SUBMERGED CITRIC ACID FERMENTATION OF WHEY PERMEATE BY *Aspergillus niger*

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ERRATUM

In the text and figures, for isocitric dehydrogenase read isocitrate dehydrogenase. On page 11, for pyruvic kinase read pyruvate kinase.

PAGE 133

Citric acid yield in whey permeate has been calculated on the basis of total lactose utilized. Separate shake-flask experiments using lactic acid as a carbon source in the medium of Kristiansen and Charley (1981) produced no citric acid, however, sparse growth did occur. Thus, the quantity of lactic acid present in the whey permeate (5.2 g/l) being metabolized by the culture, will make a small contribution to the biomass produced, but is ignored in the calculations of citric acid yield in this thesis.

PAGE 194, 200 AND 204

In this thesis the terms "inhibition" and "stimulation" of 2-oxoglutarate dehydrogenase and pyruvate carboxylase respectively are taken to mean changes in the specific activity of these enzymes. It is acknowledged that this is an incorrect use of these words, which refer strictly to rates of reaction of enzymic reactions.

Figure 6.33 contains a mis-plot. Figures 6.32 and 6.33 have been replotted, including points corresponding to zero inhibitor concentration.

NOTE: (a) competitive inhibition is shown in Figure 6.32 (page 174), (b) Figure 6.33 (page 174) shows competitive inhibition and allows the estimation of an approximate value of Ki, (c) the use of Figure 6.33 to estimate Ki is not strictly correct, since the data are obtained from batch culture; steady state chemostat data are required to analyse the inhibition in this way.

Figure 6.33 has been plotted with volumetric rate data. This is possible in this case, since the dry weight of mycelium at day 6 was the same at all inhibitor concentrations. Thus specific and volumetric rates are equivalent and the value of Ki obtained will be the same. However, under most circumstances, the dry weight might be expected to vary with inhibitor concentration and thus specific rate data should be used to determine Ki.

ABSTRACT

The feasibility of using lactic casein whey permeate as an alternative source of raw material for the production of citric acid by *Aspergillus niger* was studied.

A. niger (10 strains) and *A. carbonarius* (1 strain) were screened for their ability to produce citric acid from lactic casein whey permeate in shake-flask culture. Of the organisms tested, *A. niger* IMI 41874 produced the highest citric acid concentration of 5.0 g/l, representing a yield of 13.5% (w/w) based on lactose utilized. When the permeate was supplemented with additional lactose (final concentration 140 g/l), a concentration of 8.2 g/l was obtained, representing a yield of 15.5% (w/w). This organism was selected for further study including strain improvement work by induced mutation using UV light. A mutant strain (MH 15-15) was isolated which produced a citric acid concentration of 10.2 g/l in lactose-supplemented whey permeate. Using a sucrose-based synthetic medium a concentration of 52.8 g/l (yield 48% (w/w)) was observed, compared with 34.0 g/l (yield 33% (w/w)) produced by the parent strain. This mutant was used throughout subsequent experiments.

In fermenter culture experiments using lactose-supplemented whey permeate a citric acid concentration of 14.8 g/l was obtained. When extra nitrogen was fed to the culture after the onset of citric acid production, a concentration of 19.5 g/l was observed. Experiments with decationized whey permeate, supplemented with various amounts of different trace elements, proved unsuccessful in respect of improved citric acid production when compared with untreated whey permeate.

Experiments with different sugar sources using a synthetic medium demonstrated a marked effect of the sugar source on citric acid production. Thus, concentrations of 52.8 g/l, 31.0 g/l, 23.0 g/l, 5.0 g/l and 0 g/l were obtained from sucrose, glucose, fructose, lactose and galactose respectively. Good mycelial growth was observed with all the sugars. Similar experiments in fermenter culture showed the same trend of results, but in contrast to the experiments using whey permeate, citric

acid production was lower than in shake-flask culture. The activities of some TCA-cycle enzymes in mycelial cell-free extracts were investigated during fermenter culture experiments using the different sugar sources in synthetic medium and whey permeate. The initial activities of aconitase and both NAD- and NADP-linked isocitric dehydrogenase showed a strong relationship with subsequent citric acid accumulation. During citric acid accumulation the activities of these enzymes decreased significantly compared with those found during growth phase, but did not completely disappear. 2-oxoglutarate dehydrogenase disappeared completely when citric acid production was high but activity was maintained when production was low. The activity of pyruvate carboxylase increase considerably during citric acid production but little activity was detected when citric acid was not produced. It was concluded that accumulation of citric acid is not a consequence of the complete disappearance of the activity of aconitase or isocitric dehydrogenase (both NAD- and NADP-linked), but rather the accumulation is caused by the repression of 2-oxoglutarate dehydrogenase causing a block in the TCA-cycle, and the concomitant increase in pyruvate carboxylase activity. It was hypothesized that glucose and fructose cause repression but galactose does not.

Experiments using various combinations of glucose and galactose as sugar source demonstrated that galactose caused competitive inhibition of citric acid production from glucose. The inhibition showed a strong relationship with the levels of activity of 2-oxoglutarate dehydrogenase and pyruvate carboxylase.

The effect of methanol on citric acid production from lactose, glucose, galactose and whey permeate was investigated. In shake-flask culture, 1% (v/v) methanol caused increased production and yields of citric acid from both glucose and lactose. Citric acid production from galactose was also observed (12.5 g/l). In fermenter culture, using whey permeate, the presence of 3% (v/v) methanol gave a 69% increase in citric acid production (25.0 g/l compared with 14.8 g/l in the absence of methanol). The presence of methanol showed a general inhibitory effect on the various TCA-cycle enzymes studied,

in particular 2-oxoglutarate dehydrogenase.

Overall, it was concluded that the main obstacle to the improved production of citric acid from whey permeate is the nature of the sugar source rather than the other components of the substrate. In particular, the galactose moiety of lactose is not a favourable sugar source.

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ABBREVIATIONS

ABBREVIATIONS OF UNITS

^{o}C	degrees Celsius
cm	centimetre
d	day
g	gram
h	hour
l	litre
m	metre
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mM	millimole
nm	nanometer
rpm	revolutions per minute
µg	microgram
µL	microlitre

OTHER ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
cAMP	cyclic AMP
DNA	Deoxyribonucleic acid

DOT	Dissolved Oxygen Tension
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic acid
HPLC	High Performance Liquid Chromatography
ID	Internal Diameter
IMI	Commonwealth Mycological Institute
mRNA	messenger RNA
N	Nitrogen
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinuclotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
RNA	Ribonucleic Acid
TCA	Tricarboxylic Acid
UV	Ultra-violet

PREFACE

The Departments of Food Technology and Biotechnology of Massey University have a continuing interest in developing indigenous raw materials for use as foods or pharmaceuticals, thus making New Zealand more self-sufficient and extending the range of exports. It was apparent that large quantities of lactic casein whey were being used for spray irrigation and a potentially valuable fermentation feedstock (lactose) was being wasted. A number of chemicals which could be made from lactose are imported into New Zealand and citric acid was chosen for study on the basis of its continuing need in the food industry as an acidulant. This thesis is the result of that study.

Except where stated specifically in the text or Acknowledgements, all experimental work and chemical and physical analyses were carried out by the candidate personally.

Where the published work of others has been used, it is acknowledged in the text by the quotation of the authors' names and the date of publication. All cited work is listed in (alphabetical) order of the first author's names in the Reference section.

CHAPTER 1

INTRODUCTION

Citric acid is one of the most commonly used organic acids in the food and pharmaceutical industries, because of its ease of assimilation, palatability and low toxicity. New Zealand spends a considerable amount of money every year to import this valuable organic acid. Table 1.1 shows the citric acid import figures for the period 1976 to 1982 (New Zealand Department of Statistics, Imports, 1976 - 1982).

Overseas, citric acid is produced commercially by fungal fermentations of sugar solutions such as molasses, but for various reasons the use of molasses for production of citric acid in New Zealand is impracticable. If citric acid is to be produced in New Zealand in commercial quantities, a suitable indigenous raw material must be found. One possibility is whey.

TABLE 1.1 CITRIC ACID IMPORTS INTO NEW ZEALAND FROM 1976 TO 1982.

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

Whey is a by-product of the dairy industry produced during manufacture of cheese and casein. In the past, its disposal presented a problem to the industry as it was considered a waste material. More recently, however, two major uses have been found. First, the whey can be subjected to ultra-filtration to recover the soluble protein as a valuable product. The remaining liquor containing all the original lactose (4 - 5%) and minerals can then be disposed of by the second method, namely spray-irrigation on pastures, a process which helps to maintain fertility. An estimate of the volume of whey produced and the volume processed for the year 1982 - 83 dairying season in New Zealand is presented in Table 1.2 (Hobman, P., N.Z.D.R.I., personal communication, 1982).

TABLE 1.2 AN ESTIMATE OF THE VOLUME OF WHEY PRODUCED AND THE VOLUME PROCESSED FOR THE 1982 - 83 DAIRYING SEASON IN NEW ZEALAND.

	Base Product	
	CHEESE	CASEIN
Quantity (tonnes)	120,000	57,000
Volume of whey produced (m^3)	912,000	1,480,000
Volume of whey used (m^3) for:		
Whey cheese	2,600	-
Whey powder	100,000	108,000
Lactose	373,000	-
Whey protein extraction only	-	293,000
Whey protein extraction plus lactose utilization	60,000	371,000
Miscellaneous	500	-
Total	536,100	772,000
Percentage of whey partially or totally processed	59	52
Percentage of whey not processed	41	48

Overseas, it has been shown that whey permeate has potential as a raw material for production of various materials by fermentation. These include the use of whey permeate as a substrate for the production of yeast (Wasserman *et al.*, 1958; Wasserman, 1960; Wasserman *et al.*, 1961; Amundson, 1967; Bernstein and Everson, 1974; Moulin and Galzy, 1976; Bernstein and Tzeng, 1977), ethanol (Rogosa *et al.*, 1947; O'Leary *et al.*, 1977a, b; Moulin *et al.*, 1980), alcoholic beverages (Mann, 1972; Holsinger *et al.*, 1974; Palmer, 1978), ammoniated organic acids (Gerhardt and Reddy, 1977) and microbial polysaccharides (Charles and Radjai, 1977). Two recent reports refer to the production of citric acid from whey permeate (Chebotarev *et al.*, 1979; Somkuti and Bencivengo, 1981).

The work described in this thesis was undertaken to investigate the feasibility of using lactic casein whey permeate as an alternative source of raw material for the production of citric acid by fermentation.

CHAPTER 2

PRODUCTION OF CITRIC ACID BY FERMENTATION

2.1 INTRODUCTION

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

Among the citric acid-producing countries, the United States is by far the largest producer. The two major companies involved are Miles Laboratories, Inc. (Elkhart, Indiana) and Pfizer, Inc. (New York, N.Y.). Other important manufacturers include:

(a) John and E. Sturge, Ltd, Birmingham, England.
(b) Joh. A. Benckshiser, GmbH, Ludwigshafen/Rhein, West Germany.
(c) Citrique Belge, Tienen, Belgium.
(d) Rhone-Poulenc S.A., Paris, France.
(e) San Fu Chemical Company, Ltd, Taipei, Republic of China.

Citric acid is used for a variety of purposes. About 70% of that produced is used in the food and beverage industry, about 12% in pharmaceuticals and about 18% in other industrial applications (Atticus, 1975). Citric acid is employed as an acidulant in the food and pharmaceutical industries and it finds extensive use in the production of carbonated beverages as a flavour enhancer and preservative. The chemical and cosmetic industries also use citric acid

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

* Department of Trade and Industries, Wellington, New Zealand, 1982 (personal communication).

because of its sequestering and plasticizing properties (Meyrath, 1967). Other industrial applications include the treatment of boiler water, metal plating, detergent formulation, tanning and textiles.

A massive literature exists regarding citric acid production by fermentation. In the last 20 years more than 450 reports have been published and they have originated from various parts of the world, testifying to the world-wide interest in this chemical. However, many comprehensive reviews have been published (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). Since these authors have adequately covered the work performed over the last 50 years, no attempt will be made here to give a detailed review of the literature. Thus, this chapter attempts only to highlight those factors which have been shown to be important in the fermentation process.

2.2 HISTORY

The history of citric acid production from fungi has been extensively reviewed by Foster (1949) and Miall (1975). However, the development of a fermentation process for the production of citric acid may be divided into three phases (Perlman and Sih, 1960). The first phase was begun by Wehmer (1893), who reported on the use of *Penicillium lacteum* and *Mucor piriformis* for the production of citric acid when grown on the surface of media containing carbohydrate and inorganic salts. The manufacture of citric acid by this process was attempted at a factory in Thann, Alsace, which started up in 1893, but was abandoned ten years later, because of many difficulties including degeneration of the organism, contamination, long fermentation times and high costs. However, Wehmer did lay the ground work out of which all microbial citric acid manufacturing processes later developed. The second phase, surface fermentation using *Aspergillus niger*, was the result of Currie's research, first reported in 1917 (Perlman and Sih, 1960). He was the first to mention the importance of using pure reagents in the fermentation medium to obtain increased yields. He

joined Chas. Pfizer and Co. Inc. in Brooklyn, New York, and was partly responsible for the development of a citric acid process in this company, which was first operated on a commercial scale in 1923. The modern phase began with Perquin's thesis in 1938 and resulted in a shift of interest from surface culture to large-scale submerged culture vessels. Surface culture methods continued to be used for many years but now appear to have been replaced by the more efficient submerged culture methods. Details of the surface culture method have been adequately reviewed by Prescott and Dunn (1959) and submerged culture methods have been reviewed by Smith *et al.* (1974).

2.3 CITRIC ACID-ACCUMULATING ORGANISMS

Accumulation of citric acid is widespread among different groups of fungi, especially the genera *Aspergillus* and *Penicillium*. The various strains of *Aspergillus* and *Penicillium* used by many investigators for citric acid production have been adequately reviewed by Loesecke (1945), Foster (1949) and Perlman and Sih (1960). However, it is apparent from the literature reports that only selected strains of *Aspergillus niger* are used commercially for the production of citric acid.

It is now well documented that some yeasts are also capable of accumulating citric acid together with considerable amounts of isocitric acid. Suitable carbon sources include glucose, molasses, various alcohols, acetate, fatty acids and n-paraffins. An extensive review in this regard has been published by Kapoor *et al.* (1982). Of the various yeasts studied, the genera *Candida* and *Saccharomycopsis* appear to be the most useful, particularly when using paraffins as substrate.

Bacteria such as *Bacillus licheniformis*, *Bacillus subtilis* and *Brevibacterium flavum* have also been shown to accumulate citric acid when grown in media containing glucose, isocitric acid or hydrocarbons (Kapoor *et al.*, 1982).

2.4 FACTORS AFFECTING ACCUMULATION OF CITRIC ACID BY *A. niger*

There are a number of factors that affect the accumulation of citric acid by *A. niger* when grown in a carbohydrate-containing medium. This section aims to summarize the present knowledge, rather than provide a comprehensive literature survey.

2.4.1 BIOCHEMISTRY

Although many theories have been put forward to explain the accumulation of citric acid by *A. niger* in carbohydrate-containing media (e.g. Perlman and Sih, 1960; Meyrath, 1967), there is still no single hypothesis which fully explains the optimum physiological conditions required to obtain high yields (Smith *et al.*, 1974). It is generally accepted that the final step in the synthesis of citric acid is the condensation of acetyl-CoA and oxaloacetic acid (Figure 2.1), and this condensation is the major route of citric acid synthesis (Kapoor *et al.*, 1982). There exist, then, two problems to be solved. First, the reason why citric acid accumulates rather than being metabolized. Secondly, the source of oxaloacetic acid, since accumulation of citric acid implies some disturbances in the normal operation of the TCA-cycle, preventing production of oxaloacetic acid by this route.

Shu *et al.* (1954) studied the mechanism of citric acid formation by *A. niger* using a medium containing glucose-1-C^{14} as the sole source of carbon. As a result of mathematical analysis of their data, they concluded that 37-40% 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 conditions of fermentation were such as to give 50-70% yields of citric acid. Cleland and Johnson (1954), using radiolabelled glucose as substrate, 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.

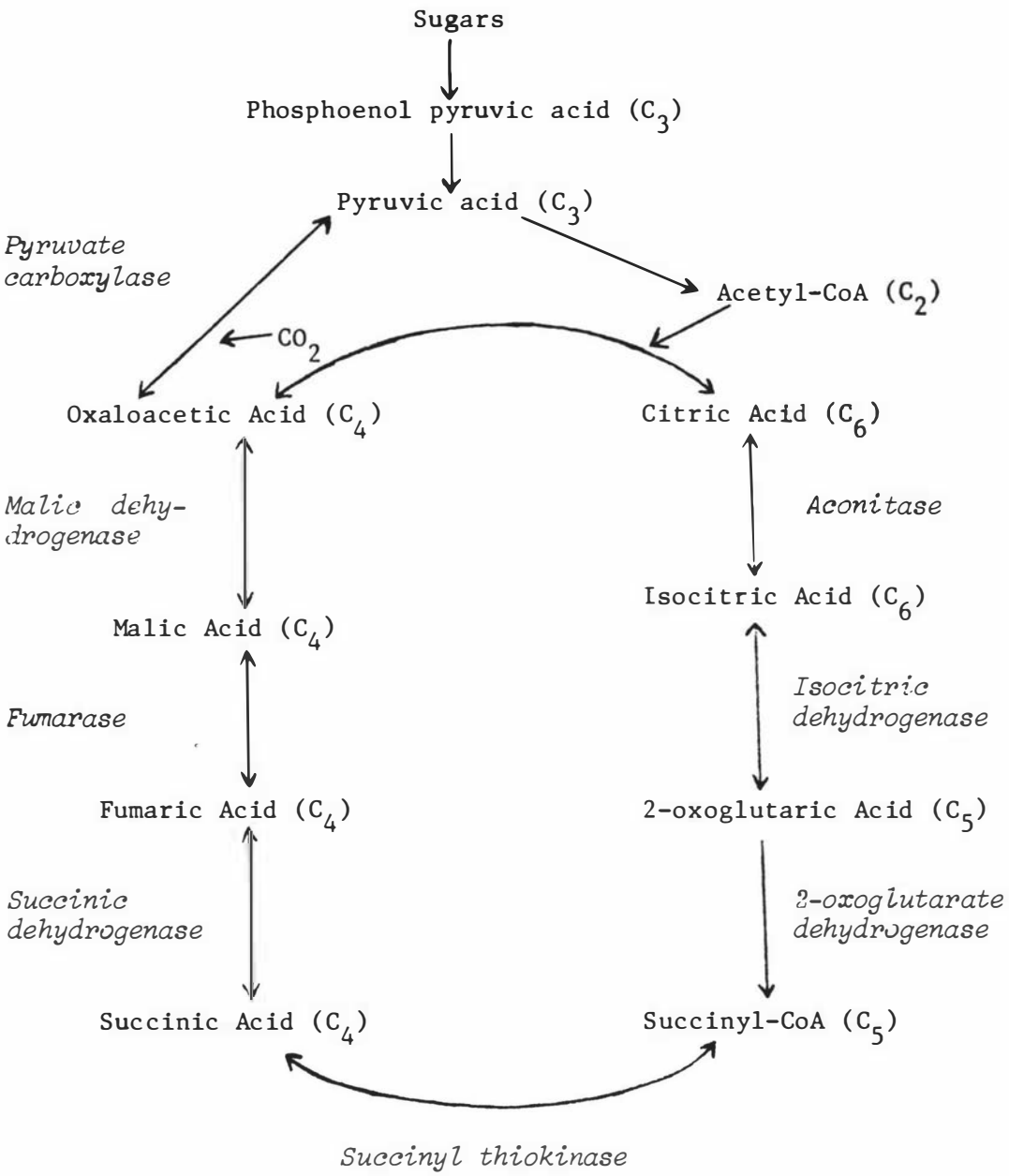

FIGURE 2.1 TRICARBOXYLIC ACID (TCA) CYCLE.

Subsequently, the enzymes phosphoenol pyruvate carboxykinase and pyruvate carboxylase were demonstrated to be active during citric acid production (Woronick and Johnson, 1960; Bloom and Johnson, 1962). In addition, Verhoff and Spradlin (1976) reported that pyruvate carboxylase, isocitrate lyase and oxaloacetate hydrolase were active in *A. niger* during oxalic acid and citric acid production. Thus, much evidence exists that a major source of oxaloacetic acid during citric acid accumulation is *via* a carboxylation of a C_3 fragment.

There are 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 in detail are aconitase and both NAD- and NADP-linked isocitric dehydrogenase. Ramkrishnan *et al.* (1955) reported that citric acid accumulated because of the disappearance of aconitase and isocitric dehydrogenase, but it was not stated which isocitric dehydrogenase was examined. They further demonstrated that during citric acid accumulation the activity of condensing enzyme increased tenfold and that the accumulated citric acid inhibited the activity of isocitric dehydrogenase. In contrast, La Nauze (1966) was able to demonstrate the presence of aconitase and both NAD- and NADP-linked isocitric dehydrogenase during the citric acid accumulation phase, although in lower activity when compared with the initial growth phase of the fungus. However, on considering these two reports, a major defect is apparent in the report of Ramkrishnan *et al.* (1955). These authors failed to mention the procedure of cell-free extract preparation, nor did they mention any precaution to avoid enzyme inactivation during preparation. It seems possible that the observed complete disappearance of these enzymes could have been the result of enzyme inactivation during preparation. Furthermore, the presence of these enzyme activities during citric acid accumulation has subsequently been reported by many investigators. Ahmed *et al.* (1972) re-examined the role of the TCA-cycle during citric acid accumulation, studying in particular, mitochondral function, TCA-cycle enzymes and also intermediates of the TCA-cycle. They demonstrated that certain TCA-cycle enzyme activities, e.g. the citric acid condensing enzyme, aconitase and both NAD- and NADP-linked isocitric dehydrogenase, were as high during the production phase as during the growth phase

of the fermentation. The presence of TCA-cycle intermediates, as demonstrated by Ahmed (1970), gave no support to the concept that the TCA-cycle is completely blocked during citric acid accumulation.

The activities of aconitase, both NAD- and NADP-linked isocitric dehydrogenase and citrate synthase (condensing enzyme) were studied in cell-free extracts of *A. niger* during citric production by Szczodrak (1981). He reported that during intensive citric acid accumulation, the activities of aconitase and both NAD- and NADP-linked isocitric dehydrogenase decreased significantly, although they did not disappear completely, compared with their activities during the mycelial growth phase. Citrate synthase activity was reported to be maintained at almost the same level over the entire fermentation period or increased slightly.

There are some reports indicating 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 the cell-free extract of *A. niger* during citric acid production on a sucrose-based synthetic medium, although they detected the presence of aconitase and both NAD- and NADP-linked isocitric dehydrogenase throughout the period of citric acid accumulation. They concluded that the accumulation of citric acid occurred due to blockage of the TCA-cycle at the step of 2-oxoglutarate dehydrogenase. Conversely, the presence of this enzyme in the cell-free extract of *A. niger* was reported by Muller and Frosch (1975) during citric acid degradation.

Kubicek and Rohr (1981), after their extensive study regarding the physiological aspects of the citric acid fermentation, 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 pyruvic kinase step, (b) uninfluenced rate of citric acid formation by poorly regulated citrate synthase, (c) incomplete operation of the TCA-cycle due to repression of 2-oxoglutarate dehydrogenase by glucose and NH_4^+ ions and inhibition of isocitric dehydrogenase and succinic dehydrogenase by several metabolites and (d) anaplerotic formation of

oxaloacetic acid by constitutive, weakly-regulated pyruvate carboxylase.

In summary, then, there is still some uncertainty with regard to the operation of TCA-cycle during citric acid accumulation.

2.4.2 Genetic Complement of Organisms

Strains of *A. niger* vary considerably in their ability to produce citric acid and to excrete it into the fermentation medium. This suggests differences in their genetic complements. Furthermore, substantial increases in the ability of a particular strain to produce citric acid have been achieved by genetic manipulation, usually by induced mutation (Gardner *et al.*, 1956; Imshenecky *et al.*, 1960; Millis *et al.*, 1963; Scherbakova, 1963, 1964; Ilczuk, 1968; Scherbakova and Lanskaya, 1971; Somkuti and Bencivengo, 1981). However, little is known of the exact nature of such mutations at the biochemical level. Das and Nandi (1965b) made the general statement that the increased citric acid production is due to favourable changes in enzyme activities, but provided no specific examples.

The idea of treating microorganisms with various mutagens (e.g. UV light, X-rays and chemicals) and to search for improved mutants among the surviving progeny has now been recognised as the best means to isolate strains with improved yields of citric acid. Kresling and Stern (1935) first reported mutants of *A. niger* producing remarkably more citric acid than the parent strain. They used UV light and radium as mutagens, but the practical implications of these observations were not realized until 1945, when Demerec (1945) reported a new mutant of *Penicillium notatum* x-162 showing improved yields of penicillin in comparison with the parent strain. Since this observation, the process of induced mutation and strain selection has been used in improving the yield of various metabolic products.

One difficulty in screening mutants for better citric acid production is the lack of a precise and quick method by which improved strains can be easily selected. Agar plates with nutrient media containing an acid/base indicator have been used for preliminary screening of survivors.

This method is, however not very precise. James *et al.* (1956) developed a technique for the isolation of high acid-yielding mutants of *A. niger* by growing them on paper disks soaked in a medium containing molasses, salts and bromcresol green. They reported that the colonies which developed on the paper disk were similar to those on agar with respect to colour, sporulation and aerial growth. The acid-yielding capacity of different colonies was determined by dividing the diameter of the acid zone by the diameter of the colony. The figure so obtained was called 'acid unitage'. Several advantages of this paper culture technique over the usual agar-containing media were claimed, e.g., colonies grow compactly on paper culture, with diameters of less than 2cm after 4 days and the diffusion of acid metabolic products is similarly restricted. Thus, they further claimed, it is possible to cultivate on the same surface area many more colonies than would be accommodated on agar media. Despite these claims, however, this method appears to be very time consuming in respect of inoculation procedure, when thousands of isolates are to be examined.

Gardner *et al.* (1956) obtained mutants of *A. niger* using multiple X-ray and UV irradiation and the best mutant was isolated by the paper culture technique. They reported that this mutant gave a sixfold higher yield of citric acid than the parent strain in submerged culture using molasses as raw material. Similarly using UV irradiation, Millis *et al.* (1963) screened about 40,000 colonies and isolated one which produced fourfold more citric acid than the parent strain in shake-flask culture, using a sucrose-based synthetic medium. Das and Nandi (1969a,b) by successive mutations with different mutagenic agents (e.g. UV, gamma rays and nitrogen mustard) obtained a mutant of *A. niger* which gave four times more citric acid than the parent. Similar increases in yields by successive mutation have been reported by other investigators (e.g. Sharma, 1973; Banik, 1975).

Somkuti and Bencivengo (1981) attempted to isolate a mutant which produced more citric acid from whey permeate by treating the spore suspension with N-methyl-N-nitroso-N-nitroguanidine (NTG). They obtained a mutant culture which produced 28 g/l citric acid

compared with 10 g/l produced by the parent in the lactose-supplemented acid-whey permeate.

Besides induction of mutation, strain improvement in *A. niger* by somatic recombination and polyploidization has been attempted, but without much success (Kapoor *et al.*, 1982).

From all these observations it can be concluded that alteration of the genetic complement by induced mutation can lead to much improved production of citric acid. No doubt, industrial laboratories have also used such techniques, but have not published their results.

One problem that is always associated with citric acid production by *A. niger* is the maintenance of cultures. There are several reports of the almost complete loss by a culture of the acid-producing ability which originally characterized it, without a noticeable change in growth rate (Yuill, 1953a,b). The reasons for this are still unclear. However, it has been suggested that such degeneration is probably due to spontaneous mutations occurring during repeated subculturing of the fungus, especially in synthetic media (Chrzaszcz and Zakomorny, 1938). It has been reported that such degeneration can be avoided by preserving cultures under a layer of mineral oil, by maintaining a dispersion of spores on sterile soil stored at low temperature (Yuill, 1953a), by freeze-drying methods (Mahritra and Hesseltine, 1958) or by periodical re-isolation from single spores and storage at low temperature (Chrzaszcz and Zakomorny, 1938; Rohr *et al.*, 1979).

2.4.3 Environmental Factors

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 by 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 attempt to summarize those factors which are known to be important.

2.4.3.1 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 through 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). However, it is not clear whether it is the pellet form *per se* which is important for citric acid production, or whether the environmental conditions causing pellet formation coincidentally favour citric acid production.

Schweiger and Snell (1949) developed a medium in which *A. niger* grew in the form of small pellets, averaging 0.1mm diameter, which were composed of short stubby, forked bulbous mycelia. They suggested that this type of pellet was desirable for citric acid production because 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 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 aicd. Clark *et al.* (1966) reported that filamentous growth of *A. niger* has little capacity to produce citric acid and itsoccurrence has always resulted in poor yields. They further demonstrated that manganese ions induced filamentous rather than pelletal growth during submerged cultivation.

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 in the yields was the different modes of growth in shake-flask culture (pellet-like) and in the stirred fermenter (filamentous). They concluded that the filamentous growth in the fermenter was due to manganese ions contamination of the medium from impurities in the stainless steel parts.

The reader is referred to the review of Whitaker and Long (1973) for further information regarding the importance of the pellet form of growth for citric acid production. However, it does appear that the favourable effect of the pellet form on citric acid production may be *via* an effect on aeration efficiency. Thus, factors such as manganese ions which are known to be detrimental to citric acid production may act through their effect on fungal morphology.

2.4.3.2 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 coincidentally minimizes the danger of 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 fermentation since this facilitates mycelial growth; thereafter the pH drops 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 low pH (1.7) both growth and acid production were greatly retarded. They

obtained the highest yield at pH 3.7-4.2. 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 upon the nature of the substrate; pH value 2.5-4.0 is optimum for defined media and an initial pH of 6.0-7.5 is required in molasses medium (Berry *et al.*, 1977).

2.4.3.3 Temperature

Temperature is an important factor in the control of citric acid production by *A. niger*. The temperature used will depend in part on the organism and the fermentation conditions. The optimum temperature range of 28-30^{o}C has been proposed for high yields and rapid rates of accumulation (Prescott and Dunn, 1959). Increasing the temperature above 30^{o}C has been found to decrease the citric acid yield and increase oxalic acid accumulation (Doelger and Prescott, 1934). The importance of the incubation temperature in determining the yield of citric acid has been emphasized by many investigators (for example, Kitos *et al.*, 1953; Martin, 1957).

2.4.3.4 Aeration

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

Karow and Waksman (1947) obtained maximum yields of citric acid when pure oxygen rather than air was supplied to the cultures. They concluded that oxygen is a limiting factor in the production of citric acid in submerged fermentation and if not supplied in sufficient amounts, alcohol will be formed and accumulate.

Buelow and Johnson (1952) studied the effect of aeration on citric acid production from a sucrose-based synthetic medium in 50-gallon tanks. They found that an increase in aeration rate from 0.9 to 3.5 millimoles of oxygen per litre/minute, obtained by increasing agitation

speed, resulted in decreasing the fermentation time by approximately 40 hours and increasing the yield from 55 to over 80% (based on utilized sugar).

Kovats and Gackowska (1976) have reported that interruption in aeration during the fermentation adversely affects citric acid production. Similarly Kubicek *et al.* (1980) reported that a short interruption of aeration (20 minutes) did not reduce the viability of the fungus but resulted in a complete and irreversible loss of ability to produce citric acid. They also reported critical DOT values of 18-21 and 23-26 mbar for the growth and production phases, respectively. The minimum DOT for citric acid production was about 25 mbar and production increased steadily between 40 and 150 mbar. The effect of DOT on the volumetric rate of oxygen consumption and citric acid production of *A. niger* is shown in Table 2.2, according to these authors. Thus, they concluded that citric acid accumulation is favoured by increasing the DOT of the fermentation medium and that any interruption in aeration adversely affects citric acid production.

2.4.3.5 Carbohydrate Source

In much of the early work on citric acid production, pure sugars (sucrose or glucose) were used as a source of carbon, but in present commercial practice the raw material is primarily beet molasses or cane molasses.

The concentration of carbohydrate material affects the yield of citric acid obtained (based on sugar utilized). Currie (1917) and Doelger and Prescott (1934) found a sucrose concentration of 100 g/l, in a synthetic medium, to be satisfactory. Porges (1932) reported that 120-200 g/l sugar solutions were necessary for higher citric acid yields, because in weak (50 g/l) solution the sugar was used as an energy source for growth and as a result less acid could be formed.

During citric acid production from whey permeate, Somkuti and Bencivengo (1981) reported that maximum citric acid yields were influenced by the initial lactose concentration. They obtained their highest yields

TABLE 2.2 EFFECT OF DISSOLVED OXYGEN TENSION (DOT) ON THE VOLUMETRIC RATE OF OXYGEN CONSUMPTION (dO_2/dt) AND CITRIC ACID PRODUCTION (dCIT/dt) OF *A. niger* (KUBICEK *et al.*, 1980)

Volumetric rates are given in µmol/l/min. The age of culture was 150 hours.

DOT (mbar)	dO_2/dt	dCIT/dt
159	106	32
132	102	28
93.5	90	23
80	-	21
50	85	14
39	-	9
32	74	3
18	63	0
8	57	0
5	43	0

when the initial lactose concentration was adjusted to 150 g/l.

In current commercial production of citric acid from molasses, using the submerged process, the concentration of sugar in the medium is usually adjusted to 160 g/l (Berry *et al.*, 1977).

There is very little comparative information available in the literature in respect of citric acid production from different sugar sources. Amelung (1927) obtained citric acid in various concentrations when glycerol, xylose, arabinose, glucose, fructose, galactose, mannose and mannitol were used in a synthetic medium in surface culture. He reported that in comparison with other sugars, very little citric acid was obtained from galactose. A similar report was published by Noguchi (1962), using data from shake-flask culture. In a comparative study with different carbon sources for citric acid production in surface culture, Bernhauer (1928) obtained variable concentrations of citric acid. The results of his experiments are summarized in Table 2.3 according to Foster (1949). Bernhauer considered that the fructose portion of the sucrose molecule was much more efficient in generating citric acid than free fructose. Similarly Doelger and Prescott (1934) obtained higher yields from sucrose than from either glucose or fructose, and concluded that the relative proportions of the two monosaccharides was important.

It is apparent from the literature reports that various crude carbohydrate sources have been used to produce citric acid using selected strains of *A. niger*, e.g. beet molasses (Gerhardt *et al.*, 1946; Martin and Waters, 1952; Clement, 1952; Steel *et al.*, 1955), cane molasses (Perlman *et al.*, 1946, Moyer, 1954; Gaden *et al.*, 1954), unrefined sucrose (Gardner *et al.*, 1956), sugar cane juice (Divekar *et al.*, 1971), potatoes (Masior, 1968), brewery waste (Hang *et al.*, 1977), cheese whey permeate (Chebotarev *et al.*, 1979) and lactic casein whey permeate (Somkuti and Bencivengo, 1981). Further, these reports suggest that pretreatment of these crude carbohydrates is essential to reduce the excessive metal ion contents which are reported to have detrimental effects on citric acid production. Thus, various

TABLE 2.3 CITRIC ACID YIELD FROM DIFFERENT CARBOHYDRATES (FOSTER, 1949).

Carbohydrate	Average (%) (w/w)	Highest Value (%) (w/w)
Sucrose	37.2	46.5
Fructose	36.9	44.5
Inulin	35.1	39.9
Glucose	24.6	31.7
Glyceraldehyde	23.9	30.2
Glycerol	20.3	30.0
Maltose	13.2	16.9
Xylose	12.0	15.0
Mannose	5.6	11.5
Arabinose	5.4	8.7
Mannitol	4.1	7.7
Galactose	1.8	6.2

techniques (e.g. cation exchange processes, treatment with ferrocyanide or other chemicals) have been used to minimize these ions in the fermentation media. Using a decationized sucrose solution, Perlman *et al.* (1946) obtained a threefold increase in the yield of citric acid when compared with crude sucrose. Similar increases in yields have been reported by Snell and Schweiger (1949) and Sanchez-Marroquin *et al.* (1970).

2.4.3.6 Nitrogen Source

The nitrogen requirement for citric acid production is generally met by the addition of ammonium sulphate, ammonium nitrate, sodium nitrate, potassium nitrate or ammonia. However, no single nitrogen source has been shown to be definitely superior to another and it is possible that the advantages occasionally observed were merely a measure of the purity of the compound used (Perlman and Sih, 1960).

In general, a low nitrogen concentration in the medium (less than 1.0 g/l) favours the production of citric acid, while a high nitrogen concentration favours abundant growth of mycelium with low acid production. However, the exact conditions seem to vary according to the carbohydrate source and fungal strain used (Loesecke, 1945).

Thus it appears that, for maximum production of citric acid, the concentration and source of nitrogen must be determined for the particular strain of fungus and fermentation conditions in use.

2.4.3.7 Trace Elements

Many variations in the requirements of some essential trace elements, such as Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} in the citric acid fermentation have been reported and these need to be investigated whenever a new strain or substrate is used (Berry *et al.*, 1977). The regulatory action of the mineral constituents of the medium on the growth and citric acid accumulation of *A. niger* is presented in Table 2.4 according to Berry *et al.* (1977).

TABLE 2.4 REGULATORY ACTION OF THE MINERAL CONSTITUENTS OF MEDIUM ON THE GROWTH AND CITRIC ACID ACCUMULATION OF *A. niger* (BERRY *et al.*, 1977).

Constituent	Concentration	Stimulation
NH_4^-, NO_3^-	Less than 1.0 g/l	Citric acid production
	High, above 1.0 g/l	Mycelium growth
PO_4^{--}	Deficiency	Citric acid production
	High, above 0.1 g/l	Mycelium growth
Trace Metals		
Fe^{2+}	Less than 1.0 mg/l	
Zn^{2+}	Less than 1.0 mg/l	
Cu^{2+}	0.1-1.0 mg/l	Citric acid production
Mn^{2+}	Less than 1.0 µg/l	

The optimum concentration of Fe^{2+} in the fermentation medium for production of citric acid is controversial. However, it is generally agreed that the concentration should be very low. Shu and Johnson (1947, 1948b) stated that in submerged culture citric acid production occured only in media deficient in Fe^{2+} and Mn^{2+}. Shu and Johnson (1948b) reported that at high Fe^{2+} levels the heavy growth of mycelium which occured consequently depressed the yield of citric acid because of the increased amount of sugar utilization as a growth substrate. Tomlinson *et al.* (1951) reported that a combination of Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} was essential for obtaining high yields of citric acid from a reagent grade sucrose-based synthetic medium. They further reported that the omission of any one of these four elements from the fermentation medium caused a marked reduction in acid formation. Similar observations were reported by Trumpy and Millis (1963) and Srivastava and Kamal (1979).

Clark *et al.* (1966) studied the effect of physiologically important metals on the morphological appearance and citric acid production of *A. niger* NRC A-1-233 during submerged fermentation of beet molasses. Their results are summarized in Table 2.5. To study the effect of Mn^{2+} in more detail, they added various concentrations of Mn^{2+} to the fermentation medium and found that the yield of citric acid was reduced by about 10% by the addition of as little as 2 ppb at the start of the fermentation. They reported that greater additions of up to 100 ppb resulted in sharp decreases in the yield of citric acid with marked changes in the pellet morphology from hard solid pellets to a filamentous form. They concluded that Mn^{2+} adversely affected the fungal morphology and citric acid production. Similar conclusions about the effect of Mn^{2+} were drawn by Kisser *et al.* (1980) and Heinrich and Rehm (1982). In addition, the effect of Mn^{2+} on some TCA-cycle enzymes has been reported by Kubicek and Rohr (1977). They demonstrated that the presence of Mn^{2+} in the fermentation medium increased the specific activities of aconitase and isocitric dehydrogenase (both NAD- and NADP-linked) and decreased the specific activity of citrate synthase.

TABLE 2.5 EFFECT OF DIFFERENT METALS ON CITRIC ACID PRODUCTION AND PELLET MORPHOLOGY (CLARK *et al.*, 1966)

Metals Added	Amount Added (ppm)	Citric Acid Yield After 96 hr (%) (w/w)	Pellet Morphology
None	-	8.8	Normal
Al^{3+}	5.0	7.3	"
Ca^{2+}	80.0	9.0	"
Co^{2+}	0.1	9.0	"
Cu^{2+}	3.0	8.6	"
Fe^{2+}	100.0	7.5	"
Mg^{2+}	20.0	8.8	"
Ni^{2+}	0.1	8.6	"
Zn^{2+}	5.0	7.5	"
Mn^{2+}	1.0	1.5	Filamentous
Mixtures of all metals	Concentrations shown above	1.4	Filamentous
Mixtures of all metals except Mn^{2+}	"	7.4	Normal

The critical influence of Fe^{2+}, Cu^{2+} and Zn^{2+} on citric acid production have also been reported. Iron has been reported to cause a stimulation of aconitase activity and concurrently decrease the yield of citric acid (La Nauze, 1966). The effect of Cu^{2+} as an antagonist to Fe^{2+} was suggested by Schweiger (1961) and Zn^{2+} has been reported to influence several enzyme systems associated with sugar metabolism in *A. niger* (Bertrand and De Wolf, 1959). It is also reported that Zn^{2+} has an important role in the regulation of growth and citric acid production, and its deficiency during growth of the fungus apparently signals the transition from the growth phase to the acid production phase (Kapoor *et al.*, 1982).

It is clearly impossible to make predictions of the precise amounts of trace elements required for the maximum yield of citric acid from any particular strain grown on a specific medium. The optimum medium composition will be found only by experimental measurement.

2.4.3.8 Presence of Methanol and other Compounds

One of the important advances 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 increased citric acid production. He employed a large number of strains of the *A. niger* group in a survey to determine the effect of various concentrations of methanol on the production of citric acid from sucrose. He reported that most of the organisms showed a marked increase in total acid production and in fermentation efficiency. He concluded that higher levels of Fe^{2+}, Zn^{2+} and Mn^{2+} could be tolerated by the organism in either surface or submerged culture if a slightly toxic concentration of methanol were present. Moyer (1953b) demonstrated that the addition of methanol (final concentration 2-4% (v/v)) at the time of inoculation greatly stimulated the production of citric acid by *A. niger* from crude carbohydrate sources, e.g. gelatinized cornstarch, beet molasses, blackstrap molasses, in either surface or submerged culture. The exact role of methanol in the stimulation of citric acid production was not elucidated by the author,

but he pointed out that methanol was not assimilated during fermentation. He emphasized that the use of methanol should find application in the commercial production of citric acid. There are several reports which are consistent with the findings of Moyer, that methanol is beneficial in increasing citric acid yield (for example Noguchi, 1960; Noguchi and Arao, 1960; Noguchi and Bando, 1960; Hang *et al.*, 1977).

Somkuti and Bencivengo (1981) reported that the addition of methanol to lactic casein whey permeate stimulated citric acid production by *A. niger* in shake flask culture. They obtained 70% and 136% yield increases when methanol was added to the fermentation medium at the rate of 2% (v/v) and 4% (v/v) respectively after 48-72 hours of incubation.

Thus, it appears that methanol is not assimilated, but when added to the growth medium at a concentration of 1 to 4% (v/v), citric acid production is stimulated. The mechanism of action is not known, but it is probably a general toxicity, causing enzyme inhibition or denaturation of cell proteins, resulting in depression of growth and possibly an increase in cell permeability.

The addition of some other chemicals to the fermentation medium has been reported to enhance citric acid production in submerged fermentations. Ohtsuka *et al.* (1975) reported that malic hydrazide (1 g/2.5 kg molasses) increased the citric acid yield from 30% to 70% under submerged culture conditions. The stimulation of citric acid production by the addition of some mild oxidizing agents such as hydrogen peroxide or naphthoquinone to the fermentation medium has been reported by Bruchmann (1966). The mechanisms by which these chemicals stimulate citric acid production have not been explained.

Some short chain carbohydrates, such as glycerol, or lipid materials have been reported to enhance citric acid production by *A. niger* when added to the fermentation substrate. Leopold (1971) stated that the addition of glycerol to molasses medium (30-50 g/l)

increased the citric acid yield by 30%. However, the glycerol was apparently metabolized and utilized as a carbon source.

Millis *et al.* (1963) studied the effect of lipids on the production of citric acid. They observed that fatty acids with a chain length of 15 carbon atoms, or natural oils with high content of unsaturated fatty acids, or oleic acid itself, when added at 2% (v/v) to a sucrose-based synthetic medium, increased the yield of citric acid by about 20%. They also observed that lipids which improved the yield of citric acid had no effect on the mycelial dry weight. They suggested that β-oxidation of the lipids could provide additional acetyl co-enzyme A (acetyl-CoA) for citric acid formation, and so increased the yield. They further suggested that the unsaturated fatty acids were acting as alternative hydrogen acceptors to oxygen during fermentation, thus allowing the organism to metabolize actively for longer and so improving the yields of citric acid.

Cyclic AMP has been reported to stimulate the accumulation of citric acid by *A. niger*. Wold and Suzuki (1973) found that when a concentration of 10^{-6} M or higher was added to a sucrose-based synthetic medium, the rate of citric acid production increased. It was suggested that citric acid accumulation in *A. niger* perhaps resulted from an abnormal cAMP metabolism.

In summary, there are many reports regarding the essential factors affecting the accumulation of citric acid. The many differences in these reports appear to be due to the different strains of organism used, with different media compositions. Obviously, for any new medium and/or strain of organism, the environment must be optimized by experimentation.

2.5 INDUSTRIAL PROCESSES

There are three basic processes used for commercial production of citric acid (Lockwood, 1975):

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

The precise technical details of these processes have been kept secret by the companies concerned. However, examination of some of the patents issued to various companies has given some information as to the processes used (Lockwood, 1975).

The Koji fermentation process:

This relatively simple fermentation process has been developed in Japan. Cooked solid vegetable residues are spread in trays and inoculated with a selected strain of *A. niger*. Sweet potato residues or wheat bran are used as substrates. During incubation, the starch is saccharified by the amylase of *A. niger* and much of the sugar produced is converted to citric acid. The temperature inside the solid mass is usually maintained at 28°C and the pH drops to 1.8-2.0 as the citric acid accumulates. The mass is extracted with water in percolators after 5-8 days of incubation and the citric acid is then purified. Estimated production by this method is only about 2,500 tons (Lockwood, 1975).

The liquid culture shallow pan process:

This method is commonly called the surface culture method and is the oldest method in use in Europe and the U.S.A. It is estimated that 30 acres of shallow pans are required in one large citric acid plant. Pans are made of high purity aluminium or stainless steel to avoid problems of corrosion and trace metal ion contamination. Beet molasses is commonly used as substrate but raw sugar or high-test syrup are also used. Molasses is usually treated with ferrocyanide to remove traces of iron and the precipitate is removed by filtration. The medium is then adjusted to a pH value of 2.5-4.0, using sulphuric acid, prior to inoculation. Spores of selected strains of *A. niger* are blown over the sterile solution in the pans. They germinate within the first 24 hours, and a thin fragile white pellicle of mycelium covers the surface of the solution. The temperature of

incubation is maintained at 30°C and sterile, humidified air is blown slowly over the surface of the solution for a period of 5-6 days. After 8-10 days of incubation, the sugar content of the culture solution is reduced from its initial level of 200 g/l to 10 g/l, and the maximum concentration of citric acid is achieved. The yield of citric acid by this process is about 85% on the basis of initial carbohydrate supplied.

The submerged fermentation process:

Figure 2.2 outlines the process used by different companies for citric acid manufacture by the submerged process (Lockwood, 1975). The raw materials used include beet molasses, commercial syrups of high glucose content and high-test cane syrup. A pelletal form of inoculum from a selected strain of *A. niger* is used to inoculate the fermenter, and the culture is agitated and aerated during the entire fermentation period. The duration of fermentation depends on the initial sugar concentration, subsequent sugar additions and the amount of growth. Usually it ranges from 6-14 days but uniformity is maintained for a given set of conditions. The yield of citric acid by this process is about 95% on the basis of total carbohydrate supplied (Lockwood and Batti, 1965).

Nowadays, the submerged fermentation process rather than surface fermentation process is used for commercial production of citric acid, because of the following advantages (Sodeck *et al.*, 1982):

- higher yield of citric acid with regard to the sugar used
- improved process control
- reduced fermentation period
- reduced floor space required
- reduced manual handling
- lower investment cost.

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

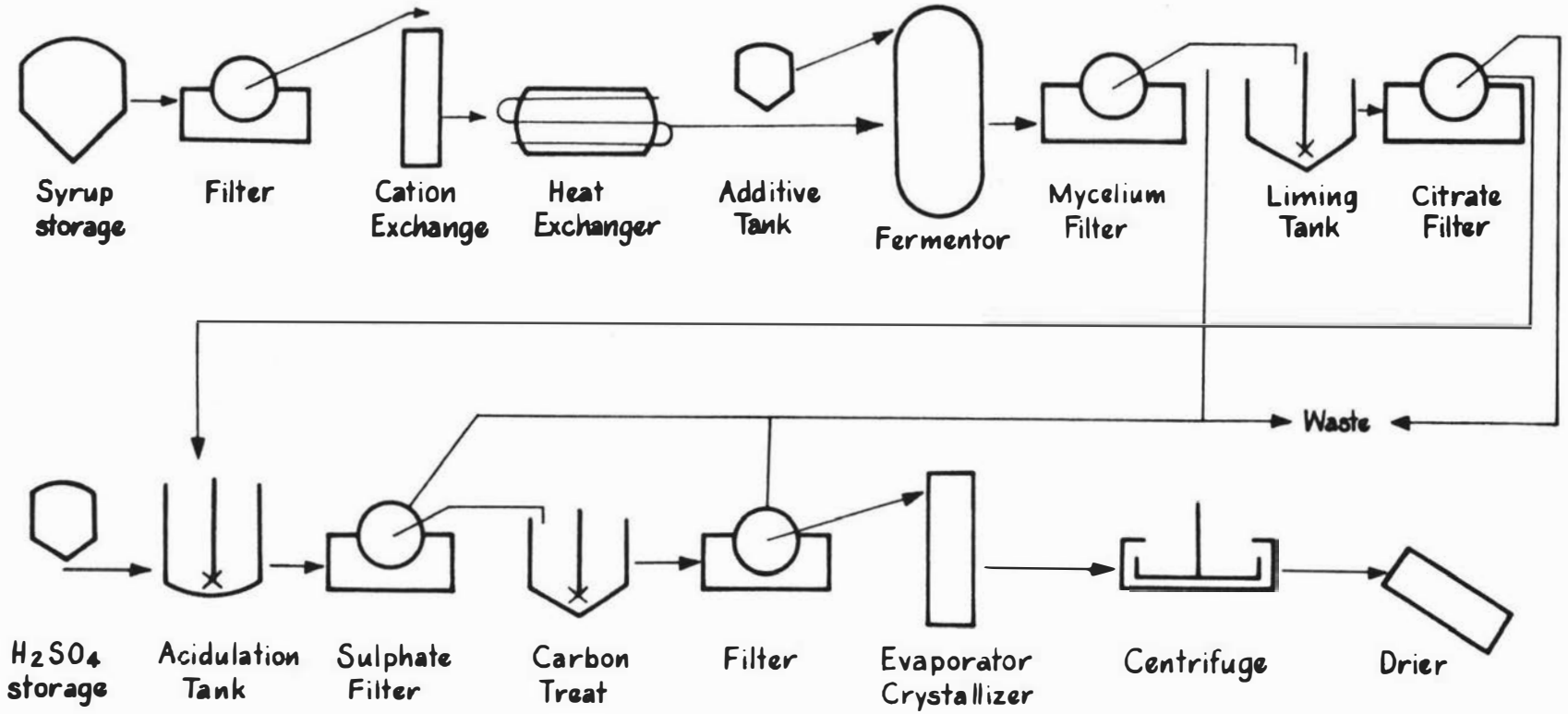

FIGURE 2.2 Flow diagram for submerged citric acid manufacture (Lockwood, 1975).

2.6 WHEY AS SUBSTRATE FOR CITRIC ACID PRODUCTION

Very little information is available with respect to citric acid production from whey. However, two reports have recently appeared in the literature, one a Russian patent by Chebotarev *et al.* (1979) and the other a paper originating in U.S.A. by Somkuti and Bencivengo (1981).

The original patent report was not available in New Zealand. However, the available abstract indicates that these authors used cheese whey as substrate and a strain of *A. niger* as a citric acid-producer. The medium was adjusted to pH 4-5 and sterilized at 130-132^{o}C prior to inoculation. The fermentation was continued for 5-6 days at a temperature of 25-28^{o}C, but no details were given of the process. The citric acid was separated from culture medium by precipitation with calcium or barium salts. No data were reported in respect of the yield or concentration of acid obtained.

Somkuti and Bencivengo (1981) used lactic casein whey permeate as a substrate and a mutant strain of *A. niger* as a citric acid-producer. The natural pH of acid-whey permeate (4.5) was used and the fermentation was performed in shake-flask culture for a period of 12-14 days at a temperature of 30^{o}C. They obtained a citric acid concentration of 28 g/l when the lactose concentration of whey permeate was adjusted to 150 g/l with additional lactose.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 MICROBIOLOGICAL MEDIA

Potato Dextrose Agar (PDA) was obtained from Oxoid Ltd, London, England. Microbiological Agar was obtained from Davis Gelatin Ltd, Christchurch, New Zealand. Yeast Extract was obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A. Bacto Beef Extract and Nutrient Broth were obtained from Difco Laboratories, Detroit, Michigan, U.S.A. The Sucrose-Beef Extract medium adopted for the sporulation of *A. niger* IMI 41874 and mutant strain *A. niger* MH 15-15 was prepared according to Sanchez-Marroquin *et al.* (1970) (Table 3.1). The liquid medium described by Kristiansen and Charley (1981) (Table 3.2) was used to investigate the effect of sugar source on citric acid production. Media used for the screening of mutants are described in Table 3.3.

Lactic casein whey permeate was supplied by the N.Z. Dairy Research Institute, Palmerston North, New Zealand. It was prepared according to the method described by Matthews (1978) and its composition is tabulated in Table 3.4.

TABLE 3.1 SUCROSE-BEEF EXTRACT MEDIUM OF SANCHEZ-MARROQUIN *et al.* (1970).

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

* The medium was made to volume with distilled water.

TABLE 3.2 MEDIUM OF 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
[a] Fe^{3+}	0.1×10^{-3}
[b] Zn^{2+}	0.1×10^{-3}
[c] Cu^{2+}	0.06×10^{-3}

* The medium was made to volume with distilled water and adjusted to pH 6.5 using 1 M NaOH.

[a] as $(NH_4)_2SO_4 . Fe_2(SO_4)_2 . 24H_2O$.

[b] as $ZnSO_4 . 7H_2O$.

[c] as $CuSO_4 . 5H_2O$.

TABLE 3.3 SCREENING MEDIA FOR MUTANT SELECTION.

No.	Component	Concentration (g/l)
(a)	Agar	20.0
	Na_2CO_3	5.0
	Ox gall	50.0 (70% solids)
	The medium was made to volume with lactic casein whey permeate.	
(b)	Medium in Table 3.2, where galactose was used as sugar source, plus the following:	
	Agar	20.0
	Na_2CO_3	5.0
	Ox gall	50.0 (70% solids)
	The medium was made to volume with distilled water.	
(c)	Citric Acid Medium	
	Citric acid	20.00
	NH_4Cl	2.00
	$MgSO_4 . 7H_2O$	0.25
	KH_2PO_4	0.25
	Yeast extract	1.00
	The medium was made to volume with distilled water and adjusted to pH 3.5 using 1 M NaOH.	

TABLE 3.4 COMPOSITION OF LACTIC CASEIN WHEY PERMEATE .

Component	Concentration (g/l)*
Lactose	43.0
Lactic acid	5.2
Citric acid	1.4
Total nitrogen	0.55
Non-protein nitrogen[a]	0.46
Metal Ions	
K^+	600.0
Na^+	372.0
Ca^{2+}	150.0
Mg^{2+}	57.0
Zn^{2+}	3.0
Mn^{2+}	0.13
Fe^{2+}	1.2
Pb^{2+}	0.27
Ni^{2+}	0.13
Cu^{2+}	0.17
pH 4.5	

* Metal concentration is expressed as mg/l.

[a] According to Kavanagh (1975), expressed as mg/g.

3.1.2 GASES

Oxygen-free nitrogen and carbon dioxide gases were supplied by New Zealand Industrial Gases Ltd, Palmerston North, New Zealand.

3.1.3 SUGARS

Sugars used in the preparation of media were all of analytical grade. Sucrose was obtained from Ajax Chemicals, Sydney, Australia. Fructose and lactose were obtained from BDH Chemicals Ltd, Palmerston North, New Zealand. Galactose and glucose were obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A.

3.1.4 CHEMICALS

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

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

  Amberlite Resin 1R-120(H); ammonium ferrous sulphate; ammonium sulphate; buffer tablets: pH 4.0 and 7.0; citric acid; copper sulphate; ethylenediaminetetraacetic acid; Folin-Ciocalteu's phenol reagent; orthophosphoric acid; 2-oxoglutaric acid; potassium dihydrogen orthophosphate; potassium cyanide; succinic acid; tris (hydroxymethyl) methylamine.

- Diversey-Wallace Ltd, (Papatoetoe, New Zealand).

  Pyroneg$^{(R)}$ detergent.

- ICI Ltd, (Wellington, New Zealand).

  Methanol.

- J.T. Baker Chemical Co., (Phillipsburg, New Jersey, U.S.A.).

  Acetonitrile (HPLC grade).

- May and Baker Ltd, (Dagenham, England).

  Lactic acid.

- N.Z. Pharmaceuticals Ltd, (Palmerston North, New Zealand).

  Ox gall.

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

  Adenosine 5-monophosphoric acid (AMP; muscle adenylic acid); adenosine 5'-triphosphoric acid (ATP, sodium salt); bovine serum albumin; β-nicotinamide adenine dinucleotide (NAD); β-nicotinamide adenine dinucleotide, reduced form (NADH, disodium salt); β-nicotinamide adenine dinucleotide phosphate (NADP, monosodium salt); β-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, malic dehydrogenase and pyruvic acid (sodium salt).

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

  Dow-corning antifoam A.F. emulsion (Food grade).

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

  Buffers for glucose and lactose analysis (using enzyme electrode method).

3.1.5 ORGANISMS

The fungi used in this work were obtained from the following sources:

Commonwealth Mycological Institute, Kew, Surrey, England.

Aspergillus carbonarius	IMI 41873
Aspergillus niger	IMI 27809
A. niger	IMI 31821
A. niger	IMI 41874
A. niger	IMI 51433
A. niger	IMI 75353
A. niger	IMI 83856
A. niger	IMI 84304

American Type Culture Collection, Rockville, Maryland, U.S.A.

Aspergillus niger	ATCC 12846
A. niger	ATCC 26036
A. niger	ATCC 26550

By mutation during strain improvement work.

A. niger	MH 15-15 (a mutant of *A. niger* IMI 41874)

All organisms, with the exception of *A. niger* IMI 41874 and *A. niger* MH 15-15, were maintained on slopes of Potato Dextrose Agar (PDA) and subcultured every two months. After inoculation, the slopes of PDA were incubated at 30°C for 5 - 6 days. After sporulation, the cultures were kept at 4°C.

A. niger IMI 41874 and mutant strain *A. niger* MH 15-15 did not sporulate well on PDA. Hence, cultures were maintained on slopes of Sucrose-Beef Extract Agar (Table 3.1), where they sporulated profusely.

Spores of *A. niger* IMI 41874 and *A. niger* MH 15-15 were preserved in the following way:

(a) The organisms were grown on slopes of Sucrose-Beef Extract medium 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 clumps and the concentration was adjusted to 1 - 2 x 10^8 spores/mL using sterile distilled water.

(c) Spore suspension (2 mL) was dispensed aseptically into 3 mL of nutrient broth containing 30% (v/v) glycerol in a 10 mL capacity screw-cap bottle (15 such bottles were prepared from the same spore suspension). 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 medium and incubated at 30°C for 5 - 6 days. One further subculture was performed on this medium (for better sporulation) prior to inoculum preparation.

3.2 MEDIA STERILIZATION

All microbiological media were sterilized by autoclaving at 121°C for 15 minutes.

3.3 CLEANING OF GLASSWARE

All glassware was washed in hot pyroneg(R) solution, rinsed in tap water, then in distilled water and hot air dried. Glassware used for enzyme assays and for the storage of HPLC solvents was treated in chromic acid solution after the detergent wash, then rinsed thoroughly with glass-distilled water prior to drying.

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 known 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 Whatman No.54 filter paper, which was previously dried at 105 ± 2°C and pre-weighed. The fungal mass was washed thrice with distilled water (about 250 mL) and dried to constant weight at 105 ± 2°C.

3.4.3 ANALYSIS OF SUGARS

The following methods were used at various times for the determination of sucrose, glucose, galactose, fructose and lactose.

3.4.3.1 The Anthrone Method

The sugar content of fermentation samples was determined by an adaptation of the anthrone method described by Ghosh *et al.* (1960). This method was used for those results described in Chapter 4.

