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NUTRIENT REQUIREMENTS FOR ACETONE-BUTANOL-
ETHANOL PRODUCTION FROM WHEY PERMEATE BY
CLOSTRIDIUM ACETOBUTYLICUM P262
IN A RANGE OF BIOREACTORS

by

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*To my father who passed away in peace,
To my beloved mother, sister 'Lek' and brother 'Som'*

ABSTRACT

The acetone-butanol-ethanol (ABE) fermentation process, using *Clostridium acetobutylicum* P262, was studied. Experiments were conducted in a traditional batch fermentation using freely-suspended cells in a defined medium with lactose as carbon source. Solvent production did occur under conditions of nitrogen-, phosphate- or iron-limitation. However, the optimum conditions were observed when all nutrients were present slightly in excess of growth requirements. A greater excess of nutrients caused the fermentation to be acidogenic rather than solventogenic.

Sulphuric acid casein whey permeate, without nutrient supplementation, proved to be a poor substrate for growth and solvent production by this organism. However, the addition of yeast extract (5 g/l) led to strong solventogenesis. The deficiency in the whey permeate was shown to be iron rather than assimilable nitrogen, phosphate or vitamins.

Experiments were also performed in a defined medium in continuous culture using freely-suspended cells in a CSTR. An inverse relationship was observed between the biomass concentration and the specific butanol productivity. It is suggested that this was due to the cell population not being homogeneous, and that a change in the nutrient *balance* led to a change in the relative proportions of acidogenic, solventogenic and inert cells (spores).

The addition of supplementary yeast extract during continuous solvent production from whey permeate using free cells in a CSTR showed that yeast extract supplementation of less than 1 g/l favoured acid production rather than solvent production. It is unlikely that high solvent productivities can

be attained with this substrate in a single stage continuous culture system with freely-suspended cells. A maximum solvent productivity of 0.05 g/l.h was observed in continuous culture compared to 0.06 g/l.h in batch culture.

A continuous fermentation process was investigated using cells immobilized by adsorption onto bonechar and operated in a packed bed reactor. Three nutrients (i.e. nitrogen, phosphate and iron) were selected for this investigation. Solvent production was favoured by high concentrations of these nutrients in the influent medium. It was not possible to restrict the supply of phosphate or iron in the reactor due to leaching of the bonechar. However, conditions where biomass growth was restricted by a restriction in the nitrogen supply were not conducive to solvent production.

This reactor was also operated using whey permeate as the substrate. The biomass build-up was controlled by minimizing the supplementary yeast extract concentration of the feed, but this had deleterious effects on solvent production. Hence, when producing solvent from whey permeate in this type of reactor a compromise must be made between strong solventogenesis and reactor longevity.

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ABBREVIATION

ABE	Acetone-Butanol-Ethanol
ATP	Adenosine 5'-tri-phosphate
CoA	Coenzyme A
°C	Degree Celcius
cm	Centimetre(s)
CSTR	Continuous Stirred Tank Reactor
D	Dilution rate(s)
g	Gram(s)
GPR	General Purpose Reagent
h	Hour(s)
l	Litre(s)
M	Molar
mg	Milligram(s)
min	Minute(s)
ml	Millitre(s)
mm	Millimetre(s)
NAD ⁺ , NADH	Nicotinamide adenine dinucleotide, and its reduced form
nm	Nanometre
OD ₆₂₅	Optical density at 625 nm
PBR	Packed Bed Reactor
rpm	Revolutions per minute
μl	Microlitre(s)
%(w/v)	Percentage weight by volume
%(v/v)	Percentage volume by volume

CHAPTER 1

INTRODUCTION

1.1 Background and Objectives of the Thesis

Recently, there has been renewed interest in the microbial production of chemicals, such as butanol, from renewable resources. Due to the finite resource of fossil fuels and the oil crisis of the mid-1970s, coupled with problems of proliferation of wastes generated by agriculture and industry, the acetone-butanol-ethanol (ABE) fermentation process has once again been considered as source of renewable energy. It is generally known that for economic reasons the fermentation process has failed to compete with the synthetic process since the 1950's. However, if the limitations of the fermentation process, such as high substrate costs, low product concentration, severe product inhibition and inefficient product recovery systems, can be relieved, the fermentation process could be more realistic.

It has been challenging to apply the achievements of modern biotechnology to overcome these limitations. However, a prerequisite to success is collaborative research among microbial physiologists, geneticists and biochemical engineers. Microbial physiologists develop knowledge of the fermentation mechanisms and regulation of solvent production, as well as product toxicity. At the same time, strain improvements are carried out by genetic engineers. The biology of a fermentation process can be understood adequately for biochemical engineers to be able to achieve optimal fermenter productivity. Finally, new techniques for selective product removal and concentration are being developed to reduce the energy costs related to downstream processing.

Much progress has been made in our understanding of the biological aspects of this fermentation process, in particular of regulation of the fermentation pathways. This knowledge can be applied by biochemical engineers to design the most suitable fermentation systems. Thus, many reactor configurations and novel fermentation technologies have been developed to improve reactor yields and productivities. However, an important factor which appears to have been neglected is the role of cell density on solvent (ABE) production. Several reports in the literature have attempted to describe the situation but the results appear to be contradictory.

Thus, the present study was conceived to investigate the nutrient status of the culture, and its effects on cell density and solvent production. In addition, the study has been extended to the use of whey permeate as a substrate because this material has the potential to be used commercially for the ABE fermentation process (Maddox, 1980). Furthermore, the effect of cell density is of fundamental importance to the development of some novel fermentation technologies. Here, it is often assumed that an increase in cell density within the reactor will give a corresponding increase in reactor productivity, but there are literature reports which suggest that this may not necessarily be true. Hence, further knowledge is required to describe the situation.

1.2 History of the Acetone-Butanol-Ethanol (ABE) Fermentation

The historical development of the industrial ABE fermentation process has been well documented (Gabriel, 1928; Gabriel and Crawford, 1930; Prescott and Dunn, 1959; Rose, 1961; Hastings, 1978; Compere and Griffith, 1979; Walton and Martin, 1979; Jones and Woods, 1986). The microbial production of butanol was first discovered by Pasteur in 1861. An additional product of this fermentation, known as acetone, was reported by Schardinger in 1905 (Jones and Woods, 1986). Due to the shortage of natural rubber around the turn of the century, the ABE fermentation process was developed, since butanol was considered a

precursor of butadiene, the starting material for synthetic rubber. The British Company Strange and Graham Ltd., became interested and recruited the services of Perkins, Weizmann, Fernbach and Schoen in 1910 to study the formation of butanol by microbial fermentation (Gabriel, 1928). This led to the remarkable development of a microbial fermentation process for butanol production. Weizmann succeeded in isolating an organism, later named *Clostridium acetobutylicum*, which was able to produce butanol and acetone from starchy materials in better yields than the organism of Fernbach which utilized potatoes. The Fernbach process was patented in 1912 and the Weizmann process in 1915.

The outbreak of World War I in 1914 caused a sharp increase in the industrial demand for acetone, since acetone was used to dissolve cordite in the manufacture of explosives. The Weizmann process was recognized by the British government and a production plant was built at the Royal Naval Cordite Factory at Poole, but production was subsequently moved to the USA and Canada. However, at the end of the war, all these plants were closed down due to reduced demand for acetone. At this time, however, the automobile industry developed rapidly and required large amounts of solvent (butyl acetate) for nitrocellulose lacquers. This allowed the microbial production of butanol to become of importance again.

The industrial production of ABE by fermentation was carried on until after World War II. It then started to decline due to the unprecedented growth of the petrochemical industry and the increased use of grains and molasses for human and animal consumption. In the 1960's, industrial production in Europe and North America ceased due to the unfavourable economics. The ABE Fermentation Plant in South Africa was able to continue operating until its closure in 1981 (Jones and Woods, 1986).

The sudden rise in crude oil prices in 1973, and again in 1979, triggered a renewed interest in the biotechnological production of butanol and acetone. So far, however, it has not led to a process which can compete with the synthetic

processes from petrochemicals, at least not in the western world. Acetone is currently produced by either the cumene hydroperoxide process or the catalytic dehydrogenation of isopropanol. Butanol is synthesized from either propylene by the oxo-process or acetaldehyde by the aldol process (Moreira, 1983).

1.3 The ABE Fermentation Process

1.3.1 Microorganisms

A number of organisms of the *Clostridium* group are known to ferment sugars to a variety of neutral end products. In certain fermentations, butanol and acetone or isopropanol are the main solvents whereas in other processes only acetone and ethanol are formed. However, two species have been developed for solvent production. They are *Clostridium acetobutylicum* and *C. beijerinckii*.

C. acetobutylicum ferments starch, hexoses, or pentoses to butanol, acetone, and ethanol in the general ratio of 6:3:1 (Spivey, 1978). *C. beijerinckii* ferments hexoses or starch and produces largely butanol and smaller quantities of isopropanol and ethanol.

There are two other species which have been isolated recently which could be potentially useful for the development of solvent production. *C. auranti-butylicum* produces both acetone and isopropanol in addition to butanol (Cummins and Johnson, 1971), and *C. tetanomorphum* produces butanol and ethanol (Nakamura *et al.*, 1979). *C. acetobutylicum* is described as a Gram-positive, straight rod (measuring 0.6 - 0.9 μm by 2.4 - 4.7 μm), with rounded ends, and existing singly, paired or in chains, depending on the growth phase and medium used. It is a strict anaerobe with an optimal growth temperature of 34°C (Cato *et al.*, 1986). The optimal pH value for growth and acid production is 6.5, while a pH of less than 5.3 is often required for solvent production (Spivey, 1978; Gottschal and Morris, 1981b; Bahl *et al.*, 1982a; Monot *et al.*, 1984). The vegetative cells are motile with peritrichous flagella. Actively motile cells are regarded as a prerequisite for good solvent production (Gutierrez and Maddox, 1987).

Division occurs by transverse fission resulting in long chains of organisms which break apart into single cells in liquid medium during vigorous fermentation. Subterminal oval spores (approximately 1 μm by 1.5 μm) are formed which usually distend the rods. Biotin and *p*-aminobenzoic acid are required as growth factors (Rubbo *et al.*, 1941). *C. acetobutylicum* differs from other butyric clostridia in that under appropriate conditions it can convert acids to neutral solvents (McNeil and Kristiansen, 1986).

1.3.2 Course of Fermentation

The progress of a 'typical' batch fermentation process using *C. acetobutylicum* is now well documented (Prescott and Dunn, 1959; Spivey, 1978; Jones *et al.*, 1982; Ennis *et al.*, 1986, and Awang *et al.*, 1988). It is characterized by two distinct phases which correspond to the two-stage mechanism of product formation. Initially, there is an acidogenic phase where active logarithmic growth of the organism occurs, producing acetic and butyric acids over the period of 7-18 h. This causes a decrease in the *pH* value of the culture. Eventually, the growth rate decreases and the second phase, known as the solventogenic phase, occurs from 18 h through to 36-60 h. The *pH* value of the culture rises slightly due to a decrease in growth rate and acids being assimilated and metabolized to solvents. The *pH* 'breakpoint' is where the acidogenic phase switches to the solventogenic phase (Davies and Stephenson, 1941; Barber *et al.*, 1979). It has been suggested that the uptake of acids functions as a detoxification process in response to an unfavourable environment, resulting in neutral solvents being produced. These solvents are less toxic to the cells than the previously synthesized organic acids (Woods and Jones, 1986). Hydrogen and carbon dioxide gases are released throughout the fermentation with gas evolution being maximal during the solventogenic phase.

According to Awang *et al.* (1988), the fermentation can be considered to proceed in three phases (Fig. 1.1). Microbial activity ceases in the third (stationary) phase

due to the accumulation of toxic concentrations of solvents (approximately 20 g/l).

Sugar is utilized throughout the fermentation. The solvents yield approximates 30% (solvents produced/weight sugar utilized). However, slight differences in yields can be observed depending on strains and cultural conditions used (Beesch, 1952; Yerushalmi *et al.*, 1983). Typical solvent productivities of batch fermentation processes are in the range of 0.2 - 0.6 g/l.h depending on the operating conditions (Spivey, 1978; Ennis and Maddox, 1985; Yu *et al.*, 1985).

1.3.3 Biochemistry of the Fermentation

The main biochemical pathways involved in the conversion of carbohydrate to fatty acids, solvents, carbon dioxide, and hydrogen by *C. acetobutylicum* (Fig. 1.2) are now well investigated and firmly established (Thauer *et al.*, 1977; Lenz and Moreira, 1980; Doelle, 1981; Volesky *et al.*, 1981; Gottschalk and Bahl, 1981; Kim *et al.*, 1984; Papoutsakis, 1984; Hartmanis and Gatenbeck, 1984; Petitdemange and Gay, 1986; Rogers, 1986; Jones and Woods, 1986).

Hexose sugars are metabolized via the Embden-Meyerhof glycolytic pathway to 2 mol of pyruvate, 2 mol of ATP and 2 mol of NADH + H⁺, from 1 mol of hexose. Pentose sugars are metabolized by way of the pentose phosphate pathway, leading via a series of steps, to fructose-6-phosphate and glyceraldehyde-3-phosphate which then enter the EMP pathway (Zeikus, 1980; Volesky and Szczesny, 1983). The fermentation of 3 mol of pentose yields 5 mol of ATP and 5 mol of NADH.

Pyruvate, resulting from glycolysis, is further oxidised by pyruvate-ferredoxin oxidoreductase in the presence of coenzyme A (CoA) to form acetyl-CoA, CO₂ and reduced ferredoxin. The acetyl-CoA formed by this phosphoroclastic reaction serves as the precursor for all the fermentation products. Two molecules

of acetyl-CoA are converted to acetoacetyl-CoA, which is further converted via the cyclic system, leading to the formation of butyrate. As the culture *pH* falls due to accumulation of acids, acetoacetyl-CoA is diverted from the normal cyclic mechanism, to yield acetoacetate, which is then converted to acetone and CO₂ via the acetoacetate decarboxylase system. This latter step is irreversible.

Diversion of the cyclic system to produce acetone prevents further butyric acid formation and eliminates the two steps generating NAD⁺. To regenerate NAD⁺ the organism must reconvert butyrate to butyryl-CoA, and then reduce the latter to butanol. In addition, further sugar utilization is forwarded to butanol rather than butyrate production, and ferredoxin is regenerated via NADH, rather than hydrogen production. Ethanol is also produced from acetyl-CoA by two steps of reactions. Acetyl-CoA is converted to acetaldehyde by acetaldehyde dehydrogenase, and then to ethanol by ethanol dehydrogenase. This results in the oxidation of 2 mol of NADH + H⁺ to NAD⁺.

1.4 Factors Affecting the Fermentation

The factors involved in the transition from the acidogenic to the solventogenic phase appear to be more complex than once thought. Several investigations have been undertaken in batch and continuous culture to understand the way in which the production of solvents is initiated and maintained, but the exact reason for the transition is not clearly understood (Gottschal and Morris, 1981a; Gottschal and Morris, 1981b; Andersch *et al.*, 1982; Bahl *et al.*, 1982a; Monot *et al.*, 1982; Monot *et al.*, 1984; Fond *et al.*, 1985; Long *et al.*, 1984b). Although these studies brought a wealth of new information, it is evident that no single factor specifically induces solvent production. Hence, all of the factors implicated in solventogenesis will be covered in this Section.

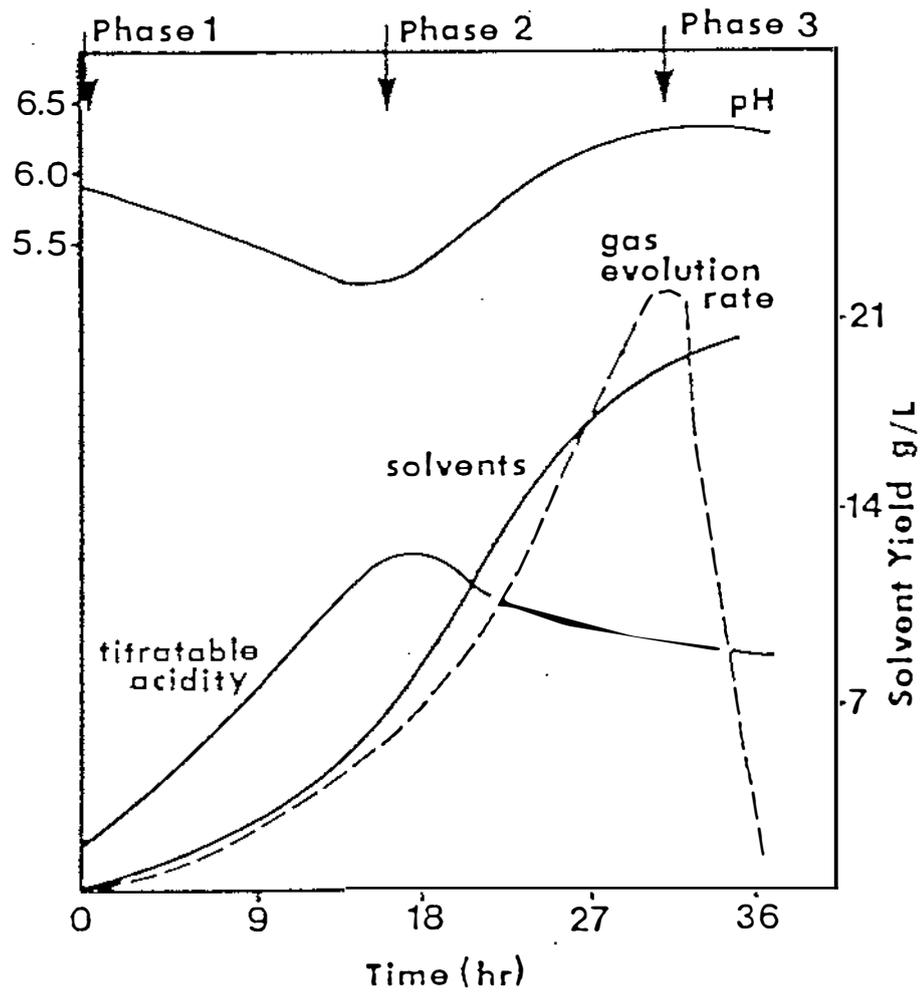


Figure 1.1 Outline of an ABE fermentation as carried out by *C. acetobutylicum* (Awang *et al.*, 1988)

1.4.1 Substrate Source and Concentration

C. acetobutylicum can utilize a great variety of sugars (Compere and Griffith, 1979; Mes-Hartree and Saddler, 1982). Traditional substrates such as corn, wheat, millet, rye or molasses were originally utilized as raw material for the commercial ABE fermentation, but due to their high costs, alternative cheaper substrates are now being studied including whey (Maddox, 1980; Gapes *et al.*, 1983; Schoutens *et al.*, 1984; Welsh and Veliky, 1984; Ennis and Maddox, 1985); lignocellulosic materials (Saddler *et al.*, 1983; Yu *et al.*, 1984; Marchal *et al.*, 1984; Yu *et al.*, 1985); Jerusalem artichokes (Marchal *et al.*, 1985); and sulfite waste liquor (Wayman and Yu, 1985).

The initial sugar concentration plays an important role in the ABE fermentation. At low (less than 20 g/l) initial sugar concentration, the fermentation tends to be acidogenic, with little solvent being formed (Monot *et al.*, 1982). However, at higher initial concentrations (up to 60 g/l) the process becomes progressively more solventogenic (Monot *et al.*, 1982; Ennis and Maddox, 1987). At concentrations above 80 g/l, considerable sugar remains unfermented, probably due to product inhibition (Monot *et al.*, 1982) while at 120 g/l, the fermentation activity of the culture is negligible (Qadeer *et al.*, 1980), probably due to substrate inhibition.

The mechanism of sugar transport is not fully understood. However, it is believed that phosphotransferase systems are responsible for uptake of glucose and fructose (Hutkins and Kashket, 1986). It is likely that other substrates of *C. acetobutylicum* are taken up by symport mechanisms driven by the trans-membrane proton gradient. Disaccharides such as sucrose or maltose might then be cleaved by an appropriate phosphorylase, and the free glucose can be converted to glucose-6-phosphate by hexokinase (Bahl and Gottschalk, 1988).

1.4.2 Temperature

The temperature of the fermentation can affect overall yield, solvent ratios, and rate of solvent production. In the molasses fermentation, solvent yields remained fairly constant at temperatures between 30° and 33°C but decreased at 37°C (McCutchan and Hickey, 1954). Similar results were observed in a synthetic medium by McNeil and Kristiansen (1985, 1986) when they investigated the effect of temperature upon growth rate and solvent production in batch cultures of *C. acetobutylicum*. They found that the total solvent yield decreased with increasing temperature, possibly because of a reduction in acetone production. It appeared that the yield of butanol was not affected by the temperature. In terms of total solvent yield and productivity, the optimum fermentation temperature was reported to be around 35°C.

1.4.3 Oxygen

C. acetobutylicum is an obligate anaerobe. Optimal growth occurs at a negative culture redox potential, E_h , in the range of -250 mV to -400 mV. Exposure of an anaerobic culture to oxygen for a short period of time is not lethal. However, if cultures are exposed to high dissolved oxygen concentrations (40 to 60 μM), the rate of glucose consumption decreases and growth, and DNA, RNA and protein syntheses are halted. Under aerobic conditions, the organism has been reported to be drained of reducing power, and the production of butyrate, but not acetate, ceased. In addition, there was a marked fall in the level of intracellular ATP. These consequences of oxygenation were all reversible and growth and metabolism resumed when cells were returned to anaerobic conditions (O'Brien and Morris, 1971).

Studies by Prescott and Dunn (1959) and Long *et al.* (1984) revealed that the exposure of a culture to oxygen also resulted in an increase in spore formation and the degree of enhancement was influenced by the culture conditions, the age

of the cells, and the duration of the exposure to oxygen. It has also been reported that short bursts of aeration to the cultures could increase the output of butanol by 3.1 to 9.1 % (Nakhmanovich and Kochkina, 1963).

1.4.4 pH and Acid End-products

The culture pH plays a crucial role in determining the outcome of sugar metabolism. A number of recent studies have reported that cultures maintained at high pH values tend to produce mainly acids, whereas cultures maintained at low pH values produce mainly solvents. However, the pH range over which solvent synthesis is likely to occur varies widely depending on the strain and culture conditions used (Jones and Woods, 1986). The range of pH value where solvent production may occur is pH 3.8 to 5.5 (Bahl *et al.*, 1982a; Nishio *et al.*, 1983; Monot *et al.*, 1984). However, the industrial strain *C. acetobutylicum* P262 can produce good levels of solvent at values approaching pH 6.5 (Jones *et al.*, 1982; Jones and Woods, 1986).

Although a prerequisite for the switch from acidogenesis to solventogenesis is the lowering of the pH of the culture medium, pH itself is not the trigger (Gottschal and Morris, 1981b; Long *et al.*, 1984a). The lowering in pH value results from a rise in the level of acid end-products and is associated with an increase in cell number and decreases in hydrogen production and the specific growth rate (Jones and Woods, 1986).

The weak organic acids, acetic and butyric acids, produced as end-products of metabolism are, by nature, toxic to the cell, and are able to partition in the cell membrane in their undissociated form (Kell *et al.*, 1981; Huesemann and Papoutsakis, 1986). At sufficiently high acid concentration, the pH gradient across the membrane collapses, resulting in the total inhibition of cell metabolic functions within the cells. At lower concentrations, the accumulation of acid end-products, and the associated decrease in pH value, result in a decrease in growth

rate until growth is eventually halted, although substrate utilization and cellular metabolism continue (Herrero, 1983; Herrero *et al.*, 1985). Hence, it has been suggested that the switch to solventogenesis is a detoxification mechanism of the cell to remove the inhibitory effects when acid end-products reach toxic levels (Costa, 1981; Bahl *et al.*, 1982a; George and Chen, 1983; Hartmanis *et al.*, 1984; Long *et al.*, 1984a).

In this way, the onset of solventogenesis is usually associated with a lowered *pH* of the medium and a critical level of the undissociated form of the acids (Häggström, 1985). Thus, the trigger concentration of butyrate has been demonstrated to be lower at low *pH* values than at high *pH* values (Holt *et al.*, 1984). It is now generally accepted that the effect of *pH* is really an effect of the undissociated butyric acid concentration, and this is likely to be the essential factor in the regulation of solvent production (Bahl and Gottschalk, 1985).

1.4.5 Nutrient Concentrations

In the ABE fermentation, in both batch- and continuous-cultures, it is now generally accepted that carbon source limitation is detrimental to solvent production (Gottschal and Morris, 1981a; Bahl *et al.*, 1982b; Monot *et al.*, 1983; Long *et al.*, 1984a; Fond *et al.*, 1984; Ennis and Maddox, 1987). Under carbon source limitation, the amount of acid end-products generated is insufficient to achieve the threshold concentration to induce solvent production.

The literature is rather confusing with regard to the effect of other nutrient concentrations on the regulation of solvent production in batch and continuous culture. Thus, in this section, the effect of nutrient concentrations on the regulation of solvent production will be critically reviewed according to the mode of the operation.

1.4.5.1 Nutrient Concentrations in Batch Culture

Many reports in the literature regarding nutrient effect on the regulation of solvent production are often difficult to interpret. Although initial nutrient concentrations are usually described, the nutritional status of the culture during and after the fermentation is often not (Maddox, 1989). According to Maddox (1989), nutrient limitation in batch culture can be defined as a situation where cellular growth is restricted (terminated) due to the exhaustion of an essential nutrient.

Monot and Engasser (1983a) reported that strong solvent production can be achieved after exhaustion of nitrogen from the medium. In contrast, Long *et al.* (1984a) concluded that nitrogen-limited cultures did not produce solvents. Their results indicated that there must be a minimum nitrogen concentration remaining after the growth phase to induce solventogenesis. However, the experiments performed by Roos *et al.* (1985), where the ratio of nitrogen to glucose was varied, suggested that an excess of nitrogen is detrimental to solvent production, and that as the ratio of nitrogen to glucose decreases, the rate of solvent production may be enhanced by a lowered availability of nitrogen. Hence, the possible role of nitrogen limitation in solvent production remains to be clarified.

Bahl *et al.* (1982a) have described batch cultures under phosphate limitation, where solvent production occurs after exhaustion of phosphate from the medium. In contrast, when phosphate was in excess, the fermentation produced acids rather than solvents. Recently, Bryant and Blaschek (1988) have described the effect of phosphate buffer concentration on solvent production. Although the nutritional (phosphate) status of the culture at the end of the process was not given, the result appears to confirm that solvent production can occur under phosphate-limited conditions.

Junelles *et al.* (1988) have described the effect of the iron concentration of the medium on the ratio of butanol to acetone in batch culture. The results showed that the butanol to acetone ratio increased from 3.7:1 to 11.8:1 as the iron concentration was reduced from 10 mg/l to 0.2 mg/l (as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$). It was suggested that iron limitation, in addition to inhibiting the enzyme hydrogenase, directly affected the carbon and electron flow. Similar results have been observed by Bahl *et al.* (1986) who observed an increase in the ratio of butanol to acetone from 2:1 to 8:1 under conditions of iron limitation. Hence, on the basis that butanol is a more valuable product than acetone, a close observation of the iron content of media as a tool to direct the metabolic pathway towards butanol formation is worth considering.

1.4.5.2 Nutrient Concentrations in Continuous Culture

In recent years continuous culture of *C. acetobutylicum* has been widely used as a research tool to define parameters responsible for changes in the physiology and activity of this microorganism. The fundamental areas of ABE fermentation such as the effect of medium components and acid end-products on solvent production, the influence of temperature, culture *pH*, dilution rate, maximum attainable solvent concentration and yield and the stability of a continuous culture with regard to solvent production have been widely investigated.

There have been conflicting reports with respect to the role of some nutrient concentrations in regulation of solvent production. In addition, some reports are difficult to interpret as it is not always clear whether a nutrient-limited condition has, in fact, been attained. Direct comparison of the results is even more difficult because of differences in the strain used, medium composition, and fermentation conditions. However, it is now generally accepted that no single growth-limiting factor specifically induces solvent production in continuous culture, and each factor must be considered in interaction with other important environmental parameters, such as culture *pH*, dilution rate, etc. Nevertheless, some nutrients

have been shown to be more suitable for growth limitations and production of solvents in high yields than others (Bahl and Gottschalk, 1988; Maddox, 1989).

Monot and Engasser (1983a) reported that it was possible to obtain solvent production in a nitrogen-limited chemostat culture as long as the fermentation was maintained at lowered *pH* and dilution rate. However, Long *et al.* (1984a) have pointed out that the ammonium concentration used by Monot and Engasser (1983a) was not limited. In fact, it was sufficient for the production of the threshold concentration of acid end-products and the initiation of solvent production. In contrast, Gottschalk and Morris (1981a) and Andersch *et al.* (1982) both failed to obtain significant levels of solvent in a nitrogen-limited chemostat. In addition to the low level of solvent production, the process was difficult to maintain. Their findings were subsequently supported by Jöbses and Roels (1983), Roos *et al.* (1985), and Stephens *et al.* (1985). Therefore, the possible role of nitrogen limitation in the induction of solventogenesis in continuous culture, and its application to an industrial process, remains to be clarified.

