PRODUCTION OF CITRIC ACID BY SUPPLEMENTED BATCH CULTURE OF FUNGI

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ABSTRACT

The effect of a single pulse of ammonium sulphate and citrate upon the progress and final outcome of batch citric acid fermentation was studied. It was found that the optimum addition time of N was in the range of 40 to 75 h. It was found that final citric acid concentration achieved was increased when the amount of supplemental N source added was between 0.25 and 0.5 kg m^{-3}.

60 kg m^{-3} citric acid addition at time 90 h, led to increase 40.3 % the final citrate production (final produced citric acid 80 kg m^{-3}).

The best initial concentration of $(NH_4)_2SO_4$ was found to be 3 kg m^{-3} which produced the highest citric acid concentration (81 kg m^{-3}) when the initial concentration of $(NH_4)_2SO_4$ was varied between 0.5 and 4.0 kg m^{-3}.

It was recognised that control of culture pH is critical and it should be kept between 1.8 and 2.5. An initial pH of 2.5 was found a optimum pH for citrate production in STR.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Citric acid is a 6- carbon containing tricarboxylic acid which was first isolated from lemon juice. It is a natural component of many citrus fruits, and was crystallized from lemon juice by Scheele in 1784. The historical development of the citric acid fermentation is illustrated in Table 1.1 (Kubicek and Rohr 1986). Approximately 70% of citric acid produced is used in the food and beverage industry for various purposes, 12% in pharmaceuticals and about 18% for other industrial uses which are summarized in Table 1.2 (Marison 1988). The estimated world production of citric acid was reported as 350 000 tons / year in 1986 (Kubicek and Rohr 1986). It however was recently reported that the world market requirement of citric acid is around 500 000 tons / year (Bu'lock 1990).

1.2 Production Process

At the present day most citric acid is produced by fungal (A. niger) fermentation. Chemical synthesis of citric acid is possible but it is no cheaper than fungal fermentation. However, a small amount of citric acid, approximately less than 1% of total world production, is still produced from citrus fruits in Mexico and South America where citrus fruits are available economically. There are basically three different types of batch fermentation process used in industry. These are the Japanese koji process, the liquid surface culture and the submerged fermentation process (Lockwood 1979), but nowadays nearly all citric

acid is produced by submerged culture fermentations because profitability is relatively low and thus the economics of the operation are very constricted (Bu'lock 1990). Continuous fermentation has been studied on the laboratory scale by some workers (Kristiansen and Sinclair 1979; Charley 1981; Stevensen 1986) but no commercial production by this type of process is known. Since cell growth and citrate production occur at different times, and for the economic reasons, the substrate sugar must fully utilized, it is necessary to use multitank systems which would increase costs. In addition continuous culture usually gives lower citrate concentration i.e recovery costs increase. Therefore continuous culture is probably not economically competitive at the present time.

1.2.1 Koji Process

This process is seldom used outside Japan where it accounts for about one-fifth of the annual Japanese citric acid production. Wheat bran or sweet potato waste is steam sterilized. The raw material picks up water from the condensing steam so that the final concentration of water in the waste is 70 - 80%. The mash is inoculated with A. niger and is spread out in trays or on the floor in a disinfected room. Air circulation is provided by fans and the temperature is kept at about 28°C . After 5 - 8 days, the mash is collected and the citric acid formed is extracted with water (Lockwood 1979).

1.2.2 Liquid Surface Process

The liquid surface process was the first one developed by Pfizer, Inc. in US for large scale production (Rohr *et al.* 1983). The fermentation is carried out in shallow pans, made of high purity aluminum or stainless steel, mounted on a rack in a disinfected room. Air is circulated over the liquid surface. Molasses is generally employed as the substrate and sterile nutrients are added prior to inoculation. The fermentation is completed within 6 - 12 days at a temperature of 30°C. The liquid is easily separated from the mycelial mat and the citric acid can thereafter be recovered. The main advantages of this fermentation process are: very little mechanical equipment is needed, ease of use, easy separation of the cells from the broth, and low energy requirements. But low oxygen transfer to cells, long fermentation times and low overall productivity, together with bulky equipment and the labour intensive nature can be listed its disadvantages. This tray system however is still useful, especially in economies where labour is relatively cheap and technology is limited.

1.2.3 The Submerged Process

This is the main process used to produce citric acid in industry (Bu'lock 1990). The medium, after sterilization and inoculation, is aerated and vigorously stirred. This method provides the culture with a high oxygen concentration in the liquid and consequently, the fermentation time is only 3 - 5 days at 25 - 30 °C. The organism used, A. niger, is same as in the surface culture

process. This process has following advantages over the other system:

high oxygen transfer rate;

shorter fermentation times and high productivity;

small equipment, less capital investment;

easier control of environmental parameters;

fairly low manual labor needs. Conversely, the process has high energy requirements and effective filtration equipment is required to separate cells.

1.3 The Aim of the Project

The aims of the work are as follows:

1: It has been shown that yields of citric acid in batch culture may be increased by the addition of nitrogen after the mycelial growth stage (Shepard 1963). It has also been reported the fermentation can be regenerated by addition of ammonia which induces new growth and excretion phases (Briffaud and Engasser 1979). It therefore seems necessary to investigate and optimize the addition time and amount of nitrogen added. Observation of the effect of citrate or sucrose additions would also be of value.

2: There is disagreement in the literature about initial amount of N source [$(NH_4)_2SO_4$] and initial pH for citric acid production. Much of the previous work has been carried out in shake flask culture with the usual associated drawbacks, thus it is often difficult to make valid comparisons. In order to examine these questions more methodically an STR will be used through this study.

3: Charley (1981) found that citric acid productivities up to 2.7 kg m^{-3} h^{-1} could be reached in a CSTR by A. niger 11414. The performance of the cells was strongly dependent on medium composition, dilution rate and pH. Stevensen (1986) has also reported that citric acid productivity was increased from 0.26 kg m^{-3} h^{-1} to 1.16 kg m^{-3} h^{-1} with A. niger CP30 by changing pH, inlet sucrose concentration and dilution rate. There was no dynamic continuous culture study. Welles and Blanch (1976) concluded that a culture of S. cerevisisae kept continuously in transient phase would produce substantially more ethanol. Their experiment was carried out over an on-off pulse cycle at different frequencies which clearly shows that pulse feeding offers an exciting possibility for increasing product yields. Productivity of citric acid may be increased via ammonia or citrate supplementation relative to steady state continuous culture.

Table 1.1: Time table of developments in citric acid fermentation (Kubicek and Rohr 1986)

1784	It was first discovered and isolated from lemon juice by Scheele.
1893	Wehmer discovers fungal citric acid accumulation in *Citromyces* (today identified as *Penicilium sp*).
1917	Systematic investigations of Currie on conditions for citric acid production
1919	First industrial plant in Belgium (Societe des Produits Organiques de Tirlemont).
1923 - 1938	Construction of further plants in England (Sturge), CSSR (Kaznejov), US. (Pfizer), USSR and Germany (Benckiser)
About 1930 and later	Bernhausers fundamental work on surface citric acid productin.
1944	First submerged process (Szusc)
About 1947 and later	fundamental work on the submerged process by Johnson
1968	First patent on citric acid production from n-alkanes.

Table 1.2 Uses of citric acid (Marison 1988)

Industry	Area	Uses
Beverage	General	Flavour enhancer
		Preservative
		Haze eliminator
		Prevents deterioration
	Wine	Prevents turbidity
		Inhibits oxidation
	Soft drinks	Cool taste
		Aids carbonation
Food	Confectionary	Flavour enhancer
	Frozen food	Anti-oxidant
	Dairy products	Colour enhancer
		pH regulator
		Inactivates trace metals
		Emulsifier
Pharmaceutical		Solvent and flavourig agent
		Effervescent with H_2CO_3
Cosmetics		Anti-oxidant and synergist
Other		Metal plating, boiler water treatment
		In detergents
		Tanning
		Textiles

CHAPTER 2

LITERATURE SURVEY

Introduction

This survey will cover only submerged fermentation processes. It contains some early literature which concentrates on fundamental aspects. There are many reports on the production of citric acid in submerged culture which show the wide variation in the conditions recommended for a succesful fermentation.

Since it has been shown that the nature and quantity of trace metals, carbon and nitrogen source and correct environmental conditions are very important for a succesful citric acid fermentation, all these factors will be discussed.

2.1 Microorganism

As discussed in section 1, nowadays most citric acid is produced using A. niger (Marison 1988). Other strains of fungi apart from A. niger have been screened for citric acid production are shown in Table 2.1 (Kapoor *et al.* 1982). The reason for choosing A. niger over other potential citrate producing organism are:

cheap raw materials (molasses) used as substrate (economical);

high and consistent yields.

Although mainly A. niger has been used in the citric acid production process, various kinds of yeast and some bacteria are known to accumulate citric acid in the medium (Table 2.2) Yeast can grow on a wide variety of substrates including glucose, acetate, hydrocarbons,

Table 2.1: Filamentous Fungi Producing Citric Acid Apart from A. niger (Kapoor *et al*. 1982).

A. awamori	Penicillium janthinellum
A. fonsecaeus	P. restrictum
A. luchensis	Acremonium sp.
A. urentii	Trichodema viride
A. saitoi	Mucor piriformis
A. usami	Ustulina vulgaris
A. fumaricus	Botrytis sp.
A. phoenicus	Ascochyta sp.
A. lanosus	Absidia sp.
A. flavus	Talaromyces sp.
	Eupenicillium sp.

Table 2.2 : Citric acid producing yeasts and bacteria (Marison 1988)

Yeast	Bacteria
Candida sp.	Bacillus licheniformis
Hansenula sp.	Bacillus subtilis
Pichia sp.	Brevibacterium sp.
Debaromyces sp.	Cornyebacterium sp.
Torulopsis sp.	
Kloeckera sp.	
Trichosporon sp.	
Rhodotorula sp.	
Endomyces sp.	
Nocardia sp.	
Saccharomyces sp.	
Zygosaccharomyces sp.	

molasses, alcohols, fatty acid and natural oils. Gledhill *et al.* (1973) stated that the hydrocarbon route to citrate offers certain advantages over the traditional carbohydrate process including lower substrate costs, higher product yield (140 to 150% ,w/w, on hydrocarbons), production rates of 1.4 $kg/m^3/h$ and final broth concentrations of up to 225 kg/m^3. Generally yeast citrate production is carried out with high aeration and agitation at 22-30 °C, pH 4.5 and fermentation period of 3-6 days. The main advantages of using yeasts, as compared with filamentous fungi, are faster growth rates, wide range of growth substrates and potential for developing continuous processes. However , the major disadvantage is that yeast generally produce considerable amounts of isocitric acid as well. Although a wide range of bacteria are able to produce citric acid, they have received little industrial interest.

2.1.1 Inoculum

Steel *et al.* (1955) recommended that between 120×10^3 and 280×10^3 pellets per litre (obtained from spore inoculated shake flasks) is a suitable inoculum level, although Kristiansen (1976) indicated that the final concentration of citric acid and dry weight is not related to inoculum size, as long as it was kept below 10^6 spores/ml culture. Charley (1981) therefore employed a less complex method for inoculum. Spores were harvested from PDA plates and incubated an orbital shaker at 30°C and 250 rpm for 20 hr.

At the end of this period the small pellets which had formed in the flasks were used as inocula for fermenter experiments, using an inoculum level of 10%.

2.1.2 Growth Form

There is a general agreement in the literature that the pelleted form is desirable for acid production. An ideal pellet configuration, pellets of 1.2 to 2.5 mm diameter after five days, was described early (Clark 1962). Gomez *et al.* (1988) showed that the pelleted form is favourable due to pellet cultures have low culture viscosity causing improving bulk mixing and aeration conditions and lower oxygen consumption than in the cultures composed mainly of filamentous (dispersed) forms. Furthermore, problems of wall growth and pipe blockage are reduced and separation of biomass from culture liquid by filtration is considerably enhanced by the pelleted growth form.

2.1.3 Strain Improvment

Strain improvment by mutation in order to achieve higher yields and higher trace metal tolerance is a continual aim of industrial producers. Its importance can be illustrated by the 500 fold increase in penicillin production from Penicillium chrysogenum due to mutation (Kelly 1988). But surprisingly little work has been published on increasing citric acid production by strain improvement. Probably production companies want to keep secret their succesful methods. McKay *et al.* (1990) increased the production of

citric acid yield from glucose by Yarrowia lipolytica IFO 1658 two fold, and by Candida guillermondii NRRL Y-448 from galactose, six fold via ultra-violet mutagenesis and subsequent selection. James *et al.* (1956) produced a mutant via multiple X-ray and UV irradiation of spores, and mutant strains showing a six fold increase in citric acid yield compared to the parent strain. The success of a strain improvment programe is dependent on rapid and accurate screening. The method of Das and Roy (1981) is a rapid method for correlating the acid producing ability of certain colonies. After a culture has been subjected to the mutation procedure, the surviving cells are plated out on complex medium on an agar plate with an acid indicator added. This indicator changes colour when in contact with acid. A cell will grow to a colony with a certain diameter ,D_1. Surrounding the colony will be a circle of indicator, detecting acid released from the cells. The diameter of the colored circle is D_2. The ratio D_2/D_1 is a preliminary measurement of the acid producing ability of the particular colony.

2.2 Biochemistry of Citric Acid Over Production

The biochemical pathways related to citric acid accumulation and the role of the tricarboxylic acid cycle (TCA) in fungi has been well established (Figure 2.1) (Marison 1988). Citric acid accumulation can be divided into three processes (Kubicek and Rohr 1986):

Fig. 2.1 The Citric Acid cycle

PK :Pyruvate kinase

PC :Pyruvate carboxylase

PEP-CK :Phospho enol pyruvate carboxy kinase

PDH :Pyruvate dehydrogenase

ME :Malic enzyme

CS :Citrate synthase

AC :Aconitase

IDH :Isocitrate dehydrogenase

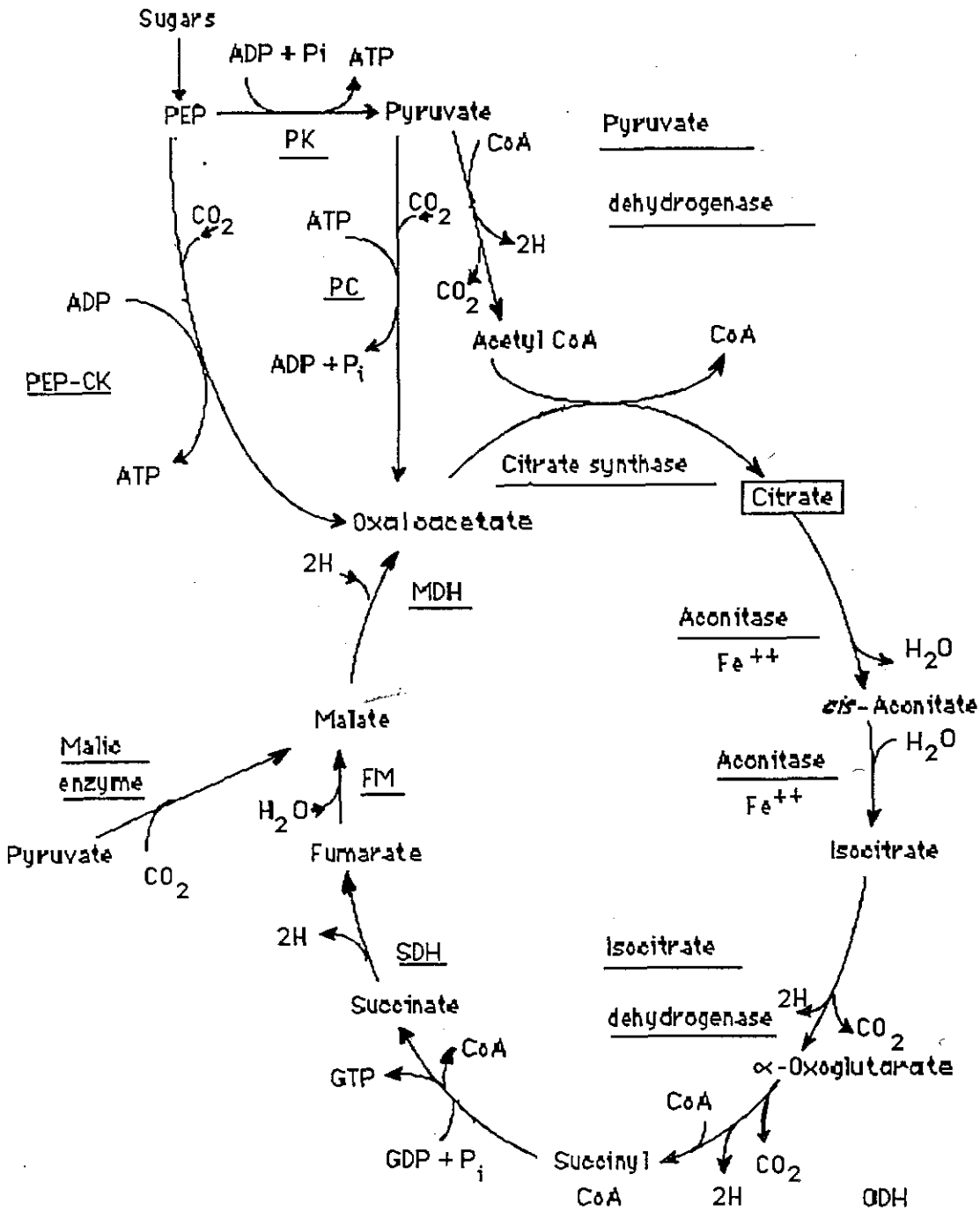
KDH :α-ketoglutarate dehydrogenase

STK :Succinate thiokinase

SDH :Succinate dehydrogenase

FM :Fumarase

MDH :Malate dehydrogenase


Sugars
ADP + Pi
ATP
PEP
Pyruvate
PK
Pyruvate
dehydrogenase
CoA
2H
CO_2
ATP
PC
ADP
ADP + P_i
PEP-CK
ATP
Acetyl CoA
CoA
Citrate synthase
Citrate
Oxaloacetate
2H
MDH
Aconitase
Fe^{++}
H_2O
cis-Aconitate
Malate
Malic enzyme
FM
H_2O
Pyruvate
Fumarate
Isocitrate
2H
SDH
Isocitrate dehydrogenase
Succinate
GTP
CoA
∝-Oxoglutarate
GDP + P_i
Succinyl CoA
2H
ODH

(1) the breakdown of hexoses to pyruvate and acetyl-CoA by glycolysis;

(2) formation of oxaloacetate;

(3) condensation of acetyl-CoA and oxaloacetate to citric acid.

As citric acid synthesis involves the condensation of an acetyl unit with oxaloacetate, it is quite important to generate sufficient oxaloacetate in order for production to continue. Regeneration of oxaloacetate involves four mechanisms (Kapoor *et al.* 1982):

(1) the direct carboxylation of pyruvate catalysed by malic enzyme provides malate which is readily oxidized into oxaloacetate through malic dehydrogenase;

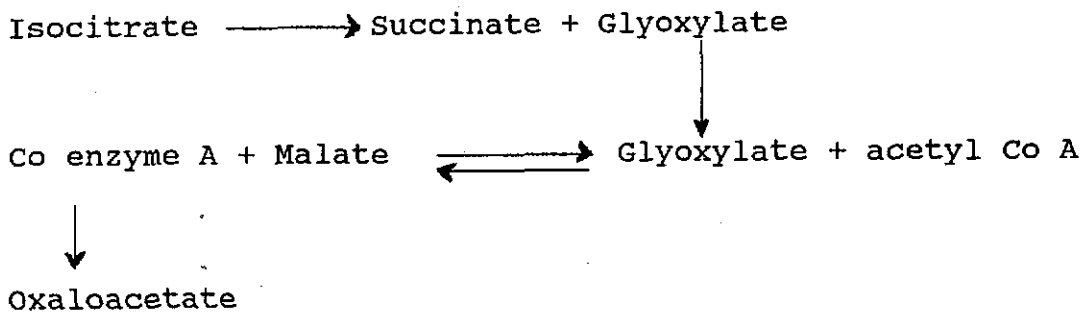
(2) the carboxylation of pyruvate catalysed by pyruvate carboxylase;

$$\text{Pyruvate} + CO_2 + H_2O + \text{ATP} \xrightleftharpoons{Mg^+} \text{Oxaloacetate} + \text{ADP} + \text{Pi}$$

(3) the carboxylation of phospho-enol pyruvate (PEP) catalysed by PEP carboxykinase;

$$\text{PEP} + \text{ADP} + CO_2 \xrightleftharpoons{} \text{Oxaloacetate} + \text{ATP}$$

(4) via the glyoxylate cycle involving the key enzymes isocitrate lyase and malate synthase;



Pyruvate carboxylase is an important enzyme for citric acid production. It is poorly regulated, only weakly inhibited by 2- Oxoglutarate and not influenced by acetyl-CoA (Feir and Suzuki 1969).

Phosphofructokinase was the regulatory enzyme of citric acid production in A. niger (Habison *et al.* 1979). The enzyme was inhibited by high concentrations of citrate and ATP but activated by ADP, AMP, inorganic phosphate and ammonium ions. During citric acid production ammonium ions overcome the inhibition of PFK by citrate and ATP.

Aconitase and isocitrate dehydrogenase are very important key enzymes in citric acid fermentation. The activity of these enzymes decrease to very low levels during the production stage which causes faulty operation of the cycle whilst the activity of citrate synthase increases (Marison 1988).

2.3 Factors Affecting The Fermentation Process

2.3.1 Medium Constituents

2.3.1.1 Trace Elements

Trace element nutrition is one of the most important factors affecting the yields (grams citric acid per gram

sugar) of citric acid fermentation. In particular, the levels of manganese, iron, copper and zinc are quite critical. If the levels of these trace elements are correct other factors have less pronounced effects. Conversely, medium will not allow high production unless the trace element content is controlled carefully.

Manganese (Mn^{2+} ions) in the nutrient medium plays a key role in the accumulation of large amounts of citrate by A. niger. When the Mn^{2+} concentration is maintained below 0.02 mM (which does not affect growth rate or biomass yield) large amounts of citric acid are produced (Rohr *et al.* 1983). Clark *et al.* (1966) observed 10% and 25% reduction in citric acid yield on adding 2 ppb and 100 ppb manganese to beet molasses respectively. Bowes and Mattey (1979) also noted that by the addition of 10 ppm Mn^{2+} in the growth medium, citrate accumulation is effectively halved by A. niger. Orthofer *et al.*(1979) found that manganese deficiency leads to significantly lower lipid content in A. niger whereas there were elevated lipid levels in manganese sufficient cultures. Jernejc *et al* (1982) stated that low mycelial lipid levels result in high yields of citric acid. Protein synthesis is also said to be inhibited by manganese deficiency (Kubicek *et al*, 1979).

Tomlinson *et al.* (1950) found that up to 1 mg iron per litre medium is essential for high yields of citric acid by A. niger, but that amounts in excess of this interferes with citric acid accumulation. Partial deficiency of iron

has also been reported to be necessary for citric acid production by several workers (Snell and Schweiger 1949; Chesters and Roninson 1951; Schweiger 1961). The presence of excess iron favours the production of oxalic acid (Lockwood 1974).

Copper ions play an important role in reducing the deleterious effect of iron on citric acid production (Tomlinson *et al.* 1950; Lockwood 1979). It has also been reported that copper ions can successfully counteract addition of manganese to citric acid fermentation media and are inhibitors of cellular manganese uptake (Kubicek and Rohr 1986). Jernejc *et al.* (1982) found that copper is an essential requirement for citric acid production and optimum concentration of Cu^{2+} is 40 ppm for high yield.

Low concentrations of zinc in the fermentation medium are generally favoured in most citric acid production media (Tomlinson *et al.* 1950). Chesters and Rolinson (1951) reported that zinc deficiencies promote citric acid production. According to Wold and Suzuki (1976), zinc plays a role in the regulation of growth and citrate accumulation. At high zinc levels (about 2 μM) the cultures are maintained in growth phase, but when the medium becomes zinc deficient (below 0.2 μM) growth is terminated and citric acid accumulation begins. Addition of zinc to citrate accumulating cultures results in their reversion to growth phase.

Since molasses (beet or cane) contains inhibitory amounts

of metal ions like zinc, iron, copper, it is absolutely necessary either to remove these ions or to render them ineffective by pretreatment. The most commonly used methods of pretreatment are the addition of ferro or ferricyanide to precipitate iron, zinc, copper and manganese (Gerhardt *et al.* 1946), decreasing the available manganese content to below 0.002 ppm (Horitsu and Clark 1966) or passing the medium over ion exchange resins (Lookwood 1974). Horitsu and Clark (1966) have carried out a detailed study of the effect of ferrocyanide on citric acid production in beet molasses and found that concentrations above 30 ppm stimulated citric acid formation in resting cells, but markedly inhibited the development of growing cells. They found no evidence that ferrocyanide favors citric acid accumulation by blocking a reaction in the Krebs cycle but stated that the beneficial effects are because of inactivation of inhibiting metals. Quader *et al.* (1966) found that addition of any chelating agent stimulated citric acid production. They obtained greatest stimulation with 0.1 mM EDTA (about 10 fold) and with 84 μM ferrocyanide (about 20 fold). They also found that ferrocyanide did not affect total uptake of zinc and copper by the mould and EDTA did not affect the iron, copper and zinc contents of the mycelium.

2.3.1.2 Sugars

Due to their rapid assimilation by the fungus the usual carbon sources are glucose, fructose, or sucrose for high

final yield of citric acid. For strain A. niger B60, maltose and sucrose, two disaccharides, were found to be better carbon sources for production of citric acid than the monosaccharides glucose and fructose (Xu *et al.* 1989). In most cases, sucrose or its cheaper commercial source molasses is used. Several sources of crude carbohydrates have been used for citric acid production, e.g., date syrup, cotton waste, whey permeate, brewery waste, beet and cane molasses, unrefined sucrose, cane juice (Kubicek and Rohr 1986) and grape pomace as a novel substrate for production of citric acid (Hang and Woodams 1985). Not only the type but also the concentration of the carbon source is important in the citric acid fermentation. Shu and Johnson (1948) showed that a maximal citric acid production rate is obtained at 14 to 22% of sugar in the medium. The results of Anderson *et al.* (1980) are in good agreement with those of Shu and Johnson. They found that 2, 6 and 10% sugar solutions give poor yields whilst getting high yield with 14, 18 and 20% sugar solutions. They obtained the greatest yield with 14%. Xu *et al.* (1989) also indicated that the optimal initial concentration of sucrose was around 10-14%.

2.3.1.3 Nitrogen Source

Usually ammonium sulfate or ammonium nitrate has been used as a nitrogen source. Physiologically, acid ammonium compounds are preferred since their consumption lowers the pH of the medium to below 2 which is an additional

prerequisite of citric acid fermentation. Xu *et al.* (1989) investigated the effect of different nitrogen sources on citric acid production by A. niger and produced 32 kg per m^3 citric acid using urea. They also noted that the optimal concentration of ammonium sulfate was 5 kg m^{-3}.

2.3.1.4 Phosphate

Shu and Johnson (1948) reported that the effect of phosphate is not very pronounced but the balance between manganese, zinc and phosphate was critical. In any cases of trace metal contamination, phosphate limitation can have a beneficial effect on citric acid yield. Requirement of phosphate for fungal growth is 0.1 to 0.2% (Kapoor *et al.* 1982). However the presence of copper in the medium could reduce the optimum phosphate concentration (Jernejc *et al.*1982). Martin and Steel (1955) found that phosphate plays a key role in secondary metabolite production. When they added 0.005% phosphate to beet molasses, it was found that 5-ketogluconic and gluconic acid replace oxalic acid as secondary products.In addition fermentation time was significantly reduced.

2.3.1.5 Magnesium

Magnesium is essential for growth and citric acid production due to its role as a cofactor in a number of enzyme reactions in the cell. It has been reported that the optimum concentration of magnesium sulphate to produce

maximum citric acid varies from 0.02 to 0.025% (Kapoor *et al.* 1982).

2.3.2 Environmental Conditions

Aeration and agitation, pH, incubation temperature and fermentation period, and their effects on citric acid production will be discussed.

2.3.2.1 Aeration

Aeration has been shown to have a critical effect on the submerged citric acid production process. Khan and Ghose (1973) suggested that aeration should be 0.6 vvm (liter air per liter medium per minute). The citric acid concentration was however raised from 30.3 to 48.7 kgm^{-3} by increasing air flow rate from 0.9 to 1.3 vvm. (Gomez *et al.* 1988). Critical dissolved oxygen tension (DOT) values are 9-10% of air saturation and 12-13% of air saturation for growth and production phases, respectively (Dawson *et al.* 1986). Clark and Lentz (1961) found that citric acid production was related to oxygen pressure. The yield of citric acid increased by increasing the flow rate of air and the oxygen pressure up to 1.7 atmospheres using pure oxygen for pressures of 1 atm and greater, beyond which citric acid production would decrease. Batti (1966) reported that interruption in aeration for one hour after 89 hours resulted in a 50% reduction in citric acid yield. The deleterious effect of interruption of aeration can be prevented by raising the pH to 3 until the production of

citric acid commences again. Dawson *et al.* (1986) observed that 20 min interruption in aeration,at fermentation time 1 and 3 days, had slightly inhibitory effect on acid production until fourty day. This effect however was quickly overcome since DOT never fell below 20% of saturation, which is well above the critical value of 12-13% and had no effect on the final citrate production and mycelial growth. When the air supply to the fermentation was interrupted for 120 minutes on days 1 and 3, DOT value decreased to zero, mycelial growth and citric acid production, but not sugar consumption, ceased. However the effect was not permanent and both the growth and acid production rates eventually recovered.

2.3.2.2 Agitation

Clark and Lentz (1963) found that agitation in stirred tank fermenters was critical. Increasing agitator speed would break up pellets, leading to dispersion of more than 95% of pellets, resulting in higher yields of citric acid. Maximum yield was obtained at agitator speeds between 400 and 700 rpm. Khan and Ghose (1973) also reported that 500 rpm was the optimal agitation speed for citric acid production. Gomez *et al.* (1988) found that the length of the fermentation for any given agitator speed was important. They produced 48 kg m^{-3} citric acid when carrying out fermentation at 1000 rpm, but the concentration of citric acid was only 30.3 kg m^{-3}, applying agitation speed 450 rpm for the first 48 h, and

1000 rpm for the rest of the fermentation period.

2.3.2.3 pH

There is no agreement in the literature about optimal initial pH. Moyer (1953) found that pH did not have any effect within the range 1.95 to 3.10. Khan and Ghose (1973) however reported that citric acid yield increased with increasing pH. The optimal initial pH was found to be 6.5. On the other hand, optimal initial pH was been reported to be 2.8 and 2.5 by Kamal *et al.* (1982), Jernejc *et al.* (1982) respectively. However in the last two studies the work was carried out in shake flask cultures with the usual associated drawbacks, thus it is often difficult to make valid comparisons. Marison (1988) reported that the pH of the initial medium should lie between 1.4 and 3.0 with A. niger growing on molasses in order to reduce contamination and suppress oxalic acid formation. Charley (1981) reported that the optimal final pH for batch fermentation was 1.7. This was supported by the findings of Kubicek and Rohr (1986) which recomended that pH should be kept low (below 2.0). According to that report at higher pH's, A. niger accumulates gluconic acid, especially when the pH is around 4.0 (1986). Marison (1988) also reported that pH should be maintained around 2.0.

2.3.2.4 Incubation temperature

According to Steel *et al.* (1955) the incubation temperature should be in the range 28 to 32°C, while Gerhardt *et al.* (1946) found 30°C was the optimum for citric acid production. Khan and Ghose (1973) also reported that 30°C was the optimum for citric acid production.

2.3.2.5 Duration of Fermentation

It has been reported that the citric acid fermentation is completed in 8 days. Extension of the fermentation period did not increase the yields of citric acid. (Palo *et al.* 1984). Kamal *et al.* (1982) also obtained maximum yield with incubation period of about 6-9 days.

2.3.3 Other Factors

Various compounds, some of them listed in Table 2.3 (Kubicek and Rohr 1986; Marison 1988), stimulate citric acid production by A. niger. The most important acid promoters which will be discussed are, alcohols, lipids, cyclic-AMP, vitamins and amino acids. The effect of the addition of toxic chemicals such as phenol will also be considered.

2.3.3.1 Alcohols

Moyer (1953) found that addition of lower alcohols, methanol, ethanol, n-propanol, to crude carbohydrate raw materials could increase the yield of citric acid. Optimal concentration of methanol, which was said to be more

effective than ethanol, varied from 1 to 4% by volume. However addition of methanol to highly purified, high yielding substrates is deleterious to acid yields. Hang and Woodams (1985) observed that the presence of methanol at a concentration of 3% in grape pomace led to an increase in the citrate yield by a factor of around 3. The exact mechanism of the alcohol effect however is unexplained, though it is postulated (Marison 1988) that addition of methanol increases the tolerance of the fungi to Fe^{2+}, Zn^{2+} and Mn^{2+}.

2.3.3.2 Lipids

Millis *et al.* (1963) found that addition of natural oils with a high content of unsaturated fatty acids and oleic acid at 2% (v/v) to fermentation media led to increases in the yield by 20% without affecting dry weight of mycelium. They suggested that unsaturated fatty acids act as an alternative hydrogen acceptor to oxygen during the fermentation, since only high levels of unsaturated fatty acids were effective in improving the yield of citric acid. Gold and Kieber (1968) found that a concentration of fatty acid of 0.05 to 0.3% had to be maintained during the fermentation.

Table 2.3: Compounds which increase citric acid production by A. niger (Kubicek and Rohr 1986; Marison 1988)

4-Methyl-umbelliferone
3-Hydroxy-2-naphtoic acid
Benzoic acid
2-naphtoic acid
Iron cyanide
Quaternary ammonium compounds
Amine oximes
EDTA
1,2-Diaminocyclohexane N,N'-tetraacetate
Diethylentriaminepentaacetate
Surface active agents
H_2O_2
Vermiculite
NaF
KF

riboflavin (4×10^{-5}M) stimulated the citric acid formation to the extent of 59.06% and 50.36% respectively. Biotin (3 10^{-5}M) produced the greatest enhancement stimulating growth and increasing the production of citric acid by 66.4%.

2.3.3.5 Amino Acids

Lal and Srivastava (1982) observed the effect of different concentrations (range 1.0×10^{-3} to 5.0×10^{-3} M) of some amino acids on growth and citric acid production in sugar medium by A. niger AL 29. According to them the presence of glutamic acid (4×10^{-3} M)and aspartic acid (3×10^{-3} M) stimulated citric acid production by 79 and 76.7% respectively. Presence of lysine (5×10^{-3} M) and serine (4×10^{-3} M) also could influence the formation of citric acid by 62.3 and 50.4% respectively. The effect of cysteine (in all concentrations) was found to be detrimental.

2.3.3.6 Toxic Chemicals

Qureshie and Qadeer have studied (1987) the effect of addition of "toxic" chemicals (range 0 to 60 ppm) such as phenol, resorcinol, hydroquinone, o-cresol, α-naphthol and β-naphthol on spore germination, mycelial dry weight and citric acid production in a based glucose medium using A. niger EU-1. There was slight increase in citric acid formation in the presence of phenol (20 ppm) and β-naphtol (20 ppm). But hydroquinone (with 30 ppm) and o-cresol

2.3.3.3 Cyclic-adenosine mono phosphate (c-AMP)

A. niger shows a higher productivity when the cyclic adenosine mono phosphate (c-AMP) concentration in the medium is greater than 10^{-6} M. ATP, GTP and adenosine also stimulate production above 10^{-4}M. Conversely AMP, GMP and guanosine either have no effect or very slightly inhibit productivity (Marison 1988). Bu'lock (1974) suggested that c-AMP promoted the complex switching of carbon utilisation which leads to citric acid accumulation. However, Wold and Suzuki (1976) showed that a relation between c-AMP and levels of Zn^{2+}. c-AMP affected the rates of growth and acidogenesis when added to cultures growing at low levels of Zn^{2+} but had no effect at high levels of Zn^{2+}. So c-AMP did not induce the phase transition but zinc deficiency did. c-AMP enhanced growth during the growth phase, but inhibited growth and augmented citrate synthesis during the production phase.

2.3.3.4 Vitamins

Lal and Srivastava (1982) reported the effect of different concentrations (range 1.0×10^{-5} to 5.0×10^{-5}M) of some vitamins on growth and citric acid production in sugar medium by A. niger AL 29. They observed that ascorbic acid and p-amino benzoic acid (PABA), at all concentrations, inhibited both growth and citric acid. Conversely, the presence of thiamine (3×10^{-5}M) and

(with 15 ppm) exhibited maximum citric acid stimulation i.e 85 and 80 kg m^{-3} respectively. Acid formation in the presence of resorcinol (with 50 ppm) was 78 kg m^{-3}. They concluded that the increase in citric acid production may be due to either the direct effect of these phenols on the growth process i.e., metabolism of A. niger, or to the inhibition of enzymes involved in further metabolism of citric acid.

2.4 Product Recovery

The recovery of citric acid is summarized and illustrated in Fig 2.2. This is the method most commonly used on a large scale. The method is based on calcium citrate precipitation. A novel method for citrate extraction has also recently been developed (Marison 1988). In this method citric acid extracted by a 2-phase extraction using tridecylamine or tri-isononylamine and a water insoluble ester, ketone or alcohol. Citric acid salts are then separated from mixed solvent extracts with NH_4OH, carbonates or bicarbonates.