The experimental sample was diluted to between 0 and 100 µg sugar per mL of solution. Ice-cold, anthrone solution (2 mL, 0.2% (w/v) in conc. H_2SO_4) was added to 1 mL of the diluted sample and mixed thoroughly. The mixed samples were heated for 15 minutes in a boiling water bath and then cooled in ice. Sulphuric acid (2 mL, 66% (v/v)) was added and the samples were again mixed thoroughly. The absorbance of the sample was read at 625 nm against a reagent blank using a Cecil CE-272 Linear Readout U.V. Spectrophotometer (Cecil Instruments, Cambridge, England). Results were calculated from a standard curve (concentration range 0 - 100 µg sugar/mL) of the particular sugar under investigation. All determinations were performed in duplicate.

3.4.3.2 Enzyme Electrode Method

An enzyme electrode method was used to determine the lactose and glucose concentration in fermentation samples. A YSI Model 27 sugar analyser (Yellow Springs Instruments Ltd, Yellow Springs, Ohio, U.S.A.) was used. This method was used for those results described in Chapter 5 (Part I).

3.4.3.3 High Performance Liquid Chromatography (HPLC)

This method was used for the results described in Chapters 5 (Part II), 6 and 7. When mixtures of sugars were used in fermentation media, they could all be separated and determined by HPLC, except for glucose and galactose mixtures. Under the analytical conditions used the retention times of these two sugars were identical. In this situation the total concentration of glucose and galactose in the mixture was determined by HPLC and then the glucose concentration was determined separately by the enzyme electrode method (Section 3.4.3.2). To obtain the galactose content of the mixture, the glucose content was deducted from the total galactose and glucose.

Quantitative analysis of different sugars (viz. sucrose, glucose, fructose, galactose and lactose) was performed 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 μ Bondapak family Carbohydrate Analysis Column (3.9 mm ID x 30 cm, Part No.84308, Waters Associates) was used for the analysis.

The detector was a Model R401 differential refractometer (Waters Associates). The response was recorded on a CR600 twin-pen, flat-bed chart recorder (J.J. Lloyd Instruments Ltd, Southampton, England). Analyses were conducted at ambient temperature. The solvent system was a mixture of acetonitrile and water (ratio 80 : 20). The solvent flow rate was 1.7 to 3.0 mL/min depending on the resolution required. A typical chromatogram of different sugars using these conditions is presented in Figure 3.1.

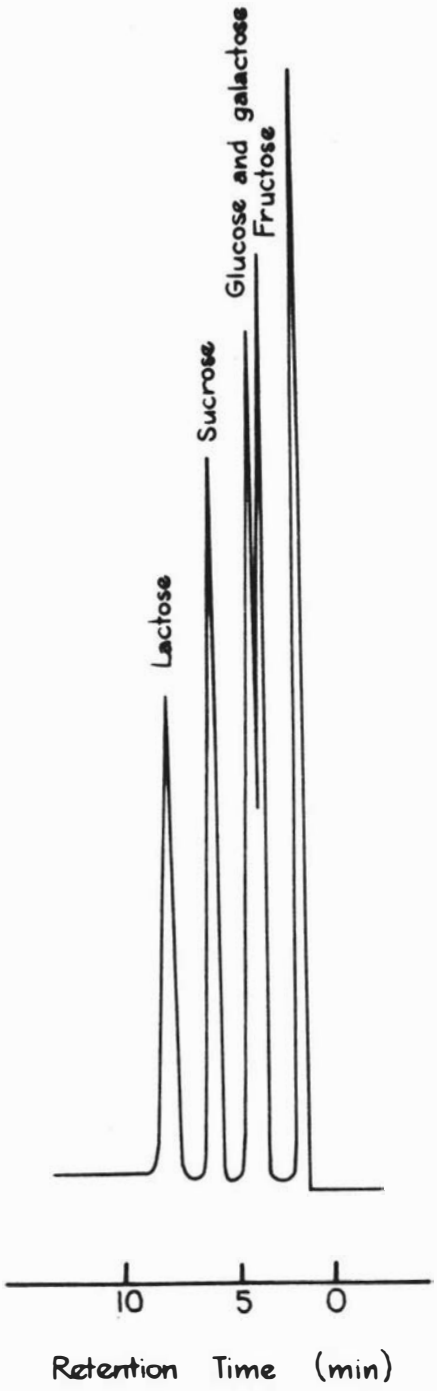

FIGURE 3.1 The HPLC separation of sugars.
Solvent flow rate = 2 mL/min.

Between 25 µL and 50 µL of sample was injected into the chromatograph, the exact amount depending on the sugar concentration in the sample.

The quantitation of individual sugars was done by measuring the peak height of sugar with reference to peak height of a standard curve. The standard curves were linear up to 10 g/l concentration (50 µL injected volume). Typical standard curves are shown in Figure 3.2.

3.4.4 Determination of Citric Acid and Lactic Acid

Citric acid and lactic acid were determined by an HPLC method (isocitric, 2-oxoglutaric and succinic acids could also be detected by this method). The equipment used was as described in Section 3.4.3.3 except a µ Bondapak C_{18} 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 deionized glass-distilled water and adjusted to pH 2.4 using orthophosphoric acid (according to Coppola *et al.*, 1978). The solvent flow rate was 0.5 to 2.0 mL/min depending on the resolution required. Typical chromatograms of appropriate substances using these conditions are shown in Figures 3.3 and 3.4.

Between 25 µL and 50 µL of sample was injected into the chromatograph. The amount of citric acid present in the experimental samples was normally calculated by measuring the peak height of acid with reference to peak height of the standard curve. The standard curve was linear up to 5 g/l concentration (50 µL injected volume). Such a standard curve is shown in Figure 3.5. This method of calculation was used for the quantitation of citric acid for Chapters 5, 6 and 7.

On certain occasions an internal standard method was used for the quantitation of citric and lactic acids. In this case, samples of 50 µL volume, containing a standard amount of citric (or lactic)

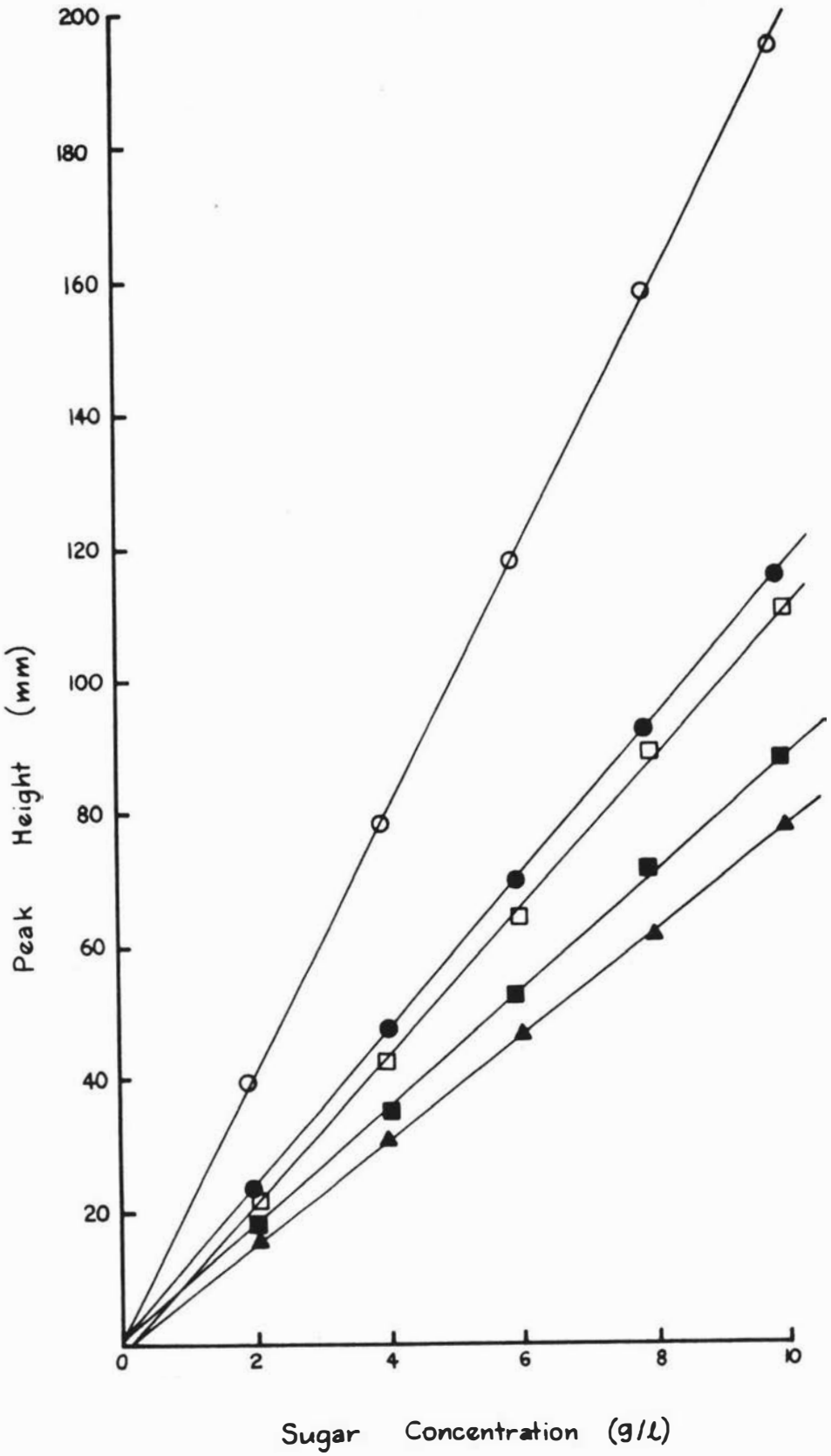

FIGURE 3.2 Standard curves for sugar analysis by HPLC. Sucrose (□), glucose (●), fructose (○), galactose (■), lactose (▲). (See Section 3.4.3.3 for chromatographic conditions).

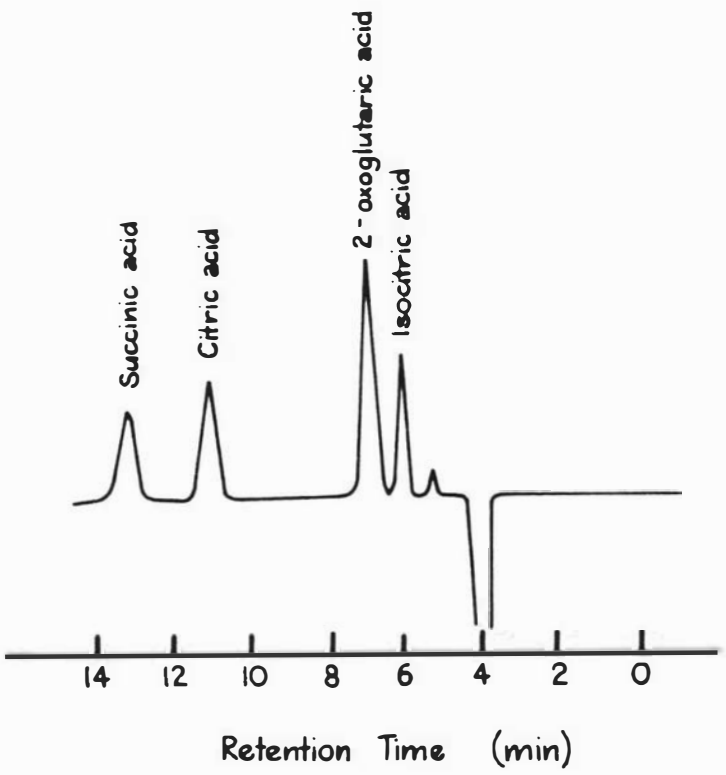

FIGURE 3.3 The HPLC separation of some TCA-cycle acids.

Solvent flow rate = 0.5 mL/min.

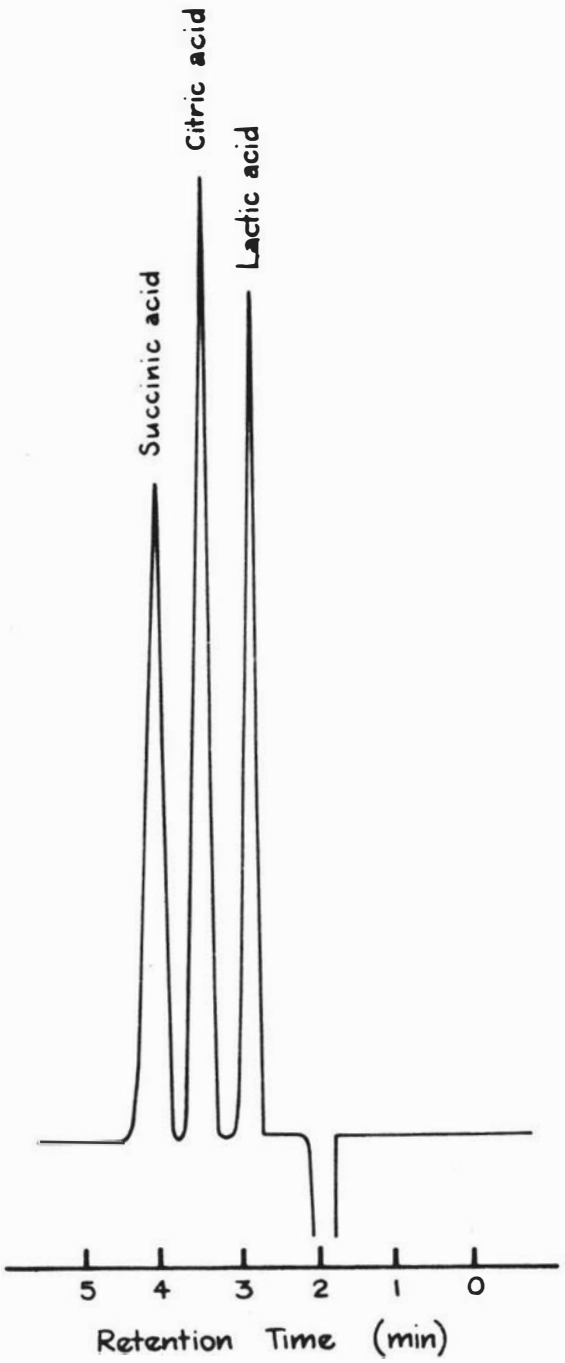

FIGURE 3.4 The HPLC separation of lactic acid, citric acid and succinic acid.

Solvent flow rate = 1.5 mL/min.

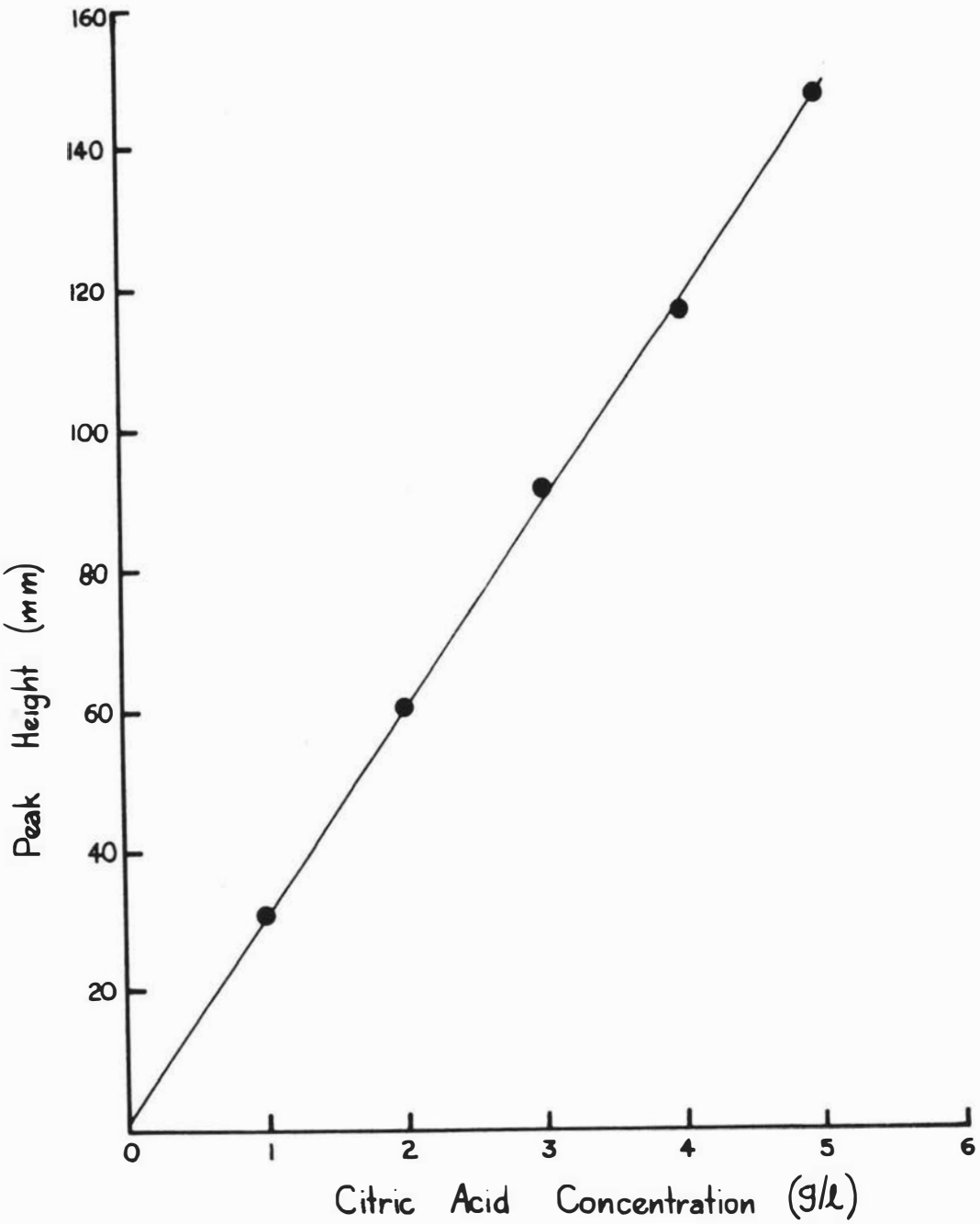

FIGURE 3.5 Standard curve for citric acid analysis by HPLC (see Section 3.4.4 for chromatographic conditions).

acid and 5 g/l succinic acid as internal standard, were injected and peak height ratios were measured. A similar quantity of the sample to be analysed, also containing 5 g/l succinic acid, was injected and the peak height ratio was compared with the standard to obtain the concentration of acid (refer Section 3.10.1 for calculation). This method was used to obtain the results described in Chapter 4.

Thorough cleaning of the HPLC column (μ Bondapak C_{18}) was necessary after the use of potassium dihydrogen orthophosphate solutions. Normally, deionized degassed glass-distilled water (2 mL/min for 1 hour) was used. This was followed by cleaning with methanol/water (50 : 50) and pure methanol for 20 and 30 minutes respectively.

In certain cases, when the resolution was poor, a more extensive cleaning procedure was adopted in the following sequence. For each step the cleaning solution was passed at a rate of 2 mL/min.

(a) Glass-distilled water for 1 hour.

(b) 0.01M oxalic acid solution for 15 minutes.

(c) Glass-distilled water for 30 minutes.

(d) Methanol/water (50 : 50) for 30 minutes.

(e) Pure methanol for 30 minutes.

(f) Tetrahydrofuran/dichloromethane (1 : 1) for 30 minutes.

(g) Then followed the steps e, d and c in that order.

3.4.5 DETERMINATION OF TOTAL NITROGEN

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

A known volume of the fermentation liquor or weighed sample of oven-dried mycelia (0.02 - 0.04 g N) was transferred to a Kjeldahl digestion flask. Sodium sulphate (2 g), mercuric sulphate solution (5 mL; of 150 g red mercuric oxide dissolved in a solution of 180 mL conc. sulphuric acid plus 1320 mL water) and conc. 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. The heating was then continued for a further 1 hour. The cooled contents were then transferred quantitatively to a 100 mL volumetric flask. The ammonia concentration of the digested samples (10 mL) was determined by steam distillation in a Markham still apparatus after the addition of sodium hypophosphite (5 mL; 15% (w/v)) and sodium hydroxide (15 mL; 60% (w/v)). The distillate containing ammonia was collected in boric acid solution (10 mL; 2% (w/v)) with 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 then titrated with 0.02 M HCl to grey-green end point. All determinations were carried out in duplicate.

3.4.6 Trace Element Analysis

Trace element analysis of whey permeate and sugars was performed by either the Chemistry Department, Massey University, Palmerston North, New Zealand or by the N.Z. Dairy Research Institute, Palmerston North, New Zealand, using a Varian Techtron Atomic Absorption Spectrophotometer type A.A.5, except for potassium and sodium which were determined using an EEL Flame Photometer.

3.5 Method of Mutation

3.5.1 Preparation of Spore Suspension

The 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. This preparation was thoroughly shaken for 1 hour on a Griffin Flash Shaker (Griffin and George Ltd, London, England) and then filtered through sterile glass wool to remove mycelium and spore clumps. The spores were counted using a haemocytometer (Assistent, Germany) and the required concentration was obtained by dilution with sterile distilled water.

3.5.2 Spore Irradiation

Spore suspension (10 mL) was pipetted into a sterile petri dish (90 mm diameter x 15 mm depth) and irradiated for a pre-determined time period (2 - 30 mins) by placing the dish 11 cm below a Universal -UV-Lamp, (Camag, Muttenz-Schweiz, 29200), operating at a wavelength of 254 nm. During irradiation the suspension was swirled gently. The entire procedure was performed in a dark room and the irradiated suspensions were shielded from light until plated out in order to minimize any photoreactivation effects.

3.5.3 Post-Irradiation Treatment

Following irradiation, the spore suspension was diluted to contain 400 - 500 viable spores/mL. 0.1 mL of the diluted suspension was then spread across the surface of a selected screening medium (Table 3.3 (a) or (b)). Control plates of spores without irradiation were similarly prepared. After 4 - 5 days of incubation at 30^{o}C, discrete colonies appeared on the plates. The acid-producing capacity of different colonies was determined by dividing the diameter of the clear zone surrounding the colony by the diameter of the colony and the figure so obtained was termed 'acid unitage' (James *et al.*, 1956). It was then possible to compare, in a semi-quantitative way, acid production by isolates with different colony size. The acid unitage was calculated for each colony and colonies which gave higher values than the parent strain were isolated and transferred to slopes of Sucrose-Beef Extract Agar for subsequent testing in liquid medium.

3.6 Medium Decationization

3.6.1 Preparation of Resin

Amberlite resin IR-120(H) was used as the cationic exchange material. Resin (200 g) was suspended in glass-distilled water to give a bed 43 cm in height and 3.2 cm in diameter in a glass column of 65 cm height. The resin was washed thoroughly with 1 litre of glass-distilled water, followed by 1 litre of 0.1 M hydrochloric acid (10 mL/min). The charged resin was washed again with glass-distilled

water until the pH value of the effluent returned to that of distilled water.

3.6.2 DECATIONIZATION

Before passing through the resin bed, the medium was filtered through Whatman No.54 filter paper to remove particulate material. The whey permeate was then supplemented with lactose (100 g/l) and the solution was passed through the resin at a flow rate of 20 mL/min and the pH of the effluent was regularly monitored. In the case of whey permeate the pH value decreased from 4.5 to pH 1.5 during the treatment. The sugar solutions were prepared at a concentration of 140 g/l before passing through the resin.

3.6.3 REGENERATION OF RESIN

The expended resin beds were regenerated for future work by thorough washing with glass-distilled water followed by charging with 0.1 M hydrochloric acid and rewashing with glass-distilled water until the pH value of the effluent returned to that of distilled water. The regenerated resins were used for three cycles before they were discarded.

3.7 CULTURE CONDITIONS

3.7.1 SHAKE-FLASK CULTURE

Experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL of medium.

The Erlenmeyer flasks were siliconized using Sigmacote$^{(R)}$ (Sigma Chemical Co., St Louis, Missouri, U.S.A.) which formed a microscopically thin film of silicone on the glass surface, thus preventing wall growth of mycelium. The flasks were coated, dried in hot air and then washed with distilled water and dried before use.

Spores (approximately 1×10^8) from a standard spore suspension (Section 3.5.1) were used to inoculate 100 mL of fermentation medium

and incubated at 30°C on an Environ-Shaker Model 3597 (Lab-line Instruments Inc., Illinois, U.S.A.) for 10 - 14 days at an operating speed of 180 rpm. For sampling, the entire contents of a flask were taken.

3.7.2 FERMENTER CULTURE

The fermenter apparatus used was constructed in the Biotechnology Department, Massey University. Figure 3.6 shows photographs of the fermenter vessel and its head assembly, while Figure 3.7 shows a schematic diagram of the fermenter vessel itself. Figures 3.8 and 3.9 show respectively a photograph and schematic diagram of the fermenter unit and its ancillary equipment.

3.7.2.1 Equipment and Instrumentation

The fermenter vessel used was a six litre capacity glass jar (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.), provided with a stainless steel head containing ports for the insertion of the probes and other facilities required. The dimensions of the fermenter vessel and impellers are shown in Figure 3.7. 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. ¼ 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 ± 0.2°C by means of hot or cold water flowing through hollow baffles and was controlled by an electronic thermostat controller. The temperature was continuously recorded using a Honeywell Varsaprint Multipoint chart recorder (Amiens, France).

Air was supplied to the fermenter vessel through a blower

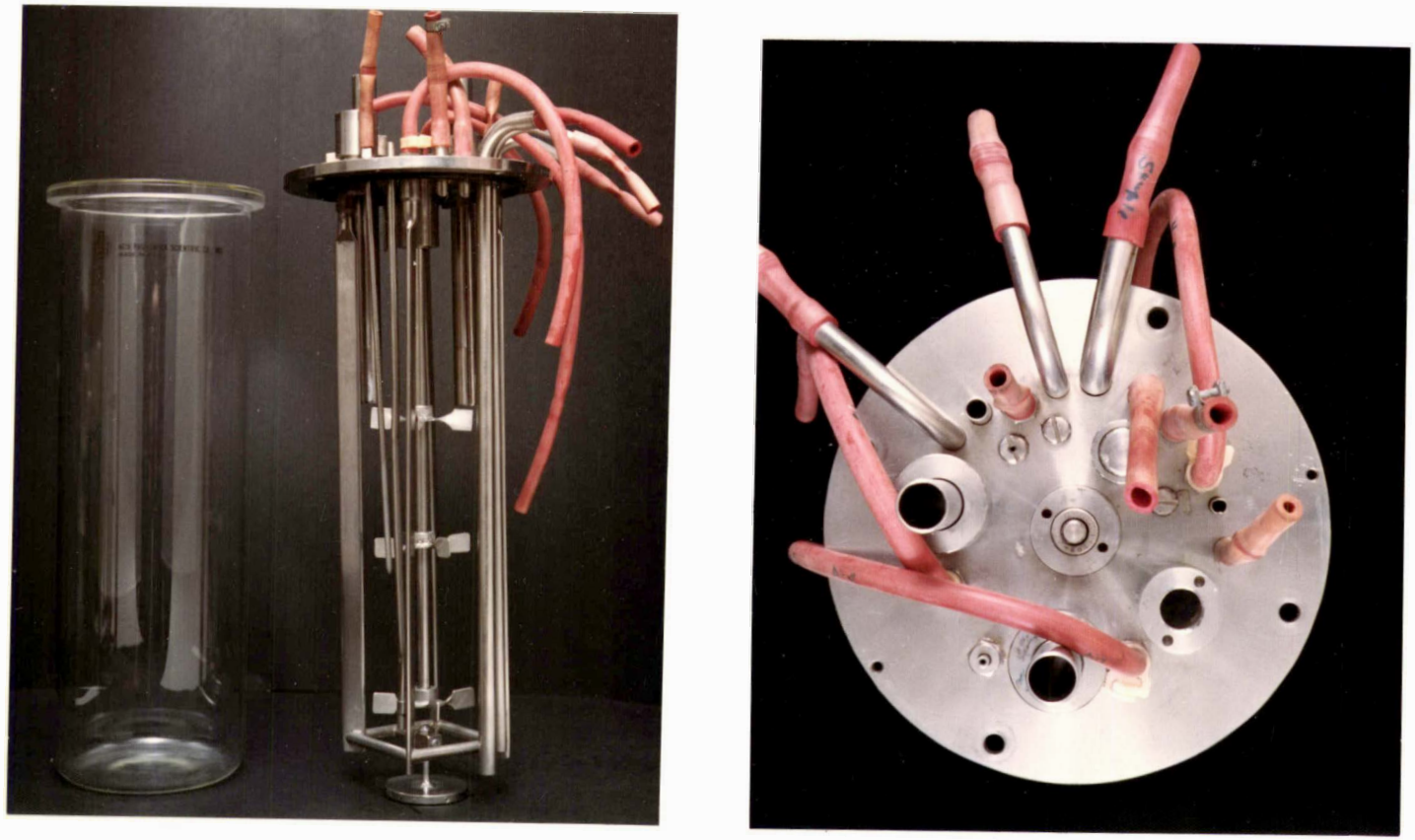

FIGURE 3.6 Photographs of fermenter vessel and its head assembly.

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

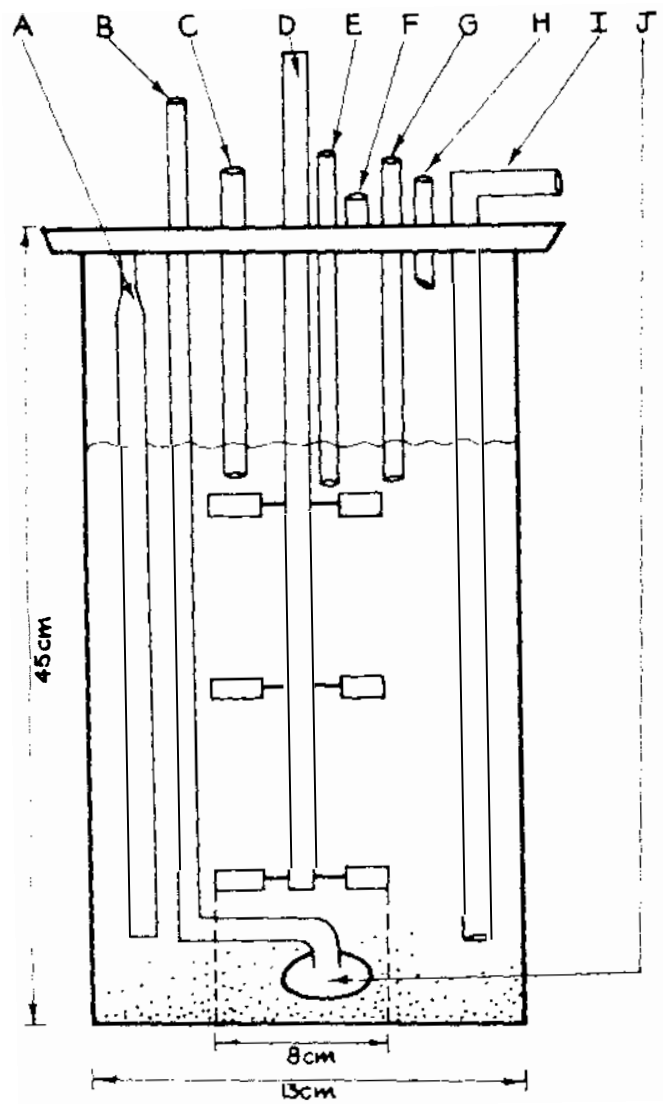

FIGURE 3.7 A schematic diagram of the fermenter vessel, showing various facilities and probes in the vessel head.

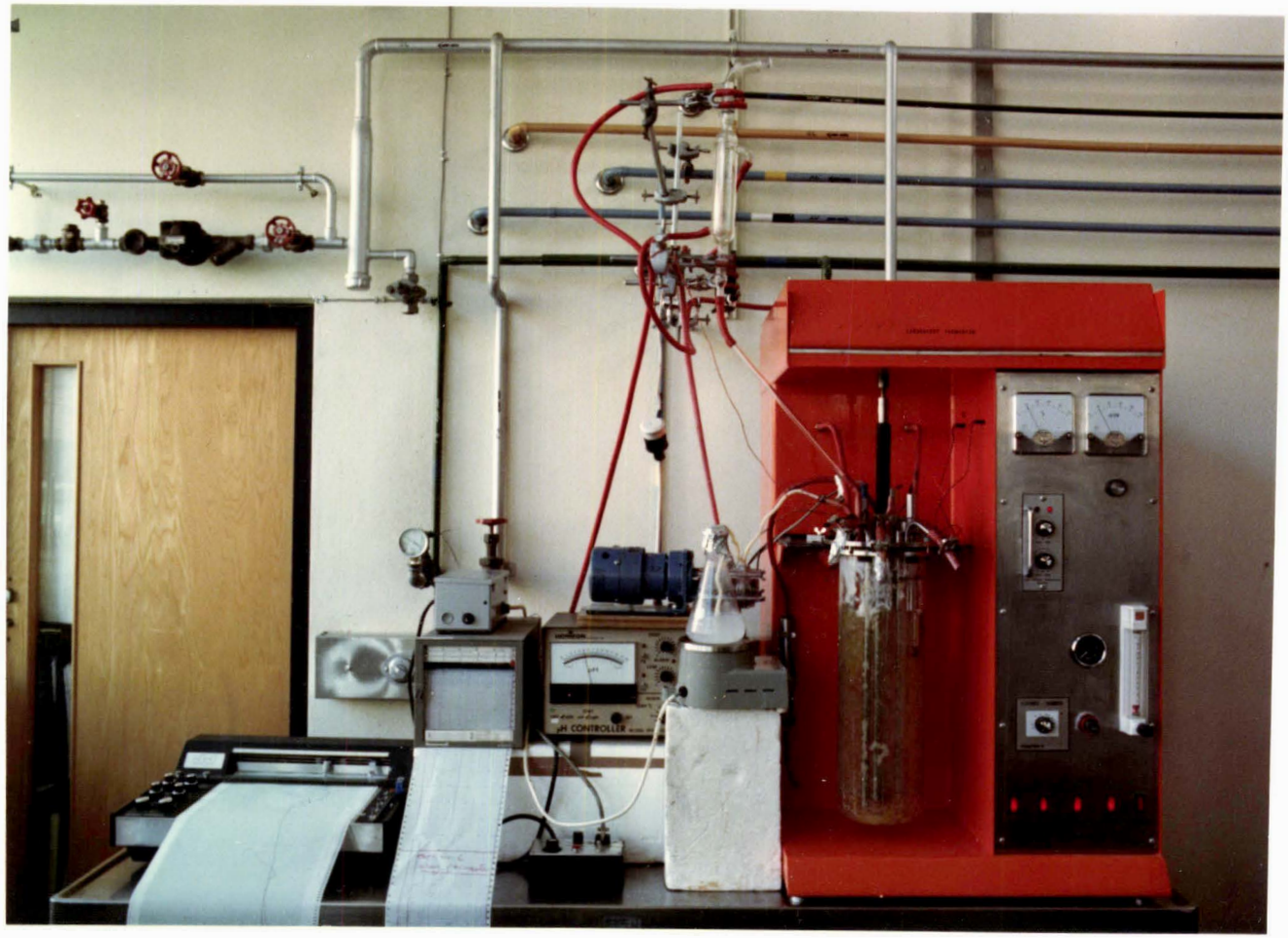

FIGURE 3.8 A photograph of the fermenter and its ancillary equipment.

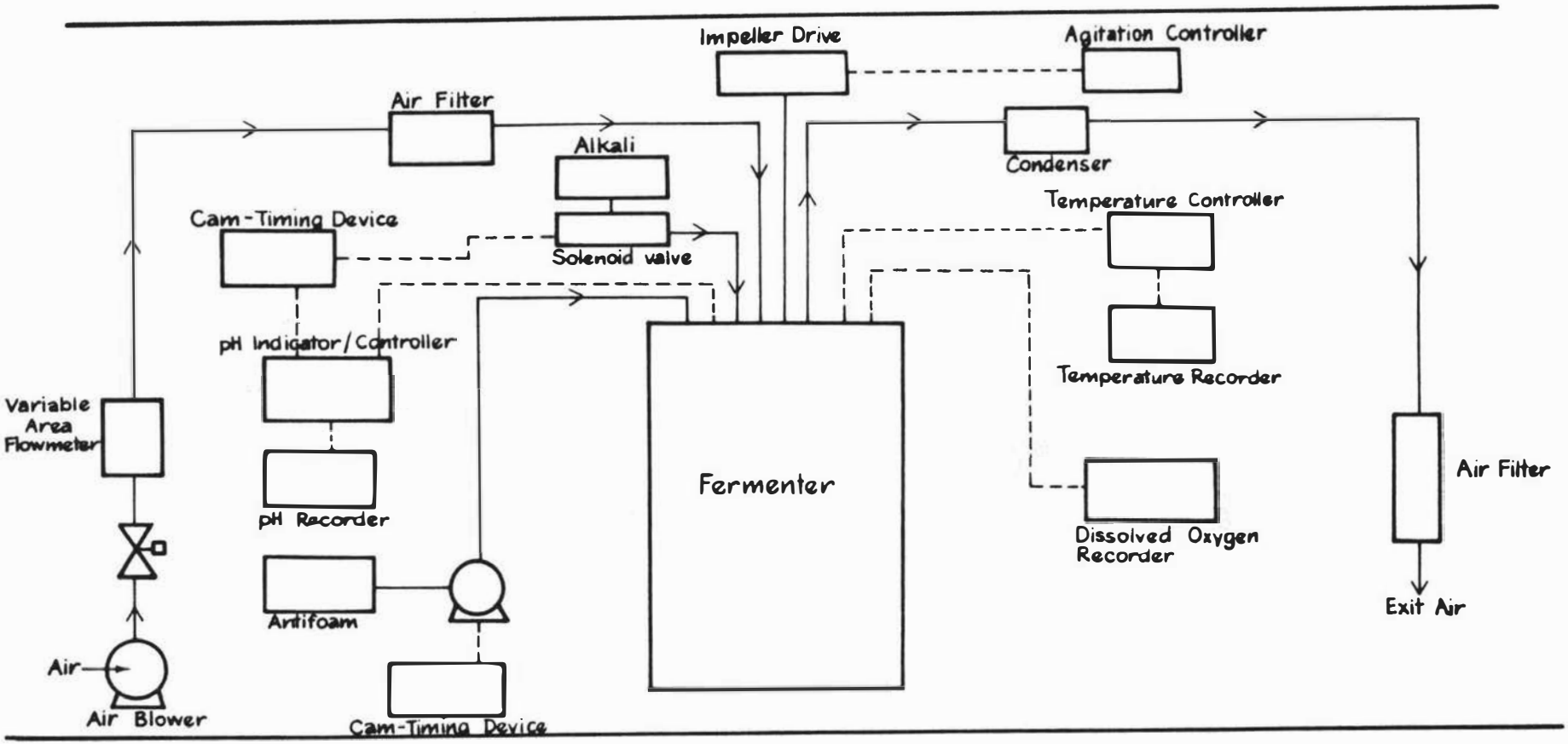

FIGURE 3.9 A schematic diagram of the fermenter and its ancillary equipment.

situated in the fermenter apparatus. The air flow rate was controlled by a needle valve and measured with a Gap variable area flowmeter (Platon Flow Control Ltd, Basingstoke, Hampshire, England) over the range of 1 - 12 litre/min. The measured air was passed through a sterile glasswool-packed filter, before entering the culture vessel through a sparger of 6 cm diameter stainless steel plate provided with 10 evenly spaced 0.3 cm holes (Figure 3.7).

Exhaust air was vented through a water cooled condenser to prevent the loss of fermentation liquid by evaporation. The condenser was ultimately connected to an exhaust air filter.

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., North Oak Park Avenue, Chicago, Illinois, U.S.A.).

In one experiment the pH of the culture broth was maintained at a desired value by the addition of 1 M NaOH through a pH meter-controlled solenoid valve (Fluorocarbon, Anaheim, California, U.S.A.). The culture pH was continuously recorded using a Honeywell Varsaprint Multipoint chart recorder (Amiens, France).

The pH value of each fermentation sample was independently measured in order to check the accuracy of the fermenter pH control system, and any discrepancies were corrected.

Antifoam emulsion (DOW-Corning Antifoam A.F) was added to the culture to supress the heavy foam generated by the high agitation and aeration rates employed during the fermentation. Heavy foaming was encountered during the first 30 - 36 hrs of fermentation and a 5% (w/v) sterile emulsion was added at a controlled rate (1 - 5 mL/min) by a pump (Cole-Parmer Inst. Co., Chicago, Illinois, U.S.A.) which was actuated for 10 seconds in every 15 minutes by a cam-timing device. No antifoam was required after 48 hours of fermentation. The rate of antifoam addition was adjusted, depending on the degree

of foaming, through a variable speed controller.

Dissolved oxygen tension was measured using a dissolved oxygen probe (Type M 1016-0202, New Brunswick Scientific Co., Inc., New Jersey, U.S.A.), and recorded using a chart recorder (J.J. Lloyd Instruments Ltd, Southampton, England). The probe was calibrated *in situ*, prior to inoculation of the fermenter, by flushing the contents of the fermenter vessel 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% saturation. The saturation condition was held for 30 minutes to ensure stable dissolved oxygen probe operation and the chart recorder span was set at 95% full scale.

3.7.2.2 Sterilization

The fermenter vessel containing the medium (4.5 litre) and all systems in the fermenter head except the pH and dissolved oxygen probes were sterilized in an autoclave for 15 minutes at $121^{o}C$.

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

3.7.2.3 Preparation of Inoculum

Spores (approximately 1×10^{8}) (refer section 3.5.1) were used to inoculate 100 mL of medium (whey permeate or Kristiansen and Charley, Table 3.2) in a 250 mL Erlenmeyer flask. The inoculated medium was then incubated at $30^{o}C$ in shake-culture for 36-40 hours. The contents of the flasks (containing small pellets of about 0.2-0.3 mm diameter) were used for inoculation of the fermenter (10% (v/v) inoculum).

3.7.2.4 Sampling

The routine withdrawal of samples (about 30 mL) was performed through the sample withdrawal system of the fermenter for mycelial dry weight, pH, citric acid and sugar determination. In some experiments samples were withdrawn for enzyme assay using cell-free extracts.

Prior to sampling, the sample withdrawal system was flushed with 10-15 ml of the culture fluid to remove any resident "dead" volume. The volumes of flush and samples withdrawn from the fermenter were recorded for volumetric analysis of the fermentation components.

3.7.2.5 Wall Growth

Mycelial growth around the vessel wall 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.

3.8 PREPARATION OF SAMPLES FOR HPLC ANALYSIS

Mycelium was removed by filtration and the filtered 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 μm) using a Swinney Filter Kit (Millipore Corporation, Bedford, Massachusetts, U.S.A.).

3.9 ENZYME ASSAYS

3.9.1 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 water (about $4^{o}C$), followed by washing with sufficient 0.1M potassium phosphate buffer (pH 7.4) to bring the pH to neutrality. The mycelium was then rewashed with cold glass-distilled water.

The washed mycelium was suspended in cold 0.1M potassium phosphate buffer, pH 7.4, containing 1mM 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 5g/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, 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 during homogenization. Shaking was continued for 1 minute at 4000 rpm at an operating temperature of about 3-4^{o}C. The homogenate was then separated from the ballotini beds by filtering through a glass sinter. The filtered liquid was centrifuged at 25000 x g for 30 minutes at 0^{o}C using a refrigerated centrifuge (Sorvall Superspeed RC 2-B Automatic Refrigerated Centrifuge). The creamy supernatant liquid was maintained at 3-4^{o}C and assayed for enzyme activity as soon as possible. Where necessary the extract was diluted using 0.1M potassium phosphate buffer, pH 7.4.

All assays were carried out at room temperature (about 23^{o}C) using a Cecil CE-272 Linear Readout UV Spectrophotometer (Cecil Instruments, Cambridge, England). Assays were conducted in triplicate and the average result was recorded.

The specific activity of the enzymes was expressed as µmoles of product/min/mg of protein. The amount of product formed during the reaction time was calculated from the standard curve of that product. Such standard curves of cis-aconitate and NADH or NADPH are shown in Figures 3.10 and 3.11 respectively.

3.9.2 Protein Estimation

The protein content of the cell-free extract was estimated by the method of Schacterle and Pollack (1973). Diluted cell-free

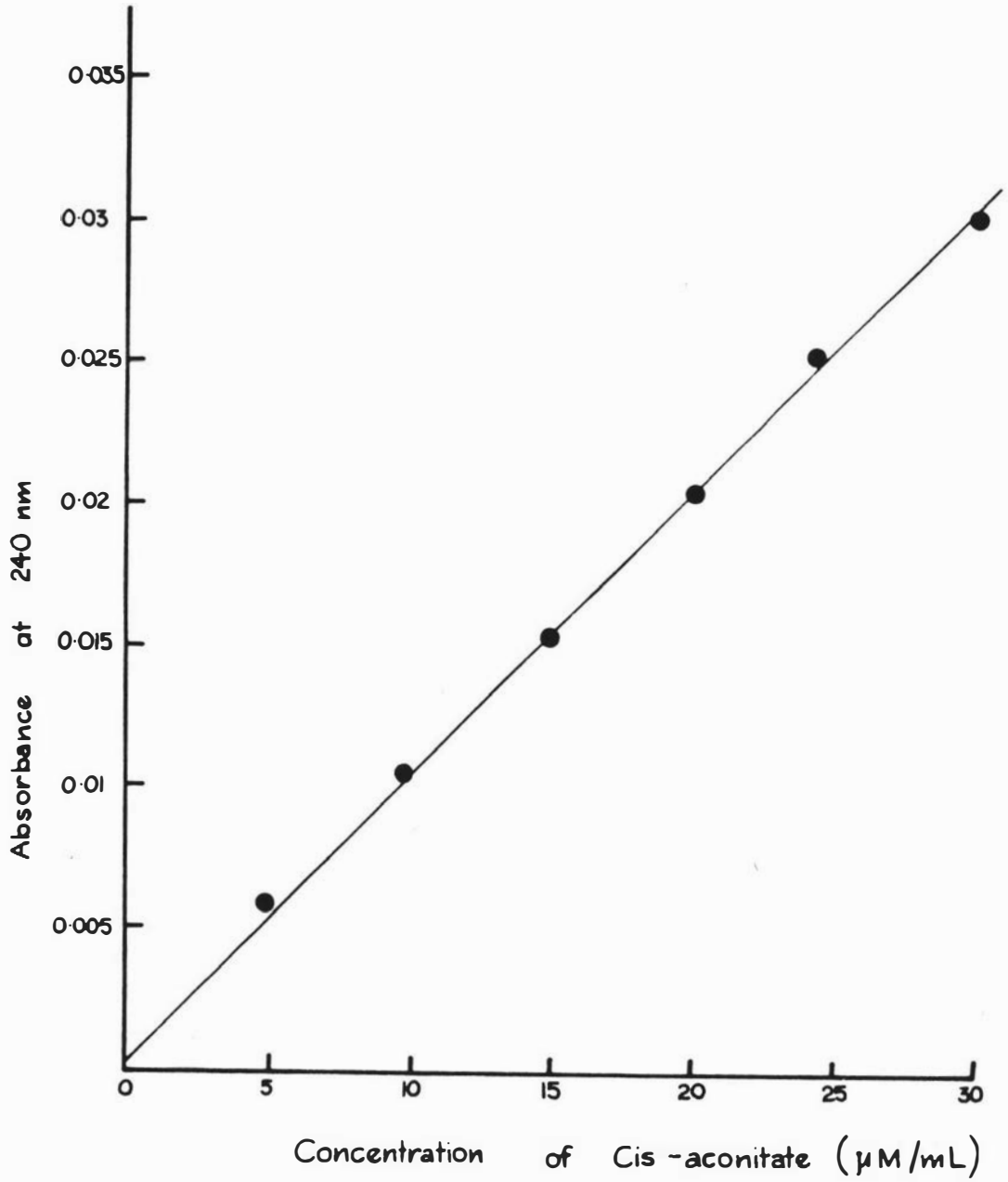

FIGURE 3.10 Standard curve for cis-aconitate at 240 nm.

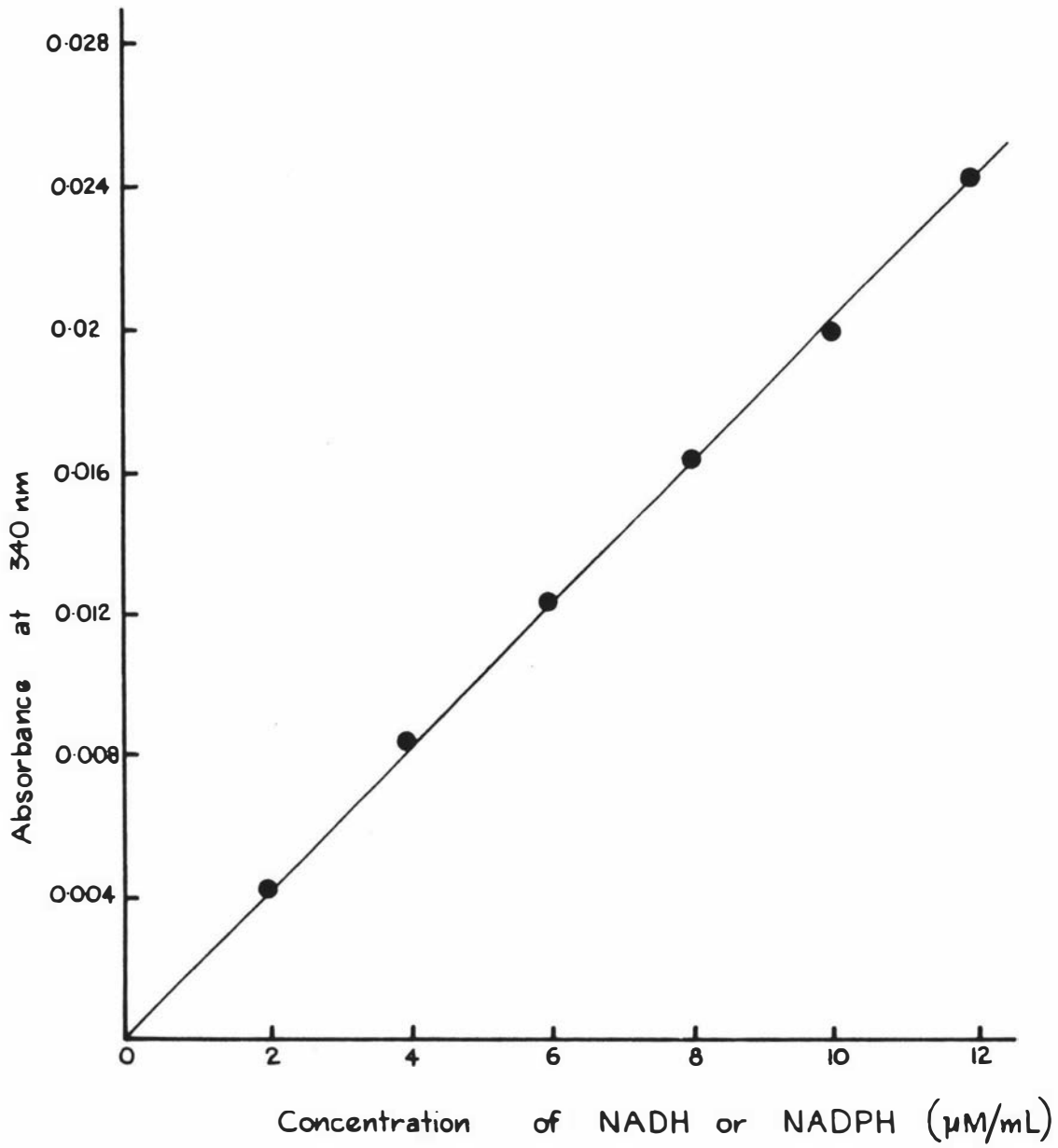

FIGURE 3.11 Standard curve for NADH or NADPH at 340 nm.

extract (1 mL containing not more than 50 µg protein/mL) was mixed with 1 mL of alkaline copper reagent (0.5M sodium hydroxide, containing 10% (w/v) sodium carbonate, 0.1% (w/v) potassium tartrate and 0.05% (w/v) copper sulphate) in a test tube and allowed to stand for 10 minutes. Folin-Ciocalteu's phenol reagent (4.0 mL, stock diluted x 18) was pipetted forcibly and rapidly into the test tube and the tubes were placed in a water bath at 55°C for 5 minutes. They were then cooled for 1 minute under tap water and the absorbance was read at 650 nm.

The equivalent amount of protein present was calculated from a standard curve of bovine serum albumin. The standard curve was linear within the range 0-50 µg/mL (Figure 3.12).

3.9.3 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: 0.1M DL-isocitrate in 0.05M potassium phosphate buffer (pH 7.4), 0.4 mL; diluted enzyme extract, 0.1 mL, and 0.05M phosphate buffer (pH 7.4), 2.5 mL. The blank contained (in 3 mL) all components other than substrate. The rate was obtained by monitoring the increase in absorbance at 240 nm over a period of 5 minutes.

3.9.4 NAD-Linked Isocitric 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: 0.5M potassium phosphate buffer (pH 7.4), 0.2 mL; 0.005M NAD, 0.3 mL; 0.0025M AMP, 0.3 mL; 0.1M $MgCl_2$, 0.1 mL; 0.3M KCN (freshly neutralized), 0.1 mL; 0.1M DL-isocitrate, 0.1 mL; diluted enzyme extract, 0.1 mL, and glass-distilled water, 1.8 mL. The blank contained (in 3 mL) all the

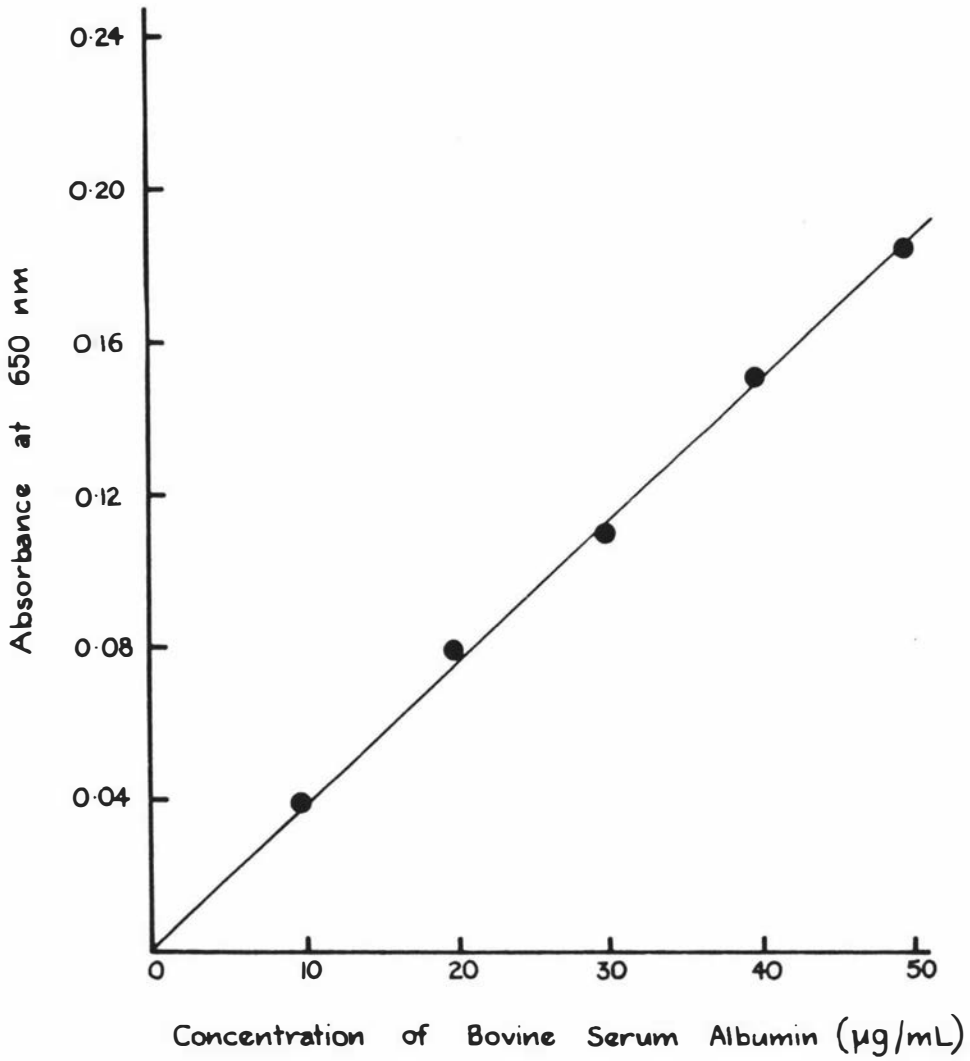

FIGURE 3.12 Standard curve for bovine serum albumin at 650 nm.

components other than the substrate. The rate was obtained by monitoring the increase in absorbance at 340 nm over a period of 5 minutes.

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

3.9.6 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: 0.5M potassium phosphate buffer (pH 8.0), 0.3 mL; 10 mM $MgCl_2$, 0.3 mL; 0.01M 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; 0.1M 2-oxoglutarate, 0.03 mL; diluted enzyme extract, 0.1 mL, and glass-distilled water, 1.58 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.

3.9.7 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 Trishydrochloride 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), and diluted enzyme extract, 0.1 mL. The blank contained (in 3 mL) all the components other than substrate. The rate was obtained by monitoring the decrease in absorbance at 340 nm over a period of 5 minutes.

3.10 CALCULATIONS

3.10.1 HPLC DATA ANALYSIS

In some experiments, quantitation of citric acid and lactic acid was accomplished by peak height measurement using an internal standard in the sample and comparison with parallel standard solutions containing known amounts of citric or lactic acid.

From the parallel standard a response factor, F_C was calculated.

The following calculation is shown for citric acid.

$$F_C = (\frac{IS}{C})_P / (\frac{IS}{C})_W$$

Where: $(\frac{IS}{C})_P$ is the peak height ratio of internal standard: citric acid.

$(\frac{IS}{C})_W$ is the weight ratio of internal standard: citric acid in the standard solution.

The response factor was used to calculate the amount of citric acid in the sample.

$$C_W = IS_W * (\frac{C}{IS})_{PX} * F_C$$

Where: C_W is the weight of citric acid in the sample (g).

IS_W is the weight of the internal standard in the sample (g).

$(\frac{C}{IS})_{PX}$ is the ratio of citric acid: internal standard peak height from the sample chromatogram.

F_C is the response factor for citric acid.

3.11 DISCUSSION OF METHODS

3.11.1 MAINTENANCE OF STOCK CULTURES

A. niger loses its ability to produce high yields of citric acid due to degeneration of conidial material during storage (Rohr and Kubicek, 1981). In the present study, when the culture was maintained by repeated sub culturing on Sucrose-Beef Extract Agar, noticeable changes were observed in the morphological characteristics. Thus, the original colour of the spores changed to a brownish colour and occasionally, 'foggy' patches of asporogenous mycelium were observed. This morphological change coincided with a drastic reduction in citric acid yield and may have been caused by spontaneous genetic changes during repeated transfers. This degeneration of the culture was prevented by preserving the spores as described in Section 3.1.5. The same stock culture was then used throughout the entire period of experimentation and regular checks proved it to be morphologically stable, with reproducible yields of citric acid.

3.11.2 SCREENING MEDIUM FOR MUTANTS

The development of a suitable screening medium to assess the acid-producing ability of large numbers of isolates after mutation was considered important in isolating a superior mutant. Initially, a medium was devised containing lactic casein whey permeate (1000 mL), agar (20 g) and $CaCO_3$ (10 g) on the basis that acid production would cause a clearing of the $CaCO_3$ in the area surrounding the colony. Unfortunately, the growing colonies spread rapidly over the surface of the agar, making it difficult to discern zones of clearing. Thus, in order to restrict the colony size, ox gall (50 g/l (70% solids)) was added to the medium (Montenecourt and Eveleigh, 1977). This successfully restricted the colony size, but interferred with the zone of clearing due to formation of a precipitate, probably the calcium

salt of bile acids, within the clear zone. This was finally overcome by the replacement of $CaCO_3$ with Na_2CO_3 (Table 3.3 (a) (b)). This medium allowed screening of 40-50 colonies on a single plate, while giving distinct zones of clearing.

On certain occasions, the medium containing galactose (Table 3.3 (b)) was used as a screening medium in an attempt to isolate mutants which could produce citric acid from galactose.

The citric acid medium (Table 3.3 (c)) was used to test the ability of selected mutants to use citric acid as sole carbon source. This test was used in parallel with the production of citric acid from lactic casein whey permeate.

CHAPTER 4

STRAIN SELECTION AND IMPROVEMENT

4.1 INTRODUCTION

Since the early discovery by Wehmer (1893) of the presence of citric acid in spent culture media of species of *Penicillium*, a variety of fungi have been investigated and screened for citric acid production. Of these various fungi, there is general agreement that only selected strains of *Aspergillus niger* are useful for commercial production of citric acid.

An available cheap carbohydrate source is always preferable (for economic reasons) for citric acid production by fungal fermentation. When a new type of raw material is to be used for the fermentation, investigations are needed to determine the exact conditions which are required for high yields of citric acid. The most important step of this investigation is to select a suitable strain of *A. niger* for that particular raw material. Foster (1949) reported that a medium ideal for one organism may be poor for another organism, yet the latter may itself produce high yields of citric acid under a different set of conditions.