Bahl *et al.* (1982b) were successful in achieving strong solvent production in a two-stage continuous process under phosphate limitation. The first stage was run at a relatively high dilution rate (0.125 h^{-1}) which resulted in the production of acids. The second stage, which was fed with only the effluent from the first stage, was run at a much lower dilution rate (0.04 h^{-1}). High yields of solvents were obtained, and stability was maintained for a long period. Similar results have been reported by Stephens *et al.* (1985) and Soni *et al.* (1986), using phosphate-limited chemostats. It was demonstrated that the phosphate-limited chemostat favours solventogenesis whereas excess phosphate favours acidogenesis. Furthermore, at low dilution rates under phosphate-limitation, culture stability was maintained for a long period with no morphological changes.

Magnesium-limitation (Stephens *et al.*, 1985; McNeil and Kristiansen, 1987) and sulphate-limitation (Bahl and Gottschalk, 1984) were reported to favour solventogenesis in continuous culture. However, Bahl and Gottschalk (1984) have

reported that a magnesium-limited chemostat culture was not appropriate for continuous solvent formation. Acids were the major fermentation products, although it was operated at the optimum conditions for solvent production. Iron-limitation has been reported by McNeil and Kristiansen (1985) to produce a poor culture with reduced biomass levels and solvent productivity.

More recently, Clarke and Hansford (1986) have demonstrated that good solvent production is independent of a carbon, nitrogen or phosphate limitation. They concluded that although solventogenesis is favoured under these conditions, such limitations are not essential for causing a metabolic shift. However, they noted the possible role of nutrient limitation in preventing culture 'degeneration' and this may represent an advantage over the non-nutrient-limited condition.

1.4.6 Product Inhibition

One of the limiting factors with respect to the ABE fermentation process is product inhibition, with butanol being the most inhibitory product (Herrero, 1983; Linden and Moreira, 1983; Ingram, 1986). Cell metabolism usually continues until solvent concentration reaches inhibitory levels of around 20 g/l. In the industrial process, solvent production is halted when the concentration of butanol reaches 13 g/l (Ryden, 1958; Walton and Martin, 1979; Spivey, 1978; Moreira *et al.*, 1981). This dilute product concentration, which, in turn, restricts the concentration of sugar that can be fermented, contributes to the high costs of product recovery. Thus, improvement in this area is critical for the economic viability of the overall process.

Butanol, acetic acid and butyric acid have been reported to be the most inhibitory of the fermentation products (Moreira *et al.*, 1981; Lin and Blaschek, 1982; Costa and Moreira, 1983; Kuhn and Linden, 1986). Growth was totally inhibited at a butanol concentration of 12 to 16 g/l, whereas cell growth was inhibited by 50 % when butanol was added at 7 to 13 g/l. The concentrations

of acetic acid and butyric acid where 50 % inhibition of cell growth occurred were reported to be at 8.0 g/l and 6.0 g/l, respectively (Costa and Moreira, 1983). Hence, the inhibitory effect to cell growth caused by butanol, acetic acid and butyric acid occurred at levels commonly observed during the fermentation. On the other hand, acetone and ethanol do not have inhibitory effects at the concentrations normally attained during fermentation (Moreira *et al.*, 1981; Costa and Moreira, 1983).

It is now known that solvent inhibition occurs at the cell membrane (Ingram, 1986). Owing to their amphipatic character, alcohols dissolve in the membrane lipids, and increase their permeability, and affect membrane fluidity (Dombek and Ingram, 1984; Vallherbst-Schneck *et al.*, 1984). Thus, high concentrations of butanol lead to a complete abolition of the *pH* gradient, lower the intracellular level of ATP, cause the release of intracellular metabolites, and inhibit sugar uptake (Moreira *et al.*, 1981; Gottwald and Gottschalk, 1985; Bowles and Ellefson, 1985; Hutkins and Kashket, 1986). These effects clearly demonstrate the importance of the cell membrane with respect to butanol tolerance. Other sites of interference, such as inhibition of glycolytic enzymes (Herrero, 1983) cannot be excluded. Simple mutation and selection experiments seem not to be very promising so far. In addition to this, butanol-tolerant strains do not necessarily produce more butanol (Jones and Woods, 1986; Bahl and Gottschalk, 1988).

1.5 Cell Density in Association with Solvent Production

1.5.1 Introduction

In recent years increasing attention has been paid to continuous culture of the ABE fermentation. This is because continuous culture has some advantages over the conventional batch process, especially in terms of increased productivity. Continuous culture of the ABE fermentation has been mainly used as a research tool to define parameters responsible for changes in the physiology and activity

of the microorganism. In fact, under steady-state conditions a constant environment is reached, and its interaction with other factors can be determined (Bahl and Gottschalk, 1988). In addition, continuous culture avoids the non-productive '*downtime*' which is a feature of batch fermentation.

It has long been recognized that continuous fermentation is a very useful means for attaining high reactor productivities. However, although the productivity is higher, the substrate concentration and the solvent yields are often low. One approach to overcome this is to maintain a high cell density within the reactor so as to allow increased productivities to be achieved with high substrate consumption. It is often assumed in this approach that each individual cell within the reactor will perform at least as well under conditions of high cell density as under conditions of low cell density. Perusal of the literature, however, suggests that this may not necessarily be so, since there are some discrepancies regarding the effect of biomass concentration on solvent productivity. At least one of these investigations has demonstrated that there is an inverse relationship between the biomass concentration and specific solvent productivity (McNeil and Kristiansen, 1987). Thus, it is the aim of this section to broadly review the effect of cell density on solvent production in chemostat, turbidostat and *pH*-auxostat culture.

1.5.2 Chemostat

A chemostat culture consists of a perfectly mixed suspension of biomass into which medium is fed at a constant rate and the culture is harvested at the same rate so that the culture volume remains constant. The biomass growth is limited by the amount of a single substrate whereas all the other nutrients are in excess (Pirt, 1975).

There have been many studies into the use of chemostats for solvent production. In addition to being a means of increasing fermenter productivity over batch

culture, chemostat culture is an excellent tool for the elucidation of biochemical mechanisms, and for studying the effects of various environmental parameters on microbial physiology. However, although chemostat culture is widely used in many reports, only a few of these reports provide sufficient evidence for a nutrient-limited situation.

Stephens *et al.* (1985) made the observation that in both ammonium- and phosphate-limited chemostat culture solventogenesis is favoured by high cell densities whereas acidogenesis is favoured by low cell densities. Similar results were observed by Soni *et al.* (1987) who found that high cell density can improve specific solvent productivity and glucose consumption rate.

In contrast, analysis of data from a range of experiments reported by McNeil and Kristiansen (1987) revealed a strong inverse relationship between biomass concentration (cell density) and specific solvent productivity under both ammonium- and phosphate-limited chemostat conditions.

1.5.3 Turbidostat

Although the chemostat technique allows precise control of biomass concentration and it is widely used for studies on the ABE fermentation, it is not particularly suitable for the long-term growth of microorganisms near their maximum growth rate. The rate of growth in the chemostat is determined by the rate of addition of culture medium containing a limiting amount of a substance required for growth (Herbert *et al.*, 1956), and, at dilution rates approaching μ_{\max} , the population tends to wash out.

The turbidostat, on the other hand, works most effectively at growth rates near μ_{\max} for it is in that range that the population density is most accurately controlled (Herbert, 1958). In fact, turbidity control by means of a photoelectric cell is stable when the biomass concentration varies rapidly with change in dilution rate, especially near to the critical dilution rate.

There are few reports describing the use of a turbidostat as a means of studying the effect of cell density on solvent production. Gottschal and Morris (1982) reported that strong solvent production by *C. acetobutylicum* NCIB 8052 in turbidostat culture was obtained only at relatively high cell densities, whereas at low cell densities the fermentation was acidogenic in nature.

1.5.4 *pH*-Auxostat

This is a method of utilizing the *pH* change brought about by growth to set the rate of addition of medium. In situations where the culture is subject to a growth-related change in *pH*, turbidostatic control is, in principle, achievable by exploiting *pH* change as a measure of increase of cell density (Martin and Hempfling, 1976).

Unfortunately, there is only one report in the literature, so far, describing the application of this technique to the ABE fermentation (Stephens *et al.*, 1985). The culture was grown in batch mode until its *pH* value reached the required set-point value. When, due to growth-related acid production, the *pH* fell below this set-point value, pumps were simultaneously activated to supply fresh medium containing all nutrients in excess, and alkali, until the *pH* returned again to the set-point value when the pumps were automatically stopped. The desired cell density was sustained by suitably adjusting the speed of the pump supplying fresh medium relative to that of the pump delivering the alkali. By this means *pH*-auxostat culture of *C. acetobutylicum* NCIB 8052 was established. A true steady state was not achievable. However, high rates of solvent production were observed at high cell density, as all nutrients were provided in excess, for periods of at least 20 days.

1.6 Utilization of Whey for the ABE Fermentation

The search for alternative fermentation substrates was and is stimulated by the high costs of the conventional starch and sugar substrates, which make up the major part of production costs. The ability of solvent-producing clostridia to utilize many of the different carbohydrates occurring in renewable resources favours most researches. Owing to a relatively low sugar content in whey (lactose 40 - 50 g/l), which is unsuitable for many fermentation processes without prior concentration but almost optimal for the ABE fermentation in which product inhibition limits the amount of sugar consumed, and to the ability of *C. acetobutylicum* to ferment lactose directly, it is reasonable to consider whey (or whey permeate) as an attractive alternative substrate for the ABE fermentation. Nevertheless, whey (or whey permeate) has proved to be a relatively poor substrate when overall reactor productivity in batch fermentations is considered, when compared with starch and molasses substrates. This, and the incomplete utilization of the lactose, are the two major problems of using whey as a substrate (Maddox, 1980; Welsh and Veliky, 1984; Ennis and Maddox, 1985; Linden *et al.*, 1986).

A number of reports have described the use of various *Clostridium* species and whey types for solvent production in conventional batch fermentation using freely-suspended cells as well as in continuous fermentation using immobilized cells (e.g. Schoutens *et al.*, 1985; Qureshi and Maddox, 1987). A brief summary of the fermentation medium, strain, culture conditions and fermentation parameters observed in batch fermentation studies is given in Table 1.1 and those of continuous fermentation studies is given in Table 1.2.

Table 1.1 Summary of the literature describing the production of solvents in batch fermentation using whey media.

Substrate	Strain	Temp (°C)	Working period (h)	Total Solvents (g/l)	Prod ^v (g/l.h)	Ratio A:B:E	Yield (g/g)	Reference
Sulphuric whey permeate + yeast extract	<i>C. acetobutylicum</i> NCIB 2951	30	120	17.0	0.14	1:10:1	0.52	1
Sulphuric whey permeate + yeast extract	<i>C. acetobutylicum</i> NCIB 2951	30	48	11.3	0.24	1.5:8:0.5	-	2,3
Sulphuric whey permeate + yeast extract	<i>C. butylicum</i> NRRL-592	30	120	11.2	0.09	2.2:7.6:0.2	0.43	4
Cheese whey permeate + yeast extract	<i>C. beyerinckii</i> LMD 27.6	30	100	5.0	0.05	1:4:0*	0.29	5
Acid whey	<i>C. acetobutylicum</i> ATCC 824	37	120	9.2	0.08	1:13:4.4	-	6
Sulphuric whey permeate + yeast extract	<i>C. acetobutylicum</i> P262	30	39	9.5	0.24	1:3:0	0.42	7
Cheese whey + yeast extract	<i>C. acetobutylicum</i> ATCC 4259	37	96	9.4	0.10	1:4.9:0	0.27	8
Cheese whey + yeast extract	<i>C. acetobutylicum</i> NRRL 596	30	96	7.8	0.08	1:3.3:0	0.35	8
Sulphuric whey permeate + yeast extract	<i>C. acetobutylicum</i> ATCC 824	37	52	4.4	0.08	1:4:0	0.21	9
Whey + ?	<i>C. acetobutylicum</i> DSM 792	37	-	10.8	-	1:129:24	-	10

References

1. Maddox (1980)
2. Maddox *et al.* (1981)
3. Gapes *et al.* (1982)
4. Gapes *et al.* (1983)
5. Schoutens *et al.* (1984)
6. Welsh and Veliky (1984)
7. Ennis and Maddox (1985)
8. Voget *et al.* (1985)
9. Ennis (1987)
10. Bahl *et al.* (1986)

Notes:

- Isopropanol is produced instead of acetone.

Table 1.2 Summary of the literature describing the production of solvent in continuous fermentation using whey media.

Substrate	Strain	Fermenter mode	D (h ⁻¹)	Productivity (g/l.h)	Yield (g/g)	Ratio A:B:E	Working period (h)	Reference
Whey permeate + yeast extract	<i>C. beyerinckii</i> LMD 27.6	Continuous/alginate entrapment	0.7	1.0	-	-	-	1
Whey permeate + yeast extract	<i>C. acetobutylicum</i> P262	Continuous/alginate entrapment	0.405	1.79	0.27	1:3.5:0	650	2
Whey permeate + yeast extract	<i>C. acetobutylicum</i> P262	Continuous/bonechar adsorption	1.0	4.1	0.23	1:2.1:0	1472	3
Whey permeate + yeast extract	<i>C. acetobutylicum</i> P262	Continuous/cell recycle	0.41	2.92	0.31	1:2.5:0	-	4

References

1. Schoutens *et al.* (1985)
2. Ennis *et al.* (1986)
3. Qureshi and Maddox (1987)
4. Ennis and Maddox (1988)

When using whey permeate as a substrate for solvent production, most workers have added supplementary yeast extract, presumably as a nutrient source. Maddox (1980) has demonstrated that batch fermentation without supplementary yeast extract performs less well. One interesting observation to arise from the use of whey as the substrate is the unusually high product ratio of butanol to acetone. Bahl *et al.* (1986) suggested that this is caused by the low iron content or presence of lactate in whey. However Linden and Moreira (1982), who observed a ratio of 20:1 using cheese whey compared with 3:1 using a glucose substrate, pointed out that the shift in ratio is more likely to be due to a combination of factors rather than to a single component present in the whey.

Schoutens *et al.* (1985) remarked on the low productivity observed when using whey permeate as substrate, and suggested that this could be due to the relatively high salt concentration in this substrate. However, no evidence was provided to support this suggestion.

Overall, the results support the view that further investigation of whey (permeate) as a substrate for the ABE fermentation is desirable.

1.7 Solvent Production Using Immobilized Cells

1.7.1 Introduction

The low productivity and the low final concentration of solvents in the fermentation broth are the major problems with the batch ABE fermentation process. Improvement in these areas is essential before a reintroduction of the ABE fermentation process can be envisaged.

Cell immobilization may be defined as any technique that confines or limits the biocatalysts or cells within a reactor system, permitting their easy reuse. The application of immobilized cells in continuous column reactors is the most

modern approach in alcohol production technology. It combines several advantages of the systems: a high cell density can be achieved, operation can be performed at a high dilution rate with little washout of cells from the system, and end-product inhibition is partially eliminated because solvents are constantly removed. Although there is a similarity, in principle, between a continuous fermentation system using immobilized cells and that using recycle of freely suspended cells, immobilized cells have the advantage that they can be used in a variety of reactor types, such as packed beds or fluidized beds, that are not readily applicable to free cells (Maddox, 1989). In addition, continuous fermentation systems using immobilized cells do not suffer from the same stability problems as those using free cells. However, factors such as cost, availability and stability of the immobilization material, simplicity and mildness of the immobilization method and ability to control as well as maintain the activity of such systems, are considered to be disadvantages of immobilized cells especially for large-scale solvent production.

1.7.2 Cell Immobilization by Entrapment

In 1979, in Sweden, Häggström (1979) was the first to report the successful immobilization in alginate of both spores and cells, and their use (after germination in the case of spores) in a non-growing mode for solvent production from glucose. Subsequently, Häggström and Molin (1980) reported superior solvent productivities compared to those using free cells, and suggested that immobilization of spores was preferable to that of cells. Later, Häggström (1981) and Häggström and Enfors (1982) reported the use of glutaraldehyde to improve the mechanical strength of the alginate beads, and use of the beads in a packed-column reactor with liquid recirculation. Reactor productivities of 2.8 g/l.h were observed, but these rapidly decreased to approximately 1 g/l.h during reactor operation. This decrease was attributed to the fact that the feed medium contained only glucose and salts, but no other nutrients. Nevertheless, the system was able to be operated for 42 days, after which time the beads remained

mechanically intact. A subsequent report from this group described a technique for maintaining a constant productivity in non-growing immobilized cells by using the intermittent nutrient-dosing technique (Förberg *et al.*, 1983). It was suggested that in order to maintain the cells in an active but non-growing state, for maximum solvent productivity and yield to be achieved, the nutrient supply must be sufficient to enable the cells to restore essential cell constituents but not so much that reproduction can proceed.

Unlike the Swedish group, Dutch workers did not use glutaraldehyde to mechanically strengthen the alginate beads, and the feed media used contained all essential nutrients for cell growth. Preferably, the Dutch group used immobilized spores (subsequently germinated) rather than cells. This is because immobilized vegetative cells of *C. acetobutylicum* lost the ability to produce solvents within 2 days, as described by Häggström and Molin (1980), whereas immobilized spores (subsequently germinated) showed no apparent activity loss. Krouwel *et al.* (1980) immobilized spores in alginate beads and used them in a packed-bed reactor for continuous solvent production. It was possible to operate the reactor for up to 9 days with a mean productivity of 1 g/l.h, representing a fourfold improvement over that of conventional batch fermentation using free cells. However, the system suffered from problems of *pH* gradients and poor gas release in the reactor bed.

On further study into a packed-bed reactor containing calcium-alginate immobilized *C. beijerinckii* cells for continuous IBE (isopropanol-butanol-ethanol) fermentation, Krouwel *et al.* (1983) observed that although the productivities were 3 to 4 times higher than that obtained in a batch fermentation using free cells, there were serious disadvantages in the long-term use of this reactor type: presence of stagnant slugs and dead zones; poor spore germination during start-up resulting from a *pH* gradient; and the system does not behave as a true plug flow. They summarized that this reactor type was not very suitable for continuous IBE fermentation.

Because of the difficulties encountered with the packed-bed reactor, Krouwel *et al.* (1983) investigated a continuous stirred tank reactor (CSTR) for continuous IBE fermentation using immobilized growing *C. beijerinckii* cells, since this type of reactor offers considerable advantages over a packed-bed reactor. These include easy gas release, no concentration gradients, and the system at steady-state is well defined allowing modelling of the system to be relatively easily achieved. Using this system, productivities of up to 4 g/l.h were observed, and the system was stable and easy to operate.

Since the ABE process suffers from product inhibition, plug flow such as that in a packed-bed reactor should be favoured. This has been confirmed in a process kinetic study performed by Schoutens *et al.* (1986) who reaffirmed that the process is controlled by product inhibition, and that a high bead fraction in the reactor favoured increased product concentration and productivity. Their subsequent reports provided a more detailed study of a fluidized-bed reactor and a gas-lift loop reactor (Schoutens *et al.*, 1986a; Schoutens *et al.*, 1986b). Both of the overall reactor models showed a low sensitivity for hydrodynamic parameters and stable operation could be attained. However, based on its overall performance, which was due to its higher reactor loading, the fluidized-bed reactor was considered to be superior.

Ennis *et al.* (1986) have reported the use of alginate-immobilized cells in a CSTR and in a fluidized-bed reactor with whey substrate. The results confirmed that the process is controlled by the inhibitory butanol concentration, and that the reactor productivities varied as a function of the dilution rate and the alginate bead fraction in the reactor. Stable operation was maintained for 17 days, with a maximum lactose utilization of 40 %. The use of pH regulation and a two-stage process in order to increase the substrate utilization were found to be detrimental to solvent production. Taya *et al.* (1986) have also described the use of alginate-immobilized cells in a CSTR, but using synthetic medium. The solvent productivities were reported to be enhanced with increased dilution rate and were comparable to other reports. In addition, a long-term operational

stability of over 2 weeks was achieved. Largier *et al.* (1985) used an alginate-immobilized, sporulation-deficient mutant in a fluidized-bed reactor, and achieved a solvent productivity of 3 g/l.h at a concentration 15 g/l. However, the system was operated for only a short period of time.

In addition to alginate, there have been studies into the use of carrageenan-immobilized cells for continuous solvent production. Anon (1981) reported a stable system with a butanol productivity of 5.2 g/l.h, but, unfortunately, no details were provided. Recently, Frick and Schügerl (1986) compared the use of three different polymers, alginate, *K*-carrageenan and chitosan, for cell immobilization. The alginate and chitosan beads were dried to improve their long-term stability, whereas the carrageenan beads were treated with diaminohexane and glutaraldehyde. A two-stage stirred tank cascade served as reaction vessel. Alginate proved to be the superior polymer matrix system, showing a solvent productivity of 4 g/l.h, corresponding to solvent concentration of 4 g/l. The system remained stable for 30 days of operation and was able to withstand high stirrer speeds.

More recently, Reardon and Bailey (1989) have investigated the effects of *pH* and added metabolites (acetate, butyrate, and acetoacetate) on the decrease in biocatalytic activity of calcium alginate-immobilized *C. acetobutylicum* in a packed-bed reactor. Non-growing (but viable) immobilized cells were used in this study since this offers several potential advantages over immobilized systems operated under growth conditions, including the potential for the conversion of a higher fraction of substrate carbon to products than in other systems. It was demonstrated that although the non-growing cells were metabolically active, the product distribution was shifted from solventogenesis to acidogenesis, and overall activity losses occurred due to cell lysis and sporulation. However, these authors used a sugar concentration of only 10 g/l so it is not surprising that little solventogenesis occurred.

The ABE fermentation has been successfully carried out by Park *et al.* (1989) in an immobilized cell trickle bed reactor comprising two serial columns packed with *C. acetobutylicum* ATCC 824 entrapped on the surface of natural sponge segments at an average cell loading of 3.58 g dry cells/g sponge. Two different feed media, which consisted of only a glucose-salt mixture and a glucose-salt mixture with yeast extract and peptone, were used to control the effluent pH value by supplying a certain fraction of each of the two feed media to the reactor. A nutrient medium fraction above 0.6 was observed to be optimal for successful fermentation in this type of reactor. A lower glucose concentration in the feed medium (10 g/l) was reported to favour acidogenesis whereas a higher concentration (60 g/l) favoured solventogenesis. The solvent productivity based on total packing volume was 4.2 g/l.h, which is 10 times greater than that obtained in an industrial batch fermentation using free cells and 2.76 times higher than that of alginate-immobilized cells in a CSTR. However, degeneration of the system used was reported, due to low nutrient concentrations, repeated exposure to oxygen and high butanol concentrations. Once the cells had degenerated, they could not be recovered. Moreover, the natural sponge which was used as the packing material was degraded by *C. acetobutylicum* due to its proteolytic and cellulolytic activity, hence polyester sponge was recommended to be used.

Overall, alginate immobilization, which represents an entrapment technique, is a useful technique for improving the productivity of the ABE fermentation, and considerably higher solvent productivities, especially over long time periods, have been achieved compared to free cell systems. The technique is simple to operate, expensive capital equipment is unlikely, and it is possibly suitable for technical substrates.

1.7.3 Cell Immobilization by Adsorption

Unlike immobilization in alginate, immobilization by adsorption of cells onto a solid surface could improve the problems of nutrient or product diffusion in the bulk liquid to or from the cells. However, accumulation of multicell layers, which results in a substantial cell leakage from the system may be a considerable disadvantage unless the use of non-growing (but viable) cells is applied. Technically, immobilization of cells by adsorption is cheap, mild, and easy to scale-up. A variety of different support materials are readily available (Förberg and Häggström, 1985).

The successful immobilization of *C. acetobutylicum* by adsorption to beechwood shavings has been described by Förberg and Häggström (1985) as giving reactor productivities comparable to those obtained with alginate immobilization. The system described was a glass reactor packed with beechwood shavings arranged as parallel sheets on a supporting base of wire netting, under which was a magnetic stirrer. The reactor was started-up using a spore suspension, and was then operated continuously using a glucose/salts feed medium with intermittent dosing of a nutrient solution. By this means, the adsorbed cell mass could be gradually increased by controlling the growth. Thus, the cells were maintained in an active, but non-growing state. The system was able to be operated under stable conditions for several weeks, with constant yield, very low cell leakage, and a productivity of 1.5 g/l.h was reported. The excellent cell adsorption that occurred was attributed to cellular polysaccharide material produced by the bacterial cells.

Welsh *et al.* (1987) adsorbed cells onto coke and reported a stable steady-state with a productivity of 1.2 g/l.h. Qureshi and Maddox (1987) have described the use of the material bonechar, as used in sugar refining, on which to adsorb the *C. acetobutylicum* P262 cells for solvent production from whey permeate. Here, a glass column reactor was packed with bonechar, and a growing culture was circulated through (inoculated) by means of a peristaltic pump. The system was

then operated as a packed-bed reactor. Initially, the reactor was operated at sufficiently high dilution rates to favour biomass accumulation, and to prevent growth-inhibitory concentrations of solvents. Once the bonechar was fully covered with biomass, the dilution rate was decreased in order that excessive biomass growth was restricted by the inhibitory solvent concentration. The maximum solvent productivity attained in this reactor was 4.1 g/l.h at a solvent concentration of 4.1 g/l. In order to maintain this solvent productivity, it was suggested that a minimum yeast extract supplementation of 1 g/l must be used when using whey permeate as a substrate. Although the system was operated successfully for 62 days, it finally suffered from problems of blockage, due to cell fouling of fluid passages, and gas hold-up in the reactor. Subsequently, a mathematical model was proposed by Qureshi *et al.* (1988) to describe this packed-bed reactor. The developed model supports the concept of different morphological/physiological cell types being present in this particular reactor, and only a small proportion of the total population actively produce solvents, the bulk of the biomass being inert. This concept of different cell types was similar to that proposed earlier by Ennis (1987) to help to describe the proportions of acids and solvents produced during continuous fermentation in a cell recycle reactor, and by Clarke *et al.* (1988) in a free cell continuous reactor. Overall, it has been pointed out by Maddox (1989) that any further increases in reactor productivities, at sufficiently high solvent concentrations, will depend on achieving a greater understanding of the factors affecting the life cycle of this organism.

Because of the problems encountered with the packed-bed reactor, further investigations were made by Qureshi and Maddox (1988) into various configurations of this type of reactor and into a fluidized-bed reactor. Although productivities of up to 6 g/l.h were achieved at solvent concentrations of 6-7 g/l, in a packed-bed reactor, blockage due to cell fouling of fluid passages, and thus gas hold-up, often became problems. The fluidized-bed reactor, on the other hand, proved to be superior in terms of operational stability with solvent productivities approaching 5 g/l.h. Moreover, based on the bonechar loading,

the fluidized-bed reactor was 6 times more productive than the packed bed, probably due to greater amounts of biomass being present.

Recently, Maddox (1988) has evaluated and compared the use of bonechar-immobilized cells, alginate-immobilized cells and a cell recycle technique, using crossflow microfiltration, all in continuous culture for solvent production from whey permeate. It was reported that bonechar-immobilized cells allowed a very simple and stable process to be developed, with superior reactor productivities. Alginate-immobilized cells were also simple and stable to operate, but reactor productivities were poorer. The recycle technique was difficult to operate and stable conditions could not be maintained.

More recently, Park *et al.* (1990), following their previous work in 1989 on immobilization by entrapment on natural sponge, have investigated immobilization by adsorption onto polyester sponge. Using a trickle-bed reactor, a butanol concentration of 5.2 g/l representing 55 % conversion of the feed (60 g/l glucose) was observed. The glucose and product concentration profiles along the column revealed that the column exhibited 'production' and 'inhibition' regions, and that the length of each region was dependent on the feed glucose concentration and feed pattern. Overall the results indicated that the strategy for long term stable operation with high solvent yield requires glucose concentrations between 40 to 60 g/l, a nutrient supply only to the inlet of the reactor, a good structured packing of porous matrix such as polyester to alleviate column clogging, and pH maintenance above 4.3 to avoid degeneration.

1.8 Summary

The main objective of this review is to provide some informative background of microbial solvent (ABE) production by *C. acetobutylicum*. A brief history of the ABE fermentation and its process is also provided in order to understand the origin of this process and the nature of this particular microorganism as well as

its complicated biochemical pathway. Further, important factors in the fermentation such as substrate source and concentration, *pH* and acid end-products, and nutrient concentrations, are discussed in order to understand the regulation systems of this fermentation process.

The role of cell density in solvent production is also considered, since the development of novel fermentation technologies to improve reactor yield and productivities is of fundamental importance, and this factor is apparently neglected in the literature.

This review is also extended to an investigation of the substrate whey and its nutritional status for the ABE fermentation process, since this substrate has potential use as a commercial substrate for the process.

Utilization of immobilized cells systems is also considered. Two approaches of immobilization technique, i.e. entrapment and adsorption, are discussed.

The ultimate aim of this thesis is to investigate the effect of nutrient composition on solvent production by *C. acetobutylicum* P262, and to relate the knowledge gained to whey permeate. In addition, the stable operation of a highly productive packed bed reactor based on bonechar-immobilized cells is a target. The approach taken was to use a fully-defined medium in three different fermenter modes, prior to performing similar experiments with the whey permeate. The three fermenter modes were traditional batch fermentation and continuous culture, both using freely suspended cells, and a packed bed reactor using cells immobilized by adsorption.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Microbiological Media

Spray-dried sulphuric acid casein whey permeate was obtained from the New Zealand Dairy Research Institute (Palmerston North, New Zealand). Yeast extract and Cooked Meat Medium were obtained from Difco Laboratories (Detroit, Michigan, U.S.A.). Reinforced Clostridial Agar (RCA) was obtained from BBL Microbiology Systems (Cockeysville, Maryland, U.S.A.). Distilled water was used for the preparation of all media.