2.5 Kinetics of Growth and Product Formation

It has been suggested (Pirt and Callow 1959) that fungi grow in the filamentous form at an exponential rate with a constant specific growth rate (μ) until some substrate becomes growth limiting, according to the Monod equation:

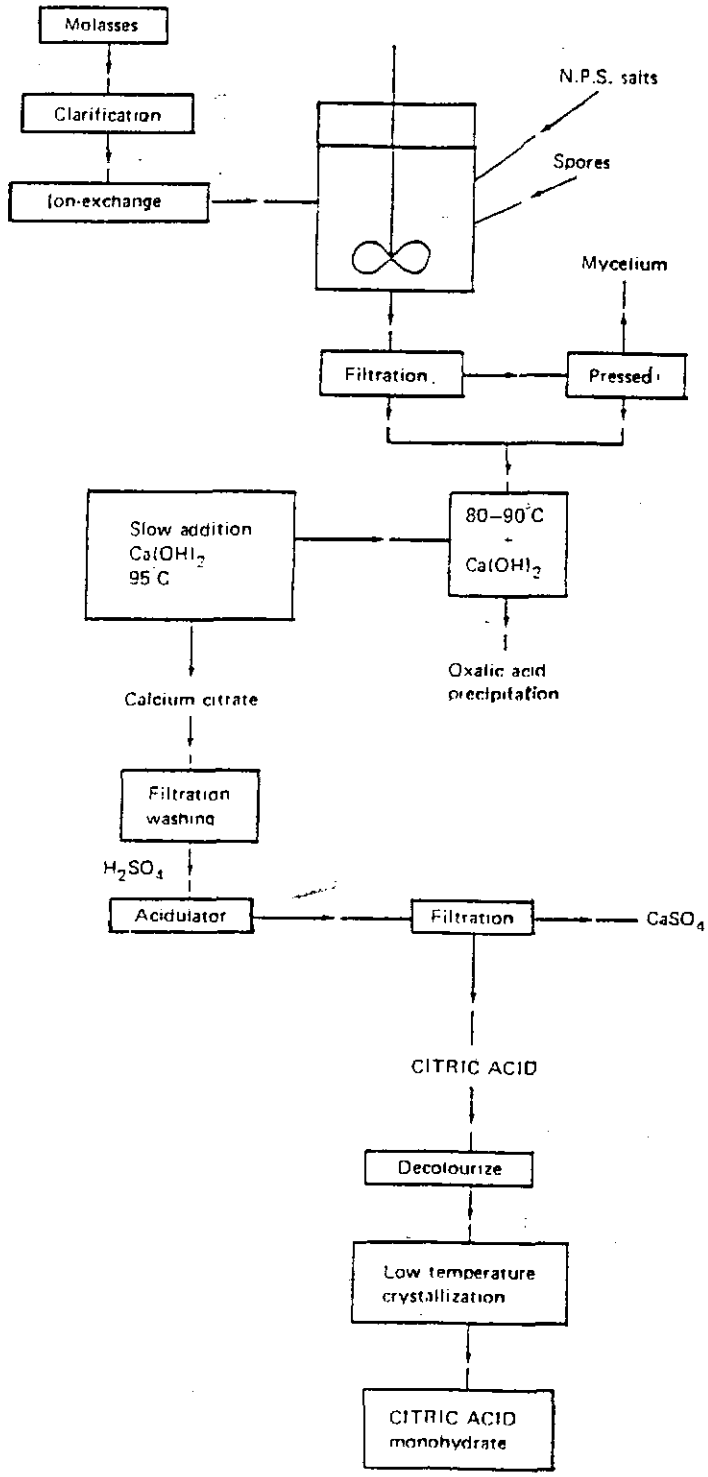


Fig. 2.2 Product Recovery

$$ln\ x = ln\ x_o + \mu\ t$$

$$\mu = \frac{\mu_{max}\ (S)}{K_s + S}$$

Where

μ_{max} : The maximum specific growth rate of the organism (in h^{-1})

K_s : Saturation constant (kg m^{-3})

S : The concentration of the limiting substrate (in kg m^{-3})

Emerson (1950), however, proposed that the filamentous mode of growth could best be described by the assumption of cube root kinetics;

$$X^{1/3} = X_o^{1/3} + Kt$$

where; X_o is cell present at t=0, K constant.

Yano *et al.* (1961) suggested that the cube root model was applicable to pellet growth due to the diffusion of oxygen into the pellet being the growth limiting step. Marshall and Alexander (1960) obtained a linear relationship between the cube root of oxygen consumption and time for a number of fungi, including A. niger. Pirt (1966) suggested that cube root kinetics was valid for growth of fungi in pellets, whilst exponential growth could be associated with the fungus growing in the filaments. Trinci (1970) found that A. nidulans grew exponentially until the pellets reached a certain thickness, after which the cube

root model was followed. Borrow *et al.* (1964) obtained a similar result for the growth of Giberella fujikuroi under nitrogen limitation, growth changed from exponential to linear and then cube root.

Kono and Asai (1969) found that different models could be modelled to describe the growth kinetics an organism of different stages in the batch fermentation. The general equation for growth is

$$dx / dt = K \Phi X$$

where

K is a constant

X is cell concentration (kg m^{-3})

Φ represents an apparent coefficient for growth and it varies according to the phase of the fermentation as follows

Induction phase $\Phi = 0$

Transient phase $\Phi = \phi$

Exponential phase $\Phi = 1$

Declining phase $$\Phi = \frac{X_c}{X_m - X_c} \frac{X_m - X}{X}$$

where, X is the cell concentration and X_m is the maximum theoretical cell concentration at the beginning of the declining phase. The value for ϕ varies from zero to unity. In the declining phase Φ drops from unity to zero.

This model has been applied to the growth of A. niger during the citric acid fermentation (Khan and Ghose 1973). During the decline phase, endogenous respiration may become important so it should be taken part into the proposed model. Chiv *et al.* (1972) proposed that the rate of decay could be modelled by a first order reaction, so cell growth became

$$dx / dt = \mu X - K_a X$$

where μ = specific growth rate and K_a = constant. A similar model was proposed by Sinclair and Topiwala (1970) which considered cell viability;

$$dx / dt = \mu X - \alpha X$$

where α = cell death constant.

Kristiansen and Sinclair (1979) assumed the existence of three cell types namely basic, storage and deactivated cells on citric acid production system. When nitrogen was present, the basic cells grew normally and no citric acid was produced. Storage cells, were produced by the transformation of basic cells under nitrogen limitation. In this phase the dry weight rose due to carbon storage. Deactivated cells were produced by storage cells losing all mitochondrial enzymes and no citric acid was produced. The equations describing the concentration of three cells types in a single stage, ideally mixed,

continuously-stirred reactor are

$$dX_b / dt = \mu_b X_b - k_t X_b - D X_b$$
$$dX_c / dt = \mu_c X_c + k_t X_b - k_d X_c - D X_c$$
$$dX_d / dt = k_d X_c - D X_d$$

where, X_b, X_c, and X_d are the concentrations of basic, storage and deactivated cells respectively; μ_b and μ_c are the specific growth rates of basic and storage cells respectively; k_t is a transformation constant; k_d is a deactivation constant; and D is dilution rate.

Product formation in fermentations was classified by Maxon (1955) and Gaden (1955) independently. Maxon related the product formation to cell growth, whereas Gaden related it to substrate utilization. The product were divided into three broad classes by Maxon (1955)

1-Growth associated products

2-Non-growth associated products

3-Intermediary products

Luedeking and Piret (1959), studying the production of lactic acid by Lactobacillus delbrueckii, proposed a model which was able to describe all of the above three types of product formation. The instantaneous rate of product formation, dP / dt, was related to the instantaneous growth rate of the organism, dX / dt, and cell concentration. The following expression was used to describe the model ;

$$dP / dt = \alpha\, dX / dt + \beta X$$

where; P = product concentration (kg m^{-3}), X = biomass concentration (kg m^{-3}); α and β are constant, the values of which determine the type of product formation

if $\alpha = 0$ then the product is nongrowth associated,

if $\beta = 0$ then the product is growth associated,

if both of them are positive, then the product is intermediary.

In some fermentations, usually when carbon becomes limiting, the organism will break down its product and use it as substrate. So Constantinides *et al.*(1970) modified the Leudeking-Piret model to account for this:

$$dP / dt = \alpha\, dX / dt + \beta X - \varphi P$$

where φ is a constant.

Kono and Asai (1969) suggested the following model for the product formation:

$$dP / dt = K_1 \Phi X + K_2 (1-\Phi) X$$

which was combined with the growth equation described earlier. By defining growing and non growing cells as:

$\Phi X \propto X$ growth

$(1-\Phi) X \propto X$ non growth

The general equation can be rewritten as:

$$dP / dt = K_1 X \text{ growth} + K_2 X \text{ non growth}$$

Khan and Ghose (1973) used the above model and found that their experimental values agreed with the theoretical model.

Kristiansen and Sinclair (1979) assumed that citric acid formation could be modelled according to the Ludeking and Piret model in single stage continuous proses:

$$dP / dt = r_p - D P$$

where

$r_p = \alpha \mu_c X_c + \beta X_c$

r_p = rate of formation of product

P = product concentration (kg m^{-3})

X_c = concentration of carbon stroge cells (kg m^{-3}), and D is dilution rate. (h^{-1})

2.6 Periodic Reactor Operation

Different types of fermenter systems have been used for production of citric acid, some of these are illustrated in Table 2.4 together with their productivity.

Table 2.4 Comparison of Various Citric Acid Process

Fermenter system	productivity kg m^{-3} h^{-1}	references	
Batch submerged fer.	0.70	Batti	1966
"	0.70	Ogawa and Fazeli	1976
Semi-continuous submerged fermentation	1.13	Lesniak and Stawicki (As Charley 1981)	1979
Continuous culture (STR)	0.43	Kristiansen and Sinlair	1979
"	2.70	Charley	1981
"	1.16	Stevensen	1985
Disk Fermenter (Medium replacement)	0.22	Anderson *et al.*	1980
Airlift fermenter (Batch with Ca alginate immobilization)	0.07	Eikmeler and Rehm	1984
Tower fermenter with two stage (continuous with polyacrylamide immobil.)	0.16	Horitsu *et al.*	1985
Repeated batch (Shake-flask)	0.46	Tsay and To	1987
Dual Hollow Fiber Bioreactor (with pure oxygen)	1.13	Chung and Chang	1987

In some respects continuous culture is superior to the other systems since it always gives higher productivity than other systems.

Although there is no work directly concerning citric acid production, a significant amount of work has been carried out to observe the behaviour of continuous cultures when subjected to cyclic operation. The major references published after 1976 will be discussed here.

Pickett *et al.* (1979) found that the growth yield and macromoleculer composition of cells vary with the frequency of nutrient cycling by studying Esherichia coli M130 grown in chemostat culture. The yield decreased by some 10% as the cycle times decreased from 6.0 to 0.25 h. The DNA and protein concentration have maxima at 2 h cycle time, while the RNA concentration is maximal at a cycle time of 1 h.

Pickett *et al.* (1980) have also examined the effect of regular variation in the input limiting nutrient concentration at different cycle amplitudes on the growth and macromolecular composition of cells of the organism mentioned above. The cycle time was kept constant at 2 h but the concentration amplitude was varied over a range of 0.2 to 5.0 kg m^{-3}. Increasing the amplitude did not effect the cell yield on glucose or the elemental composition of the organisms, but they found a large variation in the macromolecular composition of the cells.

DNA, RNA and protein contents increase 25, 100 and 41% respectively at 4 kg m^{-3} cycle amplitude. In both of above mentioned studies, square waveform were applied.

Sundstrom *et al* (1976) investigated sinusoidal variations of substrate concentrations using E. coli cultures. They observed a maximum deviation of measured substrate concentration from initial steady state concentrations near a frequency of 1 cycle per residence time.

Borzani *et al.* (1976) studied the influence of periodic variations of the feed concentration of a continuous anaerobic culture of S. cerevisiae, applying both square and sinusoidal waveform. They observed that there is no significant difference between the average yield coefficients during the steady state and transient state experiments. The average specific growth rate during the transient state was also equal to the dilution rate.

Vairo *et al.* (1977) confirmed the above conclusions that average yield coefficients during the transient state was equal to the yield coefficients obtained during the steady state experiment and the average specific growth rate during the transient state was equal to the dilution rate when the system was disturbed by oscillation of dilution rate.

Welles and Blanch (1976) concluded that a culture of S. cerevisiae kept continuously in transient phase would produce substantially more ethanol. Their experiment was carried out over an on-off pulse cycle at different frequencies which clearly shows that pulse feeding offers

an exciting possibility for increasing product yields.

Katterer *et al.* (1986) investigated transient responses of continuously growing yeast culture to step changes in dilution rate. The response was affected by the preshift conditions of the culture, the magnitude of the dilution rate increase, the organism and bioreactor type.

Abulesz and Lyberatos (1986) stated that steady state operation of continuous cultures is not necessarily the optimum type of operation. They enhanced productivity of biomass when a bioreactor was operated with a periodically varying dilution rate. They observed a time lag in the culture adaptation to new growth level whenever culture was disturbed.

Stephens and Lyberatos (1986) concluded that it was possible to modify the final fate of a mixed culture by cycling in feed substrate concentration and dilution rate which both produce the desired result.

Abulesz and Lyberatos (1988) found that Monod model is unable to predict even qualitatively the dynamic response of the culture when continuous culture of S. cerevisiae is subjected to step changes in dilution rate but incorporation of a time delay allows significant improvment in the transient fit. They found that culture had a time lag of about 3 h in adapting its growth rate, and cycling the dilution rate with a period of 3 h significantly increased the average biomass productivity.

Neil and Lyberatos (1990) have also reported that changing the environment by means of a step change in a

chemostat dilution rate or inlet substrate concentration, gives beter result than steady state operation with *S. cerevisiae.*

CHAPTER 3

MATERIALS

3.1 Introduction

Equipment used during the course of the project is listed below:

Two fermentation vessels, both stirred tanks of smilar design and dimensions, SK1 and SK3 (S.K. Fermenters, 121 Princess street Manchester, M1 7AG). Both were suitable for continuous culture of filamentous organisms

Autoclave (15 - 17 Roebuck road Hainauld Ilford Essex)

UV spectrophotometer (UV - 160, Shimadzu)

Incubator (Gallenkamp Duostat)

Orbital incubator (Gallenkamp)

Microscope (Gillett & Sibert ltd.)

Microwave (Hitachi, compact 500)

Balance (Mettler AE 100)

Only SK fermenter will be introduced here.

3.2 SK Fermenters

The SK fermenter consisted of a 10 litre glass vessel with stainless steel end plates. Temperature, pH, air flow rate, agitator speed were controlled in the fermenter. It also includes level control system for continuous culture. The fermenter containing organism is shown in Figure 3.1. General layout and instrumentation diagrams are also represented in Figures 3.2 and 3.3 respectively.

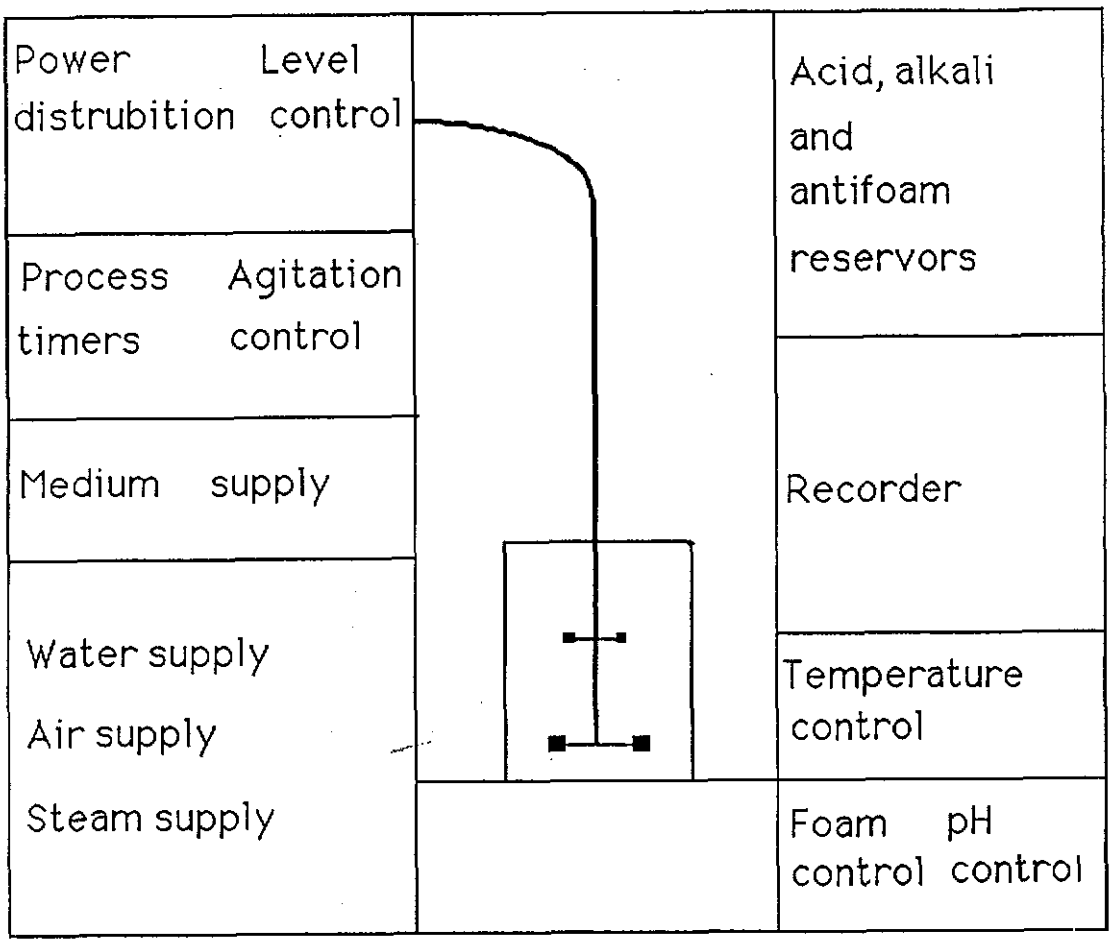


Fig. 3.2 S.K Fermenter - general layout.

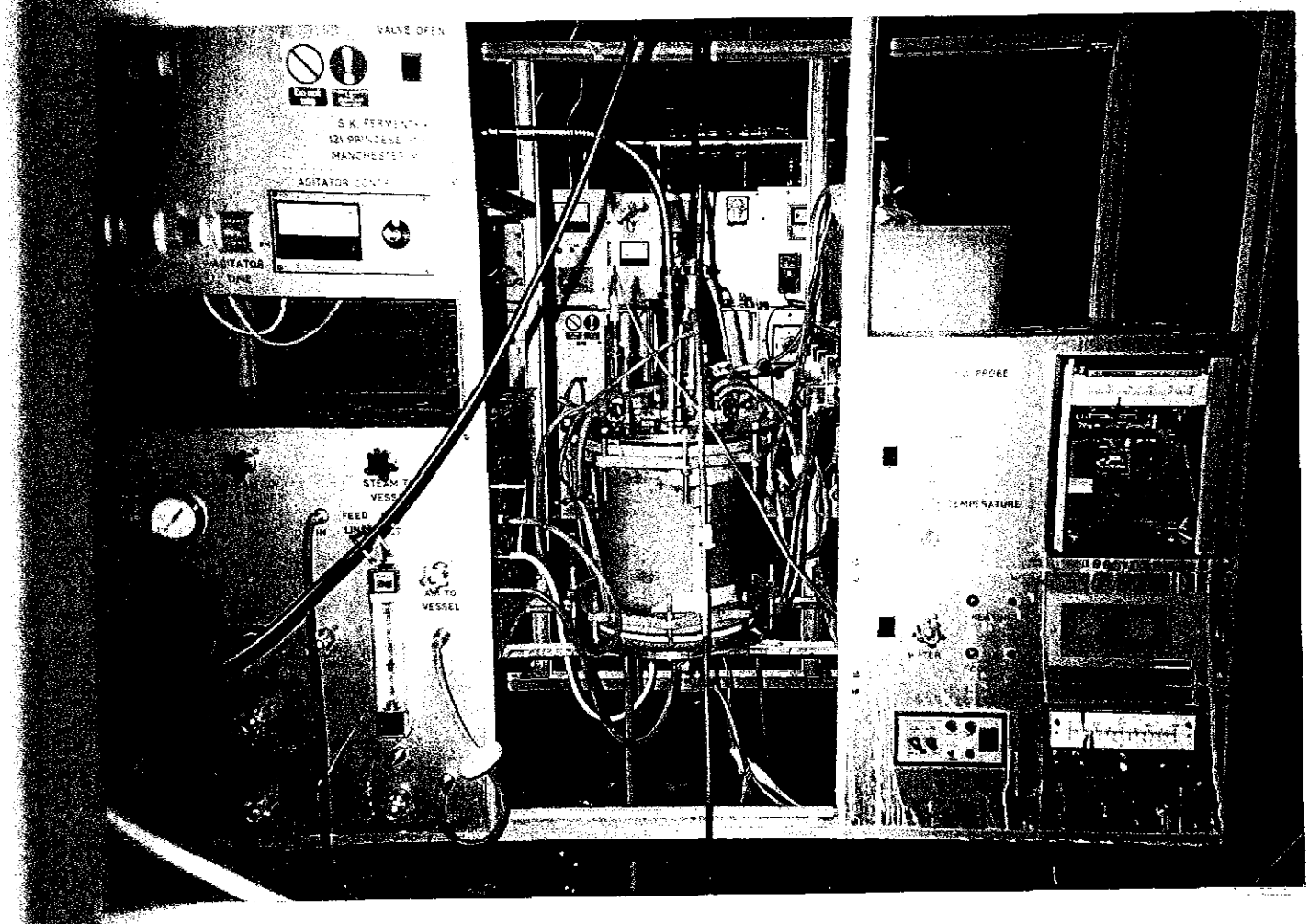


Fig 3.1 SK Fermenter

LEGEND

Pressure indicating controller

Pressure indicator

Flow controller

Level controller

Temperature indicating controller

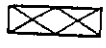
Motor controller

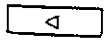
Dissolved oxygen indicator

pH indicating controller

Foam controller


Peristaltic pump


Air Filter


Variable area flowmeter


Bacterial filter


Hoffman clip


Valve


Control valve


Immersion heater

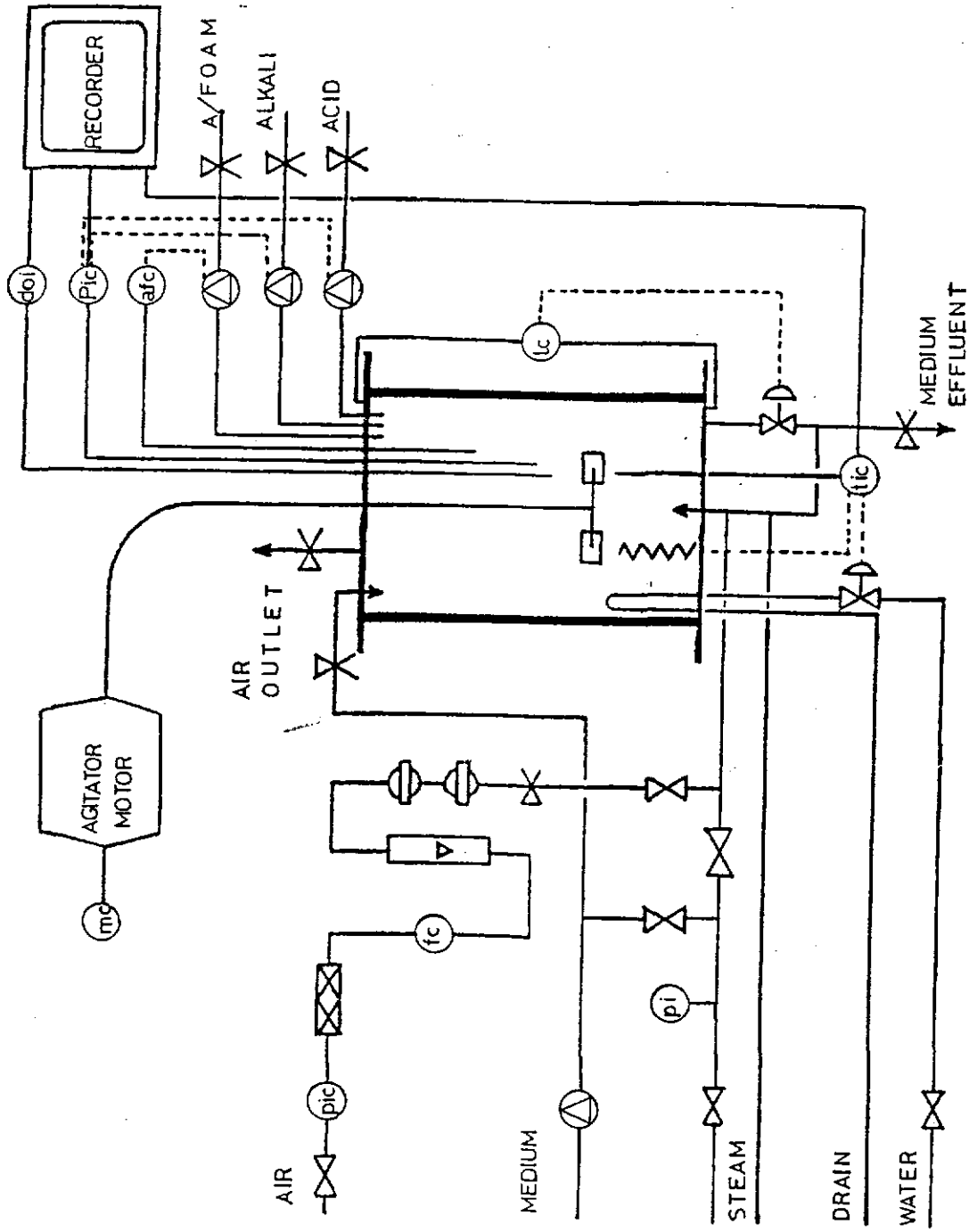


Fig 3.3 SK Fermenter - instrumentation

3.2.1 Culture Vessel and End Plates

The culture vessel was composed of a section of Pyrex glass pipe with a working volume of 10 litres. Internal diameter of vessel is 25 cm and height is 30 cm. The top plate (Fig 3.4) contains ports for inoculation / bulk titrant / water addition, pH probes, titrant, medium and antifoam inlets, an air outlet, and an agitator gland. The bottom plate (Fig 3.5) had ports for a heating element, temperature probe, cooling finger, culture effluent / sampling and air inlet.

3.2.2 Services

The fermenter was supplied with 1/4 " bulkhead couplings for connection to laboratory steam, air and water supplies. Fermenter sterilisation was carried out in-situ. The supply of mains air to the fermenter was at 25 psig. Cooling water system was divided into two separate parts. One supply went directly through a condenser on the air outlet and then to drain, and a second line to the fermenter cooling system.

3.2.3 Instrumentation and Control

3.2.3.1 pH Control

The pH of the culture was measured using non-steam sterilisable combined glass-reference electrode (Russel pH ltd. type CL8 , stem length 30 cm). The probe was connected by a coaxial cable to a pH indicating controller

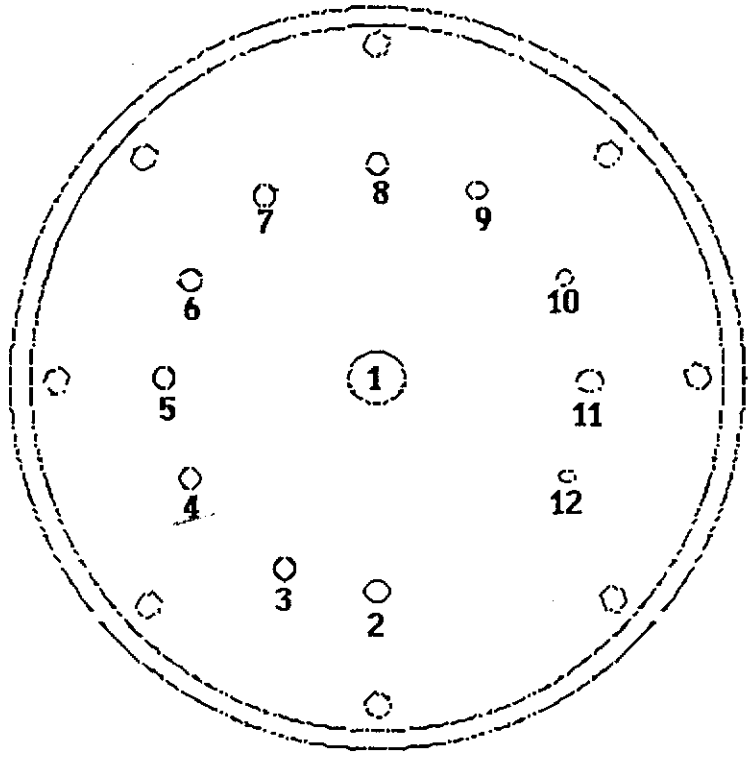


Fig 3.4 S.K. Fermenter top plate

1:agitator gland; 2:inoculation port; 3:medium inlet; 4,5,6 and 7 blanks; 8:air outlet; 9:antifoam inlet; 10:pH probe; 11:alkali inlet: 12:acid inlet

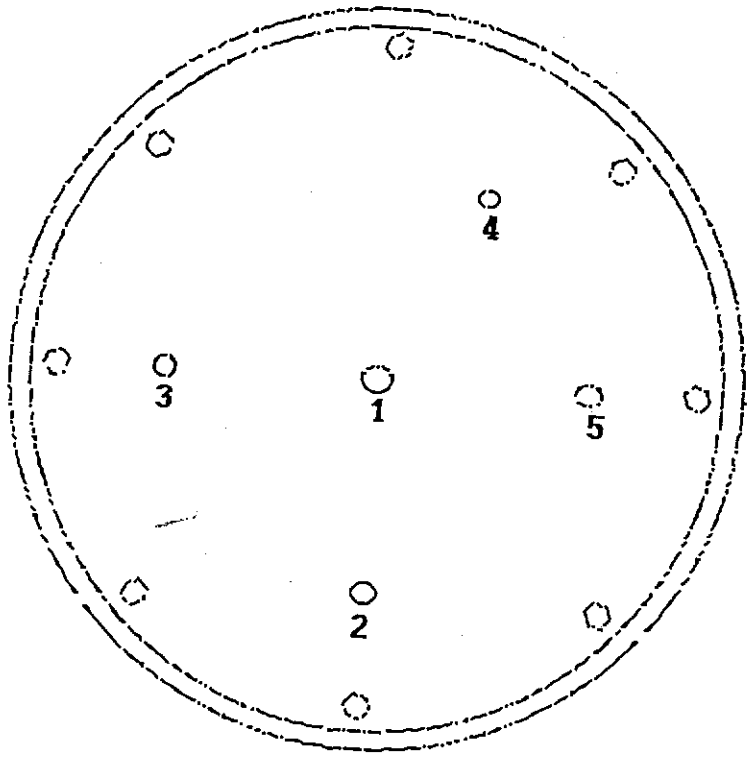


Fig 3.5 S.K. Fermenter bottom plate

1:air inlet; 2:cooling finger; 3:culture effluent;

4:temperature probe; 5:heating element

(Electronic Instruments ltd. model 9160) with a scale of 0-14. The pH controller keeps the pH within +/- 0.1 of the set point by the direct operation of two small pereistaltic pumps (Watson-Marlow ltd. type Delta), supplying 2M NaOH and 10% H_2SO_4 from 1 litre pyrex aspirators via silicone rubber tubing.

3.2.3.2 Temperature Control

The temperature indicating controller (West Guardian ltd.), connected to a platinum resistance bulb sensing element mounted in a stainless steel sheath within the fermenter, controlled both the heating and cooling system and managed to maintain temperature within +/- 0.2 °C of the set point . The heating system consisted of a 250 watt immersion heater in a stainless steel sheath and a external 150 watt heating tape (Hot Foil ltd.). Cooling water passed through a dual system of an external cooling coil and internal cooling finger which were connected in series. The flow of water through the system was controlled with a solenoid valve (Alexander Controls ltd. type AOS2).

3.2.3.3 Recorder

The fermenter was supplied with a panel mounted six channel recorder (Foster Cambridge ltd. model Clearspon P120L) and six panel mounted 1K Ω wire wound potentiometers for inputs to the recorder as well. Outputs from the pH and temperature probe were connected to

channels 3, and 5 respectively.

3.2.3.4 Level Control

The most important problem in the continuous fungal fermentation is that the level within the fermenter is kept constant. Continuous overflow weirs and air suction method are used succesfully in the bacterial and yeast fermentations. These methods are however not suitable for filamentous fungal fermentations due to the fact that the system causes retention of cells within the culture vessel (Solomons, 1972.,Kristiansen, 1976). The level control system consisted of a modification of the pressure transducer (Fig. 3.6) described by Brown and Petel (1971) and the pinch valve (Fig 3.7) described by Brown and Inkson (1972) in the SK fermenter.

The pressure transducer was made of a pyrex glass pipe reducer and a thin rubber membrane. 45 cm length of silicone rubber tubing, 6 mm outer diameter at 1.6 mm wall thickness, was fastened between 15 cm glass tubing and pipe reducer. Pipe reducer and tubing then was filled with a soapy water. 30 cm length of second silicone rubber tubing was used to connect the end of the glass tubing to a port of top plate. The system thus would allow to equilibration of the pressure on each side of the membrane. A U tube manometer was constructed one half being the silicone rubber and glass tubing, the other part is the culture vessel itself. When the fermenter was

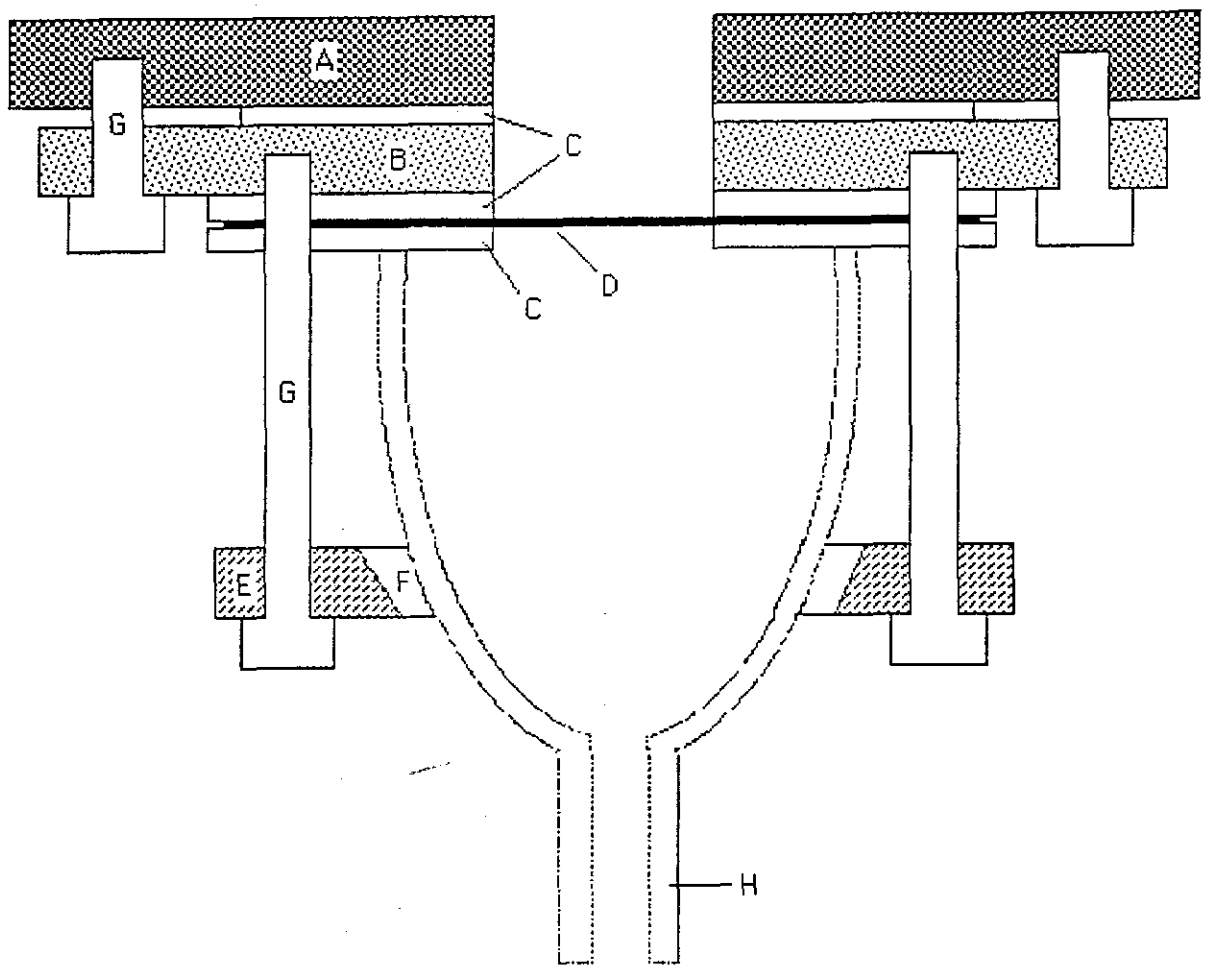


Fig. 3.6 Pressure transducer assembly

A: Fermenter bottom plate, B: Backing plate, C: Neoprene gasket,
D: Membrane, E: Backing flange, F: Insert, G: Bolt, H: QVF pipe reducer.