The main objective of the work described in this chapter was to select a particular strain of *A. niger* through a screening programme and subsequently to improve it by induced mutation.

4.2 RESULTS AND DISCUSSION

4.2.1 STRAIN SELECTION

Ten strains of *A. niger* and one strain of *A. carbonarius* were investigated for the production of citric acid from whey permeate. Experiments were conducted in shake-flask culture using lactic casein whey permeate (without any supplementation) and the fermentation was

continued for 10 days. Flasks were harvested after 2,4,6,8 and 10 days of incubation and analysed for citric acid, lactic acid, pH, mycelial dry weight and lactose concentration. The experimental results (after 10 days of incubation) are summarized in Table 4.1. The results indicate that *A. niger* IMI 75353 and *A. niger* IMI 83856 failed to grow. The remainder of the organisms grew and five of them produced citric acid. *A. niger* IMI 41874 produced the highest concentration of citric acid (5.0 g/l) which represents a yield of 13.5% (w/w) on the basis of lactose utilized. Among the non-citric acid producing strains, several caused an increase in pH value during the fermentation. The probable reason for this was the depletion of lactic acid from the medium without the production of any other acids. All the citric acid-producing strains utilized both lactose and lactic acid.

The five citric acid-producing strains were selected for further screening in shake-flask culture, where the lactic casein whey permeate was supplemented with extra lactose (100 g/l). Experimental conditions were similar to those of the previous screening programme and the results are tabulated in Table 4.2. All of the strains showed an increase in both citric acid production and yield. *A. niger* IMI 41874 again produced the highest concentration of citric acid (8.2 g/l), representing a yield of 15.5% (w/w) on the basis of lactose utilized.

Since it produced both the highest concentration and yield of citric acid, *A. niger* IMI 41874 was selected for a strain improvement programme by induced mutation.

Table 4.3 shows published examples of citric acid production on various media by some of the strains used in this screening programme. It is immediately apparent that the results obtained in lactic casein whey permeate are poor in comparison. This may be due to the nature of the sugar source, i.e. lactose, or may simply represent non-optimization of the medium, e.g. trace element composition. However, it is clear that for some strains the lactose poses a problem. For example, *A. niger* IMI 75353, which has been studied by many investigators, failed to grow on lactic casein whey permeate. To further investigate

TABLE 4.1 CITRIC ACID PRODUCTION BY DIFFERENT STRAINS OF *Aspergillus* IN LACTIC CASEIN WHEY PERMEATE AFTER 10 DAYS OF INCUBATION.

Strain	Final pH[a]	Mycelial Dry Weight (g/l)	Final Lactose Concentration[b] (g/l)	Lactose Utilized (%)	Final Lactic Acid Concentration[c] (g/l)	Citric Acid (g/l)	Citric Acid Yield[d] (%) (w/w)
IMI 27809	7.5	4.8	30.0	30	1.5	0	-
IMI 31821	7.7	4.6	35.0	19	2.5	0	-
IMI 41873	3.0	10.2	10.5	76	0	1.8	5.5
IMI 41874	2.0	12.0	6.0	86	0	5.0	13.5
IMI 51433	3.0	12.5	8.0	81	0	2.6	7.5
IMI 75353	4.5	No Growth		-	-	-	-
IMI 83856	4.5	No Growth		-	-	-	-
IMI 84304	3.5	10.5	18.0	58	1.0	0	-
ATCC 12846	2.7	7.5	13.0	70	0	2.3	7.5
ATCC 26036	7.5	4.5	36.0	16	2.5	0	-
ATCC 26550	2.5	11.0	6.0	86	0	2.5	7.0

[a] Initial pH 4.5.
[b] Initial lactose 43 g/l.
[c] Initial lactic acid 5.2 g/l.
[d] Based on lactose utilized.

TABLE 4.2 CITRIC ACID PRODUCTION BY 5 SELECTED STRAINS OF *Aspergillus* IN LACTIC CASEIN WHEY PERMEATE SUPPLEMENTED WITH LACTOSE (100 g/l).

Results are expressed after 10 days of incubation.

Strain	Final pH *	Mycelial Dry Weight (g/l)	Final Lactose Concentration (g/l) +	Lactose Utilized (%)	Citric Acid (g/l)	Yield of Citric Acid (%) (w/w) **
IMI 41873	2.8	11.8	93	35	3.3	6.5
IMI 41874	1.8	12.5	90	37	8.2	15.5
IMI 51433	3.0	12.0	100	30	4.0	9.0
ATCC 12846	2.5	8.0	105	27	2.8	7.5
ATCC 26550	2.2	11.5	85	41	5.7	10.0

* Initial pH 4.5.

\+ Initial lactose concentration 143 g/l.

** Based on lactose utilized.

TABLE 4.3 A BRIEF SUMMARY OF CITRIC ACID PRODUCTION IN DIFFERENT FERMENTATION MEDIA BY STRAINS OF *A. niger* USED IN THE PRESENT SCREENING PROGRAMME.

Strain	Sugar in the Fermentation Medium	Culture Method	Citric Acid Yield* (%)	Comments on Present Work	References
IMI 27809	Beet molasses	Submerged in shake-flask culture	60-65	+	Clement (1952)
IMI 31821	Saccharose	Surface	11-12	+	Currie (1917)
IMI 41874	Sucrose	Surface	41-49	++ 15.5	Doelger and Prescott (1934)
IMI 51433	Sucrose	Submerged in shake-flask culture	15-48	++ 9.0	Karow and Waksman (1947)
IMI 75353	Sucrose and cane molasses	Surface	32-82	-	Perlman *et al.* (1946)
	Sucrose and commercial glucose	Submerged in shake-flask culture	21-80		Shu and Johnson (1947, 1948a,b)
	Commercial sucrose	Surface	6-20		Tomlinson *et al.* (1950, 1951)
	Beet molasses	Submerged in tower type of fermenter	50-70		Martin and Waters (1952)
	Sucrose	Submerged in 50-gallon glass lined fermenter	55-81		Buelow and Johnson (1952)
IMI 83856	Cane sugar	Surface	20-25	-	Porges (1932)
IMI 84304	Glucose	Submerged in shake-flask culture	69-71	+	Moyer (1953a)
ATCC 12846	Glucose and beet molasses	Surface and submerged in shake-flask culture	12-67	++ 7.5	Moyer (1953a,b)
ATCC 26036 (Das, A. strain 'C')	Sucrose	Surface	18-20	+	Das and Nandi (1965a,b) (1969)
ATCC 26550	Beet molasses	Submerged in 36 litre glass tower fermenter	70-76	++ 10.0	Clark and Lentz (1961)
	Beet molasses	Submerged in pyrex tower fermenter	75-80		Clark (1962)

* Based on sugar utilized.
\- No growth.
\+ Grew but no citric acid produced.
++ Citric acid produced and figure expressed as percentage yield, based on lactose utilized.

this, its ability to produce citric acid from different sugars in a synthetic medium was studied. Experiments were conducted in shake-flask culture using the medium described by Kristiansen and Charley (1981) (Table 3.2), where the sugar sources used were sucrose, lactose or galactose. Fermentations were continued for 14 days. In comparison with literature reports, the production of citric acid from sucrose was poor (6.5 g/l), although good growth was observed. No growth was observed when lactose was the sugar source, and although growth occurred on galactose no citric acid was produced. Thus, the results from this experiment, as well as from the screening programme, strongly suggest that *A. niger* IMI 75353 cannot metabolize lactose. Also, the inability to produce citric acid from galactose suggests that the nature of the sugar being metabolized may affect citric acid production.

It is clear, then, that the ability to produce citric acid from lactose, as in whey permeate, does not necessarily correlate well with the ability to produce citric acid from glucose or sucrose. Thus, the use of the whey permeate as the screening medium was justified.

4.2.2 Strain Imrpovement by Induced Mutation

A. niger IMI 41874 was subjected to irradiation with UV light in an attempt to induce a mutation leading to increased production of citric acid from lactic casein whey permeate. Figure 4.1 shows a typical death curve using the conditions described in section 3.5. An irradiation time of 30 minutes was adopted as standard and used in all subsequent mutation programmes.

On the basis of 'acid unitage', 14 mutants were isolated and their abilities to utilize citric acid were examined in a citric acid medium (Table 3.3(c)). The results obtained are shown in Table 4.4, which shows that of these 14 mutants, 8 of them utilized citric acid completely within 7 days of incubation. Of the remainder, mutant MH 15-15 utilized the least amount of citric acid (about 30% of that available), indicating that it had a reduced ability to use citric acid as a carbon source.

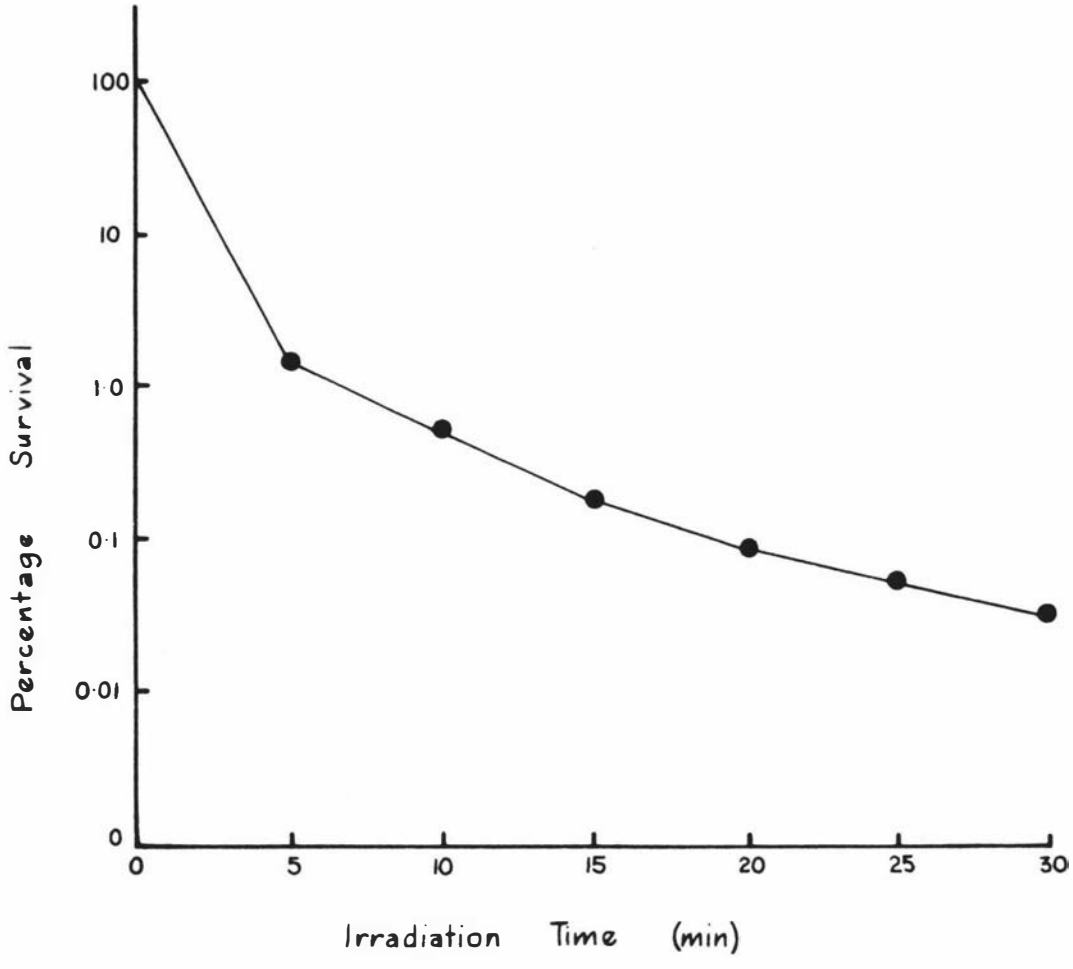

FIGURE 4.1 Death curve for spores of *A. niger* IMI 41874 when subjected to UV irradiation.

TABLE 4.4 ABILITY OF SOME OF THE SELECTED MUTANTS TO UTILIZE CITRIC ACID.

Results are expressed after 7 days of incubation.

Strain	Acid Unitage *	Mycelial Dry Weight (g/l)	Citric Acid + Utilized (g/l)
IMI 41874 (parent)	1.8	2.8	20.0
MH 2-1	2.2	1.7	18.2
MH 2-4	2.4	2.1	20.0
MH 2-10	2.5	1.6	12.0
MH 5-1	2.0	2.3	20.0
MH 5-5	2.3	2.4	20.0
MH 5-7	2.8	3.0	20.0
MH 10-2	2.6	2.1	20.0
MH 10-4	2.8	1.9	20.0
MH 10-6	2.3	2.3	20.0
MH 15-1	2.9	2.2	15.2
MH 15-2	2.5	2.2	20.0
MH 15-11	2.7	2.1	15.0
MH 15-13	2.6	2.4	18.1
MH 15-15	3.0	1.2	6.0

* Defined in Chapter 3, Section 3.5.3.

+ Initial concentration 20 g/l.

On the basis of their lower citric acid utilization, the mutants MH 2-10, MH 15-1, MH 15-11 and MH 15-15 were examined for their ability to produce citric acid in shake-flask culture using lactic casein whey permeate (without lactose supplementation). The results (Table 4.5) show that mutant MH 15-15 produced 6.5 g/l citric acid, representing an increase of 27% over the parent strain.

A further comparative study of mutant MH 15-15 with the parent strain was conducted in shake-flask culture using whey permeate supplemented with lactose (100 g/l). Flasks were harvested after 2,4,6,8 and 10 days of incubation and the results are shown in Figure 4.2. The two strains behaved similarly in all respects but the mutant produced 27% more citric acid than the parent (10.2 g/l compared with 8.0 g/l). The volumetric and specific production rates, shown in Figure 4.3, again demonstrate the superiority of the mutant strain.

A similar comparative study was performed on the parent and mutant to establish their ability to produce citric acid in a synthetic medium. The experiment was conducted in shake-flask culture using the medium described by Kristiansen and Charley, with sucrose as carbon source. The experimental results (Figure 4.4) demonstrate that both the parent and the mutant produced substantially more citric acid (34.0 g/l and 52.8 g/l respectively) than on the lactose-supplemented lactic casein whey permeate (8.0 g/l and 10.2 g/l respectively). Yields, based on sugar utilized, were 33% (w/w) and 48% (w/w) for the parent and mutant respectively, compared with 16% (w/w) and 20% (w/w) on the whey permeate. Volumetric and specific rate data, shown in Figure 4.5, again show the superiority of the mutant and are approximately five times greater than those obtained on the whey permeate (Figure 4.3).

Two major points arise from these comparative studies on lactose-supplemented whey permeate and the synthetic medium. First, it can be concluded that the mutant *A. niger* MH 15-15 is certainly superior to the parent in respect of its ability to produce citric acid. Hence, this mutant was selected for the studies described in Chapters 5, 6 and 7. Its stability was tested periodically and it was found to be stable throughout the entire experimental period.

TABLE 4.5 CITRIC ACID PRODUCTION BY 4 SELECTED MUTANTS IN LACTIC CASEIN WHEY PERMEATE.

Results are expressed after 10 days of incubation.

Strain	Final pH[a]	Mycelial Dry Weight (g/l)	Final Lactose Concentration[b] (g/l)	Lactose Utilized (%)	Citric Acid (g/l)	Yield of Citric Acid[c] (%) (w/w)
IMI 41874 (parent)	2.0	12.5	7	84	5.1	14.0
MH 2-10	2.0	12.8	10	77	4.5	13.5
MH 15- 1	2.0	13.5	8	81	5.2	15.0
MH 15-11	2.2	12.0	18	58	3.8	15.0
MH 15-15	1.8	13.5	7	84	6.5	18.0

[a] Initial pH 4.5

[b] Initial lactose concentration 43 g/l.

[c] Based on lactose utilized.

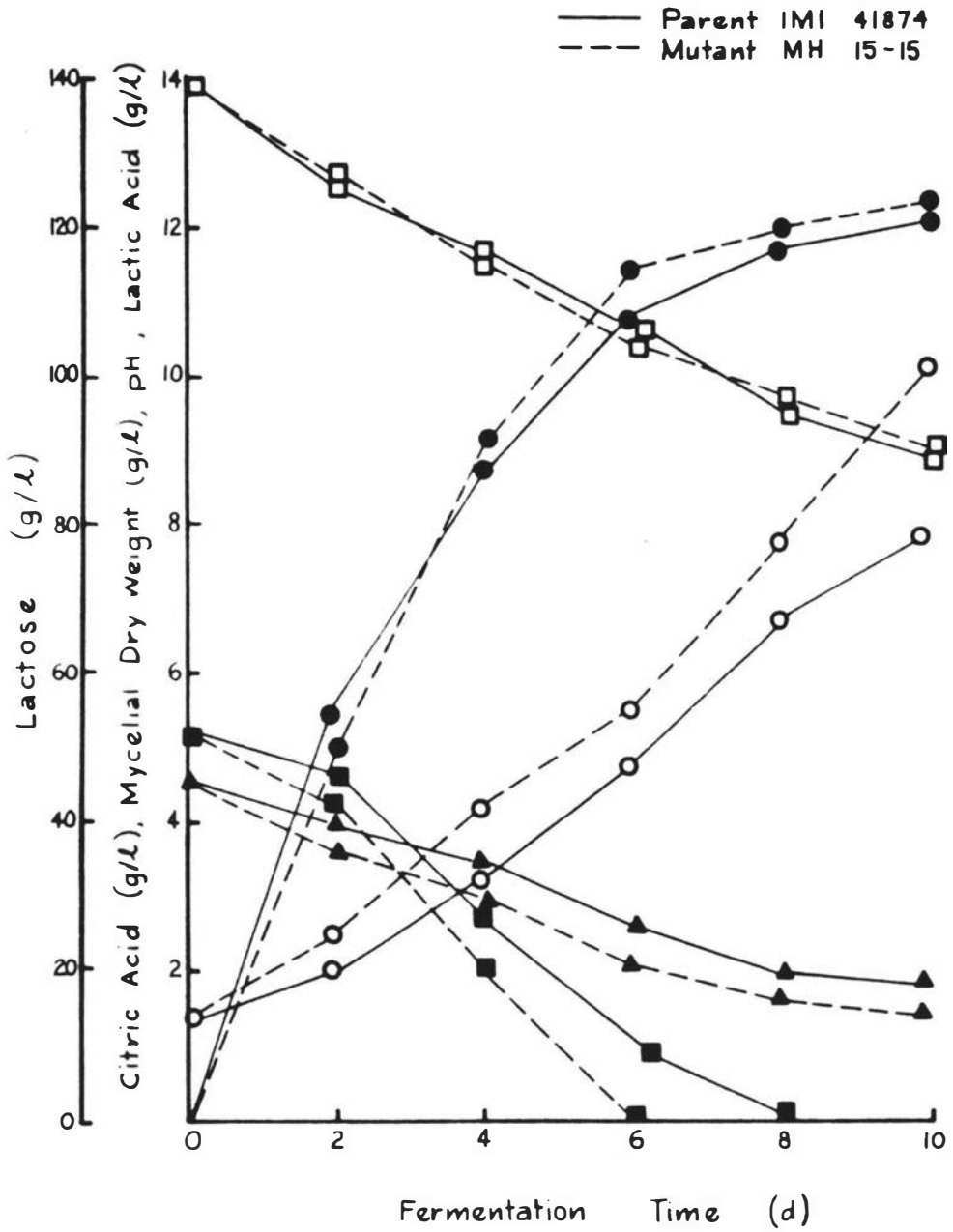

FIGURE 4.2 Production of citric acid from lactic casein whey permeate, supplemented with lactose, by parent strain IMI 41874 and mutant strain MH 15-15.

Citric acid (O), mycelial dry weight (●), lactose (□), lactic acid (■), pH (▲).

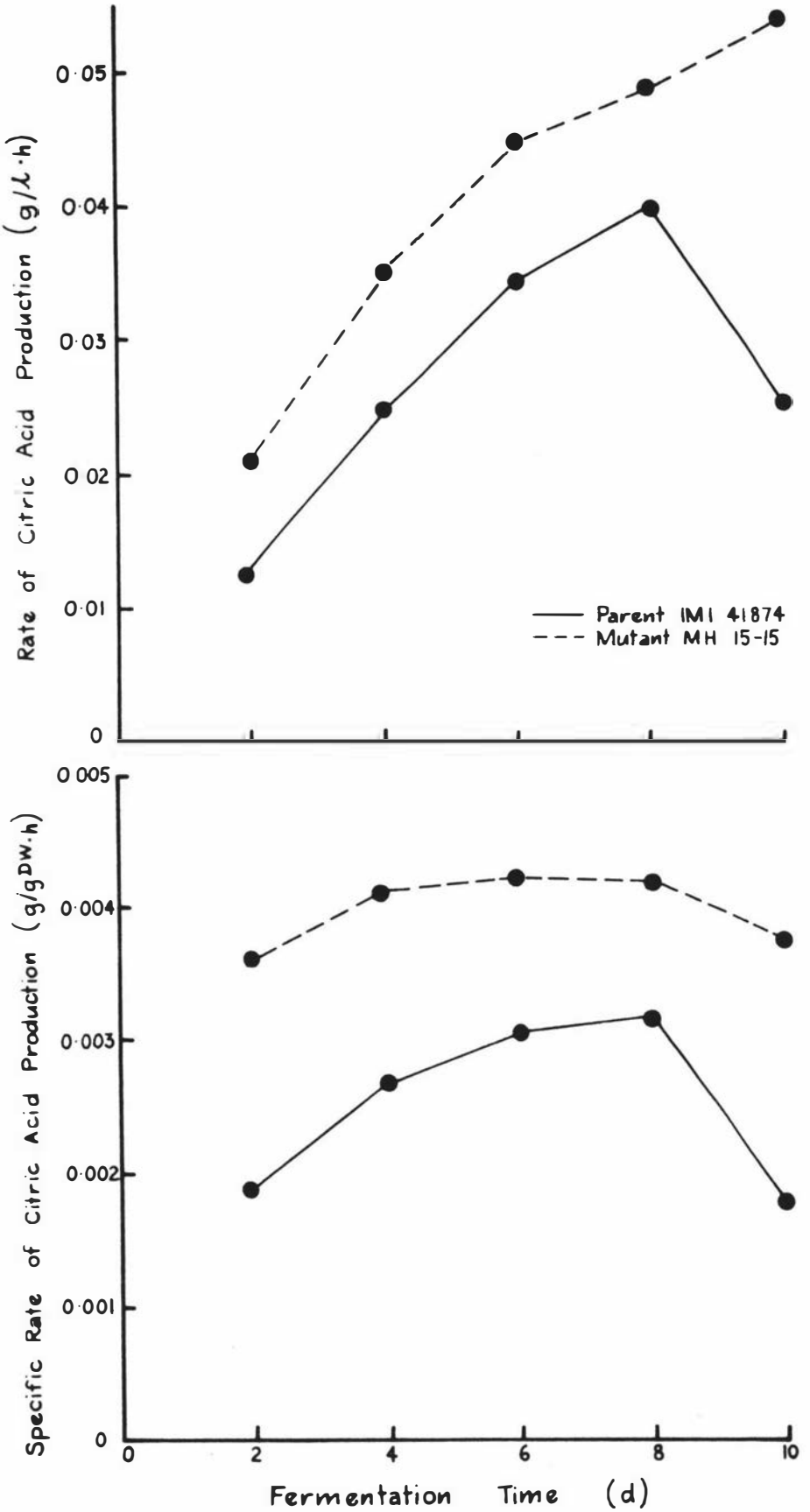

FIGURE 4.3 Volumetric and specific rates of citric acid production by parent strain IMI 41874 and mutant strain MH 15-15, in lactic casein whey permeate medium supplemented with lactose.

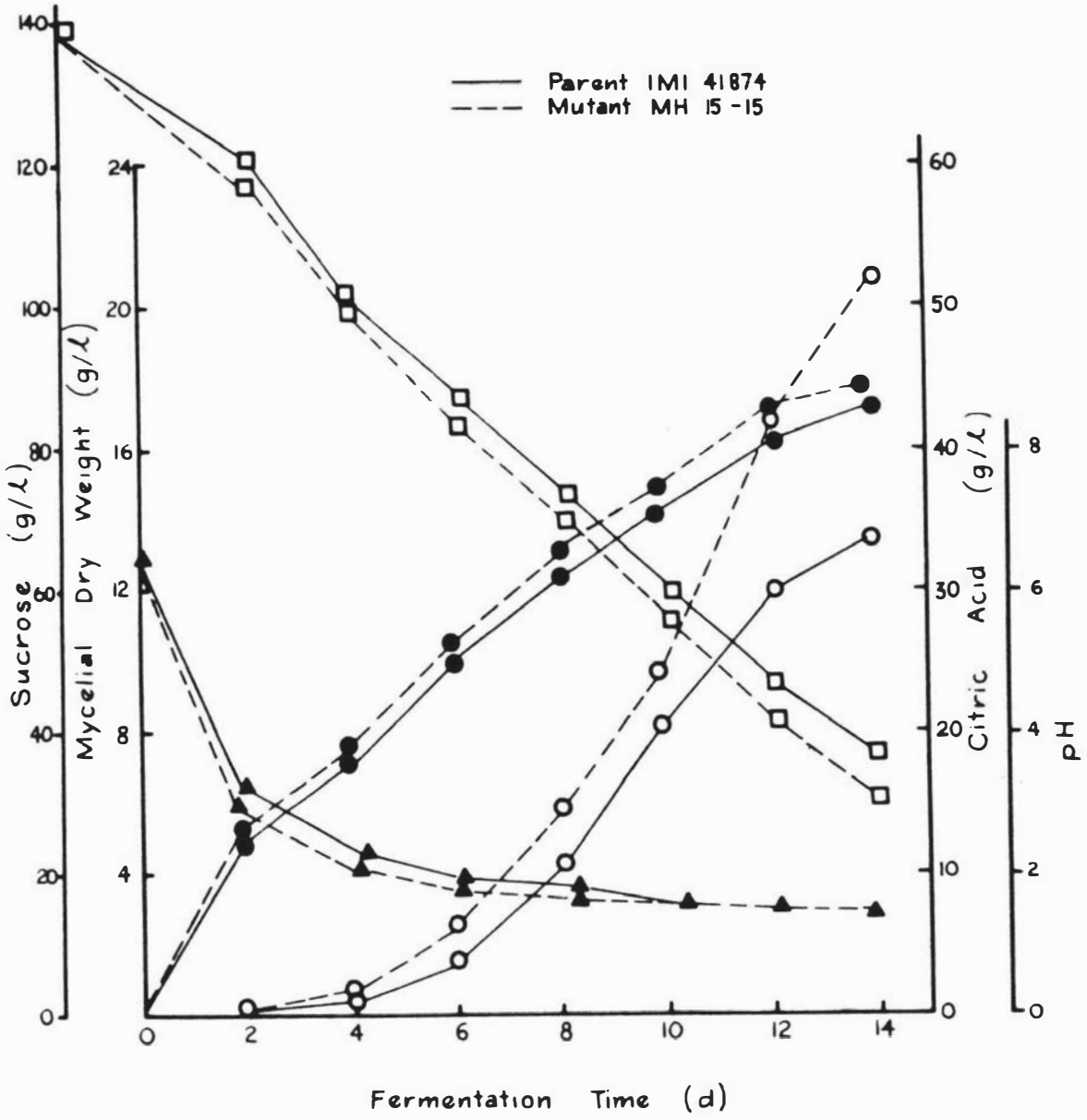

FIGURE 4.4 Citric acid production on medium of Kristiansen and Charley (Table 3.2) by parent strain IMI 41874 and mutant strain MH 15-15.

Citric acid (O), mycelial dry weight (●), sucrose (□), pH (▲).

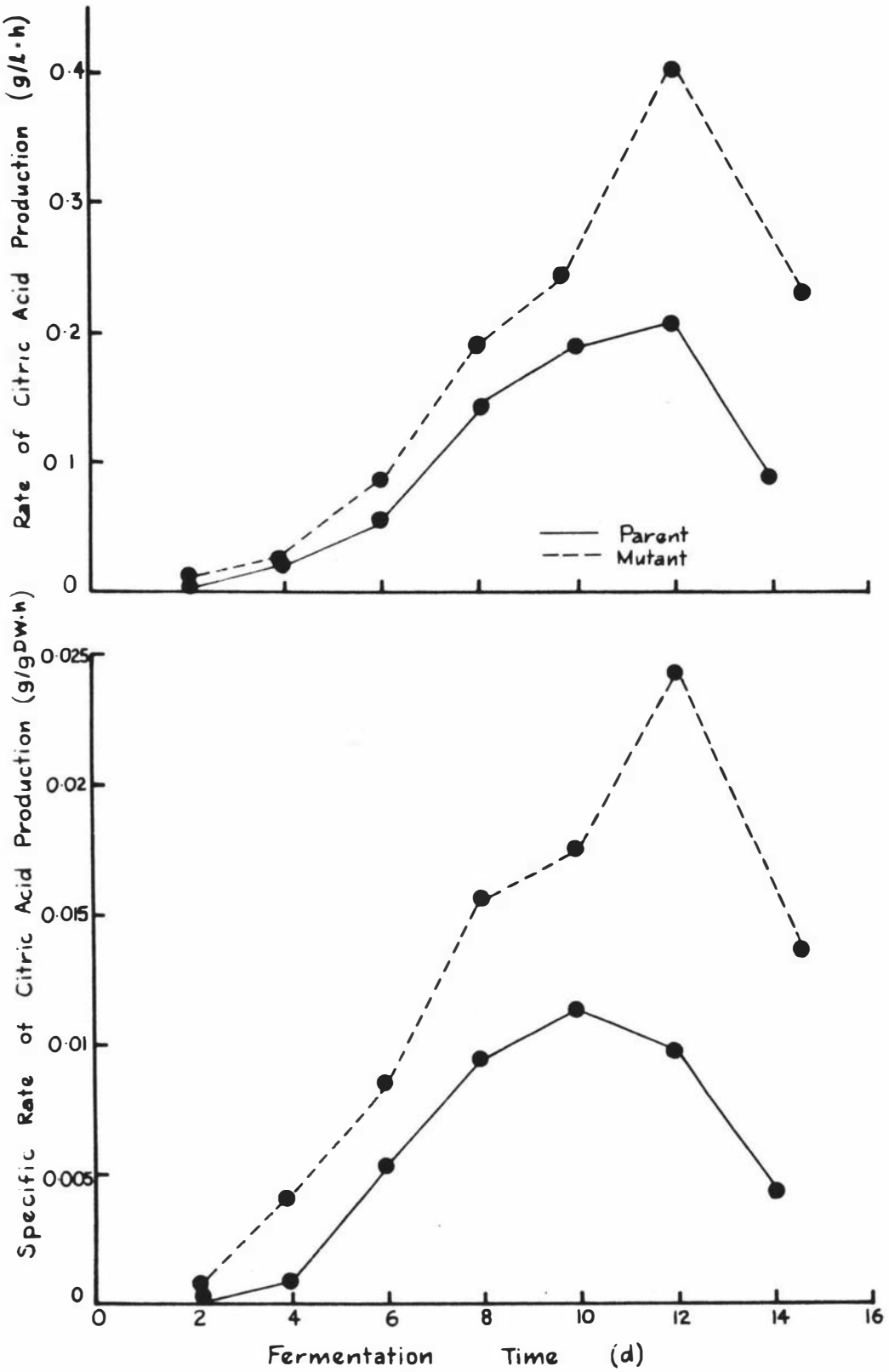

FIGURE 4.5 Volumetric and specific rates of citric acid production by parent strain IMI 41874 and mutant strain MH 15-15 on medium of Kristiansen and Charley.

Secondly, citric acid concentrations, yields, and production rates were greatly superior on the sucrose synthetic medium than on the whey permeate. Whether these effects were due to the use of the different sugar sources or to other medium constituents is investigated in Chapters 5 and 6.

In this study, the selected mutant was obtained by screening approximately 16,000 colonies in petri dishes. This mutant was subjected to a second mutation step using UV light and a further 25,000 colonies were screened, some of which were tested for their ability to produce citric acid in shake-flask, using lactic casein whey permeate. However, no further improvement was made over mutant MH 15-15. This was considered surprising since there are many reports in the literature showing that successive mutation steps can improve citric acid production severalfold (Gardner *et al.*, 1956; Das and Nandi, 1969a,b). Possibly the use of different mutagenic agent could have proved successful, as suggested by Das and Nandi (1969a,b).

It was subsequently observed (Chapter 6) that mutant MH 15-15 did not produce citric acid from galactose. This finding led to the use of the galactose medium (Table 3.3(b)) for the screening of isolates after UV irradiation of parent strain, *A. niger* IMI 41874 and mutant MH 15-15. Thus, a mutation programme was undertaken in which approximately 30,000 colonies were examined in petri dishes and many of them were isolated and tested for their ability to produce citric acid in the Kristiansen and Charley medium using galactose as carbon source. However, in no case was citric acid production from galactose observed. The failure of these attempts has no immediate explanation but the problem of galactose is further considered in Chapter 7.

4.3 CONCLUSION

The strain selection programme, using whey permeate as the test medium, identified *A. niger* IMI 41874 as being the most suitable organism for further work. A mutant strain of this organism was

subsequently isolated and proved to be superior in its ability to produce citric acid.

A comparison of lactose-supplemented whey permeate and a sucrose-containing synthetic medium demonstrated the latter to be greatly superior in supporting citric acid production by *A. niger* IMI 41874 and its mutant. Possible reasons for this include the different sugar source and a different composition of other medium constituents.

CHAPTER 5

PRODUCTION OF CITRIC ACID FROM LACTIC CASEIN WHEY PERMEATE IN FERMENTER CULTURE

5.1 INTRODUCTION

The previous chapter reported the development of an improved strain of *A. niger*. This strain was now studied in fermenter culture in an attempt to optimize the conditions for production of citric acid.

Initial experiments were directed toward the effects of lactose and nitrogen concentration and pH control (part-I), while subsequent experiments investigated the effects of trace elements using decationized whey permeate as the basic medium (part-II).

PART-I

PRODUCTION OF CITRIC ACID FROM UNTREATED LACTIC CASEIN WHEY PERMEATE

5.2 THE EFFECT OF LACTOSE SUPPLEMENTATION

5.2.1 INTRODUCTION

The shake-flask culture experiments (at the time of strain selection and improvement) indicated that lactose-supplemented whey permeate gave higher citric acid production than unsupplemented whey permeate. However, it was considered worthwhile to repeat the experiments in fermenter culture in order to set the baseline for citric acid production, as well as to provide comparison with the shake-flask cultures.

5.2.2 Results and Discussion

An initial fermentation was performed using unsupplemented lactic casein whey permeate (5 litre working volume). The initial pH value of the medium was pH 4.5 (not adjusted). The inoculum was grown in the same whey permeate according to the procedure described in Section 3.7.2.3 and a 10% (v/v) inoculum was added to the fermenter. The dissolved oxygen tension of the fermentation broth was not monitored, but the agitation speed and aeration rate were set at 200 rpm and 3 l/min respectively for the first 48 hours of fermentation and subsequently increased to 300 rpm and 5 l/min respectively. Samples were withdrawn periodically during the 10 days of fermentation and analysed for citric acid, lactic acid, pH, mycelial dry weight and lactose concentration. The time course of the fermentation is shown in Figure 5.1.

The results show that the maximum citric acid concentration (8.3 g/l) was achieved after 8 days of fermentation and then the citric acid was depleted slowly from the medium. In contrast, in shake-flask culture the maximum citric acid concentration (6.5 g/l) was obtained after 10 days of fermentation (Table 4.5, Chapter 4) and was not depleted from the medium. However, the yields, based on lactose utilized, were not significantly different (approximately 19% (w/w)). The lactose was utilized completely after 8 days of fermentation, suggesting that the citric acid depletion was a consequence of lactose deficiency. (This interpretation is consistent with the shake-flask data where no citric acid depletion was observed, but lactose was not completely utilized). Lactic acid was completely utilized after 4 days of fermentation and the pH of the medium gradually decreased from pH 4.5 to pH 1.8 as citric acid accumulated.

The volumetric and specific rate data shown in Figure 5.2, demonstrate that both the volumetric and specific rates of citric acid production reached their maximum after 5 days of fermentation (0.07 g/l.h and 0.0085 g/gDW.h, respectively) and then declined to zero after 8 days.

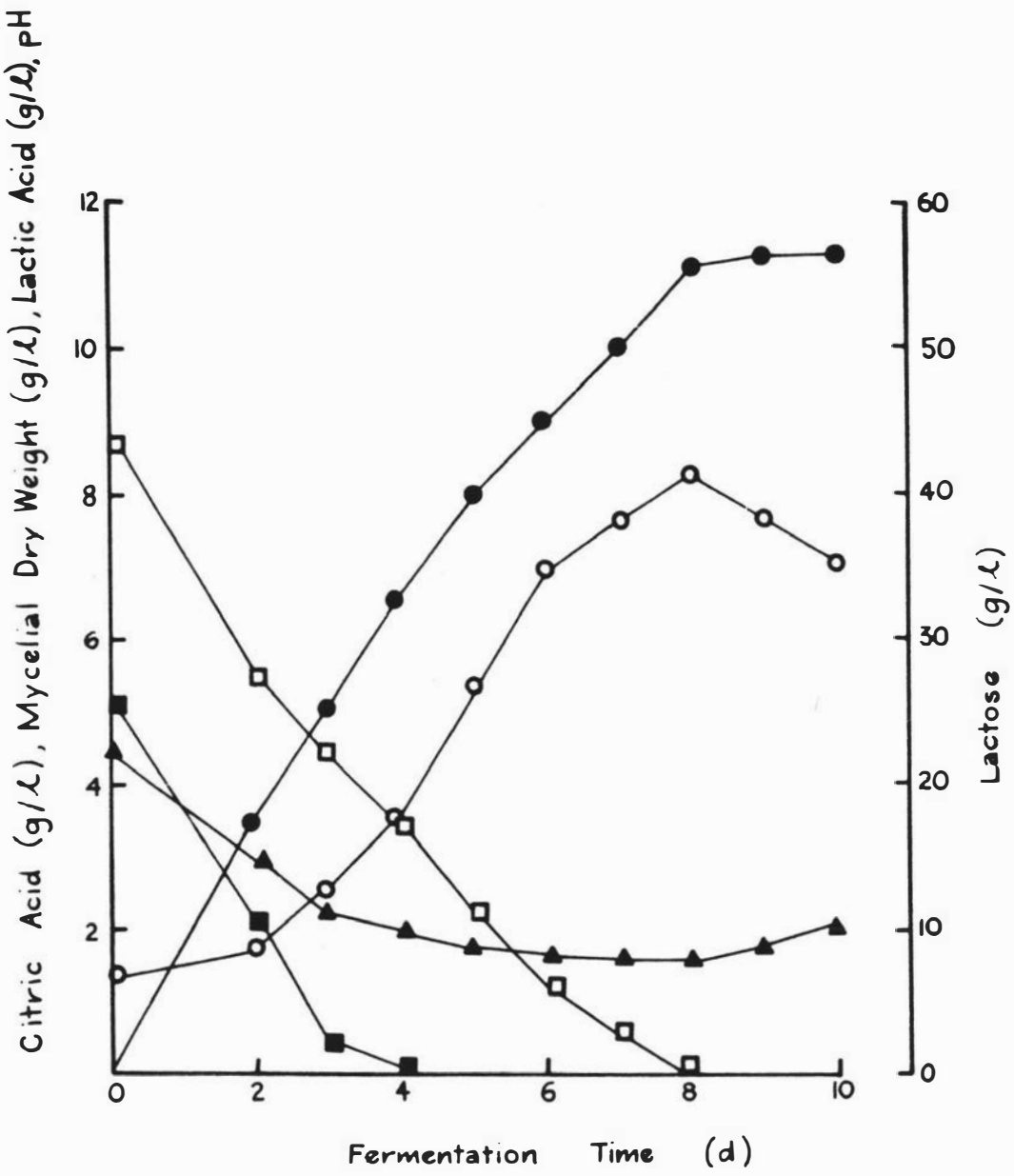

FIGURE 5.1 The production of citric acid from unsupplemented lactic casein whey permeate.

Citric acid (O), mycelial dry weight (●), lactose (□), lactic acid (■), pH (▲).

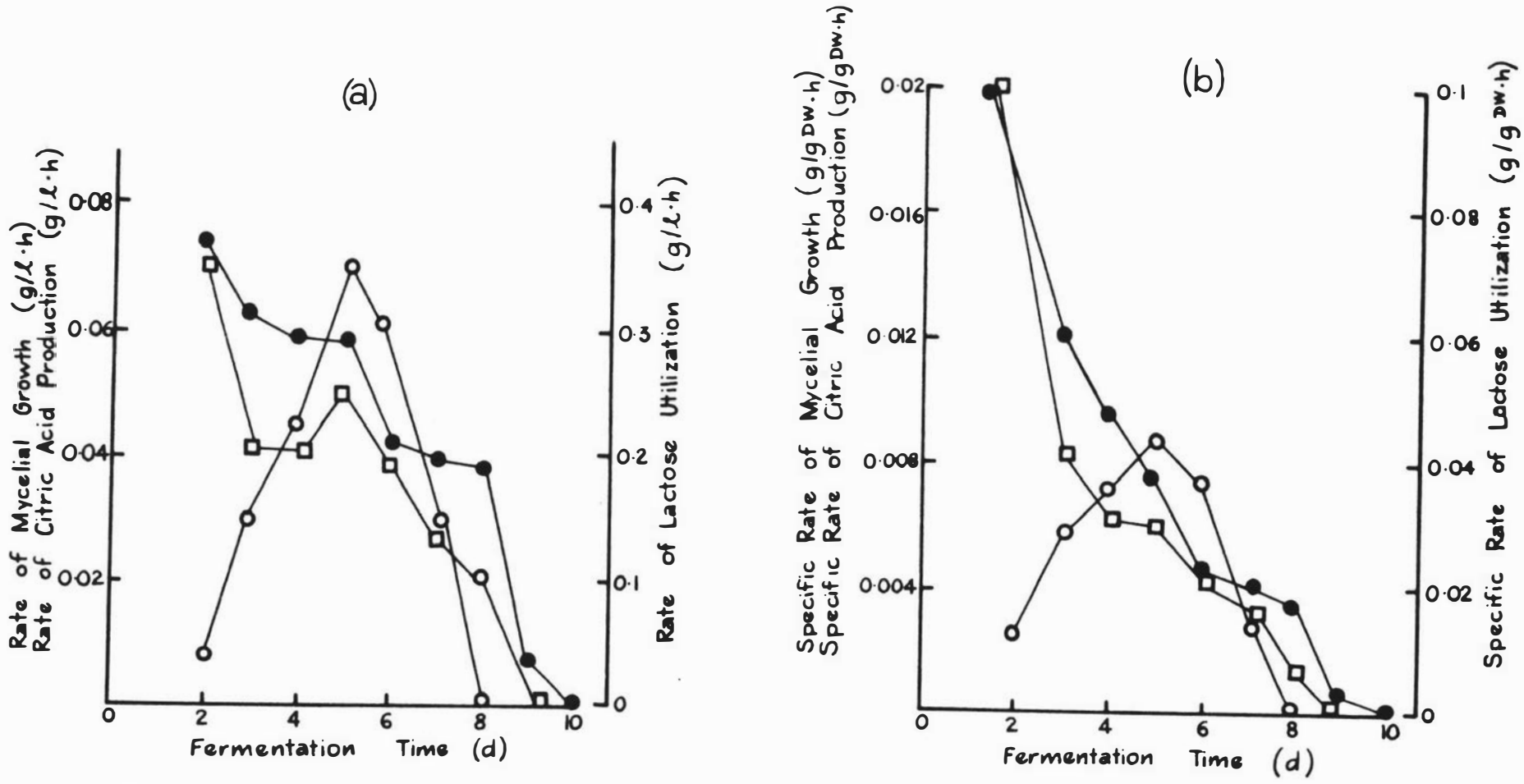

FIGURE 5.2 (a) Volumetric and (b) specific rate data during citric acid production from unsupplemented lactic casein whey permeate.

Citric acid (O), mycelial growth (●), lactose (□).

A second fermentation was performed in which the whey permeate was supplemented with additional lactose (100 g/l). The experimental conditions were identical to the previous experiment. However, in this case, dissolved oxygen tension was monitored and recorded as shown in Figure 5.3. This was the general pattern of dissolved oxygen tension in the fermenter broth for all the fermenter culture experiments.

The time course of the fermentation, presented in Figure 5.4, shows that a citric acid concentration of 14.8 g/l was obtained at the end of fermentation, representing a yield of 23% (w/w) based on lactose utilized. In contrast, using the same level of lactose supplementation (100 g/l) in shake-flask culture, the citric acid concentration and yield were 10.2 g/l and 20% (w/w) respectively. Thus, these results indicate a slight superiority of fermenter culture over shake-flask culture. A mycelial dry weight of 12 g/l was obtained at the end of the fermentation and this was approximately the same as in shake-flask culture. In comparison with Figure 5.1, using unsupplemented whey permeate, it is clear that the production of citric acid was increased in the presence of a higher initial lactose concentration. This agrees with the result of Somkuti and Bencivengo (1981), also working with whey permeate. The results also demonstrate that lactose supplementation checked the citric acid depletion from the medium. Lactic acid was completely utilized within a period of 4 days of fermentation, while the pH value gradually decreased to pH 1.8 as the citric acid accumulated. The fermented samples were analysed for glucose and galactose (hydrolysis products of lactose) but neither was detected, indicating that lactose was not hydrolysed outside the cell.

The volumetric and specific rate data, shown in Figure 5.5, indicate that the citric acid production rate (0.140 g/l.h and 0.015 g/gDW.h) reached a peak on the fifth day of fermentation. These values were approximately double those obtained using unsupplemented whey permeate in fermenter culture. The rate of sugar utilization was highest during the initial growth phase and then declined with time. This rate was similar to that observed using unsupplemented whey permeate, as was the mycelial growth rate.

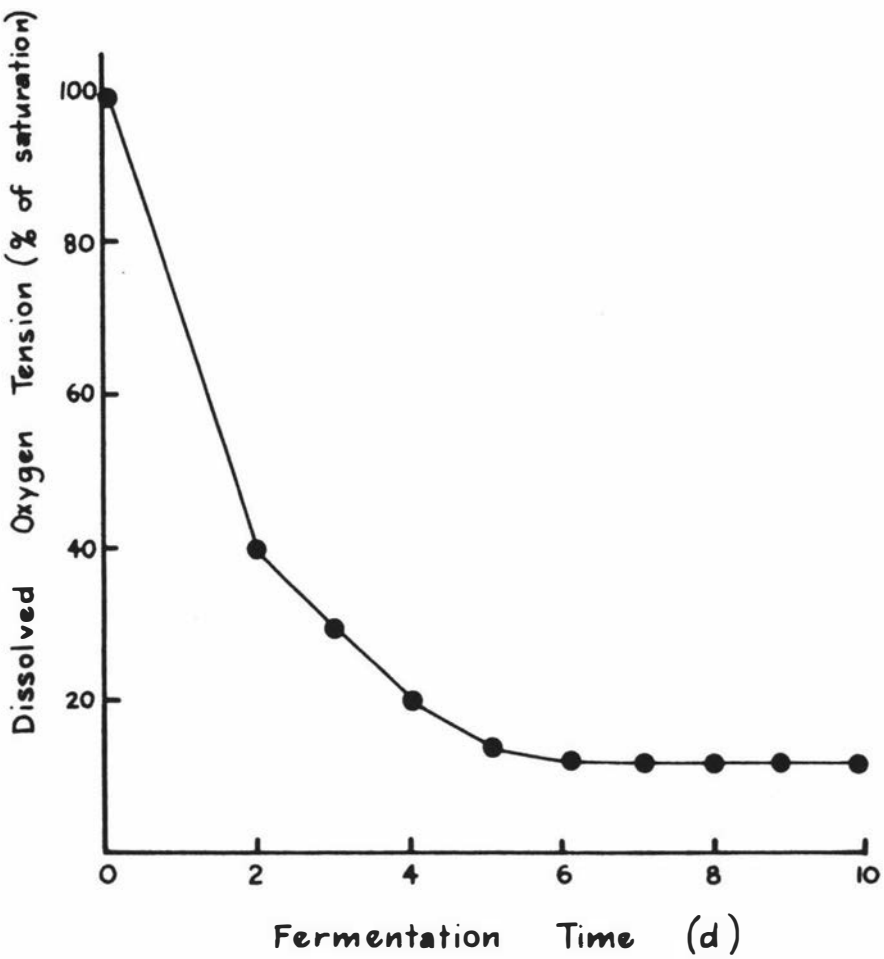

FIGURE 5.3 Dissolved oxygen tension in fermenter culture during citric acid production from lactose-supplemented whey permeate.

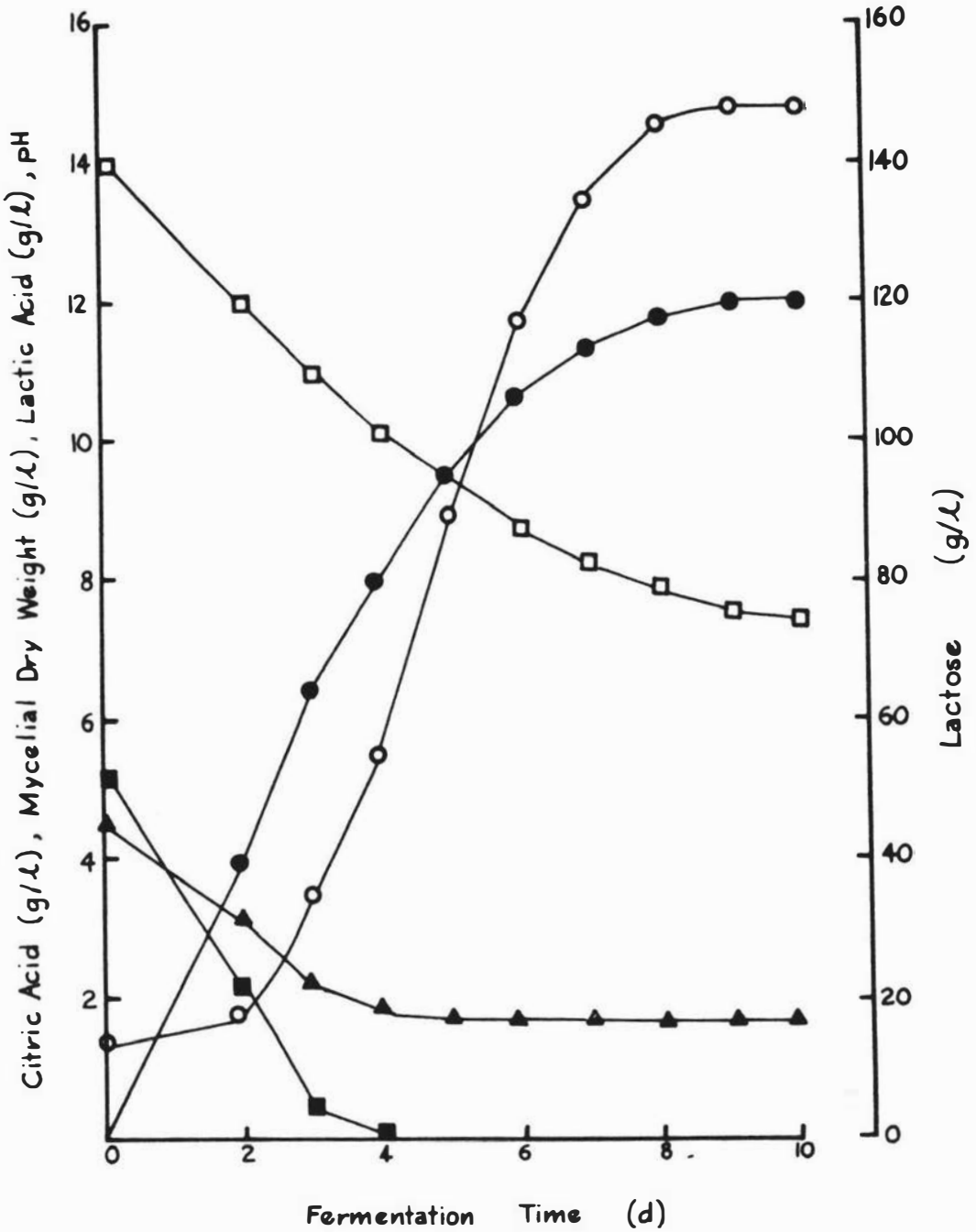

FIGURE 5.4 The production of citric acid from lactose-supplemented (100 g/1) lactic casein whey permeate.

Citric acid (O), mycelial dry weight (●), lactose (□), lactic acid (■), pH (▲).

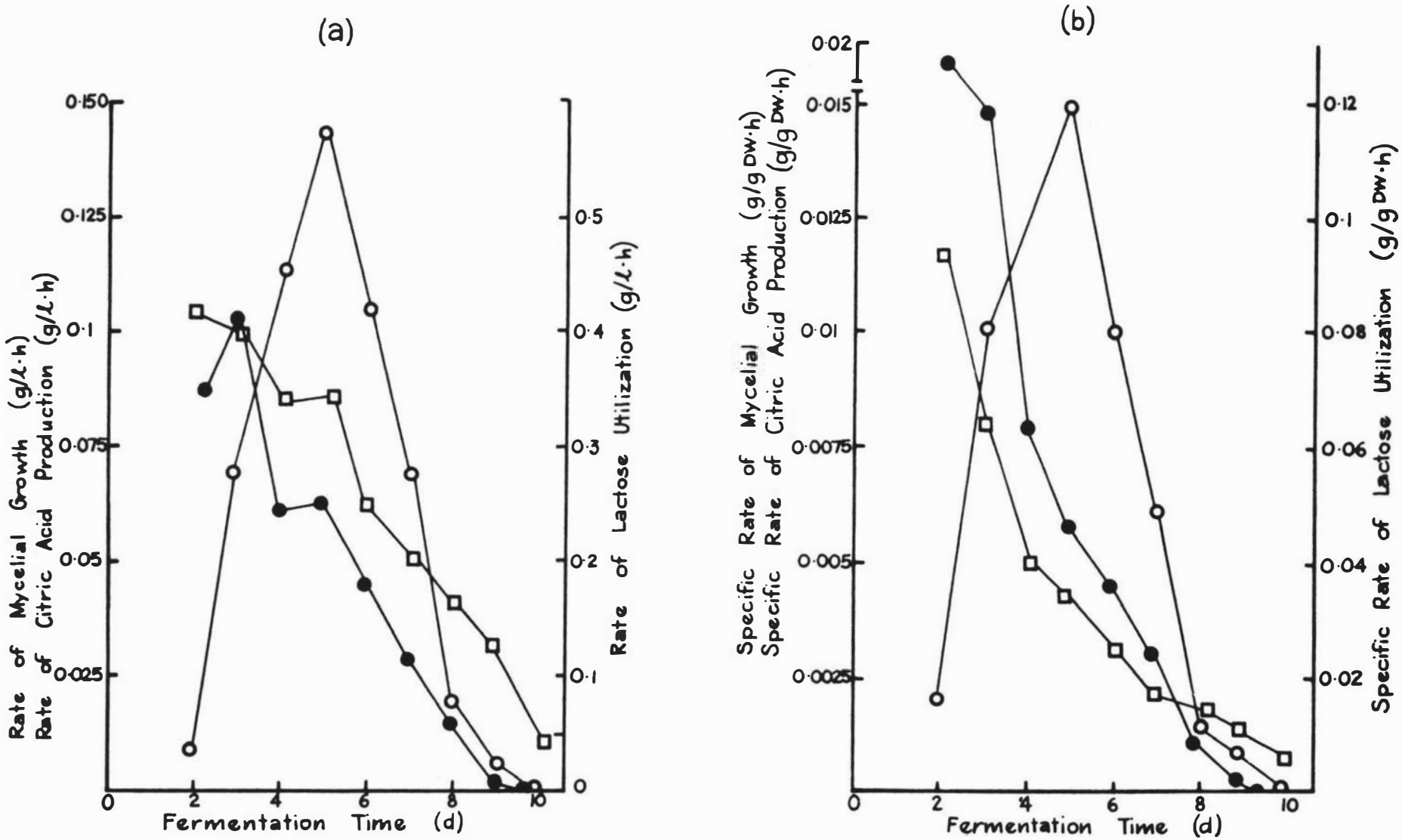

FIGURE 5.5 (a) Volumetric and (b) specific rate curves of lactose-supplemented (100g/l) lactic casein whey permeate.

Citric acid (O), mycelial dry weight (●), lactose (□).

Thus, it appears that the addition of lactose to the whey permeate caused an increase in the rate of citric acid production, without affecting either the sugar utilization rate or the growth rate. Interestingly, the citric acid production rate declined to zero at the same time as the growth rate and sugar utilization rate, despite the presence of considerable amounts (about 70 g/l) of lactose remaining in the medium. This suggests that the whey permeate may be deficient in some nutrients and that continued growth and sugar utilization may lead to continued citric acid production.

5.3 THE EFFECT OF SUPPLEMENTATION WITH NON-PROTEIN NITROGEN, LACTIC ACID, PHOSPHATE, MAGNESIUM AND TRACE ELEMENTS

5.3.1 INTRODUCTION

The previous experiment showed that a high concentration of lactose in the medium led to an increased citric acid concentration and production rate. A higher concentration of lactose in whey permeate may also be attained by concentrating the whey permeate. For example, to attain a lactose concentration of approximately 140 g/l would require about a three fold concentration of whey permeate. This would also increase the concentrations of all other components of whey permeate, including nitrogen and trace elements, which may deleteriously influence the accumulation of citric acid. Conversely, the higher concentration of such materials may enhance citric acid accumulation.

To examine the effect of such concentration, an experiment was performed by supplementing the whey permeate with three times more lactose, non-protein nitrogen, lactic acid, phosphate, magnesium and trace elements.

5.3.2 RESULTS AND DISCUSSION

Table 5.1 shows the concentrations of different components of normal whey permeate, supplementation and final concentrations set for the experiment. The fermentation was performed in a similar manner to that described previously (Section 5.2.2).

TABLE 5.1 THE DIFFERENT COMPONENTS PRESENT IN LACTIC CASEIN WHEY PERMEATE, SUPPLEMENTATION AND THE FINAL CONCENTRATION SET FOR EXPERIMENT.

Component	Present in Whey Permeate (g/l)*	Supplementation (g/l)*	Final Concentration (g/l)*
Non-protein nitrogen[a]	0.46	1.38	1.84
Lactic acid	5.20	15.60	20.80
Phosphate[b]	0.47	1.31	1.78
Mg^{2+} [c]	57	171	228
Cu^{2+} [d]	0.17	0.51	0.68
Fe^{2+} [e]	1.20	3.60	4.80
Zn^{2+} [f]	3.00	9.00	12.00
Mn^{2+} [g]	0.13	0.39	0.52
Ni^{2+} [h]	0.13	0.39	0.52

* Trace element concentration expressed as mg/l.

[a] Supplied as casein hydrolysate.
[b] Supplied as KH_2PO_4.
[c] Supplied as $MgSO_4.7H_2O$.
[d] Supplied as $CuSO_4.5H_2O$.
[e] Supplied as $FeSO_4.7H_2O$.
[f] Supplied as $ZnSO_4.7H_2O$.
[g] Supplied as $MnCl_2.4H_2O$.
[h] Supplied as $NiSO_4.7H_2O$.

There was heavy mycelial growth after 24 hours of fermentation and the pellets were broken down into a mesh-like mycelium. This thick mycelial growth made it almost impossible to maintain the aeration efficiency in the fermenter. Fermentation was discontinued after 6 days. A mycelial dry weight of 18 g/l was obtained during this time but no citric acid accumulated in the medium. The initial pH value (pH 4.5) remained unchanged while lactose utilization was less than 20% of that supplied (140 g/l).

These findings suggest that the excessive nutrient content (e.g. nitrogen and trace elements) of the medium caused the heavy mycelial growth and adversely influenced the accumulation of citric acid. Therefore, the approach of concentration of whey permeate was considered not to be useful for the present research. Further study in this area, as for example, the treatment of concentrated whey permeate for the removal of excess nitrogen, lactic acid and trace elements, could perhaps improve the situation, but this type of study was considered beyond the scope of the present research.

Hence, for all future experiments, lactose-supplemented whey permeate was chosen as the base medium.

5.4 THE EFFECT OF CONTROLLED pH

5.4.1 INTRODUCTION

The maintenance of a favourable pH value is essential for the successful production of citric acid (Kapoor *et al.*, 1982). This pH value will depend largely on the medium and the strain of fungus used (Loesecke, 1945; Kapoor *et al.*, 1982). It has been reported that the accumulation of high concentrations of citric acid in the medium may inhibit the rate of citric acid production and pH values below 2.0 have been associated with reduction of yield (Chmiel, 1977).

It was shown in Section 5.2.2 that the initial pH value 4.5 gradually decreased to pH 1.8-2.0 as citric acid accumulated. It was thought that such a low pH value at the production stage might inhibit the rate of citric acid production. Therefore, it was considered useful to control the culture pH after onset of fermentation to observe its effect on the production of citric acid.

