration 0.23 g/l

There was no residual phosphate present at steady state (initial concentration 0.06 g/l expressed as phosphate)

Table 6.10 Volumetric and specific product formation and nutrient uptake rates at steady states during phosphate-limited chemostat experiments.

DOT (% Satn)	μ (h^{-1})	Citric Acid (mg/l.h)	Citric Acid (mg/gDW.h)	Citric Acid (mg/gN.h)	Citric Acid (mg/gPO_4^{3-}.h)	Sugar (mg/l.h)	Sugar (mg/gDW.h)
25	0.045	45	15.0	360	750	405	135
75	0.045	54	6.2	560	1100	900	100
50	0.075	75	56.0	890	1250	945	710
50	0.075	63	48.0	750	1160	830	640
50	0.075	70	58.0	830	1200	870	680
75	0.12	NIL	NIL	NIL	NIL	250	500
25	0.12	NIL	NIL	NIL	NIL	520	260

DOT (% Satn)	μ (h^{-1})	Nitrogen (mg/gDW.h)	Phosphate (mg/gDW.h)	Oxygen (mM/l.h)	Oxygen (mM/gDW.h)	Carbon Dioxide (mM/l.h)	Carbon Dioxide (mM/gDW.h)	Respiratory Quotient
25	0.045	1.9	0.9	10.2	3.4	7.6	2.5	0.8
75	0.045	0.5	0.3	13.6	1.6	9.2	1.1	0.7
50	0.075	5.3	3.3	1.9	1.4	2.0	1.5	0.9
50	0.075	4.9	3.2	2.2	1.6	2.3	1.8	1.0
50	0.075	5.2	3.3	1.9	1.6	2.1	1.7	0.9
75	0.12	12.0	14.4	1.8	3.7	0.7	1.5	0.9
25	0.12	7.0	3.6	1.2	0.6	2.3	1.2	1.9

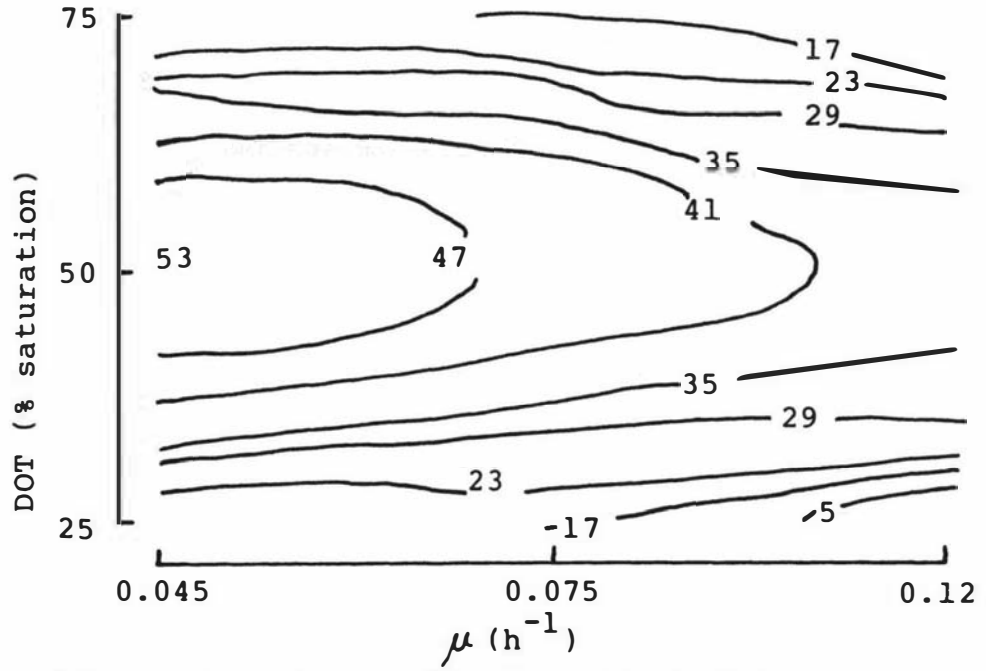
limitation. The specific citric acid production rates are slightly lower, and the yields considerably so. As a consequence of these results of the four corner points and the centre point steady state conditions, the other steady states used to make up the nine steady states of the nitrogen-limited experiment were not established under phosphate limitation.

From the results shown in Table 6.10, regression equations were obtained. Only two of these equations were used to predict response surfaces, i.e. the specific citric acid production rate and yield, shown in Figures 6.11 and 6.12, respectively. These were used as a comparison with the nitrogen-limited fermentations. All of the $\hat{Y}$ responses examined under nitrogen limitation as listed in Table 6.4 and Appendix 3, were examined under phosphate limitation.

Both of the regression models used in Figures 6.11 and 6.12 fitted the experimental data and showed no significant lack of fit. The data show that both the citric acid yield and specific production rate are favoured by a low specific growth rate. However, the DOT value need not be as high as for a nitrogen-limited fermentation.

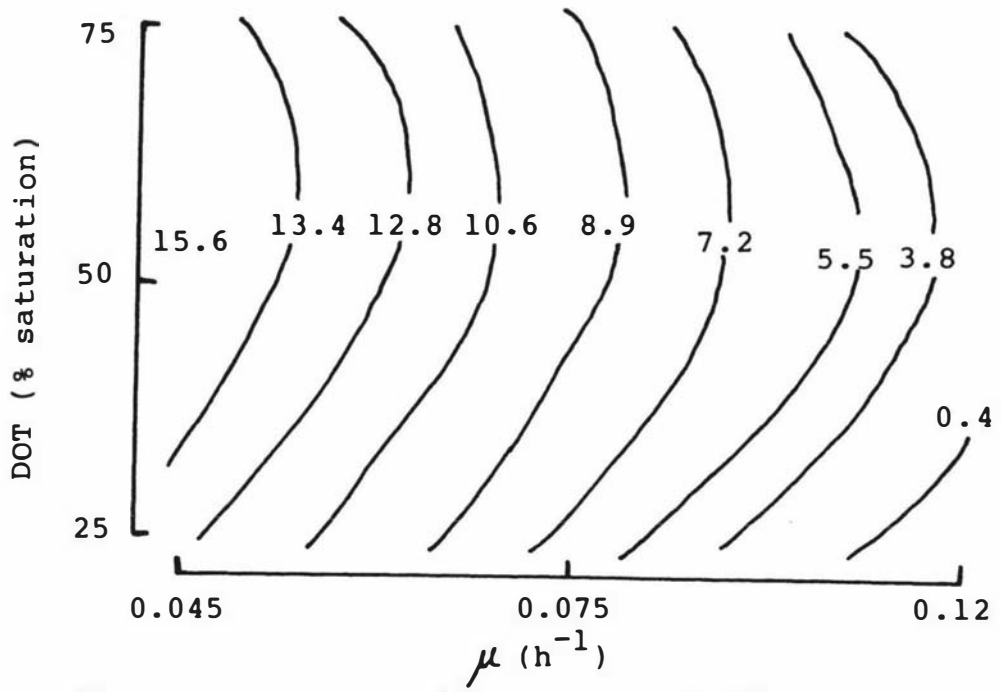
Using the correlation matrix facility of the Minitab Package, correlation coefficients were obtained for the entire matrix. The important correlation coefficients are shown in Table 6.11. In contrast with the nitrogen-limited fermentations, there was no strong relationship between the specific citric acid production rate and the DOT value of the culture. However, the strong negative relationship of

Figure 6.11 Predicted specific citric acid production rate at steady states during phosphate-limited chemostat continuous culture.



figures on contour lines are mg/gDW.h

Figure 6.12 Predicted citric acid yield based on sugar utilised at steady states during phosphate-limited chemostat continuous culture.



figures on contour lines are % yield

Table 6.11 Correlation coefficients of data from phosphate-limited chemostat cultures

Data Sets	Correlation Coefficient
Citric acid yield based on sugar used - u	-0.96
Specific nitrogen uptake rate (mg/gDW.h) - Citric acid yield based on sugar used	-0.97
Specific citric acid production rate (mg/gDW.h) - Specific sugar uptake rate (mg/gDW.h)	0.68
Specific citric acid production rate - Specific sugar uptake rate (both mg/gN.h)	0.79
Carbon dioxide yield based on sugar used - Specific sugar uptake rate (mg/gDW.h)	-0.74
Storage carbon yield based on sugar used - Specific sugar uptake rate (mg/gDW.h)	-0.92
Storage carbon yield based on sugar used - DOT	0.60

the former with the specific growth rate was observed. As in the nitrogen-limited fermentation strong negative relationships were observed between the specific sugar uptake rate and both storage carbon and carbon dioxide yields, and a positive relationship with the specific citric acid production rate. There was also a strong negative relationship between the citric acid yield and the specific nitrogen uptake rate. In contrast to the nitrogen-limited cultures, a high DOT value in phosphate-limited cultures appears to favour the production of storage carbon.

The carbon balances for the phosphate-limited cultures are presented in Table 6.12, and were calculated using the assumptions described in Section 6.2. These suggest that no other major products were formed apart from biomass, citric acid and carbon dioxide.