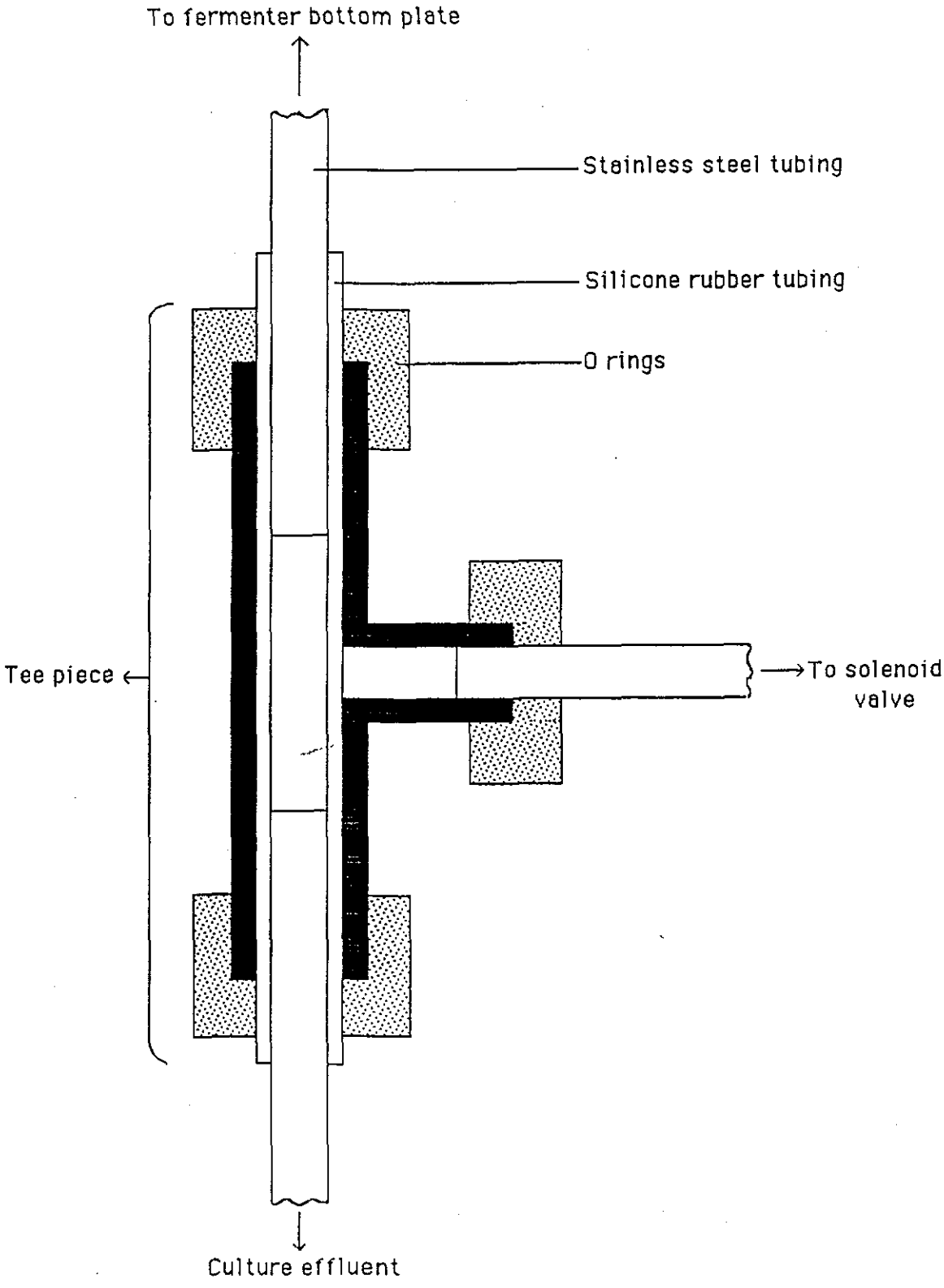


Fig. 3.7 Pinch valve assembly

filled with medium up to the working volume, the membrane was in a position of rest. Adding more medium would increase the hydrostatic head of the liquid in the vessel. The resulting reflection of the membrane would cause the liquid level in the external leg of U tube to rise. The rise in this level was detected by a flow monitor (Fisons Scientific Apparatus model: Fi monitor FM/K/D) which operated a three way normally open solenoid valve (Bellows International ltd. type 650- 27 - 2330) controlling the flow of air to the pinch valve.

3.2.4 Agitation

In the SK fermenter, baffles and an adequate agitation system was used i.e, to get sufficient oxygen transfer to the cell, to prevent oxygen limitation and to have as perfect as possible mixing in the culture. The impeller was a single flat bladed turbine with six blades which is as specified for fungal cultures by Solomons (1972), satisfying the criterion L / D = 1/2 where L is the diameter of the impeller and D the diameter of the culture vessel. The impeller was fixed to the agitator shaft by a grub screw and immersed to a depth of 2/3 of the liquid level. The agitator shaft was connected to a 1/8 HPDC shunt wound motor (Normand Electrical Co ltd.).

CHAPTER 4

EXPERIMENTAL METHODS

4.1 Micro-organism

The work was carried out with Aspergillus niger B 60 which was obtained from Prof Dr C. Kubicek, Vienna Technical University Austria, in the form of a spore suspension. Master culture were derived from the initial spore suspension and incubated at 30°C for 7 days and then stored at 4°C and renewed every month. From these master plates working cultures were prepared on PDA plates and the spores required for inoculum preparation were harvested from these plates.

4.2 Media

The commercial potato dextrose agar (PDA) -Oxoid limited- was only used as a sporulation medium, contained

Potato extract	4 g/l
D-Glucose	20 "
Agar	15 "

The sporulation medium was prepared using a ready made powder in necessary amount of distilled water which was used all the medium preparations.

The medium for initial batch fermentation in STR, shake flask and the initial growth phase of the organism before the continuous culture experiment consisted of:

	kg m^{-3}
Sucrose	140
Ammonium sulphate, $(NH_4)_2SO_4$	2.0
Potassium dihydrogen orthophosphate, KH_2PO_4	2.0

Magnesium sulphate, $MgSO_4.7H_2O$	0.5
Fe^{3+}, as ferric ammonium sulphate, $(NH_4)_2SO_4Fe_2(SO_4)_2.24H_2O$	0.1×10^{-3}
Zn^{2+}, as zinc sulphate, $ZnSO_4.7H_2O$	"
Cu^{2+}, as copper sulphate, $CuSO_4.5H_2O$	0.06×10^{-3}

pH of the medium was adjusted to 6.5 after sterilisation in the fermenter.

The initial, basic continuous culture medium as follows: .

	kg m^{-3}
Sucrose	50
Ammonium sulphate, $(NH_4)_2SO_4$	1.0
Potassium dihydrogen orthophosphate, KH_2PO_4	1.0
Magnesium sulphate, $MgSO_4.7H_2O$	0.1
Fe^{3+}, as ferric ammonium sulphate, $(NH_4)_2SO_4Fe_2(SO_4)_2.24H_2O$	0.1×10^{-3}
Zn^{2+}, as zinc sulphate, $ZnSO_4.7H_2O$	"
Cu^{2+}, as copper sulphate, $CuSO_4.5H_2O$	0.06×10^{-3}

All the chemicals used were analytical grade (B.D.H. ltd.) except for the sucrose, which was commercial grade.

4.3 Sterilisation Procedures

4.3.1 Shake Flasks

The 500 ml shake flasks containing 100 ml medium as

mentioned in 4.2 plus 5 ml distilled water to account for evaporation were used for inoculum development. The flasks were plugged with non-absorbent cotton wool (B.P.C. ltd.) and covered with brown paper then sterilised at 15 psig for 10 minutes in a small batch autoclave.

4.3.2 The Medium Aspirators

10 litres of batch medium as in 4.2 was made up in a 20 litre Pyrex aspirator. It was plugged with non-absorbent cotton wool and covered with brown paper then sterilised for 20 minutes at 15 psig in a large batch autoclave. Silicone rubber feed lines were clamped off with Hoffman clips and the ends were sealed with autoclave tape.

Continuous culture medium as described in 4.2 was prepared in amounts of 15 litres in 20 litre Pyrex aspirator, then sterilised for 40 minutes at 15 psig in a large batch autoclave. No contamination of batch or continuous culture media was observed after sterilisation. Autoclaving for a longer period of time resulted in caramelisation of the medium.

4.3.3 SK Fermenter

The SK fermenters were equipped with facilites for steam sterilisation in situ. It was possible to sterilise the vessel and the air line.

Before sterilisation all the titrant reservoirs were disconnected and the pH probe was removed from the vessel. Live steam entered the reactor via the sparger and was

allowed to run through the vessel. All inlets and outlet lines were opened for 90 minutes. These were then closed with the exception of one line which was steamed for 20 minutes. Finally the air inlet line was sterilised and a sterile air filter was placed on the non-sterile section of the air supply and the air switched on while opening the air out line. A non steam sterilisable pH probe was sterilised by immersion in 70% alcohol. Titrant reservoirs were sterilised in the small batch autoclave.

4.4 Fermentation Procedure

4.4.1 Inoculum Development

It has been reported that the final concentration of citric acid and dry weight is not related to inoculum size (Kristiansen 1976). Therefore the spore inoculum was not counted for routine fermentation and a simple inoculum method was modified as follows;

7 ml medium was aseptically removed from a 20 ml bottle using micropipette and sterile tip and added aseptically to a PDA culture plates. Spores were harvested from 7 day old plate cultures by scraping the surface with a flamed loop then aseptically transferred to the 500 ml flask which contained 100 ml sterile batch culture medium. Flasks were incubated on an orbital shaker at 30^{o}C and 250 rpm for 48 hours. After this period the small pellets formed were used as inoculum at a level of 2% v/v.

4.4.2 Fermenter Experiments

The sterile batch medium was pumped into the sterile vessel using a high speed peristaltic pump (Glen Creston ltd, type 127). The temperature was controlled at $30^{0}C$ (+/- 0.2), agitation at 400 rpm and air supplied at a rate of 1 volume of air per volume of culture per minute (1vvm). Prior to inoculation the pH probe adjusted to the required value. pH was controlled by addition of 10% H_2SO_4 and 2M NaOH.

Continuous fermentations were commenced when a reasonable cell concentration and acidogenesis had been reached, usually after 70 hours preliminary batch cultivation period. Sterilised continuous culture media was pumped in to vessel using a Watson Marlow pump at the desired flow rate. Evaporation during the batch fermentation was countered by addition of sterile distilled water. In the continuous culture it was considered that addition of titrant for controlling the pH was sufficient to cover the losses due to evaporation.

Fermentation broth samples were plated out on PDA and NA routinely to check for contamination, and also examined under a microscope (total magnification x 1000).

4.5 Sampling

Due to the dead volume in the region of the pinch valve and the bottom plate of the fermenter, an initial 25 ml sample was taken and discarded. A second 25 ml sample was

withdrawn and stored at $4^{0}C$ for dry weight determination, citrate, sucrose and ammonia assays.

4.6 Dry Weight (Biomass) Determination

5 ml samples of culture were filtered using 4.25 cm diameter Whatman GF/C filters circles and a 4.25 cm Gelman filtration unit. The filter cake was washed twice with 5ml distilled water. It was noted that there were significant differences between washing the filter cake once and washing it twice, so in all of the dry weight determinations the double washing method was applied. The filter plus cake was then dried in a microwave (Toshiba ltd. model ER558) for 20 minutes at low power and cooled in a desiccator before weighting to four decimal places on a Mettler balance (type H6). Duplicate samples were carried out.

4.7 Citric Acid Analysis

Citric acid was measured by a modification of the method described by Marier and Boulet (1958) which is based on a specific colourimetric reaction involving pyridine and acetic anhydride. 1 ml of sample with a citric acid concentration in the range 0.3 - 1.3 kg m^{-3} was placed in a test tube then 1 ml pyridine (BDH, ltd. analytical grade) was added into test tube. After mixing, 5 ml acetic anhydride (BDH, ltd. analytical grade) was added. The entire process was carried out in a fume cupboard. During

the addition of acetic anhydride, the test tube was placed into a cooling water bath since otherwise the heat released tended to cause a burst. The test tubes were closed then mixed and incubated in a water bath at $30^{0}C$ for 30 minutes. At the end of this period the absorbance of the samples was measured in a 1 cm light path cuvette, against a blank which contained 1 ml distilled water instead of sample, using a UV-160 spectrophotometer at 410 nm. A standard curve was obtained by measuring the absorbance of a series of standard samples containing known amounts of analytical grade citric acid (BDH, ltd.) (Appendix 2).

4.8 Sucrose Assay

Sucrose concentration was determined using the colourimetric method of Dubois *et al.* (1956). 1 ml of a sample which had a sucrose concentration in the range 10 - 100 x 10^{-3} kg m^{-3} was placed in a test tube. 1 ml of a 5% phenol solution (BDH, ltd. analytical grade) which was freshly prepared was added into test tube and mixed. To this was added 5 ml of 98% sulphuric acid (BDH, ltd. analytical grade) into test tube using a pipette with a broad tip. The samples were then allowed to stand at room temperature for 10 minutes. The test tubes were closed, mixed and incubated in a water bath at $30^{0}C$ for 20 minutes. The absorbance of the sample was read at 490 nm against air, in a 1 cm light path cuvette, using a UV-160 spectrophotometer. A standard curve was prepared using

samples containing known concentrations of sucrose (Appendix 2). It was noted that 96% H_2SO_4 could not be used in the assay, also when H_2SO_4 addition was made slowly, the assay failed due to mixing problems (H_2SO_4 98% is quite viscous)

4.9 Nitrogen Determination

Ammonium was determined by the method of Weichselbaum *et al.* (1969). The following procedure was employed for this purpose:

0.1 ml sample containing an NH_4^+ concentration in the range 1-70 x 10^{-3} kg m^{-3} was placed in a test tube. 1 ml Na nitroprusside solution (0.01 g Na nitroprusside dissolved in 5% phenol) was added into test tube. Then 0.1 ml 15% Na hypochlorite was added into test tube then 5 ml distilled water was added quickly into mixer. The reaction was allowed to stand for 15 minutes at room temperature. The absorbance of the sample was then read at 630 nm against air, in a 1 cm light path cuvette, using a UV-160 spectrophotometer. A standard curve was prepared using samples containing known concentrations of ammonium chloride. (Appendix 2).

CHAPTER 5

RESULTS

5.1 Batch Culture Results

In run B1, the initial pH was 6.5 and the pH of the broth was not controlled throughout the fermentation. Growth of the organism was followed by means of dry weight measurement. The second run, B2, will be used as a standard run to which other batch runs will be compared. B2 was carried out under standard conditions as described in chapter 4. In this standard run initial pH was 6.5, and was then allowed to fall to 1.8 and thereafter was controlled at 1.8 +/- 0.1.

The results of this run are presented in Fig 5.1.1 and the raw data is represented in appendix 1. The other batch experiments were divided into four sections, each of which examined the effect of changes in a single variable of interest. These were, (1) addition time of $(NH_4)_2SO_4$ (2) total amount of $(NH_4)_2SO_4$ added at 75 h, (3) initial amount of N source and of (4) pH. All the raw data from these experiments are enclosed in appendix 1.

5.1.1 Effect of Addition Time of N source on the Fermentation

2 kgm^{-3} $(NH_4)_2SO_4$ were added at different stages of the fermentation to observe the effect upon final citrate, biomass production and sucrose consumption. For this purpose seven runs were performed and all other conditions were as in Run B2 (standard). The results compared with those of the standard run (B2).

5.1.2 Effect of Total Amount of Ammonium Sulphate on the Fermentation

Different amounts of $(NH_4)_2SO_4$ were added at a fermentation time of 75 h to investigate the effect of the total amount of N added on final citric acid concentration, biomass production and sucrose consumption. The experiments were carried out for these purposes. All other conditions were as in Run B2.

5.1.3 Optimisation of Initial N level in the Medium

In order to optimise the initial concentration of $(NH_4)_2SO_4$ level, four runs were carried out All other conditions were as in B2.

5.1.4 Effect of pH

To examine the effect of initial pH a number of runs were carried out. The effect of controlling culture pH was also examined. Initial $(NH_4)_2SO_4$ was kept at 1.5 kg m^{-3} and all other conditions were as in B2.

5.1.5 Additional Batch Runs

Run B22

As B2, except 60 kg m^{-3} citric acid added to the medium at 0 h. The results are shown in Fig 5.1.21

RUN B23

As B2, except 300 ml 10% H_2SO_4 added at time 90 h. The aim

of this run is to examine effect of one shot H_2SO_4 addition on citric acid production to distinguish the effect of acidification from any specific effect due to citrate itself. The raw data is in appendix 1 and Fig 5.1.22 shows the time course of the fermentation.

5.2 Fed-Batch Culture Results

5.2.1 Feeding Sucrose

RUN FB1

Initially this was a straight forward batch in which conditions were similiar to B2, otherwise after 40 h 10 g sucrose solution per litre culture fluid was fed once every 24 h (Culture volume was kept at 9 l, thus 90 g sucrose added every day to fermenter) until 208 h. The raw data is illustrated in appendix 1 and Fig 5.2.1 shows the time course of the fermentation.

RUN FB2

As B2, otherwise after 40 h 5 g sucrose solution per litre culture fluid was fed once every 24 h (Culture volume was kept at 9 l, thus 45 g sucrose added every day to fermenter) until 184 h. The raw data is represented in appendix 1 and Fig 5.2.2 shows the time course of the fermentation.

5.2.2 Feeding Citric Acid

RUN FB3

As B2, except 30 g citric acid per litre culture fluid

was fed at time 90 h by withdrawing 0.8 l medium and adding 0.8 l concentrated (270 g citric acid in 0.8 l distilled water) citric acid solution. The raw data is given in appendix 1 and Fig 5.2.3 shows the time course of the fermentation.

RUN FB4

As FB3, except 45 g citric acid per litre culture fluid (Total 405 g citric acid added to fermenter) was fed at time 90 h. The raw data is illustrated in appendix 1 and Fig 5.2.4 shows the time course of the fermentation.

RUN FB5

As FB3, except 60 g citric acid per litre culture fluid was fed at time 90 h by withdrawing 1 l medium and adding 1 l concentrated (540 g citric acid in 1 l distilled water) citric acid solution. The raw data is represented in appendix 1 and Fig 5.2.5 shows the time course of the fermentation.

RUN FB6

As FB3, except that 120 g citric acid per litre culture fluid was added at time 90 hr by withdrawing 2 l medium and adding 2 l concentrated (1080 g citric acid in 2 l distilled water) citric acid solution. The raw data is represented in appendix 1 and Fig 5.2.6 shows the time course of the fermentation.

RUN FB7

As FB3, except that 60 g citric acid per litre culture fluid was fed at time 45 h by withdrawing 1 l medium and adding 1 l concentrated (600 g citric acid in 1 l distilled water) citric acid solution. The raw data is illustrated in appendix 1 and Fig 5.2.7 shows the time course of the fermentation.

5.3 Continuous Culture Results

RUN C1

This run was the standard continuous culture run which was carried out under the following conditions:

medium	as described in chapter 4
dilution rate	0.05 h^{-1}
culture volume	8 l (+ 0.4 l, - 0.1 l)
temperature	30^{o}C (+/- 0.2)
pH	1.8 (+/- 0.1)
air flow rate	1vvm
agitation	400 rpm

A level control probe and manometer system were examined for use in controlling the level of continuous culture. The manometer system was found to be more accurate than probe system and worked within + 0.4 / - 0.1 litre of the set point. Howewer it was found necessarry to calibrate the manometer system twice a day in order to use it successfully.

The inoculation procedure as described in chapter 4 was followed. The results from this run are presented in Table 5.1

RUN C2

This run was the same as C1 except that the dilution rate was 0.075 h^{-1} . Washout of biomass occurred in this run.

RUN C3

As C2 except 2 kg m^{-3} $(NH_4)_2SO_4$ was used in the medium instead of 1 kg m^{-3} and pH of broth kept 3. The data from this run is given in Table 5.1

RUN C4

As C3, except that the pH was controlled at 2.5. The culture was discarded in the later stages of fermentation because the citric acid concentration and dry weight were both very low. The results from the last sample taken have been included in Table 5.1 Due to the very low biomass and citric acid levels it is considered unnecessary to repeat this run.

RUN C5

As C4, except that during the batching up stage of run the culture pH was kept above 3.5. The results are shown in Table 5.1

RUN C6

As C4, except the pH during batching up stage was

initially adjusted to 6.5 and then allowed to drop to 4.0, after which it was controlled at 4.0 +/- 0.1. It was found that in order to keep pH at this high level 2 l NaOH were used which had deleterous effects on organism. It is possible that constant dropwise addition of a strong alkali damages the culture. Since the biomass obtained was so low it was not considered worthwhile progressing to continuous mode.

5.4 Cycled Operation

RUN CYC 1

On / off operation was employed by combining standard batch culture and standard continuous culture. The first 70 h as B2 then a peristaltic pump (Glen Creston ltd. type 127) was turned on thus 3 h continuous phase as C1 was applied. Then peristaltic pump was turned off for 20 h. This 20 h batch and 3h continuous cycle was repeated five times. The raw data can be located in Appendix 1 while in figure CYC 5.4.1 the results are displayed.

RUN CYC 2

As CYC 1 except that the duration of the continuous phase was 5 h instead of 3 h. The 20 h / 5 h cycle was repeated four times. The raw data can be located in Appendix 1 while in figure CYC 5.4.2 the results are displayed.

Table 5.1

Run	Dilution Rate h^{-1}	pH			Dry Weight kg m^{-3}	Sucrose Consumed kg m^{-3}	Citric Acid kg m^{-3}	NH_3 kg m^{-3}
		Batch stage		continuous				
		Initial	Controlled					
C1	0.050	6.5	1.8	1.8	3.3	8.0	4.3	0.027
C2	0.075	6.5	1.8	1.8	wash out occurred			
C3	0.075	6.5	3.0	3.0	2.5	6.0	---	0.455
C4	0.075	6.5	2.5	2.5	1.9	6.0	0.5	0.544
C5	0.075	6.5	3.5	2.5	4.4	13.0	---	0.520
C6	0.075	6.5	4.0	---	---	----	---	----

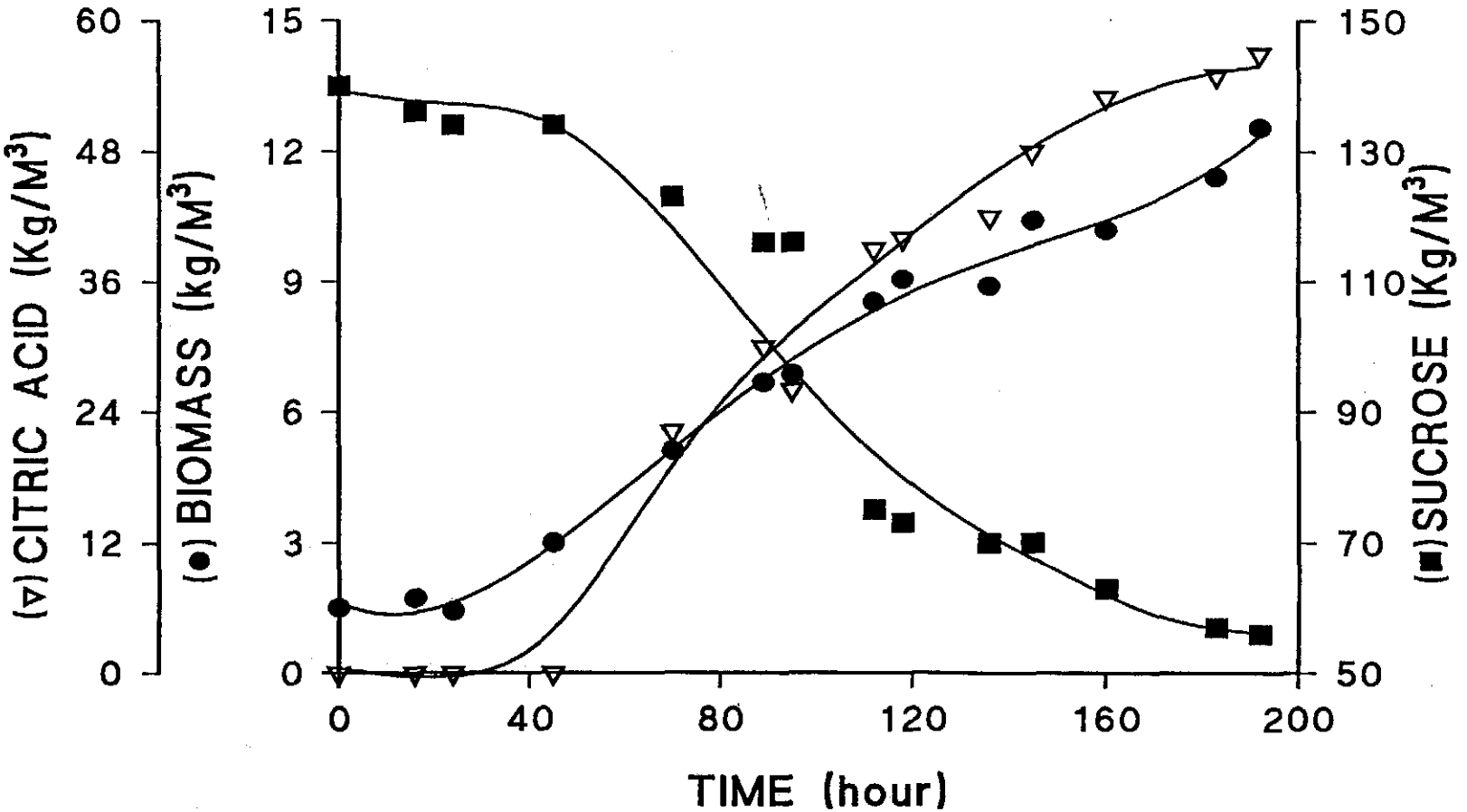


Fig 5.1.1 The effect of $(NH_4)_2SO_4$ supplementation at various times: standard run

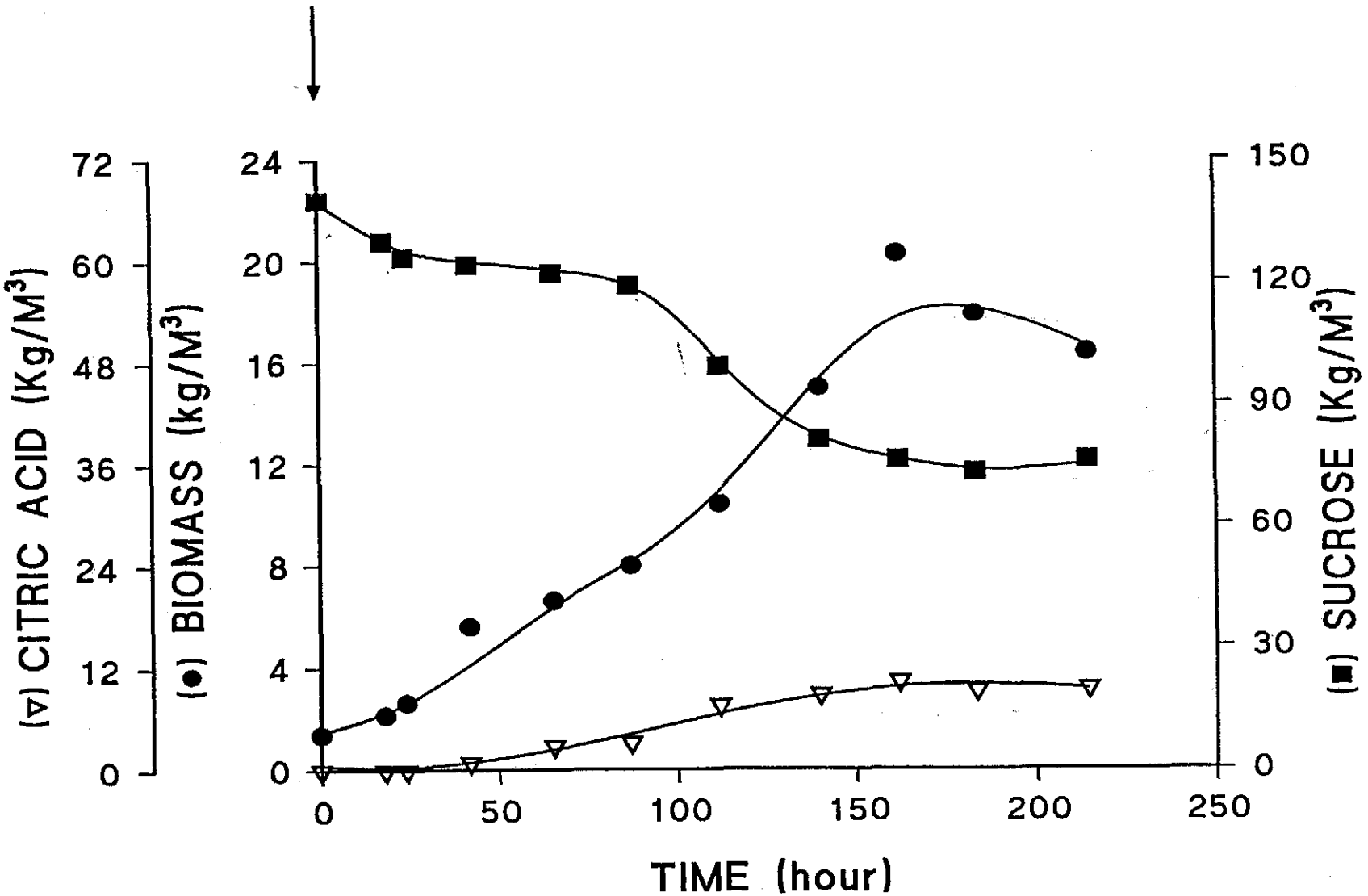


Fig 5.1.2 The effect of $(NH_4)_2SO_4$ supplementation at various times: 2 KgM^{-3} added at 0 h. (Arrow indicates addition point).

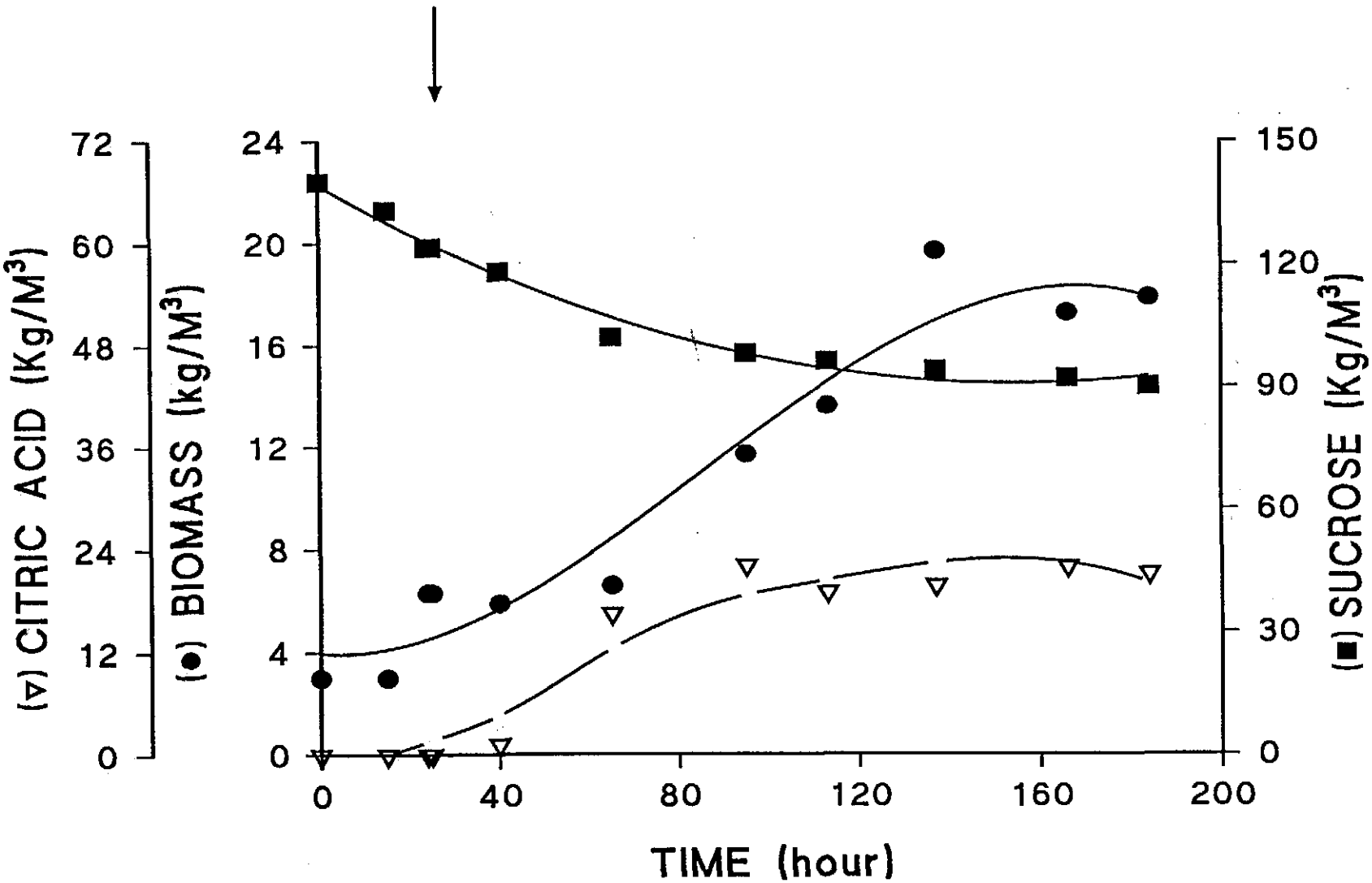


Fig 5.1.3 The effect of $(NH_4)_2SO_4$ supplementation at various times: 2 KgM^{-3} added at 25 h.

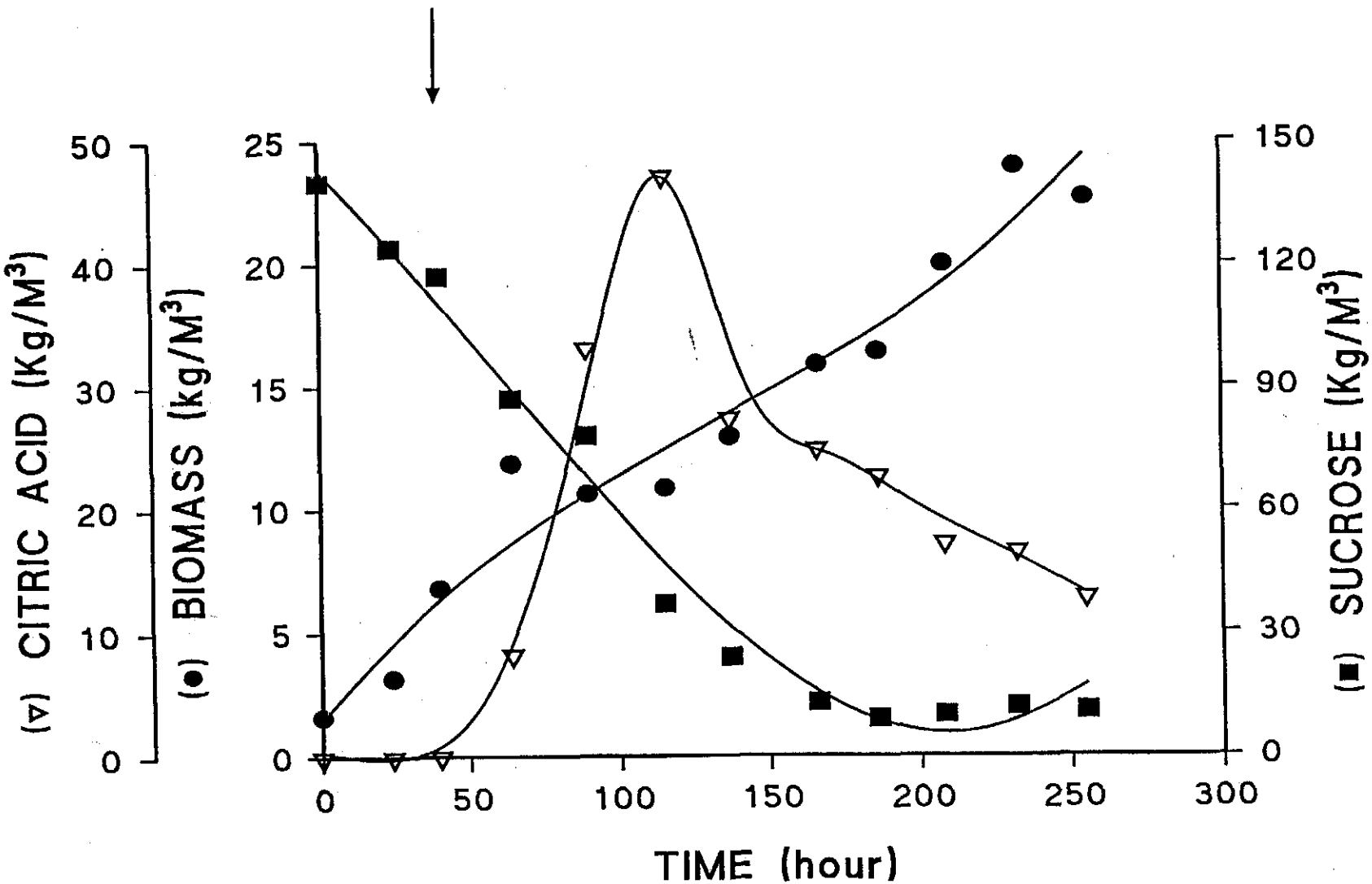


Fig 5.1.4 The effect of $(NH_4)_2SO_4$ supplementation at various times: 2 KgM^{-3} added at 40 h.