5.4.2 Results and Discussion

The basic fermentation conditions were as previously described (Section 5.2.2) and the process commenced with a pH value of 4.5. This was allowed to decrease naturally to pH 2.3 (reached on the third day of fermentation), at which time the pH value was controlled to between pH 2.3-2.5 by the automatic addition of 2M NaOH. This control was continued up to the end of fermentation. The time course of the fermentation is shown in Figure 5.6.

The results show that a citric acid concentration of 7.2 g/l was obtained at the end of fermentation, representing less than 50% of that obtained with uncontrolled pH (Figure 5.4). There was a higher mycelial dry weight (14 g/l) at the end of fermentation compared with that using uncontrolled pH (Figure 5.4), suggesting that the higher pH value in the culture medium at the later stage of fermentation led to biomass growth at the expense of citric acid production. The amount of lactose consumed remained at approximately 70 g/l.

From these results, it was concluded that to obtain higher yields of citric acid under the conditions studied, pH control is not essential (in fact, it reduces yield).

5.5 The Effect of Continuous Nitrogen Feeding After Onset of Fermentation

5.5.1 Introduction

The accumulation of citric acid has been reported to occur under nitrogen-limiting conditions (Kristiansen and Sinclair, 1978, 1979).

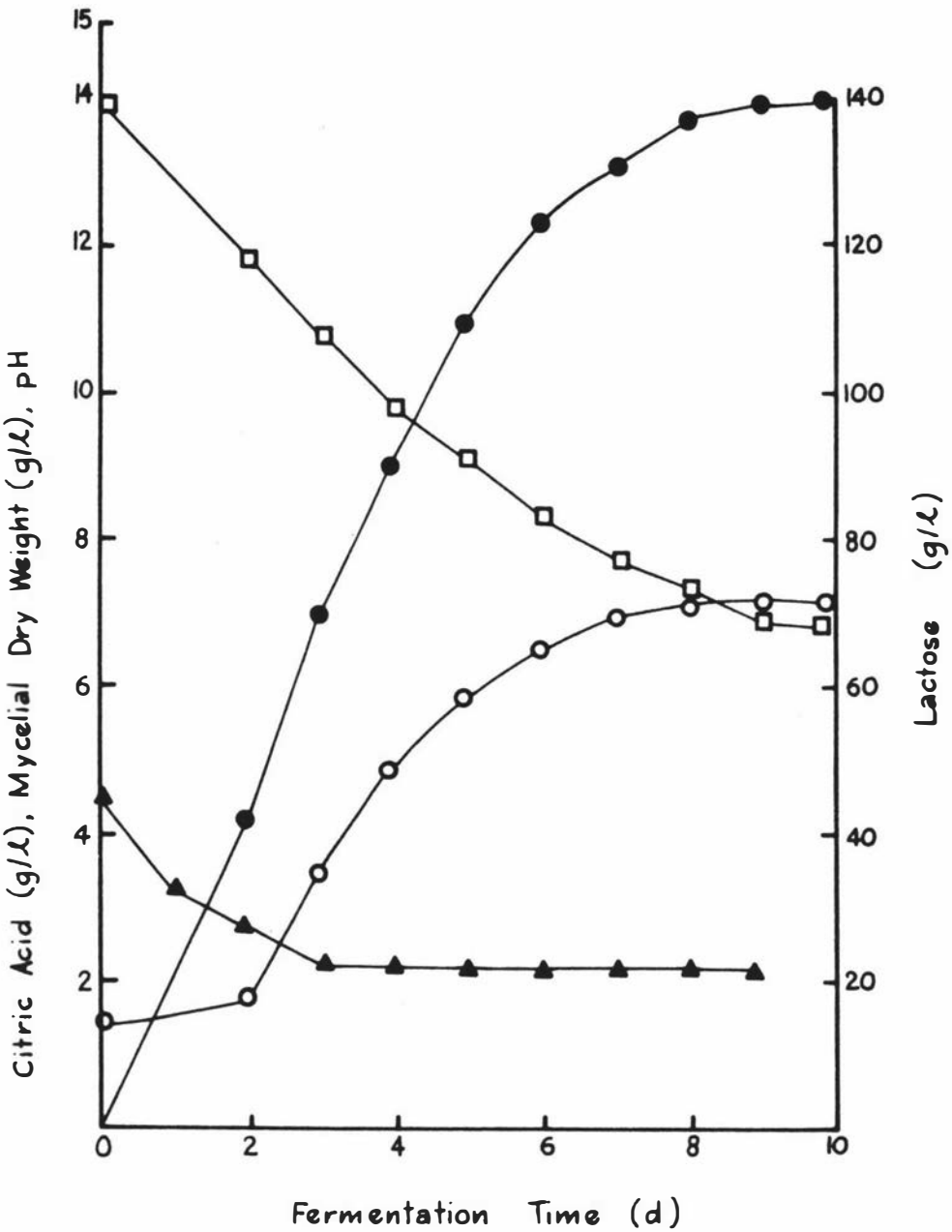

FIGURE 5.6 The production of citric acid from lactose-supplemented whey permeate at a controlled pH of 2.3.

Citric acid (O), mycelial dry weight (●), lactose (□), pH (▲).

However, the addition of small quantities of nitrogen during the production phase does stimulate citric acid production (Shepherd, 1963). In general, a low nitrogen content in the medium favours the production of citric acid, while a high nitrogen content favours the abundant growth of mycelium with low acid production. However, this condition seems to vary according to the carbohydrate source and the strain of *A. niger* used (Loesecke, 1945).

From the results of the previous experiments, it was apparent that citric acid production and lactose utilization ceased at approximately the same time as mycelial growth. This suggested that if growth could be maintained, then citric acid production would continue. To achieve this, it was decided to investigate the effect of supplying extra nitrogen to the medium, on the basis that this was the growth-limiting nutrient. In this way, it was hoped that a constant growth rate could be maintained. In order to prevent excessive mycelial growth, a continuous feed was adopted, using $(NH_4)_2SO_4$.

5.5.2 Results and Discussion

After 3½ days of fermentation, 0.007 gN/l/h was fed continuously to the culture medium and this was continued until the eighth day. The result of this experiment is presented in Figure 5.7, which demonstrates that production of citric acid, mycelial dry weight and lactose utilization were increased over that of the control experiment (Section 5.2, Figure 5.4). The final concentration of citric acid was 19.5 g/l, representing a yield of 26% (w/w) based on lactose utilized. By comparison with Figure 5.4, it can be seen that this represents a 32% increase in the production of citric acid, although the yield was only slightly higher.

The volumetric and specific rate data are shown in Figure 5.8. By comparison with Figure 5.5, it is apparent that the maximum observed rate of citric acid production increased from 0.14 g/l.h to 0.20 g/l.h, while the maximum rate of mycelial growth also increased from 0.10 g/l.h to 0.16 g/l.h. However, the specific rate of citric acid production was not significantly increased.

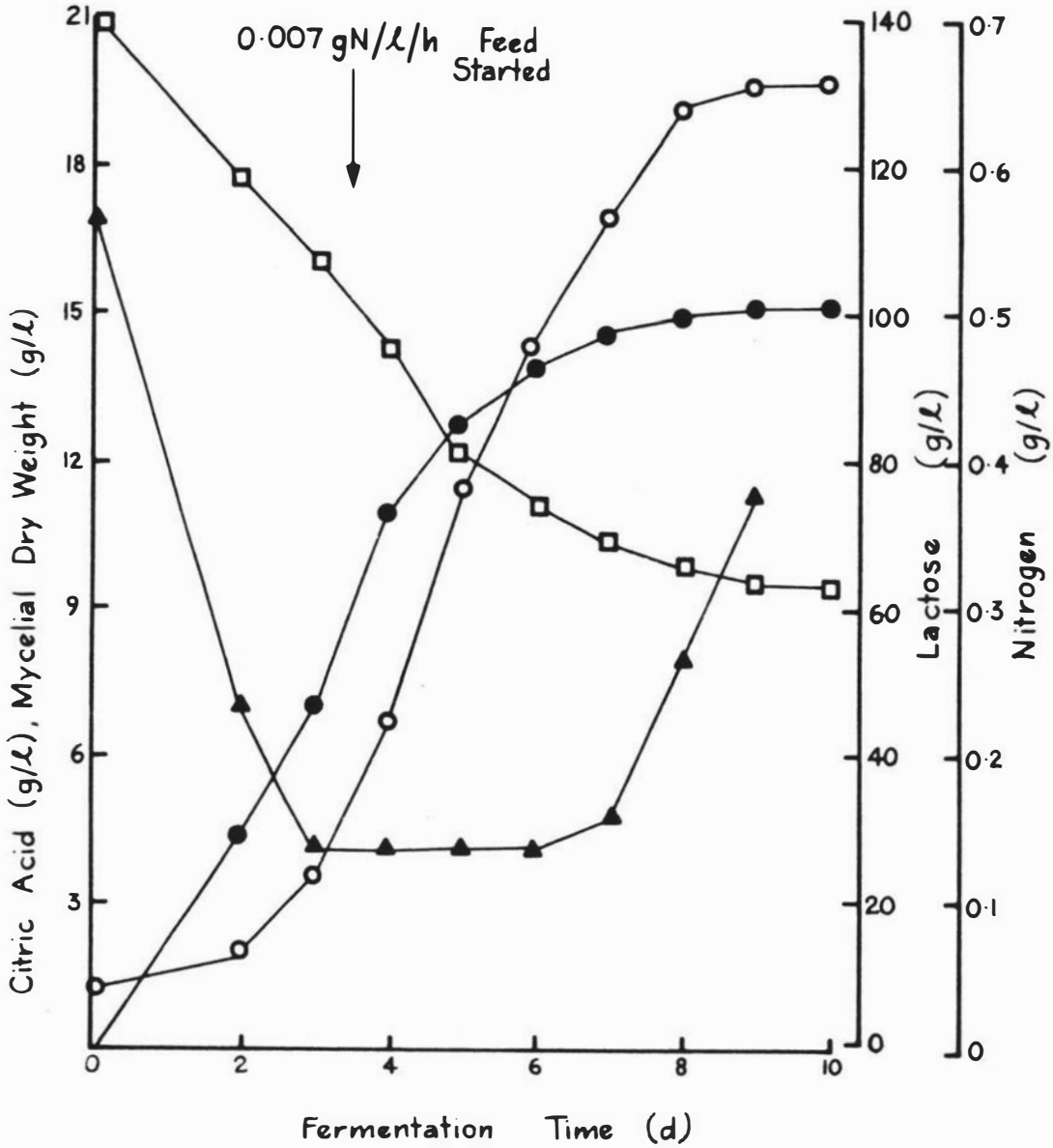

FIGURE 5.7 The production of citric acid from lactose-supplemented whey permeate, with supplementary nitrogen-feeding.

Citric acid (O), mycelial dry weight (●), lactose (□), nitrogen (▲).

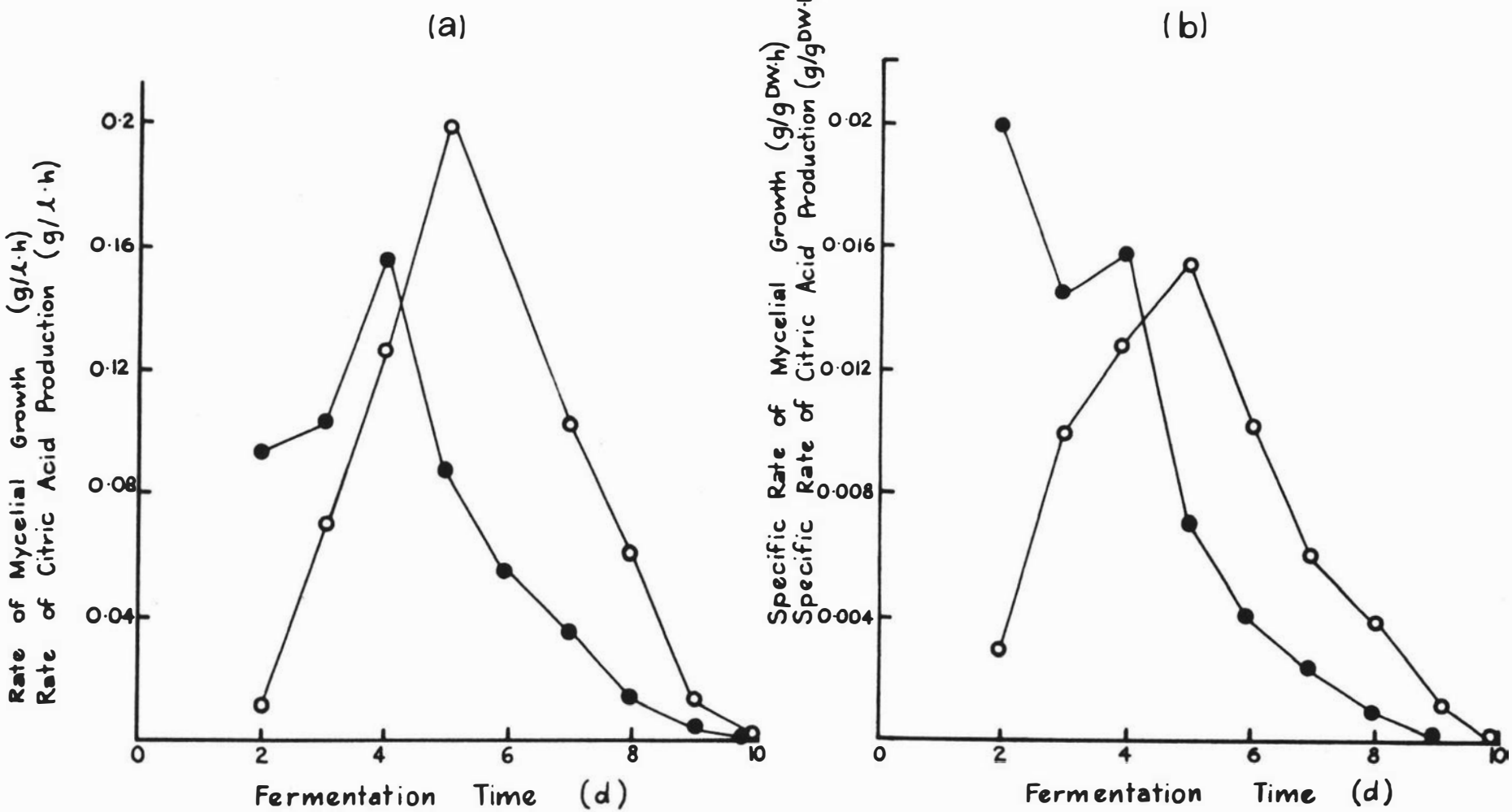

FIGURE 5.8 (a) Volumetric and (b) specific rate curves of nitrogen-feeding experiment. Citric acid (O), mycelial dry weight (●).

It is clear from Figure 5.7 that after 8 days of fermentation both mycelial growth and citric acid production ceased as in previous experiments, despite the presence of excess nitrogen and lactose. This suggests that some other nutrient had become limiting.

From the nitrogen concentration data in Figure 5.7 it can be seen that the added nitrogen was completely utilized until the seventh day of fermentation and thereafter the utilization slowed down and the residual nitrogen concentration increased rapidly.

From the experimental results it can be concluded that nitrogen feeding after onset of citric acid production can substantially increase the production of citric acid, although the yield, based on lactose utilized is only marginally affected.

5.6 THE EFFECT OF CONTINUOUS NITROGEN FEEDING AT DIFFERENT INITIAL PH VALUES OF THE MEDIUM

5.6.1 INTRODUCTION

A previous experiment had indicated that pH control was unnecessary during the fermentation. However, a substantial literature exists describing the influence of the initial pH value of the medium on the rate of citric acid production in submerged culture (for example, Shu and Johnson, 1948b; Khan and Ghose, 1973; Berry *et al.*, 1977). Therefore, it was of interest to investigate the effect of different initial pH values on the production of citric acid from whey permeate. Nitrogen feeding was also employed during the fermentations.

5.6.2 RESULTS AND DISCUSSION

The basic fermentation conditions were as previously described (Section 5.2.2) and experiments were performed using initial pH values of 3.0 and 6.0. The pH value was adjusted using H_3PO_4 or 2M NaOH. After 3½ days of fermentation, nitrogen feeding (as $(NH_4)_2SO_4$) was commenced at a rate of 0.0035 gN/l/h. The results of these two experiments are shown in Figure 5.9.

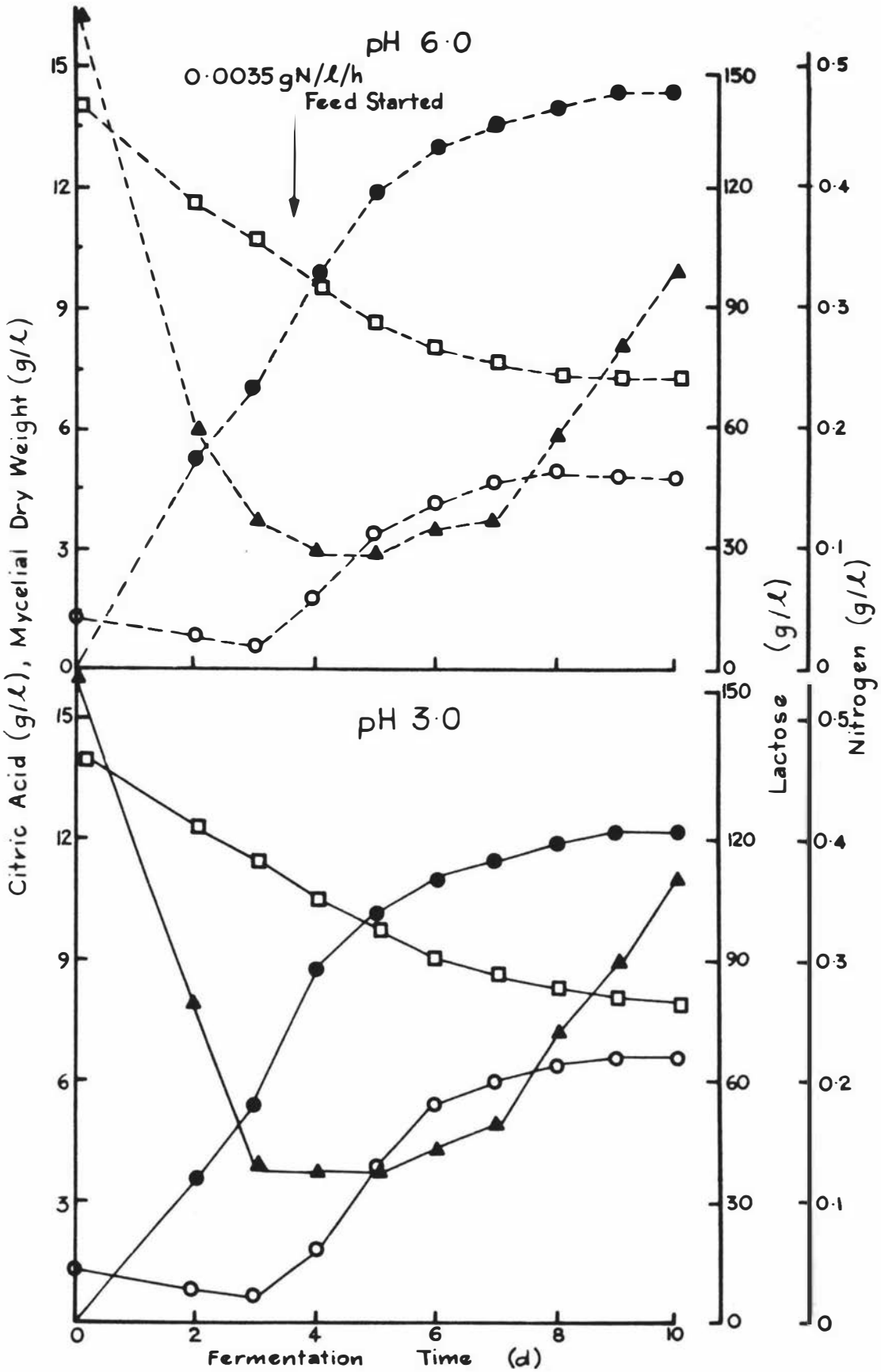

FIGURE 5.9 The production of citric acid in nitrogen-feeding experiments with different initial pH values.

Citric acid (○), mycelial dry weight (●), lactose (□), nitrogen (▲).

The results show that citric acid production was drastically reduced in both experiments when compared with Figure 5.4 (lactose-supplementation, initial pH 4.5) and Figure 5.7 (lactose-supplementation, initial pH 4.5, nitrogen feeding). However, of these two initial pH values, pH 3.0 gave the higher citric acid concentration (6.5 g/l, compared with 4.5 g/l at initial pH 6.0). The mycelial dry weight obtained was higher at initial pH value 6.0 (14 g/l) than at initial pH value 3.0 (12 g/l). This indicates that the higher initial pH value of the medium favours mycelial growth at the expense of citric acid production. During the fermentations, the pH value gradually decreased from pH 3.0 to pH 1.8 and pH 6.0 to 2.4, as the citric acid accumulated. The added nitrogen was utilized in an almost identical pattern in both of the experiments. It was anticipated that using the lower nitrogen feed rate compared with Figure 5.7, the nitrogen might be utilized throughout the entire period of fermentation. This was not the case, as the nitrogen utilization again stopped after the seventh day of fermentation.

In view of the above findings, it was decided that an initial pH value of pH 4.5, i.e. the natural pH, would be used in all subsequent experiments.

5.7 DISCUSSION AND CONCLUSIONS

The experimental results described in this section allow the following conclusions to be drawn:

(a) Lactose supplementation (100 g/l) of the permeate caused higher production of citric acid and also prevented subsequent product depletion from the medium.

(b) Lactose utilization rarely exceeded more than 50% of that supplied (140 g/l) under any experimental conditions.

(c) An initial pH value of pH 4.5 was found to be optimum when followed by a natural decrease to pH 1.8-2.0 as citric

acid accumulated. No pH control was required.

(d) Nitrogen feeding (0.007 gN/l/h) after onset of citric acid production significantly increased the citric acid production, but had little effect on yield.

The maximum observed production of citric acid was 19.5 g/l, representing a yield of 26% (w/w) based on lactose utilized. This is an improvement over the results obtained in shake-flask culture using lactose-supplemented whey permeate, but still relatively poor when compared with the use of the sucrose-based synthetic medium (Chapter 4, Figure 4.4). Therefore, at least two possibilities may be considered:

(a) The nature of the sugar source has an effect on citric acid production.

(b) The trace element composition of the whey permeate is relatively unsuitable for citric acid production.

It is also worth considering other fermentation parameters that may influence citric acid production, e.g. dissolved oxygen tension. The conditions of aeration and agitation used in the fermentation experiments maintained a maximum dissolved oxygen tension of only 12-15% of saturation (Figure 5.3). Also, these conditions had a deleterious effect on pellet morphology. Photographs of typical pellet morphology are shown in Figure 5.10. The inoculum consisted of small solid pellets (0.4-0.5 diameter), but during the course of the fermentation these broken down into a mycelial form (Figure 5.10).

The morphology of pellets of *A. niger* in submerged culture has long been realized to be important for high yields of citric acid. Small, round and hard cream coloured pellets have been suggested to be desirable for high citric acid yields (Snell and Schweiger, 1949; Martin and Waters, 1952; Steel *et al.*, 1954, 1955; Martin, 1957), while the filamentous form is reported to produce little citric acid (Carilli *et al.*, 1961; Clark *et al.*, 1966). Possibly, the degeneration

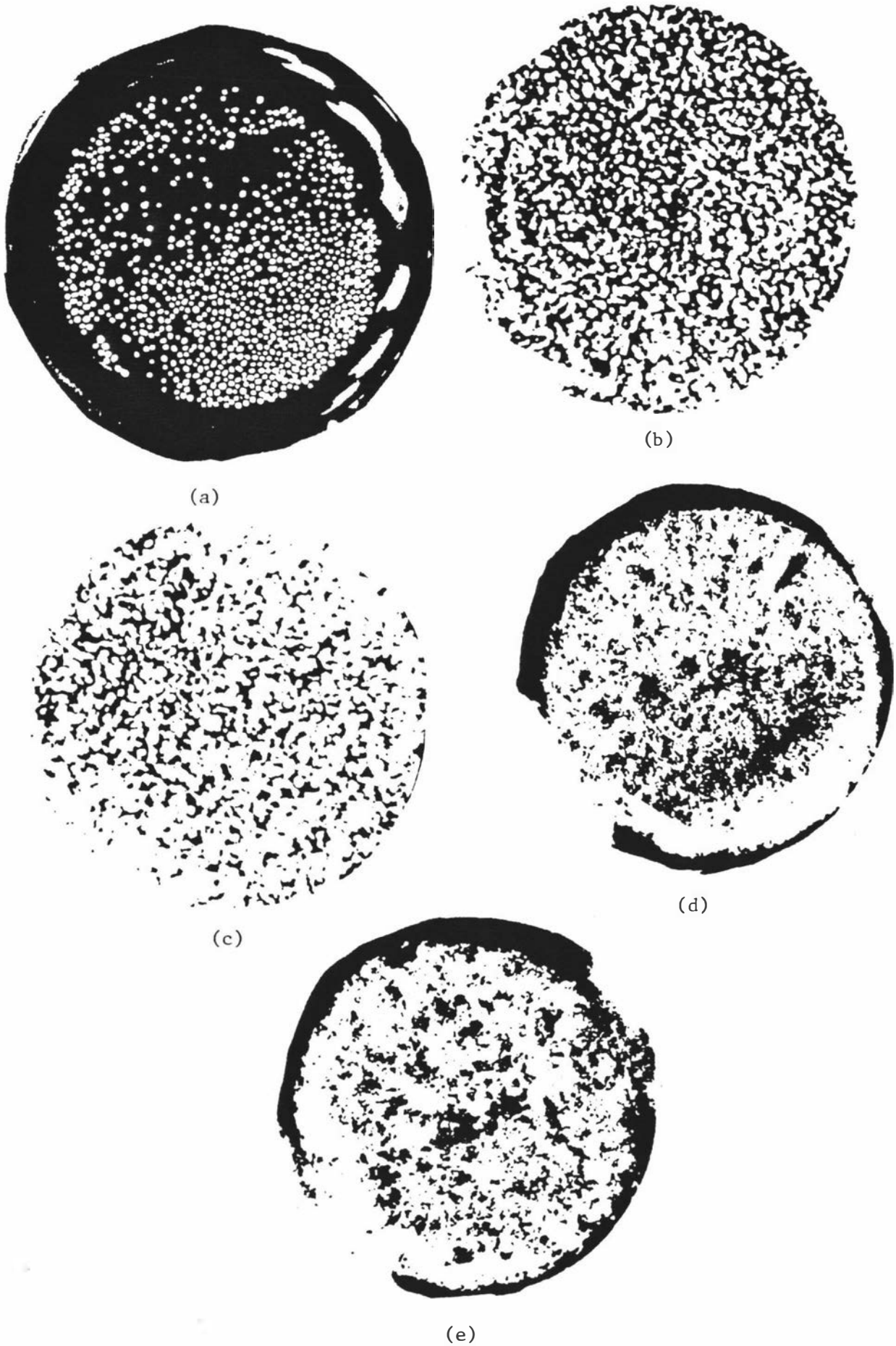

FIGURE 5.10 Pellet morphology in the fermenter at various time intervals: (a) Day 0, (b) Day 2, (c) Day 4, (d) Day 6 and (e) Day 8.

observed in the present work was due to the agitation system within the fermenter.

However, since, with whey permeate, the fermenter experiments gave higher citric acid production than shake-flask culture (where pellet morphology was maintained), it was decided that an investigation into the trace element composition of the medium might be a more fruitful approach.

PART-II

PRODUCTION OF CITRIC ACID FROM DECATIONIZED LACTIC CASEIN WHEY PERMEATE

5.8 INTRODUCTION

Since the fundamental research of Currie (1917) it has been apparent that excess quantities of many metal ions may markedly inhibit the formation of citric acid by *A. niger*. There are many conflicting reports in the literature regarding the exact trace element requirement for citric acid production (Perlman *et al.*, 1946; Shu and Johnson, 1947, 1948a,b; Tomlinson *et al.*, 1950, 1951; Trumpy and Millis, 1963; Millis *et al.*, 1963; Clark *et al.*, 1966). Nevertheless the control of trace elements concentration in the fermentation medium has ensured reasonable reproducibility of the fermentation yields (Sanchez-Marroquin *et al.*, 1970). The optimal concentrations of many trace elements (e.g. Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+}) vary so widely with different strains of *A. niger* that it is necessary to adjust the composition of the medium to avoid the inhibitory effects caused when these trace elements are present in toxic concentrations (Sanchez-Marroquin *et al.*, 1970). If higher yields of citric acid are to be obtained then the trace element composition needs to be investigated each time a new strain of *A. niger* or substrate is used (Berry *et al.*, 1977).

The literature on citric acid production from crude carbohydrate materials (e.g. cane and beet molasses, starch hydrolysate etc.) has been adequately reviewed (Perlman and Sih, 1960; Miall, 1978) and among the vast number of reports there is general agreement that a relatively high concentration of certain trace elements in these crude carbohydrates causes inhibitory effects on citric acid production. Many investigators obtained higher yields of citric acid from these crude carbohydrates by treatment with cation exchange materials (Karow and Waksman, 1947; Snell and Schweiger, 1949; Miles Laboratories, 1952; Tomlinson *et al.*, 1951), aluminium hydroxide (Shu and Johnson, 1948a) or ferrocyanide (Perlman *et al.*, 1946; Gerhardt *et al.*, 1946; Martin and Waters, 1952; Clement, 1952; Steel *et al.*, 1955; Clark and Lentz, 1961; Clark, 1962).

The experimental results in Part-I indicated relatively poor citric acid production with poor lactose utilization. It seemed possible that the low production of citric acid might be due to the effect of the concentrations of trace elements present in the lactic casein whey permeate (Table 5.2). There has been no work reported so far on the effect of trace element concentration on citric acid production from lactic casein whey permeate. Therefore, an attempt was made to investigate the effect of trace elements on citric acid production from this substrate.

5.9 THE USE OF DECATIONIZED LACTIC CASEIN WHEY PERMEATE FOR THE PRODUCTION OF CITRIC ACID

5.9.1 INTRODUCTION

The exact concentrations of various trace elements necessary to obtain high production of citric acid from whey permeate are not known. Therefore, it was decided to perform an initial experiment using decationized whey permeate to observe its effects on the production of citric acid and also to determine the guidelines for further research in this area.

5.9.2 Results and Discussion

Lactose-supplemented (100 g/l) lactic casein whey permeate was passed through cation exchange materials (see details in Section 3.6) and this decationized whey permeate was used as the fermentation medium. The content of trace elements before and after decationization is shown in Table 5.2. During the decationization process a substantial loss of total nitrogen was observed (before decationization 0.55 gN/l, after decationization 0.35 gN/l). Before measuring the content of trace elements and using the permeate in the fermenter, the pH of the decationized whey permeate was adjusted to pH 4.5 using 2M NaOH. It was found in initial investigations that the strain *A. niger* MH 15-15 grew poorly in the decationized whey permeate. Therefore, the inoculum was grown in non-decationized, lactose-supplemented whey permeate and a 10% (v/v) inoculum was used to inoculate the fermenter. The experimental conditions in the fermenter were similar to those previously described (Section 5.2.2). The time course of the fermentation is shown in Figure 5.11. The results show that a citric acid concentration of 3.5 g/l was obtained at the end of the fermentation, representing a yield of 9% (w/w) based on lactose utilized. Similarly, the final mycelial dry weight (6 g/l) was lower than that obtained using non-decationized whey permeate. The pH value gradually decreased to pH 1.8 as the citric acid accumulated, while the lactic acid was completely utilized by the fourth day of fermentation. The lactose utilization was very low (29% of that supplied). Fermentation samples were analysed for the presence of free galactose and glucose (hydrolysis products of lactose) but neither was detected. Thus, it appears that lactose is not hydrolysed extracellularly.

The volumetric and specific rate data of citric acid production, shown in Figure 5.12, indicate that the maximum rate (0.032 g/l.h) and specific rate (0.0065 g/gDW.h) were reached on the fourth day of fermentation, which was one day earlier than on non-decationized whey permeate. However, they were considerably lower in value (refer to Figure 5.5).

TABLE 5.2 TRACE ELEMENT COMPOSITION OF LACTIC CASEIN WHEY PERMEATE BEFORE AND AFTER DECATIONIZATION.

Trace Element	Before Decationization (mg/l)	After Decationization (mg/l)
K^+	600	1.2
Na^+	372	10.0
Ca^{2+}	150	15.0
Mg^{2+}	57	0.84
Zn^{2+}	3	0.13
Mn^{2+}	0.13	0.016
Fe^{2+}	1.2	0.53
Pb^{2+}	0.27	0.13
Ni^{2+}	0.13	0.017
Cu^{2+}	0.17	0.018

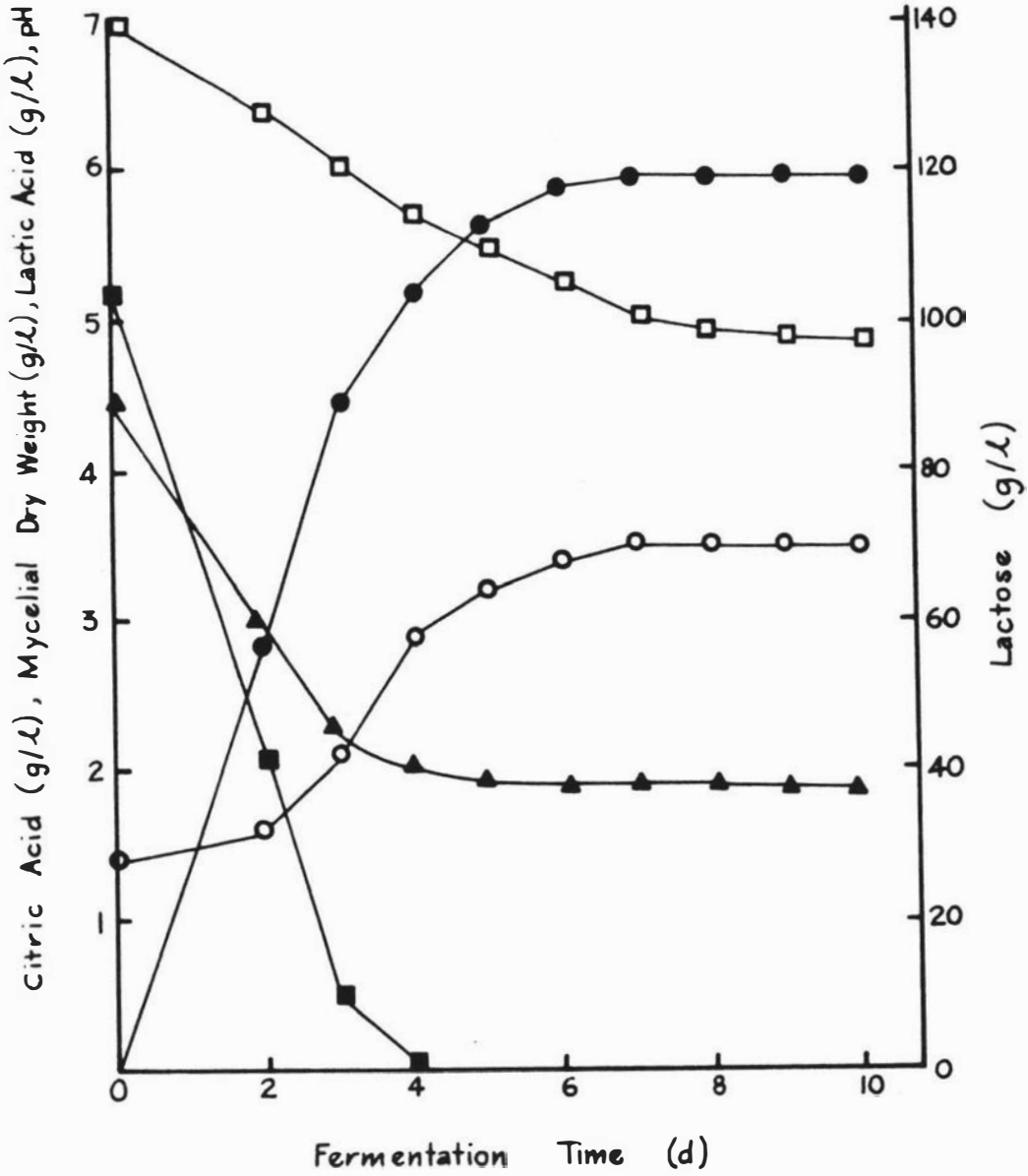

FIGURE 5.11 The production of citric acid from decationized lactose-supplemented lactic casein whey permeate.

Citric acid (O), mycelial dry weight (●), lactose (□), lactic acid (■), pH (▲).

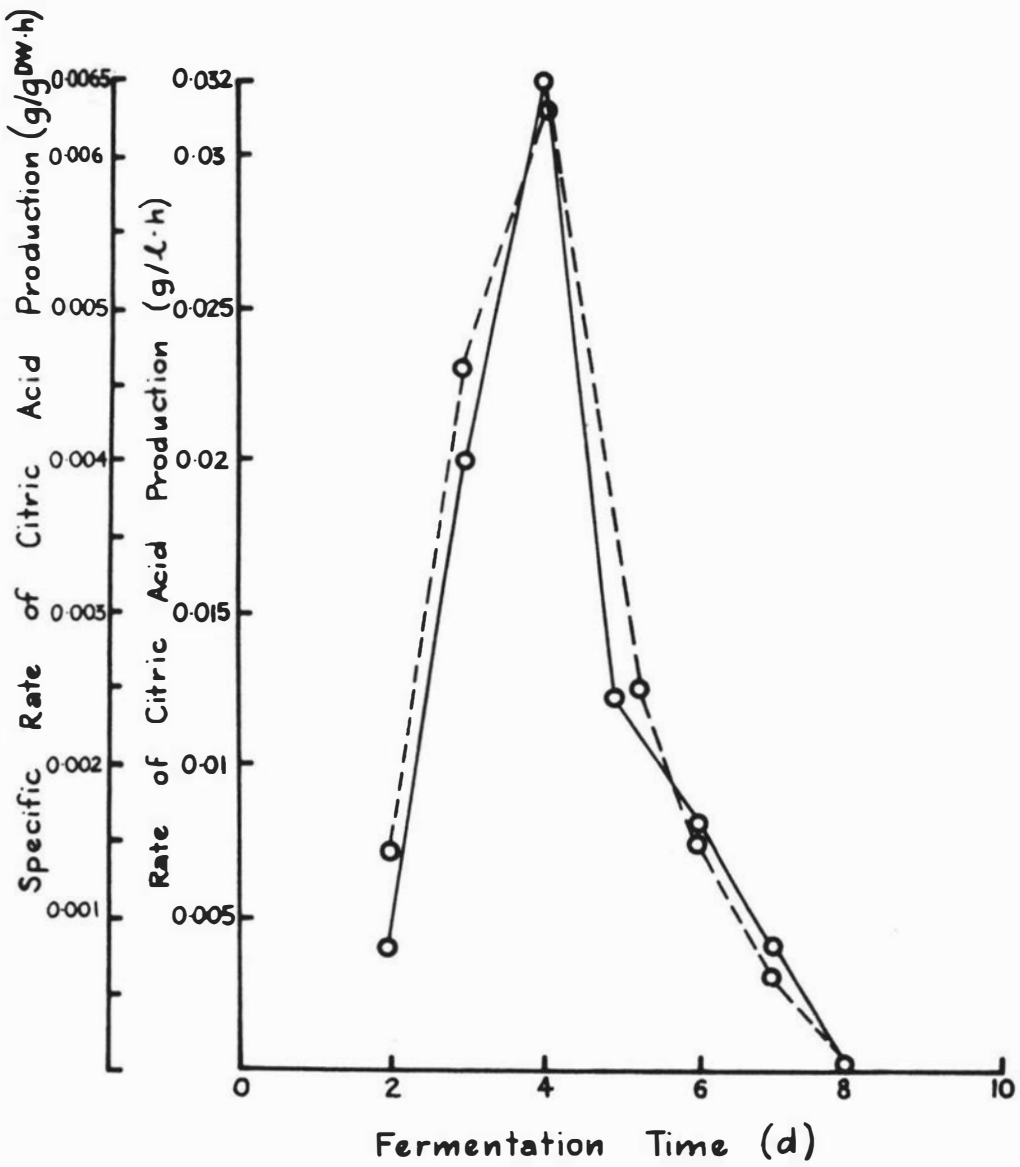

FIGURE 5.12 Volumetric and specific rate curves for citric acid production in decationized lactic casein whey permeate.

Rate (O—O), specific rate (O--O).

In view of the above findings, it can be concluded that the low level of citric acid production, mycelial dry weight and lactose utilization were the consequence of trace element deficiency in the whey permeate. Therefore, subsequent experiments were designed to supplement some essential trace elements with the aim of improving the situation.

5.10 THE EFFECT OF SUPPLEMENTATION WITH Fe^{2+}, Cu^{2+}, Zn^{2+} AND Mg^{2+}

5.10.1 INTRODUCTION

The results of the previous experiment indicated that the poor yield of citric acid in the decationized whey permeate was the effect of some trace element deficiency. The next stage, therefore, was to supplement the decationized permeate with known quantities of some trace elements.

5.10.2 RESULTS AND DISCUSSION

The decationized lactic casein whey permeate was supplemented with Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+} as shown in Table 5.3. The supplementation was based on that used in the synthetic medium of Kristiansen and Charley (1981) (Table 3.2). The inoculum was grown on non-decationized whey permeate, but before addition to the fermenter, the pellets were washed aseptically three times (to avoid trace element contamination) with sterile decationized glass-distilled water (about 1 litre). The washed pellets were suspended in 500 mL of fermentation medium and then added to the fermenter. For all subsequent experiments using decationized whey permeate, an identical inoculum preparation procedure was used. The time course of the fermentation is presented in Figure 5.13. By comparison with Figure 5.11, it is apparent that trace element supplementation significantly improved the citric acid production, lactose utilization and mycelial dry weight. However, after the maximum citric acid concentration (7 g/l) had been reached on the fourth day of fermentation, the citric acid was depleted from the medium despite the presence of lactose. In contrast, this phenomenon was not observed in unsupplemented decationized whey permeate, suggesting that a trace

TABLE 5.3 TRACE ELEMENT CONCENTRATIONS IN DECATIONIZED WHEY PERMEATE AND FINAL CONCENTRATIONS AFTER SUPPLEMENTATION.

Trace Element	Decationized Whey Permeate (mg/l)	Final Concentration* After Supplementation (mg/l)
Fe^{2+}	0.53	0.63
Cu^{2+}	0.018	0.078
Zn^{2+}	0.13	0.23
Mg^{2+}	0.84	500

* Supplementation was made to the levels used by Kristiansen and Charley (1981).

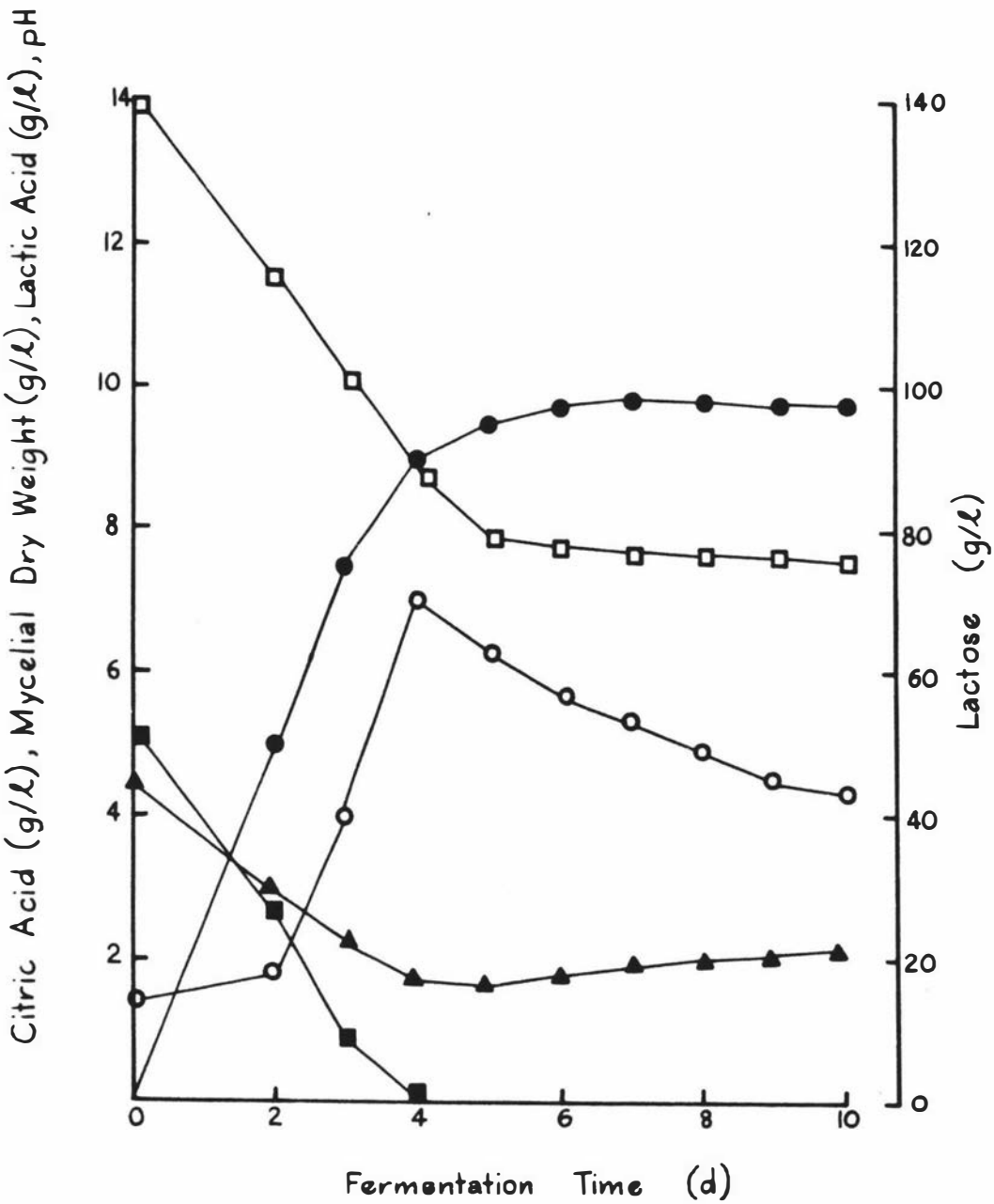

FIGURE 5.13 The production of citric acid in decationized lactic casein whey permeate, supplemented with Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+}.

Citric acid (O), mycelial dry weight (●), lactose (□), lactic acid (■), pH (▲).

elements imbalance might be the cause of citric acid depletion. The pH value decreased gradually from pH 4.5 to pH 1.8 and then increased slightly as the citric acid was depleted. Lactic acid was utilized as in the previous experiments. In comparison with Figure 5.4, using non-decationized whey permeate, citric acid production was markedly less (7.0 g/l compared to 14.8 g/l), while the mycelial dry weight was only slightly lower (10 g/l compared to 12 g/l). This latter observation suggests that although some nitrogenous materials have been removed during decationization, this does not fully explain the lower citric acid production.

The volumetric and specific rate data are presented in Figure 5.14. By comparison with Figure 5.12, it is clear that the maximum production rates observed (0.125 g/l.h and 0.013 g/gDW.h) were about four times and two times higher respectively, than without trace element supplementation. However, these rates were slightly less than those observed using non-decationized whey permeate (Figure 5.5).

From the above findings, it may be concluded that trace element supplementation improved citric acid production from decationized whey permeate, but the subsequent depletion of citric acid suggests an imbalance of trace elements. Hence, further experiments were designed in an attempt to remedy the situation and to improve on the production observed using non-decationized whey permeate.

5.11 THE EFFECT OF Mn^{2+} IN THE PRESENCE OF OTHER TRACE ELEMENTS (Fe^{2+}, Cu^{2+}, Zn^{2+} AND Mg^{2+})

5.11.1 INTRODUCTION

The presence of manganese ions in the fermentation medium has been reported to be detrimental to citric acid production by *A. niger* (Shu and Johnson, 1947; Clark *et al.*, 1966; Ilczuk and Dobrowolska, 1971; Kisser *et al.*, 1980). However, Tomlinson *et al.* (1950, 1951) and Berry *et al.* (1977) reported that the presence of a very small amount of Mn^{2+} in the fermentation medium permitted higher yields of

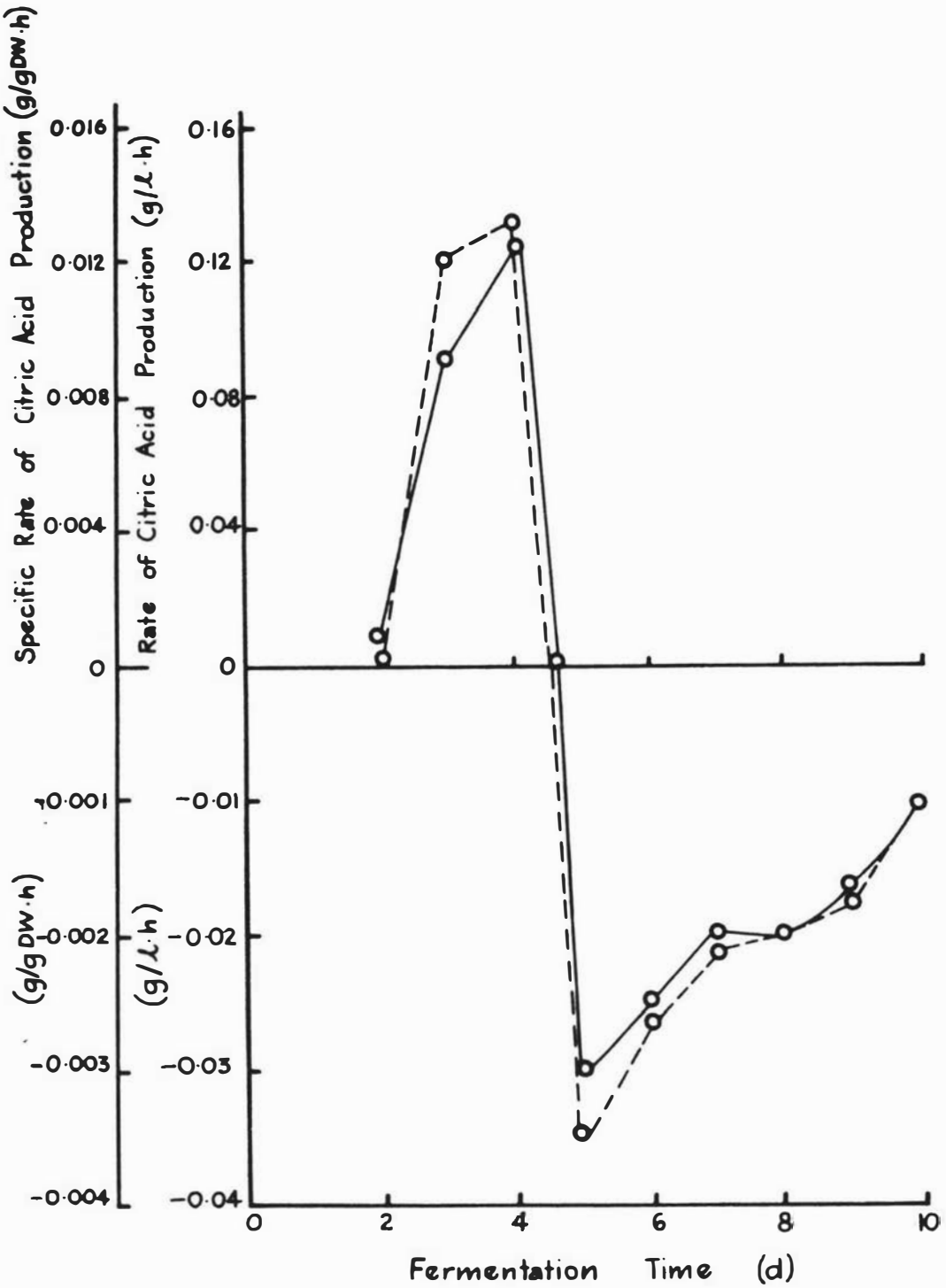

FIGURE 5.14 Volumetric and specific rate curves for citric acid production in decationized lactic casein whey permeate supplemented with Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+}. Negative values indicate the utilization of citric acid.

Rate (O—O), specific rate (O--O).

citric acid. These reports appear to be contradictory and thus it was considered useful to investigate the effect of Mn^{2+} supplementation in the decationized whey permeate, in the presence of other trace elements.

5.11.2 Results and Discussion

During decationization, the concentration of Mn^{2+} in the permeate decreased from 0.13 mg/l to 0.016 mg/l (Table 5.2). Thus, 0.1 mg/l Mn^{2+} (as $MnCl_2.4H_2O$) was added to the decationized permeate for this experiment. The other trace elements (Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+}) were also supplemented as in the previous experiment (Section 5.10). The effect of Mn^{2+} addition on the fermentation is shown in Figure 5.15. By comparison with Figure 5.13, it can be seen that there was a marked reduction of citric acid production and yield, lactose utilization, and mycelial dry weight, indicating an adverse effect of Mn^{2+} on citric acid accumulation under this study condition. The result also indicates that the added Mn^{2+} did not check the depletion of citric acid.

The mechanism by which Mn^{2+} in the medium prevents the accumulation of citric acid is not clearly understood. However, it has been suggested that in its presence, the necessary repression of TCA-cycle enzyme synthesis does not occur (Kubicek and Rohr, 1977; Bowes and Mattey, 1979).

From the result of this experiment it was concluded that citric acid accumulation was greatly reduced in the presence of added Mn^{2+} and therefore it was not included in the subsequent experiments.

5.12 The Effect of Nitrogen in the Presence of Trace Elements (Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+})

5.12.1 Introduction

It was earlier observed using non-decationized whey permeate (Section 5.5) that nitrogen-feeding after onset of citric acid production significantly improved the production of citric acid.

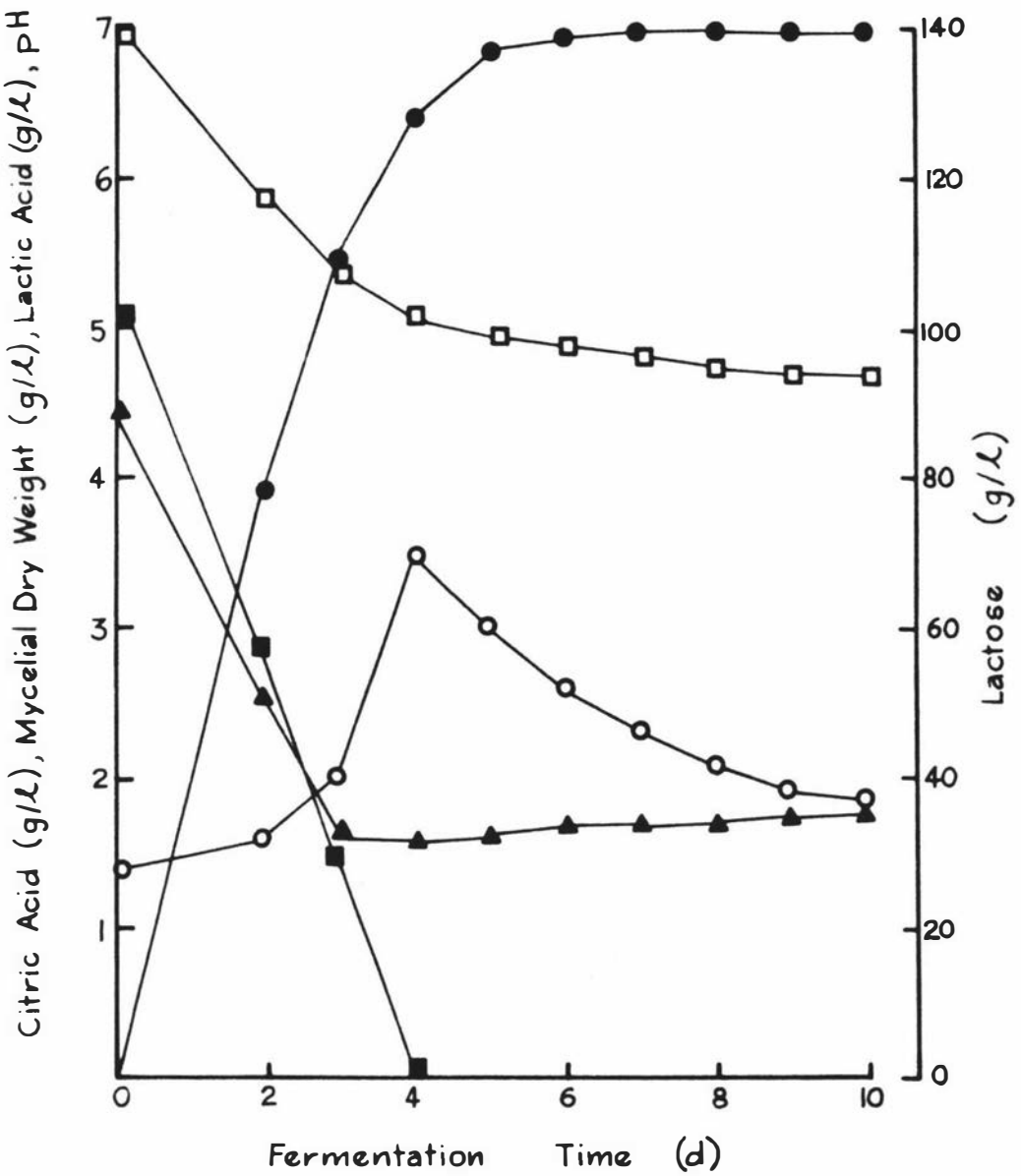

FIGURE 5.15 The effect of Mn^{2+} in the presence of other trace elements (Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+}) on the production of citric acid.

Citric acid (O), mycelial dry weight (●), lactose (□), lactic acid (■), pH (▲).

It was also observed, using decationized whey permeate, supplemented with Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+} (Section 5.10) that the process of decationization removed some nitrogenous material from the medium. Further, it may be possible that nitrogen deficiency was somehow involved in citric acid depletion (Figure 5.13). Hence some nitrogen supplementation at the point of commencement of citric acid depletion was considered useful.

5.12.2 Results and Discussion

This experiment was identical to that described in Section 5.10 with the exception that at the point of citric acid depletion (i.e. on the fourth day) 0.25 gN/l (as $(NH_4)_2SO_4$) was added to the culture medium. The course of the fermentation is presented in Figure 5.16. The results show that on the addition of nitrogen, citric acid depletion was completely checked and production continued and reached a maximum concentration of 9 g/l, representing a yield of 12% (w/w) on the basis of lactose utilized. There were also increases in lactose utilization and mycelial dry weight (in comparison with Figure 5.13). It can be seen from the nitrogen utilization curve (Figure 5.16) that the nitrogen concentration of the culture medium was very low (0.035 g/l) at the time of nitrogen addition and the added nitrogen was completely utilized.

It can be concluded from these results that nitrogen supplementation can overcome the problem of citric acid depletion, but citric acid production still compares unfavourably with that observed using non-decationized whey permeate (Figure 5.4). Hence, some imbalance in the trace element composition may remain.