Sulphuric acid casein whey permeate agar consisted of spray-dried sulphuric acid casein whey permeate (55 g/l) and Davis agar (15 g/l) reconstituted in distilled water, adjusted to *pH* 6.5 using 4M potassium hydroxide prior to autoclaving.

Media used for the various fermentation experiments are given in Tables 2.1 to 2.6 inclusive. The typical composition of sulphuric acid casein whey permeate is given in Table 2.7.

Table 2.1 Synthetic medium used for batch fermentation experiments

Component	Concentration (g/l)
Lactose	60.0
Ammonium acetate (or Ammonium sulphate)	0.5-2.0
K ₂ HPO ₄	0.02-1.0
NaCl	1.0
L-cysteine hydrochloride	0.5
MgSO ₄ ·7H ₂ O	0.2
MnSO ₄ ·H ₂ O	0.01
FeSO ₄ ·7H ₂ O	0.001-0.012
Biotin	0.001
Thiamine hydrochloride	0.02
<i>p</i> -Aminobenzoic acid	0.02

Table 2.2 Sulphuric acid casein whey permeate medium for batch fermentation experiments

Component	Concentration (g/l)
Sulphuric acid casein whey permeate	60.0
Lactose	15.0
Yeast extract	5.0

Medium was adjusted to *p*H 6.5 using 4M KOH prior to autoclaving.

Table 2.3 Synthetic medium used for continuous fermentation experiments

Component	Concentration (g/l)
Lactose	50.0
Ammonium acetate	0.046-0.30
K ₂ HPO ₄	0.22-0.94
NaCl	1.0
L-cysteine hydrochloride	0.5
MgSO ₄ ·7H ₂ O	0.2
MnSO ₄ ·H ₂ O	0.01
FeSO ₄ ·7H ₂ O	0.01
Biotin	0.0001
Thiamine hydrochloride	0.002
<i>p</i> -Aminobenzoic acid	0.008

Table 2.4 Sulphuric acid casein whey permeate medium for continuous fermentation experiments

Component	Concentration (g/l)
Sulphuric acid casein whey permeate	60.0
Yeast extract	0-5.0

Medium was adjusted to *p*H 6.5 using 4M KOH prior to autoclaving.

Table 2.5 Synthetic medium for experiments involving immobilized cells in a packed bed reactor

Component	Concentration (g/l)
Lactose	50.0
(NH ₄) ₂ SO ₄	0-3.0
K ₂ HPO ₄	0-0.4
NaCl	1.0
CH ₃ COONa	1.0
L-cysteine hydrochloride	0.5
MgSO ₄ .7H ₂ O	0.4
MnSO ₄ .H ₂ O	0.02
FeSO ₄ .7H ₂ O	0-0.02
Biotin	0.004
Thiamine hydrochloride	0.016
<i>p</i> -Aminobenzoic acid	0.04

Medium was adjusted to *pH* 6.5 using 4M KOH prior to autoclaving.

Table 2.6 Sulphuric acid casein whey permeate medium for experiments involving cells in a packed bed reactor

Component	Concentration (g/l)
Sulphuric acid casein whey permeate	50.0
Yeast extract	0-5.0

Medium was adjusted to *pH* 6.0 using 4M KOH prior to autoclaving.

Table 2.7 Typical composition of sulphuric acid casein whey permeate^a

Total solids	(g/kg)	56.4
Total nitrogen	(g/kg)	0.37
Non-protein nitrogen	(g/kg)	0.32
Ash	(g/kg)	7.9
Lactose (monohydrate)	(g/kg)	46.0
Ash/Total solids	(%)	14.0
Lactose/Total solids	(%)	82.0
Calcium	(g/kg)	1.1
Sodium	(g/kg)	0.5
Potassium	(g/kg)	1.2
Phosphate (Total)	(g/kg)	1.9
Chloride	(g/kg)	0.9
Sulphate	(g/kg)	2.2
pH		4.5-4.7 ^b

^a Based on Short (1978)

^b Based on Ennis & Higgins (1981), Higgins & Short (1980)

2.1.2 Chemicals

All chemicals used for fermentation media and analytical work were of analytical grade. Those which were commonly used in the experiments, and their sources, are listed below.

Ajax Chemicals (Sydney, Australia)

manganese sulphate.

BDH Chemicals Ltd (Palmerston North, New Zealand)

ammonium acetate; acetone; acetic acid; d-biotin; butanol; butyric acid; dipotassium hydrogen phosphate; cysteine hydrochloride; ethanol; ferrous sulphate; ferrozine; formalin; glucose; lactose; magnesium sulphate; *p*-aminobenzoic acid; potassium hydroxide; sodium acetate; sodium chloride; thiamine hydrochloride.

Lactose Company Ltd (Kapuni, New Zealand)

lactose (GPR Grade).

2.1.3 Gases and Other Materials

Oxygen-free nitrogen gas was supplied by New Zealand Industrial Gases Ltd, (Palmerston North, New Zealand). Pyroneg detergent was supplied by Diversey-Wallace Ltd, (Papatoetoe, New Zealand). BBL GasPak Anaerobic Systems were obtained from Becton Dickinson Co., (Cockeysville, Maryland, U.S.A.).

Bonechar was supplied by New Zealand Sugar Company Ltd, (Auckland, New Zealand). The acrylic clear tubing from which the packed-bed reactors were made was supplied by Mulford Plastics (NZ) Ltd, (Auckland, New Zealand).

2.1.4 Microorganism

Clostridium acetobutylicum P262 was obtained as a spore suspension from Professor D R Woods, University of Cape Town, South Africa.

2.1.4.1 Production of Spores and Culture Maintenance

All spore stock cultures were revived by heat shocking an aliquot of 0.2 ml in 20 ml Cooked Meat Medium, supplemented with lactose (10 g/l), at 75°C for 2 min, followed immediately by cooling in iced water for 1.5 min. This culture was then incubated anaerobically at 30°C for 72 h until vigorous gassing was observed.

Slopes of sulphuric acid casein whey permeate agar were streaked with the revived culture and incubated anaerobically at 30°C. Spore formation was regularly monitored by Bartholomew and Mittwer's spore staining technique using malachite green (Harrigan and McCance, 1976). After 42 days incubation,

the sporulated cultures were aseptically scraped off into sterile distilled water and dispensed at 2 ml into sterile screw-capped Kimax test tubes (16 x 100 mm). The spore count by haemocytometer was in the range 1×10^7 and 1×10^8 spores per ml. This stock spore suspension was stored at 4°C and used for inoculum preparation throughout this study.

2.1.4.2 Culture Conditions

Cultures were grown in 2 stages as follows:

An aliquot of 0.2 ml stock culture was inoculated into 20 ml of Cooked Meat Medium, heat shocked at 75°C for 2 min, then rapidly cooled in iced-water for 1.5 min. The culture was incubated anaerobically at 34°C for 17-22 h until highly motile cells were observed.

Two ml of the culture were then transferred to 100 ml of appropriate medium (identical to that used in the subsequent main fermentation), and incubated at 34°C for approximately 18 h. A portion of this culture was then used to inoculate the main fermentation [3-4% (v/v) inoculum].

2.2 Sterilization

2.2.1 Media Sterilization

All culture media were sterilized by autoclaving at 121°C for 15 min. All fermentation media in large volume containers in excess of 4 litres were sterilized under the same conditions for 20 min. For all experiments using synthetic media, the vitamins were sterilized separately, by membrane filtration, and added to the main medium prior to inoculation.

2.2.2 Equipment Sterilization

Pipettes, test tubes, bottles and glass wool gas filters were sterilized in a hot air oven at 160°C for 3 h.

pH electrodes for insertion into fermenters were sterilized in 50% (v/v) aqueous ethanol for 18 h, and rinsed with hot sterile distilled water immediately before use.

Packed-bed reactor columns were soaked in 2% (v/v) aqueous formalin for at least 12 h, and rinsed with warm sterile distilled water prior to use. Bonechar was sterilized in a hot air oven at 160°C for 3 h.

2.3 Cleaning of Glassware

All glassware was washed in hot Pyroneg solution, rinsed in tap water, then in distilled water, and hot air dried.

2.4 Anaerobic Incubation

Cultures for anaerobic incubation were placed in BBL anaerobic jars. An anaerobic condition was achieved through generation of hydrogen and carbon dioxide from a GasPak 100 disposable envelope (BBL Microbiology Systems). A BBL GasPak anaerobic indicator was used to monitor anaerobic conditions in the jar. The palladium catalyst contained in wire gauze support was activated either by placing in a hot air oven at 160°C for at least 2 h or by direct flaming until red hot, prior to use.

2.5 Analytical Methods

2.5.1 pH Measurement

All pH measurements were performed by using a Metrohm pH meter E520 (Metrohm A.G., Herisau, Switzerland) which was calibrated prior to use using pH 4.0 and pH 7.0 buffers.

2.5.2 Determination of Biomass Dry Weight

A known volume of fermentation culture (usually 10 ml) was centrifuged at about $5,500 \times g$ for 20 min using a Clandon Centrifuge (Model T52.1, Clandon Scientific Ltd, Aldershot, England). The supernatant liquid was discarded, while the cells were washed in 5 ml of distilled water, re-centrifuged, and then dried in hot air oven at 105°C for 48 h.

2.5.3 Total Cell Count

Total cell counts were performed using a standard haemocytometer (Assistant, West Germany) under $400 \times$ magnification. Both vegetative cells and spores, if any, were counted.

2.5.4 Determination of Solvents and Acids

Solvent and acid fermentation products were determined by gas chromatography (GC) using a Shimadzu GC (Model GC-8APF, Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector. A stainless steel column (1 m \times 0.15 cm internal diameter) packed with Porapak Q was used at a carrier gas (nitrogen) flow rate of 30 ml/min. The column was

maintained at 200°C, and the injector and detector temperatures at 220°C. Samples were injected as 2 µl aliquots.

Concentrations were calculated by measurement of peak height, using an internal standard in the sample, and comparison with a standard solution of similar, known solvent and acid composition. The internal standard used was *sec*-butanol (50 g/l) in 20% v/v orthophosphoric acid, added at the rate of 0.1 ml to 1.0 ml of sample or standard solution. The standard solution was composed of butanol (5 g/l), ethanol (2 g/l), acetone (2 g/l), butyric acid (2 g/l), and acetic acid (2 g/l).

A response factor (R_f) was calculated for each solvent and acid component in the parallel standard as follows:

$$R_f = \frac{\left(\frac{IS}{P}\right)_h}{\left(\frac{IS}{P}\right)_c}$$

where:

$$\left(\frac{IS}{P}\right)_h$$

is the average of the ratio of peak heights of internal standard : solvent or acid from duplicate injections.

$$\left(\frac{IS}{P}\right)_c$$

is the concentration ratio of the internal standard : solvent or acid in the standard solution.

The response factors were used to calculate the concentrations of solvents and acids in the sample.

$$C = R_f \cdot IS_c \cdot \left(\frac{CH}{ISH} \right)$$

where:

C is the concentration of solvent or acid in the sample (g/l).

IS_c is the concentration of the internal standard in the sample (g/l).

$\frac{CH}{ISH}$ is the ratio of peak heights of solvent or acid to internal standard.

2.5.5 Determination of Lactose

Quantitative analysis of lactose was performed using a Waters Associates Model ALG/GPC 244 high performance liquid chromatograph (HPLC) with a Model 590 solvent delivery system and Model U6K septumless injector (Waters Associate, Inc., Milford, Massachusetts, U.S.A.).

A Sugar-Pak I Carbohydrate column (Waters Associates) was used. The column was operated at 90°C using a solvent system of calcium acetate (20 mg/l) at a flow rate of 0.5 ml/min. The detector was a Waters Associates differential refractometer Model R401.

Fermentation samples were centrifuged (5,500 × g for 10 min) and the supernatant was suitably diluted to give a lactose content around 5 g/l. The samples were filtered through a 0.45 μm membrane using a Swinney Filter Kit (Millipore Corporation, Salmond Smith Biolab Limited, Northcote, Auckland, New Zealand) prior to injecting at 50 μl volume.

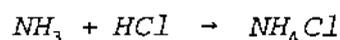
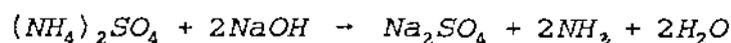
Calculation of lactose concentration was done by measurement of the peak height and reference to the peak height of a standard lactose solution.

2.5.6 Determination of Total Nitrogen by the Kjeldahl Method

The Kjeldahl method consisted of three stages: digestion, distillation and titration. A known volume of sample (usually 5 ml) was digested with excess concentrated sulphuric acid. This converted nitrogenous compounds to ammonium sulphate while carbonaceous matter was oxidised. Sodium sulphate was used as the reaction catalyst.

The ammonium sulphate formed by digestion of the sample was treated with excess alkali; this liberated ammonia which was distilled (using a Markham apparatus) into boric acid.

The ammonia absorbed by the boric acid was titrated directly with standard hydrochloric acid. From this titre the nitrogen present in the sample was calculated.



$$\text{Grams } N_2 \text{ in sample} = M_{HCl} \times V_{HCl} \times M.W. \text{ nitrogen}$$

$$\therefore \text{Concentration of } N_2 \text{ (g/l)} = \frac{M_{HCl} \times V_{HCl} \times M.W. \text{ nitrogen}}{\text{Volume of sample}} \times 1000$$

where

M_{HCl} is molarity of hydrochloric acid.

V_{HCl} is volume of hydrochloric acid used in titration.

$M.W._{nitrogen}$ is molecular weight of nitrogen.

2.5.7 Determination of Ammonium Ion

The method described in Section 2.5.6 was applied except that the digestion stage was not required, and only 2 ml of sample was used.

$$\text{Concentration (g/l) of } NH_4 \text{ in sample} = M_{HCl} \times V_{HCl} \times M.W._{NH_4\text{-salt}}$$

2.5.8 Determination of Inorganic Phosphate

This was performed by measuring the blue colour intensity formed by the reduction of molybdophosphoric acid to molybdenum blue, of uncertain composition, in the presence of hydrazine sulphate (Vogel, 1961). The intensity of the blue colour is proportional to the phosphate concentration in the sample. Sample and standard solutions containing up to 0.1 mg of phosphorous as the orthophosphate in 25 ml, were mixed with molybdate solution (5 ml; 12.5 g sodium molybdate in 500 ml of 5M sulphuric acid) and hydrazine sulphate (2 ml; 1.5 g hydrazine sulphate in 1 litre Milli-Q deionised water), diluted to the mark with deionised water in a 50 ml volumetric flask, and well mixed. The flasks were then immersed in a boiling water bath for 10 minutes. They were removed and cooled immediately in iced water. When cool, the contents were again mixed, and the volume re-adjusted to 50 ml using Milli-Q deionised water. The absorbance of the samples was measured at 830 nm against deionised water. The standard curve was linear in the range 0 to 4.0 mg/l phosphorous.

2.5.9 Determination of Iron

The method was based on that of Stookey (1970). The disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, known as ferrozine, reacts with divalent iron to form a stable magenta complex species which is very soluble in water and can be used for the direct determination of iron in water. A sample of 50 ml was mixed with 1 ml acid reagent solution (5.14 g of ferrozine and 100 g of hydroxylamine hydrochloride were dissolved in water and mixed with 500 ml concentrated hydrochloric acid, cooled to 20°C and diluted to 1 litre using Milli-Q deionised water). The solution was heated on a hot plate and held at the boiling point for 10 minutes. When cool, the contents were quantitatively transferred to a 50 ml volumetric flask and 1 ml of buffer solution (400 g of ammonium acetate in water, plus 350 ml of concentrated ammonium hydroxide, adjusted to 1 litre with Milli-Q deionised water) was added before diluting to the mark with Milli-Q deionised water. The contents were mixed and allowed to stand for 1 minute for full colour development. The absorbance of the samples was measured at 562 nm against a calibration curve prepared from standard solutions treated in the same manner.

2.6 Fermentation Culture Conditions

2.6.1 100-ml Bottle Cultures

Fermentations were conducted in 120 ml screw capped bottles containing 100 ml of medium. All cultures were incubated in anaerobic jars.

Spore stock (0.2 ml aliquot (Section 2.1.4)) was transferred to 20 ml of Cooked Meat Medium supplemented with glucose (10 g/l), heat shocked at 75°C for 2 min, followed by rapid cooling in iced water for 1.5 min, and incubated at 34°C until highly motile cells were observed (usually 17-22 h).

For experiments using synthetic medium, 1 ml of the revived culture was then transferred to 20 ml of the appropriate medium (Table 2.1) and incubated at 34°C for 17-22 h prior to transferring a 5 ml inoculum to 100 ml of the same medium.

For experiments using sulphuric acid casein whey permeate, 1 ml of the revived culture was transferred to 20 ml of Cooked Meat Medium supplemented with lactose (10 g/l) and incubated at 34°C for 17-22 h. One ml of this culture was then transferred to 20 ml of whey permeate medium and incubated at 34°C for 17-22 h prior to transferring a 5 ml inoculum to 100 ml whey permeate medium.

Samples (5 ml) were withdrawn from the fermentations at 24 h periods using a sterile pipette. Prior to sampling, the contents of the bottle were mixed to obtain a homogeneous sample.

2.6.2 Batch Fermenter Culture

2.6.2.1 Preparation of Inoculum

Spore stock (0.2 ml) was transferred to 20 ml Cooked Meat Medium supplemented with lactose (10 g/l), and heat shocked at 75°C for 2 min, followed by rapid cooling in iced water for 1.5 min prior to incubation at 34°C in an anaerobic jar. After 17-22 h incubation, vigorous gassing and a highly motile culture were observed. Five ml of this culture were used to inoculate either synthetic medium (Table 2.1) or whey permeate medium (Table 2.2) depending on the experiment, and incubated at 34°C until the most highly motile culture could be observed. This was then used as the inoculum for the final fermentation [3-4% (v/v) inoculum].

2.6.2.2 2-litre Fermentation Apparatus

The basic fermenter was a Microferm Laboratory Fermenter (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.) equipped with a 2 litre pyrex glass vessel of 1.5 litre working volume.

Continuous *pH* measurement and one-way control of *pH* were performed using an Automatic Mini *pH* Control System (New Brunswick Scientific Co.) consisting of a *pH* 40 Controller module, a *pH* 42 Pump module and a *pH* 40 Recorder module. The culture *pH* was measured using a scaled combination electrode with a silver/silver chloride reference (Broadley-James-Corporation, South Lyon, Santa Ana, California, U.S.A.). Where necessary, potassium hydroxide solution (3M or 4M) was used for *pH* control. A permanent record of culture *pH* could be printed on the strip chart recorder module.

2.6.2.3 Batch Fermenter Operation

The fermenter vessel, containing medium, was removed from the autoclave and attached to the fermenter apparatus while still hot (85°-90°C). Cooling was initiated by means of cold water flowing through hollow baffles, controlled by an electronic thermostat, and oxygen-free nitrogen gas was flushed across the medium surface. Surface flushing with gas was continued after inoculation until good gassing due to microbial growth was observed.

For experiments using synthetic medium, when the temperature was 34°C - 37°C, the vitamin solution was added into the medium. The medium *pH* was adjusted as required using potassium hydroxide solution (3M or 4M), and the fermenter was inoculated with motile cells prepared as described in Section 2.6.2.1. Agitation (50 rpm) was used to gently mix the vessel contents at this time. The culture was then left unagitated, except for brief agitation at 100 rpm prior to sampling.

Sampling was achieved by using the oxygen-free nitrogen gas to pressurize the culture vessel and, by blocking the gas outlet, force sample out. Five ml of fermentation sample were collected twice daily over a period of 5 days. The total volume of culture removed during sampling, and the volume of alkali solution added, were recorded at the time of sampling.

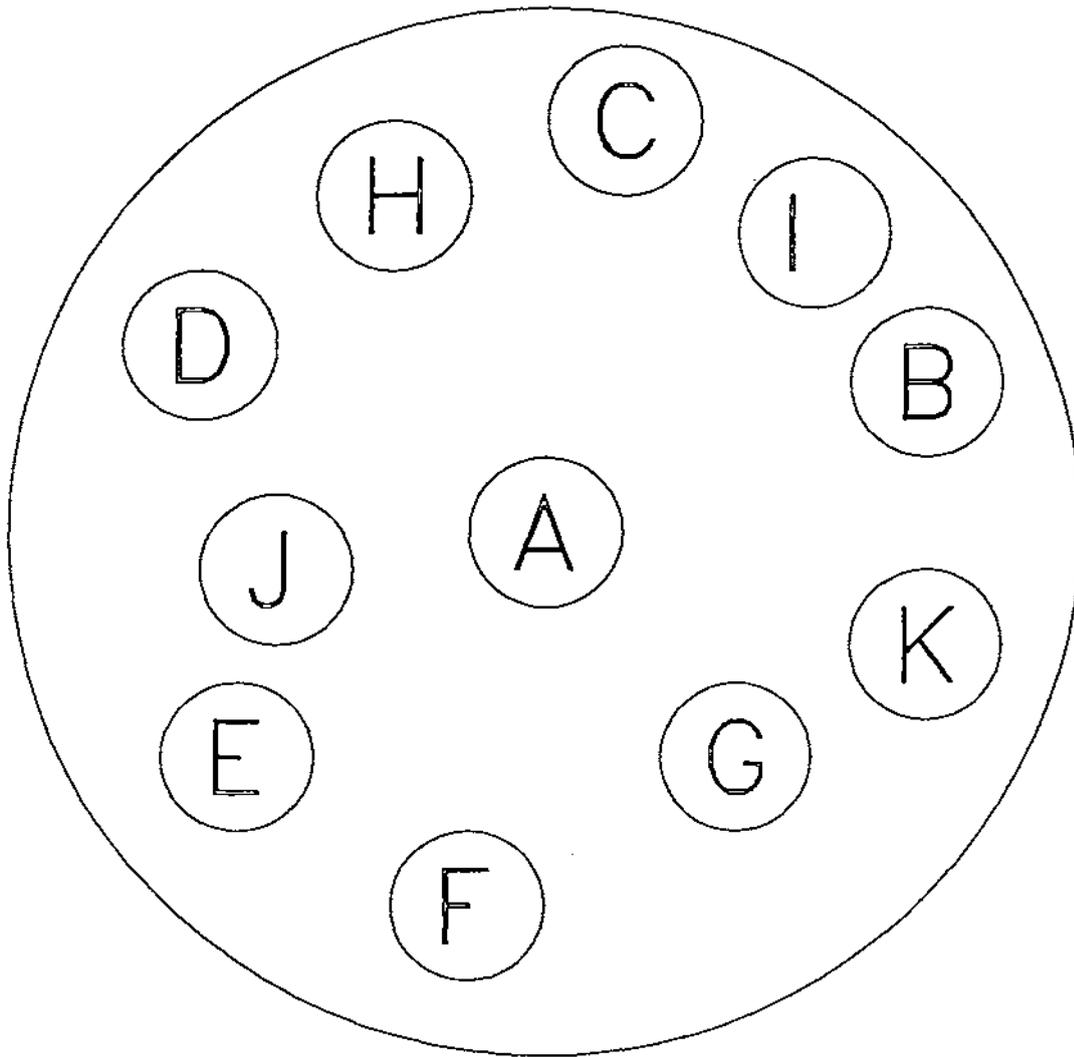
The *pH* electrodes were calibrated using *pH* 4.0 and *pH* 7.0 buffer solutions prior to each fermentation. After each fermentation the electrodes were washed thoroughly and soaked in distilled water. When not in use, the probes were stored in *pH* 7.0 buffer solution. The *pH* value of fermentation samples was independently measured (Section 2.5.1) to check the fermenter *pH* measurement, and any discrepancies were corrected.

2.6.3 Continuous Fermentation Using Free Cells

The fermenter apparatus was as described in Section 2.6.2.2. The fermenter vessel was a 2 litre pyrex glass vessel (New Brunswick Scientific Co.) of 1.0 litre working volume. Figure 2.1 gives a diagram of the fermenter head.

Temperature control was by means of internal coils. A digital thermocouple was used to check on temperature. The culture *pH* was measured and controlled using a Kent combination *pH* probe (Kent Industrial Measurements Ltd, Gloucestershire, England, Model 1117) connected to a Horizon *pH* Controller Model 5997-20 (Horizon Ecology Co., Chicago, Illinois, U.S.A.). A Masterflex peristaltic pump (Cole Palmer Co.) was used for delivery of 4M KOH to the fermenter vessel.

The fermenter contents were agitated at 50 rpm throughout experiments using a single 4 flat-bladed impeller (3.5 cm diameter) situated 4 cm above the base of the fermenter. A schematic diagram and photograph of the fermenter apparatus are depicted in Figures 2.2 and 2.3, respectively.



- A Agitator shaft
- B Thermocouple probe
- C Sampling port
- D Alkali solution inlet
- E Thermocouple/Temperature control
- F Medium feed inlet
- G pH probe
- H N₂ gas inlet
- I Heating element
- J Culture outlet
- K N₂ gas outlet

Figure 2.1 The diagram of the fermenter head.

Legend

- 1 Feed medium.
- 2 Feed medium pump.
- 3 Fermenter.
- 4 Sampling port.
- 5 Effluent pump.
- 6 Effluent container.
- 7 Agitation & on-line temperature controller.
- 8 Thermostatic controlled waterbath.
- 9 Heating and agitator control.
- 10 pH probe.
- 11 pH meter.
- 12 pH controller.
- 13 Alkali solution.
- 14 Alkali feed pump.
- 15 O₂-free nitrogen gas supply tank.
- 16 Gas filter.
- 17 Gas regulator.
- 18 Gas outlet.
- 19 N₂ gas outlet line.
- 20 Recorder.

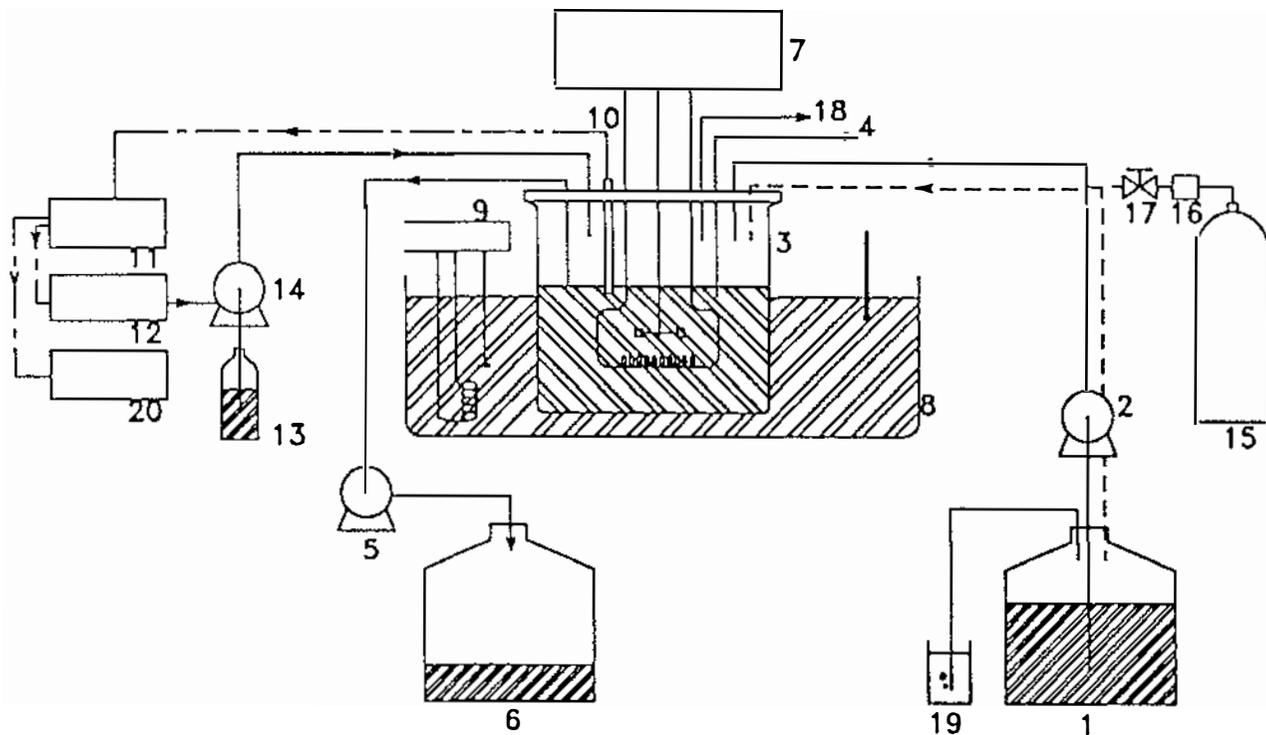


Figure 2.2 A schematic diagram of the fermenter and ancillary equipment used for continuous fermentation in a CSTR.



Figure 2.3 A photograph of the fermenter set-up for continuous (chemostat) experiments in a CSTR

Feed medium was continuously fed to the top of the fermenter using a Masterflex peristaltic pump. The level of fermenter contents was controlled using a fixed level tube, connected to a constantly running Masterflex peristaltic pump. Fermentation samples were periodically removed via this exit line.

The feed medium was kept anaerobic by surface flushing with oxygen-free nitrogen gas. The fermenter was kept anaerobic during the start-up phase by flushing nitrogen gas across the culture surface.