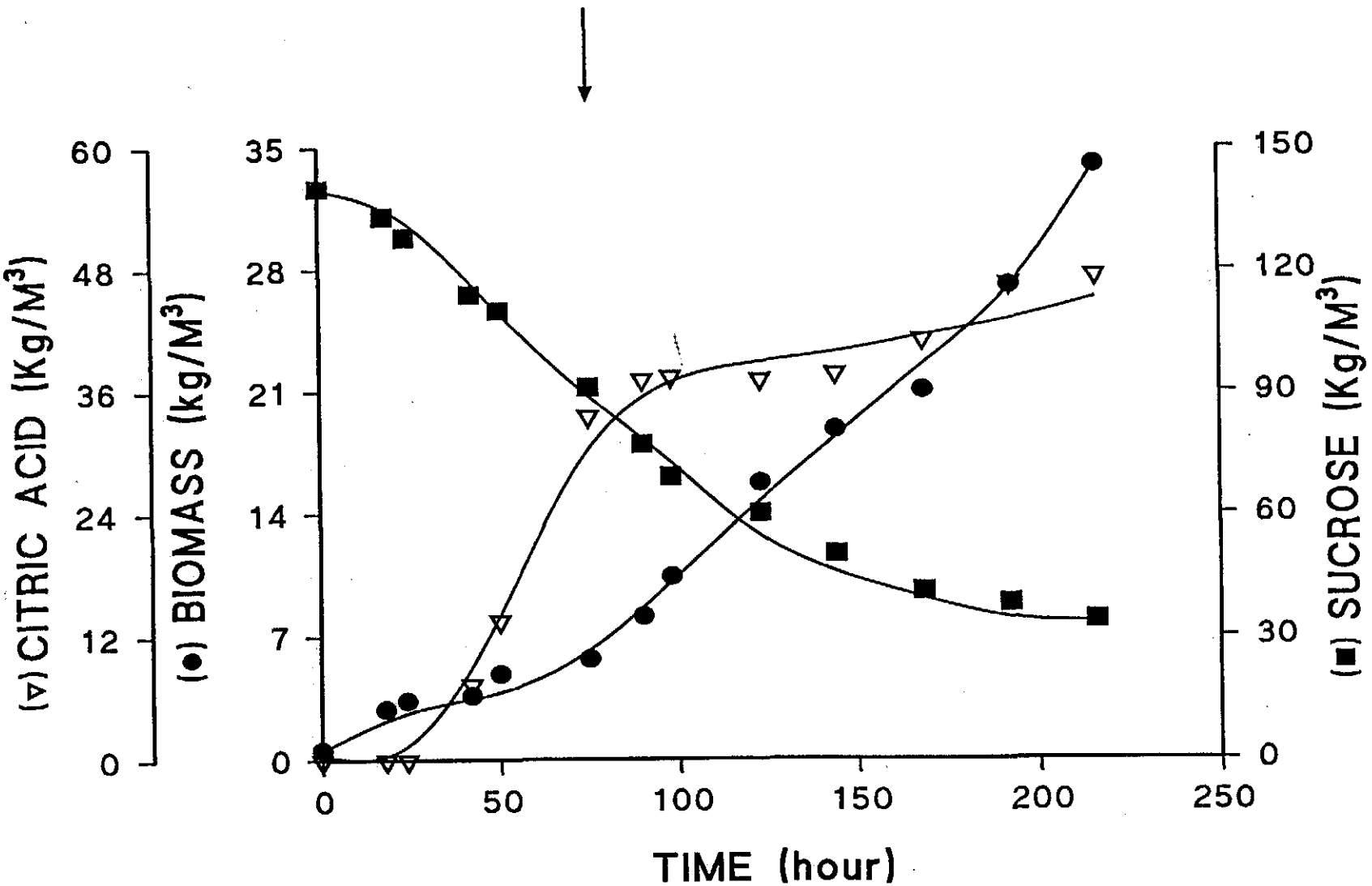


Fig 5.1.5 The effect of $(NH_4)_2SO_4$ supplementation at various times: 2 KgM^{-3} added at 75 h.

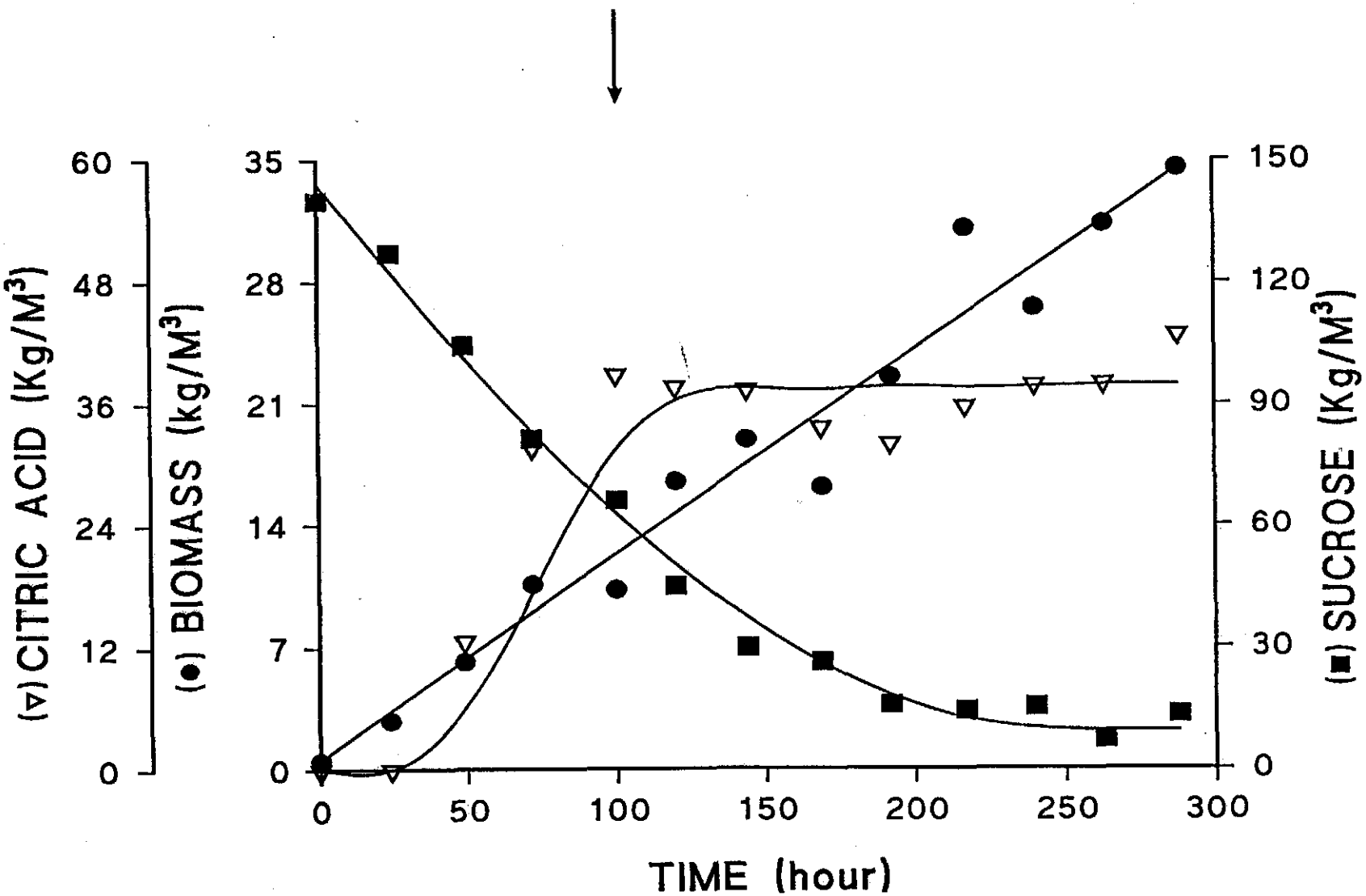


Fig 5.1.6 The effect of $(NH_4)_2SO_4$ supplementation at various times: 2 KgM^{-3} added at 100 h.

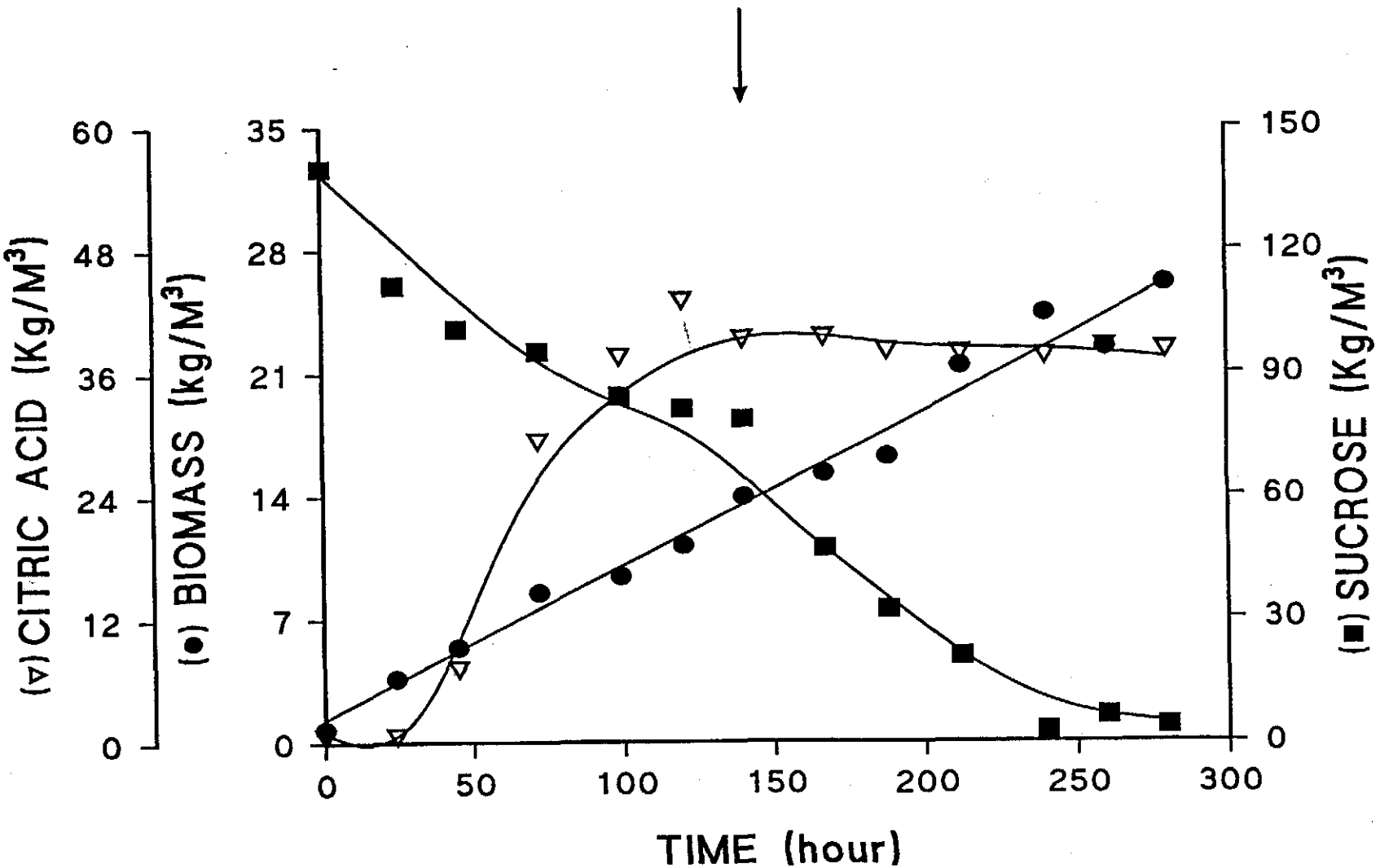


Fig 5.1.7 The effect of $(NH_4)_2SO_4$ supplementation at various times: 2 KgM^{-3} added at 140 h.

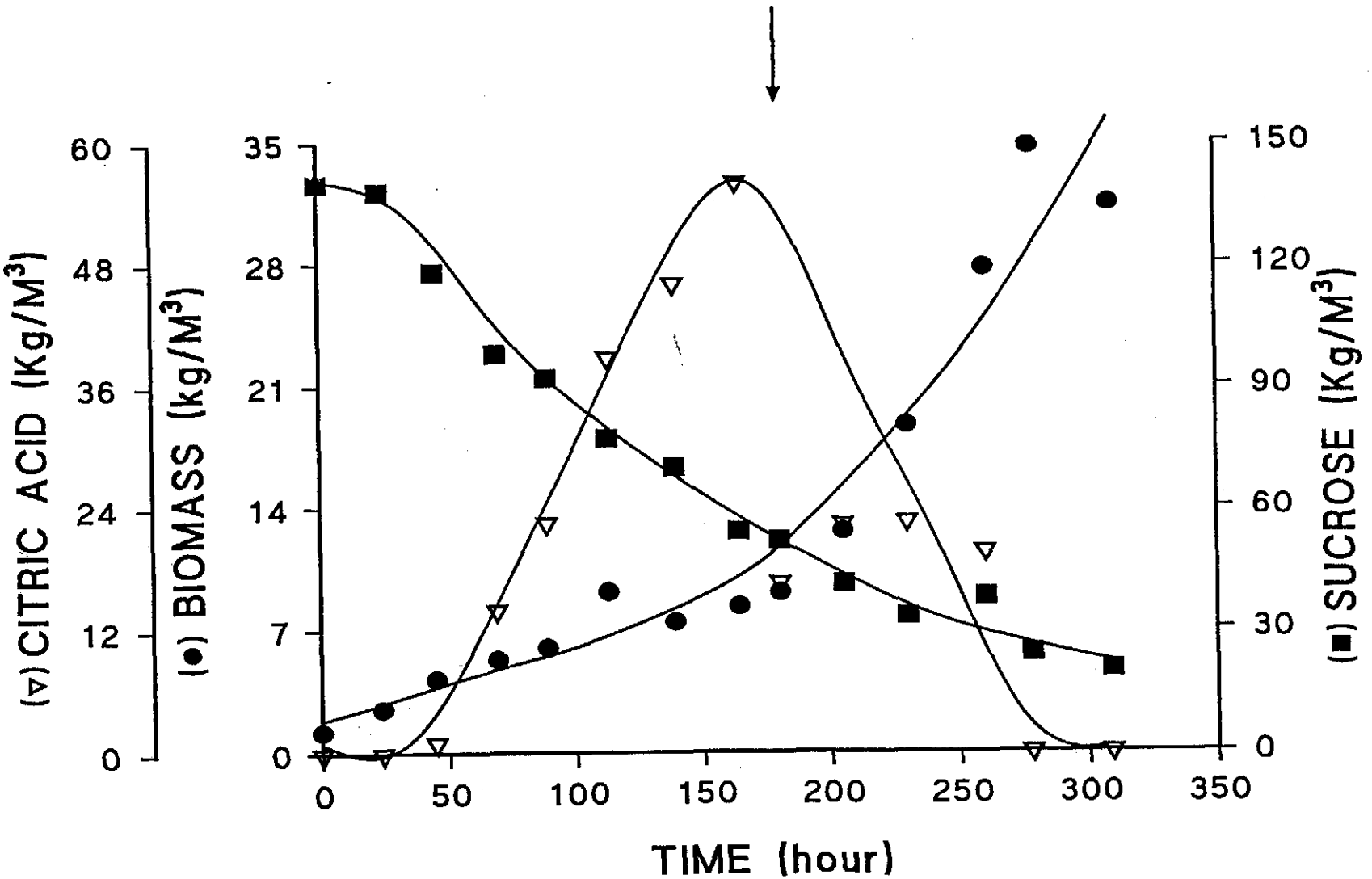


Fig 5.1.8 The effect of $(NH_4)_2SO_4$ supplementation at various times: 2 KgM^{-3} added at 180 h.

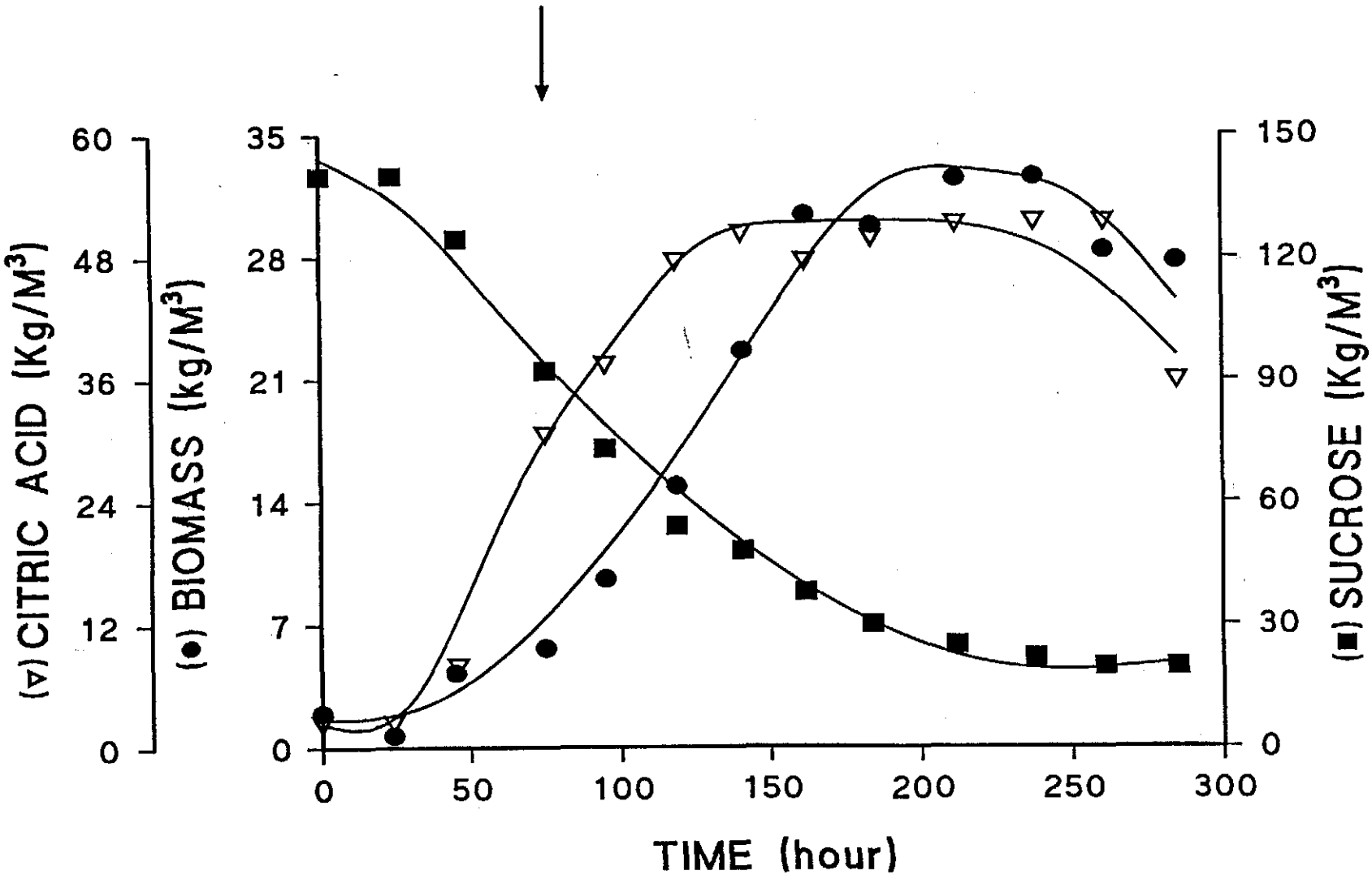


Fig 5.1.9 The effect of total amount $(NH_4)_2SO_4$ supplementation at 75 h: 1 KgM^{-3} added.

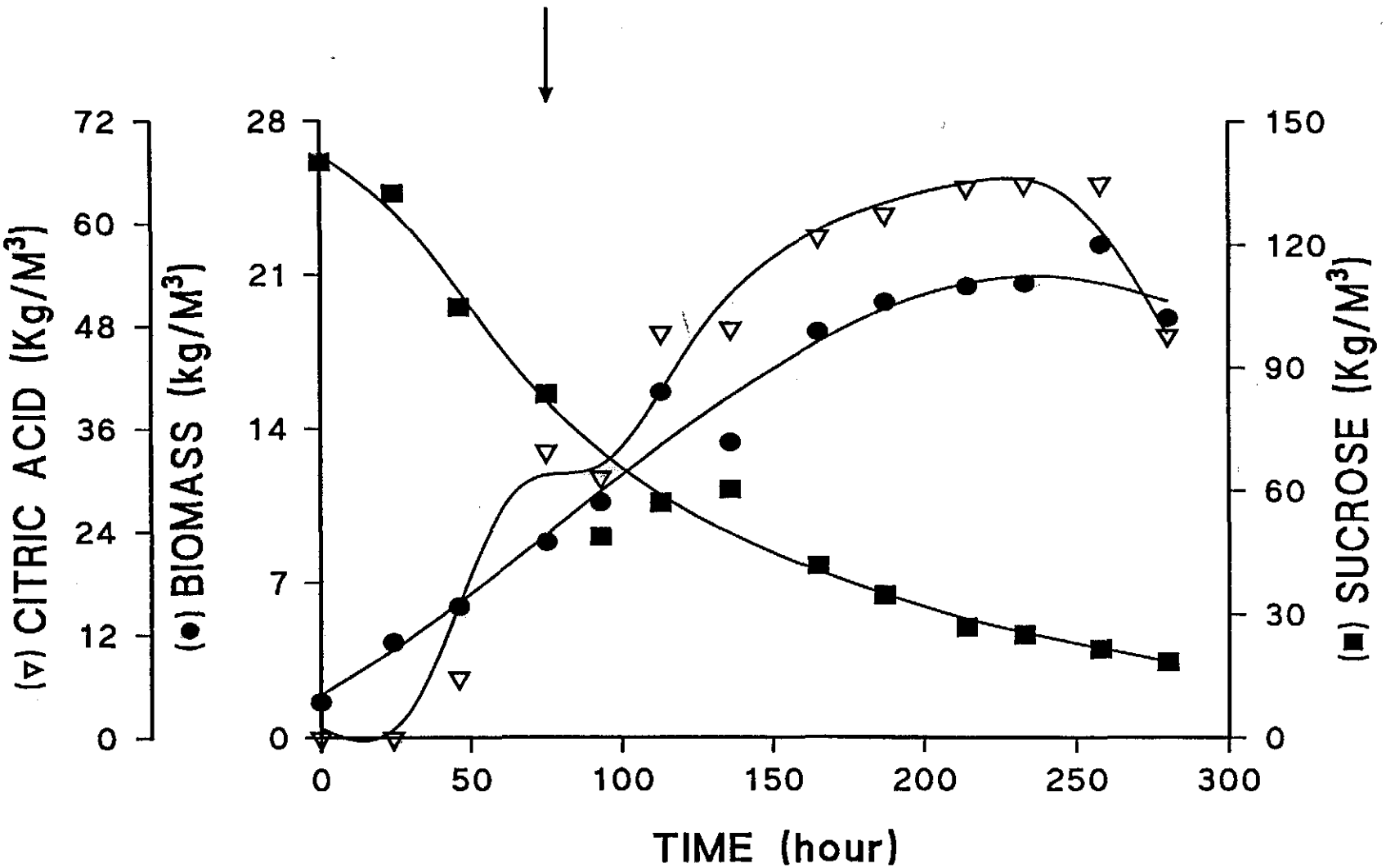


Fig 5.1.10 The effect of total amount $(NH_4)_2SO_4$ supplementation at 75 h: 0.5 KgM^{-3} added.

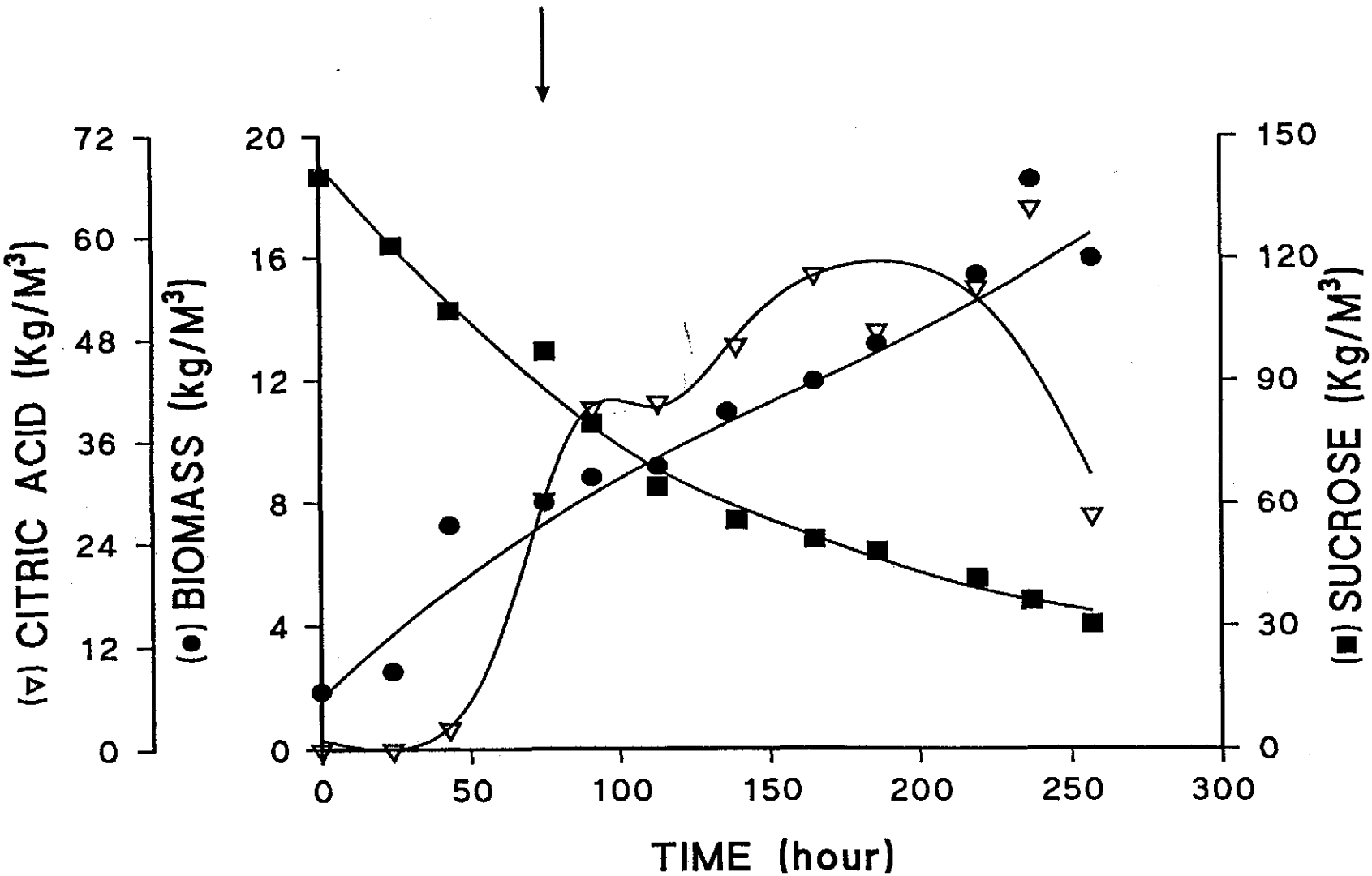


Fig 5.1.11 The effect of total $(NH_4)_2SO_4$ supplementation at 75 h: 0.25 KgM^{-3} added.

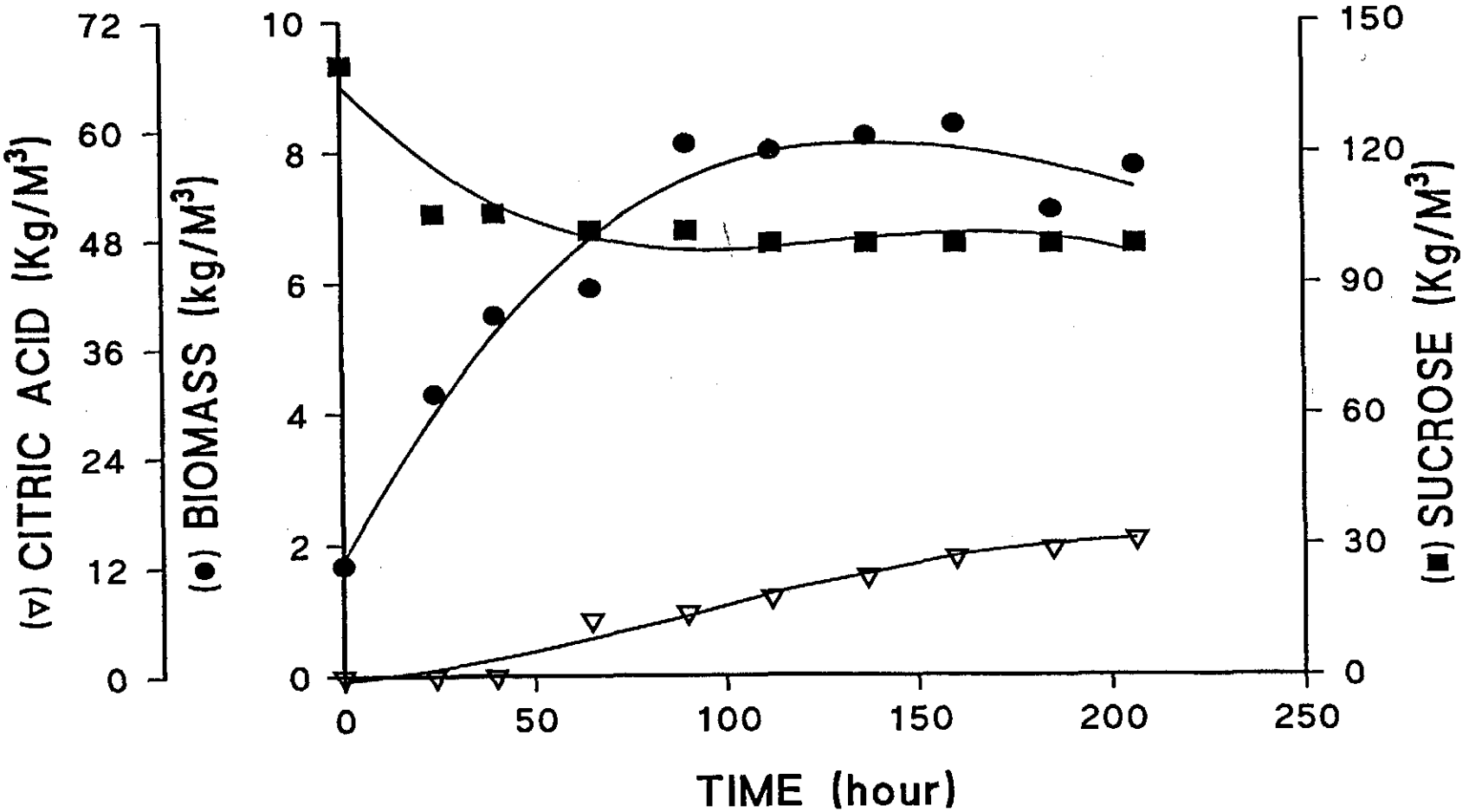


Fig 5.1.12 The effect of initial N level: 0.5 KgM^{-3} $(NH_4)_2SO_4$ used.

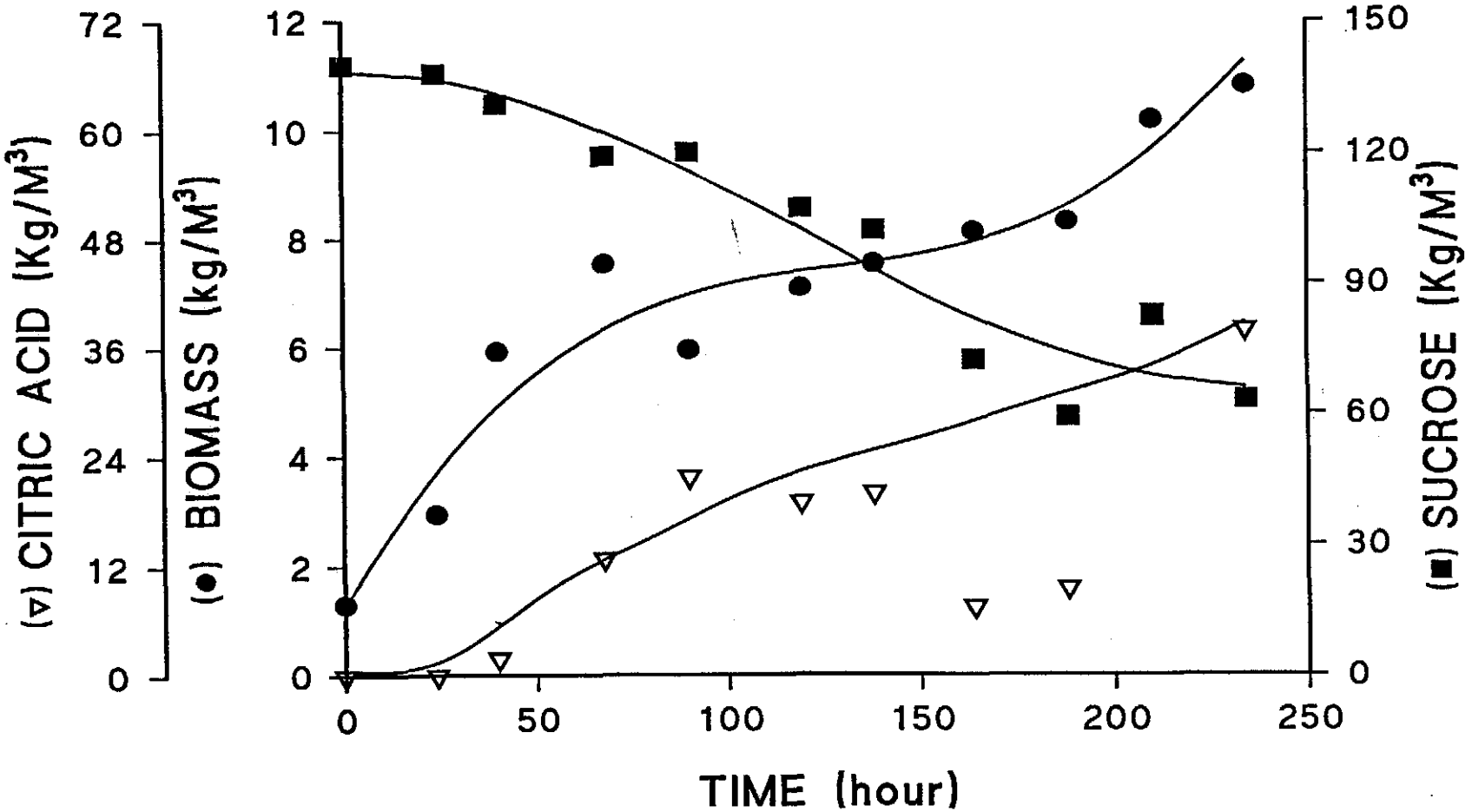


Fig 5.1.13 The effect of initial N level: 1 Kg/M³ $(NH_4)_2SO_4$ used.

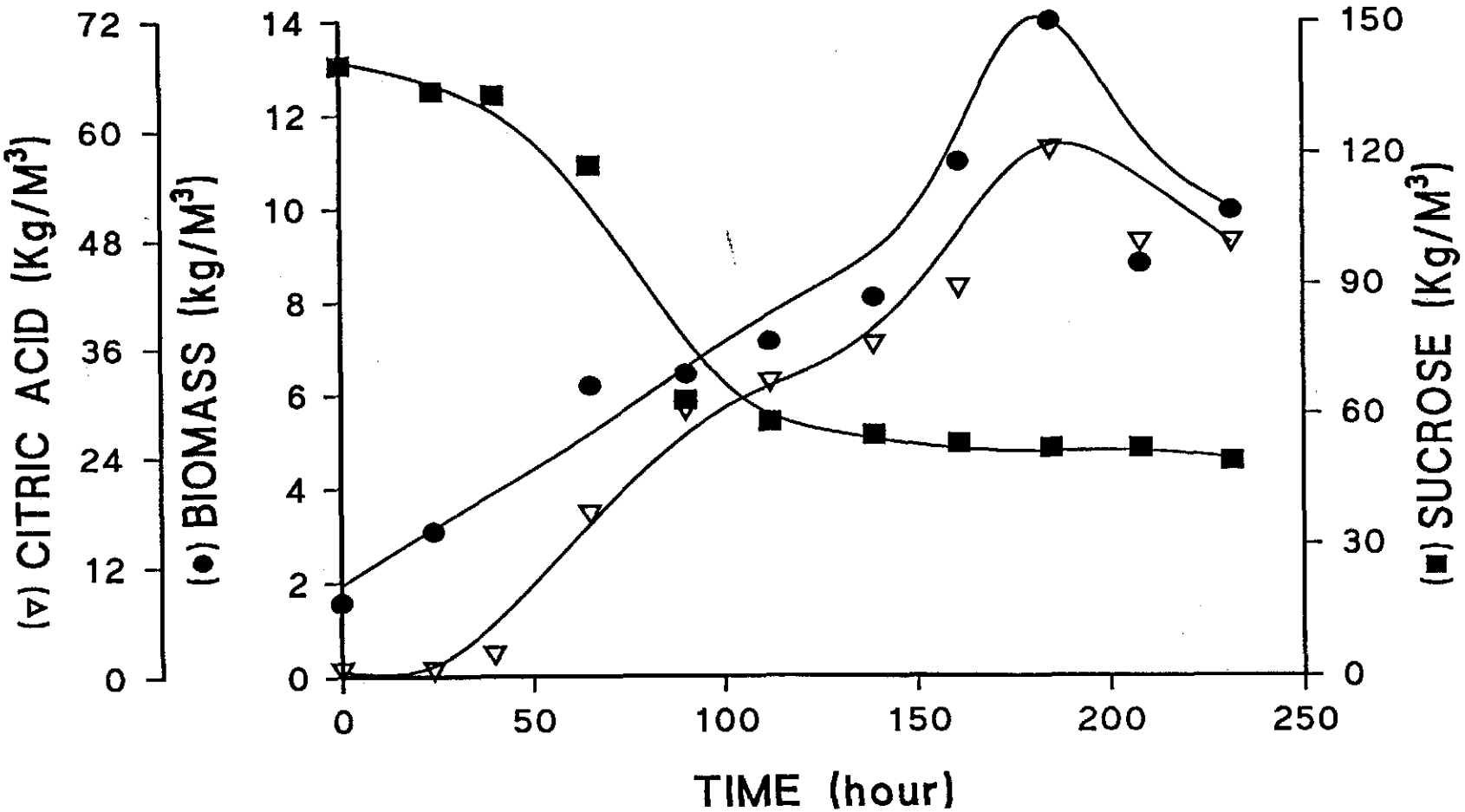


Fig 5.1.14 The effect of initial N level: 1.5 Kg/M³ $(NH_4)_2SO_4$ used.