6.4 DOUBLE NITROGEN/PHOSPHATE-LIMITED FERMENTATION

Having established DOT and specific growth rate values for maximum citric acid production rates and yields under nitrogen-limited conditions, i.e. DOT = 90% of saturation and u = 0.017 h^{-1}, the same condition was used to examine the effect of double nitrogen/phosphate limitation on citric acid production rates. The feed medium was that described in Table 3.3, modified to contain 0.2 g/l KH_2PO_4 (140 mg/l expressed as PO_4^{3-}). The results (Table 6.13) showed that neither nitrogen nor phosphate were detected in the fermentation liquor at steady state. When these results are compared with those of the nitrogen limitation (Table 6.6), it can be seen that the double limitation can be regarded as

Table 6.12 Carbon balances as % carbon used in phosphate-limited chemostat cultures

DOT (% Satn)	u (h^{-1})	Citric Acid	Carbon Dioxide	Biomass	Total
25	0.045	6.3	73.4	21.2	99.9
75	0.045	10.3	58.5	34.7	103.5
50	0.075	19.0	59.0	25.9	103.9
50	0.075	18.0	63.8	25.2	107.0
50	0.075	20.1	61.0	22.0	103.0
75	0.12	NIL	44.7	49.1	93.8
25	0.12	NIL	35.2	66.8	102.0

Table 6.13 Concentrations, and volumetric and specific product formation and nutrient uptake rates at the steady state $\mu = 0.017\ h^{-1}$; DOT = 90% of saturation in double nitrogen/phosphate-limited culture.

Citric Acid (g/l)	Biomass (gDW/l)	(gN/l)	(gPO_4^{3-}/l)	% N in biomass	% PO_4^{3-} in biomass	Sugar uptake[1] (g/l)	Citric Acid Yield (%)
22.9	4.8	0.21	0.14	4.4	2.9	35.3	65

Volumetric citric acid (mg/l.h)	Specific citric acid (mg/gDW.h)	(mg/gN.h)	(mg/gPO_4^{3-}.h)	Sugar (mg/l.h)	(mg/gDW.h)	Nitrogen (mg/gDW.h)
390	80.7	1860	2790	600	125	0.74

Phosphate (mg/gDW.h)	Oxygen (mM/l.h)	(mM/gDW.h)	Carbon Dioxide (mM/l.h)	(mM/gDW.h)	Respiratory Quotient
0.5	20.1	4.2	16.0	3.3	0.8

1 initial concentration 50 g/l

There was no residual nitrogen or phosphate present at steady state (initial concentrations of 0.21 g/l expressed as nitrogen and 0.14 g/l expressed as phosphate)

a repeat of the nitrogen limitation, i.e. the data obtained were very similar.

6.5 ANALYSIS OF SELECTED ENZYMES DURING NITROGEN-LIMITED FERMENTATION

The activities of the enzymes aconitase, NAD-linked and NADP-linked isocitrate dehydrogenases, 2-oxoglutarate dehydrogenase, pyruvate carboxylase, and isocitrate lyase were determined in mycelial cell-free extracts at each steady state for the nitrogen-limited cultures. In nitrogen-limited batch fermentation, the maximum activities of aconitase, NAD-linked and NADP-linked isocitrate dehydrogenases and isocitrate lyase coincided with the maximum citric acid production rate. Therefore, an attempt was made to relate the activities of these enzymes to the citric acid production rate in chemostat culture. The results in Table 6.14 show that all of these enzymes were active at all steady states. The enzyme activities and ratios of the activity of each pair of enzymes were modelled using the multivariate linear regression of the model described in Section 6.2. All of the regression equations showed significant lack of fit and therefore could not be reliably used in the prediction of response surfaces. Correlation coefficients of the enzyme activities, and the ratios of the enzyme activities, with all of the nutrient and product rate data obtained under nitrogen limitation were sought using the Minitab Package correlation matrix facility. The correlation coefficients were all less than 0.35.

Table 6.14 The steady state activities of selected enzymes in nitrogen-limited chemostat culture

DOT (% Satn)	u (h^{-1})	Aconitase	Isocitrate dehydrogenase, NAD-linked	Isocitrate dehydrogenase, NADP-linked	2-oxoglutarate dehydrogenase	Pyruvate carboxylase	Isocitrate lyase
		(All enzyme activities are in μmols product/min/mg protein)					
25	0.045	75.0	6.7	10.8	4.2	8.3	12.8
25	0.075	14.0	0.6	4.7	3.5	3.5	5.5
25	0.12	15.5	1.3	2.7	1.1	12.1	2.1
50	0.045	16.0	2.3	3.0	0.8	3.3	0.6
50	0.075	21.2	1.5	7.6	1.5	9.1	1.2
50	0.075	27.0	2.9	4.3	4.3	9.0	1.5
50	0.075	22.0	2.8	3.9	2.0	8.9	1.4
50	0.12	12.0	0.8	3.1	2.0	3.1	1.4
75	0.045	24.0	4.0	6.0	6.0	12.0	4.7
75	0.075	70.6	11.8	20.6	14.7	17.6	8.8
75	0.12	60.0	15.0	22.5	7.5	10.8	61.3

6.6 DISCUSSION

From the results for the nitrogen-limited cultures, a steady state for maximum citric acid production was predicted and experimentally verified to be at a low specific growth rate and a high DOT value (DOT = 90% of saturation and u = 0.017 h^{-1}). However, it must be borne in mind that DOT values higher than 90% of saturation, and specific growth rate values lower than 0.017 h^{-1} were not achieved experimentally. The volumetric and specific rates of citric acid production obtained were similar to those observed at a DOT value of 50% of saturation and a specific growth rate of 0.075 h^{-1}. However, the citric acid yield based on sugar used was significantly increased from 28% to 65%. The specific citric acid production rate under these conditions was 2.4 times higher than the maximum observed in batch fermentation, but the volumetric production rate was considerably lower, probably because of the lower amount of biomass present in the chemostat culture. An attempt to increase the volumetric citric acid production rate in chemostat culture by increasing the biomass was successful, in that an increase from 403 to 520 mg/l.h was observed. However, the greater oxygen demand of the increased biomass resulted in a lower DOT and hence a decreased specific citric acid production rate and yield. The volumetric production rate in continuous culture was still inferior to that observed in batch culture of 690 mg/l.h.

Comparing the phosphate-limited and nitrogen-limited fermentations in chemostat, the nitrogen limitation was in

all ways superior. In the chemostat experiments, the ratio of citric acid produced: oxygen used during nitrogen-limited and phosphate-limited fermentations were similar (Table 6.15). This was not unexpected, as although the growth-limiting nutrient is absent from the culture in chemostat, it is continually being replenished, and a positive nutrient uptake rate is observed, indicating the occurrence of "active" metabolic growth, rather than apparent growth from increased storage carbohydrate production. This is in contrast to the situation in batch fermentations, where prior to limiting nutrient exhaustion, the ratio of citric acid produced: oxygen used during nitrogen-limited and phosphate-limited fermentations were similar (Table 5.9). However, subsequent to the exhaustion of the limiting nutrient, the phosphate-limited fermentation exhibited a markedly higher ratio (i.e. a lower oxygen requirement). The difference in the ratio of citric acid produced: oxygen used in batch fermentations only occurred once active growth had ceased.

Higher volumetric and specific citric acid production rates and yields were obtained under nitrogen limitation at comparable steady states. For this reason, the predicted optimum condition under phosphate limitation was not experimentally verified. It was however, predicted to be the lowest specific growth rate examined, at 0.045 h^{-1}, and at a DOT value of 50 to 60% of saturation.

It is suggested that the nitrogen nutrient may be acting in the role of catabolite repressor. This was first

Table 6.15 Molar ratio of citric acid produced : oxygen used by nitrogen- and phosphate-limited chemostat cultures

DOT (% Satn)	μ (h^{-1})	N-limited	PO_4^{3-}-limited
25	0.045	0.038	0.023
25	0.075	0.119	
25	0.12	0.167	NIL
50	0.045	0.077	
50	0.075	0.150	0.21
50	0.075	0.147	0.15
50	0.075	0.137	0.19
50	0.12	0.021	
75	0.045	0.201	0.055
75	0.075	0.145	
75	0.12	NIL	NIL

suggested by Hattori *et al.* (1974) in regard to a similar effect observed in yeast during the production of citric acid, and supported by Wang *et al.* (1979) in a general discussion on continuous culture processes. It is supported in this present study by the high negative correlation of the specific nitrogen uptake rate with the citric acid yield under phosphate-limited conditions.

As was observed in batch fermentation, the double nitrogen/phosphate limitation was virtually a repeat experiment of a nitrogen-limited fermentation.

Using the results of the steady state performed to verify the predicted steady state condition for maximum citric acid production and yield of DOT = 90% of saturation/u = 0.017 h^{-1}, the stoichiometry of the fermentation was determined to be:

$$2.09\ C_{12}H_{22}O_{11} + 18.59\ O_2 \longrightarrow 100g\ \text{biomass} + 2.49\ C_6H_8O_7 + 10.06\ CO_2$$

Details of the calculations are shown in Appendix 2. The aeration and agitation requirements of the fermentation as well as the cooling load required to counter the generated heat of oxidation can be determined for scale-up to commercial scale process. Therefore the stoichiometry is regarded as the basis for design calculations for a commercial process.