Initially, the fermentation was operated in batch mode (1 litre culture volume) under pH control at a value of 5.0-5.2. After 18 h incubation the delivery of feed medium was commenced at the required dilution rate.

2.6.4 Continuous Fermentation Using Immobilized Cells

The vertical packed-bed reactor apparatus is schematically depicted in Figure 2.4 and a photograph of the experimental set-up is shown in Figure 2.5. For experiments using synthetic medium, the jacketed reactor was 25 cm high, and 2.3 cm in diameter. The total working volume of the reactor was 0.113 litre. For experiments using sulphuric acid casein whey permeate, the jacketed reactor was 24 cm high, and 3.9 cm in diameter. The total working volume of the reactor was 0.277 litre (Figure 2.6). A stainless steel (Alloy 304) sieve mesh size 40/40 per 2.54 mm was situated at the top and bottom of the reactors to support the bonechar.

Bonechar, screened to a particle size range 355-600 μm diameter, was used to pack the reactor. For experiments using synthetic medium, the reactors were packed with 0.90 g of pre-sterilized bonechar per ml of reactor volume. For experiments using sulphuric acid casein whey permeate, the reactors were packed with 0.75 g of pre-sterilized bonechar per ml of reactor volume.

Legend

- 1 Feed medium.
- 2 Feed medium pump.
- 3 Reactor.
- 4 Sampling point.
- 5 Effluent container.
- 6 Thermostatic-controlled water bath.
- 7 O₂-free nitrogen gas supply tank.
- 8 Gas filter.
- 9 Gas regulator.
- 10 Nitrogen Gas outlet line.
- 11 Water pump.

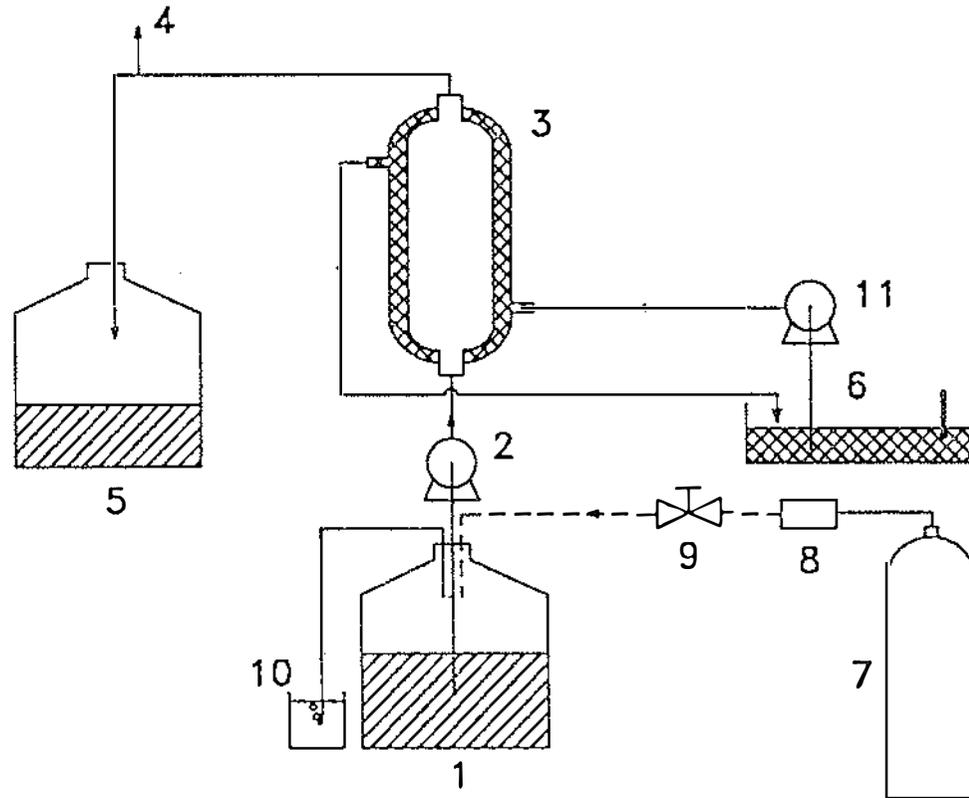


Figure 2.4 A schematic diagram of a vertical packed bed reactor and ancillary equipment.

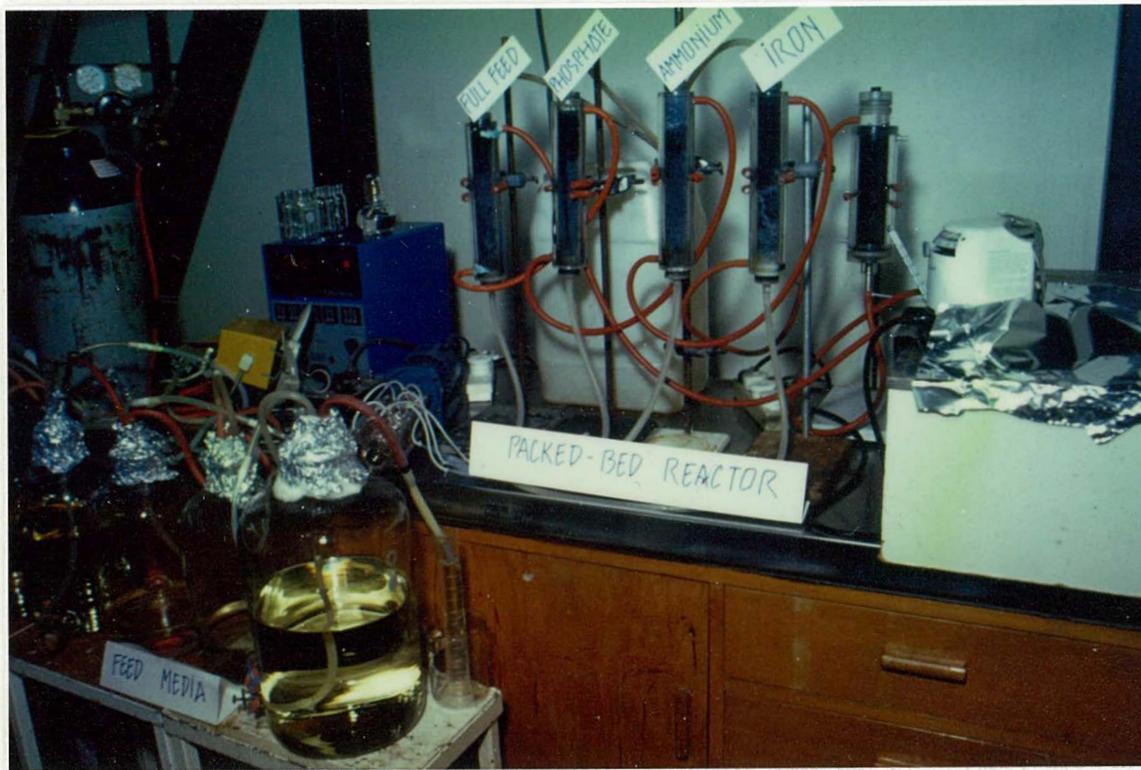


Figure 2.5

A photograph of the vertical packed bed reactor set-up for continuous culture experiments using immobilized cells in defined medium

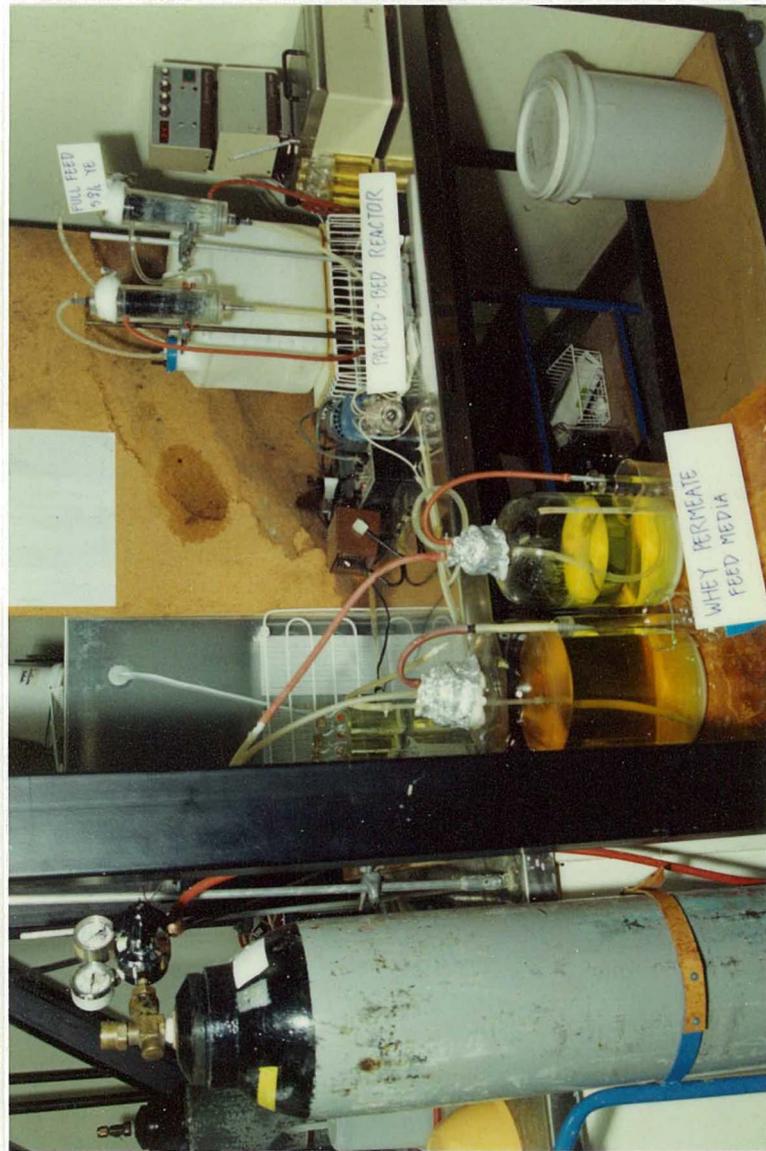


Figure 2.6 A photograph of the vertical packed bed reactor set-up for continuous culture experiments using immobilized cells in whey permeate medium

Cell immobilization was performed by pumping into the reactor, 50 ml of culture grown as described in Section 2.6.1. This was then incubated under batch conditions for 24 h, after which feed medium was fed continuously into the bottom of the reactor using a Masterflex peristaltic pump, and out from the top of the reactor from where samples were collected when necessary.

Temperature control was by means of a thermostated circulating water bath through the jacketed reactor. The feed medium was kept anaerobic by sparging with oxygen-free nitrogen gas and the *pH* was controlled at 6.5 prior to autoclaving.

2.7 Discussion of Methods

In GC analysis, errors were estimated to be 5-6% for solvents and acids analyses. The errors associated with the HPLC analysis of sugars (Section 2.5.5) were estimated to be 3-5%.

The most difficult part of the nitrogen analysis method technically was the distillation of ammonia using the Markham Still. To gain experience in this technique, a standard $(\text{NH}_4)_2\text{SO}_4$ solution was prepared and aliquots of this were distilled and the distillate titrated with standard HCl. A $\pm 2\%$ error was estimated.

In inorganic phosphate analysis, the intensity of the blue colour is proportional to the amount of phosphate initially incorporated in the heteropoly acid. If the acidity at the time of reduction is 1N in sulphuric acid and hydrazine sulphate is the reductant, the resulting blue complex exhibits maximum absorption at 820-830 nm. Ions which form heteropoly acids, such as silicate, arsenate, germanate, and tungstate, should be absent. A $\pm 5\%$ error was estimated.

In iron determination, soaking of glassware in concentrated hydrochloric acid for several hours may be necessary to obtain satisfactory and reproducible results. The estimated error during this analysis was $\pm 6\%$.

The most crucial step for the successful operation of a batch fermentation experiment was the inoculum development procedure described in Section 2.6.2.1. Slight variations in the inoculum size were used to compensate for unexplained variations in the germination rate of heat-shocked spores. The procedure of transferring a motile inoculum to freshly autoclaved and cooled medium, with the medium surface flushed with oxygen-free nitrogen gas during medium cooling and until gassing occurred due to bacterial growth, was strictly followed. This procedure gave reproducible results characterized by a small fermentation lag time and good growth.

CHAPTER 3

PRODUCTION OF SOLVENTS BY BATCH FERMENTATION

IN DEFINED MEDIUM AND IN SULPHURIC ACID CASEIN

WHEY PERMEATE

3.1 Introduction

At present, the acetone-butanol-ethanol fermentation process is considered to be uneconomic, mainly because of the low reactor productivities which can be attained, and the problem of product inhibition which contributes to the high cost of product recovery (usually by distillation).

Various approaches have been adopted to solve the problems, including the use of immobilized cells and cell recycle, and integrated fermentation/product recovery processes have also been described. However, these relatively novel fermentation techniques have so far found little industrial application for any fermentation product, and so it is possible that any commercial application of the ABE fermentation process will involve traditional batch fermentation.

The batch ABE fermentation process has been described by many authors. However, although the initial nutrient concentrations are usually described, the nutritional status of the medium during and after the solvent production phase is often not mentioned. For commercial operation in the past, it has been stated that all nutrients are generally present in excess (Jones and Woods, 1986), but it is not clear whether this is the optimum situation.

Hence, in the present study, the nutrient status of the culture and its effects on solvent production have been investigated in a batch fermentation process. Experiments were performed in a defined medium (Table 2.1), and the nutrients investigated were phosphate (Section 3.2.1), ammonium (Section 3.2.2), and iron (Section 3.2.3) ions. Lactose was used as the carbon source because of the potential use of whey permeate (Table 2.7) as a commercial substrate for the ABE fermentation process (Maddox, 1980).

3.2 Batch Fermentation in Defined Medium

3.2.1 Effect of Phosphate Ion Concentration on Solvent Production

3.2.1.1 Introduction

It has been reported by Bahl *et al.* (1982b) that solvents are produced only after phosphate exhaustion from the medium, whereas under conditions of excess phosphate acids rather than solvents are produced. The data reported recently by Bryant and Blaschek (1988) appear to support these results although the nutritional (phosphate) status of the culture at the end of the process was not given.

The purpose of this work was to further investigate the effect of the phosphate ion concentrations, in batch fermentation, on solvent production.

3.2.1.2 Results

A series of batch fermentations was performed whereby the initial phosphate ion concentration of the medium was varied, while all other nutrients were maintained constant. The results of these experiments are summarized in Table 3.1 and the progress of each fermentation is shown in Figures 3.1 to 3.8. In Runs I to IV, the phosphate nutrient was exhausted from the medium, while in Runs V to VIII all nutrients remained in excess.

Table 3.1 Effect of phosphate ion concentration on fermentation parameters, after five days of fermentation.

	Run No.							
	I	II	III	IV	V	VI	VII	VIII
Initial phosphate (mg/l) ^b	20	100	150	200	250	400	600	1000
Phosphate utilized (mg/l) ^b	20	100	150	200	200	280	300	340
Ammonium utilized (mg/l) ^a	390	1430	1450	1790	1560	1700	1610	1540
Biomass (OD ₆₂₅) (g/l)	1.05 (0.23)	1.46 (0.68)	2.22 (1.50)	2.20 (1.48)	2.35 (1.64)	2.10 (1.37)	2.36 (1.65)	2.12 (1.39)
Total solvents (g/l)	4.82	5.76	8.93	8.95	12.00	8.73	4.25	5.30
Total acids (g/l)	1.04	4.15	2.59	3.13	1.30	2.78	8.01	8.63
Lactose utilized (g/l) (%)	23.18 (41.74)	29.80 (47.45)	32.50 (50.78)	34.50 (56.56)	38.81 (63.86)	29.55 (51.65)	25.15 (41.75)	25.59 (42.32)
Total solvent yield (g solvent/g lactose utilized)	0.21	0.20	0.27	0.26	0.31	0.29	0.17	0.20
Max. observed butanol production rate (g/l.h)	0.015	0.112	0.137	0.262	0.233	0.143	0.100	0.100
Max. observed specific butanol production rate (g/g biomass phosphate.h) (g/g biomass.h)	0.75 (0.06)	1.12 (0.16)	0.91 (0.09)	1.31 (0.18)	1.16 (0.14)	0.51 (0.10)	0.33 (0.06)	0.29 (0.07)
Total solvents : total acids	4.63	1.39	3.45	2.86	9.23	3.14	0.53	0.61
Butanol : acetone	1.5:1	3.1:1	2.9:1	2.9:1	2.5:1	3.1:1	4.9:1	4.8:1

^a Initial concentration 2000 mg/l (expressed as ammonium acetate)

^b Expressed as K₂HPO₄

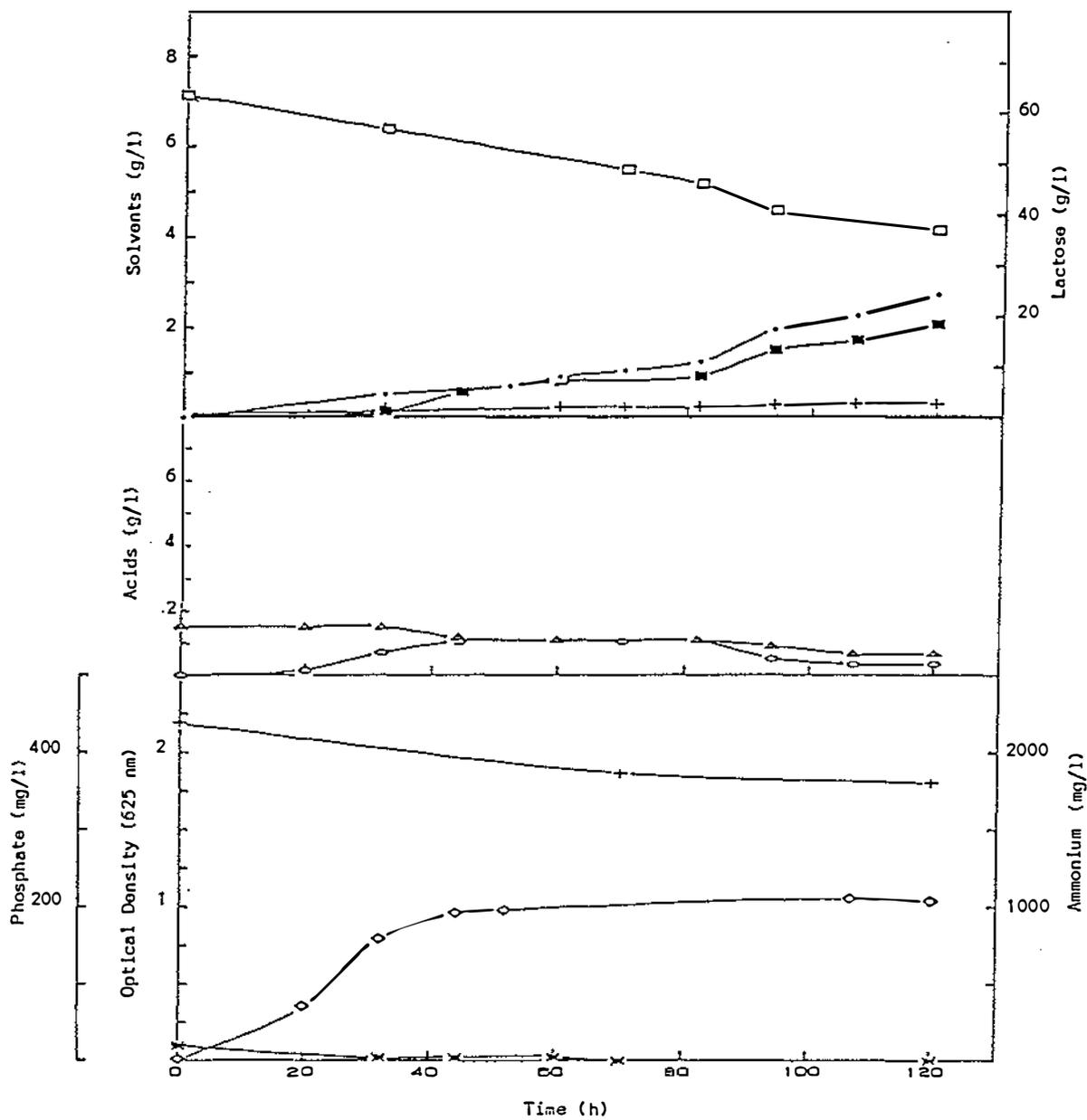


Figure 3.1

Progress of fermentation Run I. Initial phosphate 20 mg/l:
 (•) butanol; (■) acetone; (+) ethanol; (o) butyric acid;
 (Δ) acetic acid; (□) lactose; (*) phosphate; (+) ammonium;
 (o) OD₆₂₅ of culture

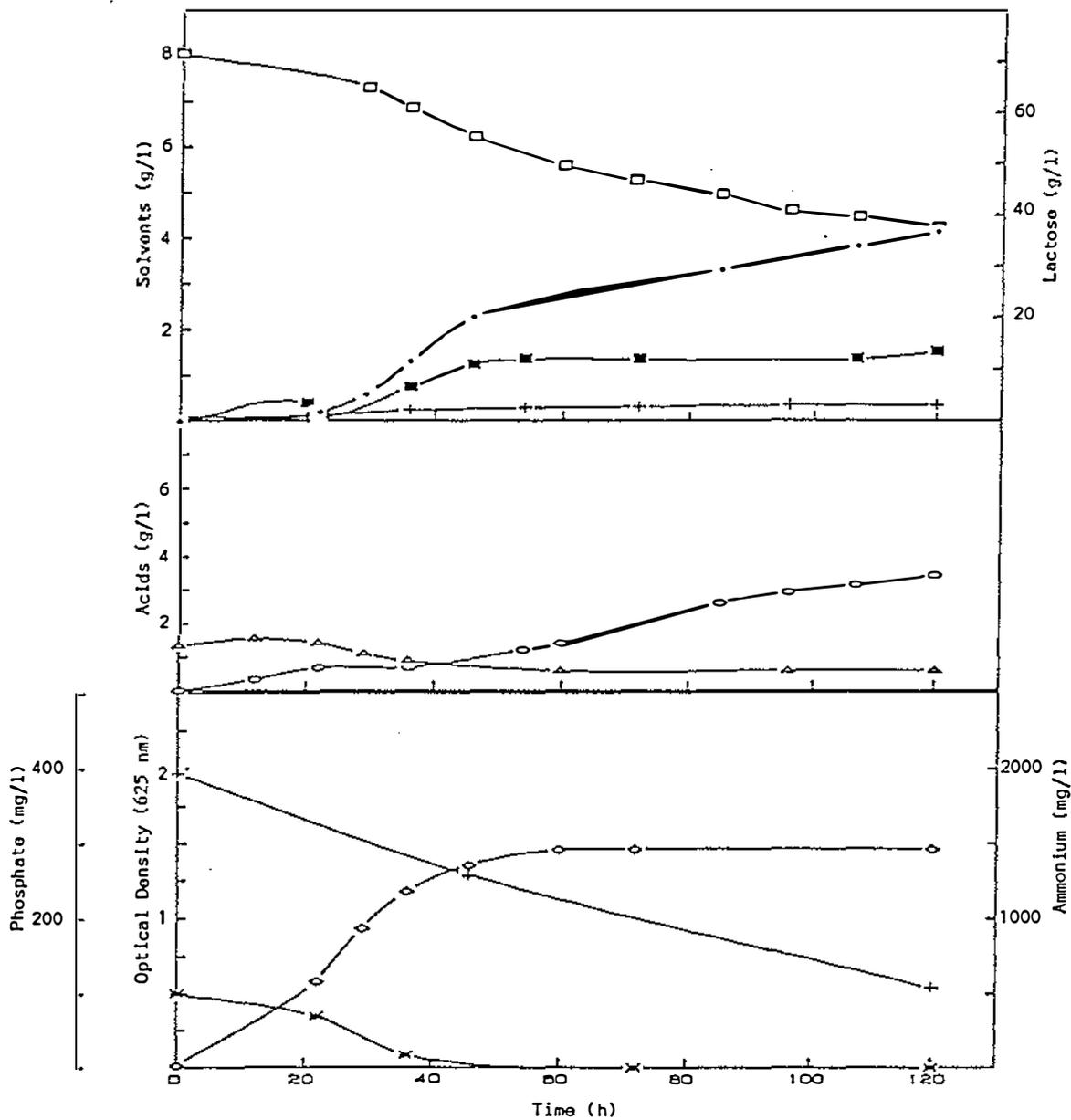


Figure 3.2 Progress of fermentation Run II. Initial phosphate 100 mg/l. Symbols as for Fig. 3.1

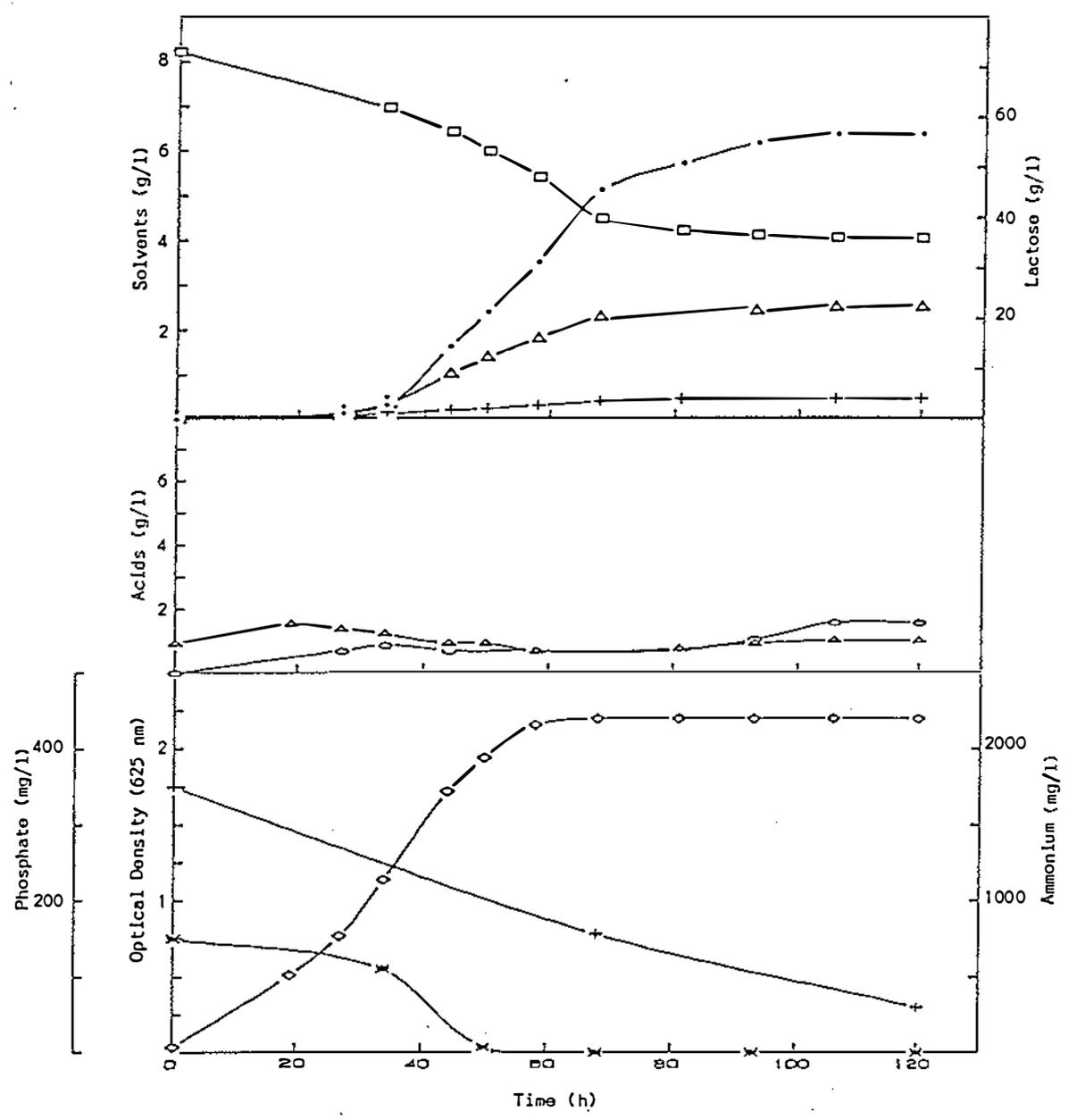


Figure 3.3 Progress of fermentation Run III. Initial phosphate 150 mg/l. Symbols as for Fig. 3.1