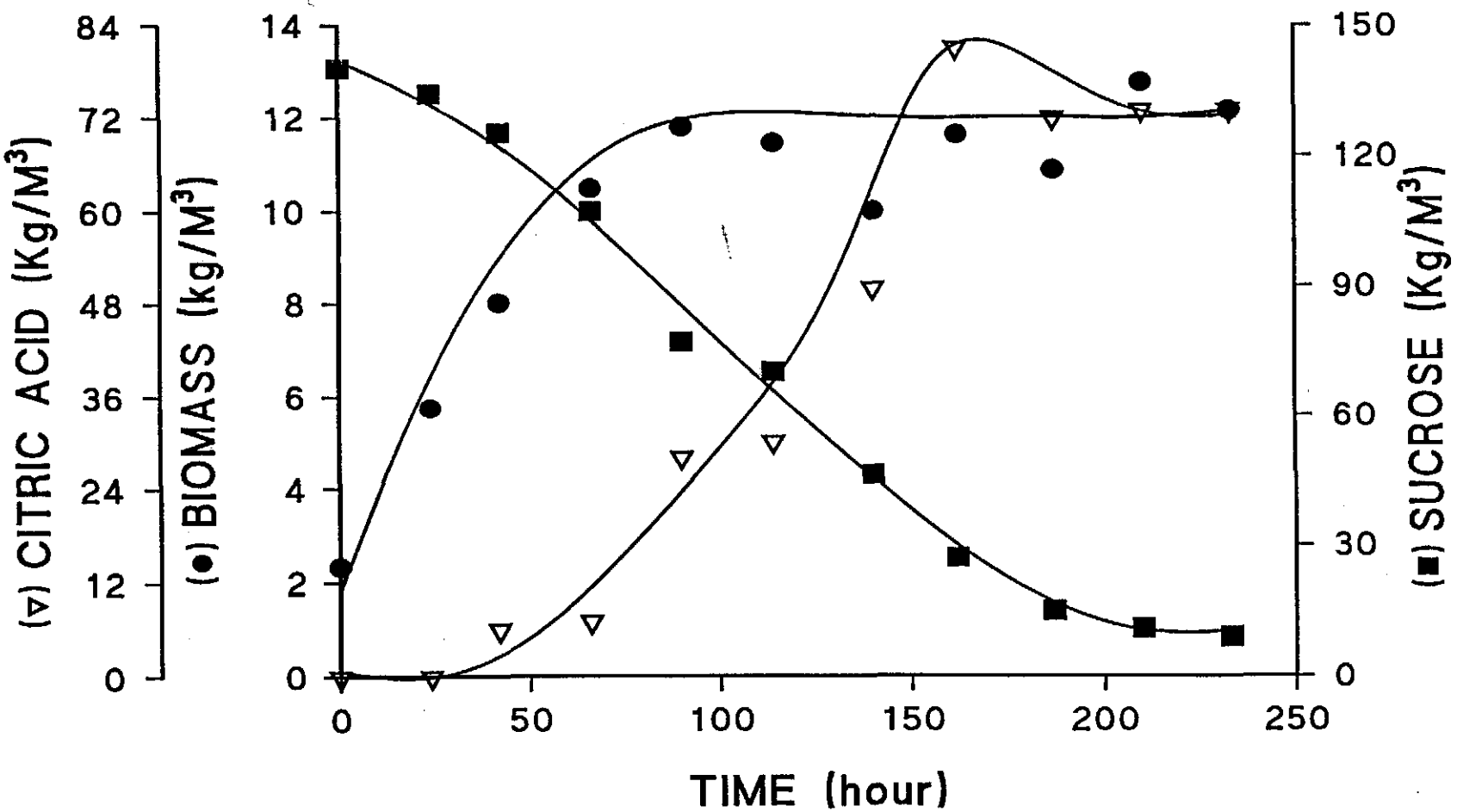


Fig 5.1.15 The effect of initial N level: 3 Kg/M³ $(NH_4)_2SO_4$ used.

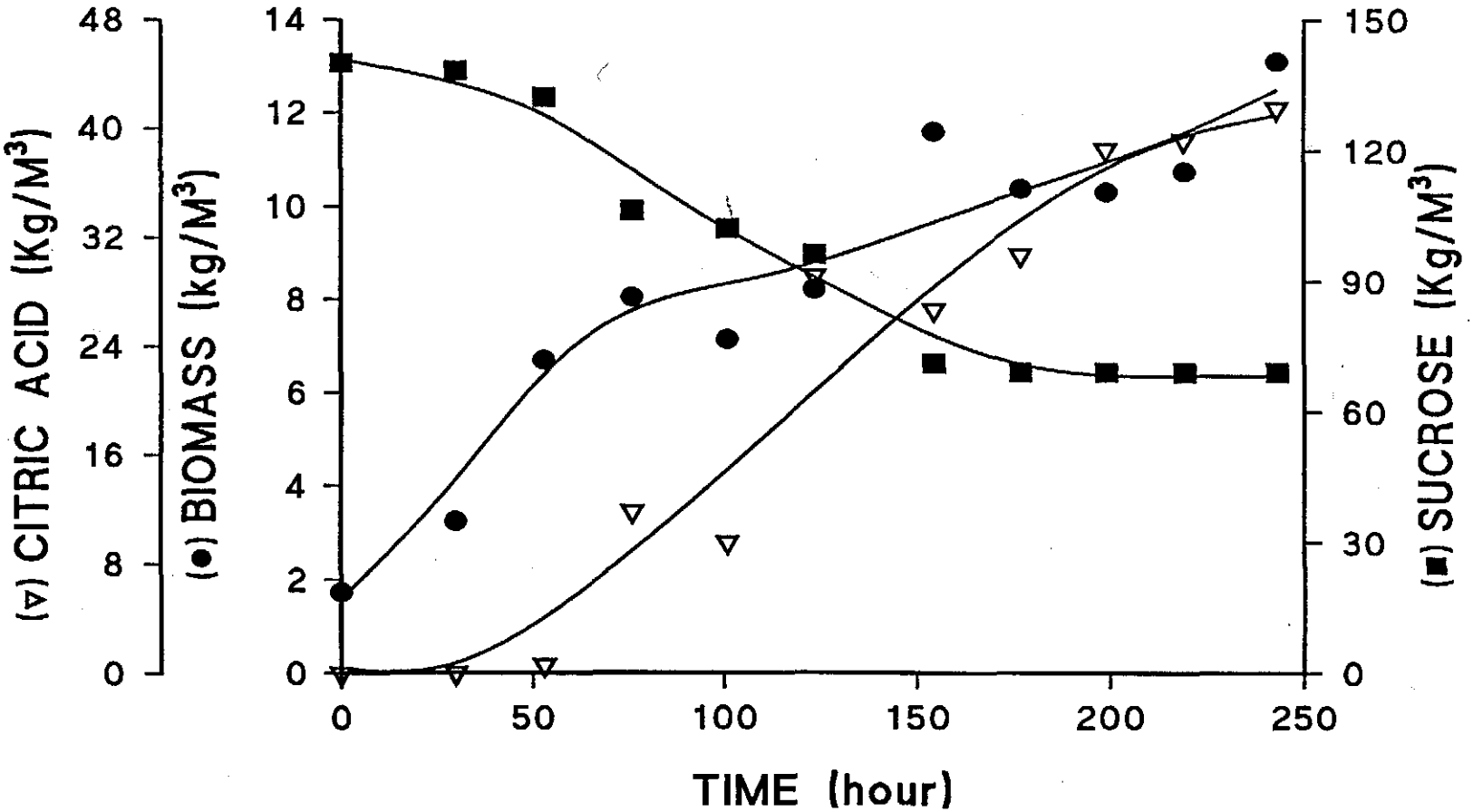


Fig 5.1.16 The effect of pH: pH adjusted to 2 throughout the fermentation.

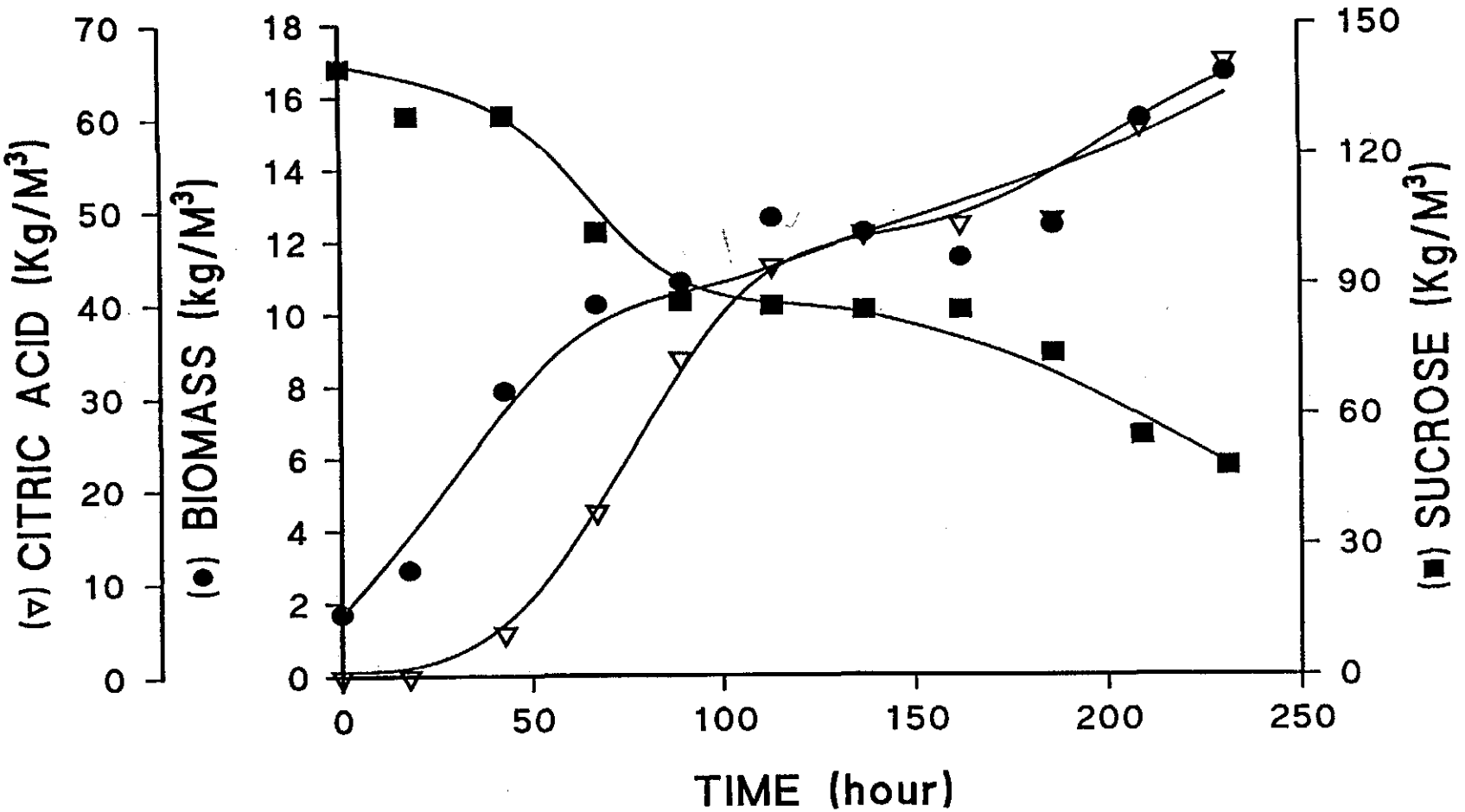


Fig 5.1.17 The effect of pH: pH adjusted to 2.5 throughout the fermentation.

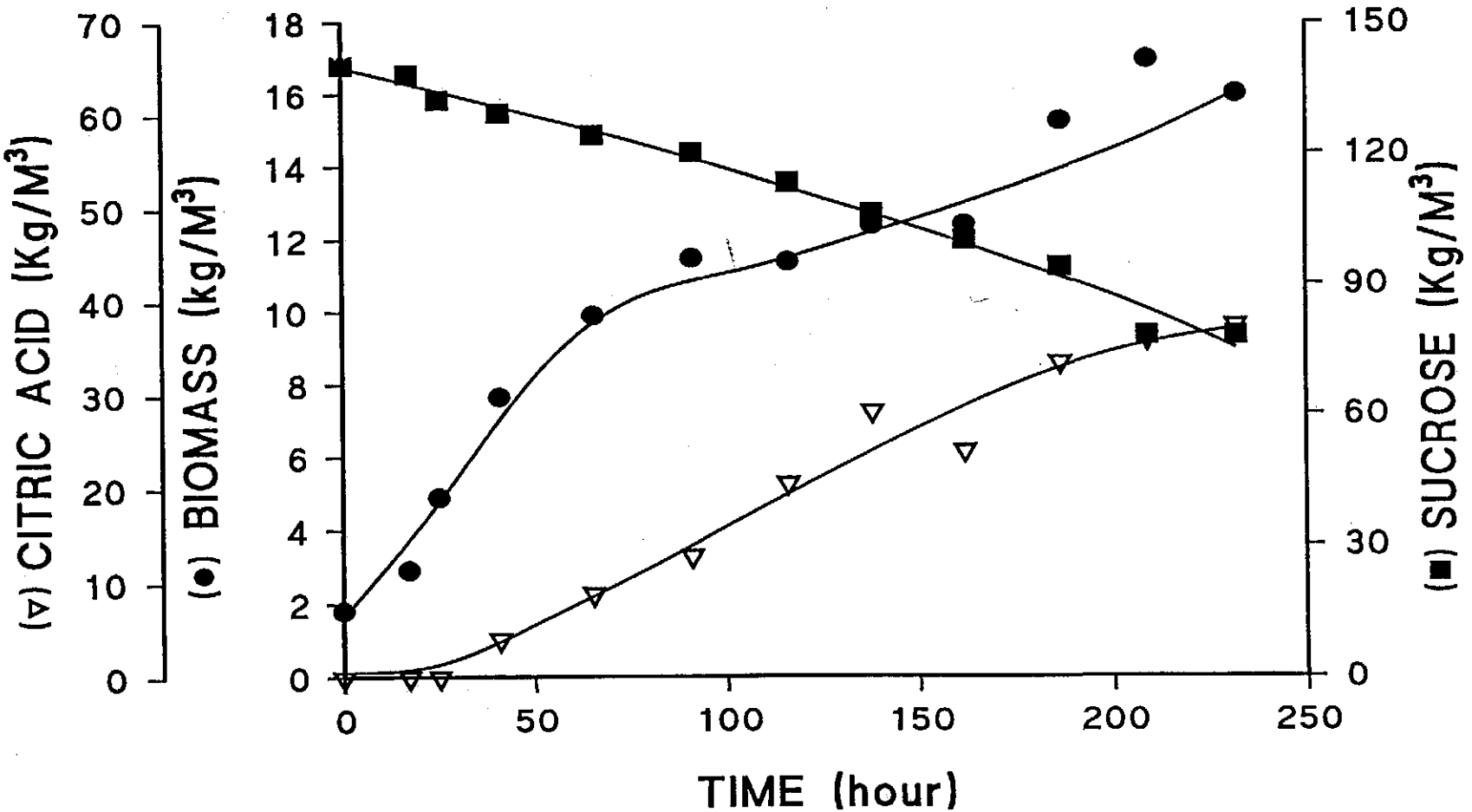


Fig 5.1.18 The effect of pH: pH adjusted to 3.0 throughout the fermentation.

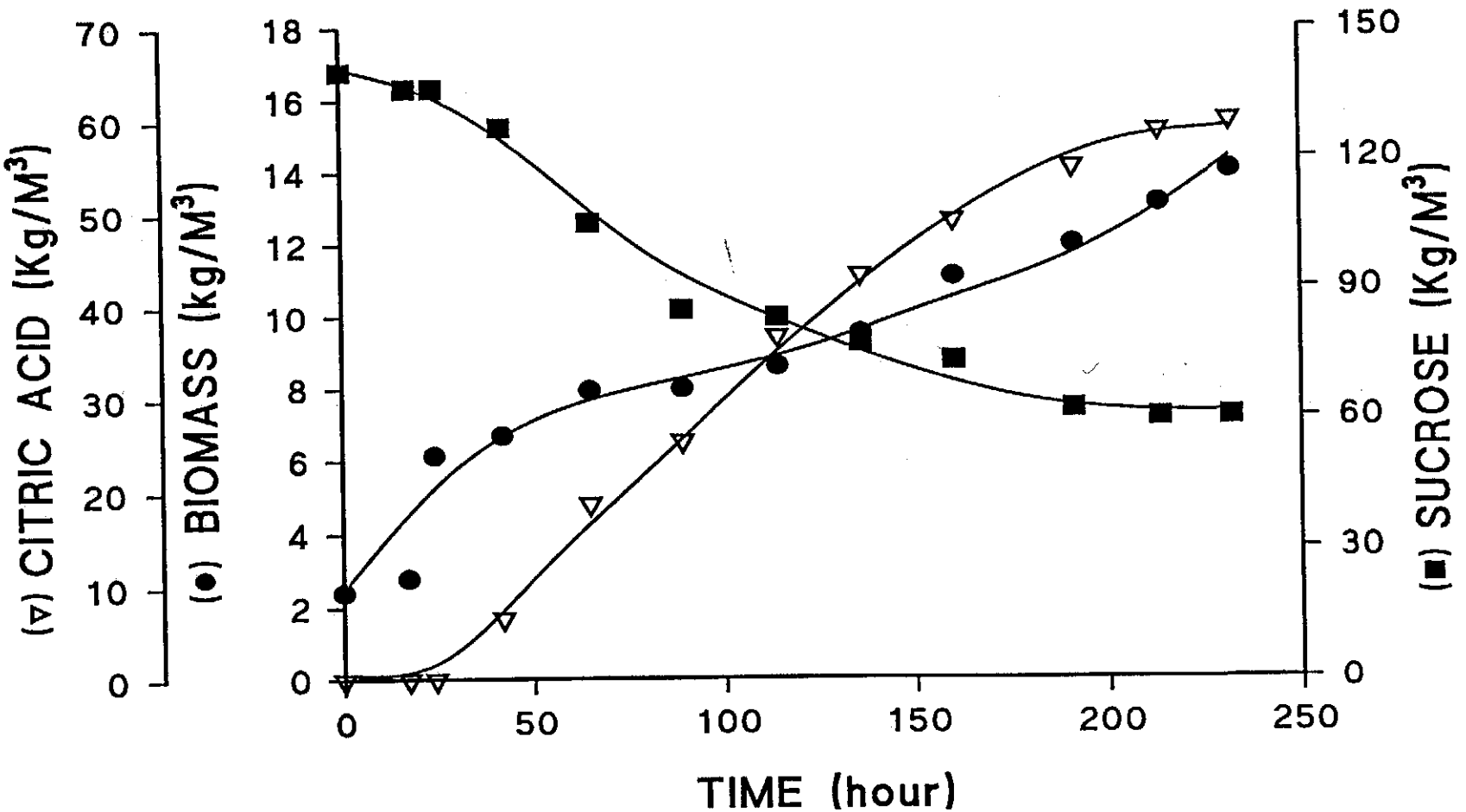


Fig 5.1.19 The effect of pH: initial pH 6.5, then controlled at 2.5

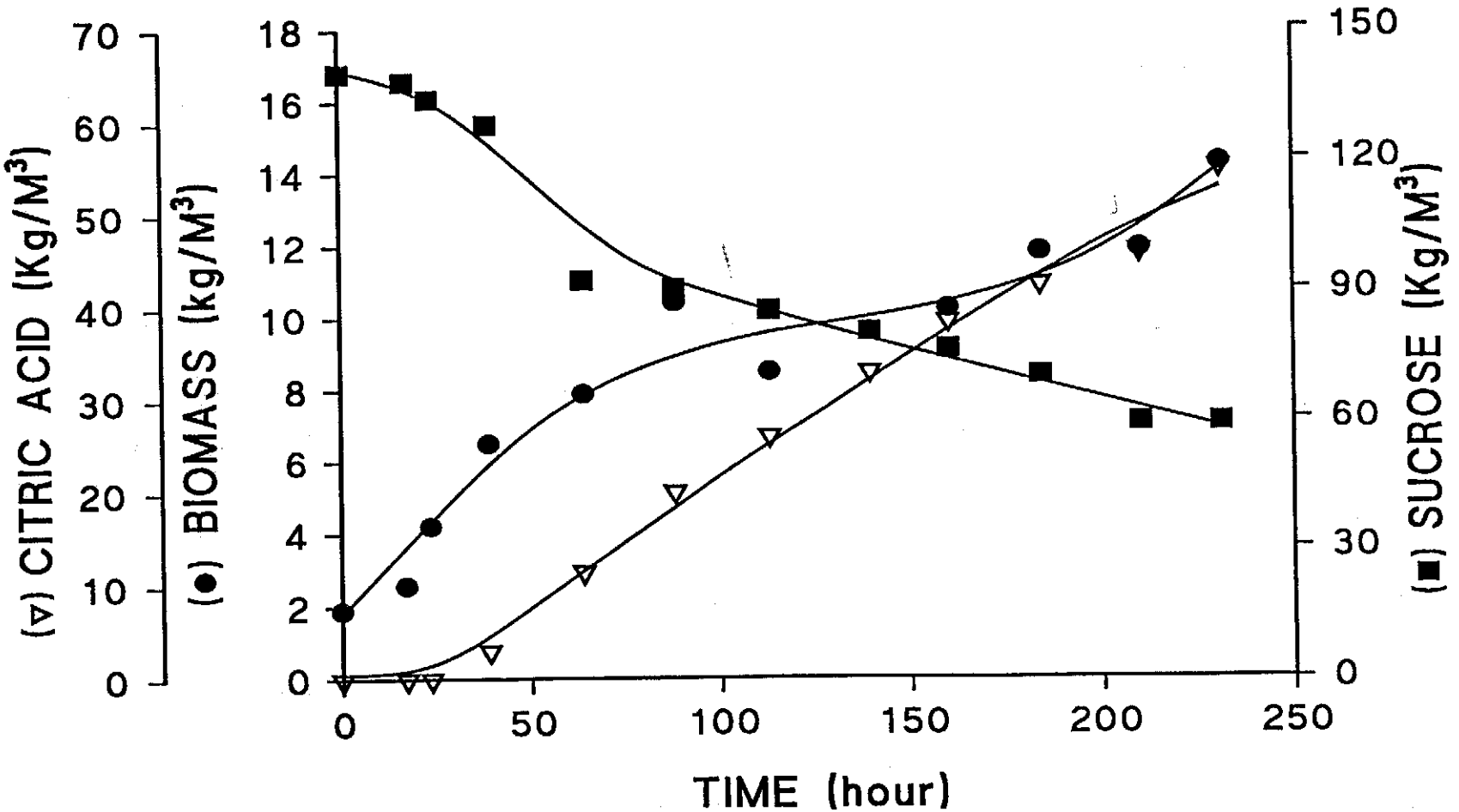


Fig 5.1.20 The effect of pH: initial pH 4.5, then controlled at 2.5

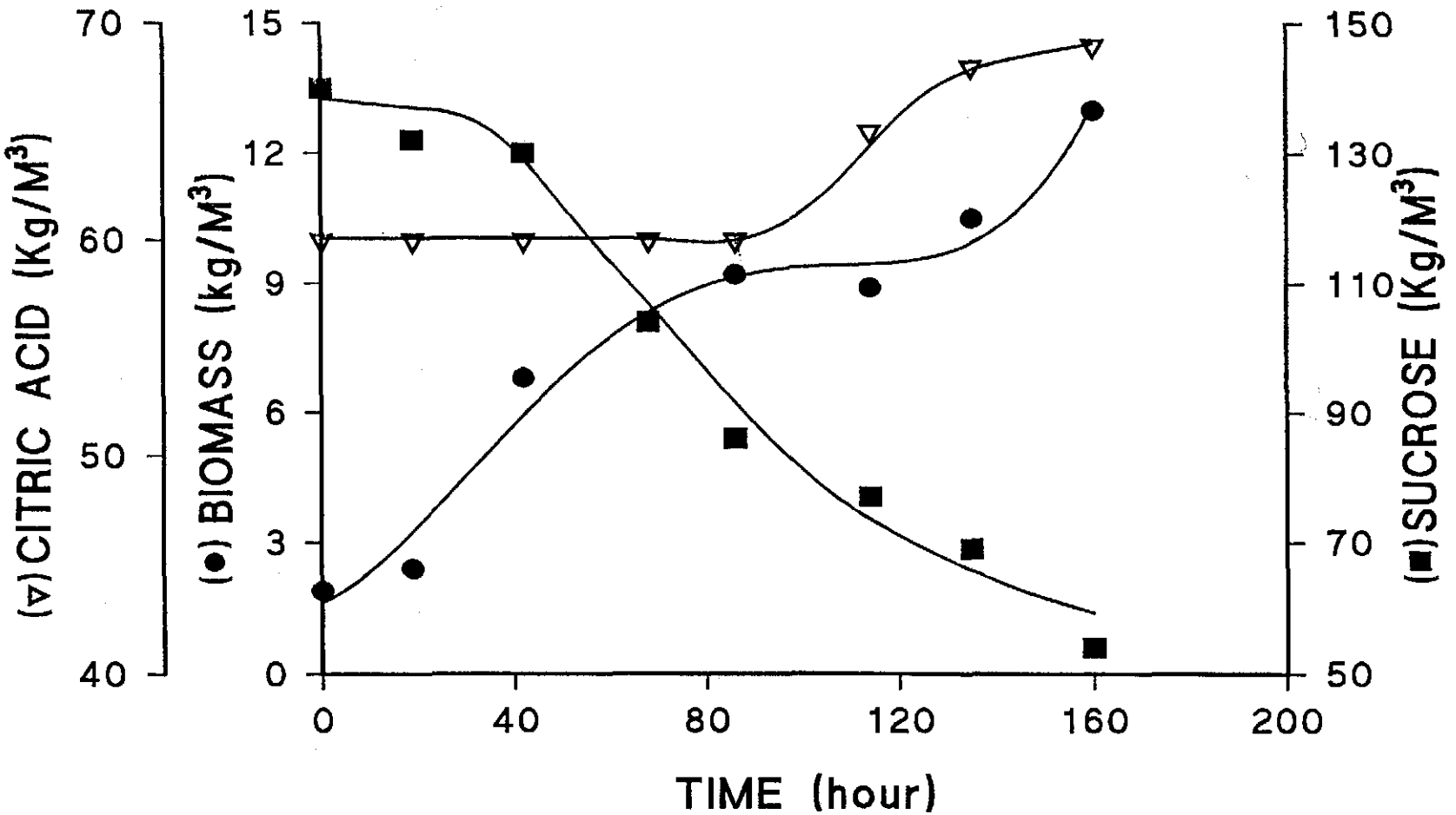


Fig 5.1.21 The effect of initial citric acid level: 60 KgM^{-3} citric acid added.

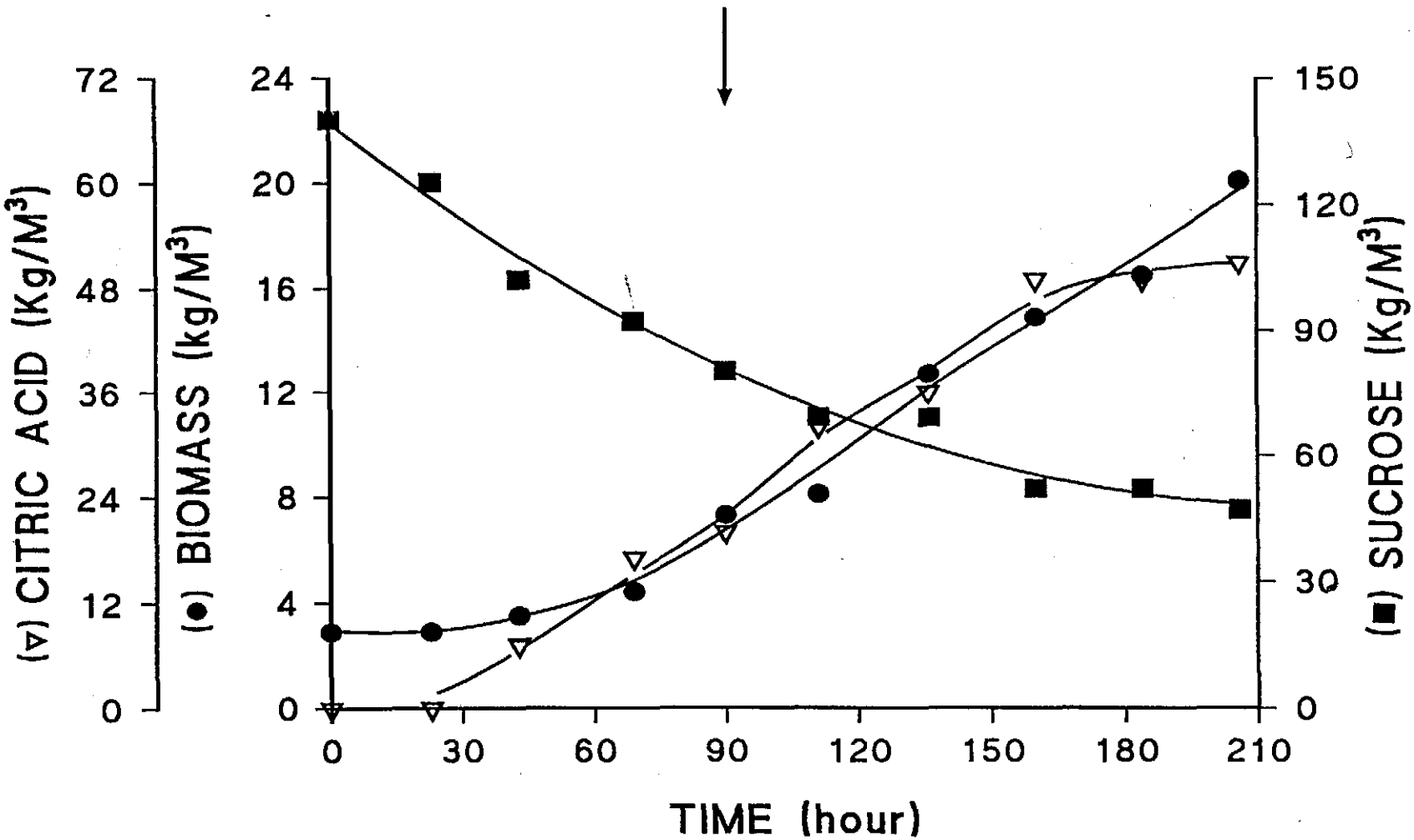


Fig 5.1.22 The effect of 300 ml, %10 H_2SO_4 addition at 90 h.

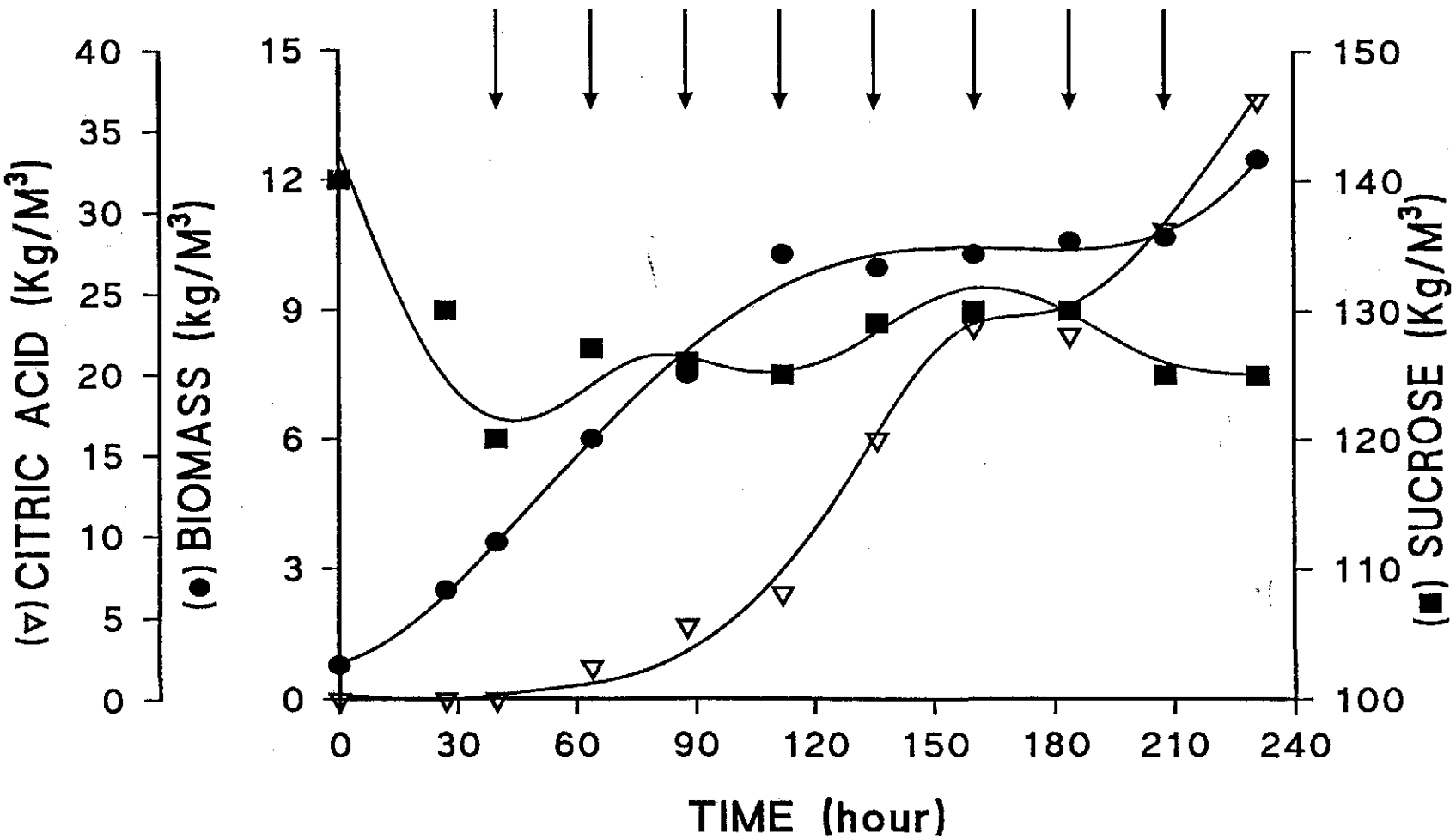


Fig 5.2.1 The effect of sucrose addition: 10 kgm^{-3} sucrose added every 24 h.

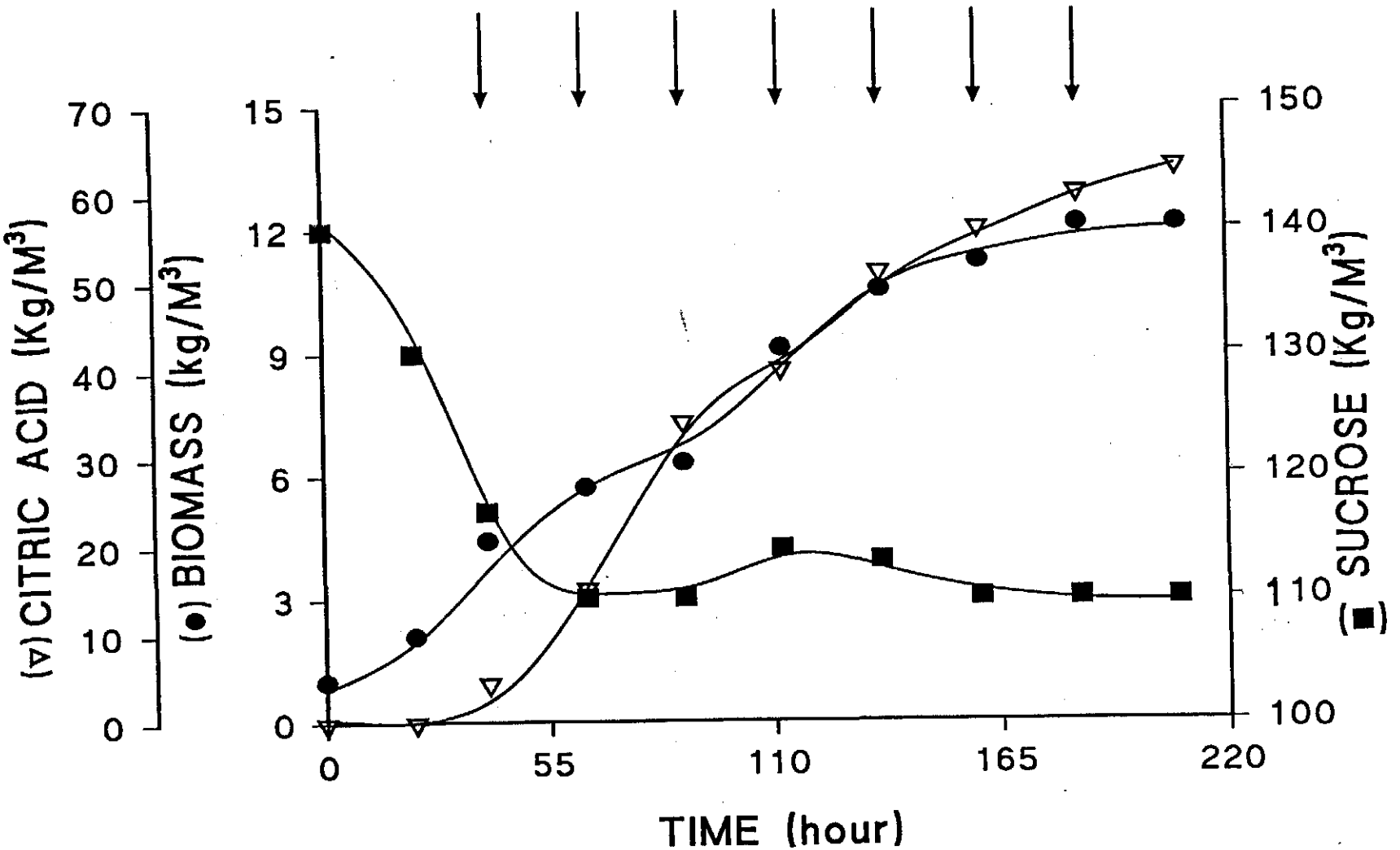


Fig 5.2.2 The effect of sucrose addition: 5 kgm^{-3} sucrose added every 24 h.