Inconclusive results were obtained from the enzyme analyses. The correlation coefficients were all less than 0.35, and hence the only conclusion to be drawn from the results is that all the enzymes are active during citric acid production, but their activities are not directly

linked to the rate of citric acid production. Thus, the results obtained, do not clarify the situation any further.

The carbon balances confirmed that citric acid, carbon dioxide and biomass are the only major products of the fermentation.

High specific citric acid production rates were obtained during both the nitrogen-limited and double nitrogen/phosphate-limited fermentations. The volumetric rates attained however, were lower than the maxima observed during batch fermentation. Although the overall fermenter productivity was higher for the chemostat culture than for the batch culture (403 mg/l.h versus 270 mg/l.h), the technical problems associated with the former technique may preclude its application on a commercial scale. Thus, the logical step is to apply the results of the chemostat culture to continuous fed-batch culture in order to achieve and then, most importantly, maintain a high volumetric production rate.

An interesting observation was that in the chemostat experiments the form of growth of the organism changed from pellets as were observed throughout the batch fermentation, to discrete pieces of mycelium approximately 1 to 2 mm in length, within 1.5 residence times of the commencement of the continuous feed. Thus, the pelletal form of growth is not essential for citric acid production to occur.

6.7 CONCLUSIONS

In chemostat culture under nitrogen-limited conditions, high citric acid production rates and yields are favoured by

operating the fermentation at a high DOT value and a low specific growth rate. A specific citric acid production rate of 79 mg/gDW.h was obtained, which is 2.4 times greater than the maximum observed in batch fermentation under nitrogen-limited conditions. Nitrogen-limited cultures are superior to phosphate-limited cultures. A double nitrogen/phosphate-limited fermentation was confirmed as being effectively a nitrogen limitation.

CHAPTER 7

CITRIC ACID PRODUCTION IN CONTINUOUS FED-BATCH CULTURE

7.1 INTRODUCTION

The results obtained in batch fermentation studies (Section 5.4) showed that the maximum observed citric acid production rate occurred prior to the exhaustion of the growth-limiting nutrient, and that subsequent to the exhaustion the production rate decreased. This implied that there is an optimum specific growth rate for citric acid production. Studies in chemostat culture (Section 6.2) subsequently showed that the maximum production rates and yields were obtained at a specific growth rate of 0.017 h^{-1} and a DOT value as high as possible.

When operating a commercial fermentation process, it is desirable to maintain the maximum production rate for as long a time as possible in order to maximise fermenter productivity. This can be achieved in chemostat culture and can also be achieved in continuous fed-batch culture by controlling the growth rate *via* the addition of the growth-limiting nutrient. The purpose of this section was to apply the knowledge gained in chemostat culture to continuous fed-batch culture.

7.2 NITROGEN-LIMITED FERMENTATION

The objective of this experiment was to achieve and maintain a high citric acid production rate, by maintaining

the specific growth rate of the organism, after initial biomass production, at a value of 0.017 h^{-1} or less. The extrapolated response surfaces shown in the previous section (Figures 6.6, 6.7 and 6.8) indicated that the lower the specific growth rate, the greater the citric acid production rates and yields that could be obtained. Following acquisition of a slow feeding pump, specific growth rates of 0.003 h^{-1} could easily be attained in fed-batch culture.

The specific growth rate was controlled by continuous addition to the culture of growth-limiting amounts of the nitrogen nutrient. During continuous addition of this nutrient, however, the volume of the culture increases continuously, resulting in a continually decreasing dilution rate and hence, growth rate. Since equipment was not available to increase the dilution rate continuously, step changes were used. The effect of this was that the dilution rate, and hence growth rate, oscillated.

The fermentation was performed as described in section 3.5.4, using the batch fermentation medium shown in Table 3.2. The initial volume of the medium was 4.0 litres and the agitation and aeration rates were maintained constant throughout the fermentation at 250 rpm and 3 l/min, respectively. After batching the fermentation, the nutrient nitrogen feed was commenced on day 3 at a rate of 10 ml/h of a $(NH_4)_2SO_4$ solution (440 mg/l), i.e. 0.92 mgN/h or, 0.23 mgN/l.h based on 4 l medium. This rate was increased to 0.35. 0.46 and 0.69 mgN/l.h on days 4,5 and 6, respectively. The time course of the fermentation is shown

in Figure 7.1. The continuous feed commenced on day 3, prior to nitrogen exhaustion, when the residual nitrogen concentration was 20 mg/l. Phosphate uptake was observed due to active growth caused by nitrogen replenishment of the medium. Citric acid production commenced after day 2, and by day 7 the concentration was 91 g/l, representing a yield based on sugar used of 65% (Table 7.1). All of the sugar was utilised by day 7, showing that the fermentation had gone to completion in 7 days, compared with 12 days in batch fermentation (Figure 5.17). The DOT value was between 40 and 50% of saturation for most of the fermentation period, rising to 55% on day 7.

From the time course data in Figure 7.1, it was possible to calculate the production rates of citric acid and carbon dioxide, and the uptake rates of phosphate, sugar and oxygen, which are shown in Figure 7.2. Nitrogen uptake rates after day 4 were calculated on the basis of the continuous feed rate, since no residual nitrogen was detected in the medium, i.e. feed rate equals uptake rate. The volumetric and specific citric acid production rates fluctuated during the period of continuous feeding. It is suggested that this was due to the oscillations in dilution rate, and hence growth rate, as described above. Nevertheless, citric acid volumetric production rates of more than 1.5 g/l.h were obtained during this period. The maximum rate observed was 2.25 g/l.h on day 7, which represented a specific production rate of 83 mg/gDW.h. These figures compare favourably with maxima observed in batch fermentation of 0.69 g/l.h and 35 mg/gDW.h,

respectively, and the maxima in chemostat culture of 0.39 g/l.h and 80.7 mg/gDW.h, respectively. Perhaps more importantly, the high rates were maintained for a longer period than in batch fermentation. The profile for the specific sugar uptake rate corresponded closely with that of the citric acid production rate. The specific growth rates at the time of the citric acid production rate peaks were between 0.005 and 0.010 h^{-1}, based on increases in mycelial dry weight, or 0.004 and 0.005 h^{-1} based on dilution rate (Table 7.2). This difference in growth rates is probably due to storage carbohydrate production in the biomass. The specific nitrogen uptake rates at the citric acid production peaks were between 0.02 and 0.03 mg/gDW.h, while the phosphate uptake rates were 0.05 to 1.5 mg/gDW.h at these times. Specific oxygen uptake and carbon dioxide production rates were approximately 0.5mM/gDW.h and 0.4 mM/gDW.h, respectively. The overall fermenter productivity was 0.54 g/l.h based on a 7-day fermentation. This compares favourably with 0.27 g/l.h obtained in batch fermentation, based on a 12 day fermentation time.

7.3 **PHOSPHATE-LIMITED FERMENTATION**

In batch fermentation and chemostat culture, phosphate-limited fermentations have been shown to be inferior to nitrogen-limitation with regard to citric acid production rates and yields. To determine whether the same was true of continuous fed-batch fermentation, it was decided to examine the effect of limiting the growth rate by means of

phosphate-limitation in fed-batch culture. As with the nitrogen-limited fermentation, the response surface predictions from chemostat culture (Figure 6.11) indicated an optimum specific growth rate of 0.045 h^{-1} or less for maximum citric acid production rates and yields. The fermentation was performed as described in section 7.2 except that the feed medium contained KH_2PO_4 at 200 mg/l (146 mg/l as PO_4^{3-}) and $(NH_4)_2SO_4$ at 600 mg/l (126 mg/l as N). The latter was included to ensure that the nitrogen nutrient was not growth-limiting. Continuous feeding commenced on day 3, after the initial batching, at a rate of 10 ml/h, i.e. 1.46 mg PO_4^{3-}/h and 1.26 mgN/h, or 0.365 mg PO_4^{3-} /l.h and 0.315 mg N/l.h. This rate was increased on days 4, 5 and 6 to 0.55, 0.73 and 1.1 mg PO_4^{3-} /l.h, respectively. The reason for the step increases in flow rate was as described previously (Section 7.2).

Figure 7.3 shows the time course of the phosphate-limited fermentation. The continuous feed commenced on day 3, prior to phosphate exhaustion. Nitrogen uptake was observed after the commencement of the continuous feed as there was sufficient nitrogen in the feed medium to ensure an excess of this nutrient. Citric acid production commenced after day 2 and by day 7 the concentration was 66 g/l, representing a yield of 55% based on sugar utilised (Table 7.1). The fermentation did not proceed to completion by day 7, as the residual sugar was 20 g/l. However, sugar utilisation was 120 g/l in 7 days compared to 90 g/l in 12 days in batch fermentation. The DOT value was 60% of saturation for most of the fermentation period.