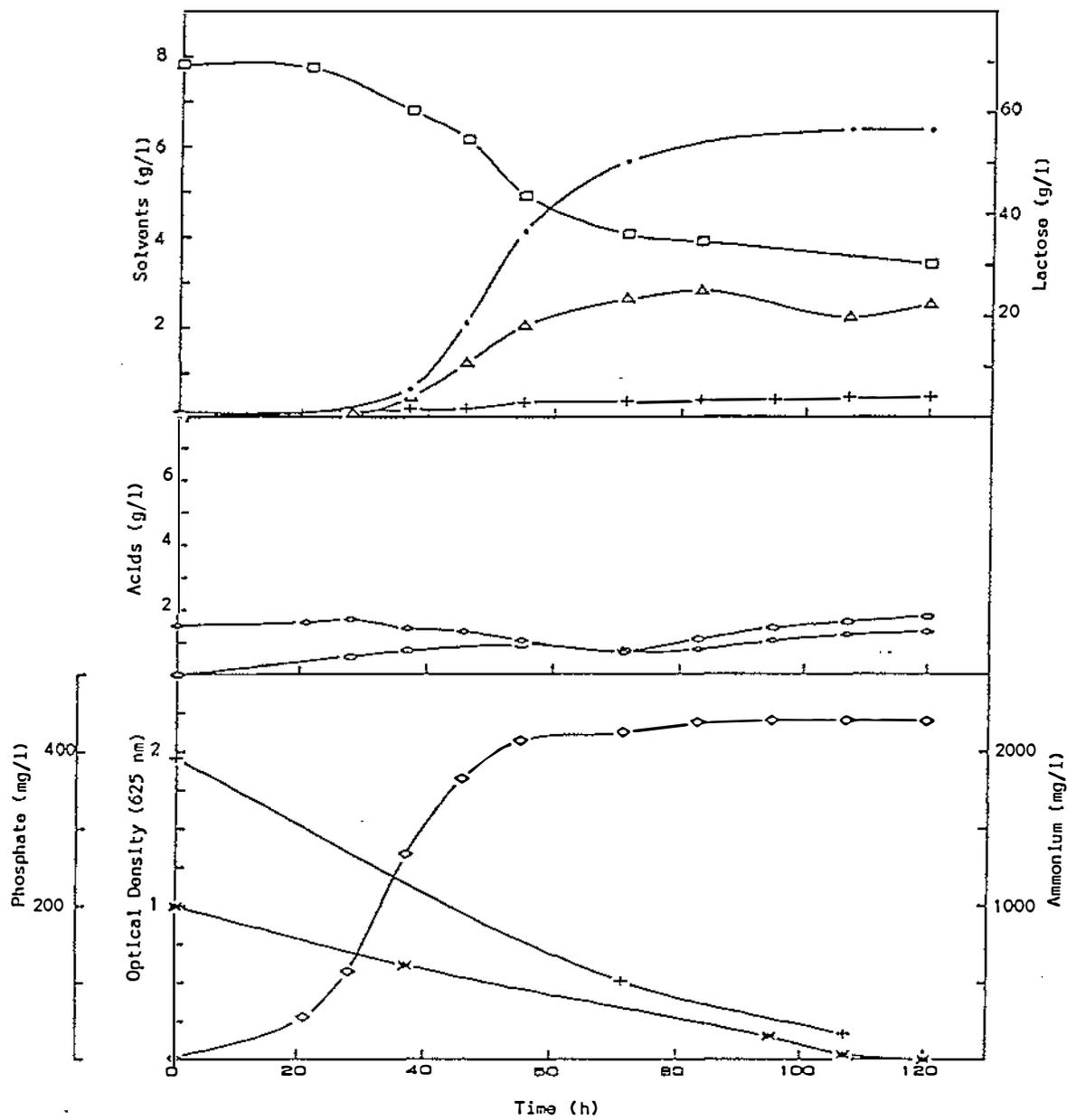


Figure 3.4

Progress of fermentation Run IV. Initial phosphate 200 mg/l.
 Symbols as for Fig. 3.1

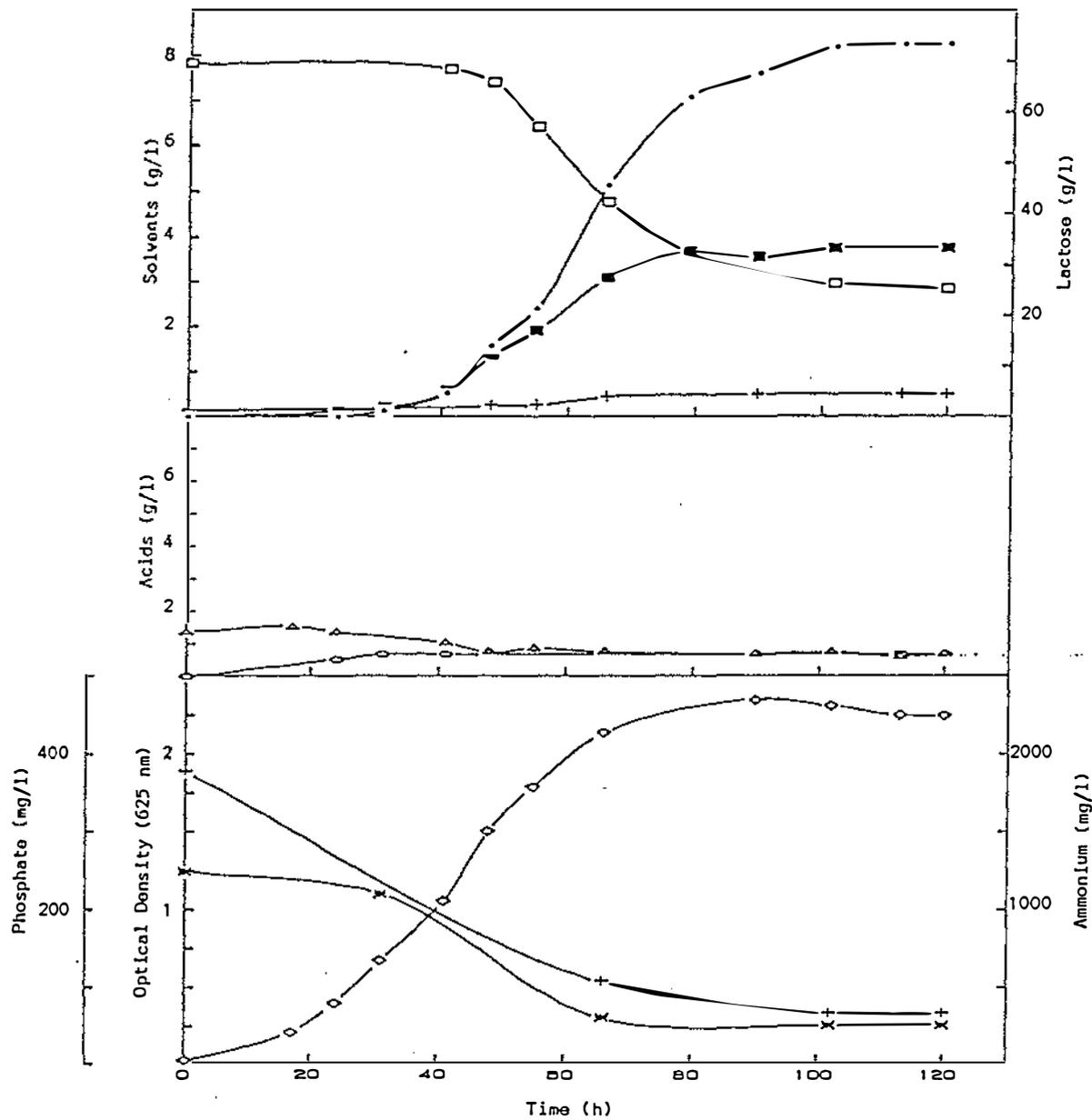


Figure 3.5

Progress of fermentation Run V. Initial phosphate 250 mg/l.
Symbols as for Fig. 3.1

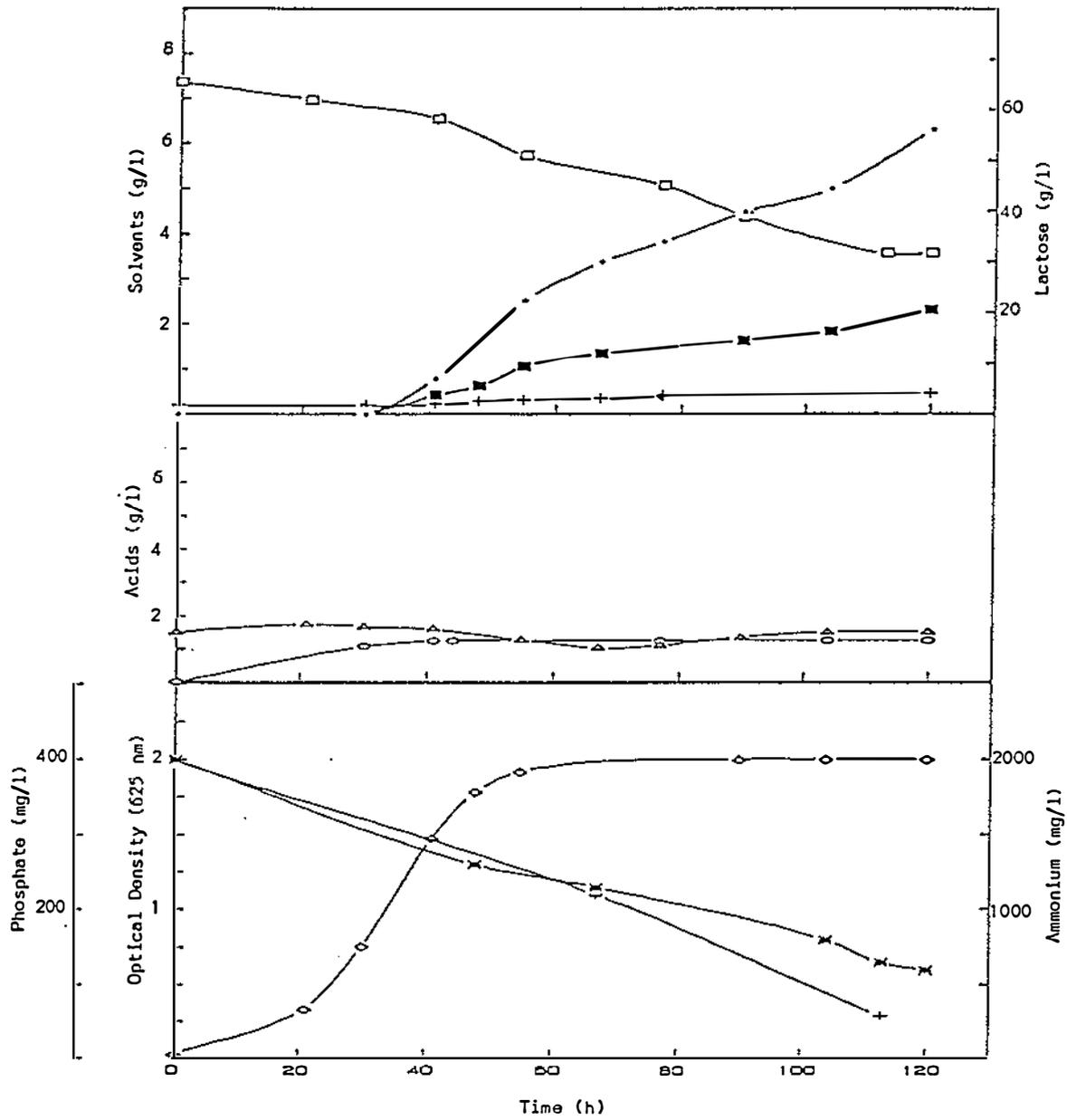


Figure 3.6 Progress of fermentation Run VI. Initial phosphate 400 mg/l. Symbols as for Fig. 3.1

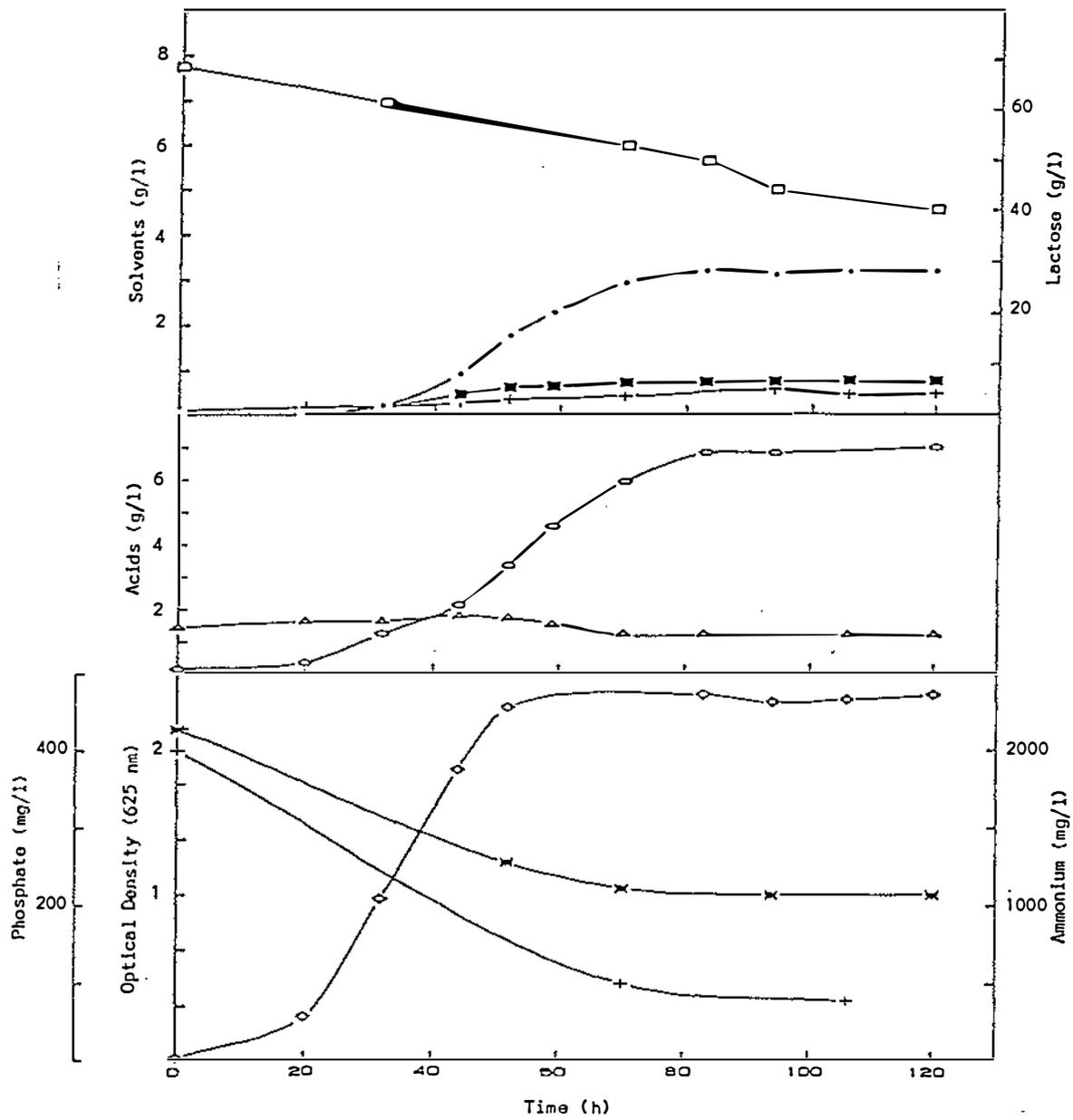


Figure 3.7

Progress of fermentation Run VII. Initial phosphate 600 mg/l.
Symbols as for Fig. 3.1

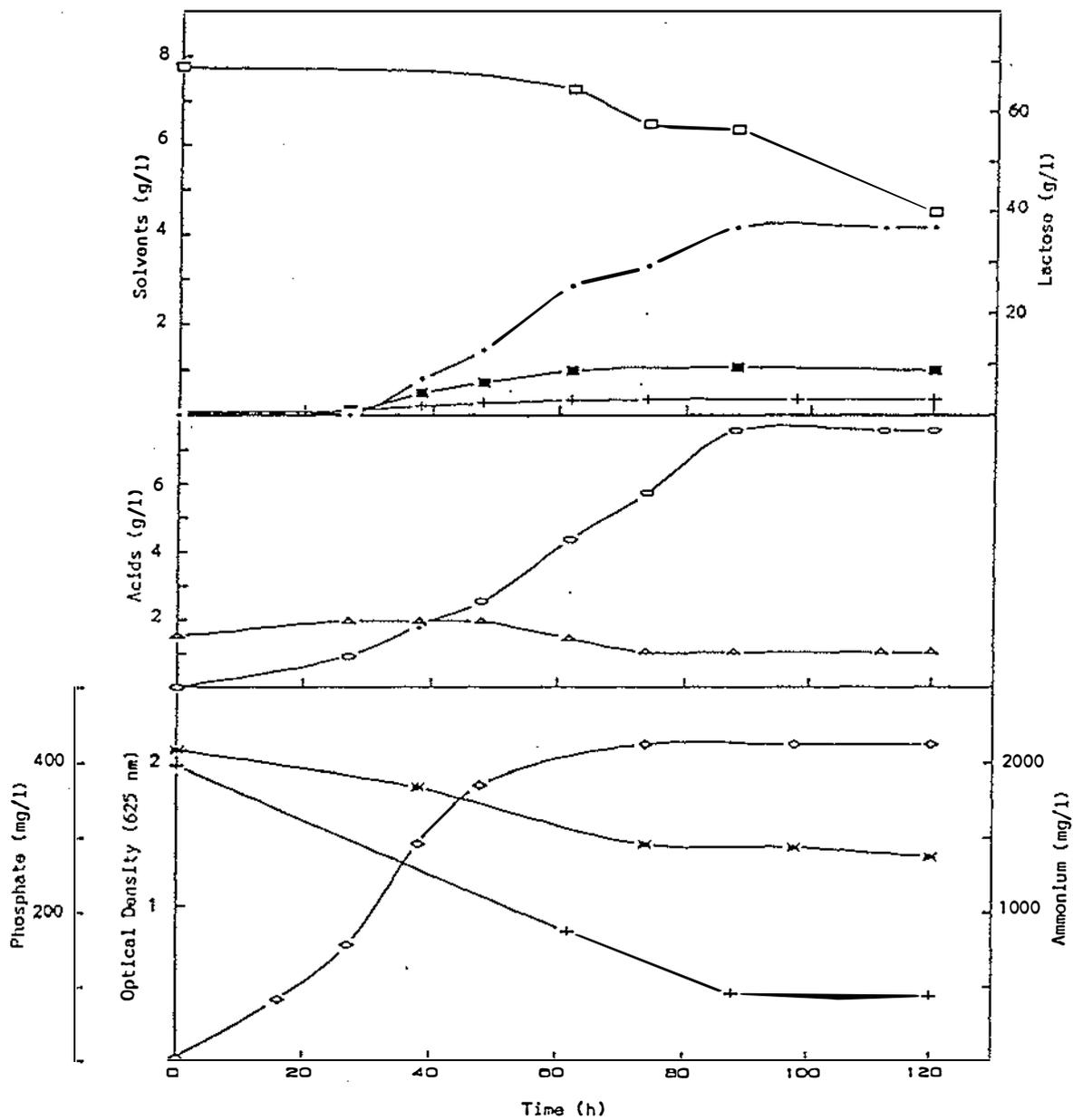


Figure 3.8

Progress of fermentation Run VIII. Initial phosphate 1000 mg/l.
Symbols as for Fig. 3.1

The initial experiment, Run I, was performed with 20 mg/l initial phosphate. Figure 3.1 shows that immediately after the phosphate ion was exhausted from the culture medium, the optical density at 625 nm reached a maximum value of 1.05, and solvent production started to increase. The total acids (acetic and butyric acid) and solvents (ethanol, acetone and butanol) after 120 h were 1.04 g/l and 4.82 g/l, respectively, representing a solvent yield of 0.21 g/g based on lactose utilized. The solvents to acids ratio was 4.63. The maximum observed butanol productivity was 0.015 g/l.h and the maximum observed specific butanol production rate was 0.75 g/g.h based on the biomass phosphate content, or the phosphate ion utilized. A total lactose utilization of 41.74 % was observed. Thus, these fermentation conditions favoured solvent yield and productivity, but the overall solvent production was relatively low. Since strain P262 is known to be capable of producing a much higher solvent concentration (up to 16 - 20 g/l), it was reasonable to suggest that solvent production was restricted by the low biomass concentration, caused by the low level of phosphate in the medium.

Run II (Fig. 3.2) was carried out with an increased initial concentration of phosphate (100 mg/l). The fermentation profile was similar to that of Run I, with phosphate being exhausted from the medium after 40h. In comparison with Run I, there was an improvement in overall production rate although the total solvent yield was approximately the same (Table 3.1). The ammonium ion utilization, the maximum observed productivity and the specific butanol production rate were significantly improved, suggesting that the amount of phosphate used in Run I was insufficient to support good growth and solvent production. An improved lactose utilization of 47.45 % was observed but this was accompanied by a higher concentration of acids, represented by a decreased solvents to acids ratio (1.39:1).

Run III (Fig. 3.3) and Run IV (Fig. 3.4) were performed under similar conditions to Run I and Run II except that the initial amount of phosphate was increased to 150 mg/l and 200 mg/l, respectively. Very similar fermentation profiles were

observed except that exhaustion of the phosphate became progressively later. In these Runs, the total solvents concentration and yield were very similar. However, there was an improvement in ammonium ion utilization in Run IV, and also of the volumetric and specific butanol production rates. The amount of lactose utilized as well as the solvents to acids ratio remained fairly constant. In comparison with Runs I and II, Runs III and IV displayed significantly stronger solvent production. In particular, Run IV displayed the highest volumetric and specific butanol production rates observed during this series of experiments.

Run V (Fig. 3.5) was performed under similar conditions to the previous Runs except that the initial concentration of phosphate in the culture medium was increased further to 250 mg/l. The fermentation profile shows that after the phosphate concentration had decreased to approximately 60 mg/l, the optical density of the culture reached its maximum level, the lactose utilization rate increased markedly, and butanol and acetone production increased rapidly. Little acetic acid or butyric acid were produced during this Run, which is evidenced in an increased solvents to acids ratio of 9.23. Throughout the fermentation, some phosphate remained unutilized in the medium. Total solvent production after 120 h (Table 3.1) was 12 g/l, representing a yield of 0.31 g/g. The volumetric and specific butanol production rates were 0.233 g/l.h and 0.14 g/g.h based on biomass dry weight, respectively. The total lactose utilization was 63.86 %. Thus, this Run demonstrated an improvement in solvent production as well as lactose utilization although there was a slight decrease in the volumetric and specific butanol production rates. The solvent yield of 0.31 g/g was the highest observed in all the Runs.

Three further Runs were performed whereby the initial phosphate concentration was further increased (Runs VI, VII and VIII). The trend in these Runs was for increasing residual phosphate in the medium, accompanied by acid rather than solvent production.

During all these Runs, there appeared to be no clear trend in the ratio of butanol to acetone produced, except for an increased ratio in Runs VII and VIII. In these Runs, however, the fermentation was acidogenic rather than solventogenic.

3.1.2.3 Discussion

The objective of this section was to determine the optimum concentration of phosphate in a defined medium for solvent production by batch fermentation. Similar studies have been performed by other workers using different substrates (predominantly glucose-based) and strains of *C. acetobutylicum*, but a precise investigation using phosphate as the limiting nutrient and lactose as the sugar source has not previously been reported.

The results show that solvent production can occur in batch fermentation under conditions of phosphate limitation, but the optimum condition is where the phosphate concentration is just slightly in excess of that required for growth. Under conditions of excess phosphate, the fermentation becomes acidogenic. A clear maximum was observed for the solvent production rate (Fig. 3.9), indicating that the cells' metabolism was being influenced in some way by the phosphate concentration of medium. These results generally support those of Bahl *et al.* (1982b) and Bryant and Blaschek (1988), although the latter authors were investigating 'buffering power' rather than phosphate as a nutrient.

The reason(s) for the observed effect appears to be complex. It has been suggested by several groups that the shift from acidogenesis to solventogenesis is induced by the accumulation of butyric acid (Gottschal and Morris, 1981b; Holt *et al.*, 1984; Gottwald and Gottschalk, 1985; Terracciano and Kashket, 1986). Monot *et al.* (1983) reported that the undissociated form of butyric acid plays a critical role in solvent formation. The results described by Terracciano and Kashket (1986) suggested that the uncharged forms of acetic and butyric acids must reach an adequate internal concentration, regardless of the total acid concentration, for initiation of solvent production to occur.

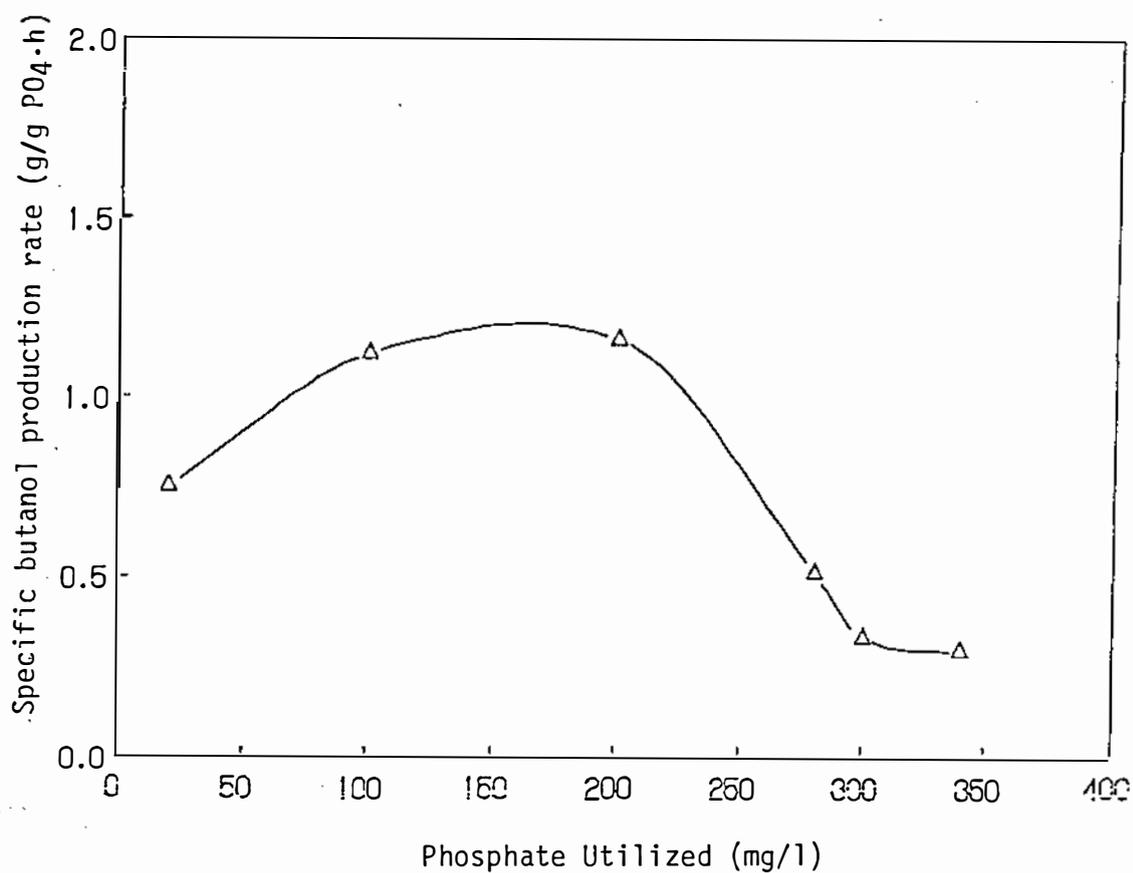


Figure 3.9 Plot of specific butanol production rate (g/g biomass phosphate·h) versus phosphate utilized, mg/l, for Runs I to VIII

It has been proposed by Gottwald and Gottschalk (1985) that as butyrate and acetate reach high levels at the end of the acidogenic phase, the levels of butyryl coenzyme A and butyryl phosphate (as well as acetyl coenzyme A and acetyl phosphate) also increase. This, in turn, results in a relative decrease in the coenzyme A and phosphate pools, which, then, is the triggering mechanism for activating or synthesizing the enzymes for solventogenesis. The substrates for the formation of butanol and acetone (butyryl coenzyme A or butyryl phosphate and acetoacetyl coenzyme A) are readily available under such conditions. Hence, growth-limiting levels of phosphate are believed to increase solvent production.

In commercial terms, it should be technically simple to apply the above results to a commercial substrate. Phosphate can be added to any deficient raw material, while an excess can be removed by precipitation using calcium hydroxide.

3.2.2 Effect of Ammonium Ion Concentration on Solvent Production

3.2.2.1 Introduction

The effect of the nitrogen status of the medium on the production of solvents is rather confusing. In a study on the effect of nitrogen limitation in batch culture, Monot and Engasser (1983a) observed that strong solventogenesis occurred after exhaustion of nitrogen from the medium. In contrast, Long *et al.* (1984a) reported that insignificant solvent production occurred unless there was a minimum nitrogen concentration remaining in the medium after the growth phase was completed. It has also been suggested that an excess of nitrogen is detrimental to solvent production, and that as the ratio of initial nitrogen to initial glucose decreases, the rate of solvent production increases (Roos *et al.*, 1985).

Therefore, the purpose of this section was to investigate in detail the effect of nitrogen (ammonium ion) concentration, in a batch fermentation medium, on solventogenesis.

3.2.2.2 Results

A series of batch fermentations was performed whereby the initial ammonium ion concentration of the medium was varied, while all other nutrients were maintained constant. The results of these experiments are summarized in Table 3.2 and the fermentation profile for each fermentation is shown in Figures 3.10 to 3.15. In Runs IX to XII, the nitrogen nutrient was exhausted from the medium, while in Runs XIII and XIV all nutrients remained in excess.