5.13 The Effect of Zn^{2+} Omission in the Presence of Nitrogen and Trace Elements (Fe^{2+}, Cu^{2+} and Mg^{2+})

5.13.1 Introduction

There are many conflicting reports in the literature regarding the exact concentration of zinc ions required for good production

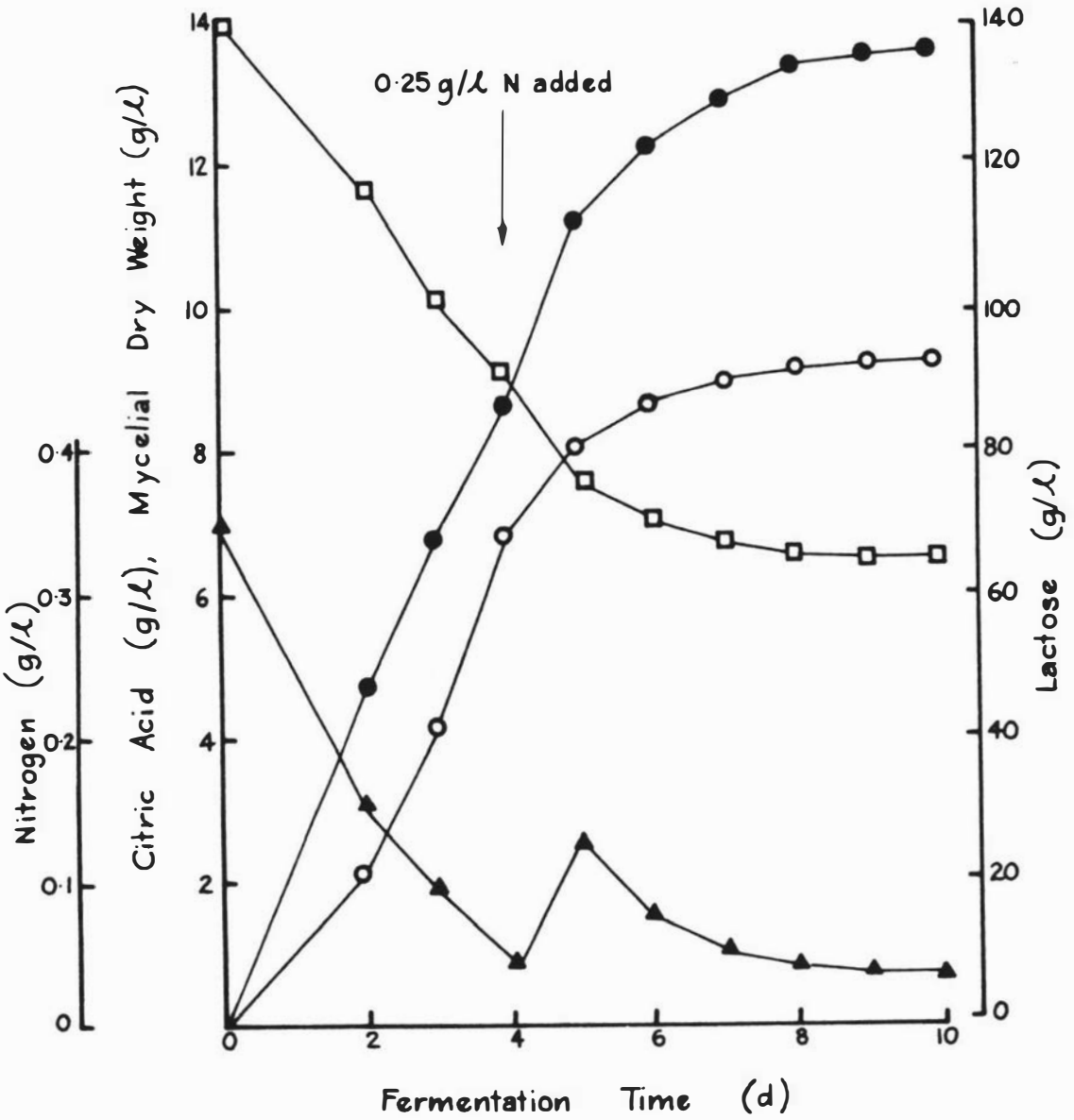

FIGURE 5.16 The effect of nitrogen in the presence of trace elements (Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+}) on the production of citric acid.

Citric acid (O), mycelial dry weight (●), lactose (□), nitrogen (▲).

of citric acid, although it is generally agreed that the concentration should be low (less than 0.065 mg/l). Kapoor *et al.* (1982) have reported that a high concentration of Zn^{2+} allows vegetative growth at the cost of citric acid production. The addition of excess Zn^{2+} to the citric acid-producing culture was found to reverse the acid production phase and its deficiency during growth apparently signals the transition from the growth phase to the acid production phase (Kapoor *et al.*, 1982). In view of this and considering the Zn^{2+} content of decationized whey permeate (0.13 mg/l, Table 5.2), it was decided to perform a fermentation without supplementation with Zn^{2+} ions.

5.13.2 Results and Discussion

The experimental conditions were identical to those described in Section 5.12 with the exception that Zn^{2+} supplementation was omitted. The course of the fermentation is shown in Figure 5.17. By comparison with Figure 5.16, it is apparent that Zn^{2+} omission resulted in an improvement in citric acid production (12 g/l compared to 9 g/l), but the yield remained at approx. 15% based on lactose utilized. However, there were no significant differences in lactose utilization mycelial dry weight or nitrogen utilization between these two experiments.

5.14 Discussion and Conclusions

The major point to arise from these results obtained using decationized whey permeate is that no condition was found which gave better citric acid production than when using non-decationized whey permeate. The use of decationized permeate without trace element supplementation clearly restricted both mycelial growth and citric acid production. However, supplementation with Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+}, to the level described by Kristiansen and Charley (1981), and used in the sucrose-based synthetic medium during the strain selection programme (Section 4.2.2), gave lower citric acid production than when using non-decationized permeate. A possible reason for this is that the decationization procedure used did not completely remove all the trace elements present (Table 5.2), and the remaining ions may have considerable effect on citric acid production. Possibly, a second decationization treatment may have removed these contaminants. However, at this stage of the

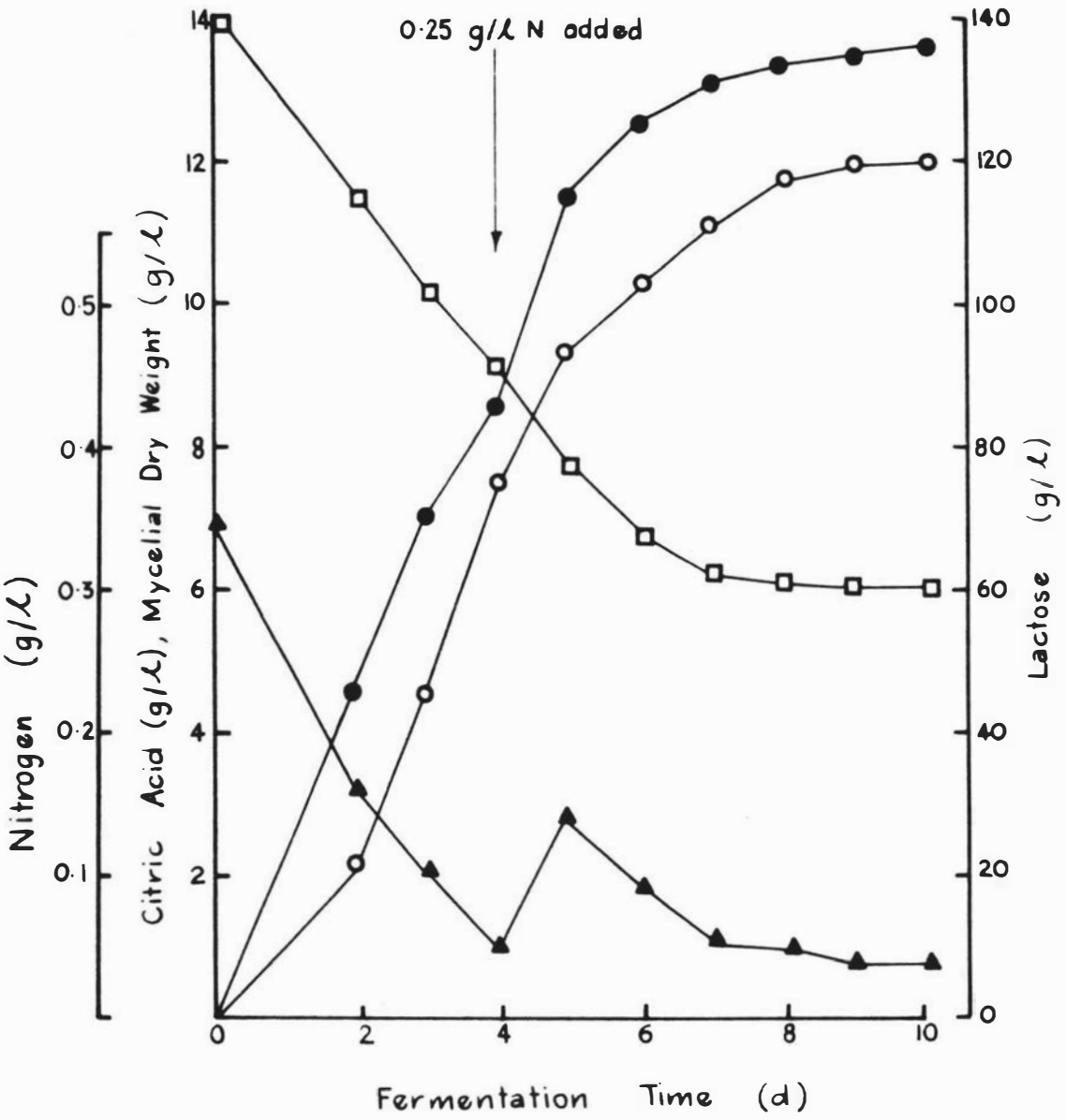

FIGURE 5.17 The effect of Zn^{2+} omission in the presence of nitrogen and trace elements (Fe^{2+}, Cu^{2+} and Mg^{2+}) on the production of citric acid.

Citric acid (O), mycelial dry weight (●), lactose (□), nitrogen (▲).

study, it was considered more appropriate to pursue a different line of investigation, i.e. the effect of sugar source, since little progress was being made with trace element composition.

Other points to arise from the results include:

(a) Depletion of citric acid from the medium can occur even when large quantities of lactose are remaining. This depletion can be prevented by the addition of nitrogen.

(b) The inhibitory effect of Mn^{2+}, described by many workers, was observed.

CHAPTER 6

THE EFFECTS OF DIFFERENT SUGAR SOURCES ON CITRIC ACID PRODUCTION AND SOME TCA-CYCLE ENZYME ACTIVITIES

6.1 INTRODUCTION

A variety of carbohydrate sources have been used by different workers for the production of citric acid. The most important of these are cane sugar, sugar beet molasses, sugar cane molasses, glucose, sugar cane juice and liquified starch obtained by acid or enzyme hydrolysis (Divekar *et al.*, 1971). The majority of the published work on citric acid production in synthetic media refers to either glucose or sucrose and very little information is available on the use of fructose, lactose or galactose. Glucose has been found to be a favourable sugar source for citric acid production, but not as good as sucrose (Karow and Waksman, 1947; Srivastava and Kamal, 1979). In his comparative study of citric acid production in surface culture using different carbon sources including sucrose, glucose, fructose and galactose, Bernhauer (1928) reported that more citric acid was produced from sucrose than from glucose or fructose and only a trace amount was produced from galactose. A similar report has appeared from Noguchi (1962), using shake-flask culture.

From the initial investigation during strain selection and improvement (Chapter 4) it was evident that the mutant strain *A. niger* MH 15-15 produced greater quantities of citric acid in a sucrose-based synthetic medium than in whey permeate. Since the investigation into the trace element composition of whey permeate provided no significant increase in citric acid production, it was decided to investigate the effects of sucrose, glucose, fructose, lactose and galactose on the production in both shake-flask and fermenter culture.

Several theories have appeared in the literature concerning the mechanism of citric acid accumulation by *A. niger* (Prescott and Dunn,

1959; Perlman and Sih, 1960; Meyrath, 1967) but apparently there is still no single hypothesis which accounts for the optimum physiological conditions required to obtain high yields (Smith *et al.*, 1974; Lockwood, 1975). The extent to which the TCA-cycle operates during the citric acid production phase is controversial. Various reports have stated that citric acid accumulation by *A. niger* is accompanied by a complete disappearance or marked reduction in the activities of aconitase and NAD-and NADP-linked isocitric dehydrogenase (Ramkrishnan *et al.*, 1955; La Nauze, 1966; Szczodrak, 1981). Conversely, Ahmed *et al.* (1972) has reported that these enzymes remain active and no changes in their activities occur throughout the period of fermentation. However, most of the published work on TCA-cycle enzyme activies has been done using sucrose-based synthetic media (La Nauze, 1966; Feir and Suzuki, 1969; Ahmed *et al.*, 1972; Mattey, 1977; Kubicek and Rohr, 1977, 1978; Bowes and Mattey, 1979), glucose (Ramkrishnan and Martin, 1954) or molasses (Ramkrishnan *et al.*, 1955; Szczodrak, 1981). There appear to have been no studies performed on TCA-cycle enzyme activities when growing the same strain of organism on different sugars, under otherwise identical fermentation conditions. Therefore, the role of some of the key enzymes of the TCA-cycle related to citric acid accumulation from different sugar sources in fermenter culture was investigated. The sugars used were sucrose, glucose, fructose, lactose and galactose.

6.2 RESULTS AND DISCUSSION

6.2.1 THE EFFECTS OF NON-DECATIONIZED AND DECATIONIZED SUGARS ON CITRIC ACID PRODUCTION IN SHAKE-FLASK CULTURE

The experiments were conducted in shake-flask using both non-decationized and decationized sucrose, glucose, fructose, lactose or galactose in the medium of Kristiansen and Charley (1981) (Table 3.2). The procedure of decationization was described in Section 3.6. The trace element content of these sugars was determined before decationization and the results are shown in Table 6.1. The data demonstrate that there was neither Cu^{2+} nor Mn^{2+} in any of these sugars but Fe^{2+} was detected in all. Zinc was not detected in sucrose or glucose but

TABLE 6.1 TRACE ELEMENT CONTENT OF DIFFERENT SUGARS BEFORE DECATIONIZATION.

Sugar	Trace Element (µg/g)			
	Fe^{2+}	Cu^{2+}	Zn^{2+}	Mn^{2+}
Sucrose	4.00	-	-	-
Glucose	2.26	-	-	-
Fructose	2.11	-	4.96	-
Lactose	1.32	-	6.20	-
Galactose	4.51	-	7.20	-

- Not detected.

it was present in fructose, lactose and galactose.

The fermentations were continued for 14 days and flasks were harvested after 2,4,6,8,10,12 and 14 days. Summarized results of the effect of both non-decationized and decationized sugars on citric acid production are presented in Table 6.2.

The time course of citric acid production from both decationized and non-decationized sugars is shown in Figure 6.1. It can be seen that citric acid was not obtained from either non-decationized or decationized galactose. Clearly, the sugar source appears to be very important in respect of citric acid production. In non-decationized sugars, at the end of fermentation, the highest concentration of citric acid was obtained from sucrose, followed by glucose, fructose and then lactose (Table 6.2).

The effect of sugar decationization on citric acid production was only slight with fructose and lactose but was quite marked with sucrose and glucose, where greater production was observed using the decationized sugar. Possibly this can be explained by the iron content of the non-decationized sugars, as excess iron has been shown to lower the yield of citric acid production (Schweiger, 1961). (Note that using non-decationized sucrose at 140 g/l represents an Fe^{2+} concentration of 0.56 mg/l, cf. 0.1 mg/l in the synthetic medium).

The volumetric and specific rate data of citric acid production from decationized and non-decationized sugars are shown in Figure 6.2 and 6.3 respectively. It is apparent from the data that the maximum rates of 0.38 g/l.h and 0.43 g/l.h were obtained from non-decationized and decationized sucrose respectively and were reached on the twelfth day of fermentation. In contrast, the maximum rates of citric acid production from glucose, fructose and lactose were reached on the eighth day. The reason for such a difference in time to reach the maximum rate is not understood. No significant differences in the specific rates of citric acid production were observed between decationized and non-decationized sugars.

TABLE 6.2 SUMMARIZED RESULTS OF THE EFFECTS OF DIFFERENT SUGARS ON CITRIC ACID PRODUCTION IN SHAKE-FLASK CULTURE.

Results are expressed after 14 days of fermentation.

Sugar	Non-Decationized Sugars					Decationized Sugars				
	pH*	Mycelial Dry Weight (g/l)	Sugar+ Utilized (g/l)	Citric Acid Concentration (g/l)	Citric Acid** Yield (%) (w/w)	pH*	Mycelial Dry Weight (g/l)	Sugar+ Utilized (g/l)	Citric Acid Concentration (g/l)	Citric Acid** Yield (%) (w/w)
Sucrose	1.8	18.8	110[a]	52.8	48.0	1.8	18.0	109	60.5	55.5
Glucose	1.9	19.5	90	31.0	34.5	1.9	19.0	94	37.0	39.5
Fructose	2.0	17.8	92	23.0	25.0	2.0	17.3	92	25.0	27.5
Lactose	2.0	9.8	68	5.0	7.5	2.0	9.4	65	5.2	8.0
Galactose	2.4	12.5	73	0	-	2.3	12.0	76	0	-

\+ Initial sugar concentration 140 (g/l).

* Initial pH 6.5.

** Based on sugar utilized.

a Result expressed as total of glucose and fructose (hydrolysis products of sucrose). See Figure 6.5(b) for details.

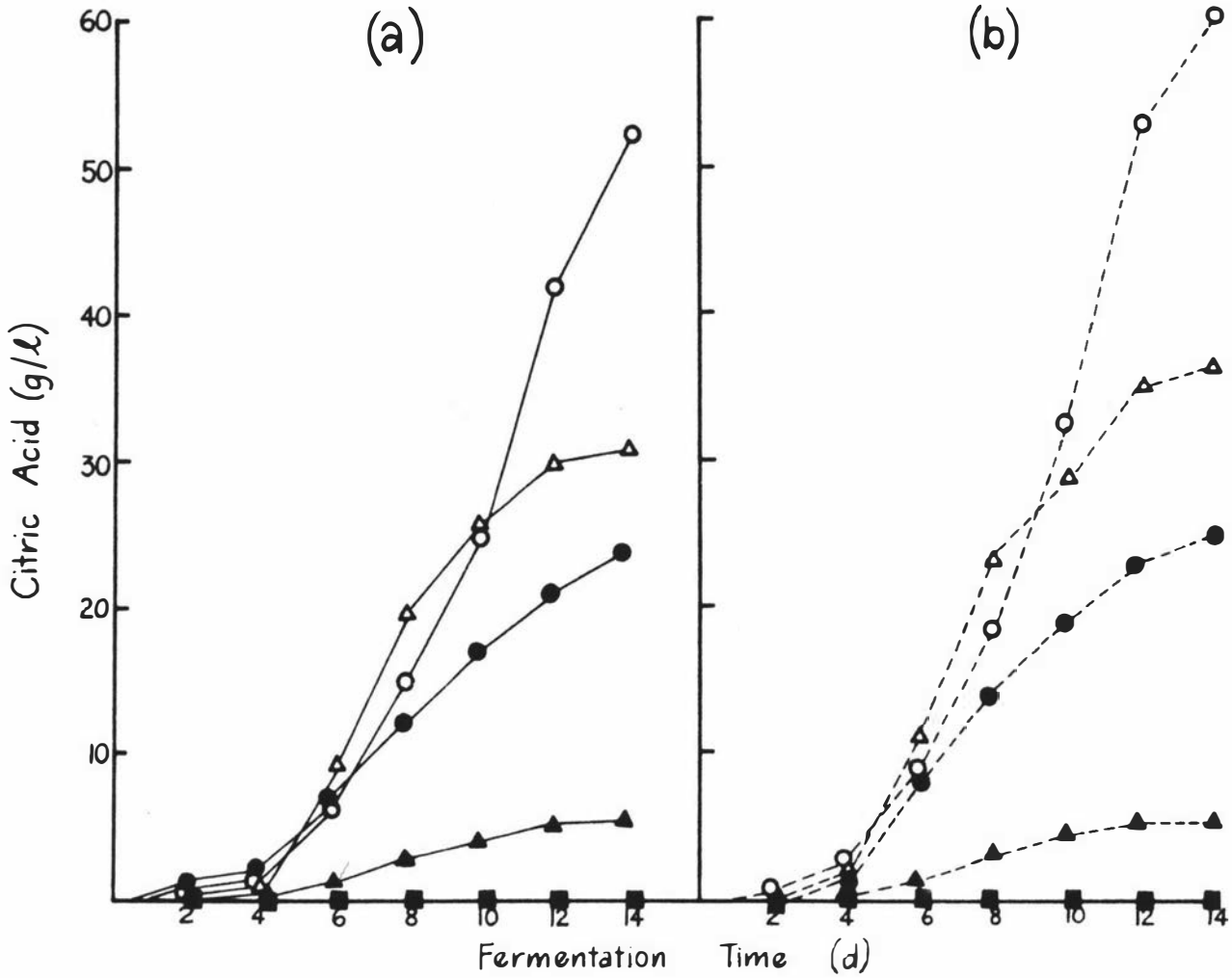

FIGURE 6.1 The effect of different sugar sources on citric acid production in shake-flask culture. (a) Non-decationized sugars and (b) decationized sugars.

Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

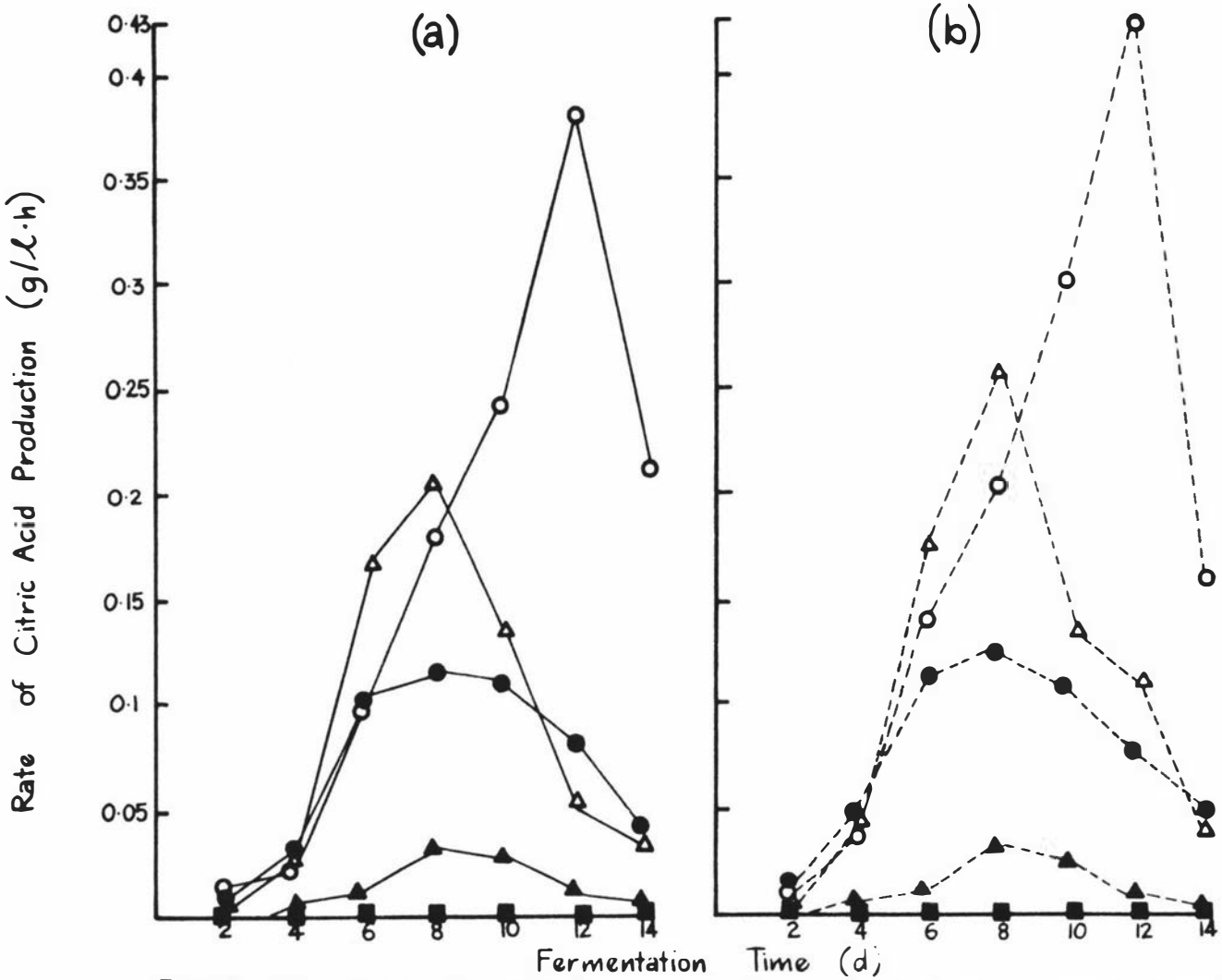

FIGURE 6.2 The volumetric rate of citric acid production from different sugars in shake-flask culture. (a) Non-decationized sugars and (b) decationized sugars.
Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

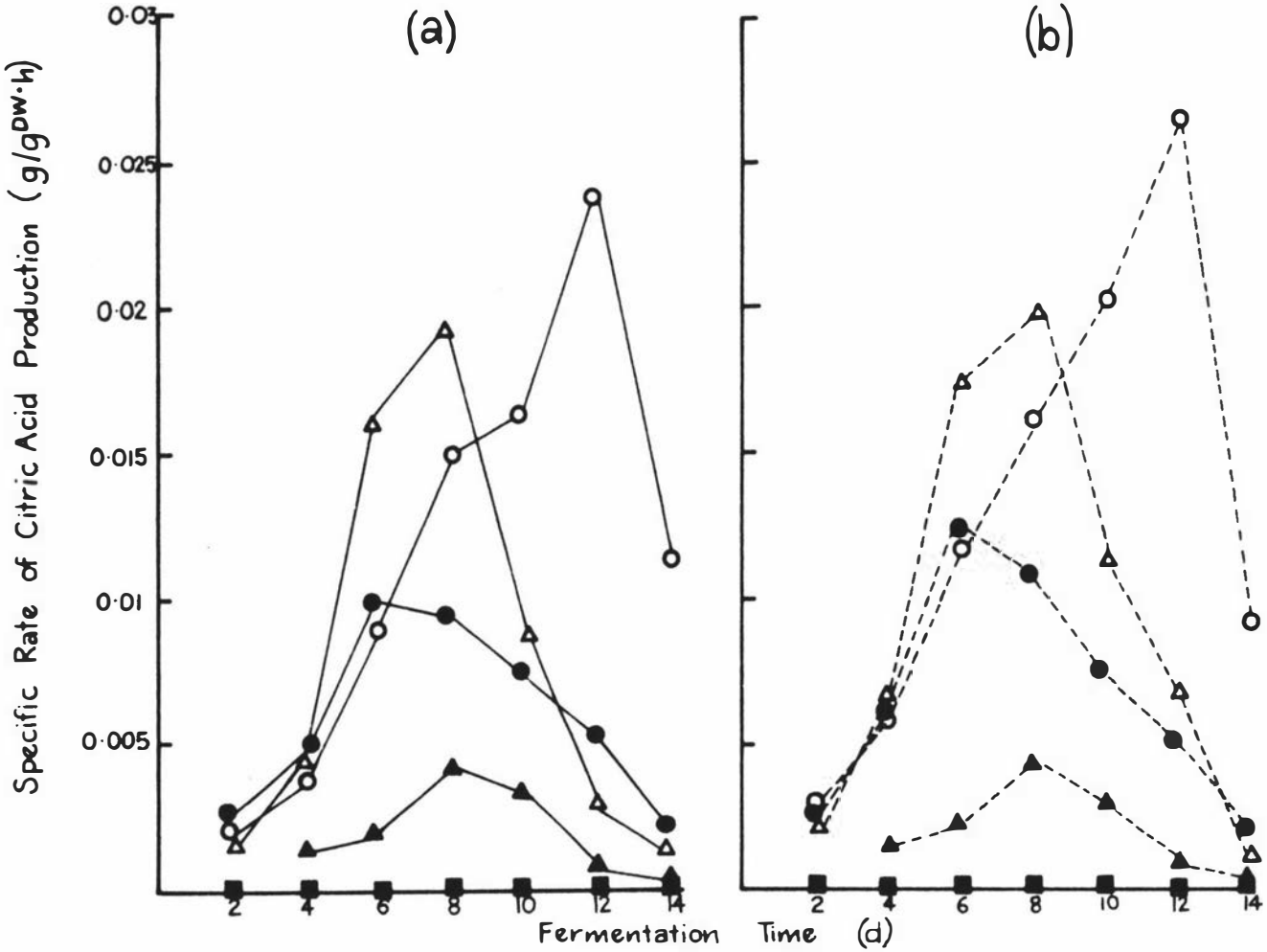

FIGURE 6.3 The specific rate of citric acid production from different sugars in shake-flask culture. (a) Non-decationized sugars and (b) decationized sugars.
Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

By comparison with Figure 4.2 (Chapter 4), it is apparent that citric acid production from lactose-supplemented whey permeate (10.2 g/l) was greater than that from the lactose-based synthetic medium (5.0 g/l) (Figure 6.1(a)). The yields were 20% (w/w) and 7.5% (w/w), respectively, based on lactose utilized, indicating that whey permeate is a superior medium for citric acid production from lactose. In terms of the volumetric production rate, a maximum rate of 0.055 g/l.h was obtained in whey permeate (Figure 4.3), which was slightly higher than that obtained in the lactose-based synthetic medium (0.040 g/l.h, Figure 6.2(a)). No significant difference in specific rates was observed (Figures 4.3 and 6.3(a)). Overall, these results indicate that the whey permeate is more suitable than the synthetic medium for citric acid production from lactose.

In terms of growth rates and mycelial dry weights obtained, there were no major differences between decationized and non-decationized sugars (Table 6.2). Therefore, the effects of non-decationized sugar sources only on mycelial dry weight are shown in Figure 6.4. It appears from the results that there was virtually no difference in mycelial dry weight production among sucrose, glucose and fructose. However, the mycelial weights produced from lactose and galactose were lower, indicating, perhaps, that these are less readily-utilizable carbon sources. Biomass yields on the basis of substrate utilized at the end of fermentation were 0.17, 0.22, 0.19, 0.14 and 0.17 g/g from sucrose, glucose, fructose, lactose and galactose respectively. These indicate that the efficiencies are fairly similar, except for lactose.

With regard to sugar utilization, no significant differences were observed between decationized and non-decationized sugars. Thus, the utilization of non-decationized sugars only is presented in Figure 6.5. Interestingly, sucrose was completely hydrolysed to glucose and fructose within the first 30 hours of fermentation and then the two monosaccharides were utilized simultaneously (Figure 6.5(b)). In contrast, the lactose was not hydrolysed outside the cell and was utilized as such. The volumetric rates of sugar utilization are shown in Figure 6.6. The

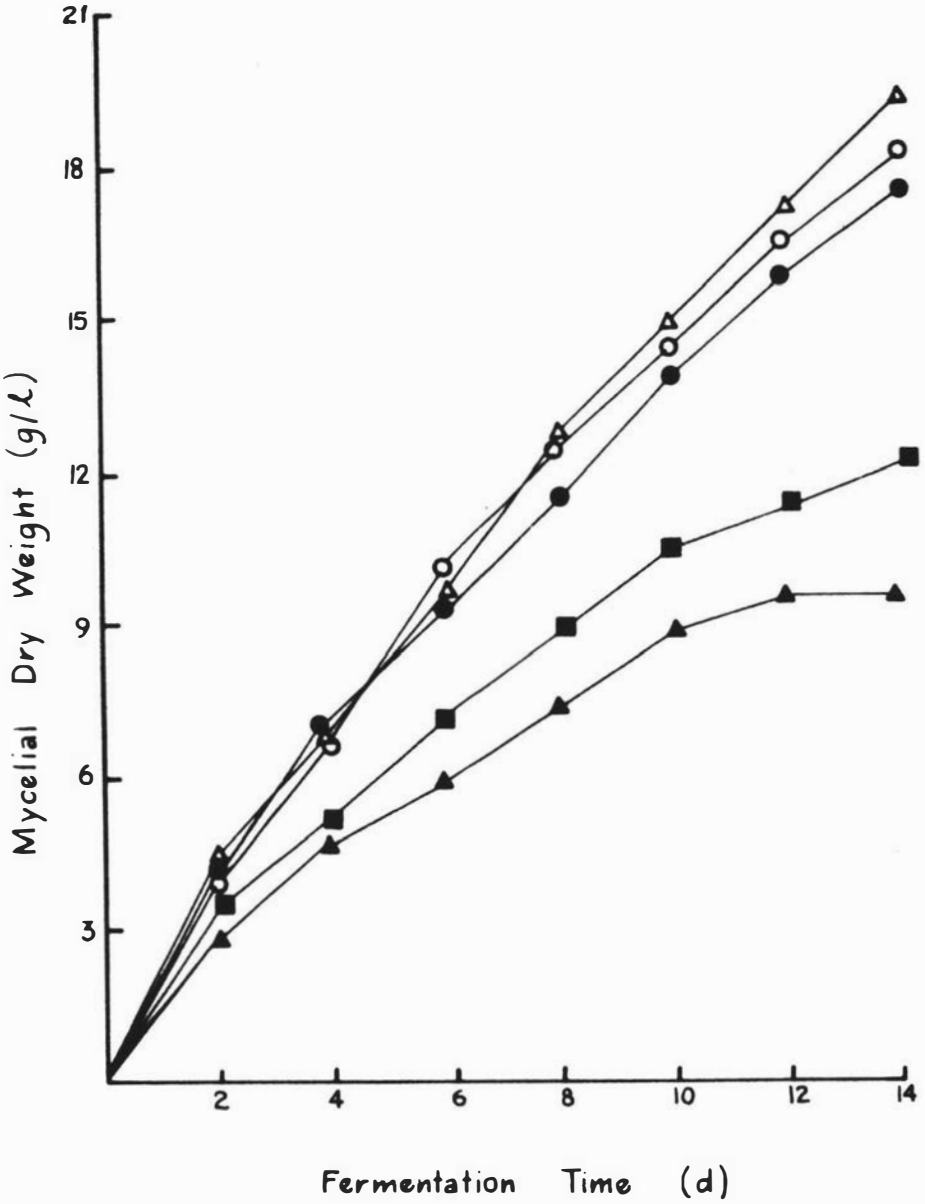

FIGURE 6.4 The effect of different sugars on mycelial dry weight production in shake-flask culture.

Sucrose (○), glucose (△), fructose (●) lactose (▲), galactose (■).

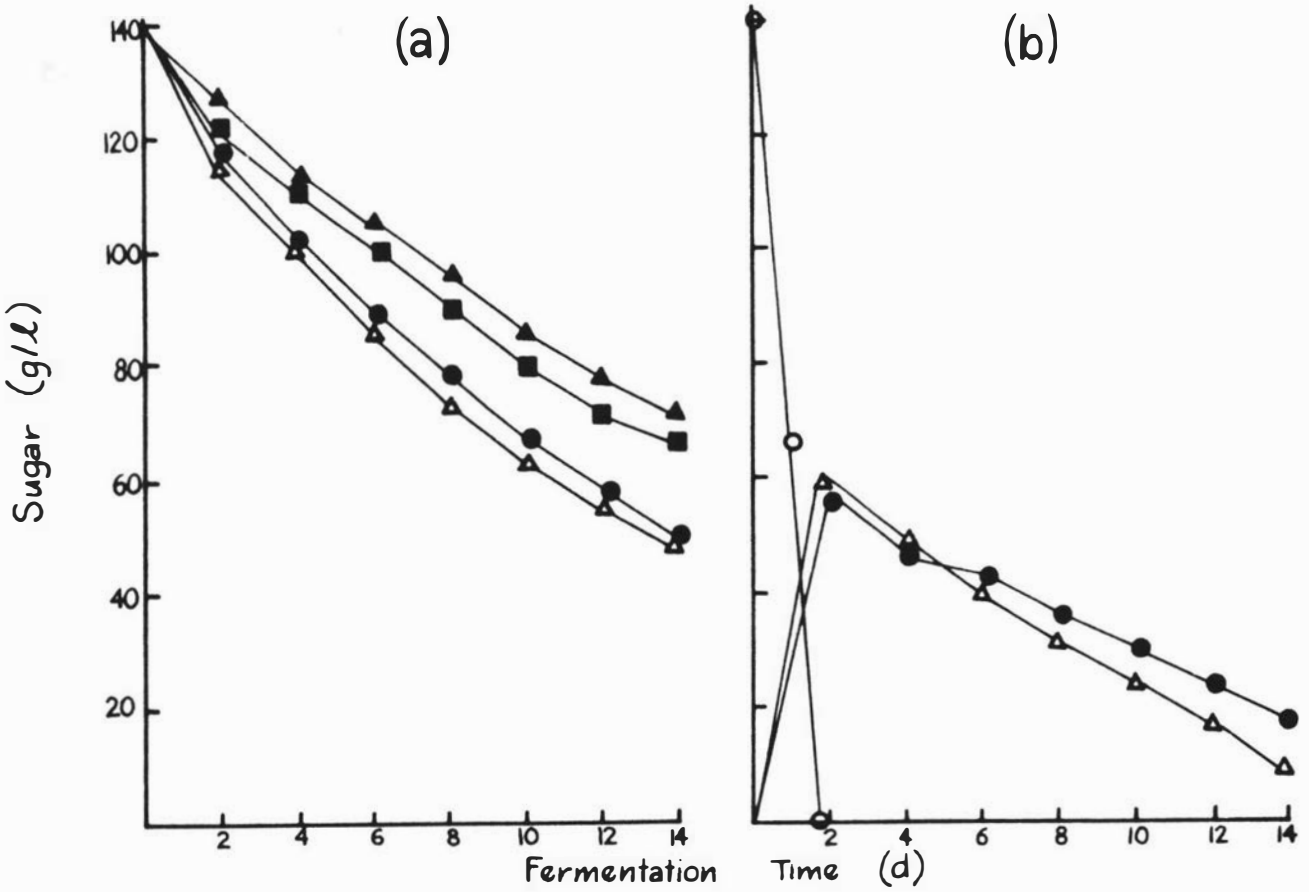

FIGURE 6.5 Utilization of different sugars in shake-flask culture. (a) Showing glucose, fructose, lactose and galactose utilization and (b) showing the utilization of sucrose and its hydrolysis products (glucose and fructose).

Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

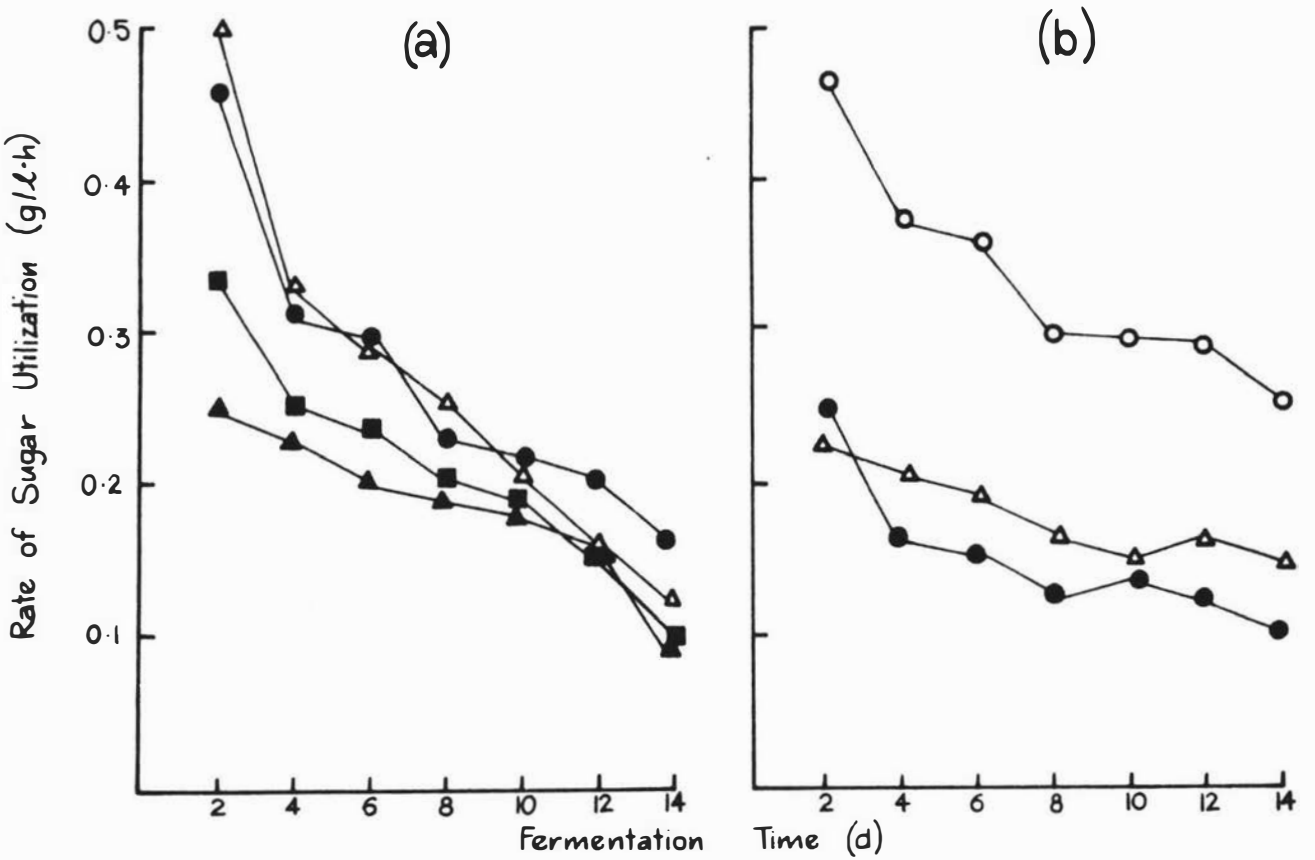

FIGURE 6.6 The volumetric rates of sugar utilization in shake-flask culture. (a) Showing glucose, fructose, lactose and galactose utilization and (b) showing utilization of glucose and fructose (hydrolysis products of sucrose).

Glucose (△), fructose (●), lactose (▲), galactose (■), combined rate of glucose and fructose (O).

rates of glucose and fructose utilization were virtually identical throughout the entire period of fermentation and were slightly higher than those of lactose and galactose. By comparison with Figure 6.6(a), however, Figure 6.6(b), indicates that the combined rate of utilization of the hydrolysis products of sucrose (glucose and fructose) was higher throughout the entire period of fermentation than that of individual glucose and fructose. This higher rate of utilization could possibly explain the higher citric acid production from sucrose. It may also be postulated that the simultaneous utilization of these two monosaccharides indicates the possibility of two uptake sites in the cell membrane.

These results of citric acid production from different sugars clearly indicate that the sugar source is important. The most significant finding is the lack of citric acid production from galactose, despite the fact that it is a utilizable carbon source. It may be concluded that this effect contributes to the relatively low production of citric acid from lactose when compared with glucose and fructose.

6.2.2 The Role of TCA-Cycle Enzyme Activities During Citric Acid Production from Different Sugar Sources in Fermenter Culture

As in the shake-flask experiments, the sugar sources used were sucrose, glucose, fructose, lactose and galactose, but they were not decationized before use. Lactose-supplemented (100 g/l) lactic casein whey permeate was also used. The fermentation conditions were similar to those described in the previous chapter in Section 5.2.2. Samples were withdrawn at intervals of 10-15 hours throughout the period of fermentation and were analysed for citric acid, mycelial dry weight, pH value and sugar concentration. For enzyme assays, mycelium was withdrawn after 2,4,6 and 8 days of fermentation. The cell-free extract was prepared according to the procedure described in Section 3.9.1 (Chapter 3) and assayed for aconitase, isocitric dehydrogenase (both NAD- and NADP-linked), 2-oxoglutarate dehydrogenase and pyruvate carboxylase. The results for the effects of different sugars and whey

permeate on citric acid production are summarized in Table 6.3.

The time course for citric acid production from the different sugar sources is presented in Figure 6.7. It is clear from the data that sucrose was again the superior carbon source for citric acid production. As in shake-flask culture, no citric acid was produced from galactose. By comparison of Tables 6.2 and 6.3 or Figures 6.1(a) and 6.7, it is apparent that lower citric acid production was obtained from all the sugars (except lactose) in fermenter culture. These reductions may be due to the fact that in the fermenter the pelletal growth broke down to a filamentous form. The importance of the pelletal form of growth in citric acid production has been discussed previously in Section 5.7. In shake-flask the pellets remained intact and solid (a prerequisite for higher citric acid production) and no filamentous form of growth was observed. No work was performed to investigate the cause of the filamentous form of growth in the fermenter, but the implication is that the agitation system was the cause. Interestingly, however, with whey permeate greater production of citric acid was obtained in the fermenter than in shake-flask culture (Chapter 5), although similar degeneration of the pelletal growth form occured in the fermenter. Possibly, this indicates some major difference between this substrate and the synthetic medium. In this respect, it is possible that in fermenter culture some trace element contamination occured from the stainless steel equipment. In the synthetic medium this may cause an imbalance in the trace element composition with a subsequent effect on citric acid production, particularly where production is expected to be high, e.g. from sucrose or glucose. In whey permeate, however, some trace element "buffering" power may be present, e.g. organic material, which prevents this imbalance.

The volumetric and specific rate data of citric acid production are presented in Figures 6.8 and 6.9 respectively. The maximum volumetric and specific rates of citric acid production were reached between the third and fourth days of the fermentation. In contrast, in shake-flask, it was on the twelfth day for sucrose and the eighth day for other sugars, and the maximum rates were slightly higher.

TABLE 6.3 SUMMARIZED RESULTS OF THE EFFECTS OF DIFFERENT SUGARS ON CITRIC ACID PRODUCTION IN FERMENTER CULTURE.

Results are expressed after 10 days of fermentation.

Sugar	pH*	Mycelial Dry Weight (g/l)	Sugar+ Utilized (g/l)	Citric Acid Concentration (g/l)	Citric Acid** Yield (%) (w/w)
Sucrose	1.7	13.5	112[a]	37.5	33.5
Glucose	1.8	14.5	100	13.0	13.0
Fructose	1.9	13.0	105	9.5	9.0
Lactose	2.0	10.5	70	5.0	7.0
Galactose	2.2	9.0	80	0	-
Whey Permeate	1.8	12.0	65	14.8	23.0

* Initial pH 6.5.

\+ Initial sugar concentration 140 g/l.

** Based on sugar utilized.

a Result expressed as total of glucose and fructose (hydrolysis products of sucrose) utilized. See Figure 6.11(b) for details.

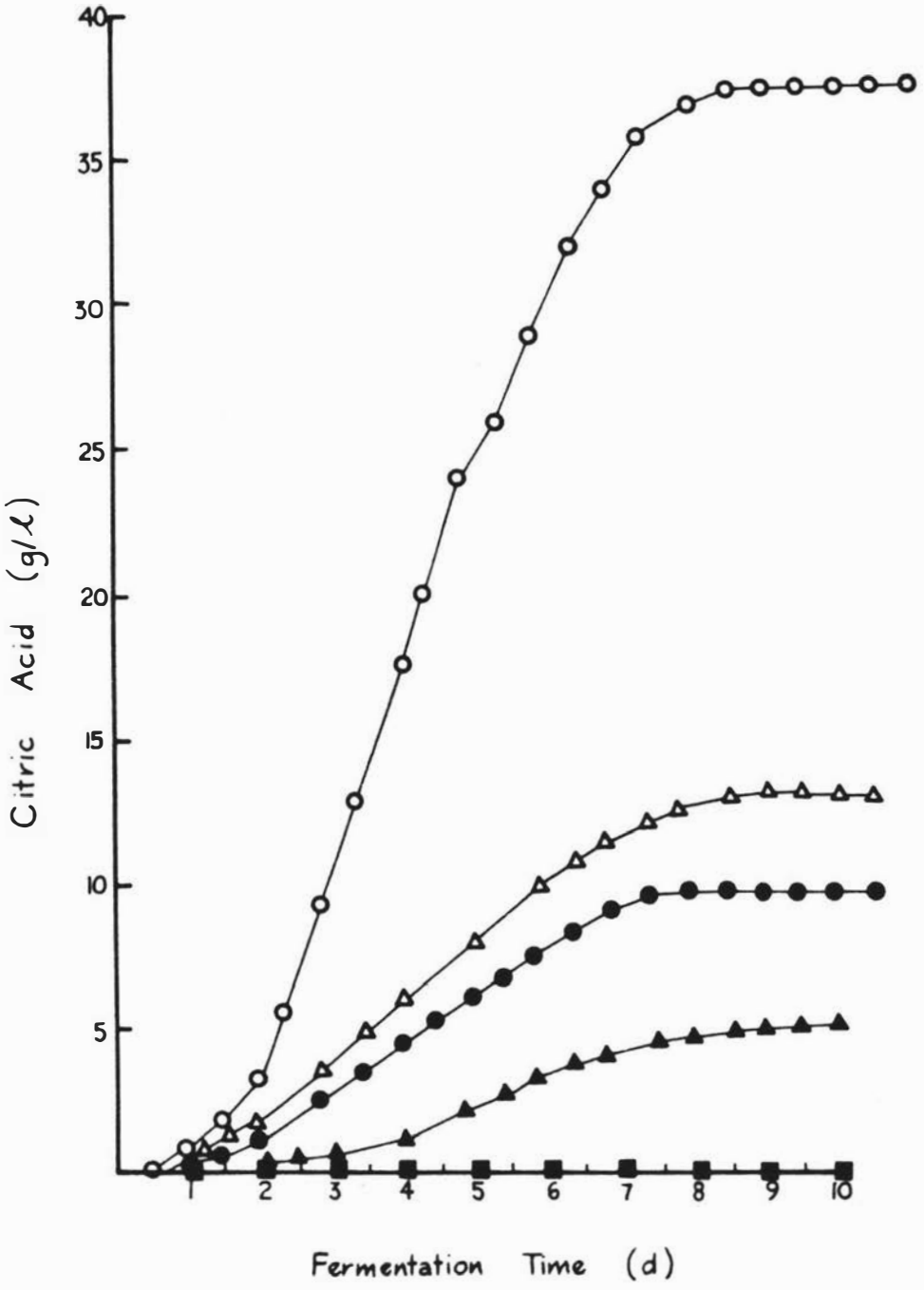

FIGURE 6.7 Production of citric acid from different sugar sources in fermenter culture.

Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

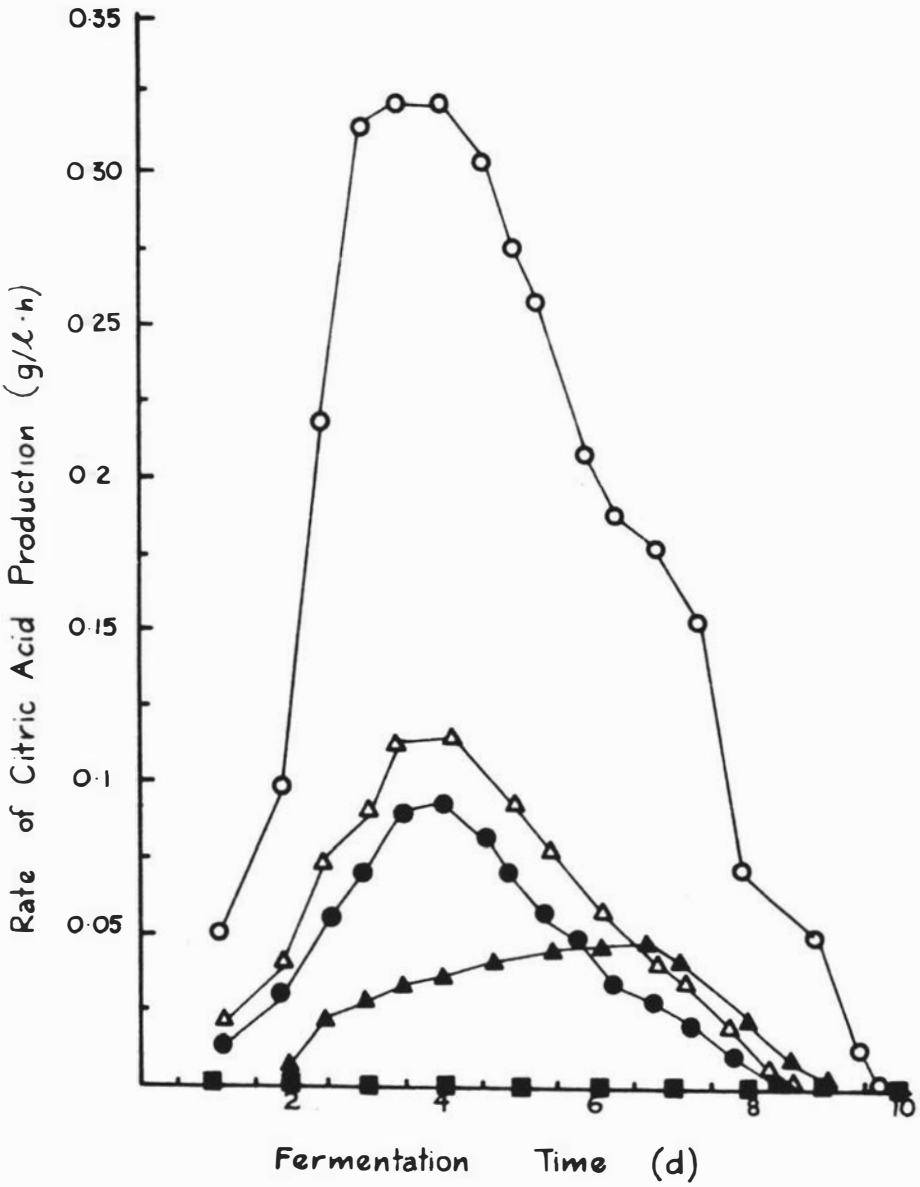

FIGURE 6.8 The volumetric rate of citric acid production from different sugars in fermenter culture.

Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

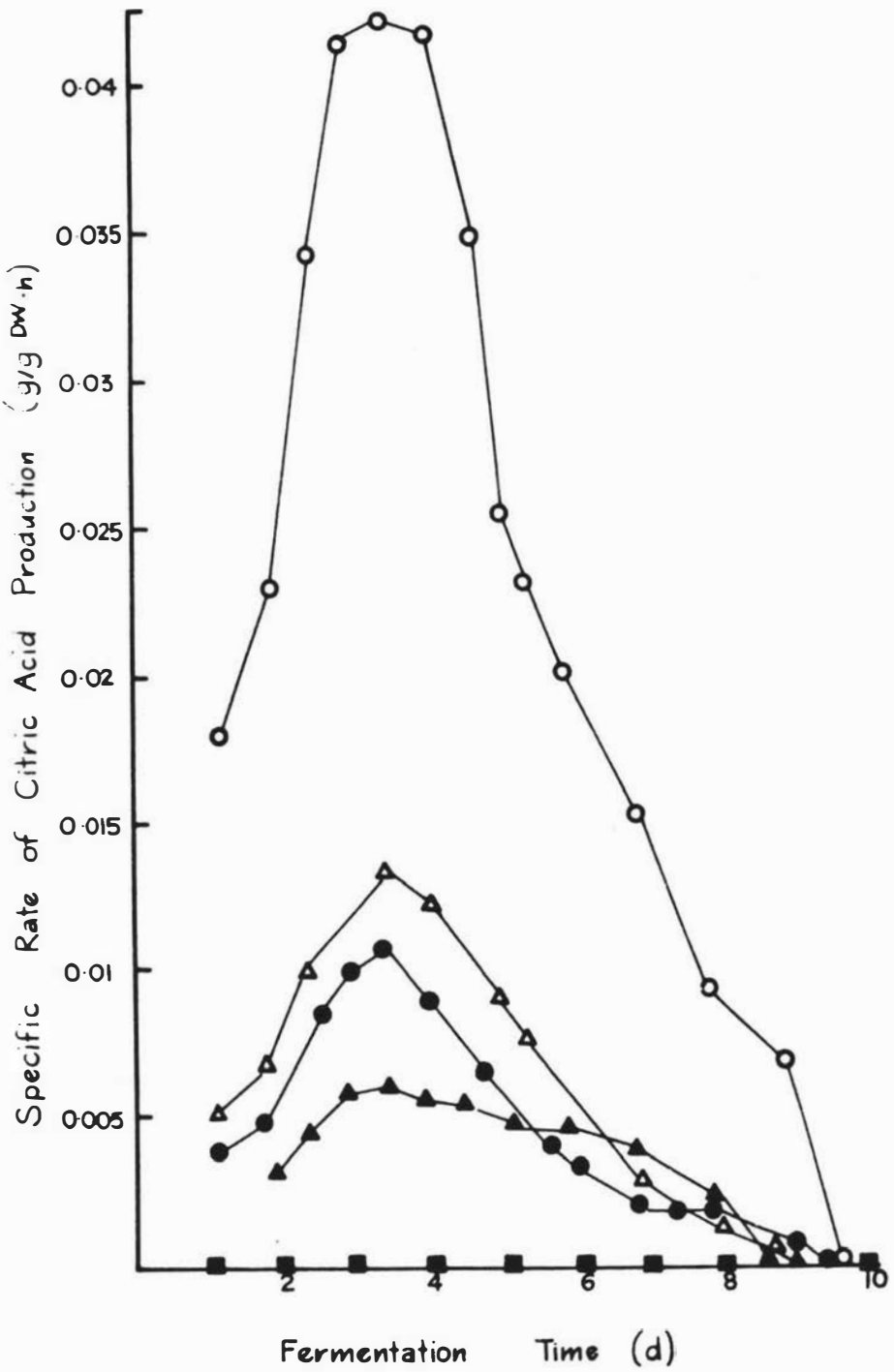

FIGURE 6.9 The specific rate of citric acid production from different sugars in fermenter culture.

Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

The effects of different sugars on mycelial dry weight production are shown in Figure 6.10. Overall, the mycelial dry weights obtained were lower than those in shake-flask and growth virtually ceased after 8-9 days of the fermentation. Again, lactose and galactose gave lower dry weights than the other three sugars.

The time course of sugar utilization is shown in Figure 6.11, which demonstrates that, as in shake-flask culture, more sucrose, glucose and fructose were utilized than lactose and galactose. Approximately 50% of the supplied lactose and galactose were utilized. Also as in shake-flask culture, sucrose was completely hydrolysed within the first 30 hours of fermentation and then the glucose and fructose were utilized simultaneously (Figure 6.11(b)). This finding is in good agreement with the result of Sibert and Schulz (1979), who reported that the first phase of growth was characterized by rapid linear degradation of sucrose with simultaneous increase in the concentrations of glucose and fructose in the medium.

The volumetric and specific rate data of sugar utilization are shown in Figures 6.12 and 6.13 respectively. From the volumetric rate data it can be seen that the combined rate of glucose and fructose (hydrolysis products of sucrose) utilization was higher than the individual glucose and fructose utilization. As in shake-flask, this may be the cause of higher citric acid production from sucrose compared with glucose or fructose. In comparison with glucose and fructose, the volumetric rates of lactose and galactose utilization were lower. On the rate curves (especially with sucrose, glucose and fructose) two peaks of sugar utilization rates are evident. The first one is presumably associated with the initial growth phase and the other with the active citric acid production phase. The second peak is absent from the lactose and galactose data, but in the case of lactose a small change of slope is evident.

The yield of citric acid and biomass per gram of sugar utilized were calculated for 2,4,6,8 and 10 days of the fermentation and the results are tabulated in Table 6.4. By taking the figures for 10 days,

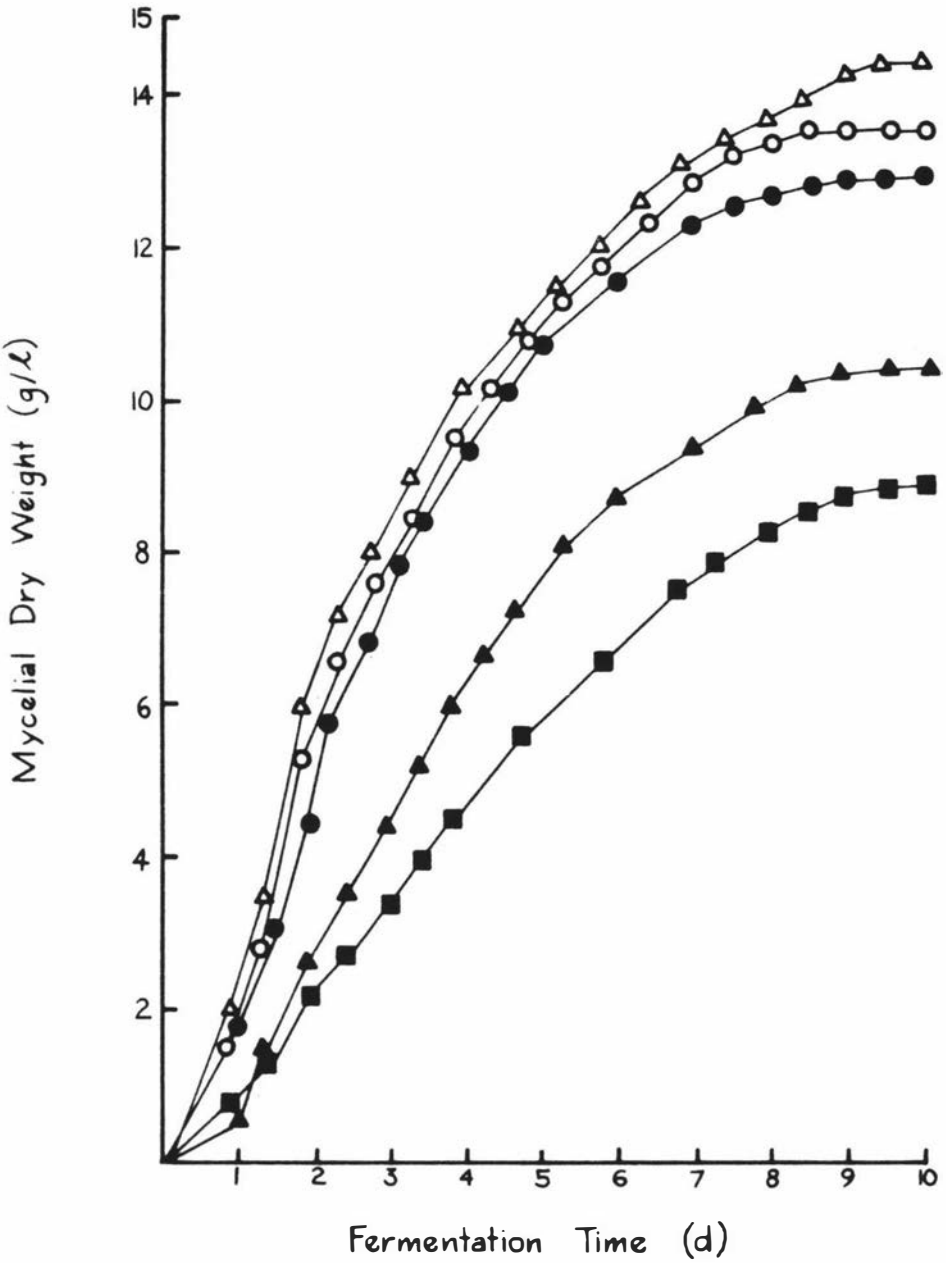

FIGURE 6.10 The effect of different sugars on mycelial dry weight production in fermenter culture.

Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

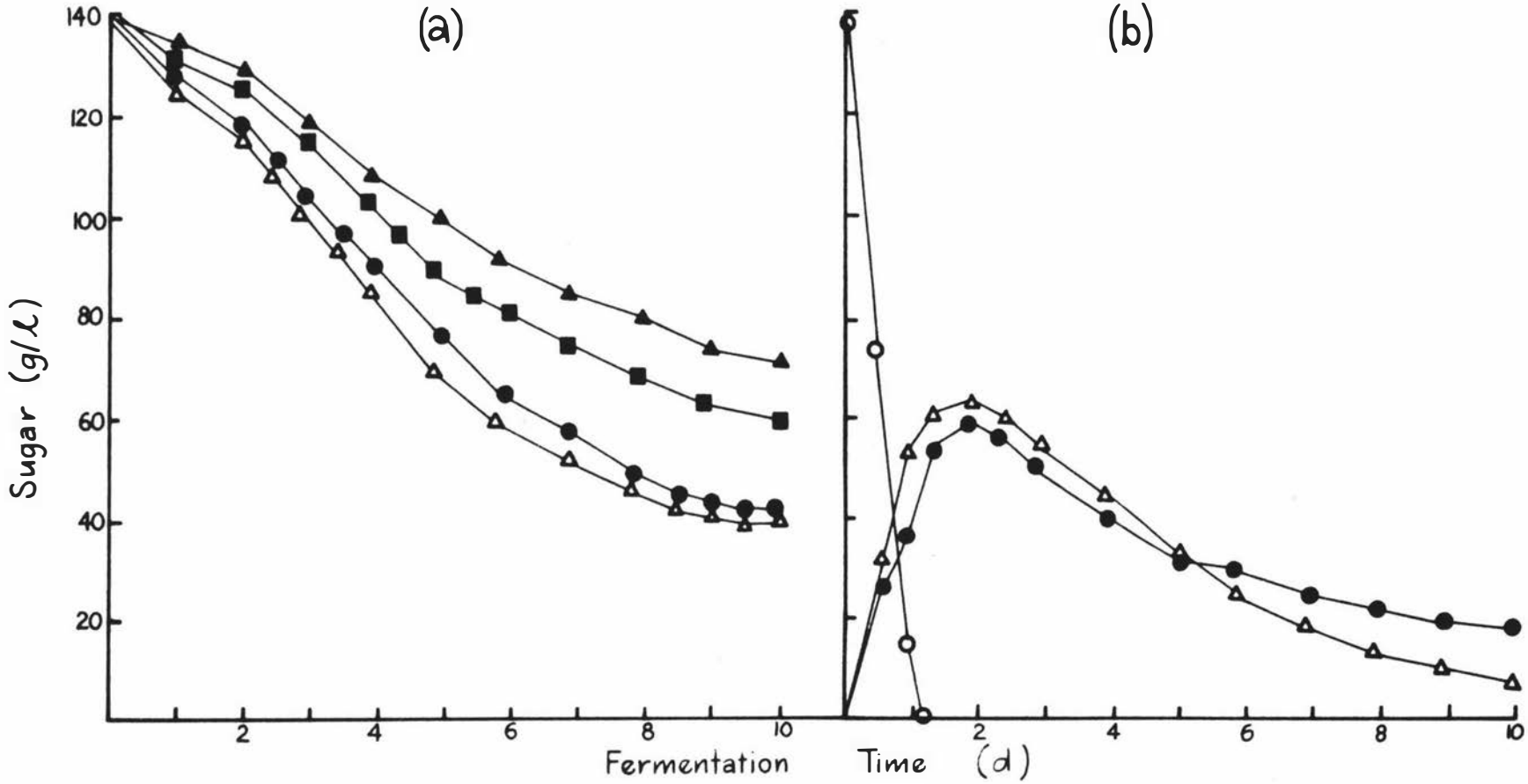

FIGURE 6.11 Utilization of different sugars in fermenter culture. (a) Showing the utilization of glucose, fructose, lactose and galactose and (b) showing the utilization of sucrose and its hydrolysis products (glucose and fructose).

Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■).

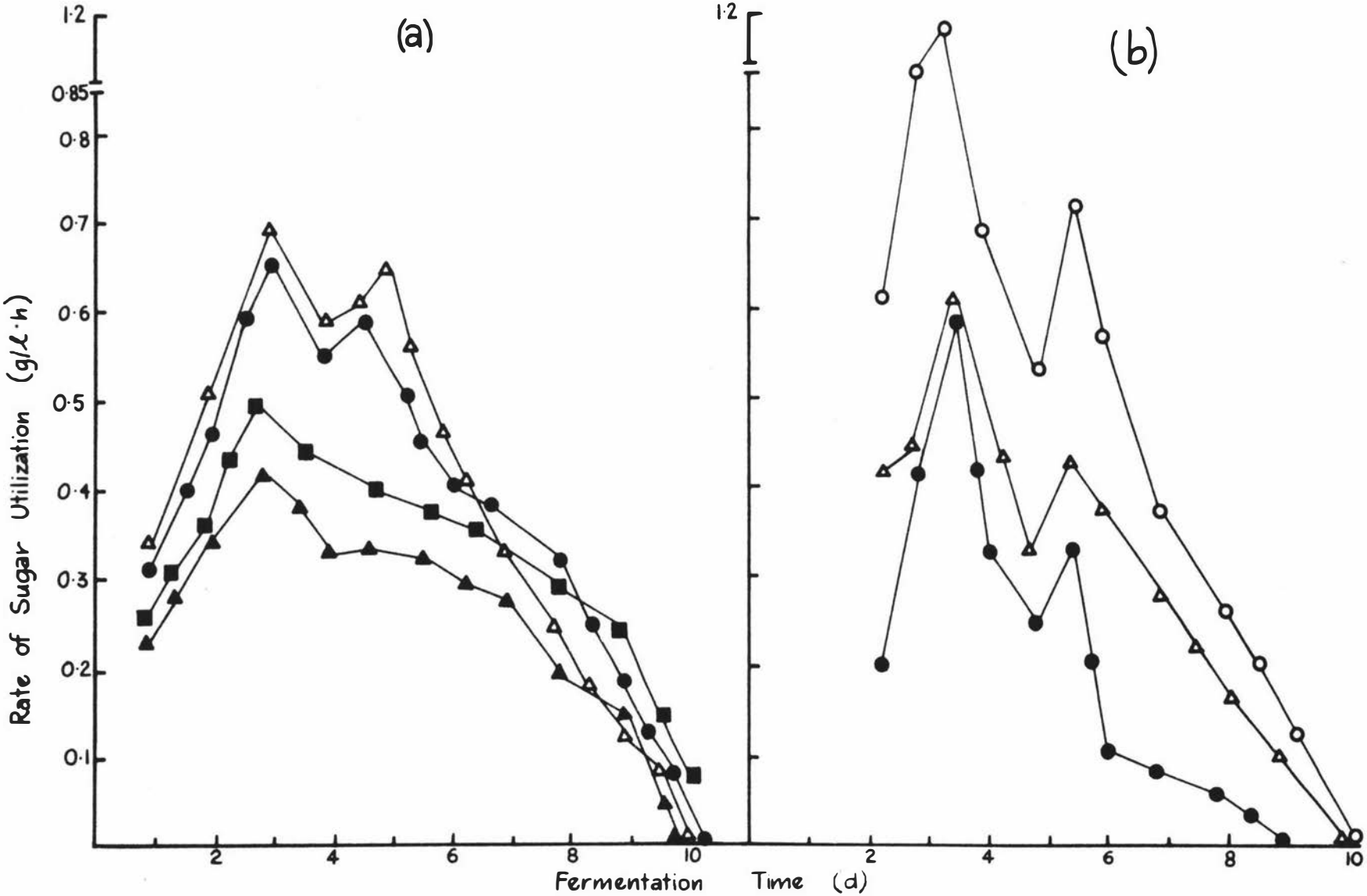

FIGURE 6.12 The volumetric rates of sugar utilization in fermenter culture. (a) Showing glucose, fructose, lactose and galactose utilization and (b) showing utilization of glucose and fructose (hydrolysis products of sucrose).

Glucose (△), fructose (●), lactose (▲), galactose (■), combined rate of glucose and fructose (O).

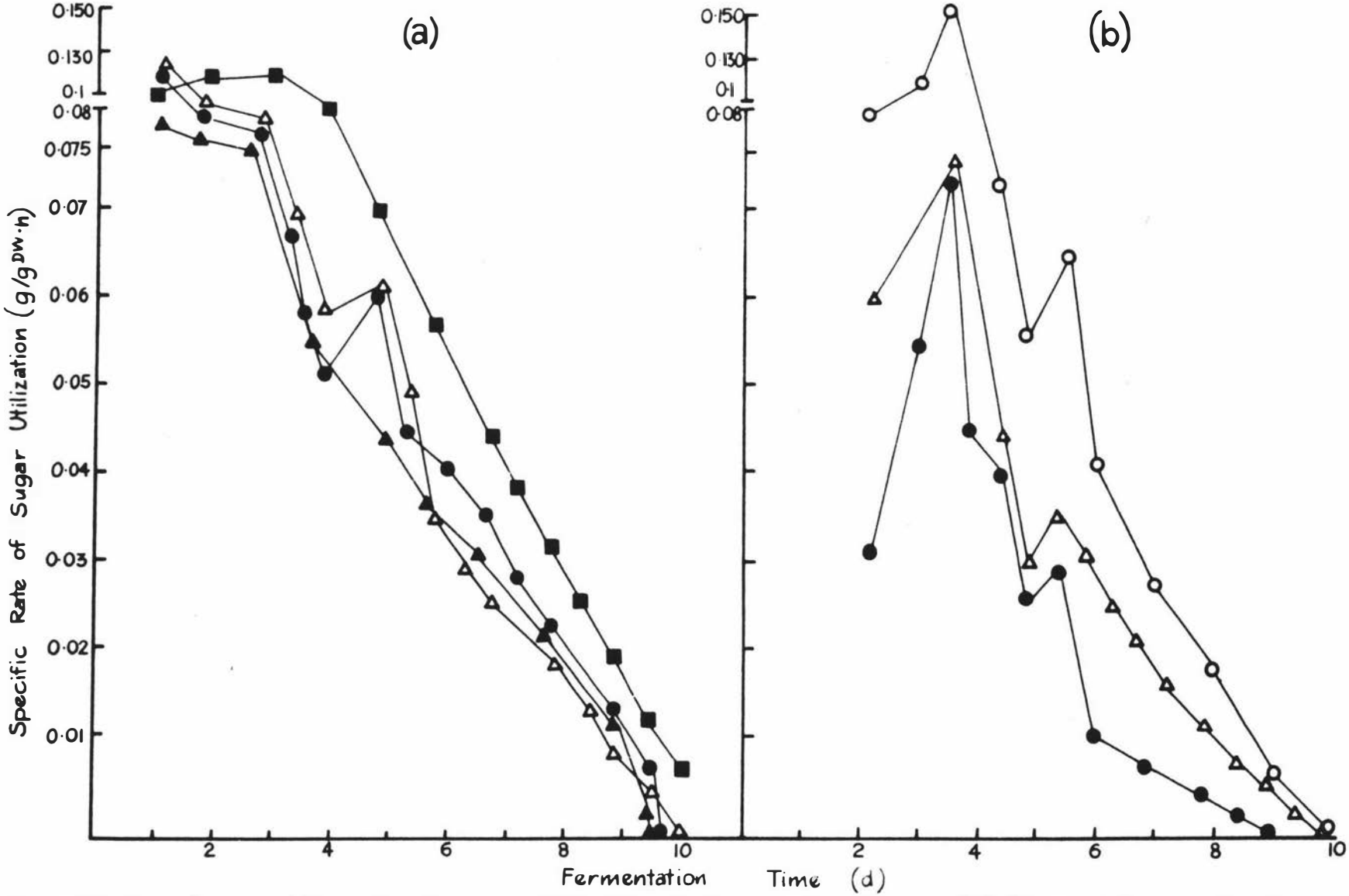

FIGURE 6.13 The specific rate of sugar utilization in fermenter culture. (a) Showing glucose, fructose, lactose and galactose utilization and (b) showing utilization of glucose and fructose (hydrolysis products of sucrose). Glucose (△), fructose (●), lactose (▲), galactose (■), combined rate of glucose and fructose (O).

TABLE 6.4 YIELD OF CITRIC ACID AND BIOMASS PER GRAM OF SUGAR UTILIZED IN FERMENTER CULTURE.

Sugar	$\frac{Y\ Citrate}{Sugar}$					$\frac{Y\ Biomass}{Sugar}$				
	Fermentation Time (d)					Fermentation Time (d)				
	2	4	6	8	10	2	4	6	8	10
Sucrose	0.185	0.315	0.350	0.340	0.340	0.300	0.167	0.140	0.123	0.120
Glucose	0.090	0.105	0.125	0.130	0.130	0.260	0.185	0.146	0.145	0.145
Fructose	0.052	0.090	0.098	0.101	0.090	0.240	0.204	0.154	0.134	0.123
Lactose	0.020	0.040	0.068	0.077	0.071	0.234	0.187	0.183	0.167	0.150
Galactose	0	0	0	0	0	0.151	0.121	0.116	0.115	0.112
Whey Permeate	0.090	0.145	0.227	0.240	0.228	0.200	0.210	0.200	0.197	0.185

it is apparent that although the citric acid yields vary with the sugar source, the biomass yields are approximately similar. The analyses carried out do not show whether other products in addition to CO_2 and biomass are produced from e.g. galactose, but it is worth noting that apart from citric acid no other TCA-cycle acids were detected.

The fermentation data using lactose-supplemented whey permeate are summarized in Table 6.3 (for details see Figures 5.4 and 5.5, Chapter 5). By comparing these data with those from lactose in the synthetic medium, it is apparent that whey permeate is a superior medium for citric acid production from lactose.

Overall, the evidence suggests that the main obstacle to obtaining improved citric acid production from whey permeate is the nature of the sugar source rather than the other constituents of the medium.

The specific activities of the enzymes aconitase and NAD- and NADP-linked isocitric dehydrogenase are shown in Figures 6.14, 6.15 and 6.16 respectively. The results show that during the initial growth phase of the organism (before citric acid production had commenced) the activities of these enzymes were higher in the presence of sucrose, glucose and fructose than in lactose, whey permeate and galactose. During the citric acid production phase the activities of all three enzymes decreased except in the presence of galactose, where the activity increased. Thus, there appears to be some relationship between citric acid production and both the level of initial activity of these enzymes, and the decrease in their activity after the second day of fermentation.

The specific activities of 2-oxoglutarate dehydrogenase and pyruvate carboxylase are presented in Figures 6.17 and 6.18 respectively. No 2-oxoglutarate dehydrogenase activity was detected in the presence of sucrose, glucose or fructose. In contrast, its activity was detected in lactose, whey permeate and galactose. In lactose and whey permeate the activity of 2-oxoglutarate dehydrogenase was highest during the initial growth phase of the mycelium and it subsequently declined as

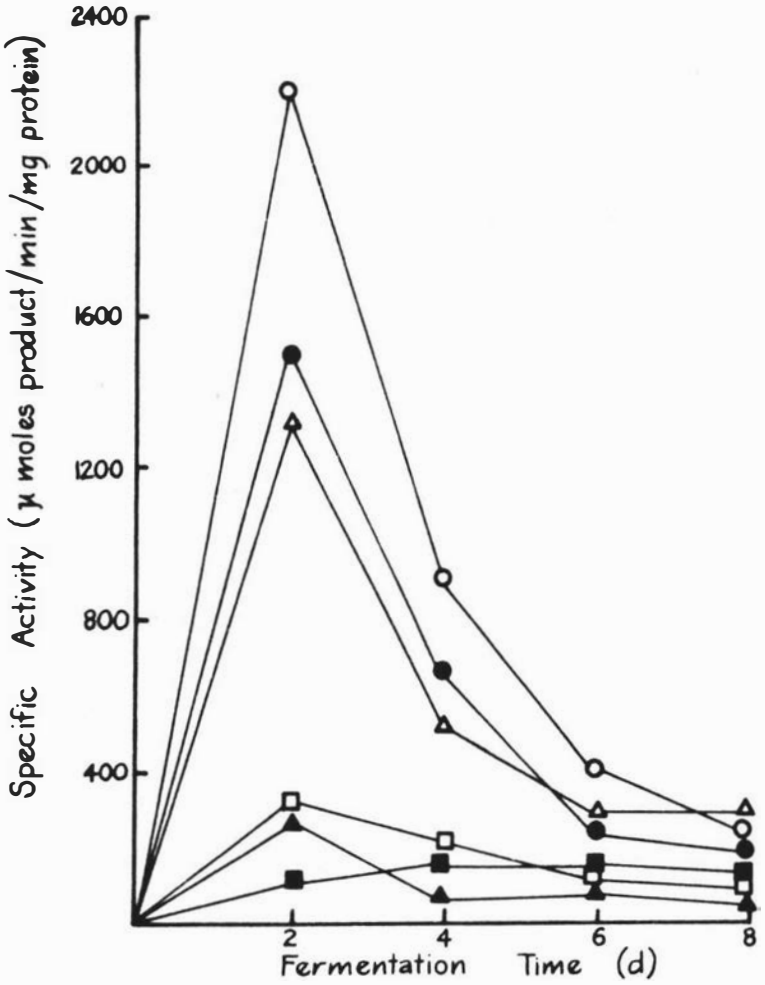

FIGURE 6.14 The specific activity of aconitase during citric acid production from different sugars.
Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■), whey permeate (□).

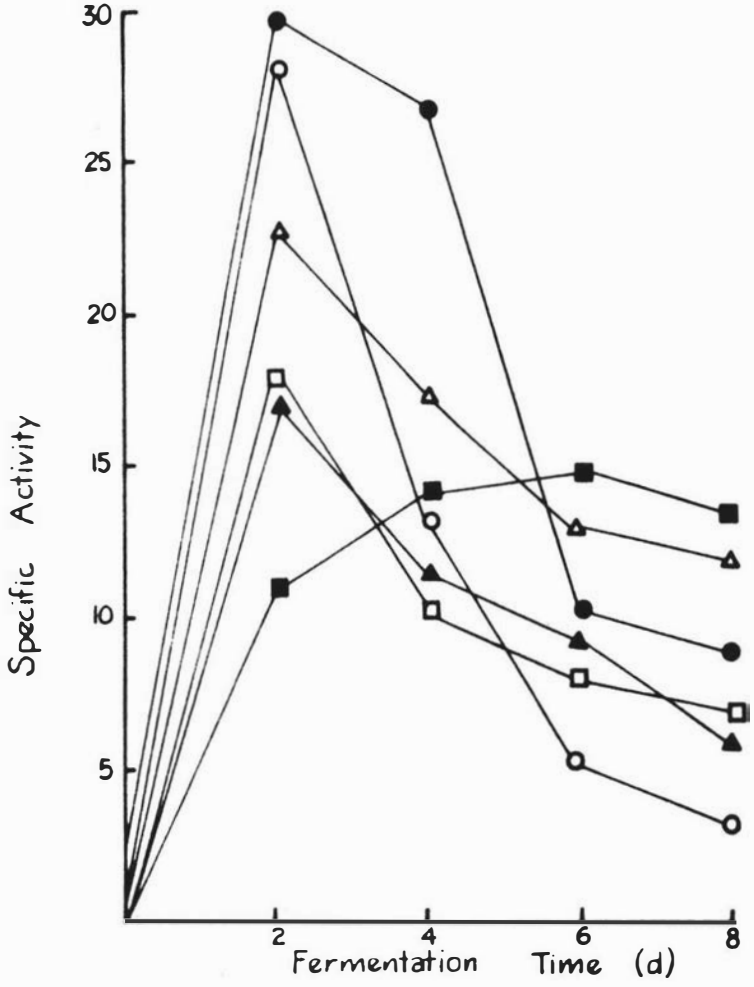

FIGURE 6.15 The specific activity of NAD-isocitric dehydrogenase during citric acid production from different sugars. Symbols as for Figure 6.14.

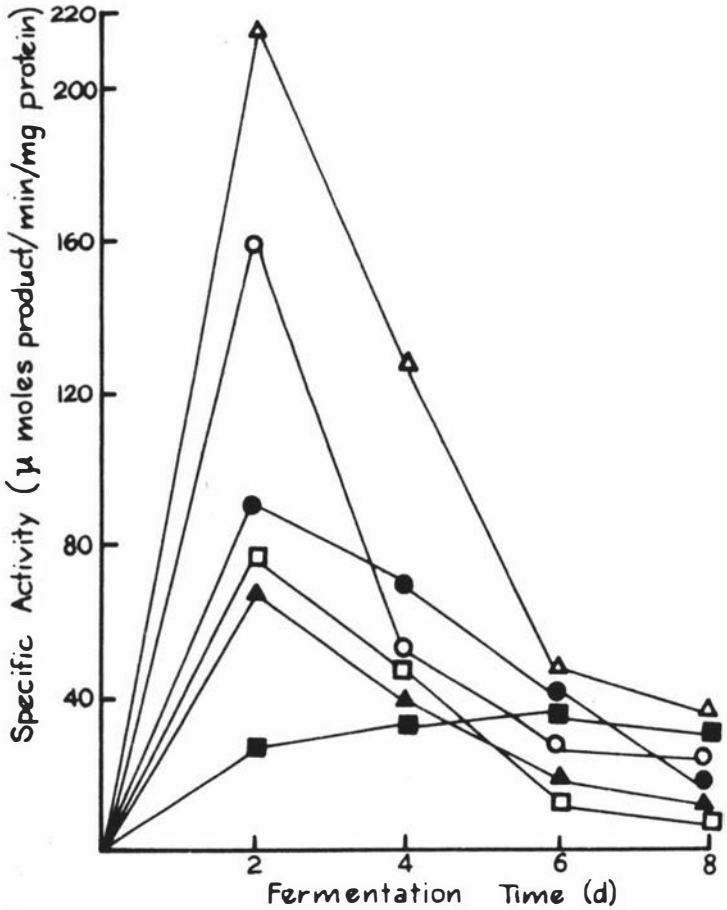

FIGURE 6.16 The specific activity of NADP-isocitric dehydrogenase during citric acid production from different sugars.
Sucrose (O), glucose (△), fructose (●), lactose (▲), galactose (■), whey permeate (□).

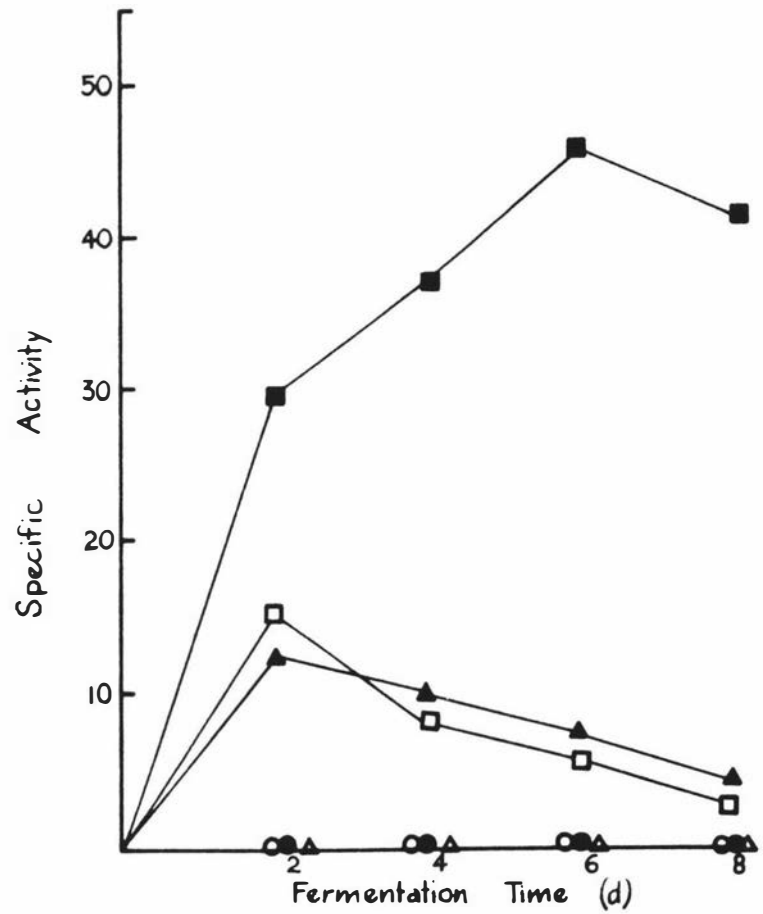

FIGURE 6.17 The specific activity of 2-oxoglutarate dehydrogenase during citric acid production from different sugars.
Symbols as for Figure 6.16.

citric acid was produced. In the presence of galactose, however, the activity continued to increase throughout the entire period of fermentation. Figure 6.18 shows that the activity of pyruvate carboxylase increased in the presence of sucrose, glucose and fructose during the citric acid production phase. In contrast, the activity of this enzyme was very low in the presence of lactose and whey permeate and very little activity was detected in the presence of galactose. Thus, it is clear from the data that both 2-oxoglutarate dehydrogenase and pyruvate carboxylase activities varied considerably with the sugar source and levels correlated well with citric acid production.

The presence of the enzymes aconitase and isocitric dehydrogenase (both NAD- and NADP-linked) throughout the entire period of fermentation indicates that the complete disappearance of these enzymes is not necessary for citric acid accumulation, although they do decline substantially. This finding is in good agreement with the results of La Nauze (1966), Kubicek and Rohr (1977) and Szczodrak (1981). The apparent relationship between the initial activity of these enzymes (at Day 2) and subsequent citric acid production is more difficult to explain. Possibly, the level of activity reflects the flux of carbon material through the TCA-cycle. In this respect, when considering growth on galactose it is pertinent to consider the fate of the carbon since no TCA-cycle acids were detected. A recent report by Heinrich and Rehm (1982) has shown that under some conditions *A. niger* can produce some gluconic acid together with citric acid, even at low pH values. Using the present analytical methods, however, gluconic acid would not have been detected, so this possibility remains speculative.

The absence of activity of the enzyme 2-oxoglutarate dehydrogenase during citric acid production from sucrose, glucose and fructose is in agreement with findings of Ng *et al.* (1973) and Kubicek and Rohr (1977). In contrast, the presence of this enzyme during citric acid degradation in *A. niger* has been demonstrated by Muller and Frosch (1975). Thus, the present data support the view of Rohr and Kubicek (1981) that citric acid accumulation is due to repression of 2-oxoglutarate dehydrogenase by glucose and fructose, causing a block in the TCA-cycle (Figure 6.19).

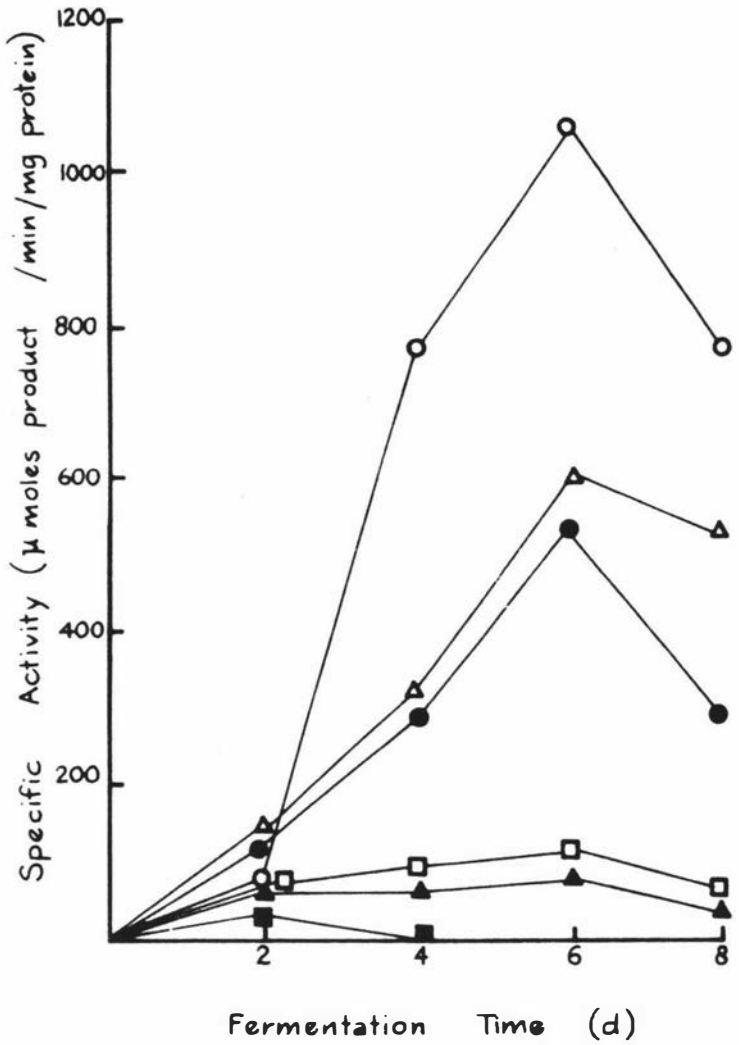

FIGURE 6.18 The specific activity of pyruvate carboxylase during citric acid production from different sugars.

Sucrose (○), glucose (△), fructose (●), lactose (▲), galactose (■), whey permeate (□).

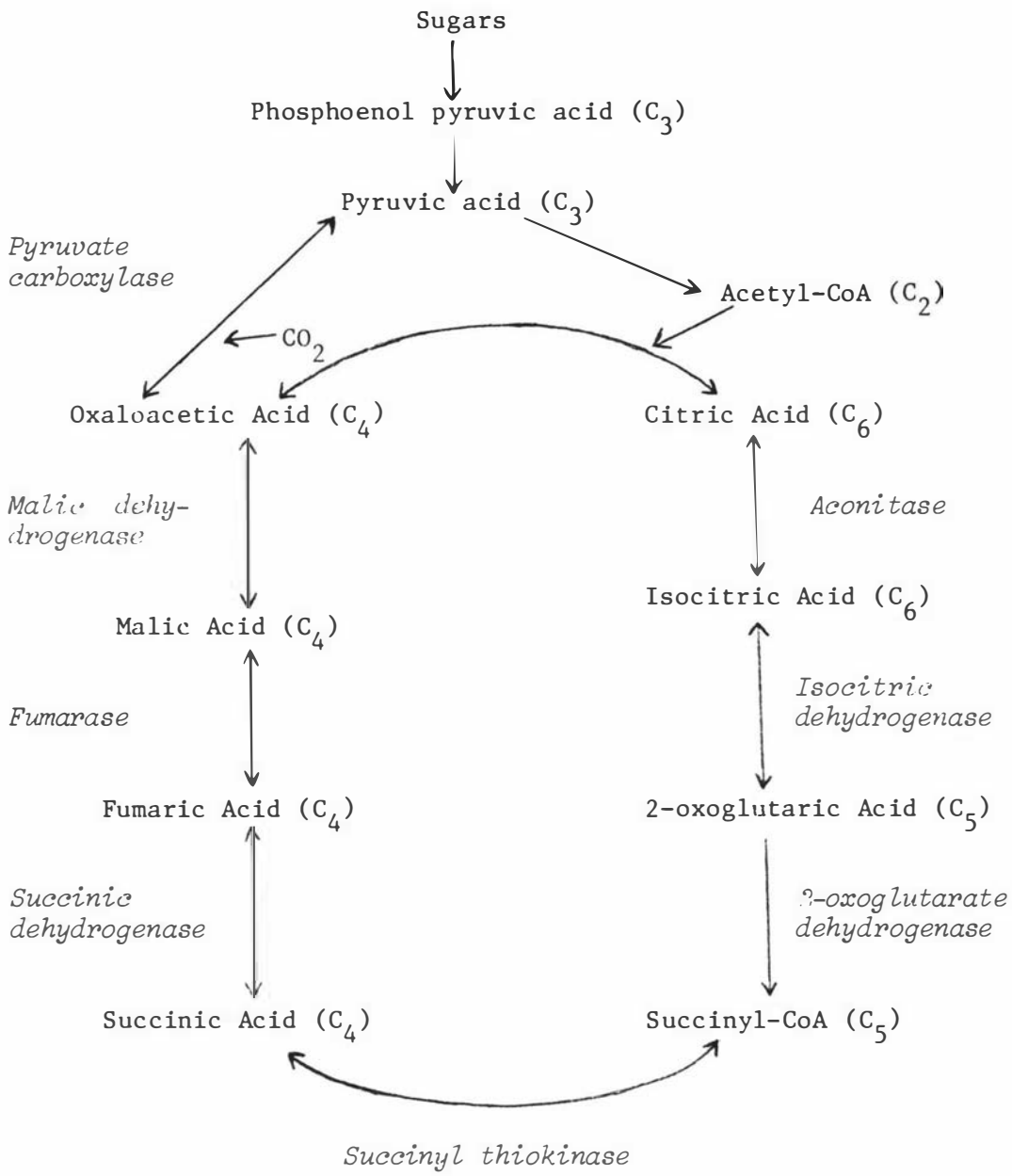

FIGURE 6.19 TRICARBOXYLIC ACID (TCA) CYCLE.

Apparently, glucose and fructose can cause repression but galactose cannot. The presence of 2-oxoglutarate dehydrogenase in the presence of galactose probably allows the TCA-cycle to continue operating, thus metabolizing citric acid. The low level of citric acid production from lactose compared with glucose is probably due to the presence of the galactose moiety.

The reason why lack of the enzyme 2-oxoglutarate dehydrogenase should cause accumulation of citric acid rather than 2-oxoglutaric acid or isocitric acid is not clear (these acids were never detected in the culture media). However, it may be due to a cell permeability effect, i.e. neither 2-oxoglutaric acid nor isocitric acid can be excreted easily from the cell, but citric acid can.

The strong relationship between the activity of the enzyme pyruvate carboxylase and citric acid production can be explained by the need to produce oxaloacetic acid, the direct precursor of citric acid. In the absence of a full TCA-cycle, carboxylation of pyruvic acid serves to produce oxaloacetic acid, thus maintaining citric acid accumulation. The increased activity is probably an effect of 2-oxoglutarate repression. This suggestion is supported by the results of Cleland and Johnson (1954) and Woronick and Johnson (1960), who reported that oxaloacetic acid in the TCA-cycle of *A. niger* was regenerated through carboxylation of pyruvic acid by the enzyme pyruvate carboxylase.

Based on these observations it may be concluded that citric acid accumulation is accompanied by a disappearance of the activity of 2-oxoglutarate dehydrogenase, with a concomitant increase in the activity of pyruvate carboxylase. At the same time, there is a reduction in the activities of aconitase and isocitric dehydrogenase.

6.2.3 The Effects of Different Mixtures of Sugars on Citric Acid Production

An investigation using various mixtures of sugars as substrate was undertaken to determine whether utilization of one sugar interfered with another and whether there were any effects of such mixtures on

citric acid production.

These experiments were conducted in shake-flask culture using the medium of Kristiansen and Charley (1981). The sugar sources used were mixtures of glucose and fructose (50:50 (w/w)), sucrose and lactose (75:25, 50:50 and 25:75), glucose and galactose (75:25, 50:50 and 25:75) and fructose and galactose (50:50). The total sugar concentration was 140 g/l. The fermentations were continued for 14 days and flasks were harvested after 2,4,6,8,10,12 and 14 days of incubation. The effects of these mixtures of sugars on citric acid production are summarized in Table 6.5 and should be compared with Table 6.2 for the effects of individual sugars.

The effect of the mixture of glucose and fructose (50:50) on the fermentation is shown in Figure 6.20, which demonstrates that the citric acid concentration, mycelial dry weight and glucose and fructose utilization were virtually identical with those obtained from sucrose in shake-flask culture (Section 6.2.1, Table 6.2 and Figures 6.1(a), 6.4 and 6.5(b)). In this event, it should be recalled that in separate shake-flask cultures, glucose and fructose produced lower citric acid in comparison with sucrose (Figure 6.1(a)), but the present finding indicates that the mixture of glucose and fructose (which also resulted from sucrose hydrolysis) produced the same amount of citric acid as from sucrose. Thus, the increased citric acid production is postulated as being due to an increased rate of total sugar utilization. The simultaneous utilization of glucose and fructose throughout the entire period of fermentation indicates the possibility of their transport into the cell through two different sites of the transport system.

The effects of different proportions of sucrose and lactose on citric acid production are presented in Figure 6.21. It is apparent from the data that citric acid production was decreased with the decreased proportions of sucrose in the mixtures. Citric acid concentrations of 42.0 g/l, 26.5 g/l, and 11.5 g/l were obtained from 75:25, 50:50 and 25:75, sucrose:lactose respectively. The yields, based on total sugar utilized, were correspondingly reduced. However, it is

TABLE 6.5 SUMMARIZED RESULTS OF THE EFFECTS OF MIXTURES OF SUGARS ON CITRIC ACID PRODUCTION IN SHAKE-FLASK CULTURE.

Results are expressed after 14 days of fermentation.

Sugar Mixture* (w/w)	Mycelial Dry Weight (g/l)	Sugar Utilized (g/l)					Citric Acid Concentration (g/l)	Citric Acid** Yield (%) (w/w)
		Glucose	Fructose	Galactose	Lactose	Sucrose[a]		
Glucose:Fructose (50:50)	19.8	63	51	-	-	-	50.0	44.0
Sucrose:Lactose (75:25)	16.2	-	-	-	4	105	42.0	38.5
Sucrose:Lactose (50:50)	14.0	-	-	-	20	70	26.5	29.5
Sucrose:Lactose (25:75)	12.5	-	-	-	43	35	11.5	14.5
Glucose:Galactose (75:25)	16.5	91	-	18	-	-	27.0	25.0
Glucose:Galactose (50:50)	16.0	70	-	32	-	-	7.5	7.5
Glucose:Galactose (25:75)	14.8	35	-	48	-	-	3.5	4.0
Fructose:Galactose (50:50)	17.0	-	56	48	-	-	11.5	11.0

* Initial total sugar concentration in the mixture 140 g/l.
** Based on total sugar utilized in the mixture.

[a] Result expressed as total of glucose and fructose (hydrolysis products of sucrose) utilized. See Figure 6.21 for details.

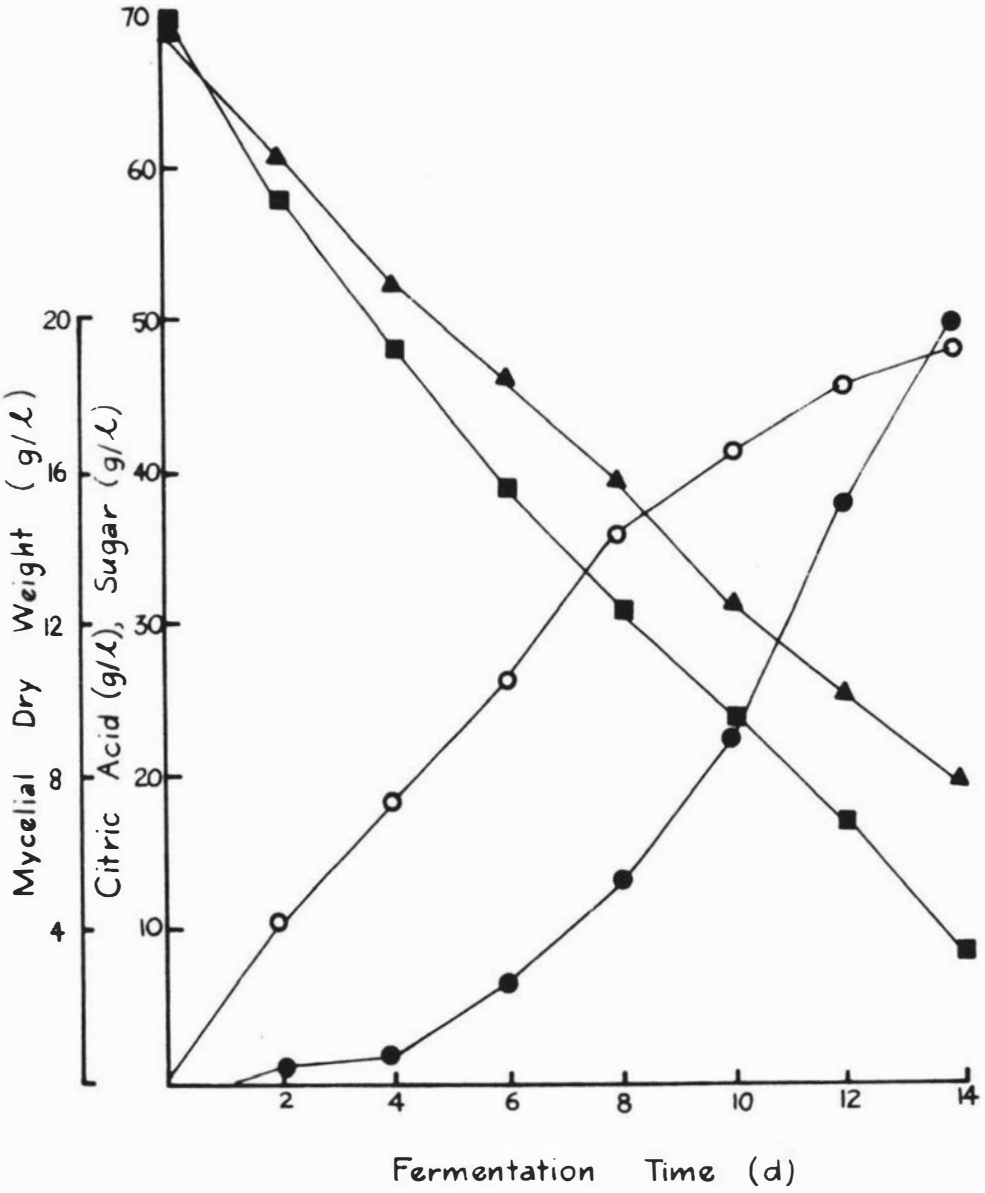

FIGURE 6.20 Production of citric acid from a glucose/ fructose mixture (50:50 w/w).

Citric acid (●), mycelial dry weight (O), glucose (■), fructose (▲).

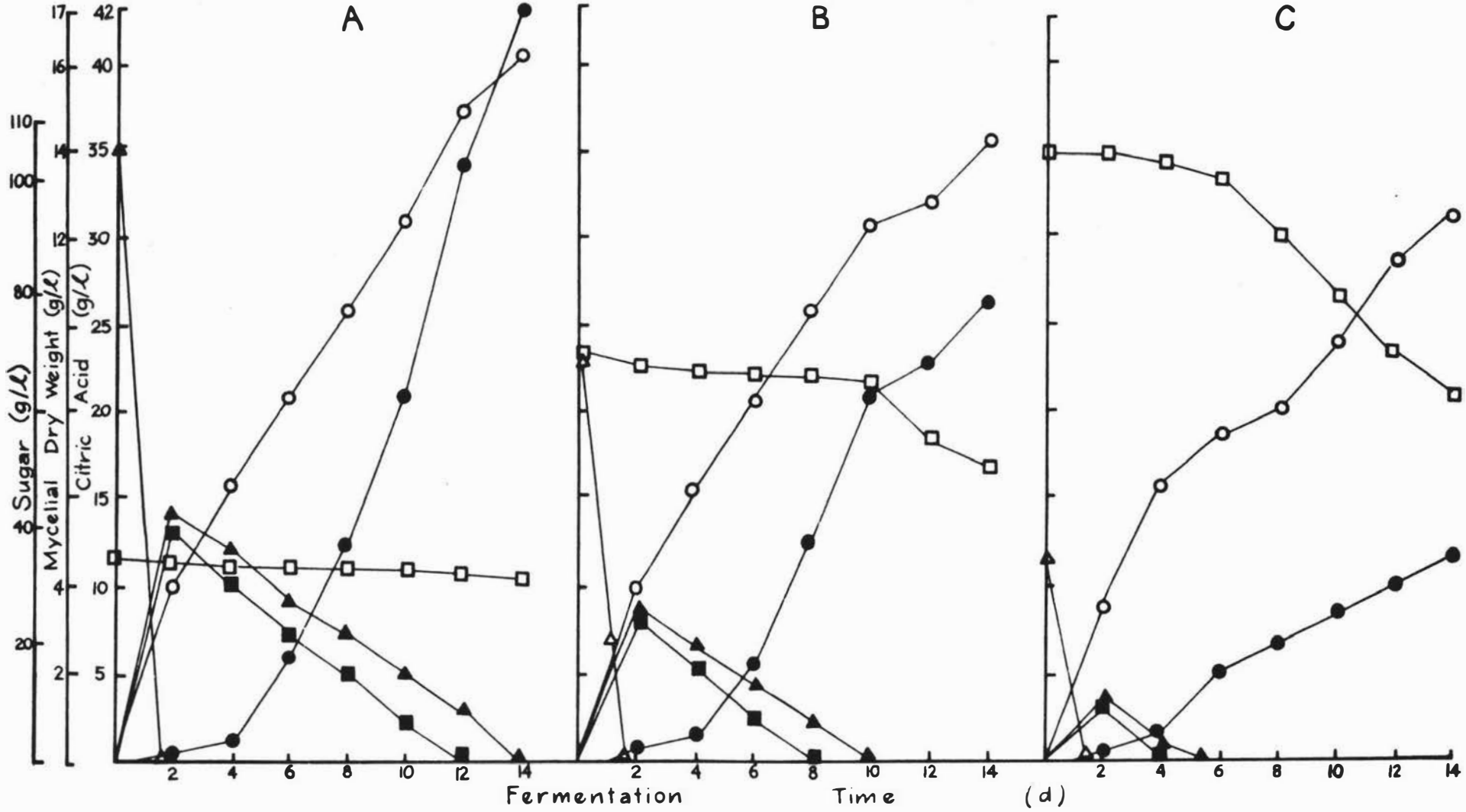

FIGURE 6.21 The effects of different proportions of sucrose and lactose on citric acid production. (A) Sucrose:lactose (75:25), (B) sucrose:lactose (50:50) and (C) sucrose:lactose (25:75). Citric acid (●), mycelial dry weight (O), lactose (□), sucrose (△), { glucose (■), fructose (▲) (hydrolysis products of sucrose) }.

impossible to determine whether the lactose interfered with citric acid production from sucrose, since different initial concentrations of the latter were used in the experiments. As in previous experiments, sucrose was hydrolysed into glucose and fructose, both of which were utilized simultaneously. It can be seen from Figure 6.21 that lactose was not utilized until glucose and fructose were exhausted from the medium, indicating a diauxie effect. The utilization by microorganisms of one sugar in the presence of another, when both are supplied in the medium, is an indication that the metabolism of the preferred sugar produces materials which interfere with the metabolism of the other sugar (Romano and Kornberg, 1969). A classic example of this is the demonstration by Monod (1947) that the adaptation to the use of lactose and galactose as growth substrate by *E. coli* is inhibited by the presence of glucose. This is seen to be the consequence of an interference by glucose with the uptake of the other sugars with consequent impairment of the induction of enzymes required for the catabolism of lactose and of galactose. From the present finding it may be inferred that the presence of sucrose (glucose and fructose) inhibits the induction of the enzyme β-galactosidase required for the catabolism of lactose and consequently no lactose was utilized in the presence of glucose and fructose.

Figure 6.22 shows the data for the effects of different glucose and galactose mixtures on citric acid production. The results show that citric acid production was decreased with increased proportions of galactose in the mixture. Concentrations of 27.0 g/l, 7.5 g/l and 3.5 g/l were obtained from 75:25, 50:50 and 25:75, glucose:galactose respectively at the end of the fermentation. From Table 6.2, glucose alone gave a concentration of 31.0 g/l, respresenting a yield of 34.5% on the basis of glucose utilized. When the yields for the data in Table 6.5 are calculated on the basis of glucose utilization alone, figures of 30%, 11% and 10% are obtained for glucose:galactose 75:25, 50:50 and 25:75 respectively. These reductions in yield and production of citric acid may be due to an inhibitory effect of galactose, but the differing initial glucose concentrations preclude such a conclusion being drawn at this stage. Interestingly, the citric

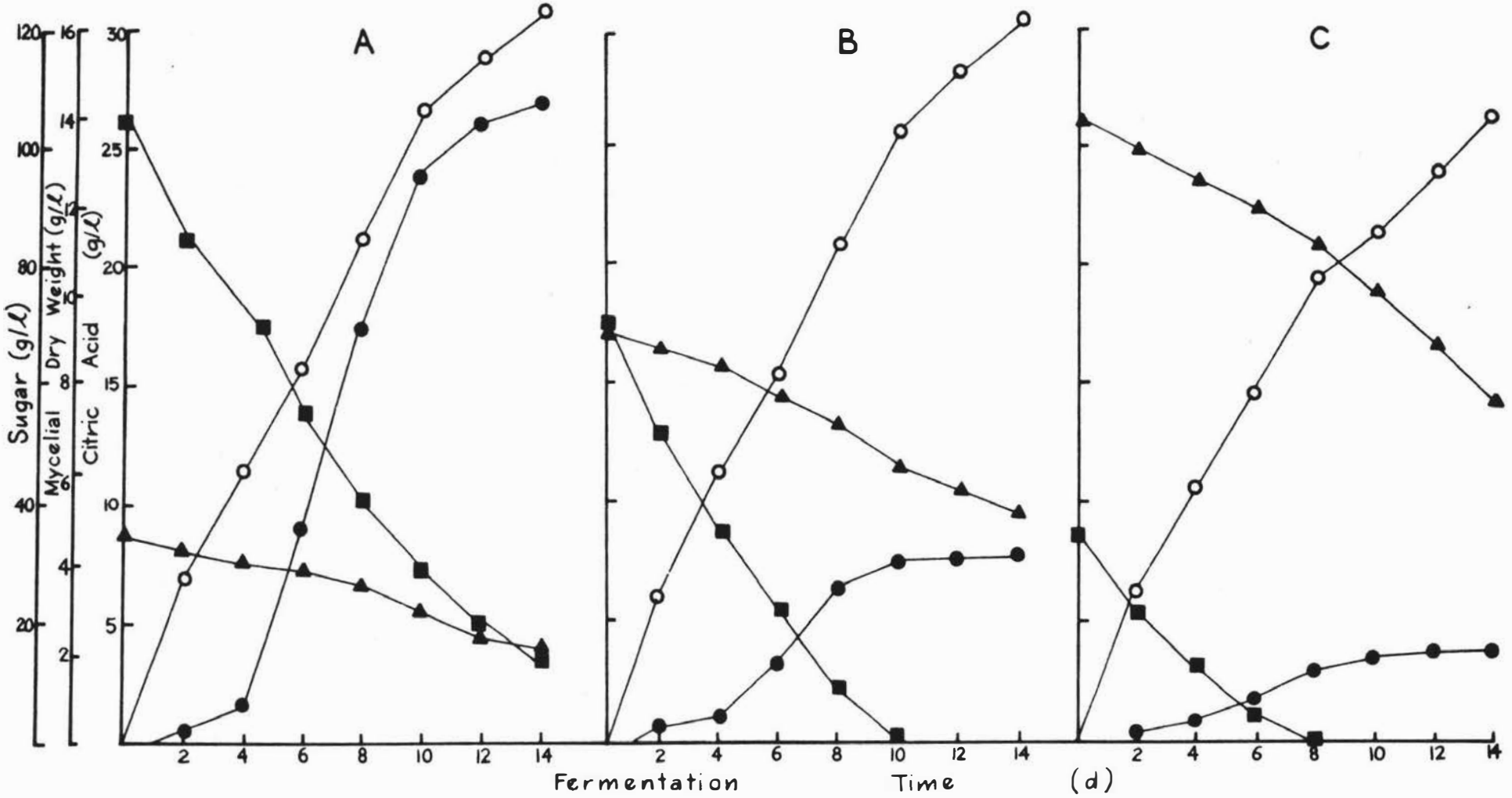

FIGURE 6.22 The effects of different proportions of glucose and galactose on citric acid production. (A) Glucose:galactose (75:25), (B) glucose:galactose (50:50) and (C) glucose:galactose (25:75). Citric acid (●), mycelial dry weight (O), glucose (■), galactose (▲).

acid production and yield from the 50:50 glucose:galactose mixture are very similar to those obtained from lactose (Table 6.2). Thus, in practical terms, there would appear to be no advantage in hydrolysing the lactose in whey permeate prior to fermentation, at least when using the present strain of organism. In terms of sugar utilization, glucose and galactose were utilized simultaneously, although the glucose utilization rate was higher. Once glucose was exhausted from the medium, citric acid production ceased, indicating that galactose did not contribute to citric acid production.

From the volumetric rate data of sugar utilization (Figure 6.23), it is clear that the rate of galactose utilization increased as the rate of glucose utilization decreased during the fermentation. Furthermore, the initial utilization rates of the sugars varied approximately in proportion to their initial concentrations. Cirillo (1961) has stated that if two sugars have widely different affinities in the transport system, the sugar with lower affinity will be transported more slowly at lower concentration but faster at higher concentration. Thus, from the present observation, it can be concluded that glucose is utilized preferentially to galactose but it is not a true diauxie effect. It can be postulated that the two sugars are transported into the cell at the same site of the transport system, but the system shows greater affinity for glucose.

For further clarification with regard to the possibility of glucose and galactose transportation into the cell through a common site, an experiment was conducted using a fructose and galactose (50:50 (w/w)) mixture. This experiment was considered on the basis of the rates of utilization of glucose/fructose and glucose/galactose in a mixture. It was previously found that, in a mixture, glucose and fructose were utilized at almost equal rates (Figure 6.6 and Figure 6.20) whereas in glucose/galactose mixtures there appeared to be competition between the sugars (Figure 6.23). These findings suggested that for the transport of these sugars there was a common site for glucose and galactose, but a different site for fructose. If this is so, then in a mixture of galactose and fructose it might be expected that the

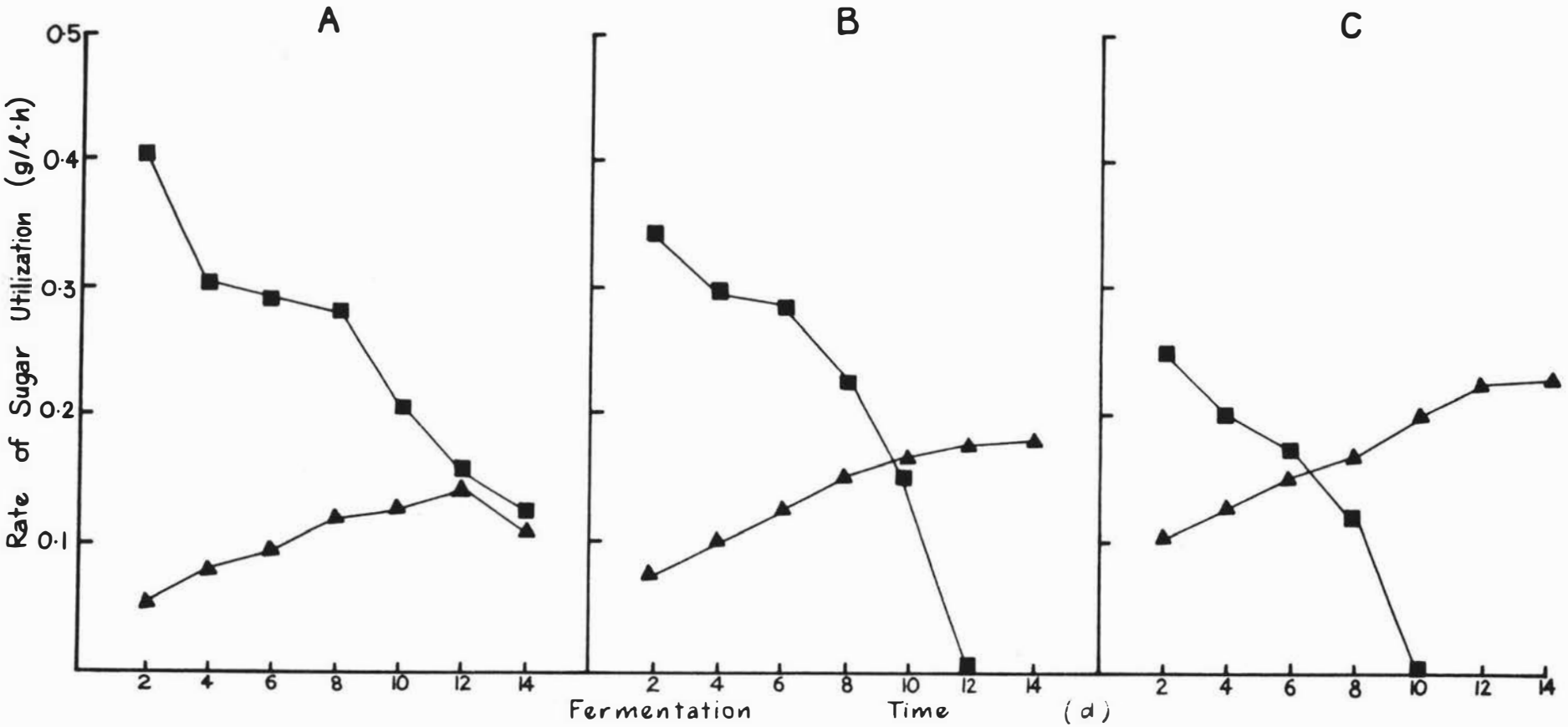

FIGURE 6.23 The volumetric rate of glucose and galactose utilization in mixtures. (A) Glucose:galactose (75:25), (B) glucose:galactose (50:50) and (C) glucose:galactose (25:75).

Glucose (■), galactose (▲).

individual sugars would be utilized at equal rates. The results of this experiment is presented in Figure 6.24, which shows that 11.5 g/l citric acid were produced from this mixture of galactose and fructose at the end of the fermentation, in contrast to the production of 23.0 g/l (Table 6.2) from fructose alone. The yield, based on total sugar utilization, was 11% (w/w) but based only on the fructose utilized it was 20% (w/w) (cf 25% (w/w) from fructose alone, Table 6.2). This finding suggests that the galactose may inhibit citric acid production from fructose, as well as from glucose, but the effect is less marked. The results show that 56 g/l and 48 g/l of fructose and galactose were utilized, respectively, at the end of the fermentation. Considering the simultaneous utilization of these sugars, combined with the rate data (Figure 6.25), it may be suggested that galactose and fructose are transported into the cell using two different sites of the transport system. It could also be equally postulated that the same transport site is used and it has equal affinity for the two sugars. However, given the data from the various mixtures of glucose, fructose and galactose, the evidence suggests that there is a common site for glucose and galactose, and another site for fructose. More data are required to clarify this.

6.2.4 Inhibition by Galactose of Citric Acid Production from Glucose

The results from the previous section indicated that the presence of galactose in glucose and galactose mixtures might cause inhibition of citric acid production from glucose. To further investigate this possibility, experiments were carried out in shake-flask using the medium of Kristiansen and Charley (1981) where two constant concentrations of glucose (70 g/l or 40 g/l) were used with three different concentrations of galactose (35 g/l, 70 g/l and 105 g/l). The effect of glucose (70 g/l) and galactose (35 g/l, 70 g/l and 105 g/l) mixtures on some TCA-cycle enzyme activities of cell-free extracts was also investigated. The fermentations were continued for 14 days and flasks were harvested after 2,4,6,8,10,12 and 14 days. The effects of these mixtures of glucose and galactose on citric acid production are summarised in Table 6.6

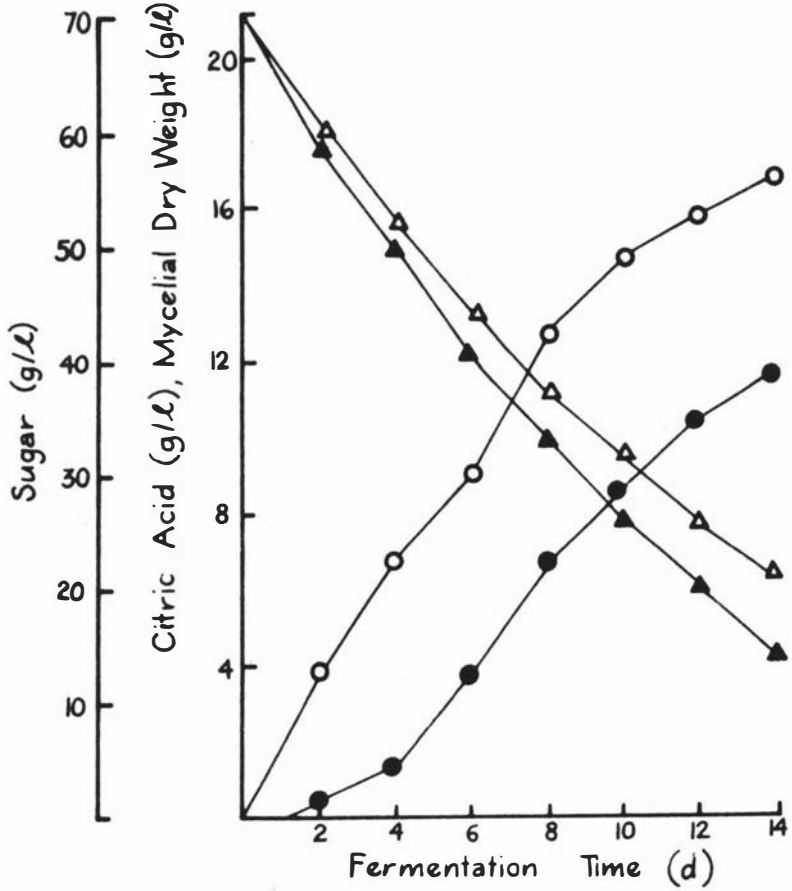

FIGURE 6.24 The effect of fructose and galactose (50:50) mixture on citric acid production. Citric acid (●), mycelial dry weight (o), fructose (▲), galactose (△).