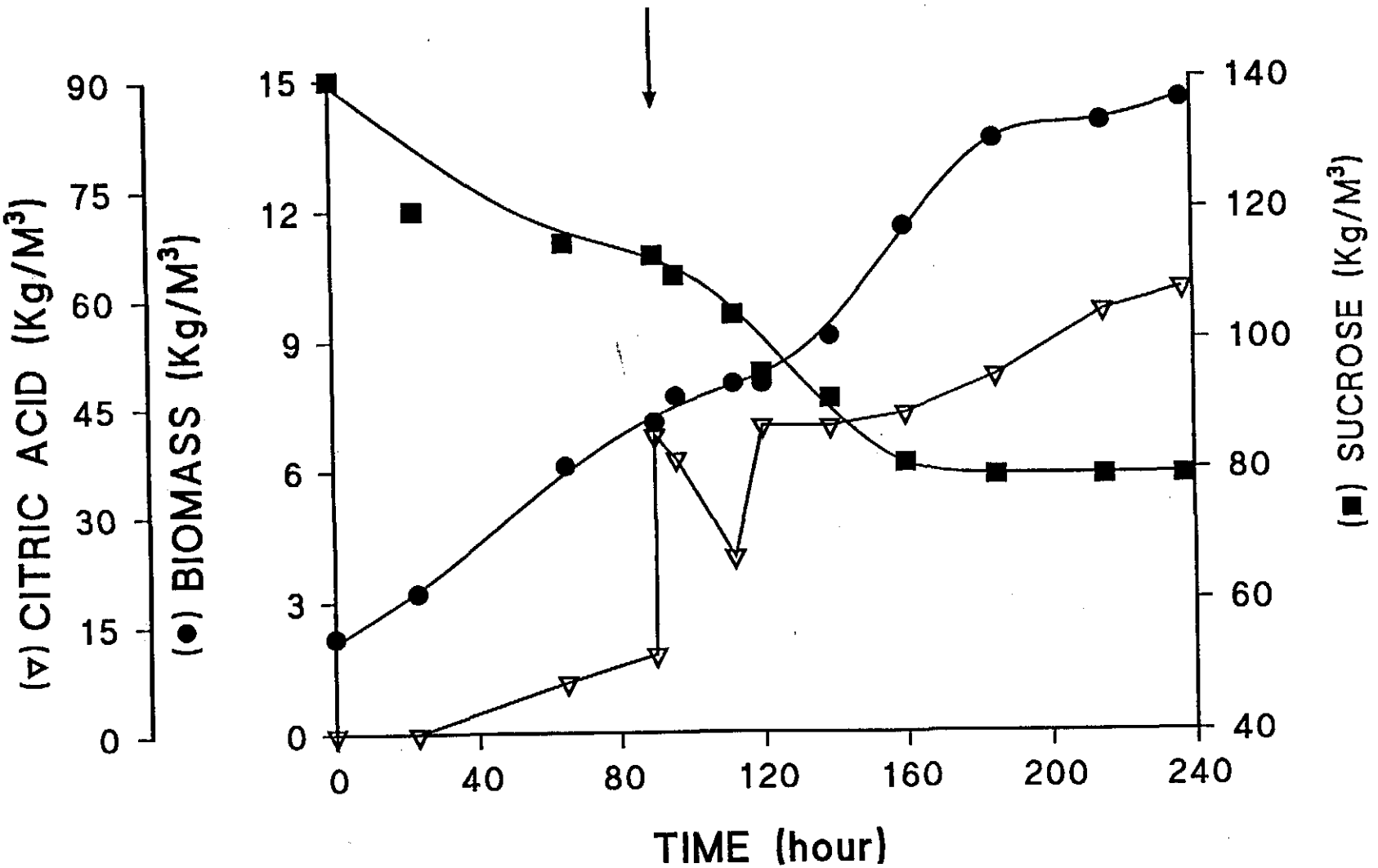


Fig 5.2.3 The effect of total amount citric acid supplementation at 90 h: 30 KgM^{-3} added.

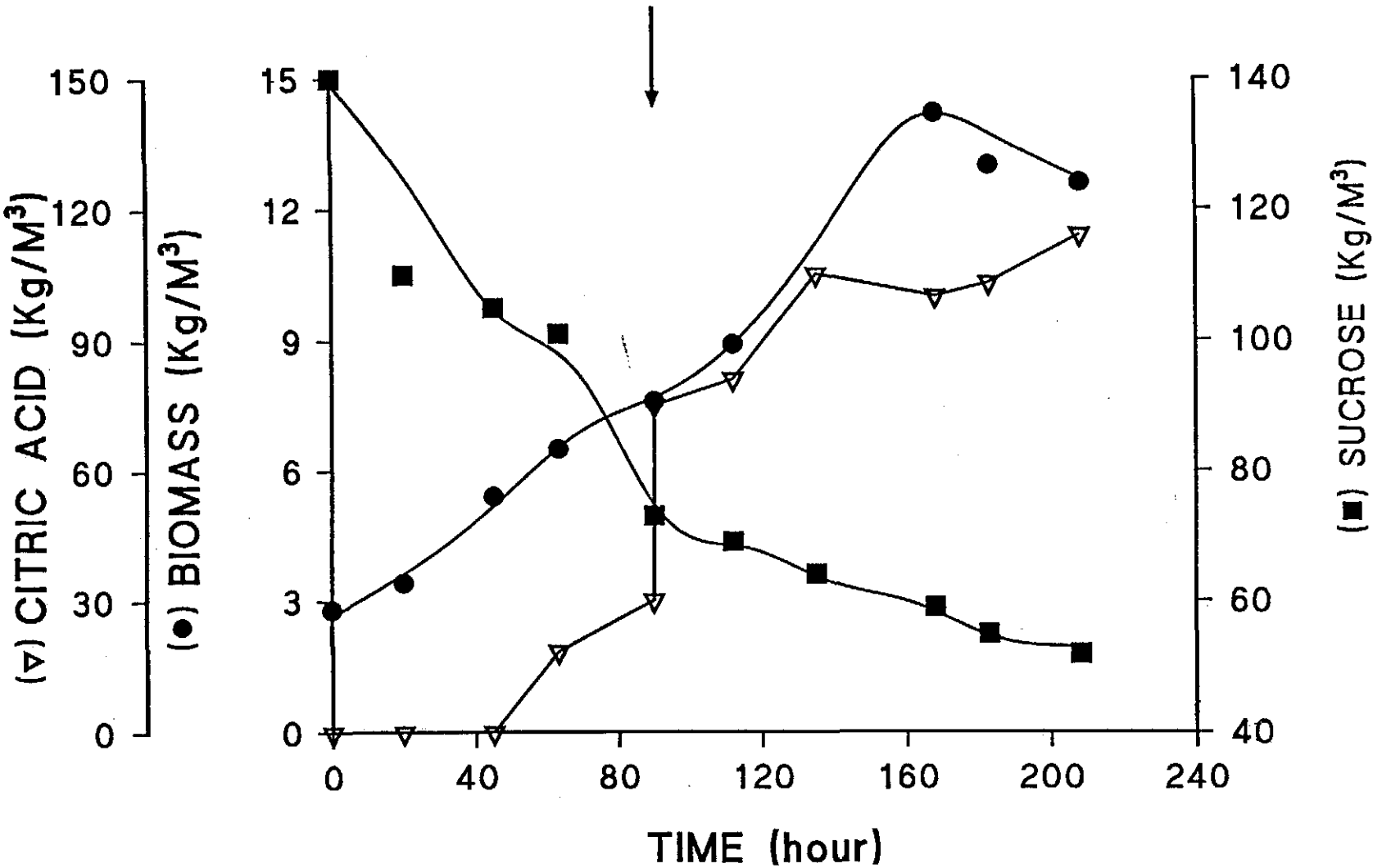


Fig 5.2.4 The effect of total amount citric acid supplementation at 90 h: 45 KgM^{-3} added.

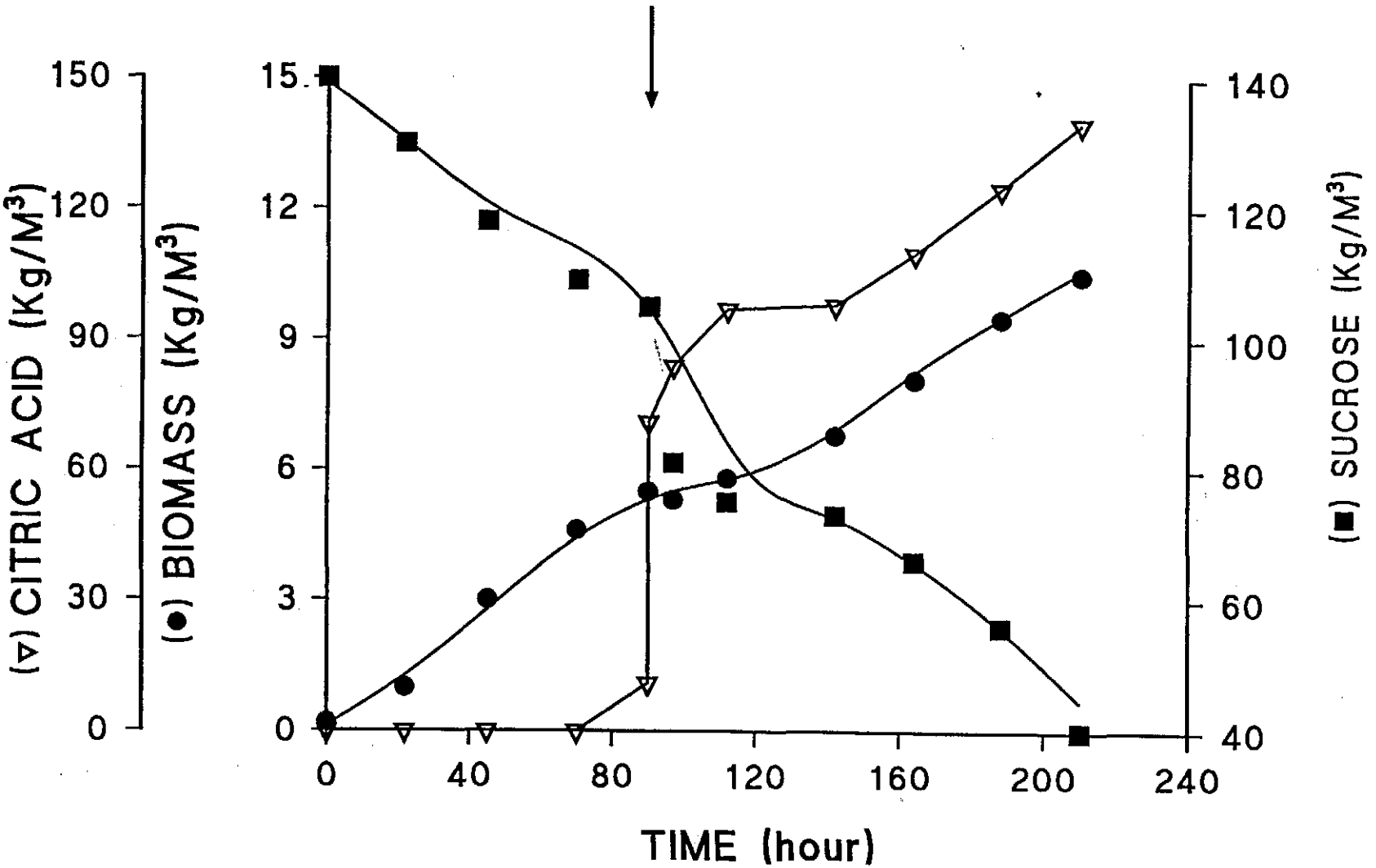


Fig 5.2.5 The effect of total amount citric acid supplementation at 90 h: 60 KgM^{-3} added.

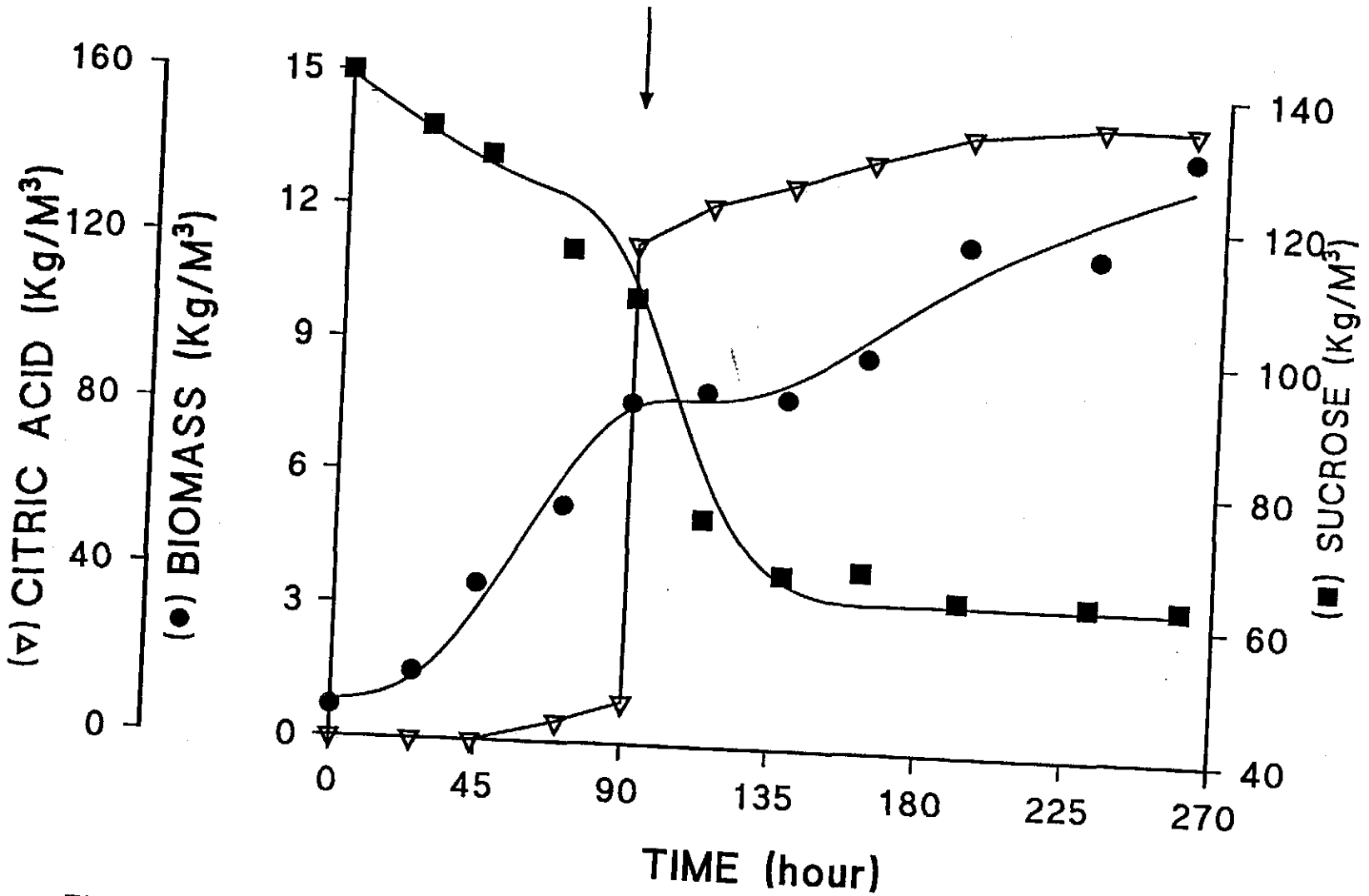


Fig 5.2.6 The effect of total amount citric acid supplementation at 90 h: 120 KgM^{-3} added.

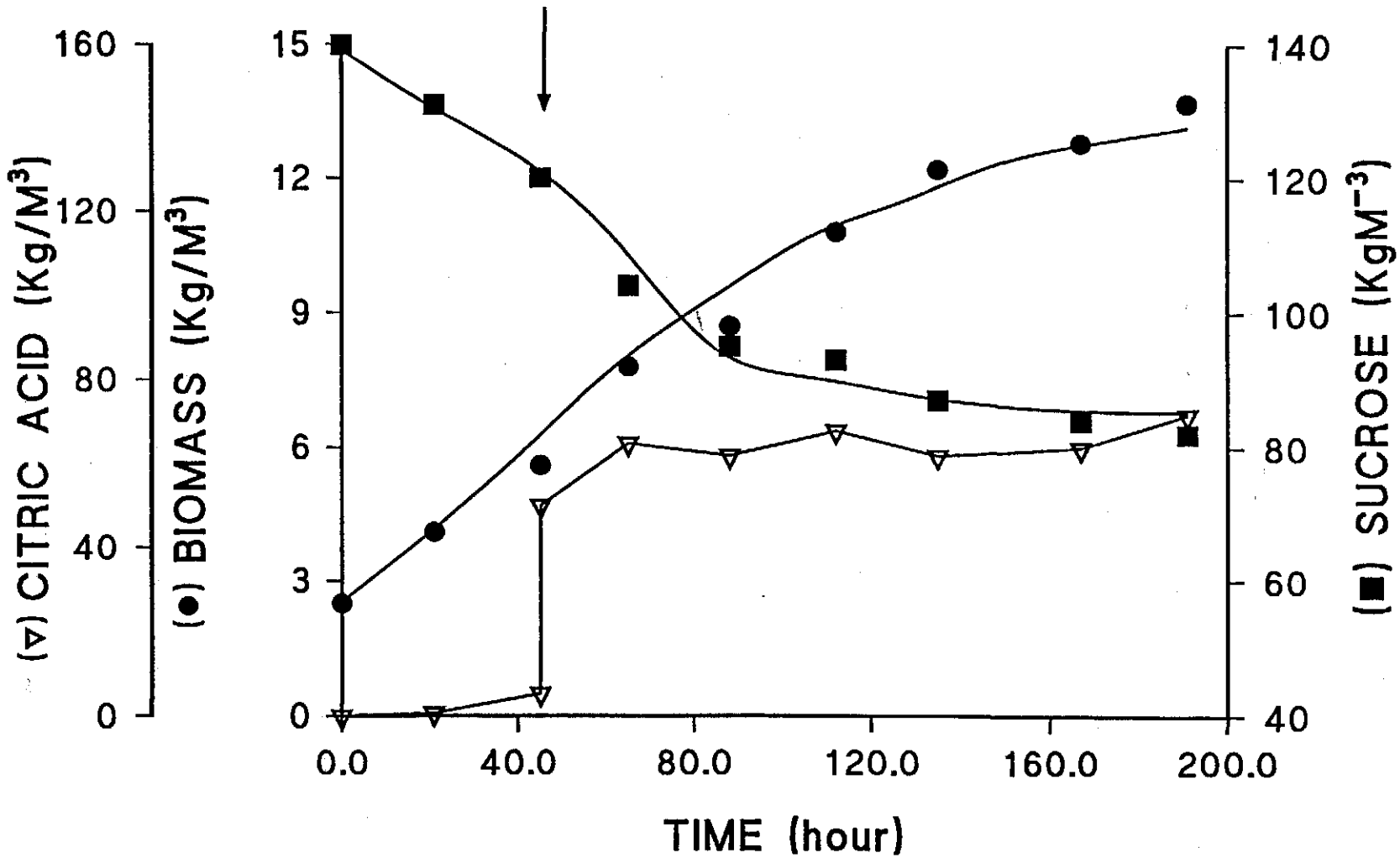


Fig 5.2.7 The effect of 60 kg/m^3 citric acid supplementation at 45 h.

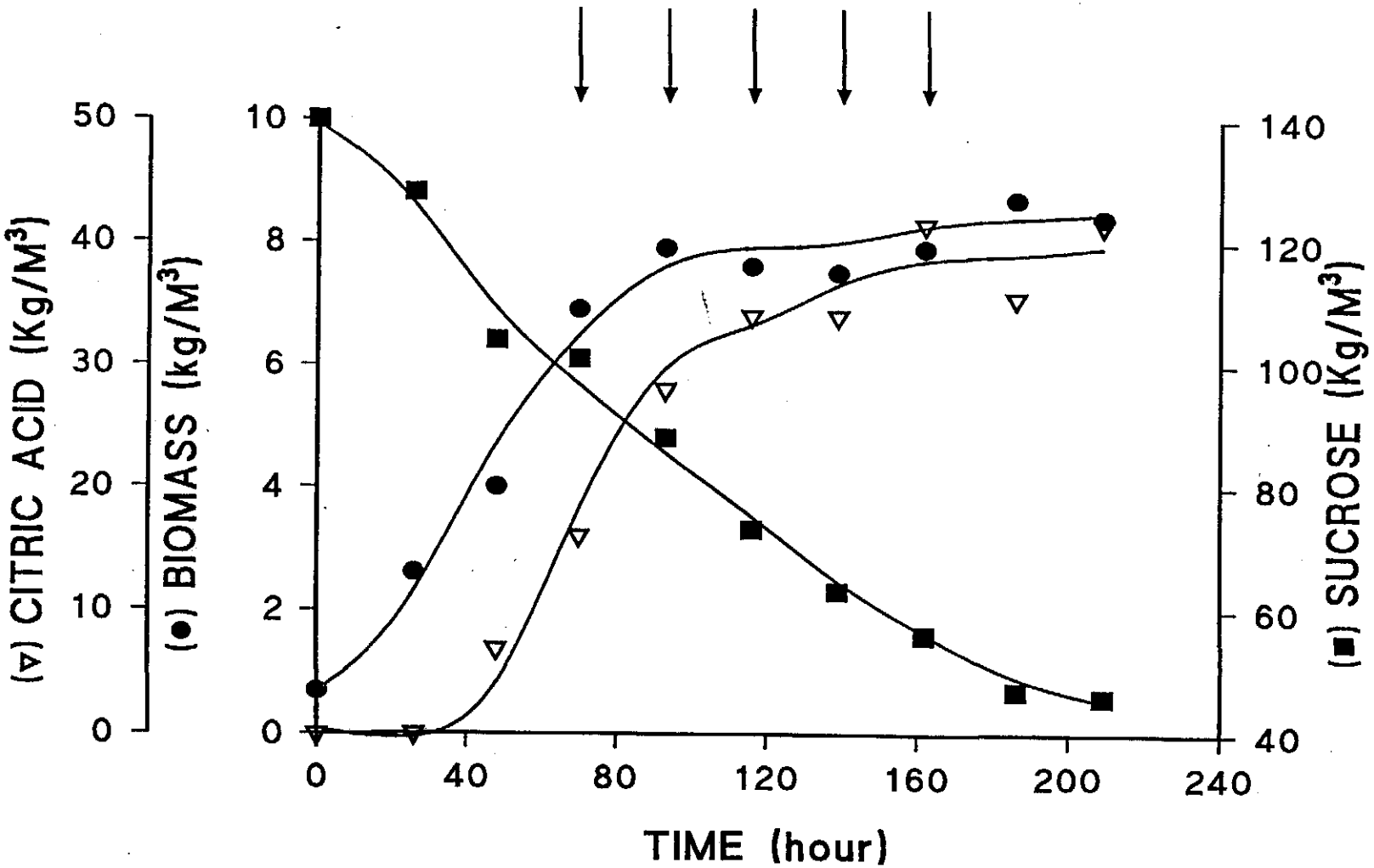


Fig 5.4.1 CYC1: 20 hr batch, 3 hr continuous phase applied.

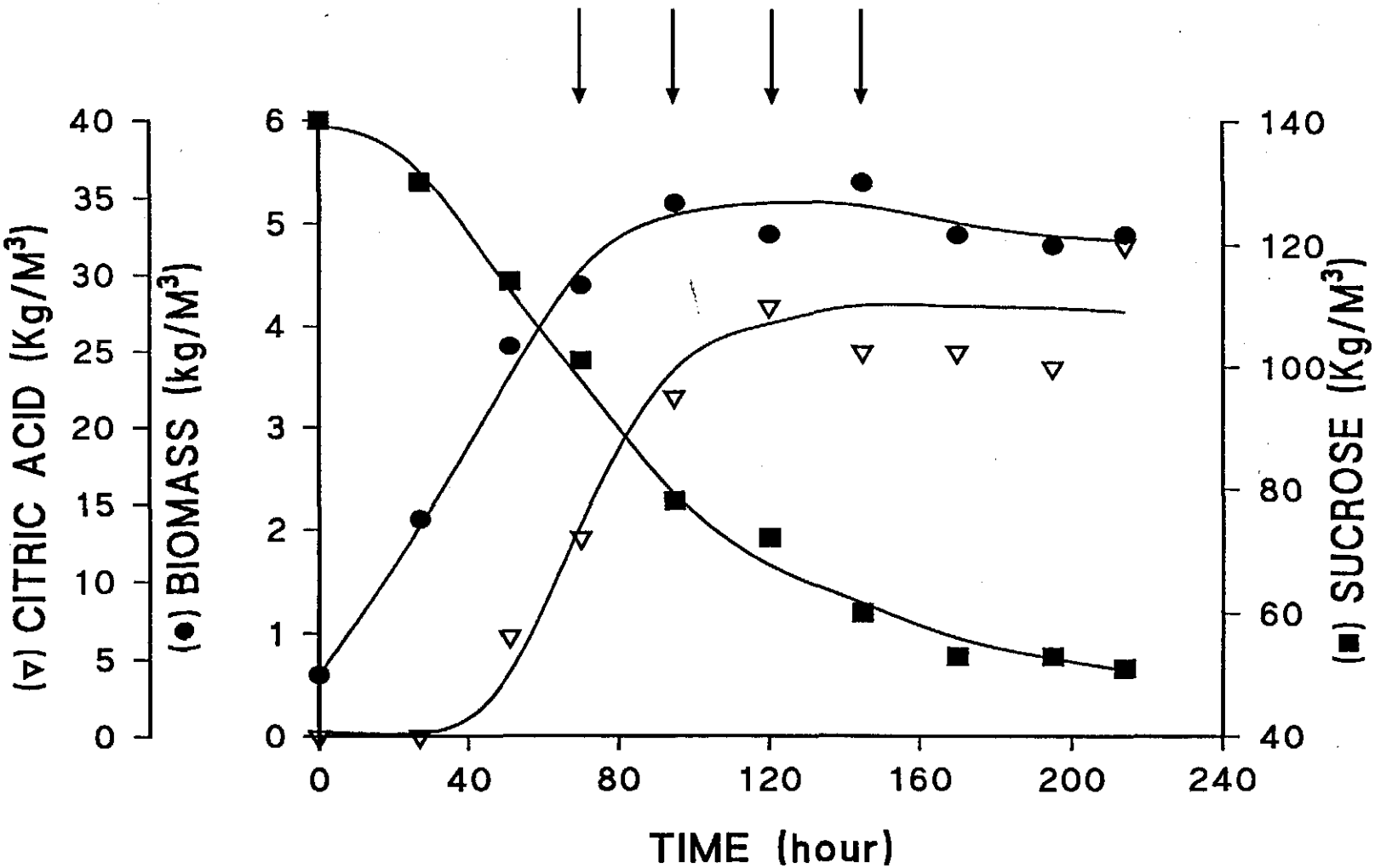


Fig 5.4.2 CYC2: 20 hr batch, 5 hr continuous phase applied.

CHAPTER 6

DISCUSSION

6.1 Standard Run

After a lag period of some 30 to 35 h biomass formation commenced. Citric acid formation appeared to have a slightly longer lag phase (40 - 50 h). The onset of citrate accumulation in the broth was associated with a greatly increased sucrose consumption rate. From the results obtained it is possible to calculate relevant yield factors and productivity.

$$Y_{x/s} = \frac{X_2 - X_1}{S_1 - S_2} = 0.131$$

$$Y_{x/N} = \frac{X_N - X_1}{N} = 7.350$$

$$Y_{P/s} = \frac{P_2 - P_1}{S_1 - S_2} = 0.678$$

$$P_{P/t} = \frac{P_2 - P_1}{t_2 - t_1} = 0.297 \text{ kg m}^{-3} \text{ h}^{-1}$$

where:

$Y_{X/S}$: Yield factor, for biomass on sucrose,

$Y_{X/N}$: Yield factor, for biomass on ammonia,

$Y_{P/S}$: Yield of citrate on sucrose,

$P_{P/t}$: Overall productivity, defined as the amount of citric acid produced divided by the time taken to produce it ($kg\ m^{-3}\ h^{-1}$),

X_2 : Final biomass concentration ($k\ g\ m^{-3}$),

X_1 : Initial biomass concentration ($kg\ m^{-3}$),

S_2 : Residual sucrose concentration ($kg\ m^{-3}$),

S_1 : Initial sucrose concentration ($kg\ m^{-3}$),

X_N : Biomass concentration at the point when N became exhausted ($kg\ m^{-3}$),

P_2 : Maximum citric acid concentration ($kg\ m^{-3}$),

P_1 : Initial citric acid concentration at time zero ($kg\ m^{-3}$),

N : Total concentration of N used ($kg\ m^{-3}$),

t_2 : Time when maximum citric acid produced (h^{-1}),

t_1 : Inoculation time (h^{-1}),

Assuming that citric acid formation follows a Luedeking and Piret (1959) type expression, then the rate of product formation (dP/dt) can be written by:

$$dP/dt = \alpha\ \mu\ X + \beta\ X$$

since

$$\mu = 1\ dX / X\ dt$$

combining the above two equations give

$$dP/dt = \alpha\ (dX/dt) + \beta\ X$$

thus

$1/X\ (dP/dt) = \alpha\ 1/X\ (dX/dt) + \beta$

By plotting 1/X (dP/dt) against 1/X (dX/dt), the values of α and β can be found. From Figure 5.1.1, the following values can be calculated by the method of numerical differentation (Sinclair and Cantero 1990).

Time h	X kg m^{-3}	P kg m^{-3}	dP/dt kg m^{-3} h^{-1}	dX/dt kg $m^{-3}h^{-1}$	1/X (dP/dt) kg^{-1}kg h^{-1}	1/X (dX/dt) h^{-1}
80	5.5	25	0.50	0.070	0.091	0.0127
90	6.4	30	0.40	0.090	0.063	0.0140
100	7.3	34	0.40	0.090	0.055	0.0123
110	8.2	38	0.30	0.080	0.037	0.0097
120	9.0	40	0.25	0.060	0.028	0.0066
130	9.5	43	0.35	0.050	0.037	0.0052
140	10.0	47	0.35	0.045	0.035	0.0045
150	10.4	50	0.20	0.040	0.019	0.0037

From Fig. 6.1.1 the values of α and β can be calculated as 3.57 kg/kg and 0.05 kg/kg h respectively. Since both α and β have positive values, citric acid production shows intermediate kinetics. Charley (1981) also noted that citric acid fermentation would show intermediate kinetics, however his value of α and β were greater than the present study.

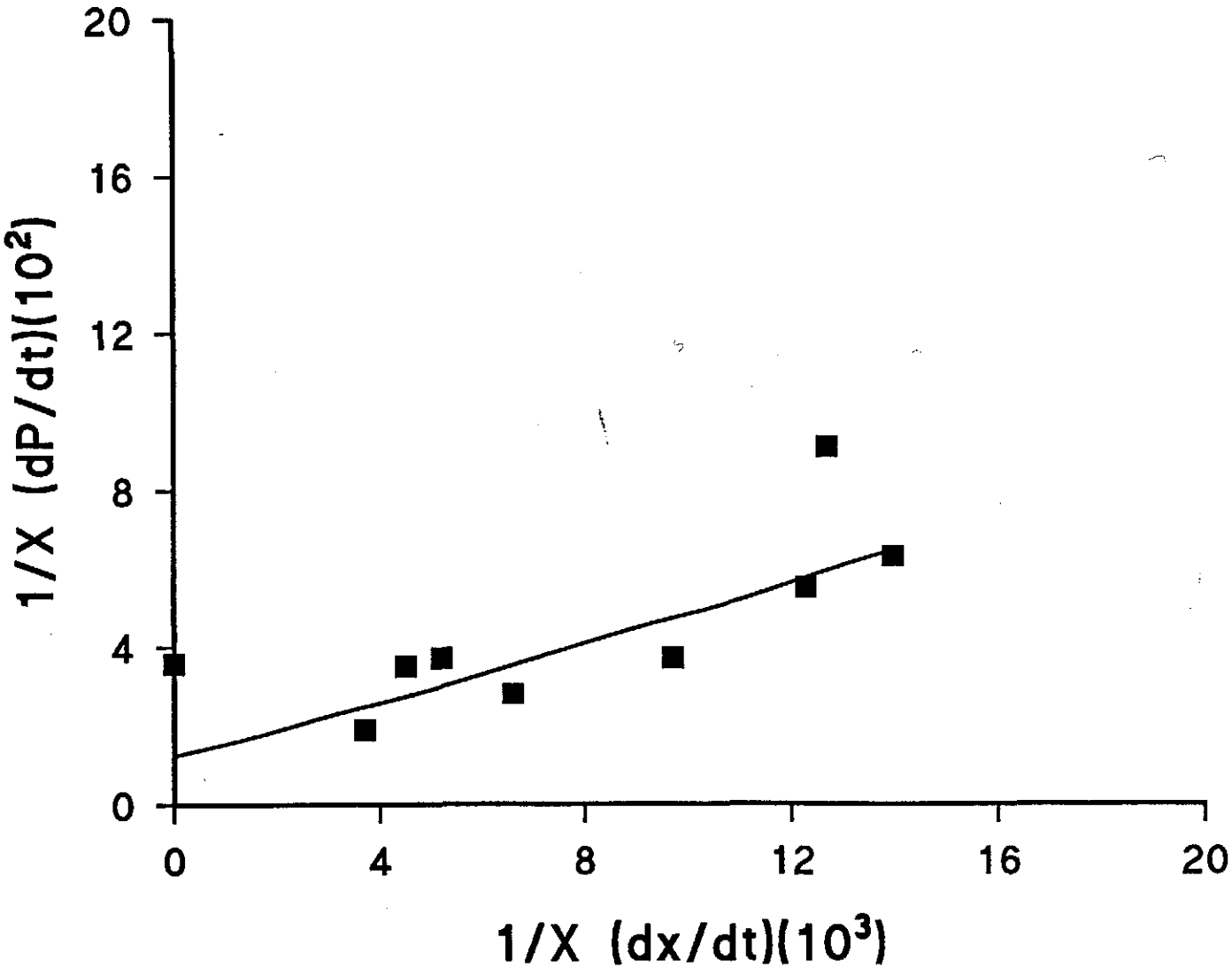


Fig 6.1.1 Determination of product formation coefficients

6.2 Effect of Ammonium Supplementation on the Citric Acid Fermentation Process

In all of the fermentations, in the series of runs with $(NH_4)_2SO_4$ added at different points during the fermentation, the maximum concentration of citric acid achieved was less than that in the standard run. Conversely, maximum dry weight increased by up to a factor of 3 (Table 6.1). Addition of 2 kg m^{-3} pulse of $(NH_4)_2SO_4$ at time 75 h led to behaviour illustrated in Fig 5.1.5. Until 75 h the fermentation process was as expected. Immediately after addition production of citric acid almost ceased. Biomass formation and sucrose consumption remained unaffected. A similar reduction in the rate of citrate formation was noted when $(NH_4)_2SO_4$ was added later in the fermentation at 100 and 140 h (Fig 5.1.6 and Fig. 5.1.7) But when addition was made at a point close the start of the acid production phase (Fig 5.1.4) production of citric acid increased sharply and productivity of 0.409 kg m^{-3} h^{-1} and yield factor ($Y_{X/S}$ for biomass on substrate) of 0.173 was reached. However, the maximum citric acid concentration still remained less than that achieved under standard conditions. It is likely that the amount of $(NH_4)_2SO_4$ added is so high (2 kg m^{-3}) that the cells do not suffer ammonium exhaustion, thus growth, but not citrate formation is stimulated. It has been reported that production of citric acid occurs only when cells suffer ammonium limitation (Kristiansen and Sinclair 1978).

From these results it can be seen that not only timing of the addition point, but also the total amount of supplemental ammonium added are important. This was examined in the following experiment. When the amount of $(NH_4)_2SO_4$ added at 75 h was reduced to 0.5 kg m^{-3} production of citric acid was inhibited until the $(NH_4)_2SO_4$ added was consumed, then citric acid concentration started to increase again (Fig 5.1.10). An increase in the maximum concentration of citric acid was achieved relative to the standard run (Table 6.2). The yield factor is 0.170 and productivity 0.326 kg m^{-3} h^{-1} with a run duration of some 187 hours. Both yield and productivity are higher than that achieved in the standard condition. This increase in the citrate concentration was associated with an increase in the maximum biomass achieved. The mechanism thus would appear to be an indirect one, mediated by formation of new cells which on ammonium exhaustion act to synthesise extra citric acid.