The rate data for this fermentation are shown in Figure 7.4. The suggested reason for the fluctuations in citric acid production rates and sugar uptake rates has been discussed above for the nitrogen-limited fermentation. The maximum observed volumetric citric acid production rate was less than that of the nitrogen-limited fermentation (Figure 7.2), at 1.25 g/l.h but the specific rate of 65 mg/gDW.h was only slightly lower. Both rates were, however, higher than those observed in batch or chemostat culture. The specific growth rate at the time of maximum production rate was 0.005 h^{-1} calculated using the increase in mycelial dry weight, compared with 0.004 h^{-1} from the dilution rate (Table 7.2). The specific sugar uptake rate peaks coincided with the peaks in citric acid production rate. The specific phosphate uptake rate was approximately 0.1 mg/gDW.h, and of nitrogen 0.03 mg/gDW.h throughout the period of continuous feeding. The specific oxygen uptake and carbon dioxide production rates were approximately 0.6mM/gDW.h and 0.3 mM/gDW.h, respectively. The former was not significantly different from that observed during the nitrogen-limited fermentation.

The overall fermenter productivity for a 7 day fermentation was 0.39 g/l.h, compared to 0.14 g/l.h in batch fermentation based on a 12 day fermentation.

7.4 DOUBLE NITROGEN/PHOSPHATE-LIMITED FERMENTATION

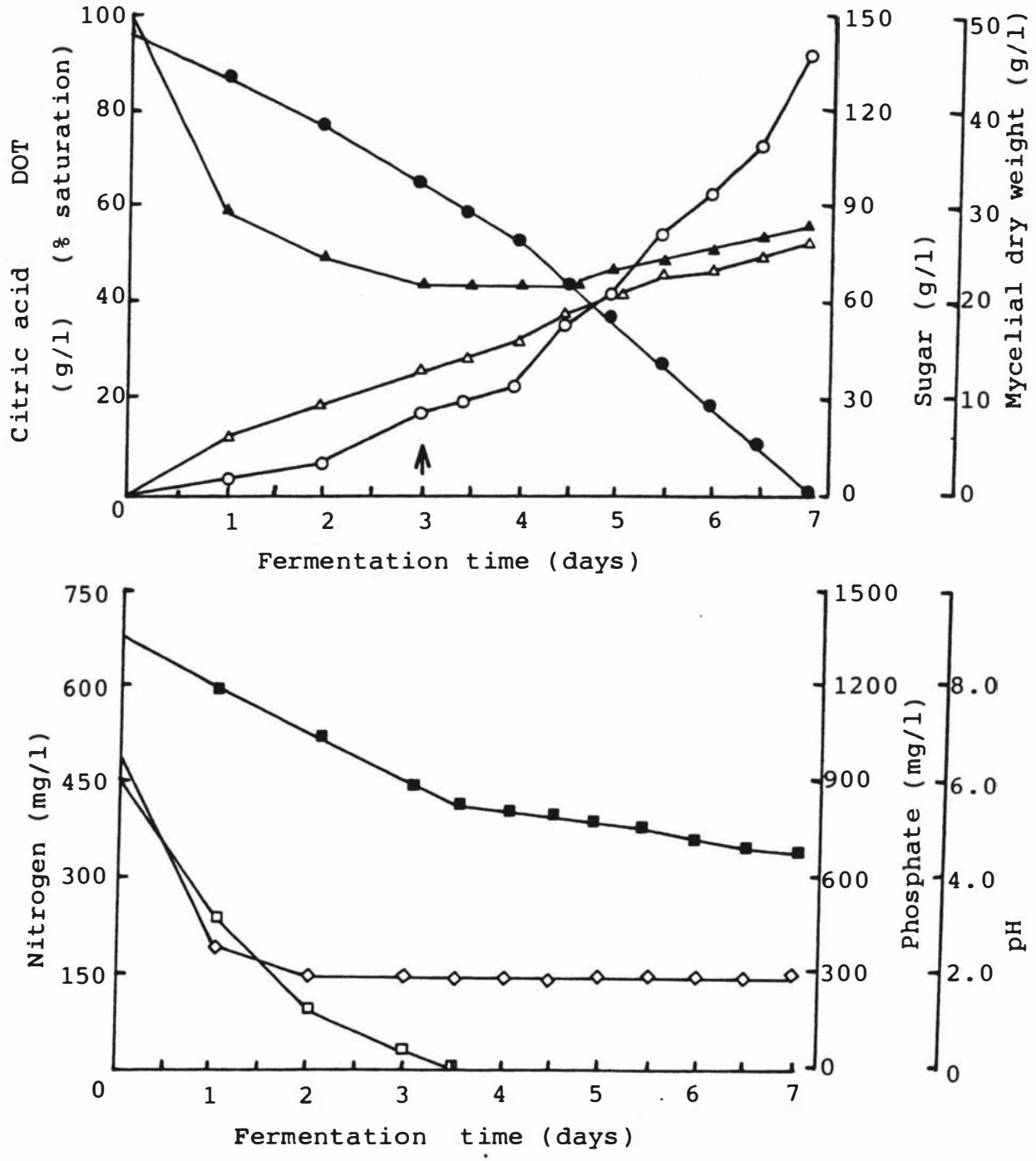
It has been shown in both batch fermentation and chemostat culture that the double nitrogen/phosphate-limited

fermentation is, in effect, a nitrogen limitation. To determine whether this is the situation in continuous fed-batch culture, this fermentation was performed.

The fermentation was conducted as described in Section 3.5.4 using the batch medium as described in Table 3.2 except that the concentration of KH_2PO_4 was 0.5 g/l. The fermentation conditions were as described in Section 7.2. The feed medium contained $(NH_4)_2SO_4$ at 440 mg/l and KH_2PO_4 at 200 mg/l and the continuous feed commenced on day 3 at a flow rate of 10 ml/h, i.e. 0.92 mgN/h or 0.23 mgN/l.h and 1.46 mgPO_4^{3-}/h or 0.365 mgPO_4^{3-}/l.h. This rate was increased on days 4, 5 and 6 to 0.35, 0.46 and 0.69 mgN/l.h and 0.55, 0.73 and 1.1 mgPO_4^{3-}/l.h, respectively. Again, due to the increasing volume and consequently decreasing dilution rate (and hence, growth rate), the step increases in flow rate caused oscillations in the growth rate.

The time course of the fermentation is shown in Figure 7.5, which shows a marked resemblance to the nitrogen-limited fermentation (Figure 7.1), except that both nitrogen and phosphate were not detected after 3 days. At 6 ½ days, the citric acid concentration was 92 g/l, representing a yield of 66% based on sugar utilised (Table 7.1). The fermentation went to completion in 6 ½ days, compared with 12 days in batch fermentation (Figure 5.18). The DOT value was between 50 and 60% of saturation for most of the fermentation period. The rate data of this fermentation are shown in Figure 7.6. The volumetric and specific citric acid production rates again oscillated during the period of continuous feeding, showing maxima of 1.75 g/l.h and 62

Figure 7.1 Time course of a nitrogen-limited fed-batch fermentation



(O, citric acid; ●, sugar; △, mycelial dry weight; ▲, DOT; □, nitrogen; ■, phosphate; ◇, pH; ↑, start of nutrient feed)

For rate data shown in Figures 7.2, 7.4 and 7.6, the following symbols were used:

- ○, volumetric citric acid production rate expressed as g/l.h
- ●, specific citric acid production rate expressed as mg/gDW.h
- △, specific sugar uptake rate expressed as mg/gDW.h^{-1}
- ▲, specific nitrogen uptake rate expressed as mg/gDW.h
- □, specific phosphate uptake rate expressed as mg/gDW.h
- ■, specific oxygen uptake rate expressed as mmol/gDW.h
- ◇, specific carbon dioxide production rate expressed as mmol/gDW.h
- ◆, specific growth rate expressed as h^{-1}

All axes, unless otherwise specified, represent the specific product formation or nutrient uptake rates.

Figure 7.2 Growth rate, product formation rates and nutrient uptake rates during a nitrogen-limited fed-batch fermentation