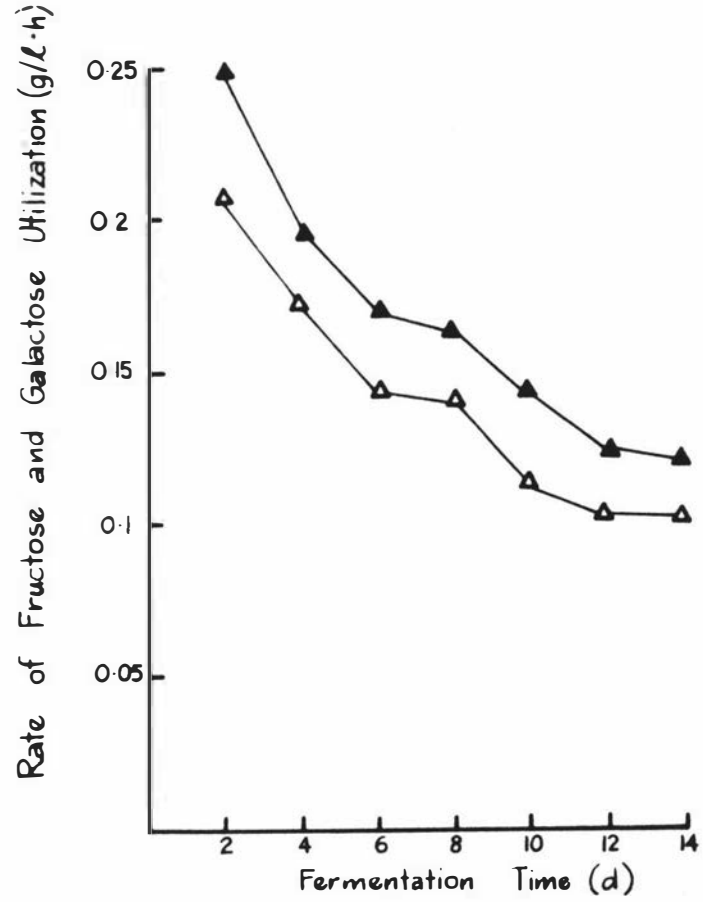

FIGURE 6.25 The volumetric rate of fructose and galactose utilization. Fructose (▲), galactose (△).

TABLE 6.6 SUMMARIZED RESULTS OF THE EFFECTS OF GLUCOSE (CONSTANT CONCENTRATION) AND GALACTOSE (VARYING CONCENTRATION) MIXTURES ON CITRIC ACID PRODUCTION IN SHAKE-FLASK CULTURE.

Results are expressed after 14 days of fermentation.

Amount of Glucose and Galactose in the Mixture (g/l)		Mycelial Dry Weight (g/l)	Sugar Utilized (g/l)		Maximum Citric Acid Concentration (g/l)	Citric Acid* Yield (%) (w/w)	Citric Acid** Yield (%) (w/w)
Glucose	Galactose		Glucose	Galactose			
70	0	15.5	70	0	27.5[a]	39.0	39
	35	16.2	70	25	22.6	24.0	32
	70	16.5	70	35	7.8	7.5	11
	105	16.8	70	42	5.7	5.0	8
40	0	11.2	40	0	10.8[b]	27.0	27
	35	15.0	40	35	6.5	8.5	16
	70	16.0	40	45	5.5	6.5	14
	105	16.0	40	50	5.3	6.0	13

* Based on total glucose and galactose utilized.

** Based on glucose utilized.

a Highest concentration of citric acid (27.5 g/l) reached on tenth day of the fermentation and then declined due to complete sugar utilization. See Figure 6.26(a).

b Highest concentration of citric acid (10.8 g/l) reached on sixth day of the fermentation and then declined due to complete sugar utilization. See Figure 6.26(b).

The data for citric acid production are presented in Figure 6.26. It is apparent that the citric acid production was dramatically reduced with an increased concentration of galactose in the mixture. Similarly, citric acid yields, based on glucose utilization alone, were reduced in the presence of galactose (Table 6.6). The reason for the decline occasionally observed in citric acid concentration was probably the exhaustion of sugar (Figure 6.28).

The volumetric rate data of citric acid production are shown in Figure 6.27. The results show that an increased concentration of galactose in the medium caused a decreased rate of citric acid production.

The glucose and galactose utilization data for the period of fermentation are shown in Figures 6.28 and 6.29 respectively. The glucose was completely utilized after 10 to 14 days (Figure 6.28(a)) and 6 to 8 days (Figure 6.28(b)) depending upon the initial glucose concentration of the medium. Increased concentrations of galactose in the media resulted in slightly slower glucose utilizations. The two sugars were utilized simultaneously, but glucose was utilized at a faster rate (Figures 6.30 and 6.31). As glucose was utilized, so the rate of galactose utilization increased. This finding suggests again that glucose and galactose undergo competition for transportation into the cell through a common site of the transport system and the transport system has a greater affinity for glucose than for galactose.

To confirm the inhibitory effect of galactose on citric acid production from glucose, plots of citric acid production rates (taken at day 6, Figure 6.27) were drawn as shown in Figures 6.32 and 6.33. The plots of Q/Qi versus i (Figure 6.32, according to Lineweaver and Burk, 1934) show that the slope of the line varies with the substrate concentration, indicating competitive inhibition. This is confirmed by the plot of 1/Qi versus i (Figure 6.33, according to Dixon, 1953), which indicates a Ki value for galactose of approximately 22 g/l

With regard to the enzyme activities, Figure 6.34 shows the specific activities of aconitase and both NAD- and NADP-linked isocitric

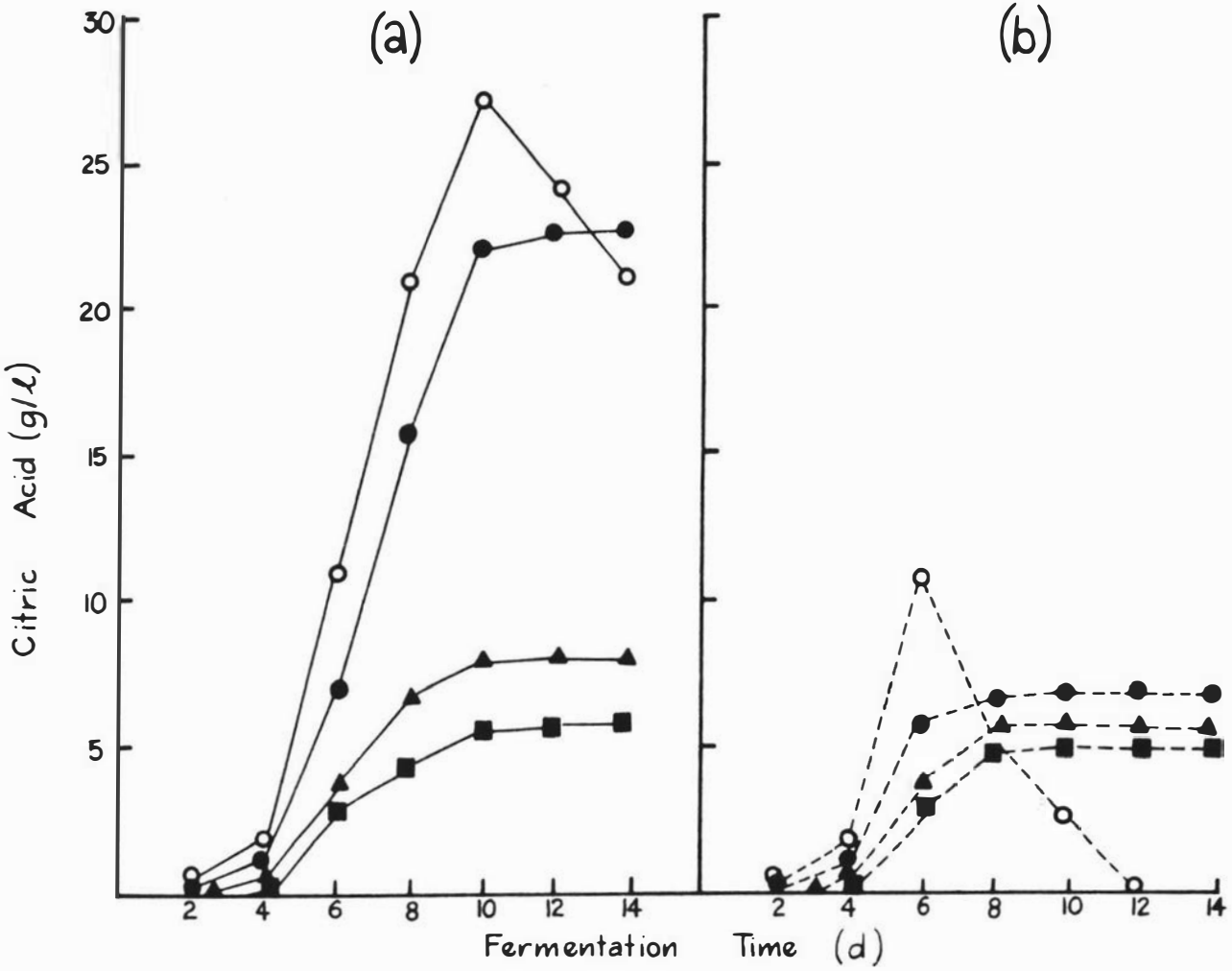

FIGURE 6.26 The effect of constant glucose with varying galactose concentration on citric acid production. (a) Glucose 70 g/1 and (b) glucose 40 g/1. Galactose absent (O), galactose 35 g/1 (●), galactose 70 g/1 (▲), galactose 105 g/1 (■).

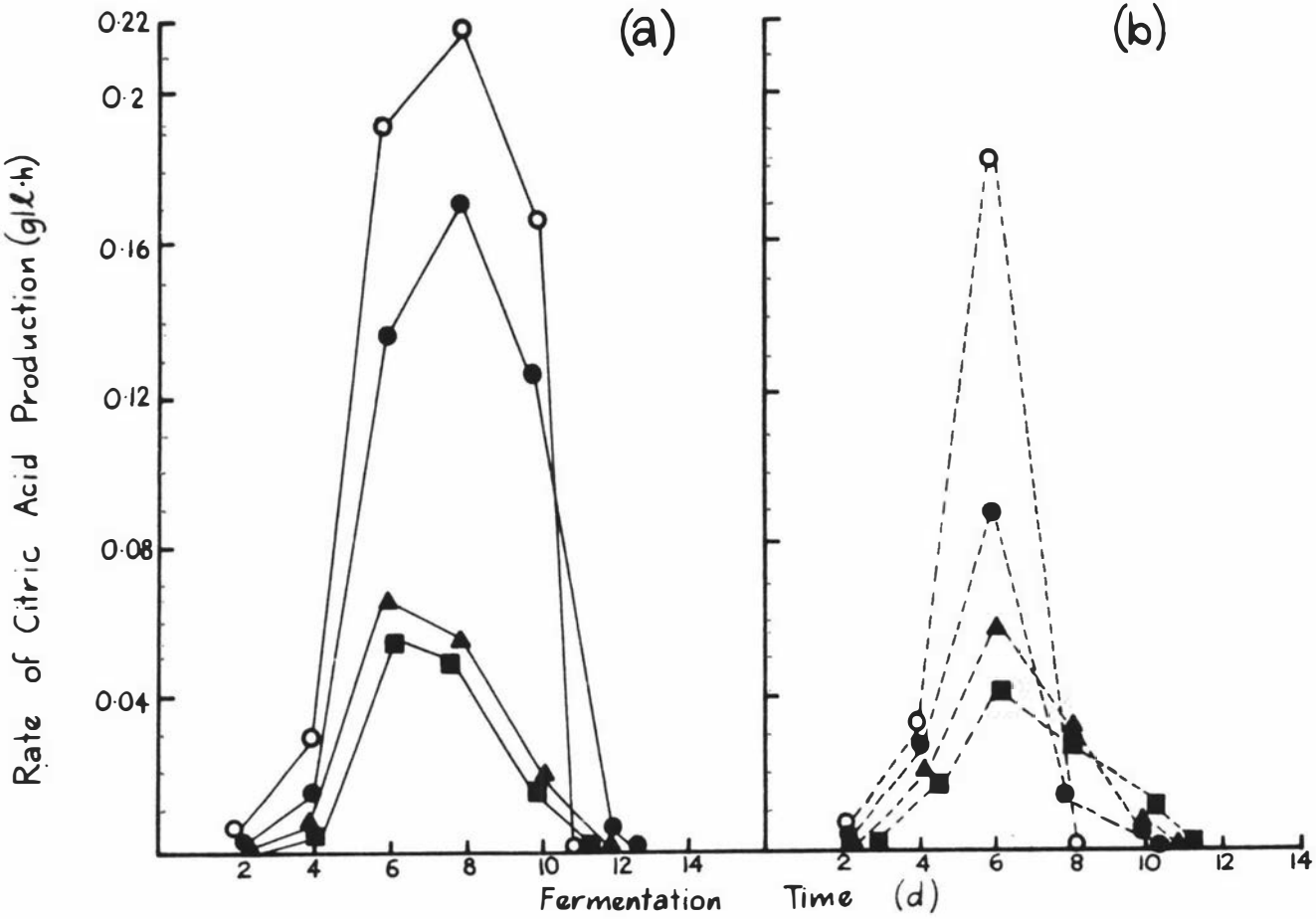

FIGURE 6.27 The volumetric rate of citric acid production in the presence of varying concentrations of galactose (constant glucose concentration). (a) Glucose 70 g/1 and (b) glucose 40 g/1. Galactose absent (O), galactose 35 g/1 (●), galactose 70 g/1 (▲), galactose 105 g/1 (■).

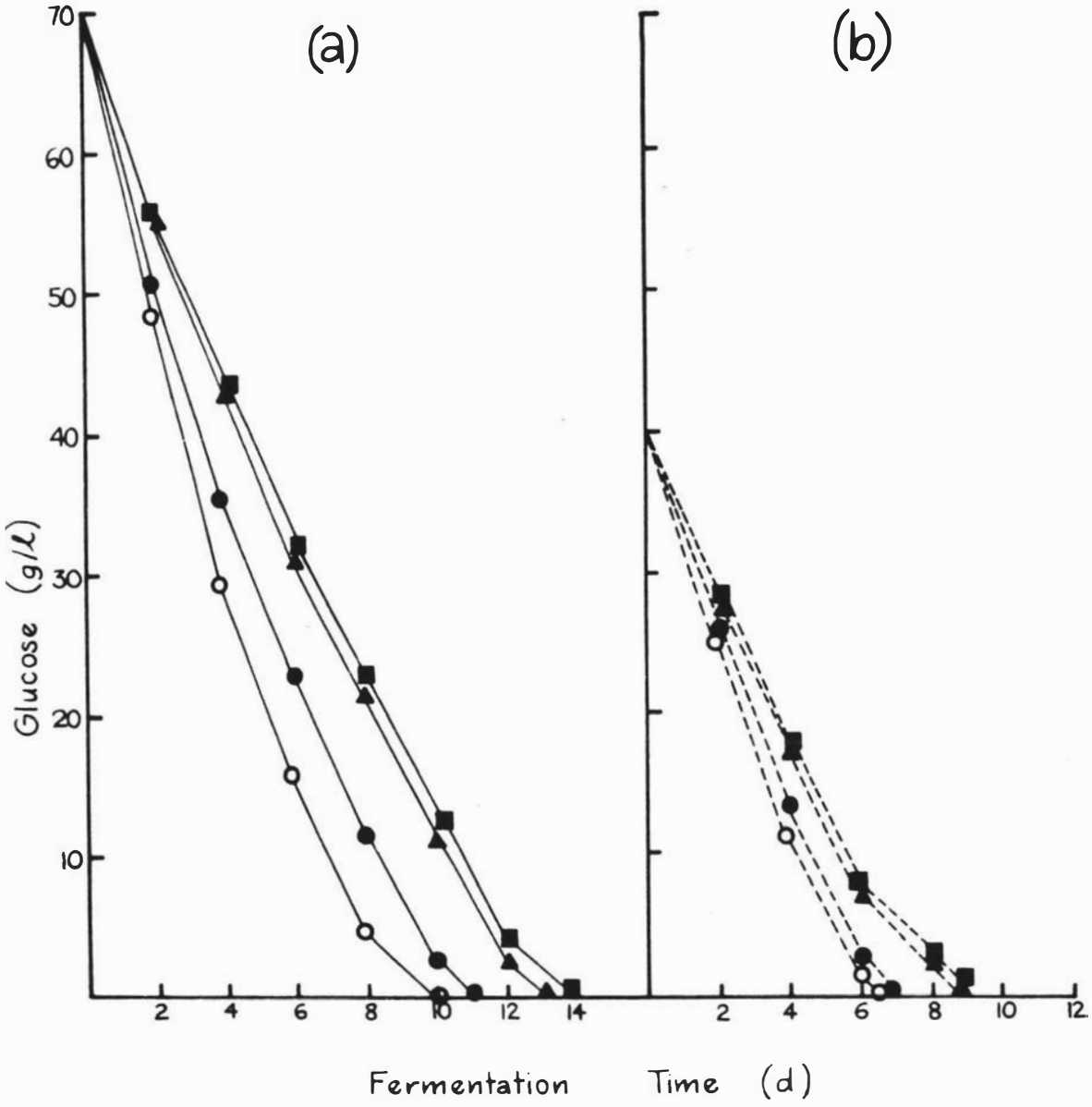

FIGURE 6.28 Glucose utilization in glucose (constant) and galactose (varying concentration) mixtures. (a) Glucose 70 g/l and (b) glucose 40 g/l. Galactose absent (O), galactose 35 g/l (●), galactose 70 g/l (▲), galactose 105 g/l (■).

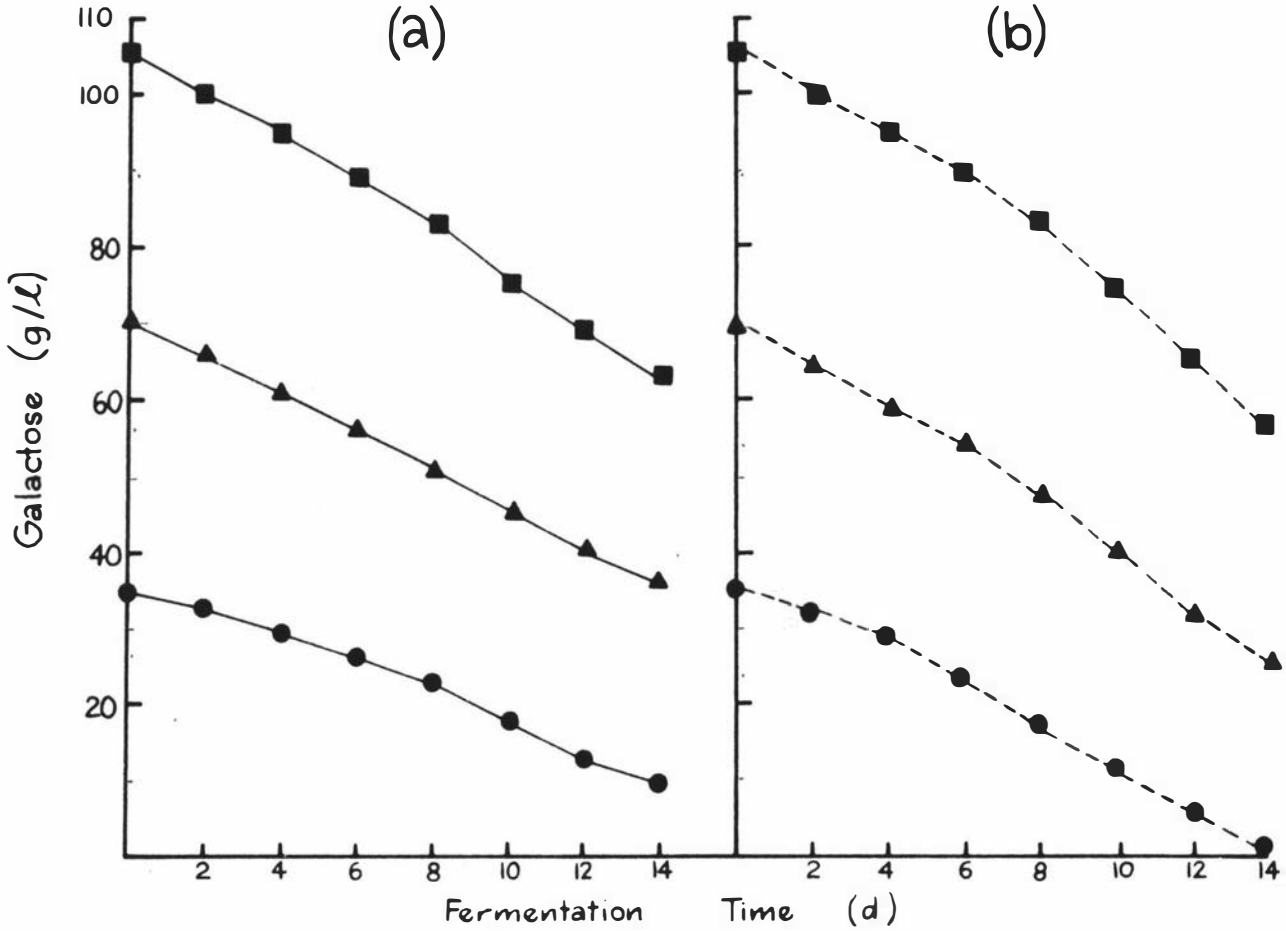

FIGURE 6.29 Galactose utilization in glucose (constant) and galactose (varying concentration) mixtures. (a) Glucose 70 g/l and (b) glucose 40 g/l. Galactose 35 g/l (●), galactose 70 g/l (▲), galactose 105 g/l (■).

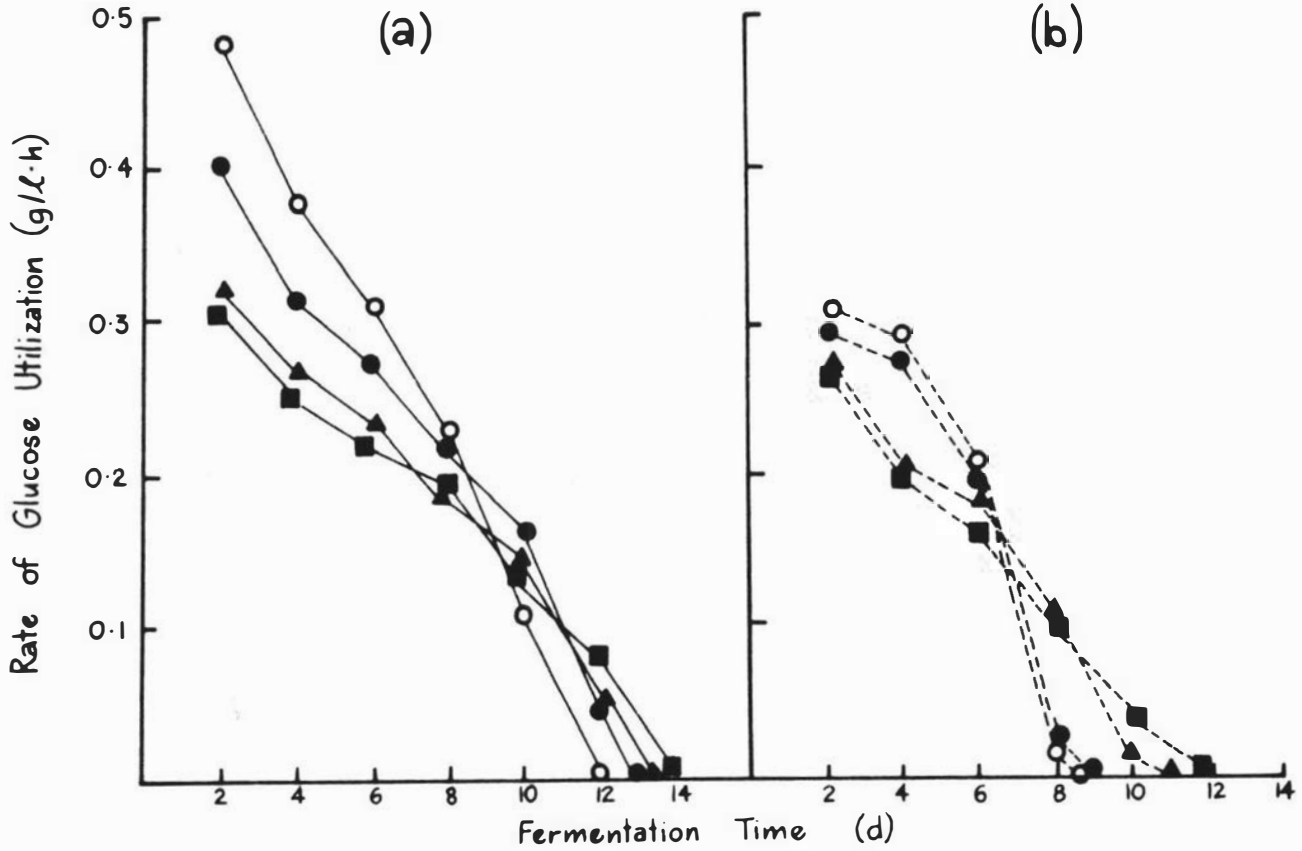

FIGURE 6.30 The volumetric rate of glucose utilization in glucose (constant) and galactose (varying concentration) mixtures. (a) Glucose 70 g/l and (b) glucose 40 g/l.
Galactose absent (O), galactose 35 g/l (●), galactose 70 g/l (▲), galactose 105 g/l (■).

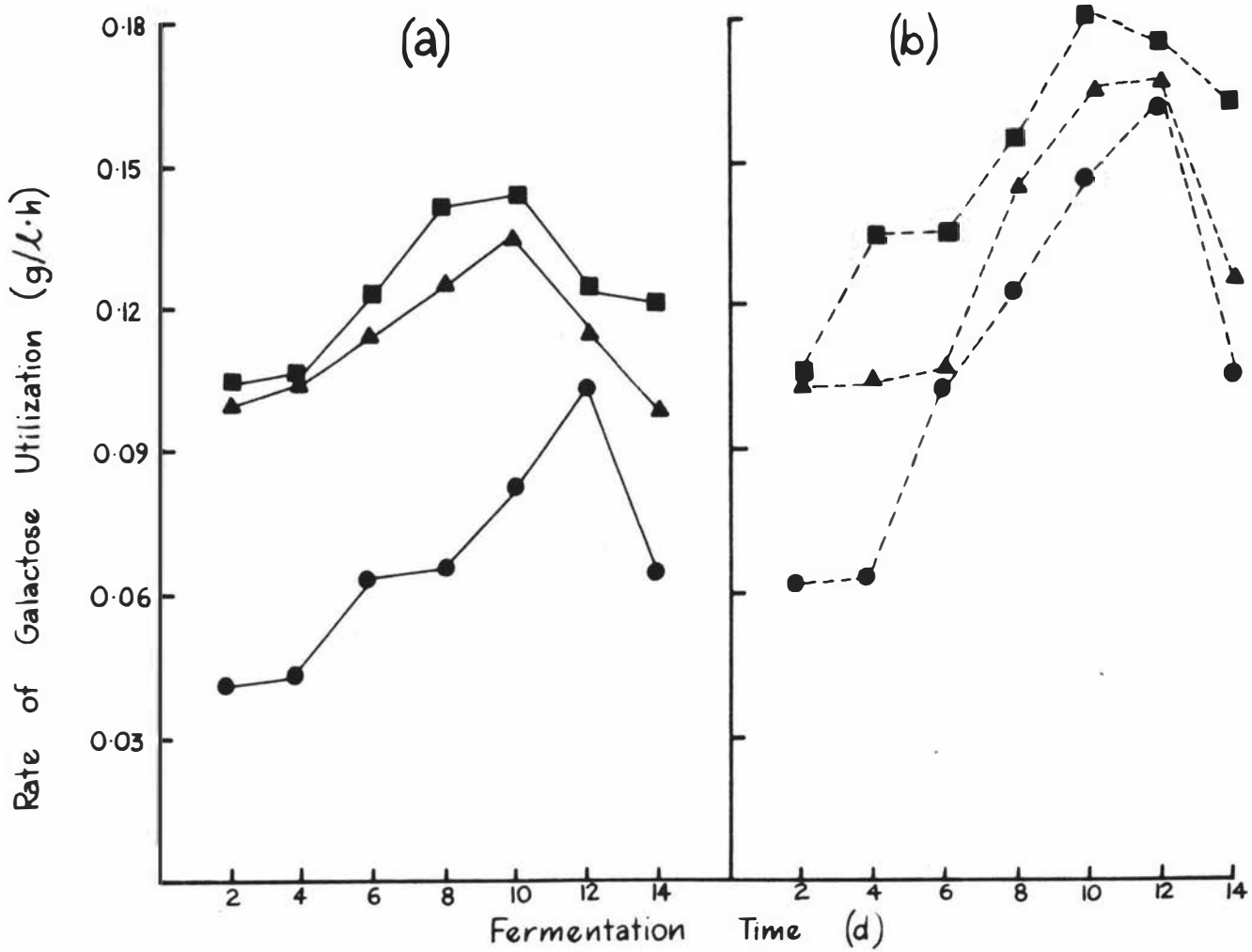

FIGURE 6.31 The volumetric rate of galactose utilization in glucose (constant) and galactose (varying concentration) mixtures. (a) Glucose 70 g/l and (b) glucose 40 g/l.
Galactose 35 g/l (●), galactose 70 g/l (▲), galactose 105 g/l (■).

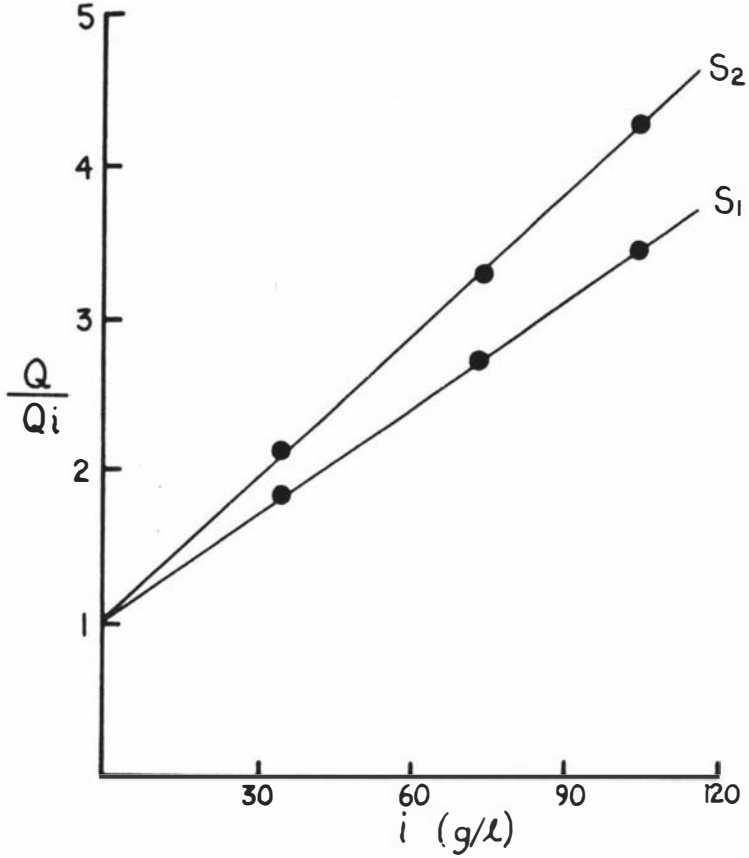

FIGURE 6.32 Plot of Q/Qi values against i (inhibitor).
Where: Q is the rate of citric acid production (g/l.h) from glucose alone.
Qi is the rate of citric acid production (g/l.h) in presence of galactose.
i is the galactose concentration.
Glucose 70 (g/l) = S_1, glucose 40 (g/l) = S_2

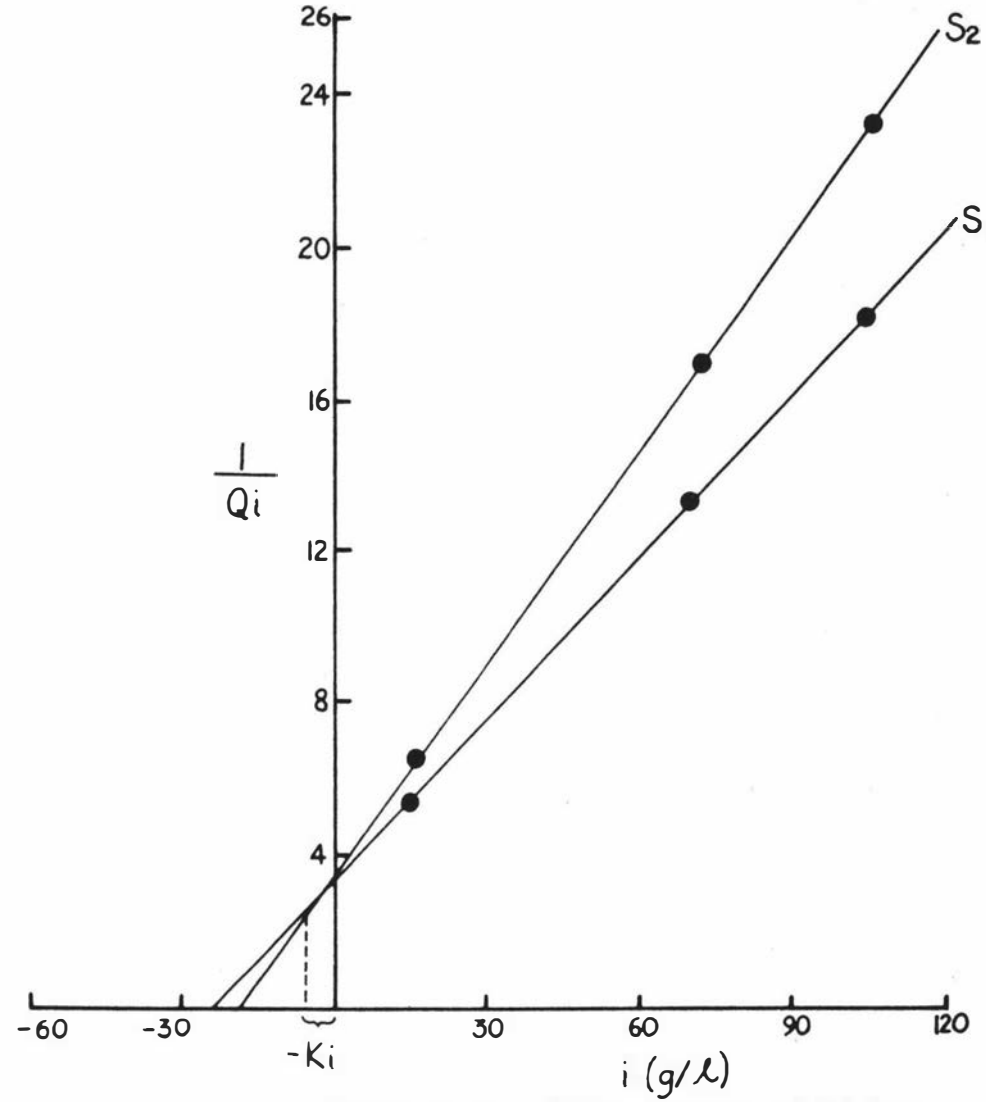

FIGURE 6.33 Determination of Ki for a competitive inhibitor (Dixon, 1953).

dehydrogenase, while Figure 6.35 shows the specific activities of 2-oxoglutarate dehydrogenase and pyruvate carboxylase. It appears from the results (Figure 6.34(a)) that during the phase of mycelial growth, aconitase activity was inversely proportional to the galactose content of the sugar mixture. However, during the citric acid production phase the activity of this enzyme decreased considerably. Similar trends were observed with both NAD- and NADP-linked isocitric dehydrogenase (Figure 6.34(b) and (c)). Thus there appears to be some relationship between the activities of these enzymes and the production of citric acid. The decline in their activities as citric acid accumulates is understandable, but the reason for the inverse relationship between activity and galactose concentration is less clear. Perhaps in the presence of galactose, carbon material is metabolised in some otherway. This possibility has been mentioned previously in Section 6.2.2 with reference to Table 6.4. However, a more conclusive relationship is seen in the behaviour of 2-oxoglutarate dehydrogenase and pyruvate carboxylase (Figure 6.35). No 2-oxoglutarate dehydrogenase activity was detected in the presence of glucose alone (this agrees with the results of fermenter culture) or in glucose with 35 g/l galactose. However, at the higher galactose concentrations, activity was detected, and was observed to increase as the fermentation proceeded (Figure 6.35(a)). This finding probably explains the lower citric acid production at the higher galactose concentrations in the sugar mixture. Thus, when 2-oxoglutarate dehydrogenase was absent high levels of citric acid were produced. However, with increased activity of this enzyme citric acid production decreased, which strongly suggests that the absence of 2-oxoglutarate dehydrogenase is a prerequisite to obtain high citric acid production under the present study conditions. As in fermenter culture (Section 6.2.2), when the TCA-cycle was blocked at the step of 2-oxoglutarate dehydrogenase, the activity of pyruvate carboxylase increased markedly during the citric acid production phase (Figure 6.35(b)), indicating that oxaloacetic acid is produced by the action of this enzyme on pyruvic acid. In contrast, the activity was very low in the presence of the higher galactose concentrations, suggesting that when the TCA-cycle is not blocked, high levels of activity of pyruvate carboxylase are not required.

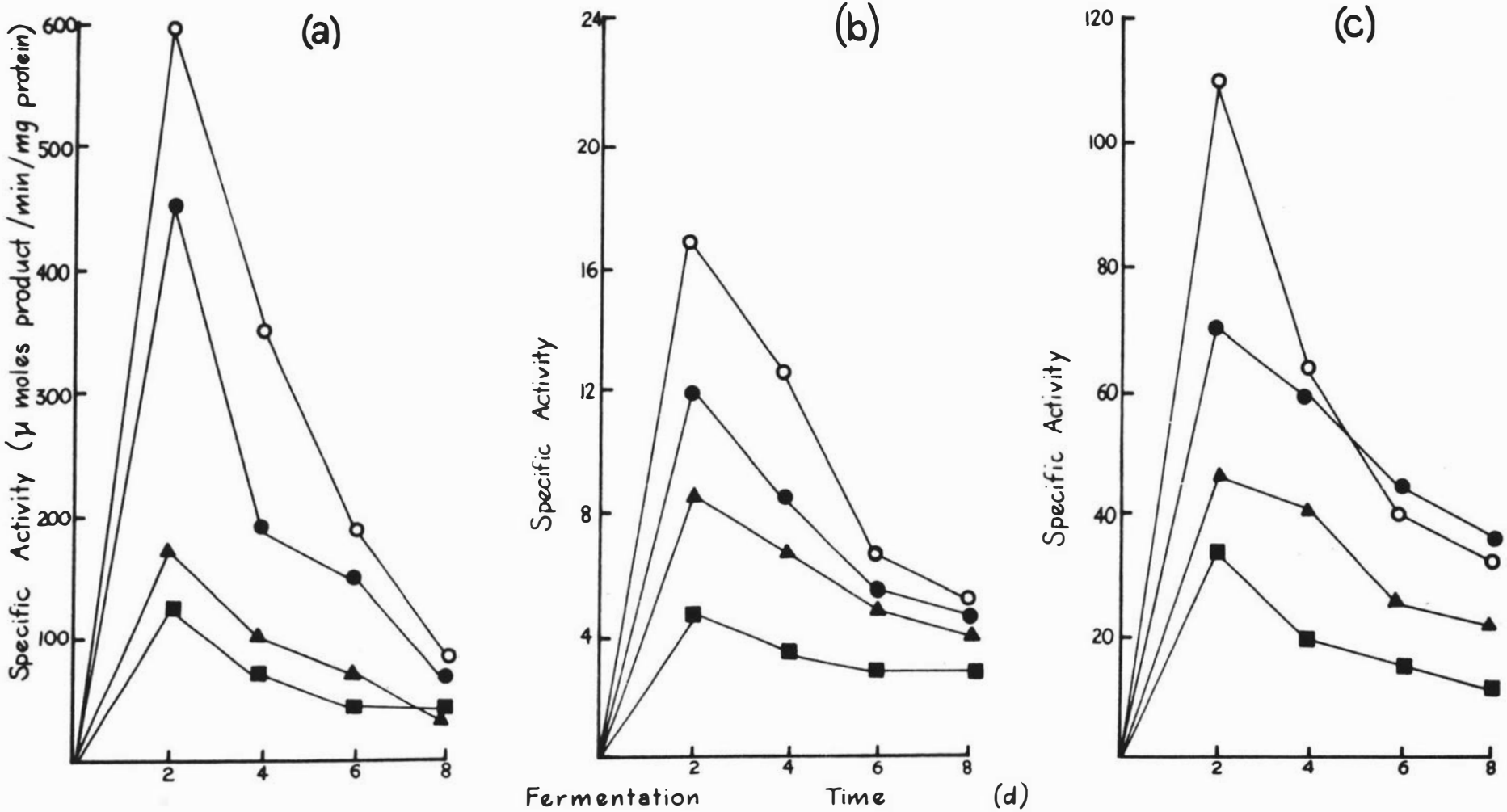

FIGURE 6.34 The specific activities of (a) aconitase (b) NAD-isocitric dehydrogenase and (c) NADP-isocitric dehydrogenase during citric acid production from glucose (70 g/l) and galactose (varying concentration) mixtures. Galactose absent (O), galactose 35 g/l (●), galactose 70 g/l (▲), galactose 105 g/l (■).

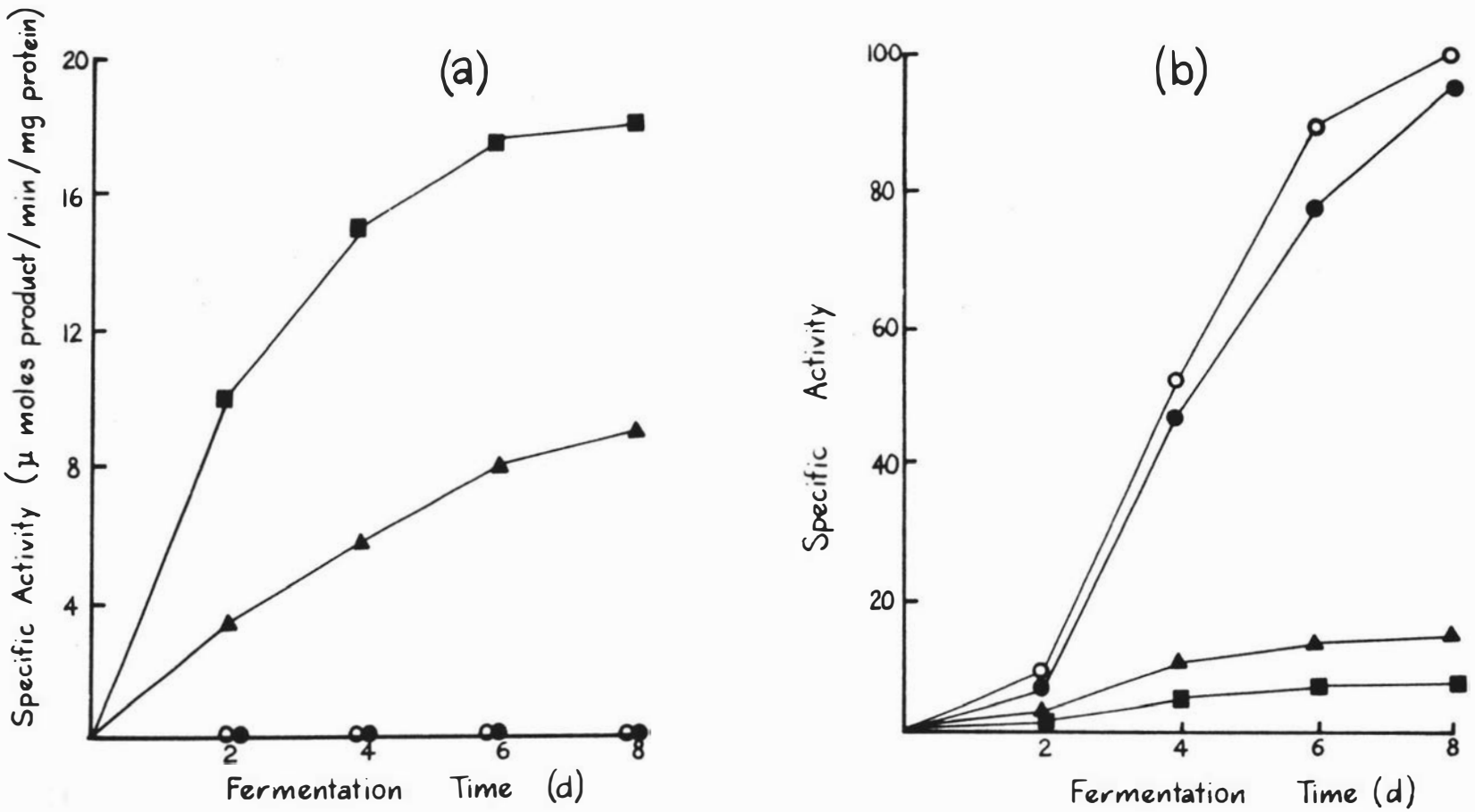

FIGURE 6.35 The specific activities of (a) 2-oxoglutarate dehydrogenase and (b) pyruvate carboxylase during citric acid production from glucose (70 g/l) and galactose (varying concentration) mixtures.
Galactose absent (O), galactose 35 g/l (●), galactose 70 g/l (▲), galactose 105 g/l (■).

The activity of 2-oxoglutarate dehydrogenase increased with the increasing proportions of galactose in the mixture. From this result it may be hypothesized that galactose inhibits the postulated repression of 2-oxoglutarate dehydrogenase caused by glucose. This assumption may be explained according to the Jacob and Monod (1961) model which is presented in Figure 6.36.

In this scheme, there are at least four genes on the chromosome (DNA) that instruct the protein synthetic machinery to make a particular enzyme. The regulator gene (R) produces a repressor protein molecule which in the presence of glucose is activated and binds to the operator gene (O), thus preventing protein synthesis by the neighbouring structural gene (S). The promoter gene (P) is the initiating site of RNA polymerase, the enzyme which catalyzes transcription of the DNA into messenger RNA (mRNA). When the repressor molecule combines with O, RNA polymerase cannot move and no mRNA complementary to the DNA sequence of S is made. Hence, no enzyme is synthesized (e.g. 2-oxoglutarate dehydrogenase). In contrast, in the presence of galactose, the aporepressor protein remains inactive and thus it cannot combine with O. Consequently the RNA polymerase can move from P and transcribe gene S and the enzyme is synthesized. This mechanism remains a hypothesis, but it serves to illustrate the principle.

6.3 CONCLUSIONS

Using a synthetic medium the effects of different sugar sources on citric acid production and some TCA-cycle enzyme activities have been studied in both shake-flask and fermenter culture.

Of the five sugar sources (i.e. sucrose, glucose, fructose, lactose and galactose) examined in shake-flask, sucrose appears to be the best sugar source for high citric acid production. Citric acid was not produced from galactose, despite the fact that it is a carbon source utilizable by *A. niger* strain MH 15-15. Results of citric acid production from decationized and non-decationized sugars did not

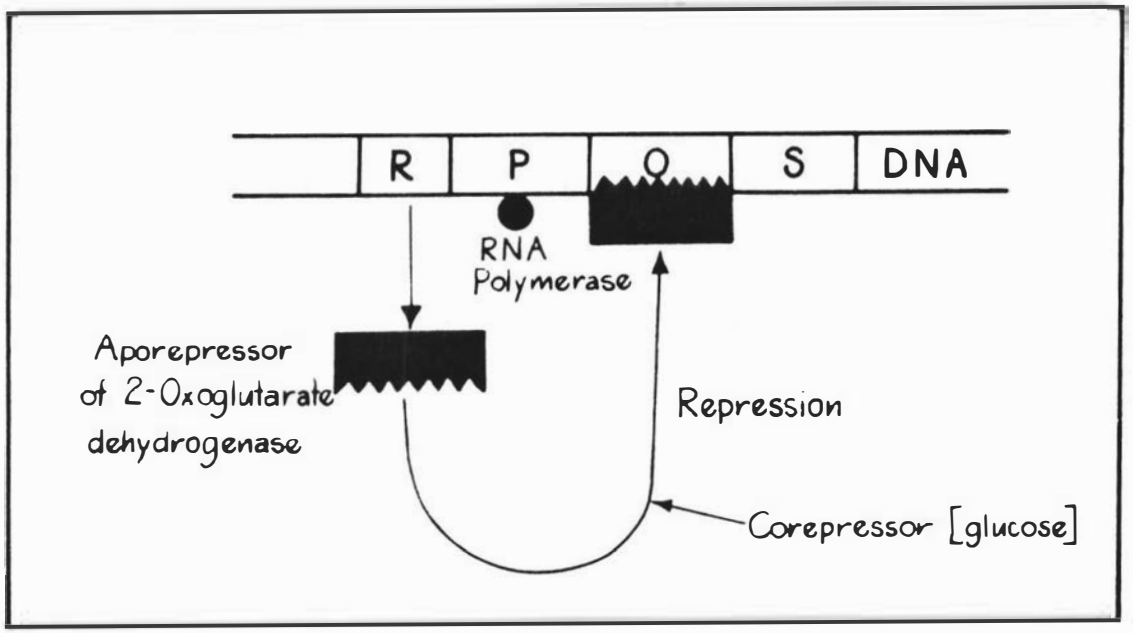

FIGURE 6.36 Scheme depicting the repression of 2-oxoglutarate dehydrogenase (according to model of Jacob and Monod, 1961).

show any significant difference for lactose, but there were a slight difference for fructose and marked differences for sucrose and glucose, where more citric acid was obtained from the decationized than the non-decationized sugar. From the results, it is concluded that the nature of the sugar has a profound effect on citric acid accumulation and that this is more important than the presence of contaminating metal ions in the sugar sources.

In fermenter culture, no citric acid was obtained from galactose, while the citric acid concentrations obtained from the other sugar sources were significantly lower than in shake-flask culture. It is suggested that the form of mycelial growth induced by the agitation method may be the cause of this effect. In both the shake-flask and fermenter culture the sucrose was completely hydrolysed to glucose and fructose within the first 30 hours of fermentation and then the two monosaccharides were utilized simultaneously. Lactose was not hydrolysed outside the cell and was utilized as such. From the results of both shake-flask and fermenter culture it is concluded that the sugar source is important in determining yields of citric acid. It may be further inferred that the relatively low production of citric acid from lactose in comparison with glucose was due to the contribution of the galactose moiety.

Aconitase and isocitric dehydrogenase (both NAD- and NADP-linked) activities in cell-free extracts showed some relationship with citric acid accumulation. However, the enzymes 2-oxoglutarate dehydrogenase and pyruvate carboxylase varied considerably with the sugar sources and their levels correlated well with citric acid production. 2-oxoglutarate dehydrogenase activity was not detected when citric acid production was high, but activity was present when production was low. The activity of pyruvate carboxylase increased considerably during citric acid production, but little activity was detected when citric acid was not produced. It is concluded that accumulation of citric acid is not a consequence of the complete disappearance of the activities of aconitase or isocitric dehydrogenase (both NAD- and NADP-linked), but rather the accumulation is caused by the repression of 2-oxoglutarate dehydrogenase, causing a block in the TCA-cycle and the concomitant increase in

pyruvate carboxylase activity. It is postulated that glucose and fructose cause repression, but galactose does not.

Experiments using mixtures of glucose and galactose as the sugar source have demonstrated that galactose inhibits citric acid production from glucose. The inhibition correlated well with the levels of activity of 2-oxoglutarate dehydrogenase and pyruvate carboxylase. The inhibition has been shown to be competitive.

It is clear from the results of this chapter that the galactose moiety of lactose presents a problem to the production of citric acid from lactose. As mentioned in Chapter 4, attempts to produce a mutant capable of producing citric acid from galactose proved unsuccessful. An alternative approach to mutation is to search for a metabolic inhibitor of 2-oxoglutarate dehydrogenase. Thus, the experiments in the next chapter were designed with this aim in mind.

CHAPTER 7

THE EFFECT OF METHANOL ON CITRIC ACID PRODUCTION AND SOME TCA-CYCLE ENZYME ACTIVITIES

7.1 INTRODUCTION

There are various reports in the literature showing that the production of citric acid by *A. niger* can be increased by the presence of 1-4% (v/v) methanol in the fermentation medium, in both surface and submerged culture (for example, Moyer, 1953 a,b; Taha and El-Zainy, 1959; Noguchi and Bando, 1960; Noguchi *et al.*, 1960; Hang *et al.*, 1977; Chaudhary *et al.*, 1978; Somkuti and Bencivengo, 1981). The exact role of methanol in stimulating citric acid production is not clear, but Moyer (1953 a) has suggested that the presence of methanol and the acidity of the medium bring about an alteration in the normal carbohydrate metabolic pathways, so that citric acid accumulates.

In the previous chapter it was shown that citric acid was not produced from galactose and that little was produced from lactose when compared with glucose. The TCA-cycle enzyme studies revealed that the enzyme 2-oxoglutarate dehydrogenase remained active in the presence of galactose and lactose but not glucose. If this were the cause of not obtaining citric acid from galactose and little citric acid from lactose, then searching for a metabolic inhibitor of 2-oxoglutarate dehydrogenase may be a realistic approach to overcome the problem. Hence, it was decided to investigate the effect of methanol on citric acid production from these various sugars and whey permeate. At the same time, the effect of methanol on the various TCA-cycle enzyme activities was studied for any inhibitory effects.

7.2 RESULTS AND DISCUSSION

7.2.1 THE EFFECT OF METHANOL ON CITRIC ACID PRODUCTION AND SOME TCA-CYCLE ENZYME ACTIVITIES IN SHAKE-FLASK CULTURE

The experiments were performed in shake-flask culture, using the medium of Kristiansen and Charley (1981), where lactose, glucose and galactose were used as sugar sources. The effect of methanol was investigated at two concentrations, 1% (v/v) and 3% (v/v), added on the third day of fermentation. Fermentations were continued for 14 days and flask were harvested after 2,4,6,8,10,12 and 14 days of incubation. For the enzyme assays, mycelium harvested after 2,4,6 and 8 days of fermentation only was studied. The effects of methanol on citric acid production are summarized in Table 7.1. The data obtained in the absence of methanol (Chapter 6, Section 6.2.1) are also included in this Section for comparison.

The effects of methanol on the time course, volumetric rate and specific rate of citric acid production are presented in Figures 7.1, 7.2 and 7.3 respectively. The most interesting and significant finding is that citric acid was produced from galactose in the presence of methanol. Also, higher citric acid concentrations were obtained from glucose and lactose in the presence of 1% (v/v) methanol, as compared with the data obtained with 3% (v/v) methanol and in the absence of methanol (Table 7.1).

It is apparent from the volumetric rate data (Figure 7.2) that the maximum rates obtained were higher with 1% (v/v) methanol as compared with the data obtained in the absence of methanol. On glucose, the maximum rate obtained with 1% (v/v) methanol was slightly higher than that obtained in the absence of methanol, while in lactose the rate was approximately three times higher than the value obtained in the absence of methanol. At 3% (v/v) methanol, rates were lower than at 1% (v/v) methanol. Similar effects were observed with specific rates (Figure 7.3). These findings indicate a stimulatory effect of 1% (v/v) methanol and an inhibitory effect of 3% (v/v) methanol in respect of citric acid production. The stimulatory effect on

TABLE 7.1 EFFECTS OF METHANOL ON CITRIC ACID PRODUCTION IN SHAKE-FLASK CULTURE.

Results are expressed after 14 days of fermentation.

Sugar	0% Methanol				1% (v/v) Methanol				3% (v/v) Methanol			
	Mycelial Dry Weight (g/l)	Sugar+ Utilized (g/l)	Citric Acid Concentration (g/l)	Citric* Acid Yield (%) (w/w)	Mycelial Dry Weight (g/l)	Sugar+ Utilized (g/l)	Citric Acid Concentration (g/l)	Citric* Acid Yield (%) (w/w)	Mycelial Dry Weight (g/l)	Sugar+ Utilized (g/l)	Citric Acid Concentration (g/l)	Citric* Acid Yield (%) (w/w)
Lactose	9.8	68	5	7.5	10.5	65	18.5	29	7.5	46	8.0	17
Glucose	19.5	90	31	34.5	14.0	85	42.0	49	8.5	55	17.0	31
Galactose	12.5	73	0	-	10.5	60	12.5	21	7.0	45	4.6	10

+ Initial sugar concentration 140 g/l.

* Based on sugar utilized.

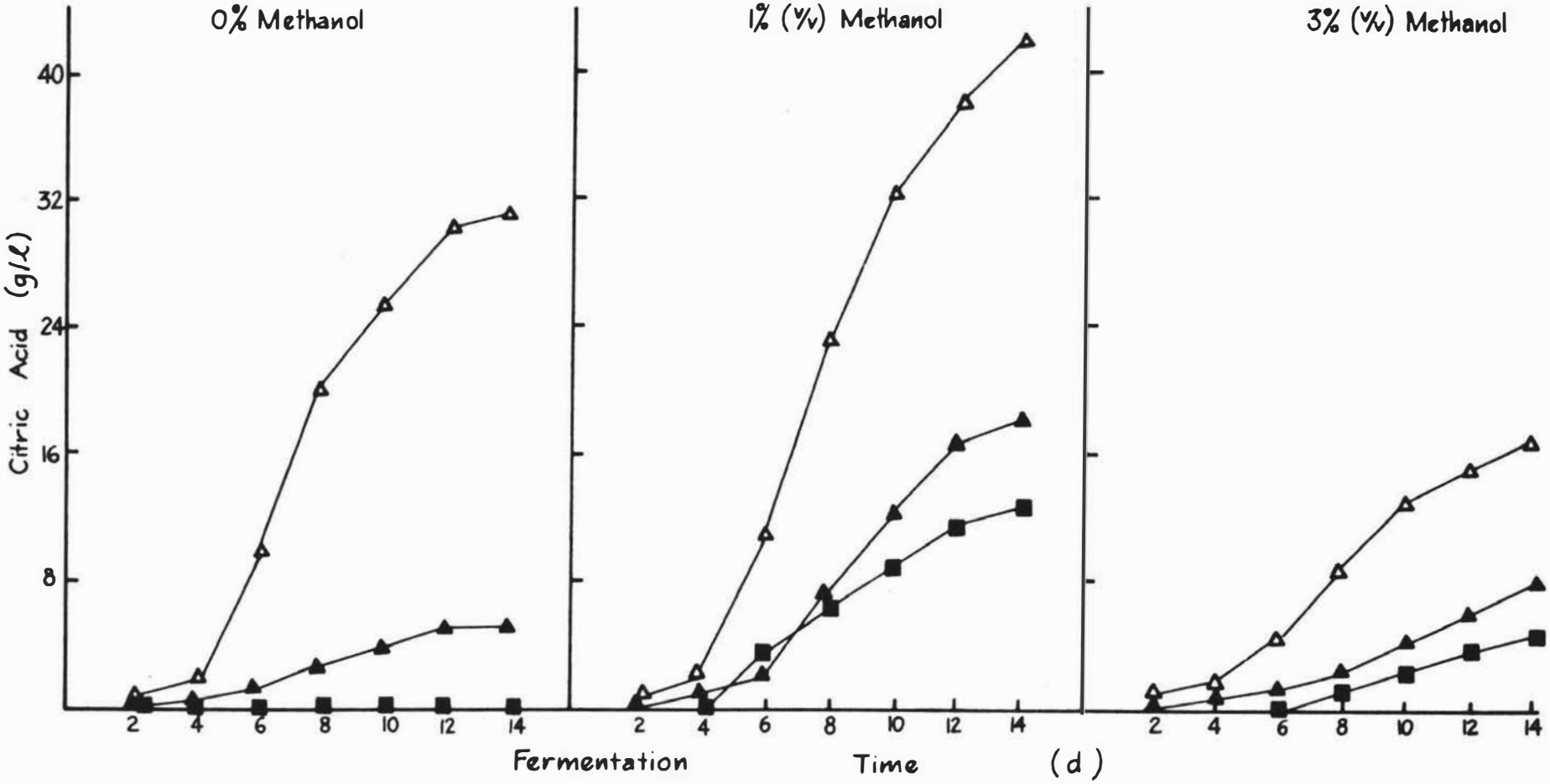

FIGURE 7.1 The effect of methanol on citric acid production in shake-flask culture. Lactose (▲), glucose (△), galactose (■).