The initial experiment, Run IX, was performed with an initial ammonium sulphate concentration of 500 mg/l. Figure 3.10 shows that the optical density (at 625 nm) rose quickly while ammonium ion was available, but it decreased sharply after the ammonium ion was exhausted from the culture medium. Butyric and acetic acids were produced during the early stage of fermentation (20 - 40 h) and then remained constant until the end of fermentation. Butanol and small amounts of acetone and ethanol were produced soon after the ammonium ion had been exhausted from the medium. The total acids (acetic and butyric acid) and solvents (acetone + butanol + ethanol) after 120 h were 4.26 g/l and 2.33 g/l, respectively, representing a solvent yield of 0.10 g/g based on lactose utilized. The solvents to acids ratio was 0.55. The maximum observed volumetric and specific butanol production rates were 0.033 g/l.h and 0.08 g/g.h based on biomass dry weight, respectively. A total lactose uptake of 39.6 % was observed. Overall, these fermentation conditions favoured acidogenesis rather than solventogenesis. In Run X (Fig. 3.11), the initial concentration of ammonium sulphate was increased to 650 mg/l. A similar fermentation profile to Run IX was observed although there was some improvement in the uptake of phosphate and lactose, as well as in the volumetric butanol production rate.

Table 3.2 Effect of ammonium ion concentration on fermentation parameters, after five days of fermentation.

	Run No.					
	IX	X	XI	XII	XIII	XIV
Initial ammonium (mg/l) ^b	500	650	1000	1200	1500	2000
Ammonium utilized (mg/l) ^b	500	650	1000	1200	1450	1450
Phosphate utilized (mg/l) ^a	140	185	222	250	280	260
Biomass (OD ₆₂₅) (g/l)	1.20 (0.39)	1.83 (1.08)	2.16 (1.44)	2.16 (1.44)	2.30 (1.58)	2.50 (1.80)
Total solvents (g/l)	2.33	2.55	5.88	5.33	7.26	4.95
Total acids (g/l)	4.26	4.46	5.23	5.35	5.12	8.33
Lactose utilized (g/l) (%)	22.92 (39.56)	26.55 (43.76)	26.97 (45.40)	24.35 (40.55)	32.85 (53.30)	26.70 (43.59)
Total solvent yield (g solvent/g lactose utilized)	0.10	0.10	0.22	0.22	0.22	0.18
Max. observed butanol production rate (g/l.h)	0.033	0.056	0.092	0.283	0.312	0.169
Max. observed specific butanol production rate (g/g biomass ammonium.h) (g/g biomass.h)	0.07 (0.08)	0.09 (0.05)	0.09 (0.06)	0.24 (0.20)	0.21 (0.20)	0.12 (0.09)
Total solvents : total acids	0.55	0.57	1.12	1.00	1.42	0.59
Butanol : acetone	7.0:1	7.7:1	4.7:1	6.8:1	6.1:1	8.2:1

^a Initial concentration 400 mg/l (expressed as K₂HPO₄)

^b Expressed as (NH₄)₂SO₄

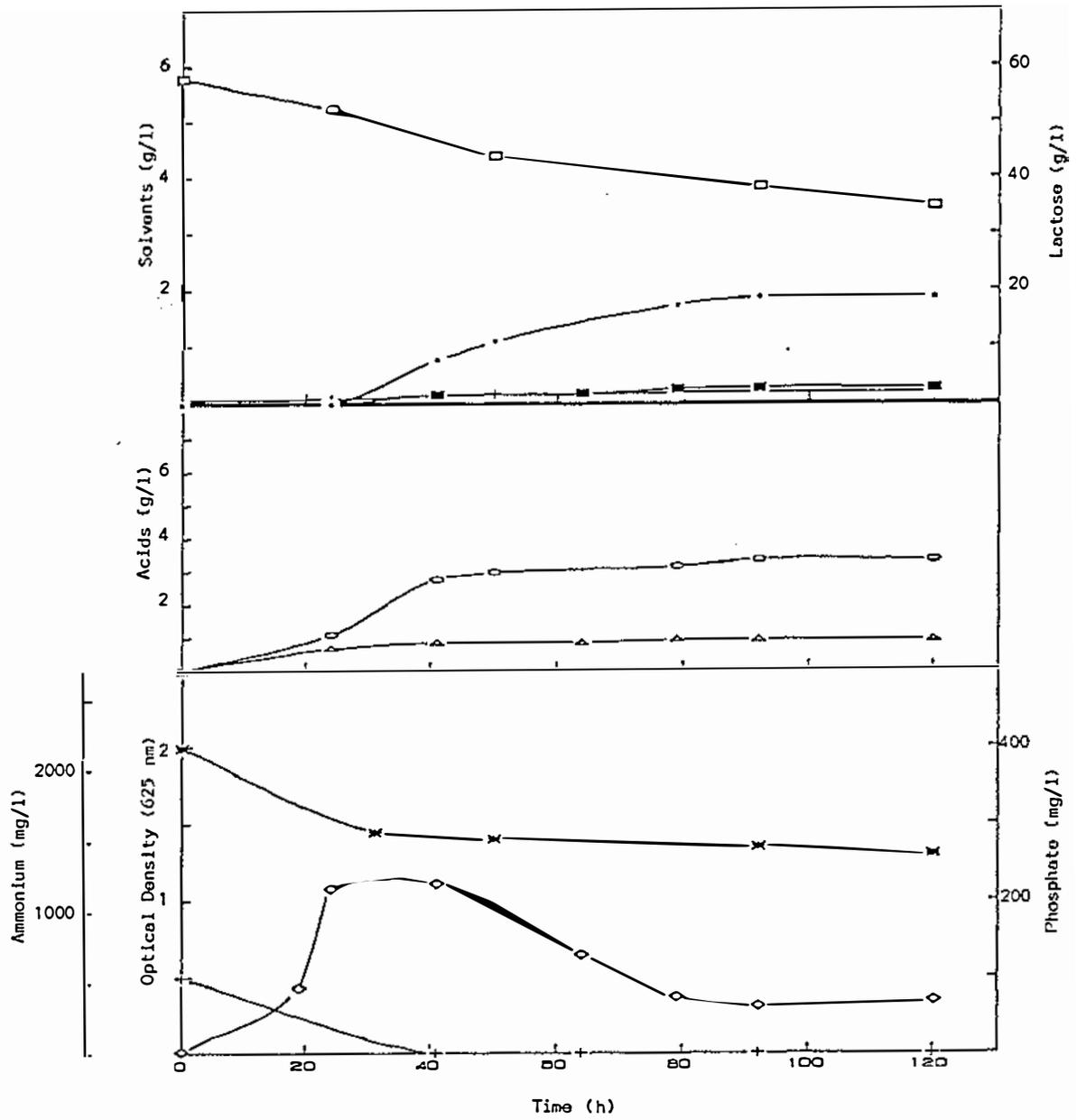


Figure 3.10

Progress of fermentation Run IX. Initial ammonium 500 mg/l.
Symbols as for Fig. 3.1

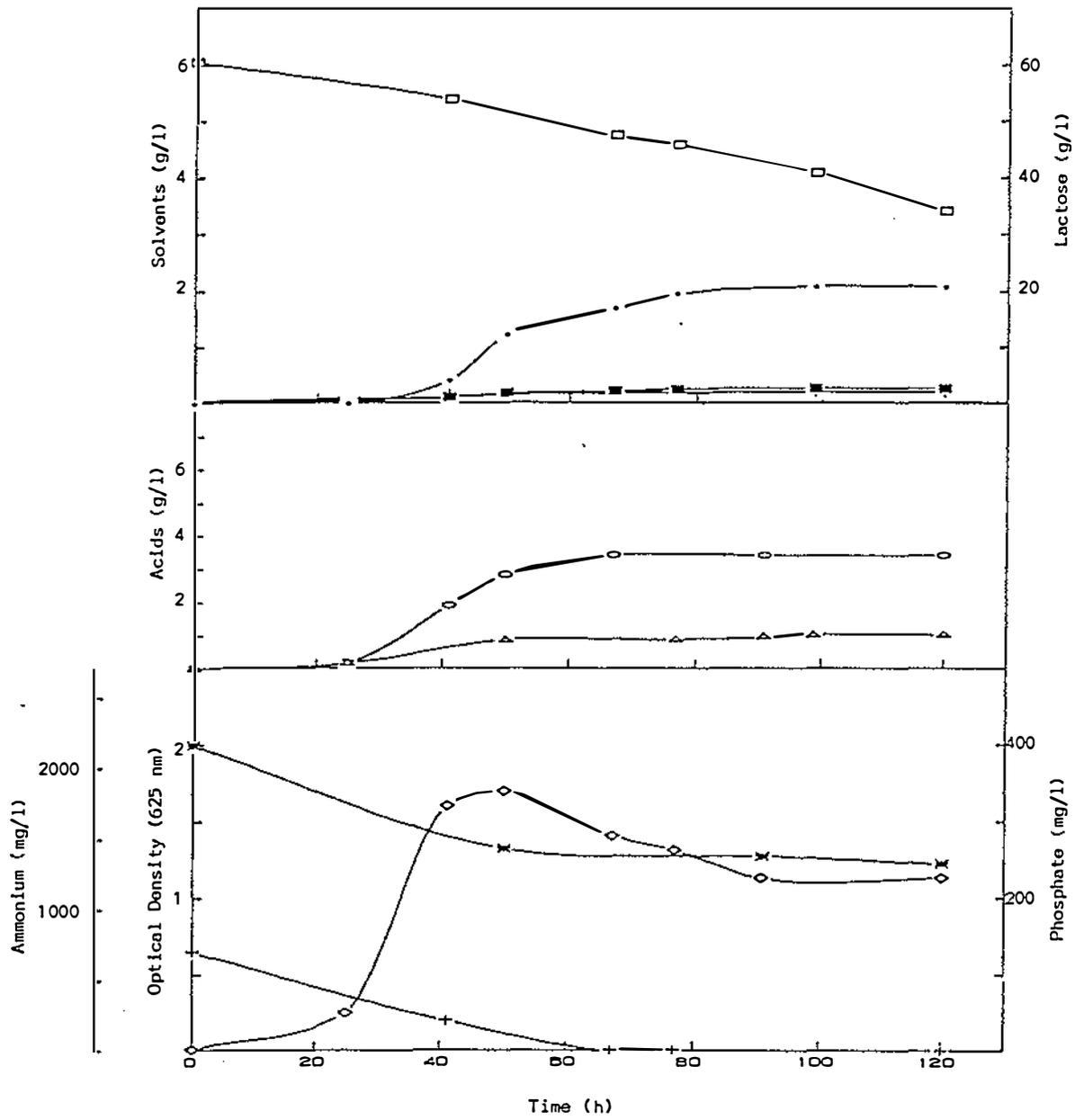


Figure 3.11 Progress of fermentation Run X. Initial ammonium 650 mg/l. Symbols as for Fig. 3.1

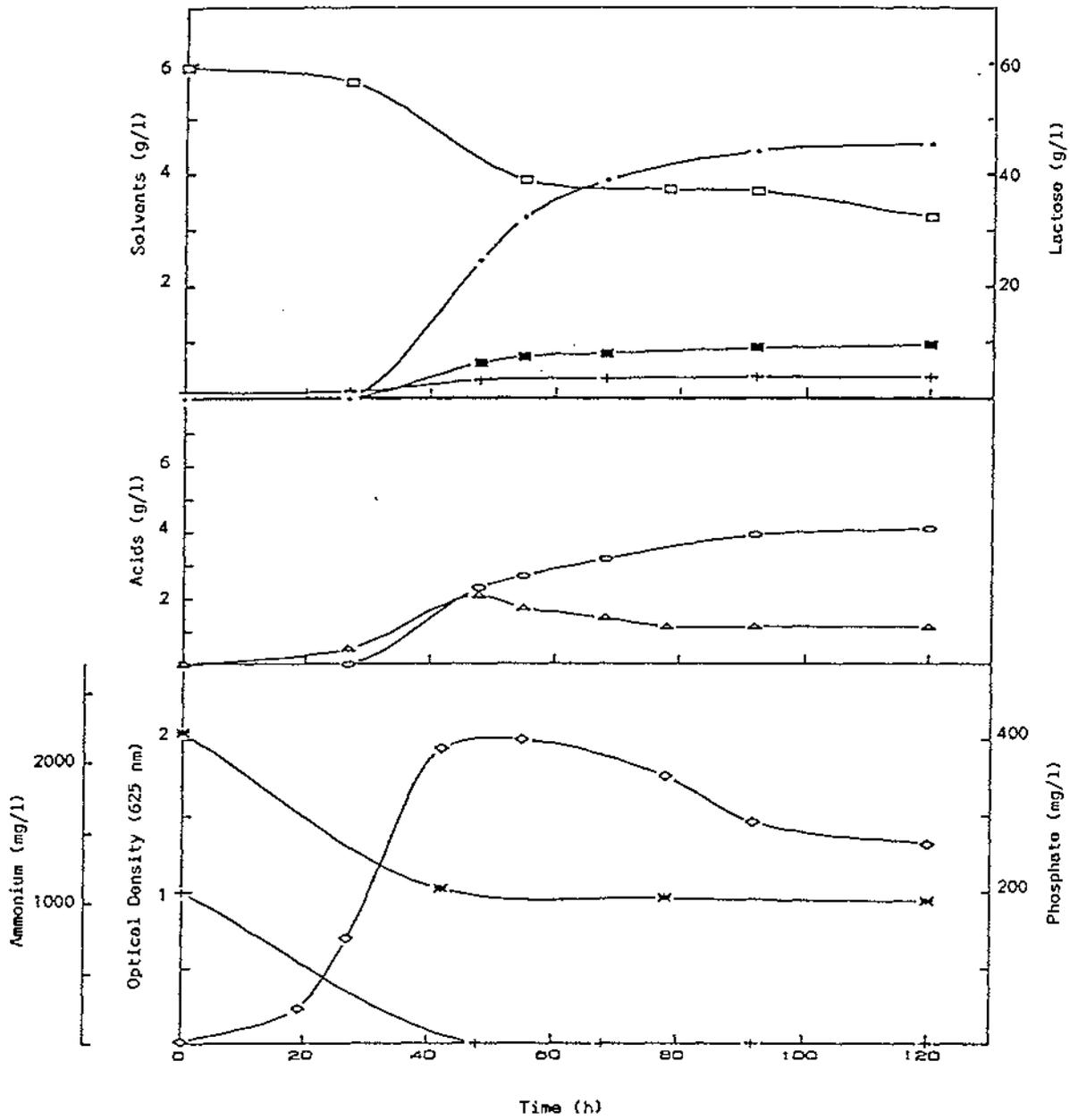


Figure 3.12

Progress of fermentation Run XI. Initial ammonium 1000 mg/l.
 Symbols as for Fig. 3.1

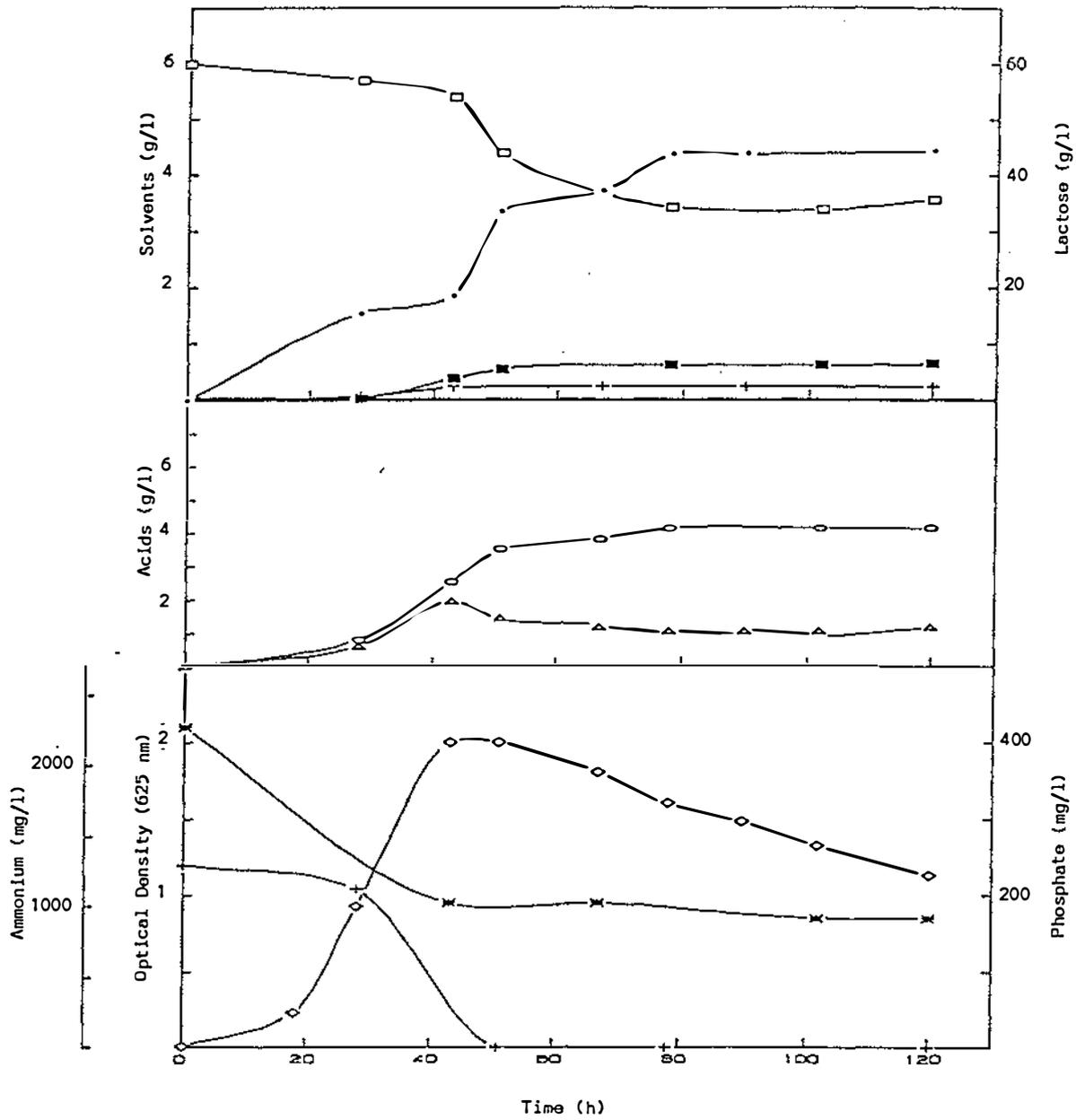


Figure 3.13 Progress of fermentation Run XII. Initial ammonium 1200 mg/l. Symbols as for Fig. 3.1

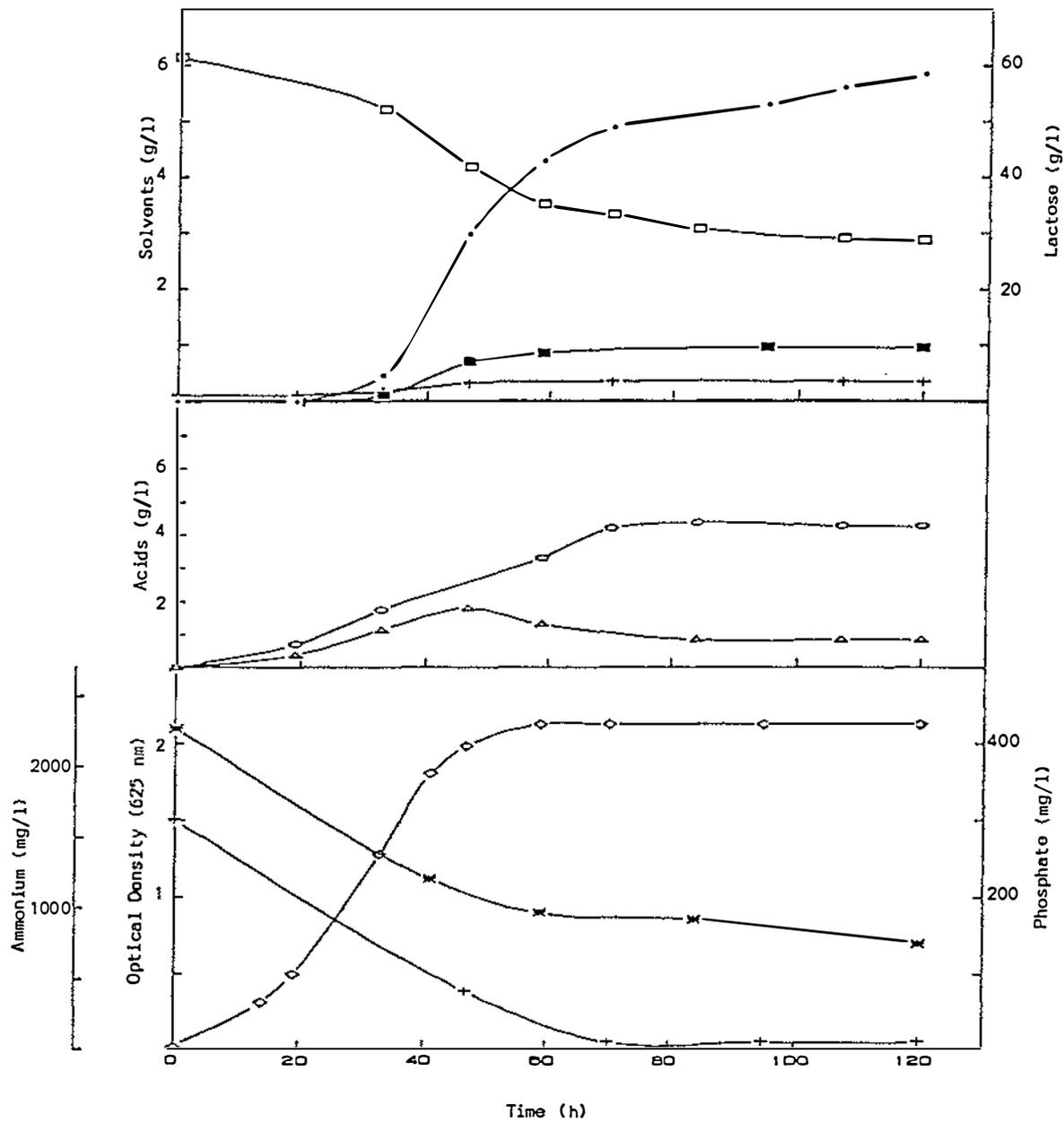


Figure 3.14 Progress of fermentation Run XIII. Initial ammonium 1500 mg/l. Symbols as for Fig. 3.1

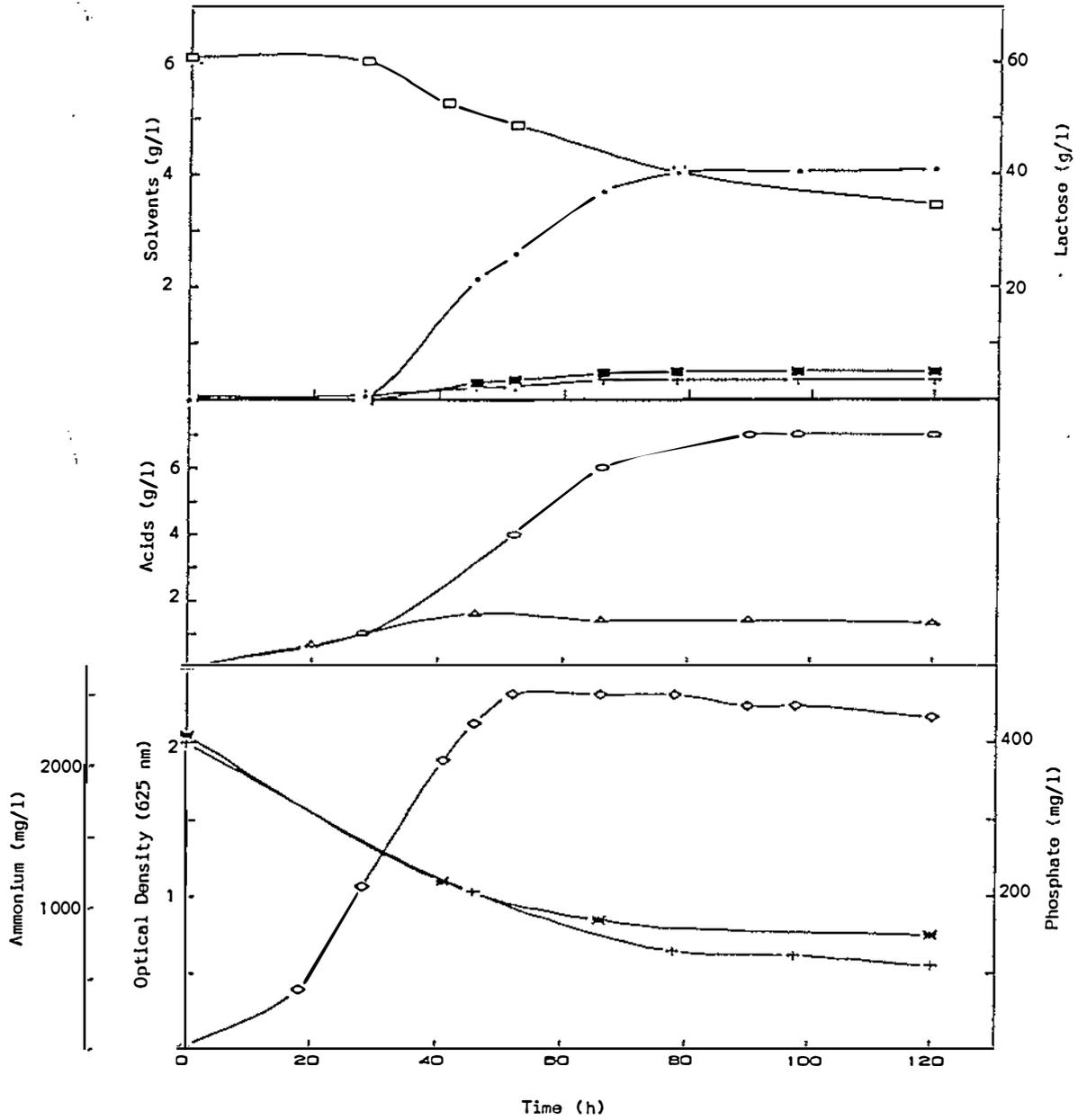


Figure 3.15

Progress of fermentation Run XIV. Initial ammonium 2000 mg/l.
Symbols as for Fig. 3.1

Run XI (Fig. 3.12) and Run XII (Fig. 3.13) were performed under similar conditions to Runs IX and X except that the initial concentration of ammonium sulphate was further increased to 1000 mg/l and 1200 mg/l, respectively. Very similar fermentation profiles were observed in these Runs. The optical density (at 625 nm) rose sharply and reached the maximum value just before the ammonium ion was exhausted from the culture medium, and then declined sharply as soon as the ammonium ion was exhausted. The total solvent and acid concentrations as well as the solvent yields, were very similar (5.88 g/l solvents with 5.23 g/l acids in Run XI and 5.33 g/l solvent with 5.35 g/l acids in Run XII). However, the maximum observed volumetric and specific butanol production rates in Run XII were significantly higher than in Runs IX to XI.

In comparison with Runs IX and X, Runs XI and XII showed improvement in terms of cell growth (represented by increases in biomass concentration), products, lactose uptake, and yield, probably reflecting the increased availability of nitrogen nutrient.

In Run XIII, with an initial ammonium sulphate concentration of 1500 mg/l, there was a small amount of ammonium ion remaining throughout the fermentation. Solvent production and the solvents to acids ratio were slightly improved over that observed in Run XII.

Finally, Run XIV (Fig. 3.15) was performed in the presence of an initial concentration of ammonium sulphate of 2000 mg/l. The fermentation profile was similar to that of Run XIII although the residual ammonium ion concentration was higher. In comparison with Run XIII, however, decreases were observed in the total solvent concentration, the lactose uptake and the solvent yield, as well as the volumetric and the specific butanol production rates. Hence, this fermentation condition favoured acidogenesis rather than solventogenesis. In all these experiments, there was no significant trend in the butanol to acetone ratio.

3.2.2.3 Discussion

The purpose of this study was to determine the optimum concentration of nitrogen in a defined medium for solvent production by batch fermentation. Similar studies have been performed by other workers using different substrates (mostly glucose-based) and strains of *C. acetobutylicum*. A defined nitrogen status during and after fermentation, using lactose as a substrate, has not previously been reported in the literature.

Results in these experiments showed that although solventogenesis can occur under nitrogen limited conditions, the maximum solvent production occurred when there was a slight excess of nitrogen beyond that required for growth. The specific butanol production rate, based on ammonium ion uptake, also showed an increase with increased biomass, and then decreased as the nitrogen nutrient became in excess (Fig. 3.16). The total solvent yield, based on lactose utilized, followed the same trend. Under conditions of nitrogen excess and that of extreme nitrogen limitation, the fermentation was acidogenic rather than solventogenic.

Factors such as the culture *pH*, concentration of acetic and butyric acids (Gottschal and Morris, 1981b; Martin *et al.*, 1983; Monot *et al.*, 1983; Monot *et al.*, 1984; Gottwald and Gottschalk, 1985), and the residual sugar concentration at the beginning of the solventogenic phase (Long *et al.*, 1984a and Roos *et al.*, 1985) are considered to have an affect on the control of solvent production. The interactive effects of these factors remains largely unelucidated and the relative magnitude of the effect of each factor is likely to be strain-specific.

Monot *et al.* (1982) reported that excess amounts of ammonium acetate in batch cultures of *C. acetobutylicum* growing on a totally synthetic medium resulted in the production of acids only. The present results support the concept that excess nitrogen leads to acidogenic conditions. Further, Monot and Engasser (1983a)

found that nitrogen-limited batch cultures allowed the production of solvents, and this is also supported by the present results.