6.3 Effect of Citrate Supplementation on Citric Acid Fermentation Process

In Table 6.1 and 6.2, it can be seen that there are differences between maximum and final citric acid concentration in some runs. A similar decline in citrate concentration in the broth has been noted by other workers (Srivastava and Gupta 1982, Diokno-palo *et al.* 1984, Roukas and Kotzekidau 1986). The decline in citric acid concentration in these runs after the maximum is achieved

Table 6.1 Effect of time of addition of 2 kg m^{-3} $(NH_4)_2SO_4$

Time of Addition pulse h	Maximum Citric Acid kg m^{-3} (at time h)	Final Citric Acid kg m^{-3} (at time h)	Maximum Dry Weight kg m^{-3}	Residual Sucrose kg m^{-3}	Yield Factor $Y_{P/S}$ $Y_{X/S}$	Yield kg m^{-3}	Productivity h^{-1}
0	10.3(162)	9.5(214)	20.7	76.0	0.294	0.161	0.061
25	22.1(95)	21.2(184)	19.7	90.0	0.326	0.442	0.233
40	47.1(115)	12.8(255)	23.9	11.0	0.172	0.365	0.409
75	47.4(216)	47.4(216)	34.0	34.0	0.315	0.447	0.219
100	43.8(288)	43.8(288)	26.0	13.3	0.268	0.366	0.152
140	43.6(164)	38.4(280)	26.0	3.8	0.199	0.320	0.154

Table 6.2 Effect of amount $(NH_4)_2SO_4$ added at 75 hours

$(NH_4)_2SO_4$ Added kg m^{-3}	Maximum Citric Acid kg m^{-3} (at time h)	Final Citric Acid kg m^{-3} (at time h)	Maximum Dry Weight kg m^{-3}	Residual Sucrose kg m^{-3}	Yield Factor $Y_{X/S}$	Yield $Y_{p/S}$	Productivity kg m^{-3} h^{-1}
0.00	57.0(192)	57.0(192)	12.5	56.0	0.131	0.678	0.297
0.25	63.5(237)	27.5(257)	18.5	30.3	0.151	0.577	0.268
0.50	64.7(233)	47.0(280)	22.4	18.4	0.170	0.532	0.278
1.00	51.7(238)	36.1(285)	32.6	19.7	0.255	0.429	0.189
2.00	47.4(216)	47.4(216)	34.0	34.0	0.315	0.447	0.219

could be due to utilisation of citrate (especially where sucrose levels are low or limiting) and re-entry of citrate into the mycelium. Althought Charly (1981) has reported that the citrate is not metabolised, Mattey has indicated that A. niger (72-4) can oxidise citrate (1977). Similarly, it has been shown that citrate can re-entre the mycelium under certain conditions, thus effectively lowering external concentration (Mattey 1977). The addition of various concentrations of exogenous citric acid during acidogenesis in batch fermentations may allow us to ascertain whether uptake of citric acid can occur.

A number of runs were carried out involving addition of citric acid in various amounts. As can be seen in Table 6.3, addition of 30 and 120 kg m^{-3} citric acid at 90 h had negative effects on citric acid production and final concentration of produced citric acid reached only 32 and 31 kg m^{-3} respectively. Consideration of Fig 5.2.3 leads to the conclusion that citrate may be metabolised or may be reentering the mycelium.

Addition of 45 kg m^{-3} citrate led to a 21% increase (final citric acid, 69 kg m^{-3}) and addition of 60 kg m^{-3} citrate led to a 40.3% increase in the final concentration (80 kg m^{-3}).

The time at which citrate addition occurred is also critical (Table 6.4). When 60 kg m^{-3} citrate was added at time 0 and 45 h only 9 and 12 kg m^{-3} of citric acid was produced respectively but there was no effect on maximum biomass concentration. Other workers noted that addition

of citric acid at time 0 h, inhibited citric acid production rate (Charley 1981). Charley also claimed that the addition of 25 and 50 kg m^{-3} citric acid led to an inhibition of the growth rate which is calculated as μ_{max} from Fig 6.3.3 , 0.257, 0.098 and 0.022 for any added citric acid, in the presence of 25 and 50 kg m^{-3} added citric acid respectively.

Chemiel (1977) studied the effect of adding various concentrations of citric acid on product formation rate and mycelial growth in shake flask cultures and found that citric acid addition inhibited both product formation rate and mycelial growth. The effect on product formation rate was modelled by the equation

$$dP/dt = V_p \text{ —— } k_pP$$

where

V_p : theoretical maximum rate of citric acid production ($kgm^{-3}h^{-1}$),

k_p : inhibition constant (h^{-1}).

Chemiel (1977) suggested that citric acid caused inhibition not only by decreasing the pH of the medium but also by a specific metabolic effect. Whereas in the present study decreasing the pH of the culture from 1.8 to 1.3 by a one shot addition of 300 ml 10 % H_2SO_4 (Run B23, Fig 5.1.22.) led to increased biomass levels of 20.1 kg m^{-3} but final citrate concentration decreased to 51 kg m^{-3}.

Table 6.3 Effect of amount citric acid added at 90 h.

Addition of Citric Acid kg m^{-3}	Produced Citric Acid kg m^{-3}	Maximum Dry Weight kg m^{-3}	Sucrose Consumed kg m^{-3}	Yield $Y_{P/S}$	Productivity kg m^{-3} h^{-1}
30	31	14.5	61	0.51	0.13
45	69	12.6	88	0.78	0.33
60	80	10.5	100	0.80	0.38
120	32	11.3	77	0.42	0.14

Table 6.4 Effect of addition time of 60 kg m^{-3} citric acid

Time of added Citric Acid h	Produced Citric Acid kg m^{-3}	Maximum Dry Weight kg m^{-3}	Sucrose Consumed kg m^{-3}	Yield $Y_{P/S}$	Productivity kg m^{-3} h^{-1}
0	9	13.2	86	0.105	0.056
45	12	13.7	58	0.207	0.063
90	80	10.5	100	0.800	0.380

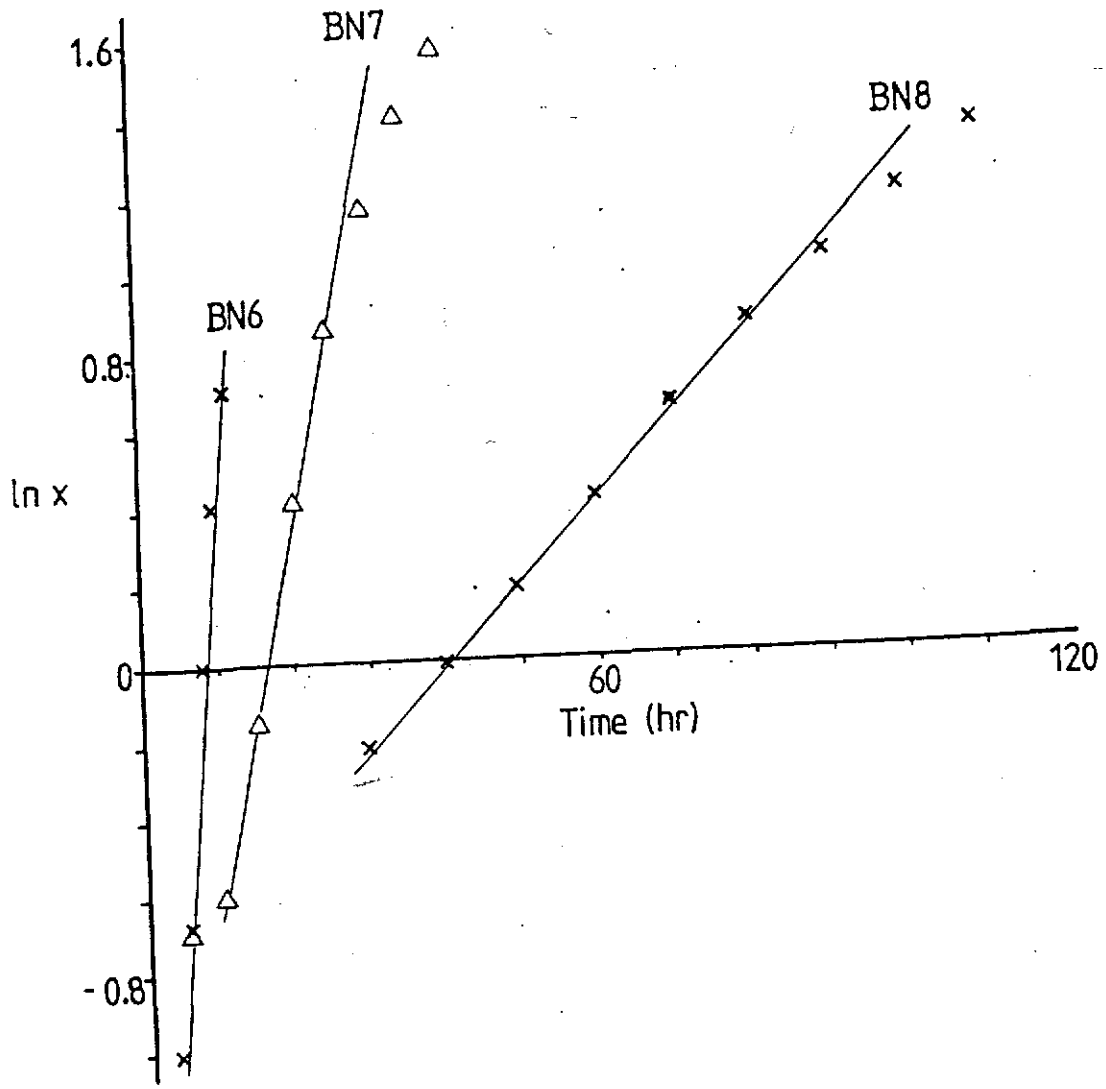


Fig 6.3.3 : Determination of μ_{max} for absence of any added citric acid (BN6) and in the presence of 25 and 50 kg m^{-3} added citric acid (BN7 and BN8) taken from reference (Charley 1981)

6.4 Effect of Sucrose Supplementation on Citric Acid Production Process

The addition of sucrose to a broth at concentration of 10 kg m^{-3} once every 24 hours after 40 hours led to a 35% decrease in citric acid concentration with final citrate concentration being 37 kg m^{-3} (Fig 5.2.1). During the course of the fermentation a total of 95 kg m^{-3} sucrose consumed. So the yield on sucrose, $Y_{P/S}$, is 0.39 which is 43 % less than that achieved in standard run. One possible explanation for the observed decrease in citrate concentration and yield on sucrose might be inhibition due to high levels of residual sucrose in the broth. In almost all fermentations carried out citrate production rate increased when sucrose level fell below 110 kg m^{-3} (appendix 1). It also was noted that in FB1 sucrose level was never less than 125 kg m^{-3} (Fig 5.2.1). This high level of sucrose should have led to decreased rates of production.

The addition of sucrose to a broth at concentration of 5 kg m^{-3}once every 24 hours after 40 hours led to a 10.5% increase in citric acid concentration with final citrate concentration being 63 kg m^{-3} (Fig 5.2.2). During the course of the fermentation a total of 85 kg m^{-3} sucrose consumed. So the yield ($Y_{P/S}$) is 0.74 which is 9 % greater than that of standard run in which the citric acid concentration was 57 kg m^{-3} and yield, $Y_{P/S}$, was 0.68.

6.5 Effect of Initial Ammonium Concentration on Citric Acid Production

The work of Xu *et al.* (1989), which examined the effect of nitrogen sources on citric acid production by A. niger, confirmed that ammonium sulphate or ammonium nitrate were the most suitable N sources for citric acid production. But there is a disagrement in the literature about the most suitable initial concentration of $(NH_4)_2SO_4$. Xu *et al.* (1989) noted that the optimal concentrations of ammonium sulphate were 5.0 and 2.5 kg m^{-3} for the submerged and the filter paper cultivation system, respectively by A. niger B60 after 5 day cultivation. However, Kubicek and Rohr (1986) reported that the concentration of the nitrogen source is 0.1 to 0.4 g N per litre, which is equivalent to 0.48 and to 1.88 kg m^{-3} $(NH_4)_2SO_4$.

In the present study, a series of fermentations was carried out varying the initial concentration of $(NH_4)_2SO_4$, in the medium between 0,5 and 4.0 kg m^{-3}. Dry weight increased with ammonium sulphate concentration. However, increasing the $(NH_4)_2SO_4$ concentration above 3.0 kg m^{-3} resulted in a drastic reduction in citric acid concentration (Table 6.5) The best initial $(NH_4)_2SO_4$ level was found to be 3 kg m^{-3} which produced the highest citric acid concentration (81 kg m^{-3}). Productivity (P/t) also increased with increasing initial concentration of $(NH_4)_2SO_4$ up to 3.0 kg m^{-3}, but beyond this point both productivity (P/t) and yield ($Y_{P/S}$) were severely reduced (Fig 6.5.1). As can be seen from Fig 6.5.2, when the

initial $(NH_4)_2SO_4$ level is 4 kg m^{-3} there is an ammonium excess in the fermentation liquor. These results supports the citric acid production theory of Kristiansen and Sinclair (1978, 1979) that citric acid production occurs only under conditions of nitrogen limitation. Thus production occurs only when the ammonium supply is fully used up.

6.6 Effect of pH on Citric Acid Fermentation Process

After adjusting initial pH of culture to 6.5, the culture pH was allowed to drop to 1.8, Fig 5.1.14 and 2.5, Fig 5.1.19 then controlled at that value. Under these conditions maximum citric acid concentration reached 58.2 and 60.0 kg m^{-3} respectively. It is clear that there is no significant difference in terms of the maximum citric acid concentration achieved between pH 1.8 and 2.5, but when final pH of culture was kept at 3.0 (Fig 5.1.18), maximum citric acid concentration was reduced to only 37.5 kg m^{-3}.

It can be seen that (Table 6.6) culture pH should thus be controlled between 1.8 and 2.5. This result differs somewhat from the results of other workers (Charley 1981, Kubicek and Rohr 1986) who have indicated that pH should be kept below 2.0 to achieve highest citric acid production. Charley (1981), who studied pH effects over the range 1.7 to 2.9 found that final yield of citric acid increased almost linearly with decreasing pH and the optimal final pH for batch fermentation was 1.7. This was supported by the findings of Kubicek and Rohr who

recommended that pH should be kept low (below 2.0). According to that report at higher pH's, A. niger accumulates gluconic acid, especially when the pH is around 4.0 (1986). Marison (1988) also reported that pH should be maintained around 2.0.

Initial pH was found to be critical for citric acid production. When the initial pH was adjusted to 2.0 (Fig 5.1.16), the maximum citric acid concentration was 41.5 kg m^{-3}. When it was adjusted to 2.5 (Fig 5.1.17), maximum citrate concentration reached 66 kg m^{-3} which is 15.8 % higher than that standard conditions (Fig 5.1.1.). Although there is a marked difference between 2.0 and 2.5 in terms of final product concentration no important changes were observed in yield or final product concentration above 2.5 (Table 6.7). Thus an initial pH of 2.5 is the clear optimum. However there is no agreement in the literature about optimal initial pH. Moyer (1953) found that initial pH did not have any effect within the range 1.95 to 3.10. Khan and Ghose (1973), however, reported that citric acid yield increased with increasing pH. The optimal initial pH was found to be 6.5. on the other hand, optimal initial pH has been reported to be 2.8 and 2.5 by Kamal and Gupta (1982), Jernejc *et al.* (1982) respectively. However, in the last two studies the work was carried out in shake flask cultures with the usual associated drawbacks, thus it is often difficult to make valid comparisons.

Table 6.5 Efffect of initial concentration of $(NH_4)_2SO_4$

Initial Amount of $(NH_4)_2SO_4$ kg m^{-3}	Maximum Citric Acid kg m^{-3} (at time h)	Maximum Dry Weight kg m^{-3}	Sucrose Consumed kg m^{-3}	Yield $Y_{P/S}$	Productivity kg m^{-3} h^{-1}
0.5	15.0(207)	8.4	41	0.365	0.072
1.0	40.0(210)	10.9	72	0.556	0.190
1.5	58.2(185)	14	91	0.639	0.314
2.0	57.0(192)	12.5	84	0.678	0.297
3.0	81.0(162)	12.7	131	0.618	0.500
4.0	10.3(162)	20.3	64	0.161	0.063

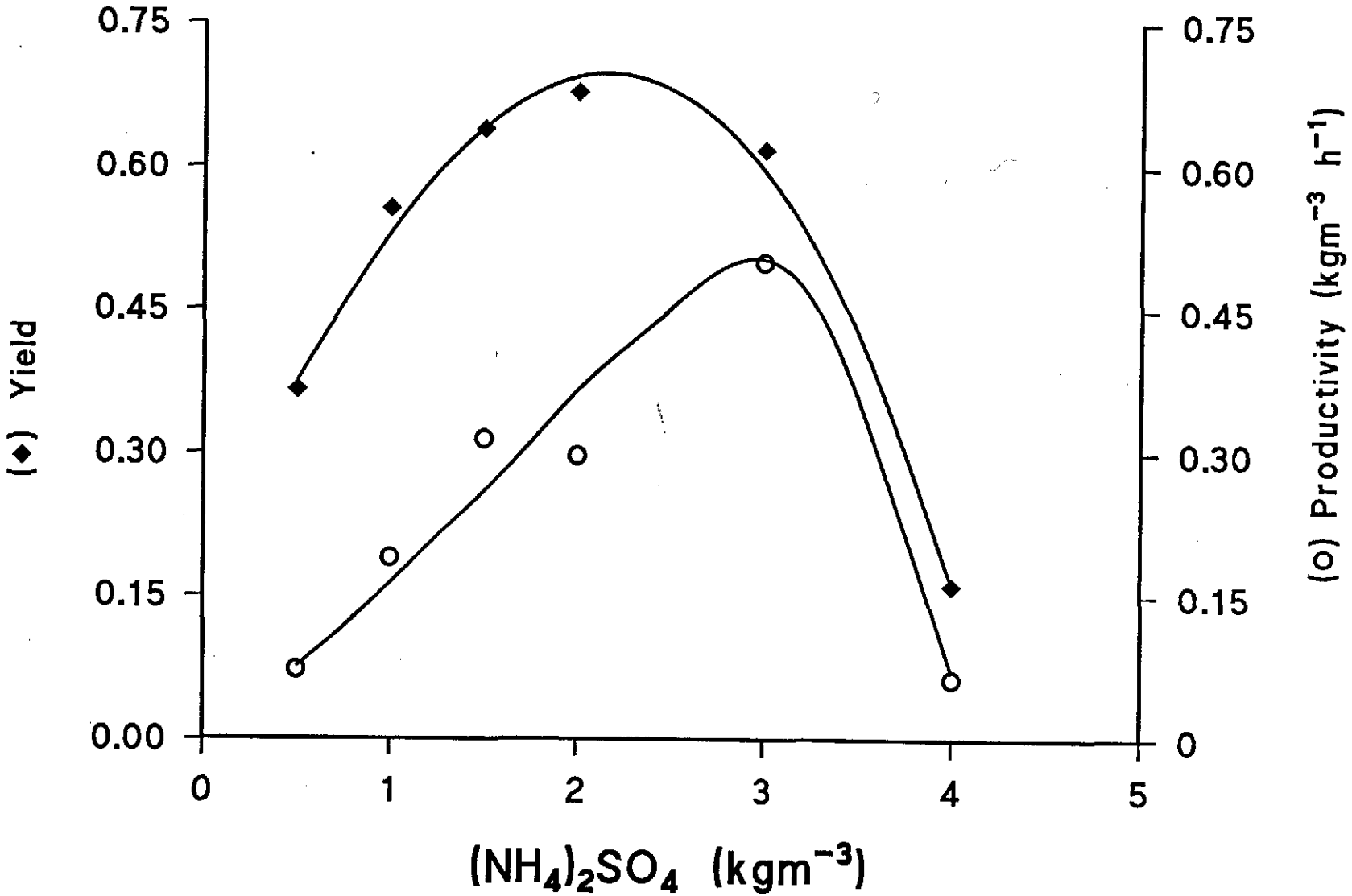


Fig 6.5.1 Effect of initial concentration of $(NH_4)_2SO_4$ on yield and productivity

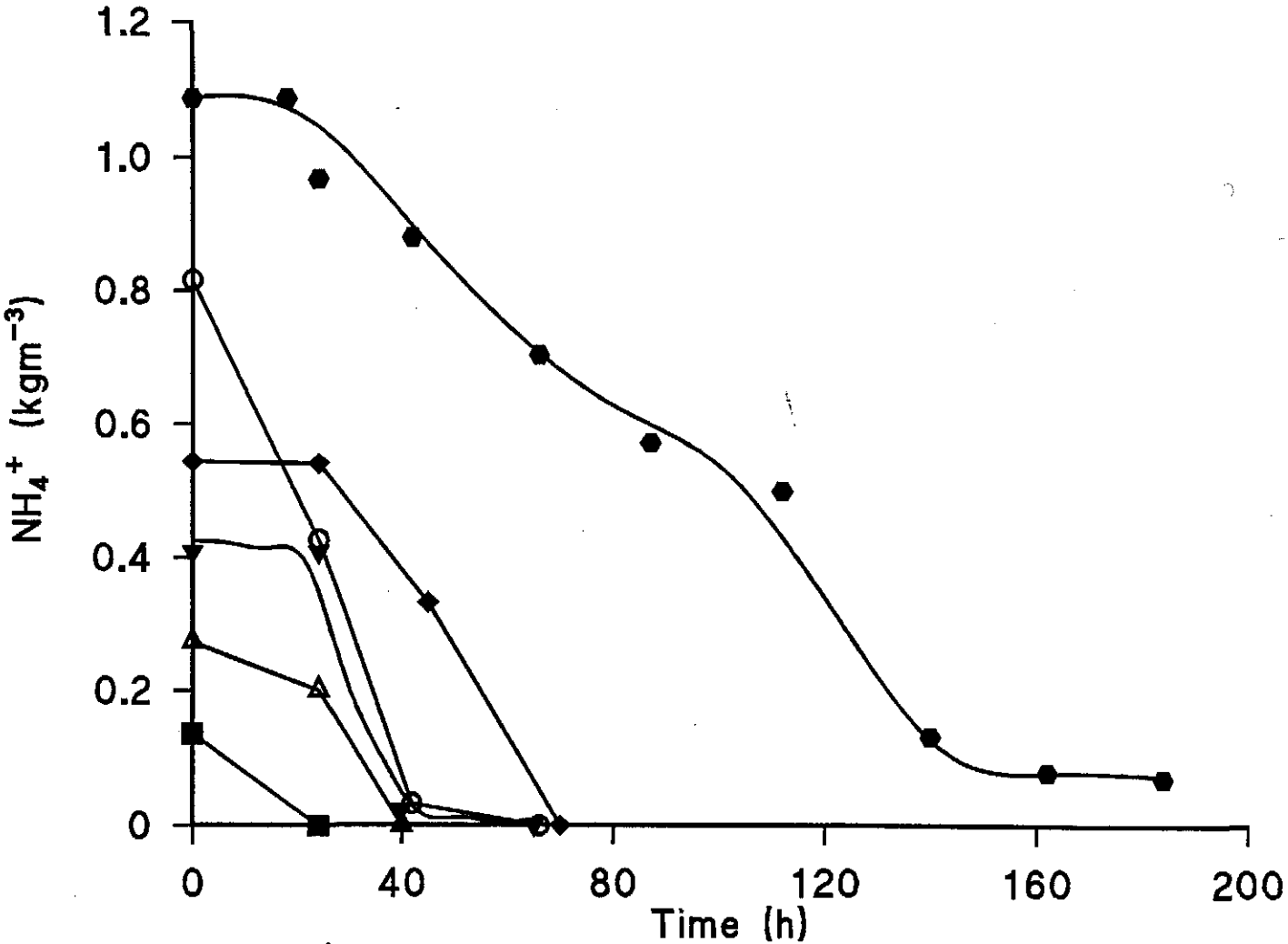


Fig 6.5.2 NH_4^+ concentration versus time [Initial concentration of $(NH_4)_2SO_4$ is 0.5(■), 1.0 (△), 1.5 (▼), 2.0 (◆), 3.0 (○) and 4.0 (●) kgm^{-3}

Table 6.6 Effect of final pH on citric acid production

Final (and Initial) pH	Maximum Citric Acid kg m^{-3}	Maximum Dry Weight kg m^{-3}	Sucrose Consumed kg m^{-3}	Yield $Y_{P/S}$	Productivity kg m^{-3} h^{-1}
1.8(6.5)	58.2	14.0	91	0.640	0.309
2.5(6.5)	60.0	13.2	82	0.750	0.258
3.0(3.0)	37.5	16.0	62	0.605	0.162

Table 6.7 Effect of initial pH on citric acid production

initial (and final) pH	Maximum Citric Acid kg m^{-3}	Maximum Dry Weigh kg m^{-3}	Sucrose Consumed kg m^{-3}	Yield $Y_{P/S}$	Productivity kg m^{-3} h^{-1}
6.5(1.8)	58.2	14.0	91	0.640	0.309
4.5(2.5)	55.0	14.3	81	0.679	0.238
2.5(2.5)	66.0	16.7	92	0.717	0.286
2.0(2.0)	41.5	13.1	71	0.584	0.170

6.7 Continuous Culture and Periodic Operation

In order to carry out continuous cultivation it is useful to know the μ_{max} of the strain to be cultivated. If μ_{max} is smaller than dilution rate, D, washout occurs. Thus μ_{max} estimation can be carried out by washout operation using the following equations.

$$dX/dt = (\mu - D)\ X$$

$$\mu = \mu_{max} \frac{N}{k_n + N} \qquad \text{and}$$

if $N >> k_n$ (k_n is saturation constant) then $\mu = \mu_{max}$

thus

$$dX/dt = (\mu_{max} - D)\ X$$

becomes

$$\ln X = (\mu_{max} - D)\ t + \ln X_o$$

As can be seen from above, during the washout the slope of ln X against t will equal $(\mu_{max} - D)$, thus μ_{max} can be calculated. At the end of the washout period, cells continue to grow at the maximum rate during the initial period of batch fermentation. Therefore during this period the slope of ln X against t is equal to μ_{max} as well.

μ_{max} was established at pH 1.8, 2.5 and 3.0 via washout/batch cycle and was found to be 0.09 h^{-1} (Fig 6.7.1), 0.11 h^{-1} (Fig 6.7.2) and 0.12 h^{-1} (Fig 6.7.3) respectively. There is broad agreement between the present study and the results of other workers (Kristiansen 1976, Charley 1981) who have shown that μ_{max} depends on pH and

increases with increasing pH. However, a close examination of the first 10 hours of a standard batch run reveals a lower specific growth rate of around 0.065 h^{-1} (Fig 6.7.4). This somewhat lower growth rate may be a result of the sudden change of pH and nutrient conditions the culture is subject to, as it is transferred from the inoculum flask to the fermenter. (pH in the inoculum flask at the end of this phase is around 1.8, while that in the vessel is 6.5, likewise there is a large difference in sucrose concentrations between the two vessels. Exposure to such large differences will certainly have metabolic consequences.) After an initial acclimatization period it is likely that specific growth would rise to around 0.1 h^{-1}. All raw data which is related to μ_{max} estimation is enclosed in appendix 1.

In run C1, 4.2 kg m^{-3} citric acid was produced consuming 8 kg m^{-3} sucrose. The productivity, is 0.215 kg m^{-3} h^{-1} and yield, $Y_{P/s}$, is 0.538.

In order to increase productivity, dilution rate was increased to 0.075 h^{-1} in C2, but washout occurred although dilution rate is smaller than μ_{max} which is 0.09 h^{-1} at working pH, 1.8. A similiar problem was noted by Novak and Fencl (1973) and Carter and Bull (1969) who were studying A. niger and A. nidulans respectively. In both cases washout occurred at less than half the μ_{max} value found in batch culture. Novak and Fencly (1973) concluded that at a dilution rate of 0.075 h^{-1} the accumulation of ammonium ions stopped and nitrite appeared in the mycelium

which diffused into the medium. It was proposed that nitrite caused culture intoxication and washout at lower dilution rates.

In runs C3, C4 and C5, although working at higher pHs and thus higher μ_{max}'s the amount of formed citric acid was negligible since excess ammonium sulphate in the culture inhibited the production process (Table 5.1).

On / off operation was employed by combining standard batch culture and standard continuous culture. After initial 70 h batch phase, 3 h continuous culture was carried out. This cycle was repeated 5 times and it was observed that citrate production decreased steadily during the cycling operation. 5 h continuous / 20 h batch phase cycle (repeated 4 times) also led to a decrease in the citrate concentration compared with the standard run. Although Welles and Blanch (1976) increased ethanol production yield by a culture of S. crevisiae carring out an on / off operation pulse cycle which was similar in concept to the present study. Since the continuous medium includes $(NH_4)_2SO_4$, it might expected to lead to reduced production due to the strict requirement for N limitation before citrate synthesis can proceed.

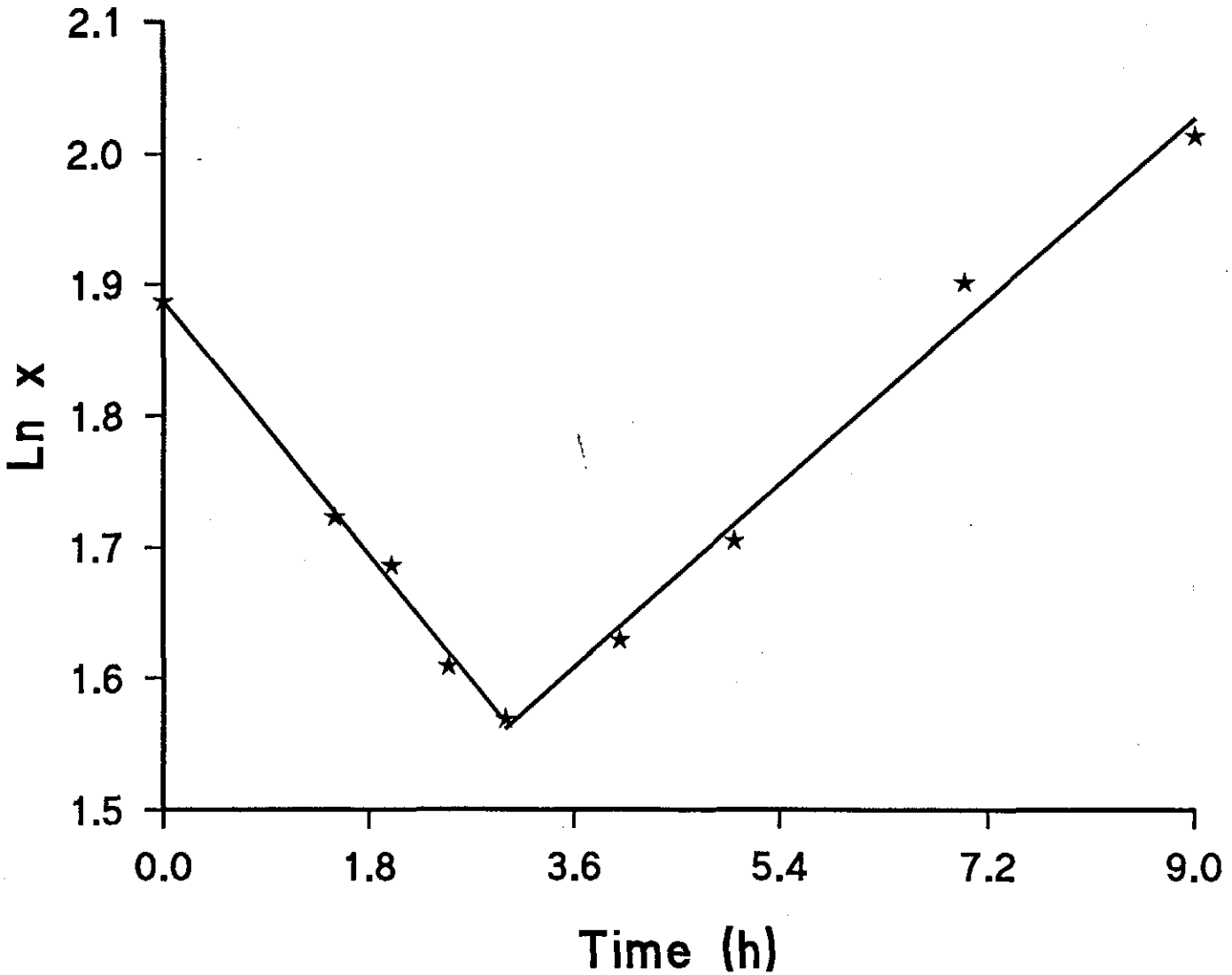


Fig 6.7.1 Washout / Batch at pH 1.8, (D= 0.2 h^{-1})

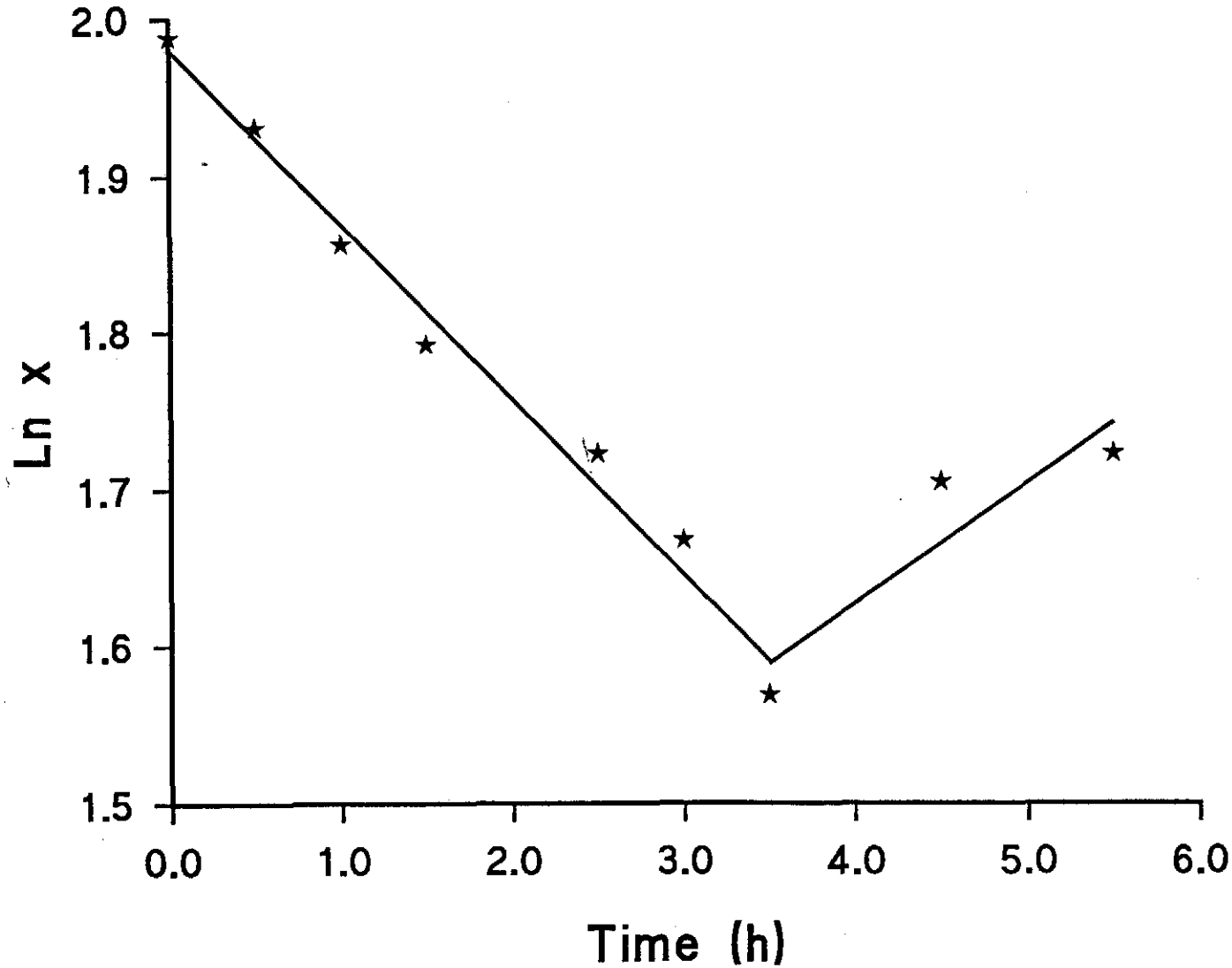


Fig 6.7.2 Washout / Batch at pH 2.5, (D= 0.2 h^{-1})

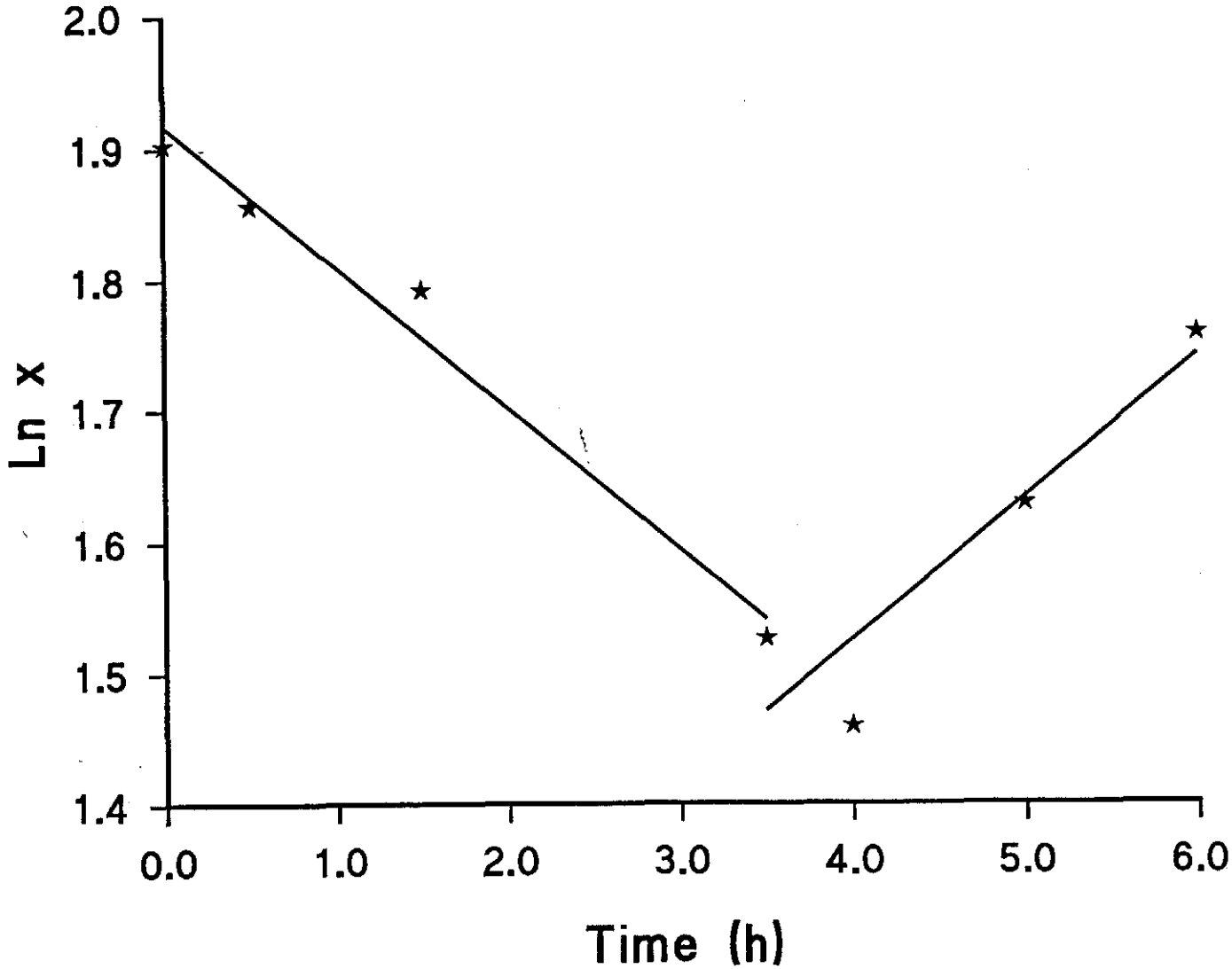


Fig 6.7.3 Washout / Batch at pH 3.0, (D= 0.2 h^{-1})