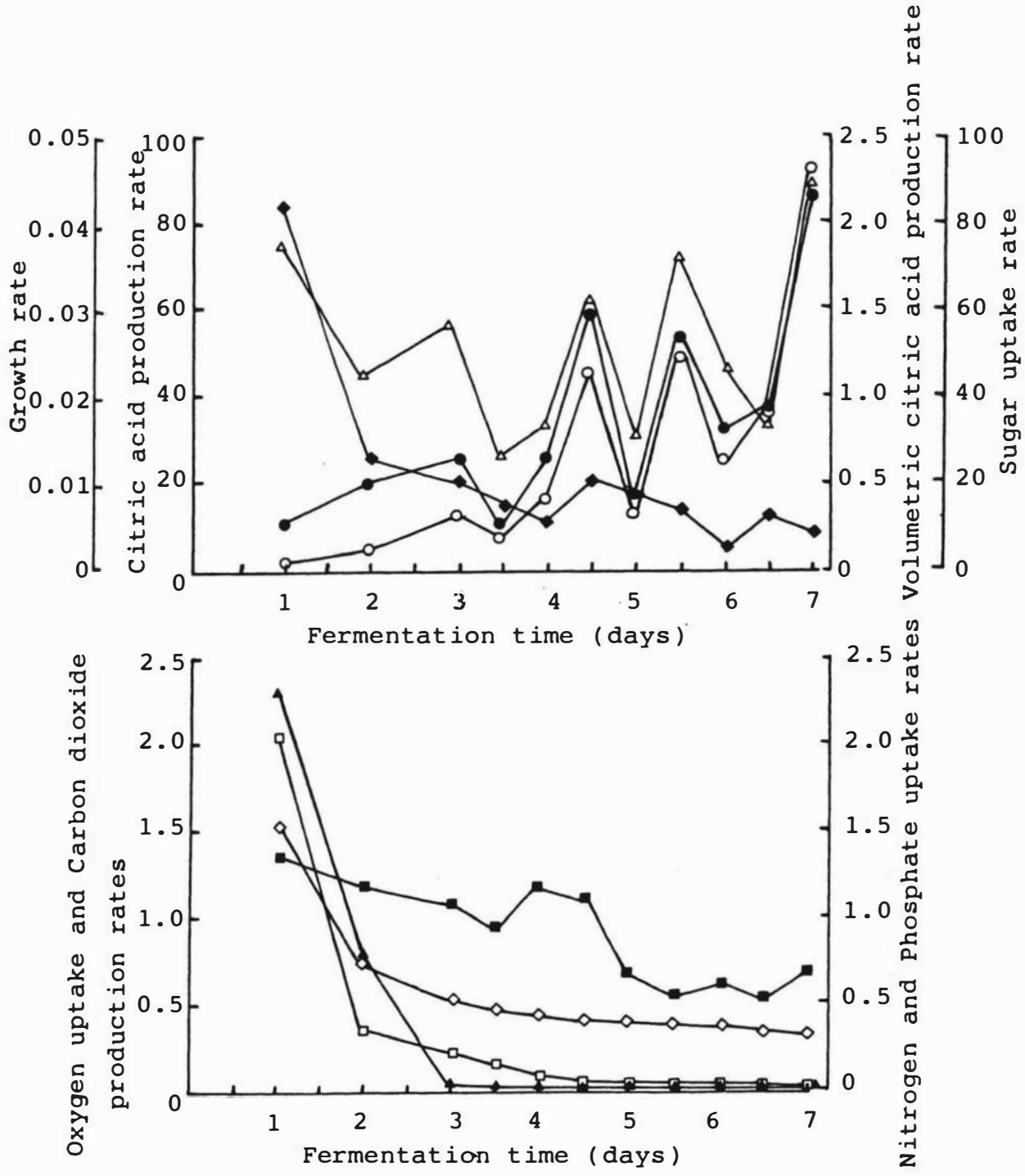
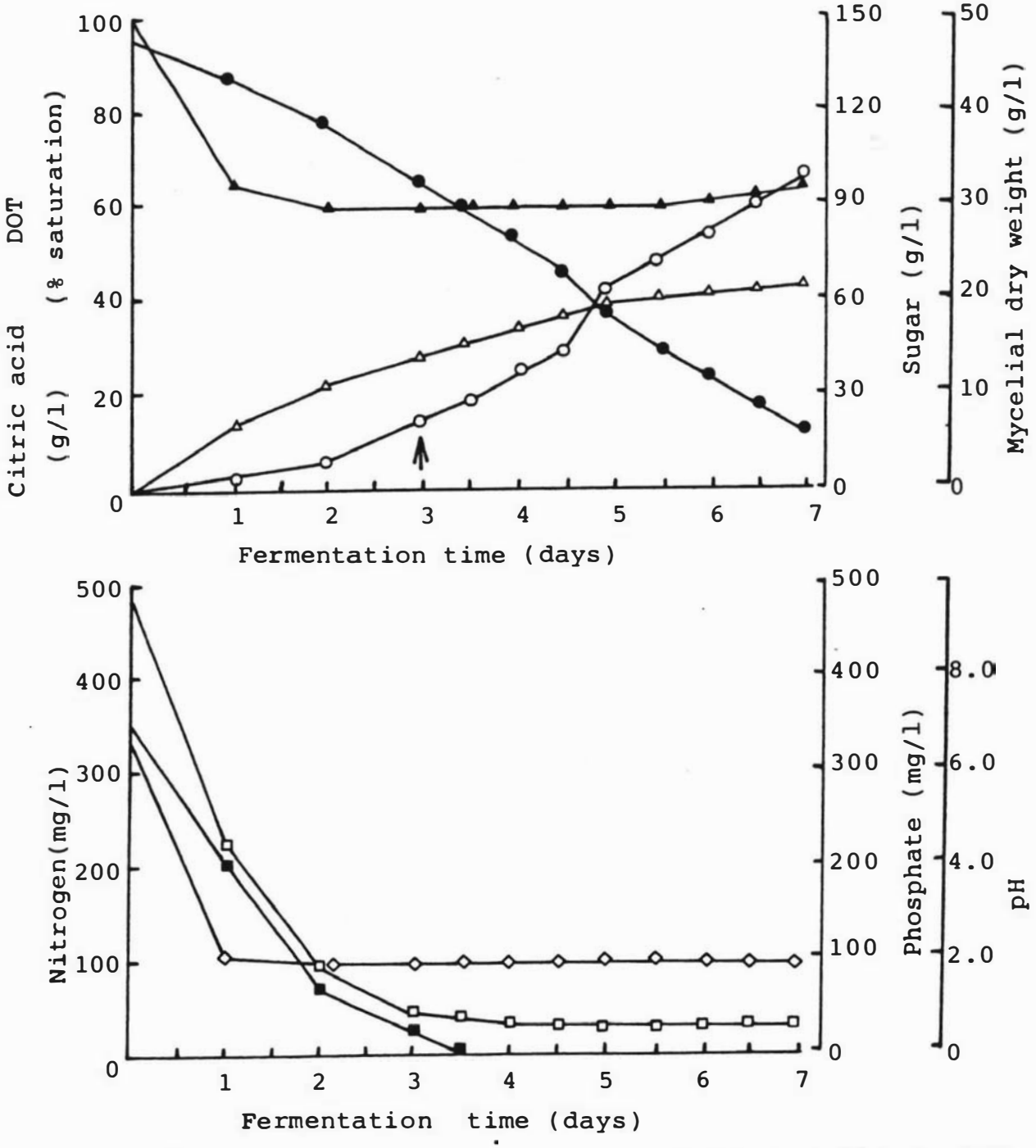


Figure 7.3 Time course of a phosphate-limited fed-batch fermentation



(O, citric acid; ●, sugar; △, mycelial dry weight; ▲, DOT; □, nitrogen; ■, phosphate; ◇, pH; ↑, start of nutrient feed)

Figure 7.4 Growth rate, product formation rates and nutrient uptake rates during a phosphate-limited fed-batch fermentation