In contrast, Long *et al.* (1984a) using strain P262 with a synthetic medium containing glucose, reported the absence of solvent production in ammonium limited batch cultures. The only apparent difference between the work of Monot and Engasser (1983a) and that of Long *et al.* (1984a) is the different strain used, but Long *et al.* (1984a) suggested that the ammonium concentration used by Monot and Engasser (1983a) was sufficient for the production of the threshold concentration of acid end-products and the initiation of solvent production. The same might be true of the present study. Further, Long *et al.* (1984a) suggested that although a threshold concentration of acid end-products, and the correct *pH* levels, are essential for the shift to solventogenesis to occur, they are not sufficient in themselves to ensure the development of the solventogenic phase. This development requires the presence of residual glucose and a minimum nitrogen concentration in the medium after the growth phase has been completed. It has also been suggested by Roos *et al.* (1985) that an excess of nitrogen is detrimental to solvent production, and that as the ratio of initial nitrogen to glucose decreases, the rate of solvent production increases.

Interestingly, the phosphate concentration used by many workers during studies into nitrogen limitation appears to be well in excess of that required for growth. This may be the reason for the difference between the results described by Long *et al.* (1984a) and those of the present study. The former used 7.8 g/l of K_2HPO_4 and KH_2PO_4 while 0.4 g/l K_2HPO_4 was used in the latter. An excess of phosphate is now known to favour acidogenesis over solventogenesis, and this might override any solventogenic effect of nitrogen limitation.

Papoutsakis (1984) has proposed a mechanism by which the cell regulates its metabolism. He proposed that solvent production is predominantly regulated by the availability and demand of biosynthetic power (ATP) and reduction energy [$NAD(P)H_2$, FdH_2]. Glycolysis of sugar usually results in insufficient ATP but

sufficient NAD(P)H₂ for growth. Acid production is the main source of ATP, but it uses very little of the available reduction energy. This excess reduction energy is released in the form of hydrogen. Solvent production requires significant amounts of reduction energy but produces very little ATP. Thus, glucose-limited cultures result in the formation of acids only. If the culture is non-ATP limited (as in nitrogen-limited cultures), a mixture of solvents and acids is produced in response to a required lower rate of ATP production per unit of sugar utilized. Similarly, when growth is inhibited by high acid concentrations or low pH, the demand for a lower rate of ATP production per unit substrate utilized is apparently met by the production of a mixture of solvents and acids. Logically, this hypothesis indicates that the shift to solventogenesis is possible under nitrogen-limited cultures.

The results of the present study show that when using synthetic medium with lactose as the sugar source, solvent production occurs under conditions of nitrogen limitation but the optimum condition is where the nitrogen concentration is just sufficient to support good growth. An excess of nitrogen leads to conditions becoming acidogenic rather than solventogenic.

Interestingly, in this present work, the specific butanol production rate, based on ammonium ion uptake, showed an increase with increased biomass, and then decreased as the nitrogen nutrient became in excess. This is similar to the result described above for the effect of phosphate concentration. The specific butanol production rate is plotted against ammonium ion uptake, or cell density in Figure 3.16, and shows a clear optimum. This result could be of significance in the development of novel fermentation technologies, as it demonstrates that the environmental conditions affect the metabolism of the cell.

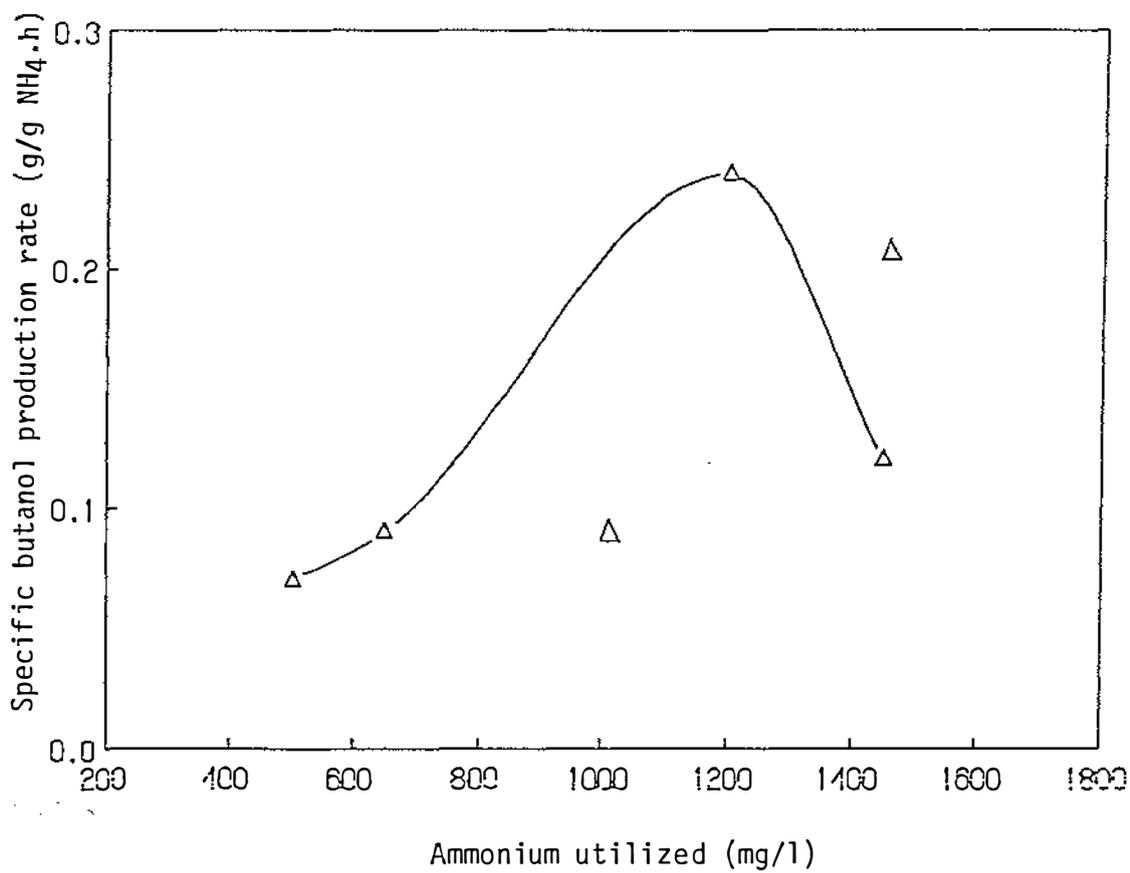


Figure 3.16 Plot of specific butanol production rate (g/g biomass ammonium.h) versus ammonium utilized, mg/l, for Runs IX to XIV

3.2.3 Effect of Iron Concentration on Solvent Production

3.2.3.1 Introduction

Few reports in the literature have investigated the effect of iron limitation on solvent production in batch culture. Monot *et al.* (1982) demonstrated that cell growth was dependent on the presence of Fe^{2+} in the medium. It was suggested that at least 1 mg/l FeSO_4 must be supplied to the culture to support good growth and normal solvent production. Bahl *et al.* (1986) reported that under iron limitation, the ratio of butanol to acetone was increased from 2:1 to 8:1. Their results have been supported by Junelles *et al.* (1988). This result could be of commercial significance if it was wished to increase the proportion of butanol in the product mix.

The purpose of the present work was to further investigate the effect of iron concentration in batch culture on solvent production. Conceivably, some commercial substrates may have a deficiency or an excess of this metal ion that will require adjustment in order to achieve maximum solvent production.

3.2.3.2 Results

A series of batch fermentations was performed whereby the initial ferrous ion concentration of the medium was varied, while all other nutrients were maintained constant. The results of these experiments are summarized in Table 3.3 and fermentation profiles for each fermentation are shown in Figures 3.17 to 3.21. In Run XV, the iron nutrient was exhausted from the medium, while in Runs XVI to XIX all nutrients remained in excess.

Table 3.3 Effect of iron concentration on fermentation parameters, after five days of fermentation.

	Run No.				
	XV	XVI	XVII	XVIII	XIX
Initial iron (mg/l) ^a	1.08	1.93	3.63	6.25	11.56
Iron utilized (mg/l) ^a	1.08	1.80	2.13	2.31	2.19
Ammonium utilized (g/l) ^b	0.17	0.48	0.35	0.43	0.46
Phosphate utilized (g/l) ^c	0.10	0.15	0.16	0.15	0.15
Biomass (OD ₆₂₅) (g/l)	1.46 (0.68)	2.08 (1.35)	2.22 (1.50)	2.26 (1.55)	2.30 (1.59)
Total solvents (g/l)	3.0	4.5	3.8	3.4	2.6
Total acids (g/l)	2.7	3.6	4.7	5.4	7.8
Lactose utilized (g/l) (%)	15.59 (25.27)	20.00 (36.15)	20.20 (34.90)	19.71 (34.82)	18.44 (32.21)
Total solvent yield (g solvent/g lactose utilized)	0.19	0.23	0.18	0.17	0.14
Max. observed butanol production rate (g/l.h)	0.023	0.064	0.054	0.052	0.033
Max. observed specific butanol production rate (g/g biomass iron.h) (g/g biomass.h)	21.30 (0.03)	35.55 (0.05)	25.35 (0.04)	22.51 (0.03)	15.07 (0.02)
Total solvents : total acids	1.11	1.25	0.81	0.62	0.33
Butanol : acetone	2.6:1	2.7:1	4.0:1	3.9:1	5.2:1

^a Expressed as FeSO₄·7H₂O

^b Expressed as (NH₄)₂SO₄

^c Expressed as K₂HPO₄

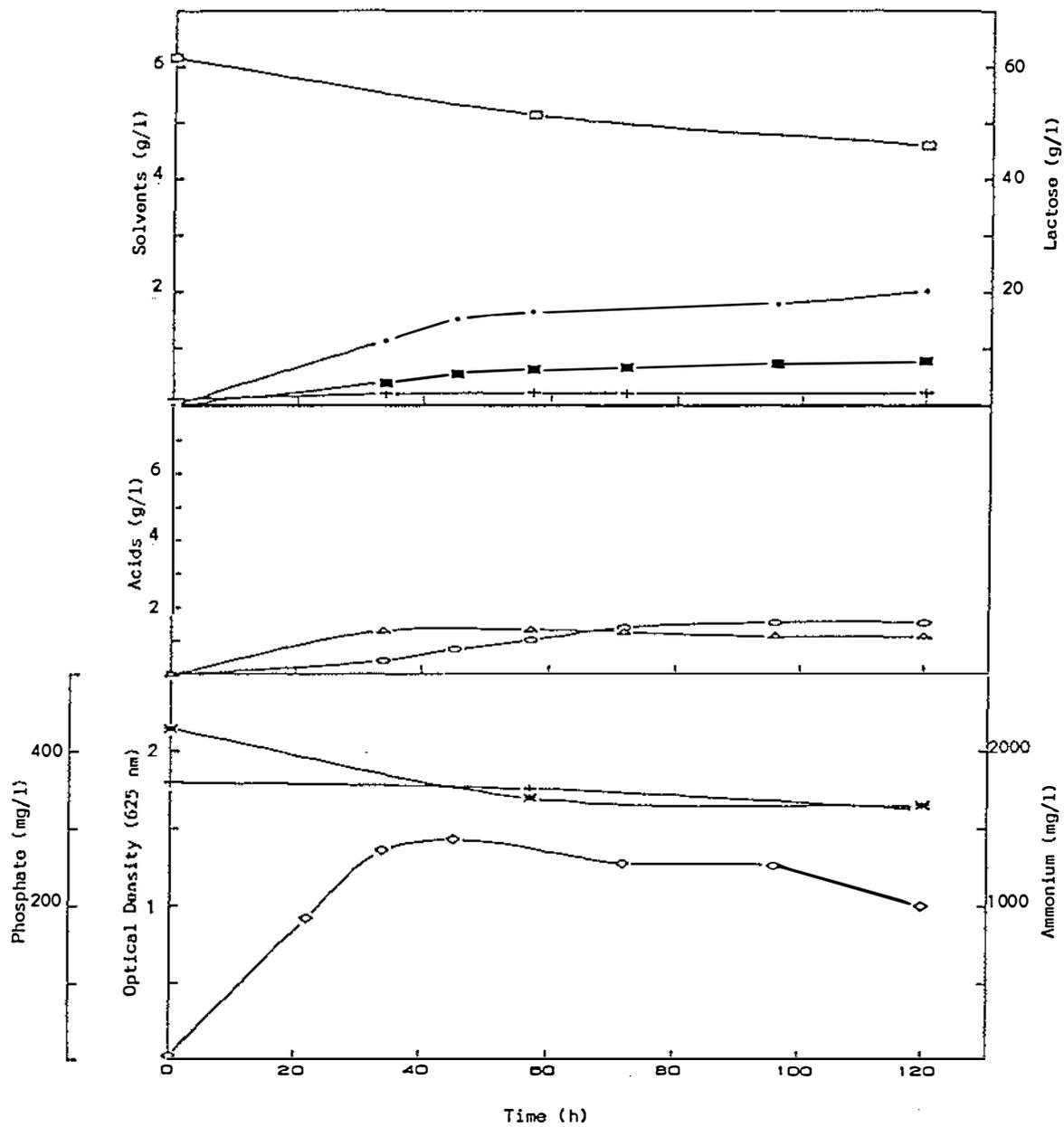


Figure 3.17

Progress of fermentation Run XV. Initial iron content 1.08 mg/l.
Symbols as for Fig. 3.1

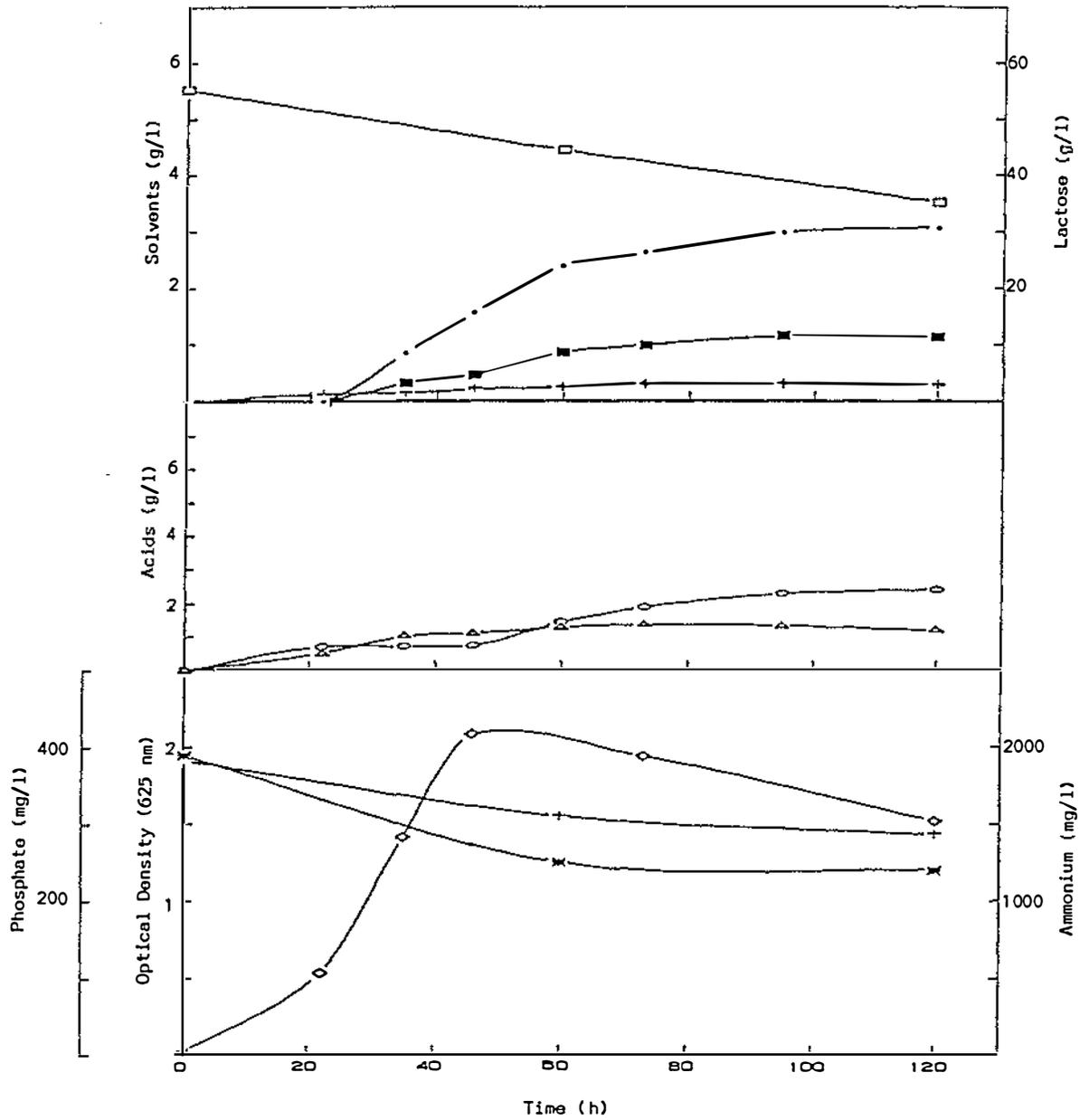


Figure 3.18

Progress of fermentation Run XVI. Initial iron content 1.93 mg/l.
 Symbols as for Fig. 3.1

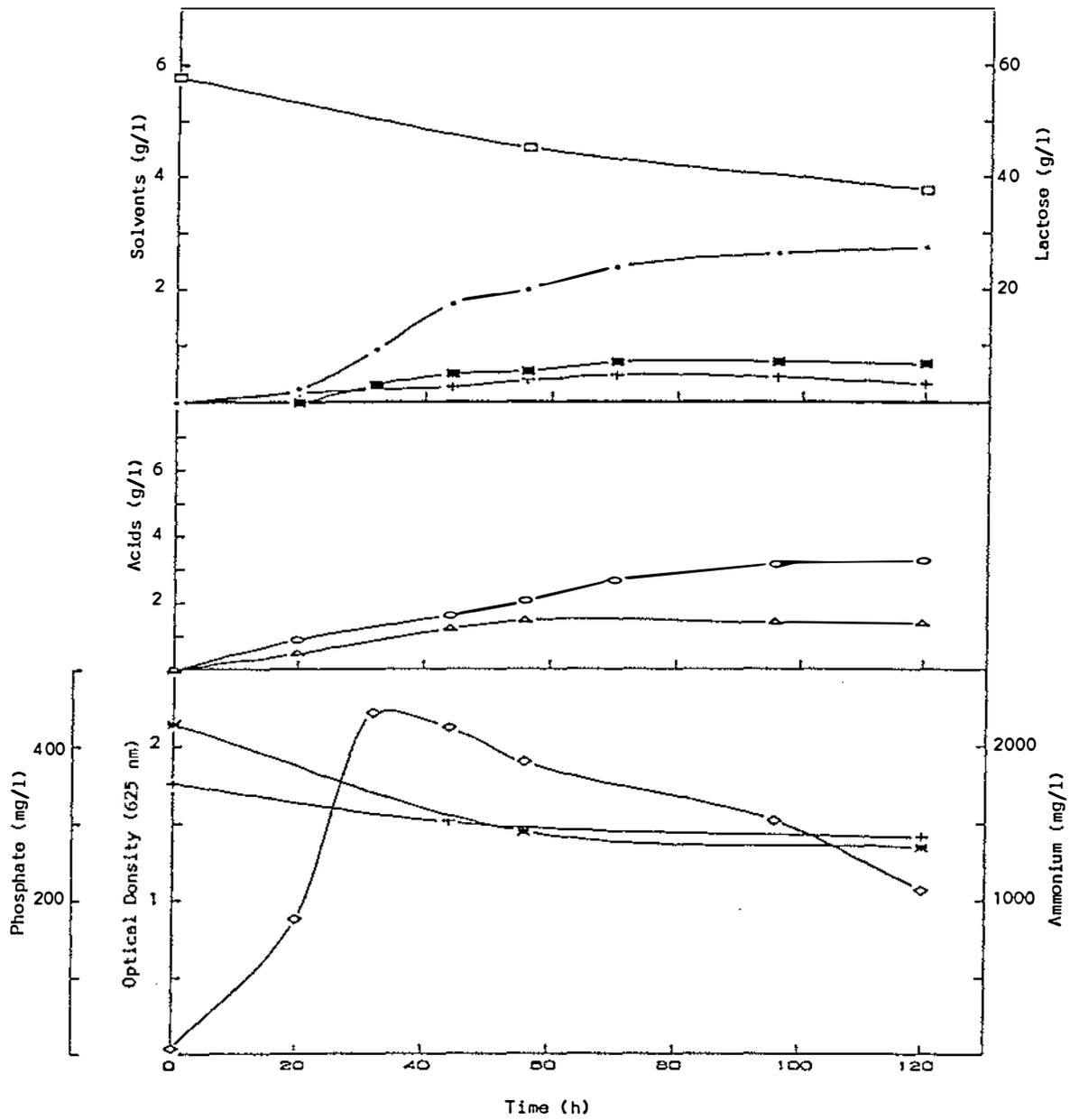


Figure 3.19

Progress of fermentation Run XVII. Initial iron content 3.63 mg/l.
 Symbols as for Fig. 3.1

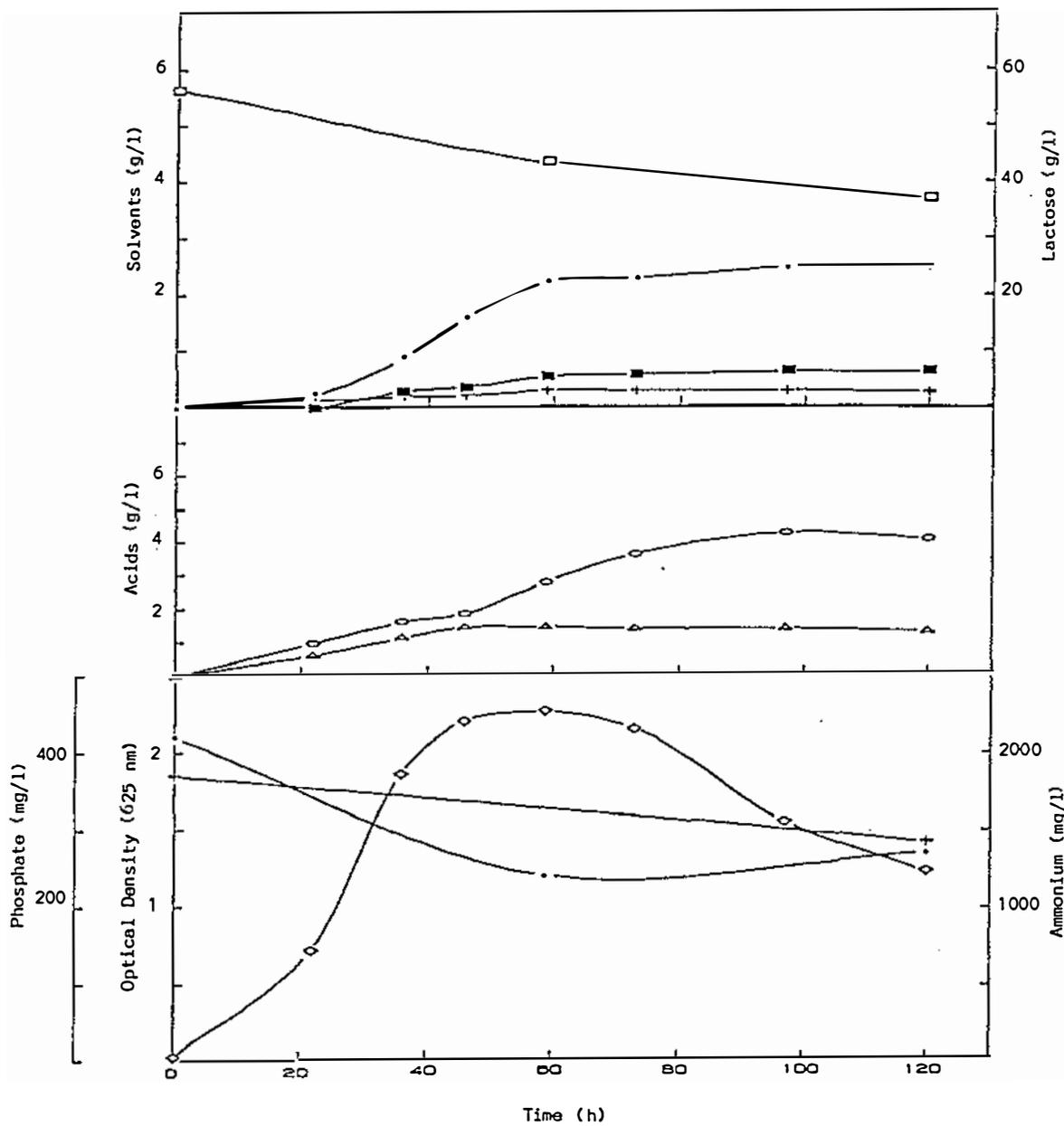


Figure 3.20

Progress of fermentation Run XVIII. Initial iron content 6.25 mg/l. Symbols as for Fig. 3.1

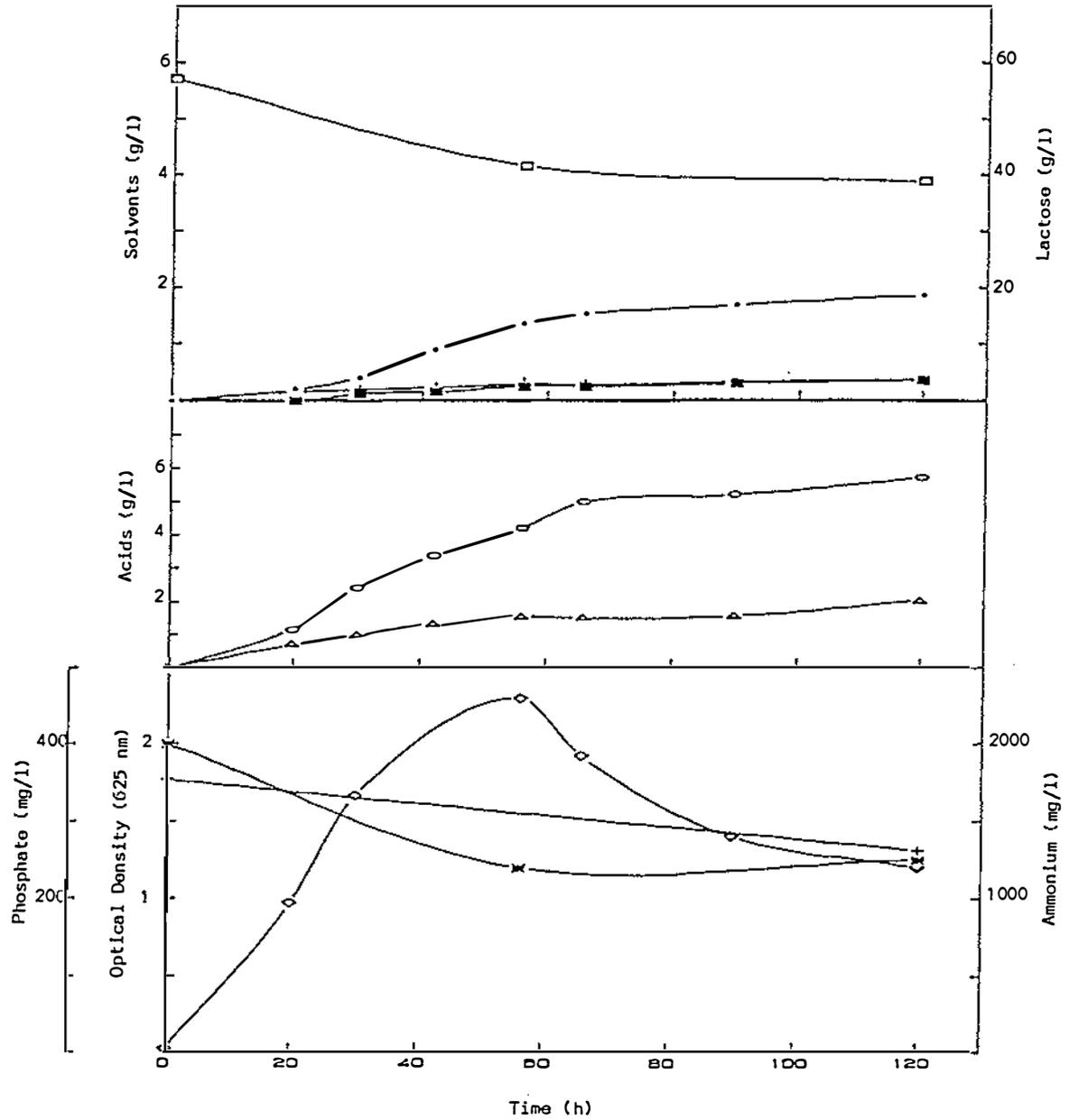


Figure 3.21 Progress of fermentation Run XIX. Initial iron content 11.56 mg/l. Symbols as for Fig. 3.1

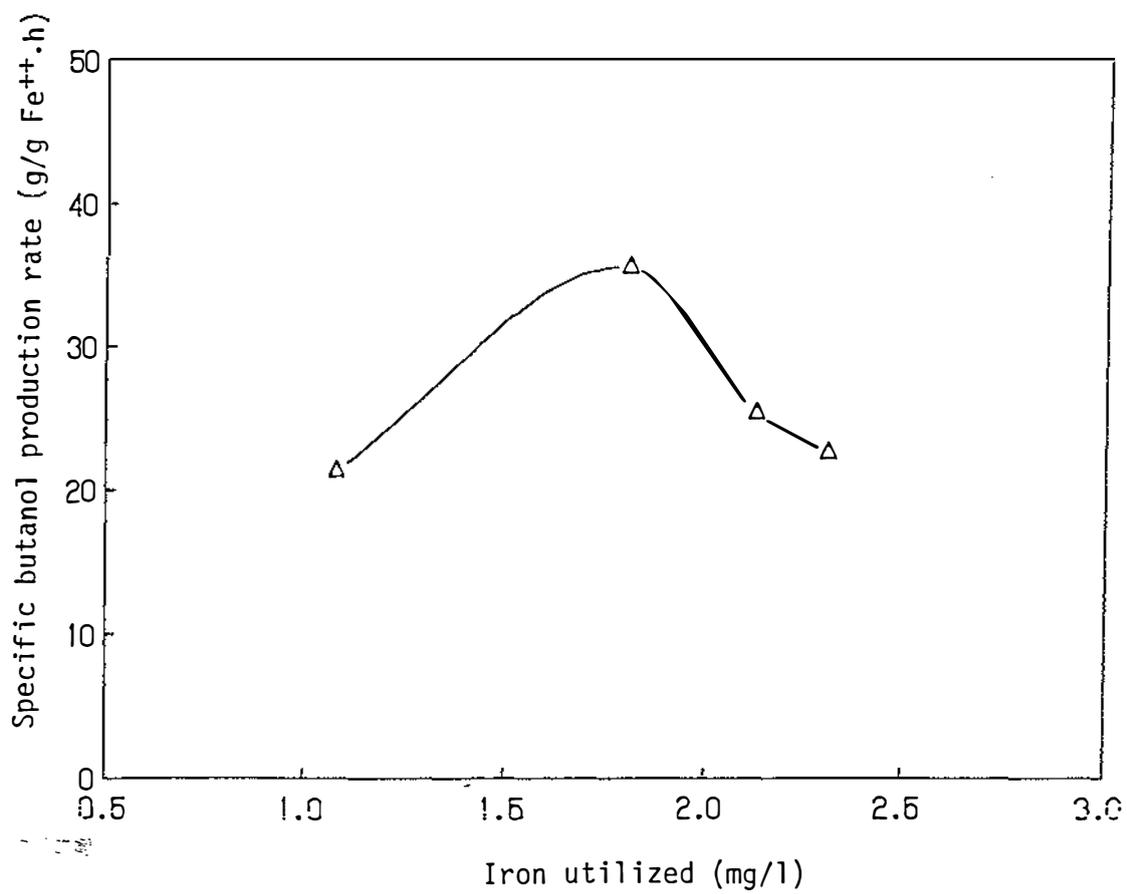


Figure 3.22 Plot of specific butanol production rate (g/g biomass iron·h) versus iron utilized, mg/l, for Run XV to XIX

The initial experiment, Run XV, was performed with 1.08 mg/l initial iron. Figure 3.17 shows that soon after the biomass concentration reached its maximum, solvent production started to increase. A total acids concentration of 2.7 g/l and a total solvents concentration of 3.0 g/l were observed. The ratio of total solvents to total acids and that of butanol to acetone were 1.11 and 2.6:1, respectively. with a solvent yield of 0.19 g/g based on lactose uptake. The volumetric butanol production rate was 0.023 g/l.h and the specific butanol production rate was 0.03 g/g.h based on biomass concentration. A lactose utilization of 25.27 % was observed. Although these fermentation conditions favoured solventogenesis, the overall solvent production was rather poor.