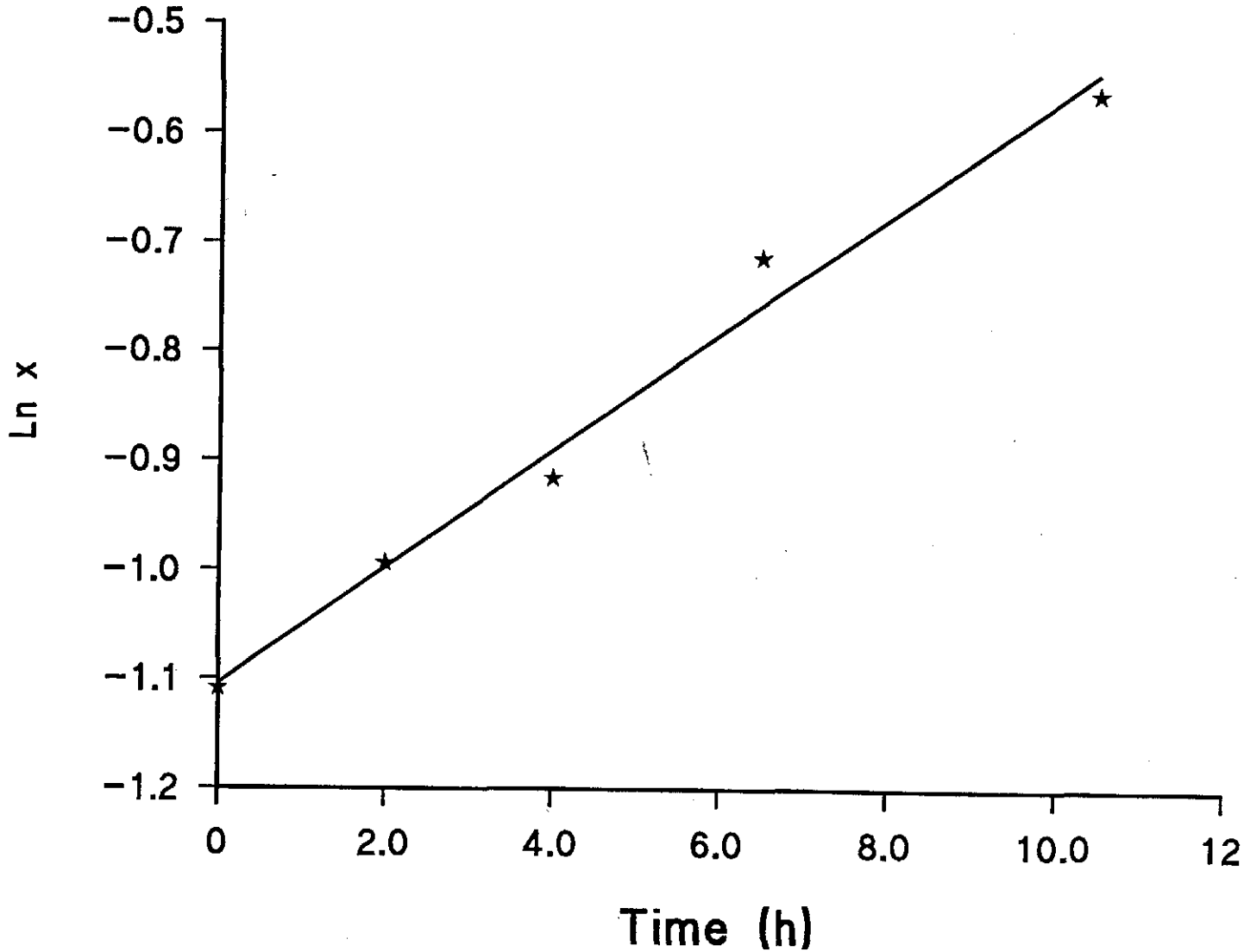


Fig 6.7.4 μ_{max} estimation during the early stage of batch run

CHAPTER 7
CONCLUSIONS

1: Applying the standard conditions described in section 4, 57 kg m^{-3} citrate, 12.5 kg m^{-3} biomass were produced and 84 kg m^{-3} sucrose consumed in batch culture.

2: It was observed that citrate production began immediately after nitrogen consumption.

3: The effect of a single pulse of $(NH_4)_2SO_4$ upon the progress and final outcome of batch citric acid fermentation was studied. It was found that the optimum addition time was in the range of 40 to 75 h.

4: From results obtained in this work it is clear that not only is the timing of the addition important, but the total amount of ammonium source added must be considered. It was found that final citric acid concentration achieved was increased when the amount of supplemental N source added was between 0.25 and 0.5 kg m^{-3}.

5: 12% more citric acid (64.7 kg m^{-3}) was produced, when 0.5 kg m^{-3} $(NH_4)_2SO_4$ was supplemented at 75 h relative to the standard run. Addition of supplemental N above 0.5 kg m^{-3}, or out with the specified time band led to negative effects on final product concentration.

6: It was recognised that citrate formation ceased immediately after addition of N, and did not recommence until all the added N was consumed.

7: It was observed that there are differences between maximum and final citric acid concentration in some runs. The decline in citric acid concentration in these runs after the maximum is achieved could be due to re utilisaton (especially where sucrose levels are low or limiting, see B9/B11) and re entry of citrate into the

mycelium.

8: The initial amount of ammonium was found to be critical. 3 kg m^{-3} was the optimum within the range of initial concentration of $(NH_4)_2SO_4$ between 0.5 and 4.0 kg m^{-3} for citric acid production (81 kg m^{-3}). Also the yield ($Y_{P/S}$, 0.618) is close to maximum yield (0.678) and productivity (P/t, 0.500 kg m^{-3} h^{-1}) is highest value at this initial N concentration of N.

9: When the initial pH was adjusted to 2 at the beginning of the fermentation, maximum citric acid concentration was 41.5 kg m^{-3}. When the initial pH was adjusted to 2.5, citrate concentration reached 66 kg m^{-3} Although there is a marked difference between 2 and 2.5 in terms of final product concentration no important changes were observed in yield or final product concentration above 2.5. Thus an initial pH of 2.5 is the clear optimum, which gave a maximum yield ($Y_{P/S}$, 0.717).

10: It was recognised that control of culture pH is also important. 58.2 and 60 kg m^{-3}citrate was produced at pH 1.8 and 2.5 respectively but when culture pH was kept at 3.0, maximum citric acid concentration reduced to 37.5 kg m^{-3}. Culture pH should thus be kept between 1.8 and 2.5.

11: μ_{max} was established at pH 1.8, 2.5 and 3.0 via washout-batch cycle and reported as 0.09, 0.11 and 0.12 h^{-1} respectively.

12: Employing the standard conditions (dilution rate 0.05 h^{-1},sucrose 50 kg m^{-3}, $(NH_4)_2SO_4$ 1 kg m^{-3} and other nutrition) 4.3 kg m^{-3}, citrate and 3.4 kg m^{-3} biomass were produced and 8 kg m^{-3} sucrose utilized in continuous

culture by Aspergillus niger B60. Under these conditions, productivity was 0.215 kg $m^{-3}h^{-1}$. Altering pH and dilution rate had no effect on the productivity.

13: On / off operation was employed by combining standard batch culture and standard continuous culture. After initial 70 h batch phase, 3 h continuous culture was carried out. Then 20 h batch phase was applied. This cycle was repeated 5 times and it was observed that citrate production decreased in the cycling operation. 5 h continuous / 20 h batch phase cycle (repeated 4 times) also led to a decrease in the citrate production compared with the standard run. Final biomass concentration in both 3 h continuous /20 h batch and 5 h continuous / 20 h batch cycles were less than those standard conditions

14: The addition of sucrose to a broth concentration of 5 kg m^{-3} once every 24 h after 40 h led to a 10.5 % increase in citric acid concentration with final concentration being 63 kg m^{-3} while 10 kg m^{-3} sucrose addition, after 40 h in every 24 h, nearly halved the final citric acid concentration (37 kg m^{-3}).

15: 30 and 120 kg m^{-3} citric acid addition at time 90 h had a negative effect on citric acid production ; Final acid concentration reached only 32 and 31 kg m^{-3} respectively. But 45 kg m^{-3} citrate addition led to increase 21% (final produced citric acid 69 kg m^{-3}) and 60 kg m^{-3} citrate addition 40.3% (final produced citric acid 80 kg m^{-3}) extra citric acid.

CHAPTER 8

FUTURE WORK

1. The supplementation of other medium constituents, ie. $MgSO_4$, KH_2PO_4 on the present process should be studied.

2. In order to generalize the effect of supplementation of $(NH_4)_2SO_4$, citrate and sucrose on the fungus and final citric acid production, a new producer organism should be tested.

3. Another fermenter system especially Tubuler Loop Fermenter (TLF) should be tested for citrate production and supplementation (as above)

4. Since the level control system has some disadvantages i.e, sudden malfunction causing loss of culture; the membrane would occasionally rupture in mid-fermentation; membrane replacement is only possible out with fermentation; the system needed calibration at least twice a day which causes an increase in culture volume may affect time taken to reach steady state. It is thus essential to change level control system. Ideally, a sensitive load all system would be in future studies.

5. After setting up a new level system and choosing a new high citrate producer strain running the following experiments would be worthwhile:

a) optimisation of pH, dilution rate, carbon source and limiting substrate;

b) pH cycling;

c) Carbon source cycling;

d) Limiting substrate cycling;

e) Dilution rate cycling.

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APPENDIX 1 : RAW DATA

BATCH DATA

FED-BATCH DATA

CYCLING DATA

μ_{max} ESTIMATION DATA

BATCH DATA

B2

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.5	140.0	---	0.544
16	5.8	1.7	136.0	---	0.544
24	1.9	---	134.0	---	0.544
45	1.9	---	134.0	---	0.331
70	1.9	5.0	123.0	22.2	0.000
89	1.8	6.7	116.0	30.0	0.000
95	1.8	6.9	116.0	26.0	0.000
112	1.8	8.5	75.0	39.0	0.000
118	1.8	9.0	73.0	40.0	0.000
136	1.8	8.9	70.0	42.0	0.000
145	1.8	10.4	70.0	48.0	0.000
160	1.8	10.2	63.0	53.0	0.000
183	1.8	11.4	57.0	55.0	0.000
192	1.8	12.6	56.0	57.0	0.000

B3

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.4	140.0	---	1.088
18	3.0	2.2	130.0	---	1.088
24	2.8	2.6	126.0	---	0.967
42	1.4	5.6	124.0	0.9	0.880
66	1.8	6.6	122.0	2.8	0.704
87	1.8	8.0	119.0	3.2	0.572
112	1.8	10.4	99.0	7.5	0.500
140	1.8	15.1	81.0	8.8	0.132
162	1.8	20.3	76.0	10.3	0.079
184	1.8	17.9	73.0	9.3	0.070
245	1.8	16.4	76.0	9.5	0.000

B4

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	3.0	140.0	---	0.544
15	2.8	3.0	133.0	---	0.520
24	2.3	6.3	124.0	---	0.384
25	2.0	6.3	124.0	---	0.930
40	1.8	5.9	118.0	1.2	0.725
65	1.8	6.6	102.0	16.5	0.523
95	1.6	11.7	98.0	22.1	0.063
113	1.8	13.6	96.0	19.0	0.000
137	1.8	19.7	94.0	19.8	0.000
166	1.9	17.3	92.0	22.0	0.000
184	1.9	17.9	90.0	21.2	0.000

B5

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.6	140.0	---	0.544
24	2.6	3.2	124.0	---	0.196
40	2.0	6.8	117.0	---	0.544
64	1.8	11.9	87.0	8.2	0.273
89	1.8	10.6	78.0	33.1	0.000
115	1.8	10.9	37.0	47.1	0.000
137	1.5	13.0	24.0	27.4	0.000
166	1.9	15.9	13.0	24.9	0.000
186	1.8	16.4	10.0	22.7	0.000
208	1.8	20.0	10.0	17.2	0.000
232	1.8	24.0	10.0	16.6	0.000
255	1.8	22.7	10.0	12.8	0.000

B6

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	0.6	140.0	---	0.544
18	2.8	2.9	133.0	---	0.544
24	2.3	3.4	128.0	---	0.404
42	1.8	3.7	114.0	7.4	0.000
50	1.8	4.9	110.0	13.6	0.000
75	2.0	5.7	91.0	33.5	0.544
90	2.0	8.2	77.0	37.1	0.075
98	2.0	10.4	69.0	37.4	0.000
123	2.0	15.8	60.0	37.1	0.000
144	2.0	18.8	50.0	37.7	0.000
168	2.0	21.0	41.0	41.1	0.000
192	2.0	27.1	38.0	46.6	0.000
216	2.1	34.0	34.0	47.7	0.000

B7

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	0.5	140.0	---	0.544
24	2.2	2.8	127.0	---	0.501
49	1.4	6.2	104.0	12.5	0.000
72	1.8	10.6	81.0	31.6	0.000
100	1.8	10.3	66.0	38.8	0.544
120	1.8	16.5	45.0	37.6	0.062
169	1.8	16.2	26.0	33.6	0.000
192	1.8	22.5	16.0	32.0	0.000
217	1.8	31.0	15.0	35.7	0.000
240	1.8	26.5	15.0	37.8	0.000
288	1.8	34.5	13.0	43.8	0.000

B8

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	0.8	140.0	0.9	0.544
24	2.3	3.6	111.0	0.9	0.525
45	1.8	5.4	101.0	7.5	0.000
72	1.8	8.5	95.0	29.6	0.000
99	1.8	9.4	84.0	37.8	0.000
120	1.8	11.1	81.0	43.3	0.000
140	1.8	13.9	79.0	39.5	0.544
167	1.8	15.2	47.0	39.7	0.000
188	1.8	16.2	32.0	38.4	0.000
212	1.8	21.4	21.0	38.2	0.000
240	1.8	24.4	----	37.9	0.000
260	1.8	22.5	6.0	38.8	0.000
280	1.8	26.0	4.0	38.4	0.000

B9

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.3	140.0	---	0.544
24	2.2	2.5	138.0	---	0.428
45	1.8	4.2	118.0	1.0	0.000
69	1.8	5.3	98.0	13.9	0.000
89	1.8	6.0	92.0	22.4	0.000
113	1.8	9.1	77.0	38.8	0.000
139	1.8	7.4	70.0	46.0	0.000
164	1.8	8.3	54.0	56.0	0.000
180	1.8	9.0	52.0	----	0.544
205	1.7	12.6	41.0	22.3	0.090
230	1.8	28.7	33.0	22.5	0.000
260	1.8	27.8	33.0	19.6	0.000
278	1.8	34.7	24.0	19.0	0.000

B10

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	2.0	140.0	2.7	0.544
24	2.7	---	140.0	2.7	0.435
46	1.8	4.3	124.0	8.2	0.000
75	1.8	5.7	92.0	30.8	0.272
95	1.8	9.6	73.0	37.6	0.025
119	1.8	14.9	54.0	47.8	0.000
141	1.8	22.6	48.0	50.6	0.000
162	1.8	30.4	38.0	47.8	0.000
184	1.6	29.8	30.0	50.1	0.000
212	1.8	32.6	25.0	51.5	0.000
238	1.8	32.6	22.0	51.7	0.000
261	1.8	28.5	20.0	51.7	0.000
285	1.9	27.8	20.0	36.1	0.000

B11

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.6	140.0	---	0.544
24	3.6	4.3	132.0	---	0.497
46	1.8	5.9	105.0	7.0	0.000
75	1.8	8.8	83.0	33.3	0.136
93	1.8	10.6	49.0	30.3	0.000
113	1.8	15.2	57.0	47.2	0.000
136	1.8	13.5	56.0	47.7	0.000
165	1.8	18.4	42.0	58.5	0.000
187	1.8	19.8	35.0	61.1	0.000
214	1.8	20.5	27.0	64.3	0.000
233	1.8	20.6	25.0	64.7	0.000
258	1.8	22.4	22.0	64.7	0.000

B12

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.9	140.0	---	0.544
24	2.4	2.5	123.0	---	0.459
43	1.8	7.3	107.0	2.4	0.000
75	1.8	8.0	97.0	29.2	0.068
91	1.5	8.8	79.0	39.9	0.000
113	1.9	9.2	64.0	40.5	0.000
139	1.8	11.0	56.0	47.3	0.000
165	1.8	12.0	51.0	55.6	0.000
186	1.8	13.2	48.0	49.0	0.000
219	1.8	15.5	41.0	54.1	0.000
237	1.8	18.6	36.0	63.5	0.000
257	1.8	16.0	30.0	27.4	0.000

B13

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.7	140.0	---	0.136
24	2.3	4.3	106.0	---	0.000
40	2.0	5.5	106.0	---	0.000
65	1.8	5.9	102.0	6.2	0.000
90	1.8	8.1	102.0	6.9	0.000
112	1.8	8.0	99.0	8.7	0.000
137	1.8	8.3	99.0	11.0	0.000
160	1.8	8.4	99.0	13.0	0.000
185	1.8	7.1	99.0	14.0	0.000
207	1.8	7.8	99.0	15.0	0.000

B14

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.3	140.0	---	0.272
24	2.6	3.0	138.0	---	0.200
40	2.0	5.9	131.0	1.9	0.000
68	1.8	7.5	119.0	12.8	0.000
90	1.8	6.0	102.0	21.8	0.000
119	1.8	7.1	107.0	19.0	0.000
138	1.8	7.5	102.0	20.0	0.000
164	1.8	8.1	72.0	---	0.000
188	1.8	8.3	---	---	0.000
210	1.8	10.2	---	40.0	0.000
234	1.8	10.9	68.0	38.0	0.000

B15

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.6	140.0	1.0	0.408
24	2.7	3.0	134.0	1.0	0.408
40	1.8	6.4	133.0	2.7	0.023
65	1.8	6.2	117.0	18.0	0.000
90	1.8	6.4	63.0	29.4	0.000
112	1.8	7.1	58.0	32.5	0.000
139	1.8	8.1	55.0	36.7	0.000
161	1.8	11.0	53.0	43.0	0.000
185	1.8	14.0	52.0	56.2	0.000
208	1.8	8.8	52.0	48.0	0.000
232	1.8	10.0	49.0	48.0	0.000

B16

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	2.3	140.0	---	0.816
24	2.4	5.6	134.0	---	0.427
42	1.8	8.0	125.0	6.0	0.033
66	1.8	10.5	107.0	7.0	0.000
90	1.7	11.8	77.0	28.0	0.000
114	1.4	11.4	70.0	30.0	0.000
140	1.8	10.0	46.0	50.0	0.000
162	1.8	11.6	27.0	81.0	0.000
187	1.8	10.9	15.0	72.0	0.000
210	1.8	12.8	11.0	73.0	0.000
233	1.8	12.2	9.0	73.0	0.000

B17

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	1.8	1.7	140.0	---	0.408
30	2.0	3.2	138.0	---	0.000
53	2.0	6.7	132.0	0.6	0.000
76	2.0	8.0	106.0	11.8	0.000
101	2.0	7.1	102.0	9.6	0.000
124	2.0	8.2	96.0	29.2	0.000
155	2.0	11.6	71.0	26.7	0.000
177	2.0	10.4	69.0	30.8	0.000
199	2.1	10.3	69.0	38.5	0.000
219	2.2	10.8	69.0	39.2	0.000
243	2.2	13.1	69.0	41.5	0.000

B18

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	2.5	1.7	140.0	---	0.408
18	2.5	2.9	129.0	---	0.408
43	2.5	7.9	129.0	4.6	0.082
67	2.5	10.2	102.0	17.5	0.000
89	2.5	10.9	86.0	34.0	0.000
113	2.5	12.6	85.0	44.0	0.000
137	2.5	12.2	84.0	47.5	0.000
162	2.5	11.5	84.0	48.5	0.000
186	2.5	12.4	74.0	49.0	0.000
209	2.5	15.4	55.0	59.0	0.000
231	2.5	16.7	48.0	66.0	0.000

B19

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	3.0	1.8	140.0	---	0.408
17	2.8	2.9	138.0	---	0.408
25	3.0	4.9	132.0	---	0.142
41	3.0	7.7	129.0	4.0	0.000
66	3.0	9.9	124.0	8.8	0.000
91	3.0	11.5	120.0	12.8	0.000
116	3.0	11.4	113.0	20.5	0.000
138	3.0	12.4	106.0	28.2	0.000
162	3.0	12.4	100.0	24.0	0.000
187	3.0	15.3	94.0	33.5	0.000
209	3.0	17.0	78.0	36.0	0.000
232	3.0	16.0	78.0	37.5	0.000

B20

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	2.4	140.0	---	0.408
17	2.8	2.8	136.0	---	0.408
24	2.5	6.2	130.0	---	0.122
42	2.5	6.7	127.0	6.6	0.000
65	2.5	8.0	105.0	18.7	0.000
89	2.5	8.0	85.0	25.4	0.000
114	2.5	8.6	83.0	36.7	0.000
136	2.5	9.5	77.0	43.4	0.000
160	2.5	11.1	73.0	49.3	0.000
191	2.5	12.0	62.0	55.0	0.000
214	2.5	13.1	60.0	59.0	0.000
232	2.5	14.0	60.0	60.0	0.000

B21

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	4.5	1.9	140.0	---	0.408
17	3.1	2.6	138.0	---	0.395
24	2.5	4.2	134.0	---	0.128
39	2.5	6.5	128.0	3.0	0.056
64	2.5	7.9	92.0	11.4	0.000
88	2.5	10.4	90.0	20.0	0.000
113	2.5	8.5	85.0	26.0	0.000
140	2.5	9.6	80.0	33.0	0.000
160	2.5	10.3	76.0	38.5	0.000
184	2.5	11.8	70.0	42.5	0.000
210	2.5	11.9	59.0	46.0	0.000
231	2.5	14.2	59.0	55.0	0.000

B22

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	3.7	1.9	140.0	60.0	0.544
19	3.7	2.4	132.0	60.0	0.448
42	3.5	6.8	130.0	60.0	0.263
68	3.2	8.1	104.0	60.0	0.000
86	3.0	9.2	86.0	60.0	0.000
114	3.0	8.9	77.0	65.0	0.000
135	2.9	10.5	69.0	68.0	0.000
160	2.8	13.0	54.0	69.0	0.000

RUN B23

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	2.9	140.0	---	0.544
23	2.4	2.9	125.0	---	0.487
43	1.8	3.5	102.0	7.2	0.090
69	1.8	4.4	92.0	17.0	0.000
90	1.8	7.3	80.0	20.0	0.000
111	1.8	8.1	69.0	32.0	0.000
136	1.7	12.7	69.0	36.0	0.000
160	1.7	14.9	52.0	49.0	0.000
184	1.7	16.5	52.0	49.0	0.000
206	1.7	20.1	47.0	51.0	0.000

FED BATCH DATA

FB 1

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	0.8	140.0	---	0.544
27	2.2	2.5	130.0	---	0.417
40	1.8	3.6	120.0	---	0.392
64	1.8	6.0	127.0	2.0	0.000
88	1.8	7.5	126.0	4.5	0.000
112	1.8	10.3	125.0	6.5	0.000
136	1.8	10.0	129.0	16.0	0.000
160	1.7	10.3	130.0	23.0	0.000
184	1.7	10.6	130.0	22.5	0.000
208	1.7	10.7	125.0	29.0	0.000
231	1.8	12.5	125.0	37.0	0.000

FB 2

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	1.0	140.0	---	0.544
22	2.8	2.1	130.0	---	0.378
40	1.8	4.4	117.0	4.3	0.141
64	1.8	5.7	110.0	15.0	0.000
88	1.8	6.3	110.0	34.0	0.000
112	1.8	9.1	114.0	40.0	0.000
136	1.8	10.5	113.0	51.0	0.000
160	1.8	11.2	97.0	56.0	0.000
184	1.8	12.1	90.0	60.0	0.000
208	1.8	12.1	90.0	63.0	0.000

FB 3

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	2.2	140.0	---	0.544
23	2.1	3.2	120.0	---	0.432
65	1.4	6.1	115.0	7.0	0.266
90	1.8	7.1	113.0	10.7	0.000
90.1	1.4	---	-----	40.7	0.000
96	1.8	7.7	110.0	37.5	0.000
112	1.8	8.0	104.0	24.0	0.000
120	1.8	8.0	95.0	42.0	0.000
139	1.8	9.1	91.0	42.0	0.000
160	1.8	11.6	81.0	43.8	0.000
185	1.8	13.6	79.0	49.0	0.000
215	1.8	14.0	79.0	58.0	0.000
237	1.8	14.5	79.0	61.0	0.000

FB4

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	2.8	140.0	---	0.544
20	2.4	3.4	110.0	---	0.290
45	1.8	5.4	105.0	4.3	0.000
63	1.7	6.5	101.0	18.5	0.000
90	1.8	7.6	73.0	30.0	0.000
112	1.7	8.9	69.0	81.0	0.000
135	1.8	15.1	64.0	105.0	0.000
168	1.8	14.1	59.0	100.0	0.000
183	1.8	13.0	55.0	103.0	0.000
208	1.8	12.6	52.0	114.0	0.000

FB 5

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	0.2	140.0	---	0.544
22	2.2	1.0	130.0	---	0.480
45	1.8	3.0	118.0	---	0.080
70	1.8	4.6	109.0	---	0.000
90	1.8	5.5	105.0	11.0	0.000
90	1.3	---	----	71.0	0.000
97	1.7	5.3	81.0	84.0	0.000
112	1.7	5.8	75.0	97.0	0.000
142	1.8	6.8	73.0	98.0	0.000
164	1.8	8.1	66.0	110.0	0.000
188	1.8	9.5	56.0	125.0	0.000
210	1.7	10.5	40.0	140.0	0.000

FB 6

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	0.7	140.0	---	0.544
25	2.4	1.5	132.0	---	0.435
44	1.8	3.5	128.0	---	0.181
70	1.8	5.3	114.0	5.0	0.000
90	1.8	7.7	107.0	10.0	0.000
90	1.3	---	----	120.0	0.000
113	1.8	8.0	74.0	130.0	0.000
138	1.8	7.9	66.0	135.5	0.000
162	1.8	8.9	67.0	142.0	0.000
192	1.8	11.5	63.0	149.0	0.000
232	1.8	11.3	63.0	152.0	0.000
260	1.8	13.6	63.0	152.0	0.000

FB 7

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	2.5	140.0	---	0.544
21	2.5	4.1	131.0	0.6	0.375
45	2.1	5.6	120.0	53.0	0.313
65	1.8	7.8	104.0	65.0	0.000
88	1.8	8.7	95.0	62.0	0.000
112	1.8	10.8	93.0	68.0	0.000
135	1.8	12.2	87.0	62.0	0.000
167	1.8	12.8	84.0	64.0	0.000
191	1.8	13.7	82.0	72.0	0.000

CYCLING DATA

CYC 1

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	0.7	140.0	---	0.544
26	2.2	2.6	128.0	---	0.344
48	1.8	4.0	104.0	6.9	0.021
70	1.8	6.9	101.0	16.0	0.000
93	1.8	7.9	88.0	28.0	0.000
116	1.8	7.6	73.0	34.0	0.000
139	1.8	7.5	63.0	34.0	0.000
162	1.8	7.9	56.0	41.5	0.000
186	1.8	8.7	47.0	35.5	0.000
209	1.8	8.4	46.0	41.5	0.000

CYC 2

Time h	pH	Biomass (kgm^{-3})	Sucrose (kgm^{-3})	Citric acid (kgm^{-3})	NH_3 (kgm^{-3})
0	6.5	0.6	140.0	---	0.544
27	2.6	2.1	130.0	---	0.387
51	2.1	3.8	114.0	6.5	0.161
70	1.8	4.4	101.0	12.8	0.000
95	1.8	5.2	78.0	22.0	0.000
120	1.8	4.9	72.0	28.0	0.000
145	1.8	5.4	60.0	25.0	0.000
170	1.8	4.9	53.0	25.0	0.000
195	1.8	4.8	53.0	24.0	0.000
214	1.8	4.9	51.0	32.0	0.000

μ_{max} Estimation Data

1 Dry weight, during the initial batch phase

Time(h)	0	2.0	4.0	6.5	10.5
Dry weight (kgm^{-3})	0.33	0.37	0.40	0.49	0.57

2 Dry weight, at pH 1.8

Time(h)	0	1.5	2.0	2.5	3.0	4.0	5.0	7.0	9.0
Dry weight (kgm^{-3})	6.6	5.6	5.4	5.0	4.8	5.1	5.5	6.7	7.5

3 Dry weight, at pH 2.5

Time(h)	0	0.5	1.0	1.5	2.5	3.0	3.5	4.5	5.5
Dry weight (kgm^{-3})	7.3	6.9	6.4	6.0	5.6	5.3	4.8	5.5	5.6

4 Dry weight, at pH 3.0

Time(h)	0	0.5	1.5	3.0	3.5	4.0	5.0	6.0
Dry weight (kgm^{-3})	6.7	6.4	6.0	5.6	4.6	4.3	5.1	5.8

APPENDIX 2

STANDARD CURVES

Citric Acid Assay

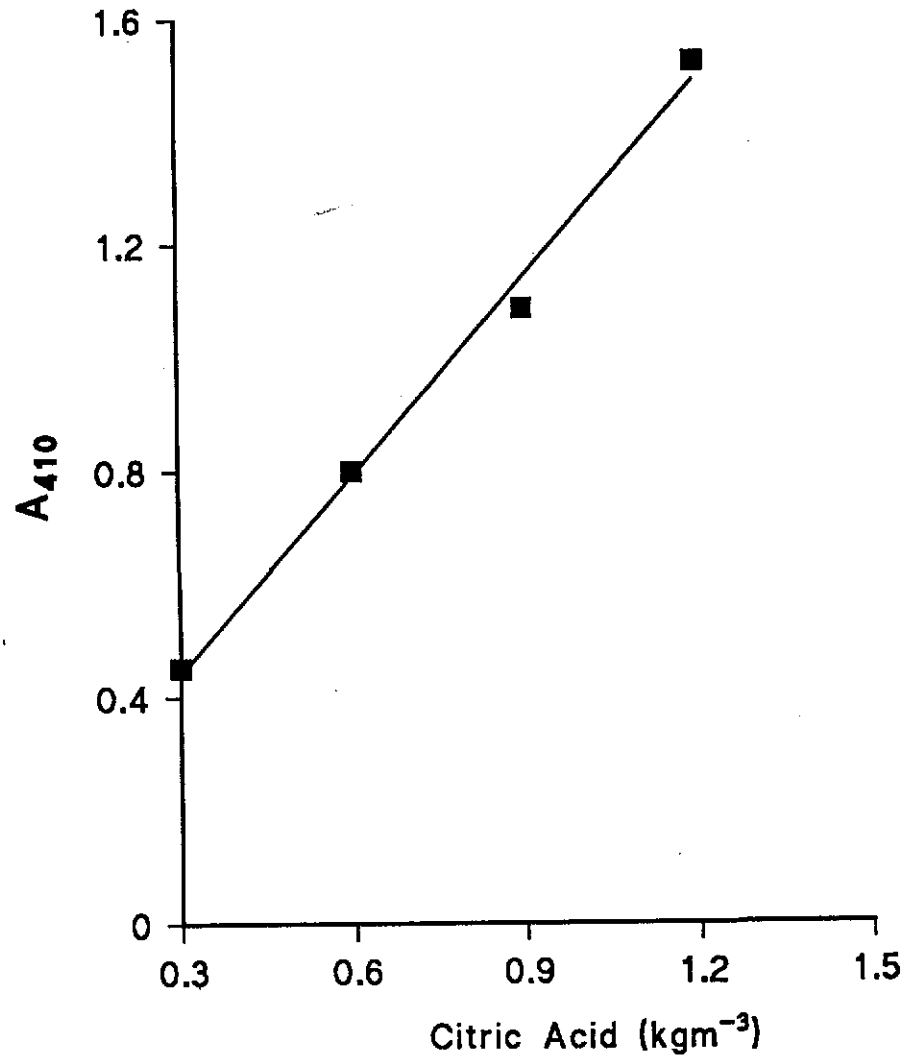
Sucrore Assay

Ammonia Assay

A typical standard curve (fig A.2.1) was produced by measuring the absorbance of standards, in table A.2.1, at 410 nm for citric acid assay.

Conc. kgm^{-3}:	0.3	0.6	0.9	1.2
Abs_{410} :	0.452	0.799	1.086	1.522

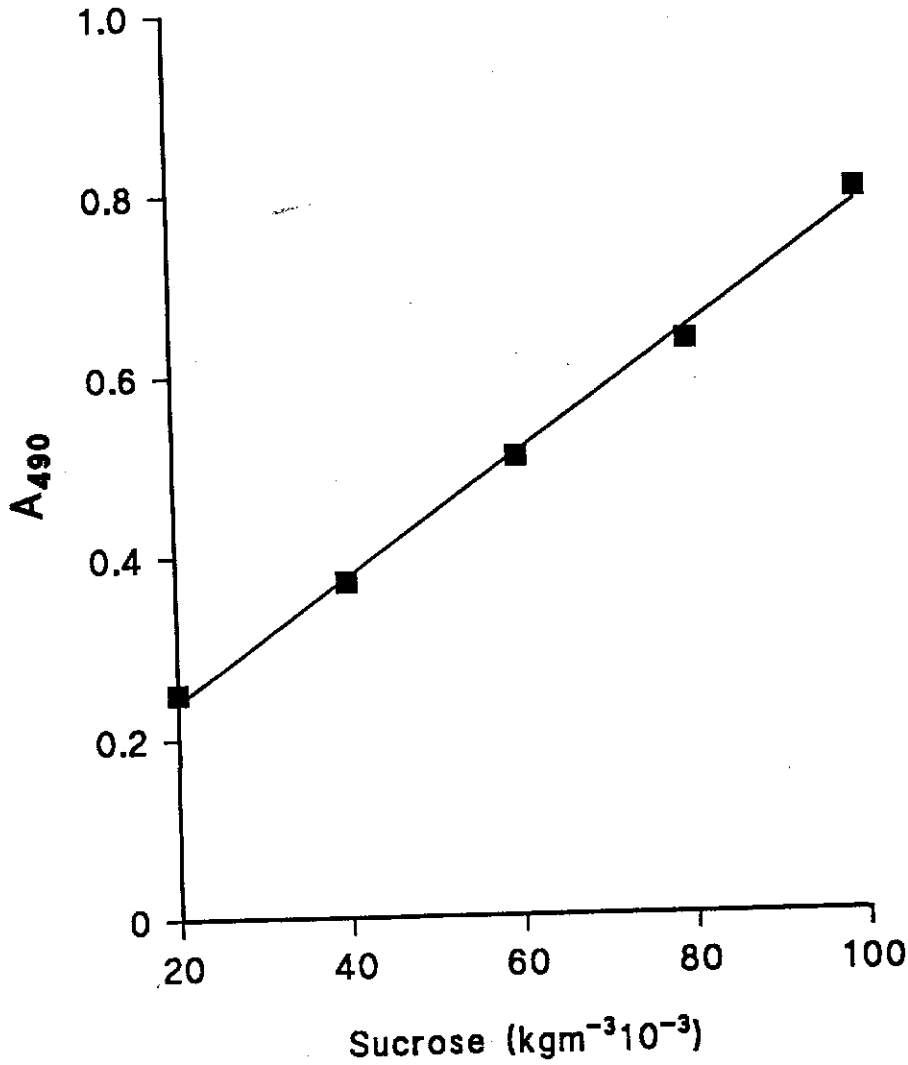
Table A.2.1



A typical standard curve (fig A.2.2) was produced by measuring the absorbance of standards, in table A.2.2, at 490 nm for sucrose assay.

Conc. $kgm^{-3} \times 10^{-3}$:	20	40	60	80	100
Abs_{490} :	0.250	0.351	0.507	0.634	0.797

Table A.2.2



A typical standard curve (fig A.2.3) was produced by measuring the absorbance of standards, in table A.2.3, at 630 nm for ammonia assay.

Conc. $kgm^{-3}x10^{-3}$:	15	30	60	75
Abs_{630} :	0.087	0.104	0.157	0.181

Table A.2.3