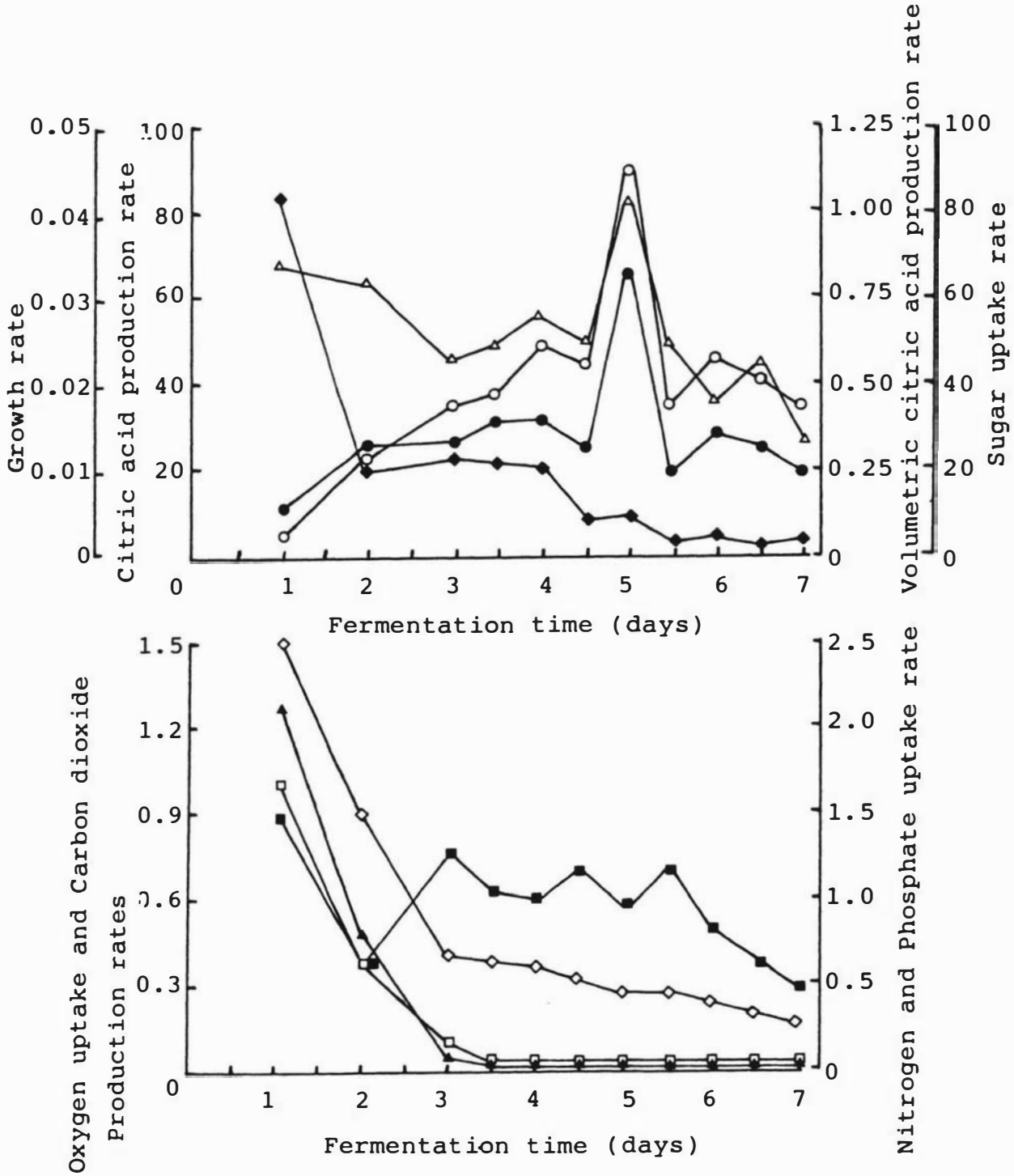
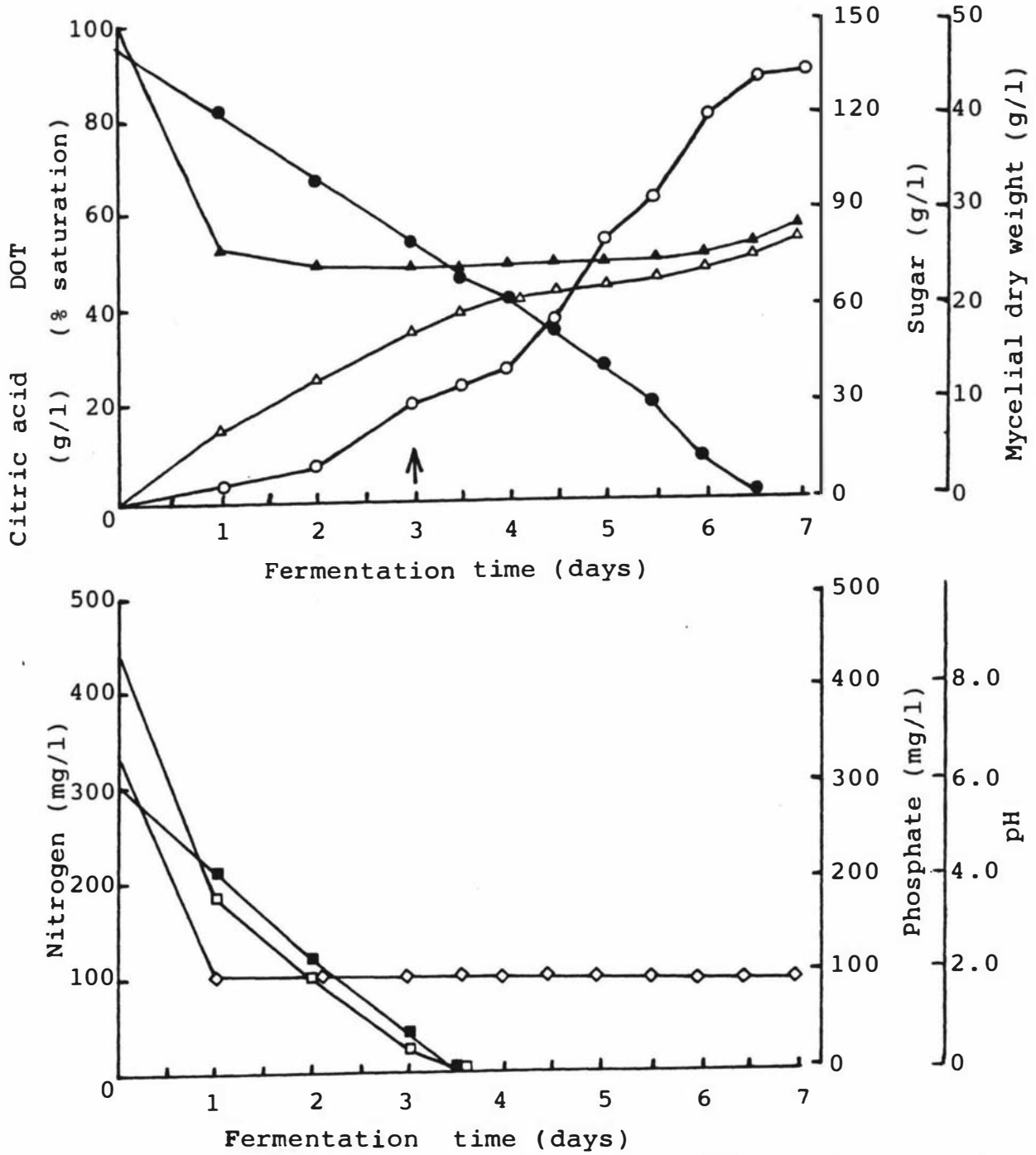


Figure 7.5 Time course of a double nitrogen/phosphate-limited fed-batch fermentation



(○, citric acid; ●, sugar; △, mycelial dry weight; ▲, DOT; □, nitrogen; ■, phosphate; ◇, pH; ↑, start of nutrient feed)

Figure 7.6 Growth rate, product formation rates and nutrient during a double nitrogen/phosphate-limited fed-batch fermentation

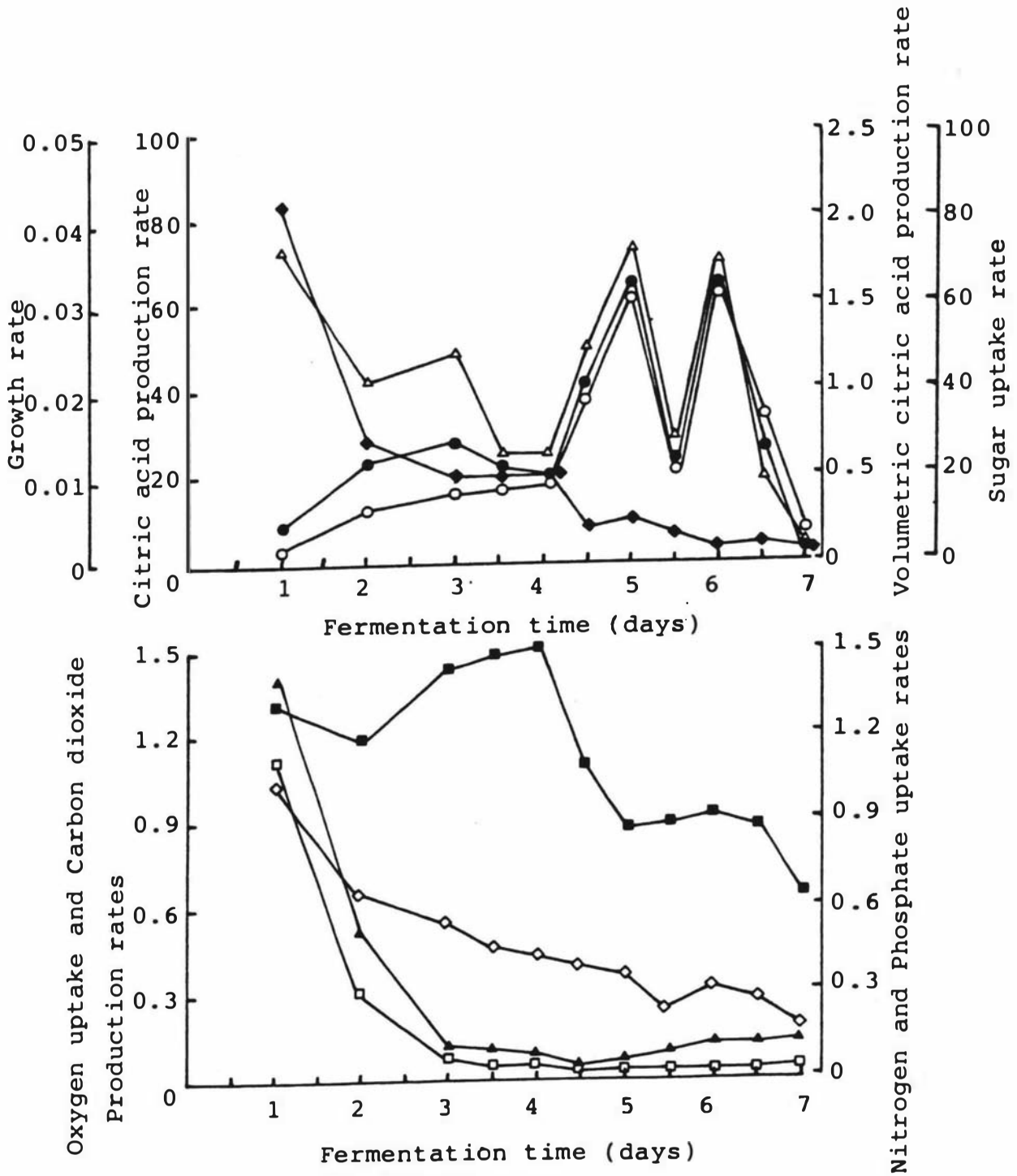


Table 7.1 Summary of fed-batch fermentation results at day 7

Limiting Nutrient	Biomass (g/l)	Citric Acid (g/l)	Sugar Used (g/l)	Citric Acid Yield (%)
Nitrogen	27	91	140	65
Phosphate	22	66	120	55
Nitrogen/Phosphate	27	92	140	66

mg/gDW.h, respectively. Again, the specific sugar uptake rate peaks coincided with the citric acid production rate peaks. The specific growth rate was 0.001 to 0.005 h^{-1}, calculated on the increase in mycelial dry weight, compared to 0.003 to 0.005 h^{-1} calculated using the dilution rate (Table 7.2). The fluctuations in citric acid production rates and the sugar uptake can be explained as suggested in Section 7.2. The specific nitrogen uptake rates were between 0.02 and 0.03 mg/gDW.h and the phosphate uptake rates were in the range 0.05 to 0.10 mg/gDW.h. The specific oxygen uptake and carbon dioxide production rates were 0.9 mM/gDW.h and 0.3 mM/gDW.h, respectively.