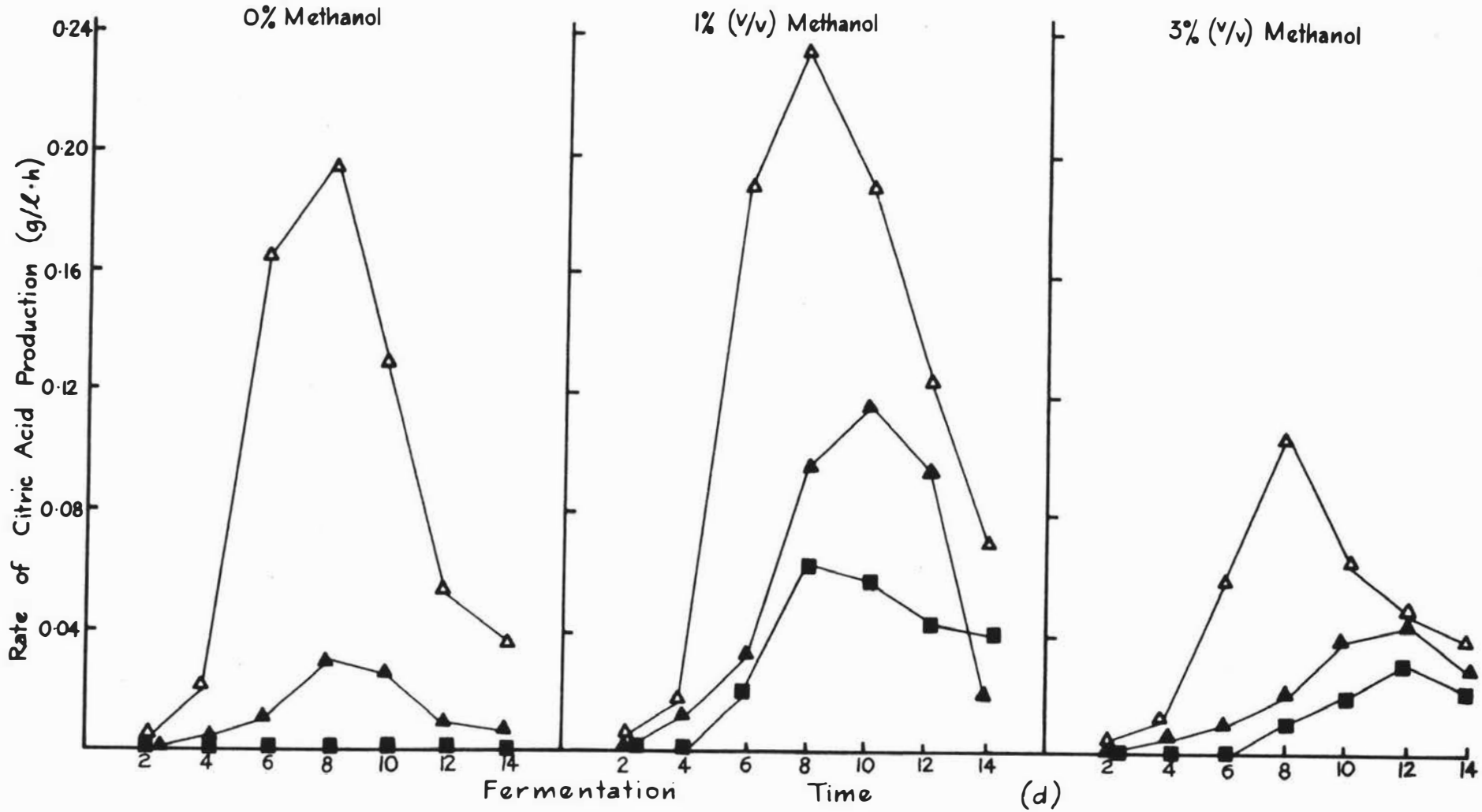

FIGURE 7.2 The effect of methanol on the volumetric rate of citric acid production. Lactose (▲), glucose (△), galactose (■).

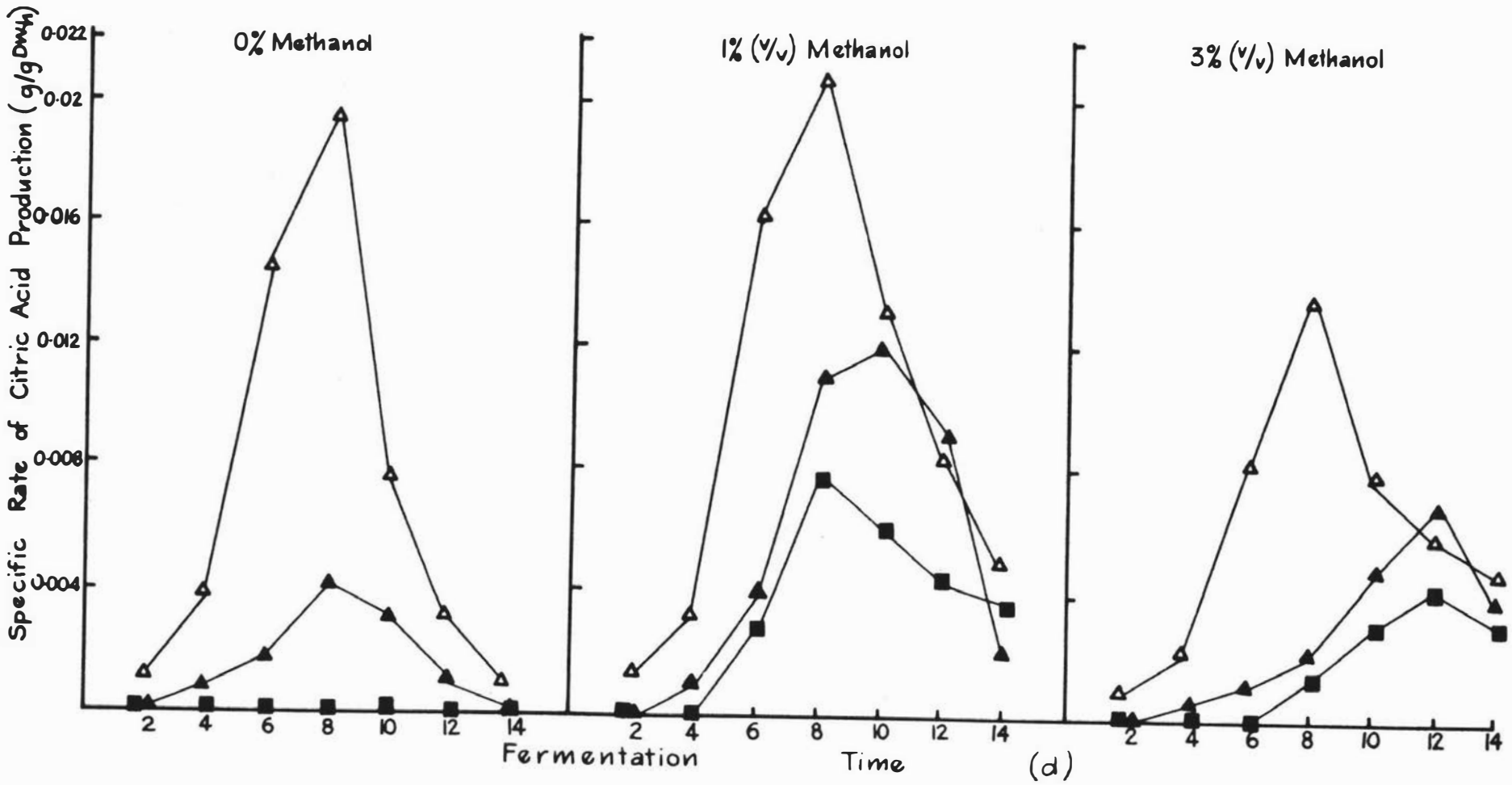

FIGURE 7.3 The effect of methanol on the specific rate of citric acid production. Lactose (▲), glucose (△), galactose (■).

production from glucose and lactose has been previously reported (Moyer, 1953 a,b; Somkuti and Bencivengo, 1981) but the effect on production from galactose has not.

The effect of addition of methanol on mycelial dry weight production is shown in Figure 7.4. The results show that increasing concentrations of methanol have an inhibitory effect on mycelial growth.

The effect of methanol on sugar utilization is presented in Figure 7.5, which demonstrates a slight inhibitory effect of 1% (v/v) methanol on sugar utilization and this is more pronounced in the presence of 3% (v/v) methanol.

The effect of methanol on the specific activities of aconitase, NAD-isocitric dehydrogenase, NADP-isocitric dehydrogenase, 2-oxoglutarate dehydrogenase and pyruvate carboxylase are presented in Figures 7.6, 7.7, 7.8, 7.9 and 7.10 respectively. Unfortunately no directly comparable experiments were performed in shake-flask culture to investigate the levels of TCA-cycle enzyme activities in the absence of methanol. However, the data obtained in fermenter culture (Chapter 6, Section 6.2.2) may be taken as an index of general behaviour in the absence of methanol. Thus, these data are presented for comparison. The present results show that during the initial growth phase of the fungus, the specific activities of aconitase and isocitric dehydrogenase (both NAD- and NADP-linked) were significantly higher in glucose than in lactose or galactose (similar findings were observed in fermenter culture, Figures 7.6, 7.7 and 7.8 respectively and the possible significance has been discussed previously). However, after the addition of methanol, the activities of these enzymes decreased to levels much lower than those observed in the absence of methanol. In the case of galactose, the effect of methanol was particularly marked, since in its absence the activities of these three enzymes continued to increase throughout the entire fermentation (Figures 7.6, 7.7 and 7.8). These data suggest a general inhibitory effect of methanol on the enzyme activities. With regard to 2-oxoglu-

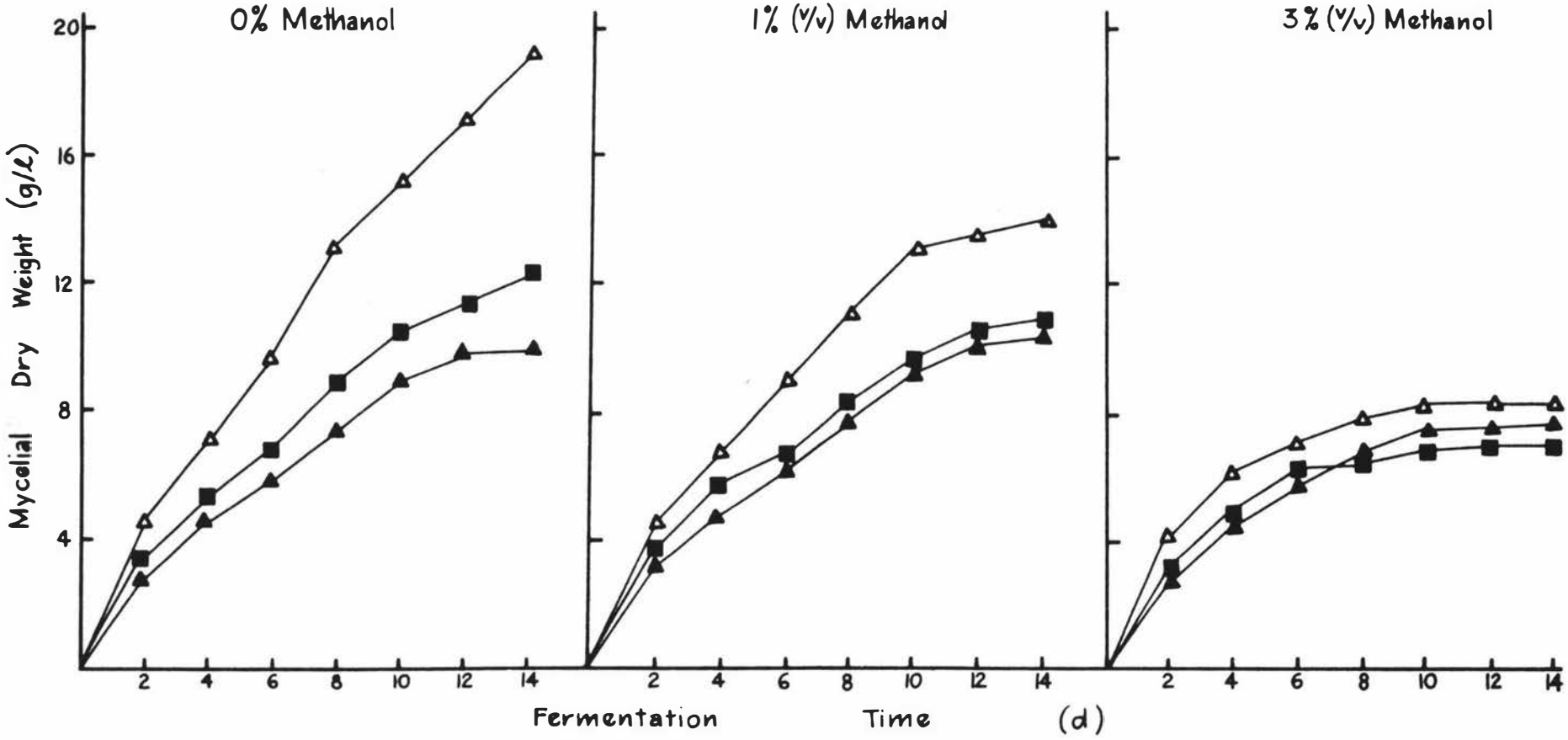

FIGURE 7.4 The effect of methanol on mycelial dry weight production in shake-flask culture. Lactose (▲), glucose (△), galactose (■).

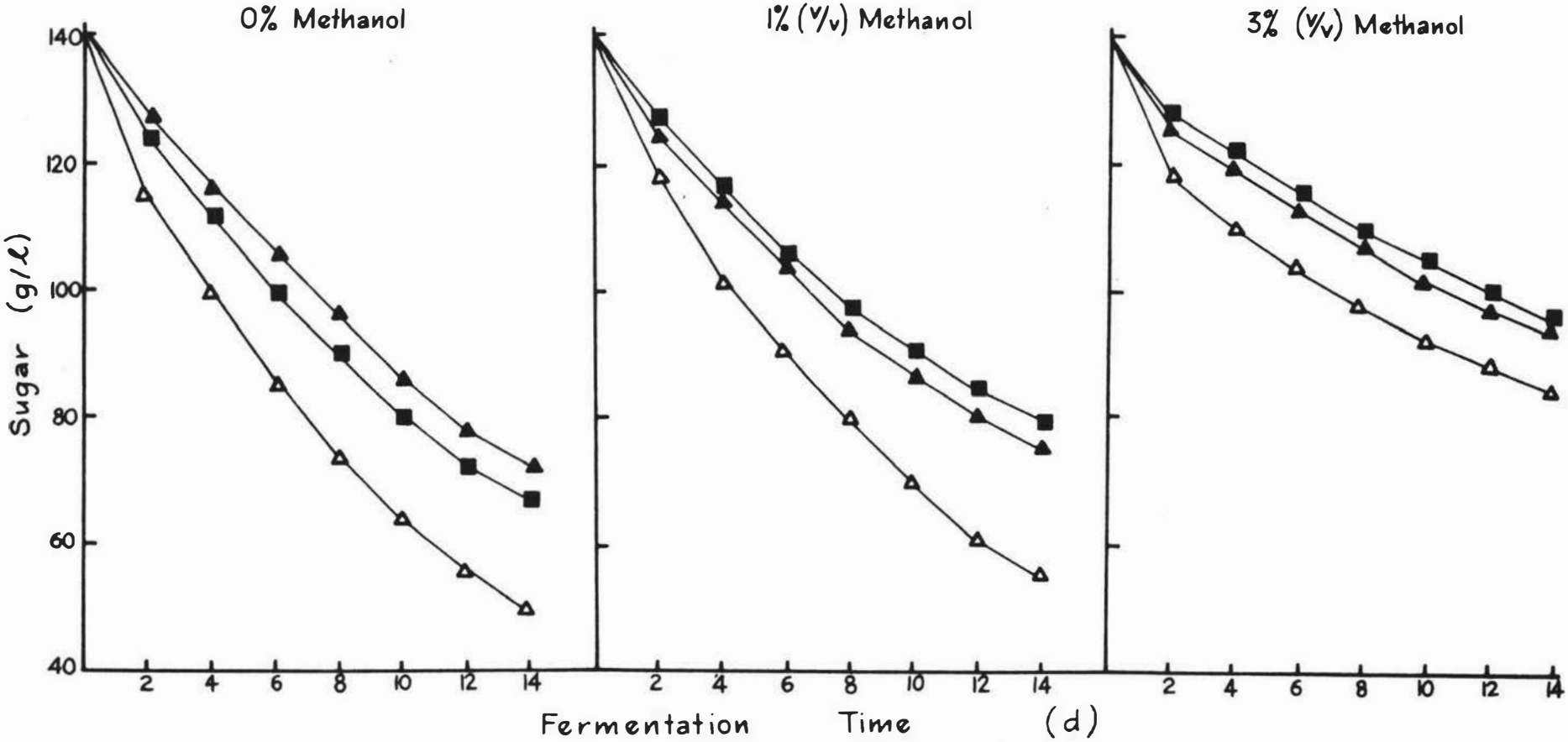

FIGURE 7.5 The effect of methanol on sugar utilization.
Lactose (▲), glucose (△), galactose (■).

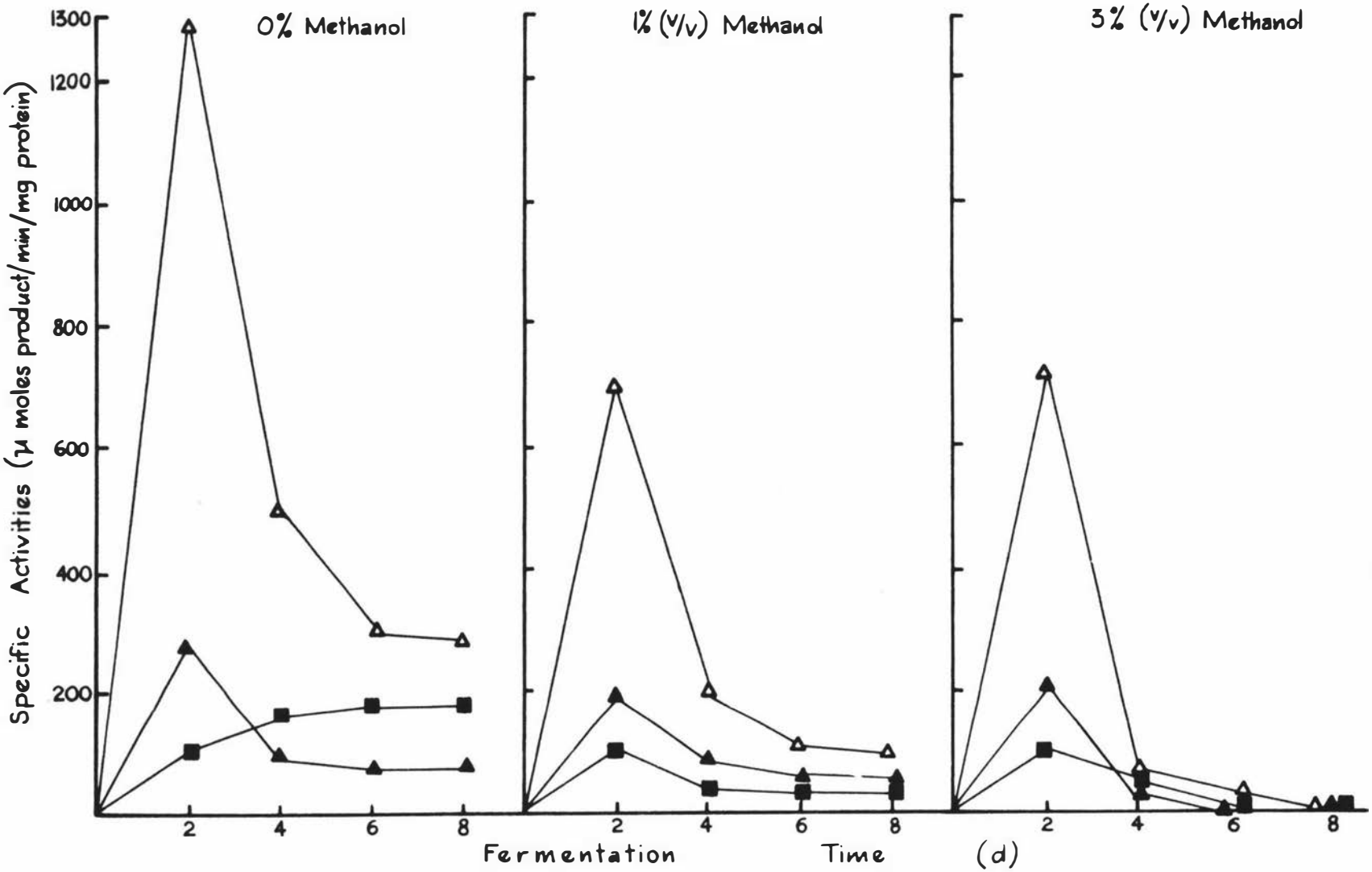

FIGURE 7.6 The effect of methanol on the specific activity of aconitase during citric acid production from various sugars.

Lactose (▲), glucose (△), galactose (■).

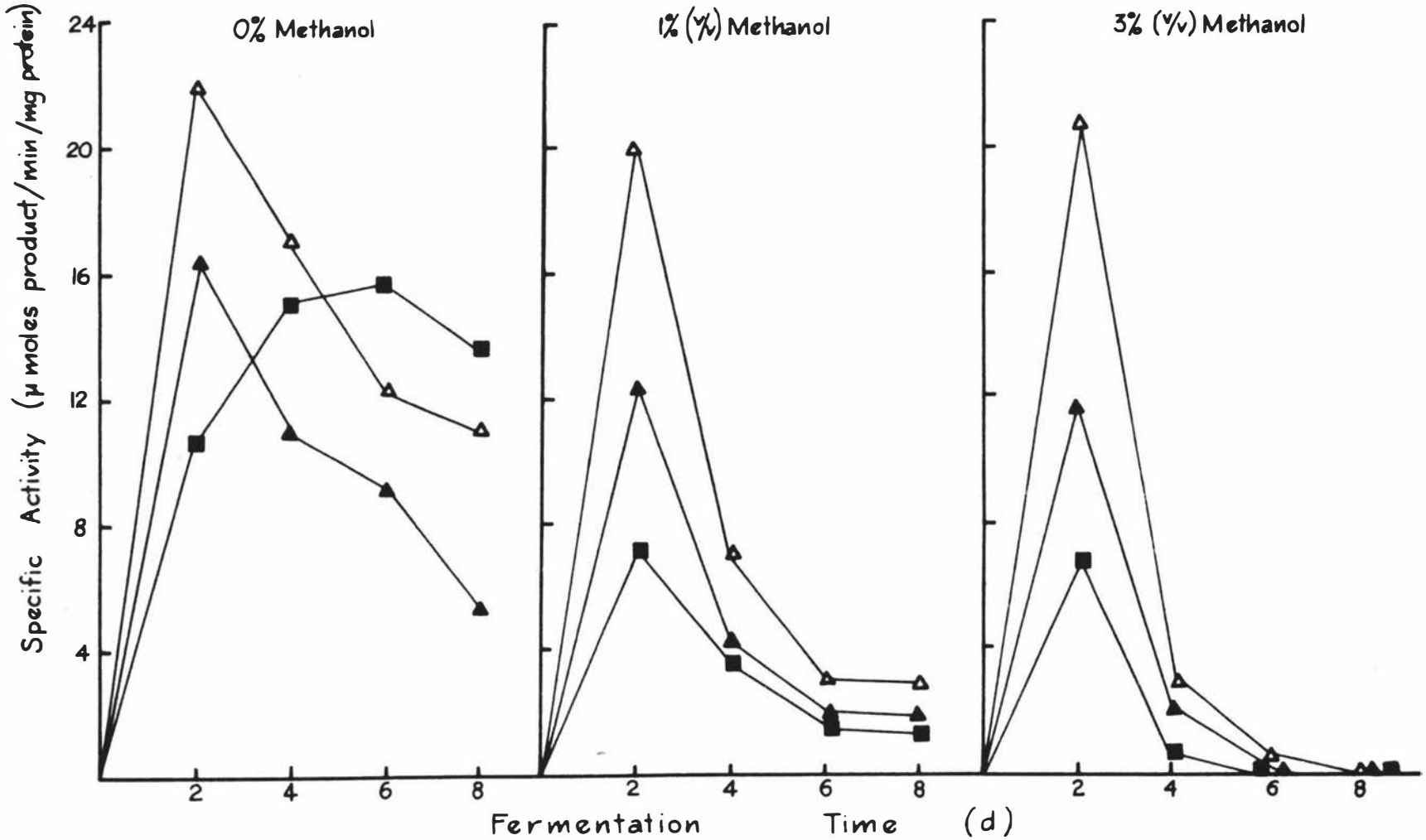

FIGURE 7.7 The effect of methanol on the specific activity of NAD-isocitric dehydrogenase during citric acid production from various sugars.

Lactose (▲), glucose (△), galactose (■).

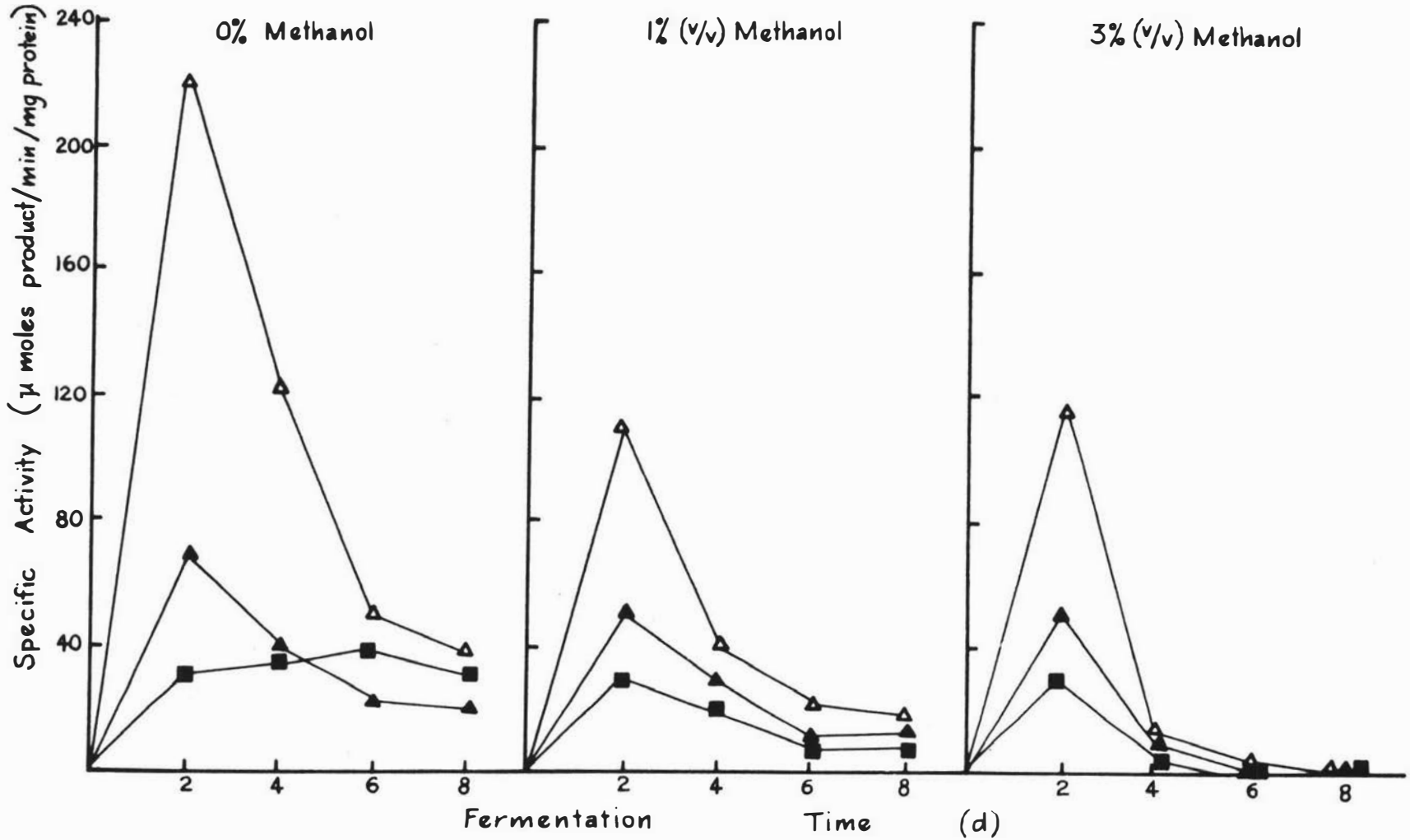

FIGURE 7.8 The effect of methanol on the specific activity of NADP-isocitric dehydrogenase during citric acid production from various sugars.

Lactose (▲), glucose (△), galactose (■).

tarate dehydrogenase, this was never detected with glucose as substrate (Figure 7.9). With lactose and galactose, however, the activity of this enzyme was detected throughout the entire fermentation in the absence of methanol (Figure 7.9). On addition of methanol, however, the activity decreased to zero by the sixth day of fermentation. This finding suggests that methanol inhibits the activity of 2-oxoglutarate dehydrogenase. The complete disappearance of the activity of this enzyme during citric acid production from galactose provides further evidence for its being a key enzyme in controlling citric acid accumulation. The enzyme pyruvate carboxylase was detected for all sugar sources throughout the period of fermentation (Figure 7.10). In glucose, after the addition of 1% (v/v) methanol the activity of pyruvate carboxylase continued to increase, although its activity was approximately fourfold less compared with that in the absence of methanol (Figure 7.10), while its activity was approximately halved by the addition of 3% (v/v) methanol. However, in the presence of lactose and galactose, the enzyme activity was approximately the same in both 1% (v/v) and 3% (v/v) methanol. In the case of galactose, the activity of pyruvate carboxylase persisted throughout the fermentation in the presence of methanol, whereas in its absence the enzyme was not detected after the fourth day (Figure 7.10).

Similar experiments were performed where methanol was added at the time of inoculation. The results obtained did not show any significant differences from the data described above.

The results suggest that methanol has a general inhibitory effect on the various enzyme activities. The effect on 2-oxoglutarate dehydrogenase seems to relate well to citric acid production from galactose. The only instance where an enzyme activity was increased in the presence of methanol was pyruvate carboxylase when citric acid was produced from galactose. This suggests that the block caused by methanol in the TCA-cycle at the point of 2-oxoglutarate dehydrogenase had a stronger effect in stimulating pyruvate carboxylase activity than did methanol in inhibiting it.

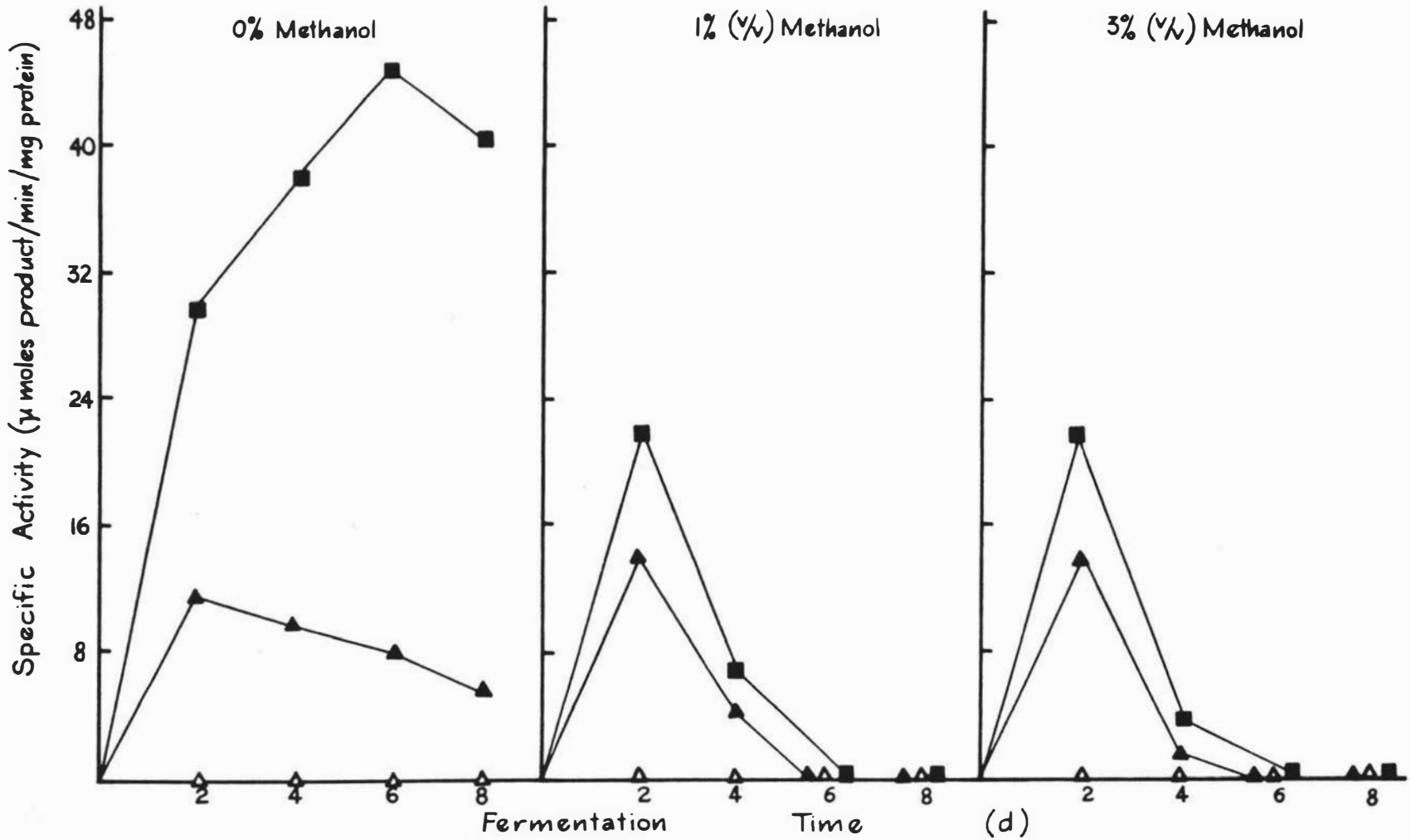

FIGURE 7.9 The effect of methanol on the specific activity of 2-oxoglutarate dehydrogenase during citric acid production from various sugars.

Lactose (▲), glucose (△), galactose (■).

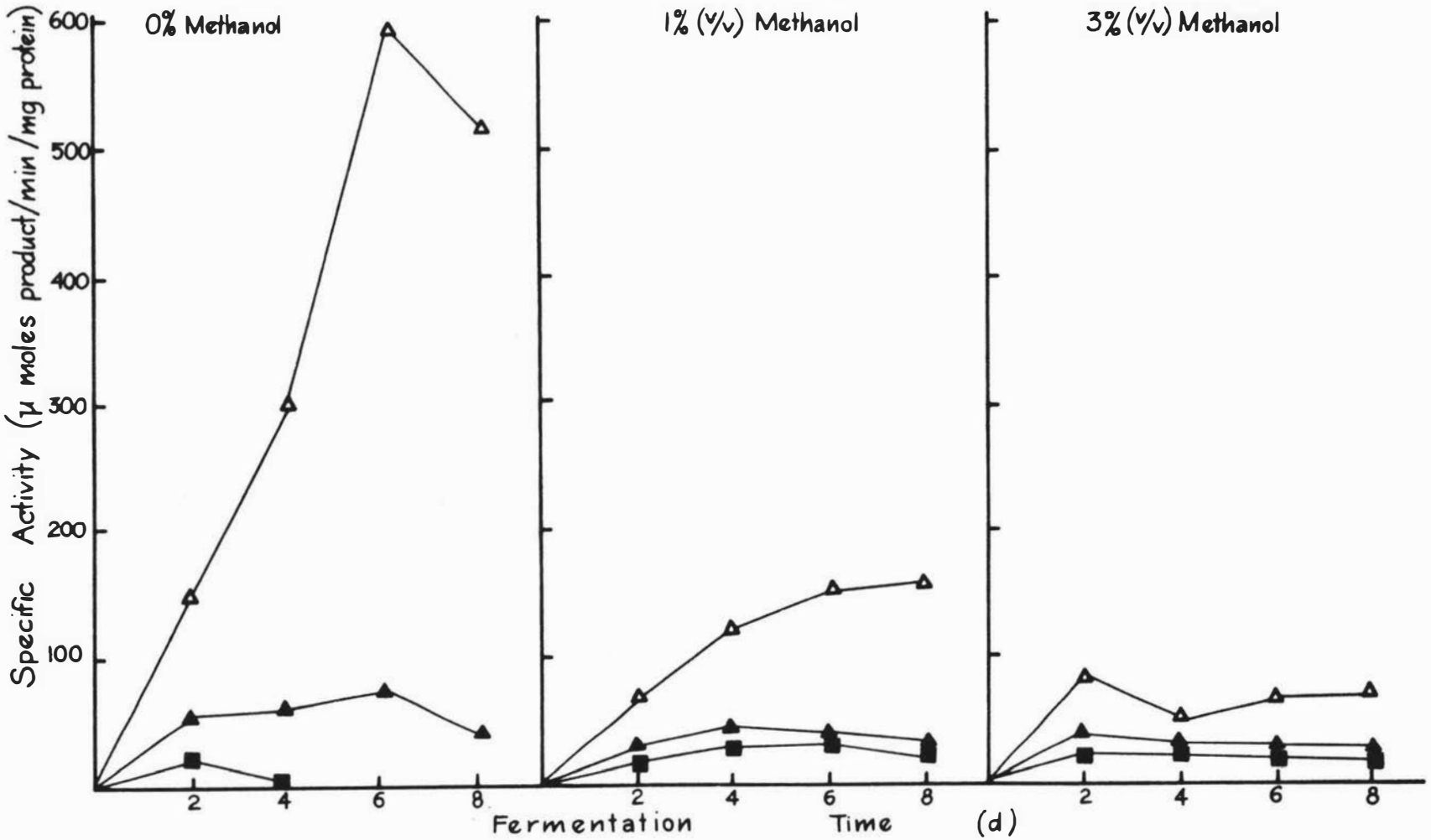

FIGURE 7.10 The effect of methanol on the specific activity of pyruvate carboxylase during citric acid production from various sugars.

Lactose (▲), glucose (△), galactose (■).

7.2.2 The Effect of Methanol on Citric Acid Production and Some TCA-Cycle Enzyme Activities, Using Lactose-Supplemented Lactic Casein Whey Permeate in Fermenter Culture

On the basis of the observation that the presence of methanol led to increased production of citric acid from lactose, an experiment was performed using lactose-supplemented lactic casein whey permeate in the presence of methanol, in fermenter culture.

Lactic casein whey permeate was supplemented with lactose (100 g/l) and the fermentation conditions were identical to those described in Section 5.2.2 (Chapter 5). On the third day of fermentation methanol was added to the fermenter to a final concentration of 3% (v/v). Samples were withdrawn daily throughout the fermentation period. For the enzyme assays, mycelium was withdrawn after 2,4,6 and 8 days only of fermentation. The time course of the fermentation is presented in Figure 7.11. The data show that a citric acid concentration of 25 g/l was achieved compared with 14.8 g/l obtained in the absence of methanol (Figure 5.4, Chapter 5). The yield was 33% (w/w) compared with 23% (w/w) in the absence of methanol. On comparison with the data obtained using the lactose-based synthetic medium in the presence of methanol in shake-flask culture (Table 7.1), both the production and yields were significantly higher in the present experiment. A slightly lower mycelial dry weight was observed in the presence of methanol but approximately 15% more lactose was utilized by the end of the fermentation (as compared with Figure 5.4). The toxic effect of methanol on mycelial growth in the fermenter culture was not so marked as in shake-flask culture. This may be due to loss of methanol from the fermenter *via* the aeration system.

The volumetric and specific rate data of citric acid production and lactose utilization are presented in Figures 7.12 and 7.13 respectively. By comparison with Figure 5.5 (Chapter 5), the maximum observed volumetric and specific rates of citric acid production were 60% and 100% greater, respectively, in the presence of methanol. On the volumetric rate curve of lactose utilization (Figure 7.13) two peaks are apparent. The first (at Day 2) is presumably associated

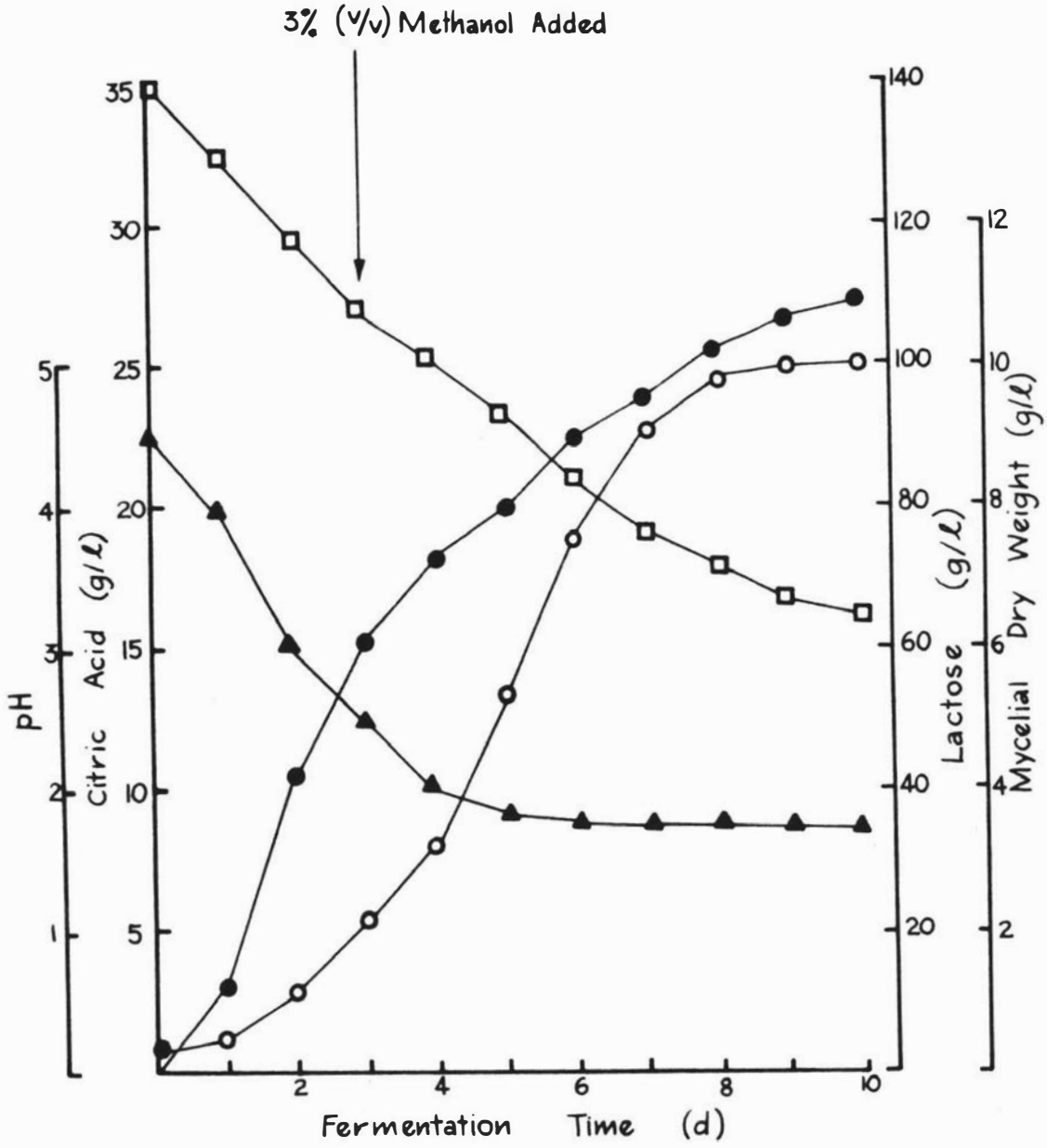

FIGURE 7.11 Effect of 3% (v/v) methanol on citric acid production from lactose-supplemented lactic casein whey permeate in fermenter culture.

Citric acid (O), mycelial dry weight (●), lactose (□), pH (▲).

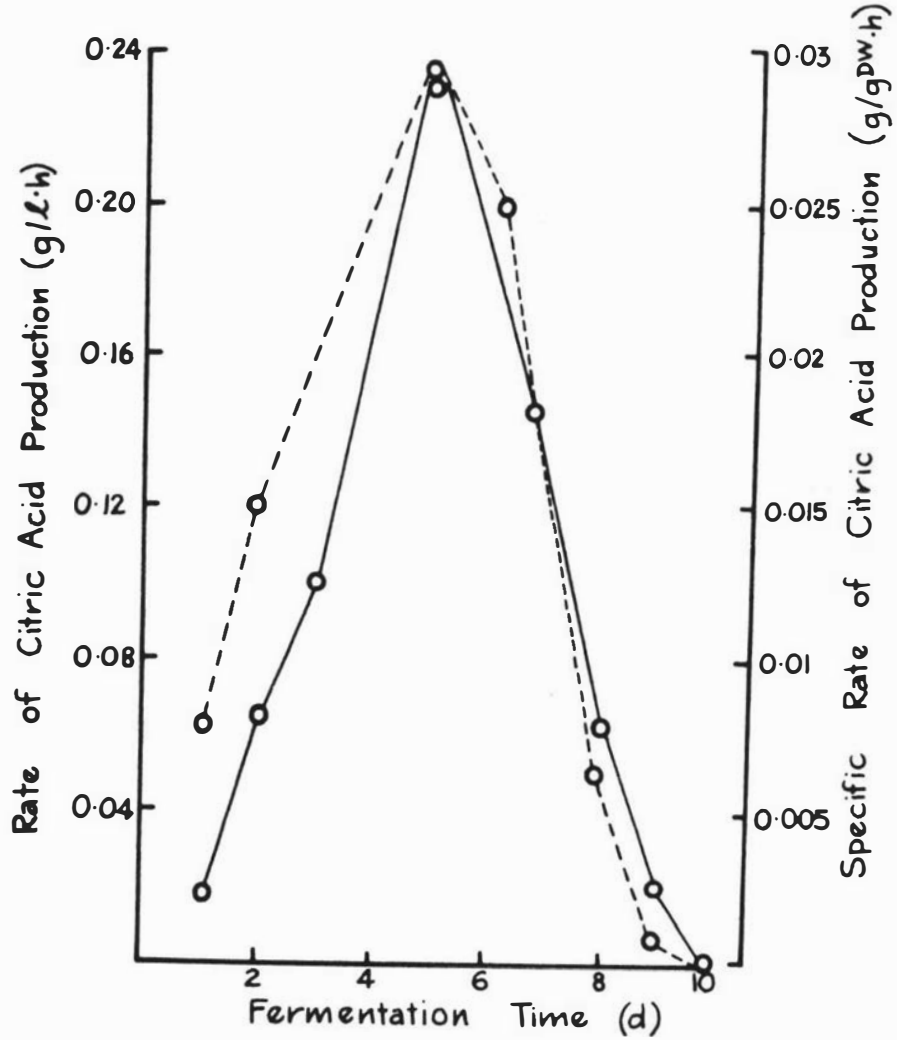

FIGURE 7.12 The effect of 3% (v/v) methanol on the volumetric (——) and specific rate (---) of citric acid production in fermenter culture.

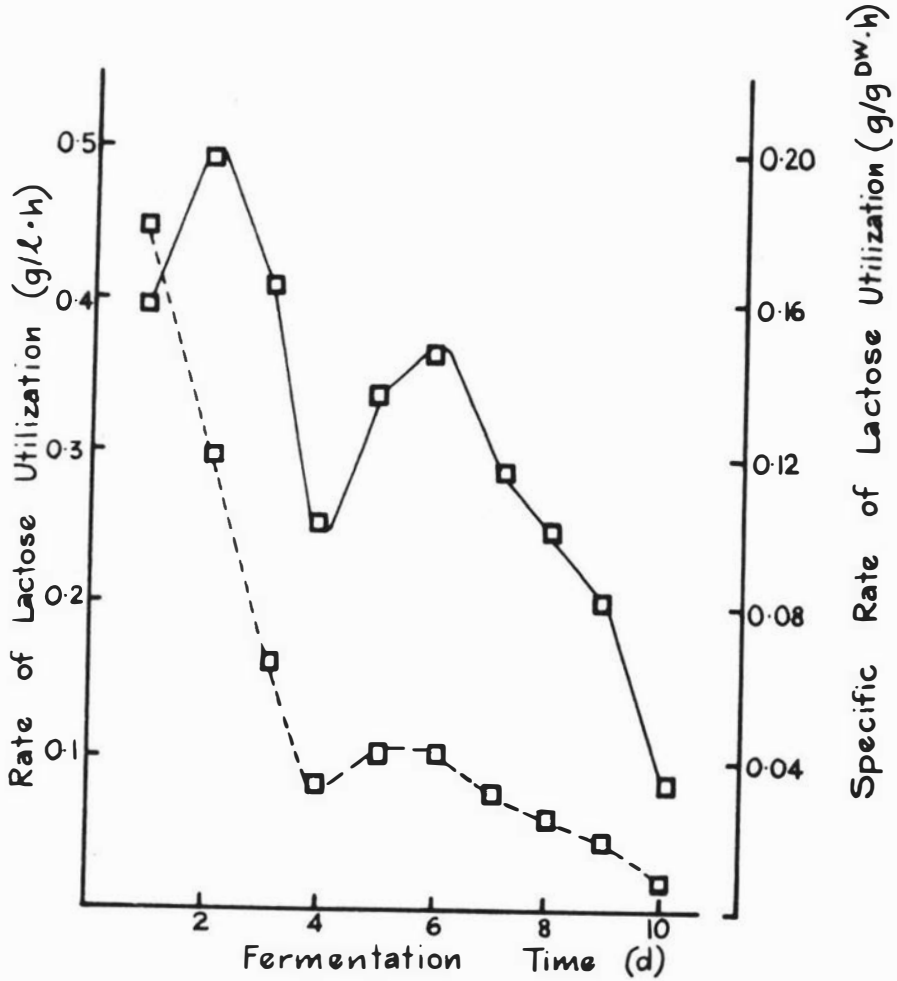

FIGURE 7.13 The effect of 3% (v/v) methanol on the volumetric (——) and specific rate of lactose (---) utilization in fermenter culture.

with active mycelial growth, while the second (at Day 6) coincides with the maximum rate of citric acid production. In the absence of methanol, the second peak was much less pronounced. When compared with the data in the absence of methanol (Figure 5.5) a slightly higher rate of lactose utilization was observed in the presence of methanol.

The effects of methanol on the specific activities of some TCA-cycle enzymes are presented in Figure 7.14. The data for the enzyme activities observed in the absence of methanol (Chapter 6, Section 6.2.2) are included in Figure 7.14 for comparison. The results show that the activities of all these enzymes were slightly lower in the presence of methanol. In particular, 2-oxoglutarate dehydrogenase was not detected after the sixth day, whereas in the absence of methanol it persisted throughout the fermentation. This finding again suggests that methanol inhibits the activity of 2-oxoglutarate dehydrogenase and this correlates well with the higher citric acid production observed in the presence of methanol.

Somkuti and Bencivengo (1981) observed a similar increase in citric acid production from whey permeate in the presence of methanol, but they did not investigate any enzyme activities.

7.3 CONCLUSIONS

The effects of methanol on production of citric acid from lactose, glucose, galactose and lactose-supplemented lactic casein whey permeate have been studied. It was observed that, in shake-flask culture, 1% (v/v) methanol caused increased production and yields from both glucose and lactose. Production of citric acid from galactose was also observed. In fermenter culture, using whey permeate, the presence of 3% (v/v) methanol led to a 69% increase in citric acid production. Apparently, the presence of methanol had a general inhibitory effect on the various enzymes studied with the exception of pyruvate carboxylase, the activity of which increased when galactose was used as the sugar source. The inhibition of 2-oxoglutarate dehydrogenase in the presence of methanol relates well with citric acid accumulation.

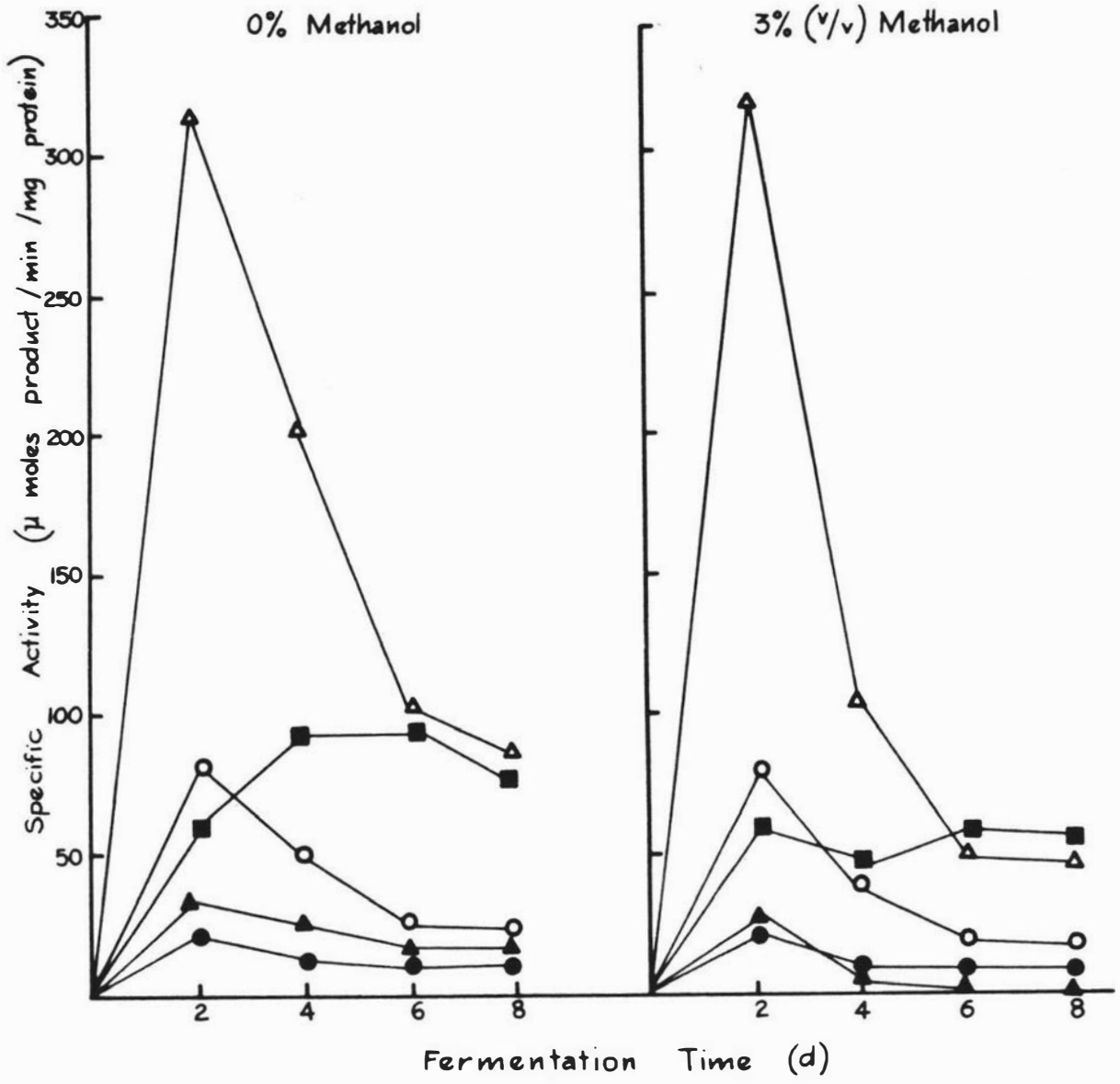

FIGURE 7.14 The effect of methanol on the specific activities of some TCA-cycle enzymes during citric acid production from lactose-supplemented lactic casein whey permeate in fermenter culture.

Aconitase (△), NAD-isocitric dehydrogenase (●), NADP-isocitric dehydrogenase (O), 2-oxoglutarate dehydrogenase (▲), pyruvate carboxylase (■).

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

The feasibility of citric acid production from lactic casein whey permeate was investigated. From the industrial point of view, this investigation has considerable interest for the dairy industry in New Zealand, for economical utilization of surplus whey permeate, the disposal of which can cause serious problems. As part of this investigation, the production of citric acid from different sugar sources and the role of some TCA-cycle enzymes, was also investigated. Finally, the effect of addition of methanol to the fermentation medium was studied.

The starting point of the investigation was the examination of ten strains of *A. niger* and one strain of *A. carbonarius* for their ability to produce citric acid from lactic casein whey permeate. The data obtained during this screening programme are presented in Chapter 4. The best citric acid-producer (*A. niger* IMI 41874) was selected for further work, including a strain improvement programme.

Strain improvement was performed by induced mutation using ultraviolet light and a suitable method was developed for screening the maximum number of survivors in a single petri dish. Out of several thousand isolates, a mutant (*A. niger* MH 15-15), which produced more citric acid than the parent, was selected. Further attempts to improve this mutant, using ultraviolet light, were not successful. Possibly, a change in mutagenic agent could have been useful. When it became apparent that both the parent and the mutant were incapable of producing citric acid from galactose (except in the presence of methanol), a mutation programme was undertaken to isolate mutants with this capability. Many isolates were collected and examined but none produced citric acid from galactose.

A comparison of lactose-supplemented whey permeate and a sucrose-containing synthetic medium demonstrated the superiority of the latter in supporting citric acid production by both the parent and mutant organisms. Whether this effect was due to the use of the different sugar sources or to the other medium constituents was investigated in Chapters 5 and 6.

The experiments in Chapter 5, describing an investigation into the medium constituents, particularly metal ions, failed to produce significant improvements in citric acid accumulation. However, the experiments in Chapter 6, describing an investigation into the effect of the sugar source, revealed marked differences in citric acid production. Sucrose proved to be the most effective sugar source, followed by glucose and fructose and then lactose. No citric acid was produced when using galactose as the sugar source, although good mycelial growth was observed. Thus, when using whey permeate, it appears that, it is the nature of the sugar, rather than the other medium constituents, which presents the major problem to improved production of citric acid.

When investigating the effect of the sugar source using synthetic medium in fermenter culture, production of citric acid was somewhat lower than in shake-flask culture. It was suggested that this was due to a breakdown of the fungal pellets to a filamentous form, caused by the agitation system in the fermenter. However, using whey permeate as substrate, fermenter culture was superior to shake-flask culture. Clearly, further work is required to investigate these effects.

The activities of some TCA-cycle enzymes in cell-free extracts were investigated when studying citric acid production from the various sugars and whey permeate in fermenter culture. For the enzymes aconitase and isocitric dehydrogenase (both NAD- and NADP-linked), there was a strong relationship between their activities during the growth phase and subsequent citric acid production. The significance of this relationship is not clear, but it may reflect how effectively the TCA-cycle is operating and the ultimate fate of the carbon source. During the citric acid production phase, the activities of

these enzymes decreased considerably, except when galactose was used as the sugar source when the activity increased. There were strong relationships between citric acid accumulation and the activities of 2-oxoglutarate dehydrogenase and pyruvate carboxylase. When citric acid was being actively produced, 2-oxoglutarate dehydrogenase was not detected, suggesting that this may be the key enzyme in citric acid accumulation. When citric acid was not produced, as when galactose was used as sugar source, this enzyme remained active. Thus, it was postulated that glucose and fructose, but not galactose, are able to repress 2-oxoglutarate dehydrogenase. During accumulation of citric acid, pyruvate carboxylase activity increased considerably, suggesting that this is a major route for the formation of oxaloacetic acid, a direct precursor of citric acid.

The effect of galactose on citric acid production from glucose was investigated and the data demonstrated that galactose caused competitive inhibition of citric acid production from glucose. The biochemical mechanism of the inhibition is not certain, but the data from enzyme activity studies are consistent with the hypothesis that galactose inhibits the repression of 2-oxoglutarate dehydrogenase by glucose.

The effect of methanol on citric acid production from different sugar sources and whey permeate was investigated, and its stimulatory effect was demonstrated. Most importantly, in the presence of methanol, citric acid was obtained from galactose. The mechanism of action of methanol is not entirely clear, but the results suggest a general inhibitory effect on the activity of all enzymes studied, particularly 2-oxoglutarate dehydrogenase.

In conclusion, the major aim of this work was to assess the feasibility of using lactic casein whey permeate as an alternative source of raw material for citric acid production. Although the present work has not developed an industrial process, the data obtained have provided valuable information which will be useful to future investigators. The main problem is with the conversion of galactose to citric acid. Thus, research in the following areas

would be useful:

(a) Screening of different strains of *A. niger* for their ability to produce citric acid from galactose. If such a strain were found, it should be possible to improve it by mutation. (In this regard, Maddox (1983) has recently screened the 11 strains of *Aspergillus* detailed in Chapter 4 of this thesis, but without success).

(b) Searching for chemical metabolic inhibitors of the enzyme 2-oxoglutarate dehydrogenase. Such work could be performed rapidly using cell-free extracts and any useful compounds could then be tested in fermentation.

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