The overall fermenter productivity was 0.59 g/l.h based on a 6 day fermentation period, compared to 0.28 g/l.h in batch fermentation based on a 12 day fermentation period.

7.5 DISCUSSION

The aim of this work, which was to determine the feasibility of citric acid production in fed-batch culture, was achieved. The volumetric and specific citric acid production rates attained in fed-batch culture were significantly higher than those in batch fermentations, with consequent reduction in fermentation times. There were, however, fluctuations in the citric acid production rates. This is explained on the basis that the dilution rate, and hence growth rate, fluctuated throughout the period of continuous feeding. This implies that there is a narrow range of optimum growth rate for maximum citric acid

Table 7.2 Comparison of specific growth rates calculated from increase in mycelial dry weight and feed-medium flow rate

Day	1	2	3	3½	4	4½	5	5½	6	6½	7
Nitrogen-limited fermentation:-											
$u_{(DW)}$	0.042	0.013	0.011	0.008	0.006	0.011	0.008	0.007	0.003	0.006	0.005
$u_{(D)}$*				0.003	0.003	0.004	0.003	0.004	0.004	0.005	0.005
Phosphate-limited fermentation:-											
$u_{(DW)}$	0.042	0.010	0.012	0.011	0.010	0.010	0.005	0.005	0.002	0.002	0.002
$u_{(D)}$*				0.003	0.003	0.005	0.004	0.004	0.005	0.006	0.005
Double nitrogen/ phosphate-limited fermentation:-											
$u_{(DW)}$	0.042	0.014	0.010	0.010	0.010	0.004	0.005	0.004	0.002	0.002	0.002
$u_{(D)}$*				0.003	0.003	0.004	0.003	0.005	0.005	0.006	0.005

* Medium flow commenced on day 3, so this method of calculation was not available before day 3½.

production rate. Nevertheless, it is clear from the data that high production rates can be maintained for extended time periods. Further experimentation using equipment which would allow maintenance of a constant dilution rate should result in improved results.

The specific citric acid production rates attained were comparable with those obtained in chemostat culture (Tables 6.1, 6.2 and 6.3), and the volumetric rates were significantly higher than those obtained in batch fermentation or chemostat culture. Hence, the best aspects of both of these techniques are combined in fed-batch culture. The only cost of this technique is a total of 10% extra nitrogen nutrient added in the feed medium. For this cost, the fermentation period is nearly halved.

To increase the citric acid production rate and yield further, the DOT value of the culture should be increased, as shown in chemostat culture. While this is technically possible, economics will decide whether it is financially viable, as significantly greater power costs, or the use of pure oxygen, would be required.

Interestingly, the biomass concentration attained in fed-batch culture was lower than that in batch fermentations, despite there being a higher concentration of growth-limiting nutrient in the former. This was probably due to less storage carbohydrate being present in the biomass, as predicted from the data in chemostat culture for low growth rates.

Another interesting observation was that the pelletal form of growth of the organism disappeared and was replaced

by discrete mycelial fragments approximately 1 to 2 mm in length within 24 hours of the commencement of continuous feeding, as was observed in chemostat continuous culture (Section 6.6)

The double nitrogen/phosphate-limited fermentation was again demonstrated to be effectively a nitrogen-limitation. The higher citric acid yield based on sugar used and the fact that the nitrogen-limited fermentation went to completion, shows the nitrogen limitation to be superior to the phosphate-limited fermentation for citric acid production. The overall fermenter productivities clearly show that in these experiments the fed-batch culture was superior to both batch and chemostat cultures (Table 7.3), although it must be borne in mind that in chemostat culture, growth rates of less than 0.017 h^{-1} were not investigated. If they were, there is no reason to believe that similar productivities to those attained in fed-batch culture could not be achieved. The main advantage of fed-batch over chemostat culture is the reduced complexity, and therefore cost, for commercial scale production.

7.6 CONCLUSIONS

The main conclusion to be drawn from these results is that citric acid production in continuous fed-batch culture fermentation is feasible. Thus, high citric acid production rates can be achieved and maintained using this technique, resulting in shortened fermentation times and thus increased fermenter productivities. It may be possible to reduce the

Table 7.3 Overall fermenter productivities for citric acid in batch, chemostat and fed-batch fermentations

Limiting Nutrient	Batch Fermentation	Chemostat Culture	Fed-Batch Culture
Nitrogen	0.27	0.40	0.54
Phosphate	0.14	0.14	0.39
Nitrogen/Phosphate	0.27	0.39	0.59

- All productivities are in g/l.h
- The values for the chemostat culture represent the highest attained experimentally.

fermentation period further by using a microprocessor controlled medium feed pump to increase the flow rate exponentially, and so maintain the growth rate constant at the optimum for citric acid production. Thus, there are commercial possibilities for this fermentation technology with regard to citric acid production.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

The major aim of this work was to seek detailed information on the course of a typical citric acid fermentation process, with a view to understanding the process. Initially, experiments were performed to investigate the effect on the fermentation of interruptions to aeration. Provided that the DOT value of the culture remained above 20% of saturation, no gross effect was observed, but when the DOT fell to zero, citric acid production ceased. This was not unexpected since the DOT_{crit} for citric acid production has been reported to be 20% of saturation (Kubicek *et al.*, 1980). However, the fact that citric acid production recovered, even after a period of 85 minutes at zero DOT value was surprising, since oxygen starvation may have been expected to kill the organism. There are two possible explanations for this observation. The first is that the organism has some facultative anaerobic capability. There are no reports in the literature regarding *A. niger* fermentations under anaerobic conditions. However, anaerobic filamentous fungi have been reported (Windham and Akin, 1984). The second possibility is that the oxygen starvation initiated the sporulation process of the organism. This process may be reversible (Baxter, pers. comm., 1986) and the delay observed in the recovery of citric acid production may have been due to the reversal of the biochemical mechanisms of sporulation and

subsequent recommencement of the biochemical mechanisms associated with citric acid production. However, spores were not observed in the culture. With this in mind, and the fact that sporulation is an aerobic process, the most likely explanation is the first, involving anaerobic metabolism.

The experiments investigating citric acid production in batch fermentation under both nitrogen and phosphate limitation revealed that the maximum citric acid production rate occurred prior to the exhaustion of the limiting nutrient. This implied that there was an optimum growth rate for citric acid accumulation and led to experiments being performed in chemostat culture. During these experiments, the effect of the DOT value of the culture was also investigated. The results showed that the maximum production rates and yields occurred at low growth rates but high DOT values. It is now possible to suggest an explanation for these observations.

At high growth rates the organism would have a high demand for energy and biosynthetic intermediates, in which case it would be expected that citric acid would be further metabolised rather than accumulated. At low growth rates, however, because of the poorly regulated key enzyme phosphofructokinase, the flux of carbon material through glycolysis is maintained high, but the organism has a much lower demand for energy and biosynthetic intermediates. Thus citric acid accumulates. Further, at the low growth rate the nitrogen (NH_4^+) uptake rate easily satisfies the

biosynthetic demand of the organism, and the excess NH_4^+ ion may contribute to the deregulation of phosphofructokinase.

The reason for the requirement of a high DOT value is less clear, but may be related to the operation of the alternative oxidation pathway (SHAM-sensitive pathway) as suggested by Kubicek *et al.* (1980) (Section 2.5). This pathway allows the reoxidation of glycolytic NADH without concomitant ATP production, and requires a high DOT value. Given that during intensive citric acid accumulation the energy demand of the organism is low, sufficient ATP is formed by substrate-level phosphorylation. However, reoxidation of NADH is essential for glycolysis to be maintained.

The problem still remains regarding the mechanism of citric acid accumulation. No relationships of citric acid production with any enzyme activities were established in chemostat culture, despite relationships being observed for physiological parameters. However, the observation that 2-oxoglutarate dehydrogenase is active during citric acid production questions the hypothesis of a metabolic blockage of the TCA-cycle at the point of this enzyme. No progress was made in the further clarification of the situation regarding biochemical mechanisms of citric acid accumulation. This would suggest that citric acid accumulation is not totally enzyme controlled. It is possible that cell membrane permeability has a role in citric acid accumulation, in conjunction with metabolic control.

An interesting observation during the fermenter

experiments was the form of growth of the organism. When inoculated into the batch, chemostat or fed-batch fermenter vessels the organism was in the form of pellets 0.2 to 0.3 mm diameter, which increased to 0.5 to 1.0 mm diameter within 24 hours. In the batch fermentation the organism remained in the pellet form throughout the fermentation. However, in both continuous culture fermentations (chemostat and fed-batch), within 12 hours of commencement of the continous feed, the proportion of pellets to discreet fragments of mycelial hyphae (approximately 1.0 mm in length) was less than 1:5, and within 24 hours no pellets were observed. Thus, it is apparently coincidence that the optimum conditions for the production of citric acid in batch fermentation also encourage the retention of the pelletal form of growth (Section 2.6.1). The pelletal form of growth is *not* a requirement for the production of citric acid.

Fermentation under nitrogen limitation was superior to phosphate limitation in batch, chemostat and fed-batch culture techniques. A possible reason for this is catabolite repression of citric acid production by nutrient nitrogen. This has been observed with yeasts (Hattori *et al.*, 1974), while Kristiansen *et al.* (1982) have reported that for *A. niger* under phosphate-limited conditions, the lower the amount of excess nitrogen in the medium the more citric acid is produced. The evidence from the present study is the strong negative relationship of citric acid yield with nitrogen uptake rate observed under phosphate

limitation, but not under nitrogen limitation. Further evidence is that a combined nitrogen/phosphate double limitation behaved as if it were a nitrogen limitation.

In conclusion, maximum citric acid production rates occurred at a low but positive growth rate and a high DOT. This information, gained from chemostat culture, was applied to a continuous fed-batch system, and fermenter productivity was doubled compared with a batch fermentation (Table 8.1). As far as can be determined, this is the first reporting in the scientific literature of the use of a continuous fed-batch fermentation system for citric acid production.

Table 8.1 Summary of comparison of batch, continuous fed-batch and chemostat fermentations

Fermentation	Volumetric citric acid production rate (mg/l.h)	Specific citric acid production rate (mg/gDW.h)	Citric acid yield (%)	Overall productivity (h^{-1})
Batch				
N-limited	690	35	56	0.27
PO_4^{3-}-limited	150	25	44	0.14
N/PO_4^{3-}-limited	700	32	57	0.28
Chemostat				
N-limited	403	83	68	0.40
PO_4^{3-}-limited	140	58	12	0.14
N/PO_4^{3-}-limited	390	80	67	0.39
Fed-batch				
N-limited	2250	80	65	0.54
PO_4^{3-}-limited	1150	60	55	0.36
N/PO_4^{3-}-limited	1750	65	65	0.58

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APPENDIX 1

The gas balances were calculated using the equations in Section 3.8.3 to calibrate the flow meters in the first instance.

Calibration

A measured 0.4 litres of water was dispensed into a glass tube and the 0.4 1 point marked. Using the equation

$$\frac{P_1 V_1}{T_1} = \frac{P_2 V_2}{T_2}$$

and substituting the actual atmospheric pressure and temperature for P_1 and T_1, respectively, and standard pressure and temperature for P_2 and T_2, respectively,

$$\frac{762.23 \times 0.4}{295.75} = \frac{760 \times V_2}{293.15}$$

V_2 = 0.39765 litres air at standard temperature and pressure (which equates to the 0.4 litres of water above).

Thus, the flow rate was calculated for each sample by timing the period required by a soap bubble to travel the marked distance (0.4 litres). It was assumed that oxygen is 20.95% of air, and carbon dioxide is 0.03% of air in the aeration inflow. The effluent air was analysed for oxygen and carbon dioxide as described in Section 3.5.3.

Worked Example

The equation used above was again used to calculate the oxygen and carbon dioxide flowrate in the influent air, and the readings from the gas analysers used to calculate the

concentrations of these two gases in the effluent air. A worked example is presented using the steady state DOT = 90% of saturation/u = 0.017 h^{-1}. Effluent oxygen was 19.9%, effluent carbon dioxide was 0.87%, the air temperature was 22.5 °C and the atmospheric pressure was 760.3 mm Hg. The water lute created a back-pressure of 53 mm Hg on the fermenter. At the aeration rate of 0.5 l/min (rotameter setting), 28.1 seconds were required for the bubble to travel the distance marked on the 397.65 ml glass tube in calibration.

$$V_2 = \frac{760 \times 295.65 \times 397.65}{(760.3 + 53) \times 293.15}$$

$$= 375 \text{ ml/28.1 sec}$$

$$= \frac{375 \times 60}{28.1}$$

$$= 800 \text{ ml/min at STP}$$

O_2 in = 0.295 x 800 = 167.6 ml/min

O_2 out = 0.199 x 800 = 159.2 ml/min

CO_2 in = 0.0003 x 800 = 0.24 ml/min

CO_2 out = 0.0087 x 800 = 7.0 ml/min

O_2 in - O_2 out - CO_2 out + CO_2 in = 0

in this example

167.6 - 159.2 - 7.0 + 0.24 = 1.64

and 1.64/167.6 = 0.009 or 0.9% error. Samples with an error of 2.0% or more were discarded.

APPENDIX 2

The calculations for the determination of the stoichiometry of the citric acid fermentation in the present study were as follows:

the steady state used was DOT = 90% of saturation/u = 0.017 h^{-1}

and in 1.0 litres of culture medium, in 1 hour

O_2 uptake was 15.8 mM = 505 mg

CO_2 production was 8.5 mM = 374 mg (269mg as O_2, 105 mg as C)

Yields based on oxygen used

Citric acid yield

$$\frac{403 \text{ (mg/l.h) citric acid}}{505 \text{ (mg/l.h) } O_2} = 79.8\%$$

Biomass yield (Mycelial dry weight)

Production rate = 85.3 mg/l.h

$$\frac{85.3 \text{ (mg/l.h)}}{505 \text{ (mg/l.h)}} = 16.8\%$$

Carbon dioxide yield

$$\frac{374}{505} = 74.1\%$$

Carbon yields

C $\longrightarrow$ cells = 14.5%

C $\longrightarrow$ CO_2 = 21%

C $\longrightarrow$ citric acid = 60%

C unaccounted for = 4.5%

Carbon used = 248 mg/l

From 1 g O, obtain 0.798 g citric acid

0.168 g biomass

0.741 g CO_2

So from 16 g O, obtain 12.8 g citric acid

2.7 g biomass

11.9 g CO_2

From sucrose

1 g sucrose ⟶ 0.67 g citric acid

0.14 g biomass

0.62 g CO_2

1 mole sucrose (342 g) ⟶ 120.6 g citric acid

25.2 g biomass

111.6 g CO_2

therefore, for 100 g biomass

714.29 g sucrose + 595.23 g O ⟶ 100 g biomass + 478.38 g citric acid + 442.68 CO_2

divide by molecular weight

2.09 $C_{12}H_{22}O_{11}$ + 18.59 O_2 ⟶ 100 g biomass + 2.49 $C_6H_8O_7$ + 10.06 CO_2

APPENDIX 3

Full regression Models for Nitrogen-Limited Chemostat Cultures which exhibited a Significant Lack-of-Fit

$\hat{Y}$	B_0	B_1	B_2	B_3	B_4	B_5	B_6	B_7	B_8
Specific sugar uptake rate (mg/gN.h)	7027	165	354	-2723	-2593	-4837	5056	1968	1160
(mg/gPO_4^{3-}.h)	31284	23817	1308	-3852	32466	-28052	-21936	4765	20820
Specific nitrogen uptake rate (mg/gPO_4^{3-}.h)	336	241	83	110	454	-206	-284	-16	207
Volumetric oxygen uptake rate (mM/l.h)	15.8	2.83	-0.55	-9.75	-3.45	1.1	-1.75	2.69	6.85
Specific oxygen uptake rate (mM/gDW.h)	2.18	0.37	0.05	0.38	-0.98	0.07	-0.24	-0.58	0.55
(mM/gN.h)	64.1	15.6	-9.3	-5.4	-33	-7.6	-2.9	-2.6	12.5
(mM/gPO_4^{3-}.h)	305	224	7.7	44.2	215	-228	-211	-62.5	196
Volumetric carbon dioxide production rate (mM/l.h)	12.1	-1.2	1.55	5.8	-8.3	-4.7	-3.0	3.8	2.69
Specific carbon dioxide production rate (mM/gDW.h)	1.72	0.2	0.4	0.68	-0.93	-0.32	-0.21	0.051	0.251
(mM/gN.h)	48.9	-2.9	3	23.8	28.6	-15.9	-19	19.5	12.5
(mM/gPO_4^{3-}.h)	219	194	16	-64	109	-171	-42	-107	85

$\hat{Y}$	B_0	B_1	B_2	B_3	B_4	B_5	B_6	B_7	B_8
Respiratory quotient	1.07	0.91	0.15	-1.17	0.37	-0.42	1.99	-1.27	-0.73
Volumetric phosphate uptake rate (mg/l.h)	4.33	-0.29	4.25	1.08	-0.81	8.32	-6.22	-2.87	-1.6
Specific phosphate uptake rate (mg/gDW.h)	0.67	0.165	0.95	0.425	-0.045	1.69	-0.53	0.075	-0.41
(mg/gN.h)	17.6	-1.2	12.2	-11.9	-1.5	37.8	-21.1	-5.27	-6.74