Run XVI (Fig. 3.18) was carried out by increasing the initial amount of iron to 1.93 mg/l. In this Run, there was a slight excess of iron remaining in the medium throughout the fermentation. A similar profile to Run XV was observed except that more nitrogen and phosphate nutrients were consumed. The total solvent concentration was 4.5 g/l, that of acids 3.6 g/l, while the solvents to acids ratio was 1.25 and the butanol to acetone ratio was 2.7:1. Increases were observed in the extent of lactose utilization (36.15 %) and the volumetric butanol production rate (0.064 g/l.h) as the amount of biomass present increased. In particular, the specific butanol production rate (0.05 g/g biomass.h), also showed an increase with increasing biomass.

The initial amount of iron was increased further to 3.63 mg/l as shown in Run XVII (Fig. 3.19). Total acids and solvents concentrations of 4.7 g/l and 3.8 g/l, respectively, were observed. Decreases occurred in the solvent yield (0.18 g/g lactose), the ratio of solvents to acids, and the volumetric and the specific butanol production rates as the iron nutrient became in excess. Hence, this fermentation condition favoured acidogenesis rather than solventogenesis.

In Runs XVIII (Fig. 3.20) and XIX (Fig. 3.21), the initial concentrations of iron were increased further to 6.25 mg/l and 11.56 mg/l, respectively. As the iron nutrient became more in excess, the fermentations became progressively more acidogenic rather than solventogenic and the ratio of butanol to acetone was slightly increased. However, decreases were observed in the solvent yield, the ratio of solvents to acids, lactose uptake and the volumetric and the specific butanol production rates (Table 3.3). Figure 3.22 shows the effect on the specific butanol production rate of the concentration of iron utilized.

3.2.3.3 Discussion

The purpose of this section was to determine the optimum concentration of iron and its effects in a defined medium on solvent production during batch fermentation. The study was stimulated by the need to know whether any potential commercial substrate, such as whey permeate could be improved by the addition or removal of this nutrient.

This series of Runs displayed a similar trend as when phosphate and nitrogen nutrients were under investigation. Increased solvent production was observed as the concentration of the growth-limiting nutrient was increased, and maximum production occurred when the initial iron concentration was in slight excess of that required for growth. As the iron concentration was increased further (Runs XVII to XIX) the fermentation became acidogenic rather than solventogenic.

These results are in general agreement with those reported by Junelles *et al.* (1988), albeit using a different strain of organism (ATCC 824) with a probable different optimum pH range for solventogenesis, on a glucose-containing synthetic medium.

The mechanism of the observed effects is not completely clear. However, iron is known to be involved in the enzyme hydrogenase and also ferredoxin (Chen and Mortenson, 1974). Junelles *et al.* (1988) reported that under iron limitation, the specific activity of hydrogenase was decreased, concomitant with a shift to solventogenesis. This can be explained on the basis of reducing power being retained in the form of NAD(P)H rather than being lost as hydrogen gas. Kim *et al.* (1984) inhibited the activity of hydrogenase by purging carbon monoxide into a fermentation, and observed a similar shift to solventogenesis. Hence the retention of reducing power is believed to favour solvent production rather than acid production.

In this present work, the results showed a direct relationship between the ratio of butanol to acetone and iron utilized. This is in contrast to Bahl *et al.* (1986) who observed an increase in the ratio of butanol to acetone as iron became limiting. The reason for this contradictory result is not clear but may be due to differing nutrient *balances* being used by the different groups. In the present work, the result is similar for both phosphate and iron concentration, i.e. the ratio of butanol to acetone increases as these nutrients became progressively in excess, and the fermentation becomes more acidogenic.

In practical terms, the results demonstrate that the iron content of the fermentation medium can influence the products obtained, and so attention should be paid to this in a commercial situation. Sufficient iron must be present to allow biomass production, but an excess is deleterious to solvent production.

3.3 Conclusions

The nutrient status of the culture has a profound effect on cell density and solvent production in batch culture medium. For all three nutrients investigated in this study, an optimum concentration for solventogenesis was identified, i.e. their concentrations should be just slightly in excess of those required for growth. A greater excess of nutrients resulted in increased acid production at the

expense of solvents. Thus, it is clear that the *balance* of nutrients is important in determining the extent of solventogenesis, and this may require investigation for each particular commercial substrate.

3.4 Effect of Nutritional Status of Sulphuric Acid Casein Whey Permeate on Solvent Production

3.4.1 Introduction

Several reports in the literature have described the use of whey or whey permeate (deproteinated whey) as a substrate for the production of solvents by batch fermentation using various strains of *Clostridium acetobutylicum* (Maddox, 1980; Gapes *et al.*, 1983; Welsh and Veliky, 1984; Ennis and Maddox, 1985, and Ennis, 1987). Because of its relatively low sugar content (lactose 45 - 50 g/l), this is not a favoured raw material for many fermentation processes. However, it is suitable for the ABE process since product inhibition restricts the amount of sugar utilization to 50 - 60 g/l. Generally, long fermentation times (80 - 120 h), with much lower productivities than achievable on industrial media, e.g. molasses, or simple glucose media, have been reported with this substrate, even when supplemented with various nutrients.

On comparing whey permeate with the optimum levels of nitrogen, phosphate and iron identified in the previous sections, it would appear to contain sufficient nitrogen, perhaps excess phosphate but most likely insufficient iron. It could also be deficient in other nutrients such as vitamins. Hence, the objective of this section was to compare the fermentation of whey permeate without supplementation, and with added vitamins and with yeast extract which contains vitamins and trace minerals.

3.4.2 Results and Discussion

Experiments were performed in batch fermentation to investigate the effect of supplementary yeast extract (5 g/l), and a combination of vitamins (biotin 1

mg/l, thiamine hydrochloride 20 mg/l, and *p*-aminobenzoic acid, 20 mg/l) on solvent production from sulphuric whey permeate. The results are presented in Table 3.4.

In these experiments, the inocula were developed in such a way that any ambiguity in the results due to 'inoculum carry-over' could be avoided (Refer to Section 2.6.1 and Section 2.6.2). The results in Table 3.4 show that when sulphuric whey permeate was supplemented with yeast extract, strong solvent production (6.9 g/l total solvents) and lactose consumption (35 g/l lactose) were observed. The solvent yield was 0.2 g solvent/g lactose utilized. However, when sulphuric whey permeate was supplemented with a combination of vitamins in lieu of yeast extract, poor solvent production (0.1 g/l solvents) and poor lactose uptake (6 g/l lactose) were observed. Similar results to the latter were observed when sulphuric whey permeate was used without any supplements (neither yeast extract nor a combination of vitamins). Thus, solvent production (0.2 g/l) and sugar uptake (14 g/l) were both low. These results suggested that the combination of vitamins could not substitute for yeast extract, even though it had been demonstrated in the previous sections that in a fully defined medium containing lactose, this vitamin combination allowed normal solventogenesis to occur. Moreover, since it is clear that whey permeate is not deficient in assimilable nitrogen or phosphate, the results indicated a deficiency of metal ion(s).

Therefore, subsequent experiments were performed in small-scale fermentations, to investigate this possibility. In these experiments, sulphuric whey permeate was supplemented with various nutrients, including metal ions, which are known to be adequate for vigorous fermentation in a fully defined lactose-containing medium (refer to Section 3.2). The results of these experiments are shown in Table 3.5.

Table 3.4 Effect of yeast extract and vitamins on solvent production from whey permeate.

	Supplement		
	None	Vitamins ^a	Yeast Extract
Initial ammonium (mg/l)	262	230	394
Ammonium utilized (mg/l) ^b	131	131	197
Initial phosphate (mg/l) ^{c,d}	1960	1715	1710
Lactose utilized (g/l)	14	6	35
Solvent produced (g/l)	0.2	0.1	6.9
Acid produced (g/l)	2.9	3.0	5.7
Solvent yield (g solvent/g lactose utilized)	0.01	0.02	0.20

^a Concentrations used were 1.0 mg/l biotin; 20.0 mg/l thiamine-HCl; and 20.0 mg/l PABA

^b Expressed as $(\text{NH}_4)_2\text{SO}_4$

^c Whey permeate, after autoclaving, contains a precipitate of mineral phosphate. There was a large excess of soluble phosphate at the completion of all fermentations.

^d Expressed as K_2HPO_4

Table 3.5 Effects of various supplements on solvent production from whey permeate.

Run No.	Supplement (g/l)				Lactose uptake (g/l)	Solvent Prod ⁿ (g/l)	Acid Prod ⁿ (g/l)	Solvent Yield (g/g)
	Vitamins ^a	NH ₄ acetate (1.1)	FeSO ₄ (0.01)	MgSO ₄ (0.2)				
I	✓	✓	✓	✓	20	4.1	2.4	0.21
II	✓	✓	X	✓	7	0.8	1.5	0.11
III	✓	✓	✓	X	21	5.2	2.5	0.20
IV	X	X	✓	X	17	3.1	2.3	0.18
V	X	X	X	X	0	0	0	0

✓ Present

X Absent

^a Concentrations used were 1.0 mg/l biotin; 20.0 mg/l thiamine-HCl; and 20.0 mg/l PABA

In the initial experiment (Run I), where the whey permeate were supplemented with all of the nutrients, solvent production (4.1 g/l solvents) and lactose consumed (20 g/l lactose) was reasonable, with a solvent yield of 0.21 g/g based on lactose uptake. In Run II where all the nutrients were supplemented except iron, poor solvent production and poor lactose uptake were observed. Run III showed that omission of magnesium had no effect on solvent production. However, in Run IV, where iron was the only nutrient that was supplemented, a solvent production of 3.1 g/l and lactose consumption of 17 g/l, representing a solvent yield of 0.18 g/g based on lactose utilized were observed. In Run V where none of the nutrients were supplemented to whey permeate medium, no solvents were produced or lactose consumed.

These results demonstrated that ammonium ion, magnesium ion and vitamins are not deficient in whey permeate. Rather, the results indicate that sulphuric whey permeate is deficient in iron, and possibly, some other metal ion.

3.4.3 Conclusions

Although the experiments described in this section were performed to investigate only major metal ions, the following general conclusions can be made:-

Sulphuric whey permeate without supplementation is a poor substrate for the ABE fermentation by *C. acetobutylicum* P262. This result is in apparent contrast to that previously reported by Maddox (1980) who observed strong solvent production from whey permeate without any supplementation. The difference in these results, however, can be explained on the basis of 'inoculum carry-over' in Maddox's experiments.

Sulphuric whey permeate is deficient in iron, rather than in assimilable nitrogen, phosphate, or vitamins. The iron content has been reported as 0.4 µg/g (New

Zealand Dairy Research Institute) which is equivalent to 0.12 mg/l as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. This amount of iron is insufficient to support good growth and solvent production, confirming an early claim of Monot *et al.* (1982) who observed poor cell growth and solvent production when FeSO_4 was present at less than 1 mg/l.

When whey permeate is supplemented with nutrients which are known to be sufficient for a fully defined medium containing lactose, solventogenesis is not necessarily as strong as in that defined medium alone. This emphasizes the importance of the *balance* of nutrients to achieve high solvent concentrations, and also that our knowledge of the nutrient requirements of *C. acetobutylicum* remains incomplete.

The data obtained concerning the nutrient status of whey permeate will be applied later in this thesis (Chapter 5), as it is now apparent that biomass growth on this substrate can be readily controlled by manipulation of the amounts of nutrient supplements that are added.

CHAPTER 4

CONTINUOUS SOLVENT PRODUCTION USING FREELY-
SUSPENDED CELLS OF *C. ACETOBUTYLICUM* P262 IN A
CONTINUOUS STIRRED TANK REACTOR

4.1 Introduction

Continuous culture of *C. acetobutylicum* has been widely used to study the physiology and activity of this microorganism. Once a constant environment (steady-state condition) is established, the influence of a single parameter and its interaction with other factors can be determined.

Continuous culture techniques have been used by a number of workers to investigate fundamental aspects of the ABE fermentation, including the effects of different nutrients and metabolites and to evaluate the effects of various physical factors such as culture *pH*, dilution rate and temperature. These studies provided useful information about the regulatory mechanisms and kinetics of solvent production. Conditions for maximizing solvent concentrations, yields, and productivity have also been investigated.

The use of continuous fermentation processes also provides a way of improving the productivity of the ABE fermentation for possible commercial exploitation. A variety of types of single-stage systems have been utilized to obtain solvent production during continuous culture.

The purpose of the work described in this chapter, was to investigate the production of solvents in defined medium and in sulphuric acid casein whey permeate, during continuous culture of freely-suspended cells in a continuous stirred tank reactor (CSTR).

4.2 Continuous (Chemostat) Solvent Production During Nutrient Limitation in Defined Medium

4.2.1 Introduction

Several reports in the literature refer to the use of chemostats for solvent production, since chemostat culture is an excellent tool for the elucidation of biochemical mechanisms, as well as for studying the effect of various environmental parameters on microbial activity and physiology. Unfortunately, some reports are difficult to interpret as it is sometimes in doubt as to whether a nutrient-limited condition has, in fact, been attained. Nevertheless, solvent production has been reported using chemostats run under nitrogen, phosphate, sulphate, and magnesium limitation (Andersch *et al.*, 1982; Monot and Engasser, 1983a; Jöbses and Roel, 1983; Stephens *et al.*, 1985), as well as in product-limited cultures and turbidostats operated under conditions of nutrient excess (Gottschal and Morris, 1982; Monot and Engasser, 1983b; Fick *et al.*, 1985; Clarke and Hansford, 1986).

One of the justifications for studies into novel fermentation technologies (e.g. cell immobilization, cell recycle techniques) is that the attainment of a high cell density within the reactor will allow increased productivities to be achieved. It is often assumed that each individual cell within the reactor will perform at least as well under conditions of high cell density as under conditions of low cell density. However, perusal of the literature suggests that this may not necessarily be so. For example, during studies using a phosphate-limited chemostat, it was shown that at higher phosphate concentrations the fermentation became more

solventogenic, suggesting that solventogenesis is favoured at high cell densities (Bahl and Gottschalk, 1984). However, by calculation of specific production rates from the data provided, it appears that the reverse may be true, i.e. as the concentration of the limiting nutrient, and thus biomass, increased, so the specific rate of solvent production decreased. Similar data have been reported by McNeil and Kristiansen (1987), using both nitrogen- and phosphate-limited chemostats. Thus, there is evidence of an inverse relationship between the biomass concentration and the specific rate of solvent production, at least in chemostat culture.

In contrast, there are reports showing that in a nitrogen-limited chemostat there is a direct relationship between the limiting nutrient concentration and the specific solvent production rate (Soni *et al.*, 1987; Stephens *et al.*, 1985). Stephens *et al.* (1985) have also stated that under phosphate-limited conditions the maintenance of a suitably high biomass concentration is a prerequisite for solvent production.

Hence, the purpose of the following chemostat studies was to investigate the affect of biomass concentration (cell density) on specific solvent productivity. This effect is important in the development of novel fermentation technologies in that there may be little point in maximising the cell density within a reactor if each individual cell performs only poorly. Further, it is fundamental to fermentation studies that techniques be developed to maximise the performance of each individual cell within the reactor. A defined medium was used in which lactose was the sugar source. This sugar was chosen because of the potential use of whey permeate as a commercial substrate for the ABE fermentation process (Maddox, 1980). The technique of chemostat culture was used since this allows precise control of biomass concentration.

4.2.2 Results

Preliminary experiments had established that a dilution rate of 0.056 h^{-1} and a culture $p\text{H}$ of 4.5 were suitable to allow both solvent and acid production to occur. Hence these parameters were kept constant in all experiments.

4.2.2.1 Effect of Ammonium (Nitrogen)-limited Chemostat

A series of single-stage continuous culture experiments was performed whereby the initial ammonium ion concentration of the medium was varied for each individual Run, while all other nutrients were maintained constant, in order to monitor the effect of the nitrogen concentration (and thus cell densities) on solvent production. The results (at steady state) of these experiments are summarized and depicted in Figures 4.1 and 4.2. Figure 4.1 represents the relationship between volumetric productivity and ammonium ion utilized whereas Figure 4.2 represents that of specific productivity and ammonium ion utilized. Steady-state conditions were defined as constant concentrations of lactose, biomass and solvents in the effluent stream and this normally took place after 3 residence times.

The initial experiment was performed using ammonium acetate at 46 mg/l in the feed medium (influent). At steady state, the concentration of ammonium ion in the culture effluent was equal to that of distilled water, indicating that the culture was truly nitrogen-limited. The volumetric rates of solvent (acetone + butanol + ethanol) production and of acid (acetic + butyric acid) production were observed to be 6.5 mg/l.h and 22.9 mg/l.h , respectively. The rate of lactose uptake was 200 mg/l.h at a cell concentration of 0.13 g/l . The specific rates of the same parameters were $50.0 \text{ mg/g biomass.h}$ for solvents, $176.1 \text{ mg/g biomass.h}$ for acids and $1.5 \text{ g/g biomass.h}$ for lactose uptake.

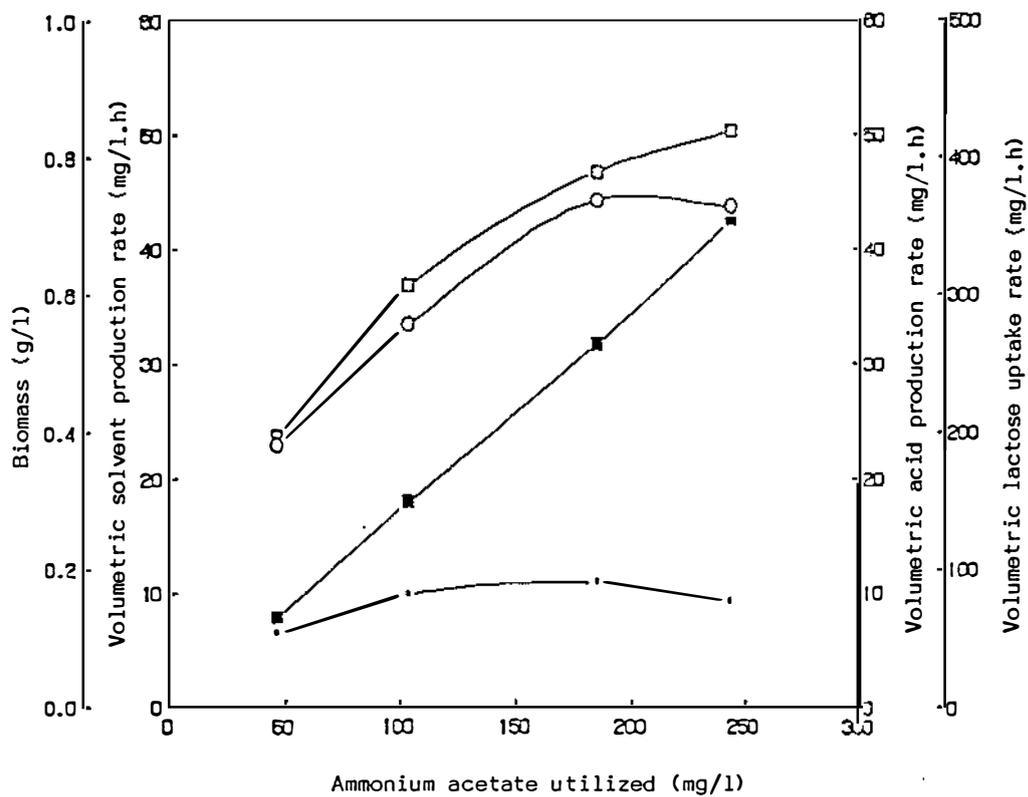


Figure 4.1

Plot of volumetric production rate (mg/l.h) versus ammonium acetate utilized (mg/l) in continuous culture at pH 4.5 and $D = 0.056 \text{ h}^{-1}$: (•) solvent; (○) acid; (■) biomass; (□) lactose uptake.

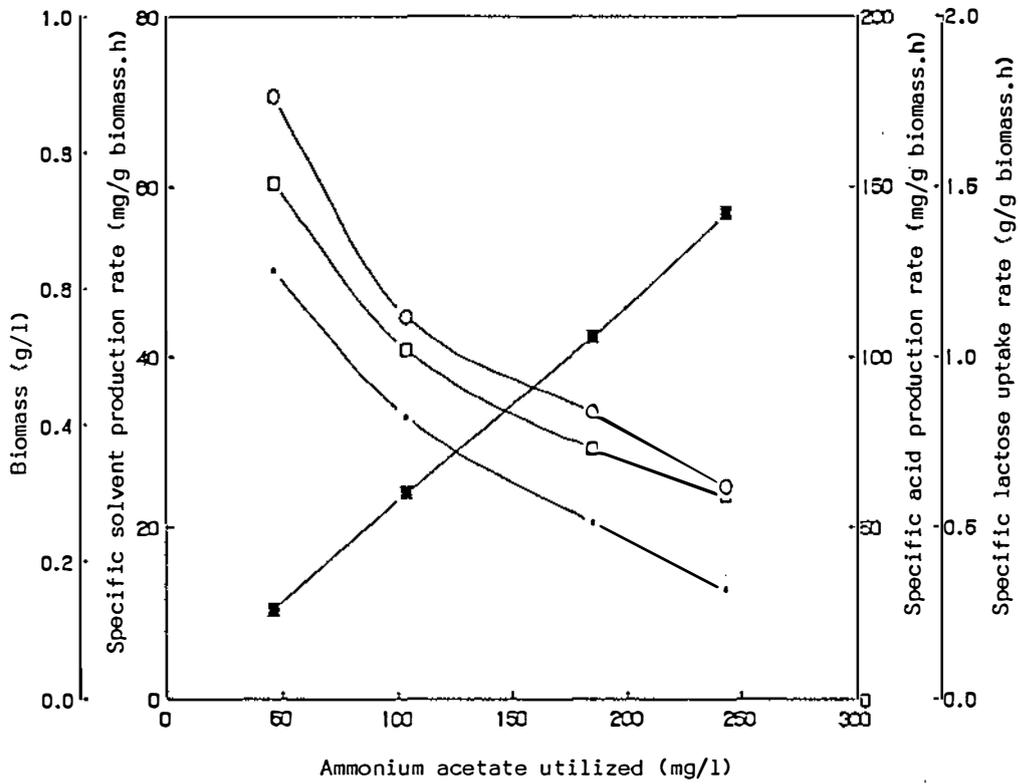


Figure 4.2

Plot of specific production rate (mg/g biomass.h) versus ammonium acetate (mg/l) in continuous culture at pH 4.5 and $D = 0.056 \text{ h}^{-1}$. Symbols as for Fig. 4.1.

The next experiment was performed by increasing the concentration of ammonium acetate in the influent to 104 mg/l. Results at steady state revealed that there was an improvement in the volumetric productivities of solvents (9.9 mg/l.h), acids (33.5 g/l.h) and lactose uptake (310 mg/l.h) while biomass concentration was increased to 0.30 g/l. As before, ammonium ion could not be detected in the reactor effluent, hence this experiment was again nitrogen-limited. However, the specific productivities of solvents, acids and lactose uptake were decreased to 32.9 mg/g biomass.h, 111.6 mg/g biomass.h and 1.0 g/g biomass.h, respectively (Fig. 4.2).

In the third experiment the concentration of ammonium acetate in the influent was increased to 185 mg/l. This run was also limited in nitrogen as the ammonium ion could not be detected in the effluent. As expected, increases in volumetric productivities of solvents, acids and lactose uptake were observed, but decreases in the specific productivities of these parameters were recorded. The biomass concentration was 0.53 g/l at steady state.

Finally, the concentration of ammonium acetate in the influent was increased to 300 mg/l. This time, there was a small amount of ammonium ions in the effluent, indicating that this fermentation was not limited in nitrogen. Although the biomass concentration continued to increase in this experiment, the increases in volumetric productivities of solvents, acids and lactose uptake were much less significant. In terms of specific productivities, further decreases in solvents and acids productivities, and lactose uptake were observed.

4.2.2.2 Effect of Phosphate-limited Chemostat

A series of single-stage continuous culture experiments was performed whereby the initial phosphate concentration of the medium was varied between each individual run, while all other nutrients were maintained constant, in order to monitor the effect of the phosphate concentration, and hence cell density, on solvent production. The steady state results of these experiments are

summarized and depicted in Figures 4.3 and 4.4. Figure 4.3 shows the relationship between volumetric productivity and phosphate utilized whereas Figure 4.4 represents specific productivity and phosphate utilized.

The initial experiment was performed with 22 mg/l phosphate concentration in the influent. At steady state, the volumetric productivities of solvents, acids and lactose uptake were 9.8 mg/l.h, 30.4 mg/l.h and 184 mg/l.h, respectively. The specific productivities of solvents, acids and lactose uptake, based on a biomass concentration of 0.26 g/l, were 37.6 mg/g biomass.h, 116.9 mg/g biomass.h and 0.70 g/g biomass.h, respectively. This culture was truly phosphate-limited since the concentration of phosphate ion in the culture effluent was equal to that of distilled water.

The next run was performed by increasing the concentration of phosphate ion in the influent to 37 mg/l. At steady state, increases in volumetric productivities of solvents, acids and lactose uptake to 8.5 mg/l.h, 33.3 mg/l.h, and 314 mg/l.h were observed. In contrast, decreases in specific productivities of solvents and acids, based on a biomass concentration of 0.43 g/l, to 19.6 and 77.5 mg/g biomass.h, respectively, were noted. No significant change in specific lactose uptake rate was observed. Again, no phosphate ion was detected in the effluent, thus this culture was also a truly phosphate-limited culture.

The concentration of phosphate ion was increased further in the third run to 59 mg/l. Again, the culture effluent was free of phosphate ions. As expected, increases in the the volumetric productivity and decreases in specific productivities of solvents, acids, and lactose uptake were observed.

In the fourth Run, the phosphate ion concentration in the influent was increased to 94 mg/l. At steady state, there were a small amount of phosphate ions in the effluent, indicating that this culture was not limited in phosphate. Increases in volumetric productivities and decreases in specific productivities were observed with increasing biomass concentration.

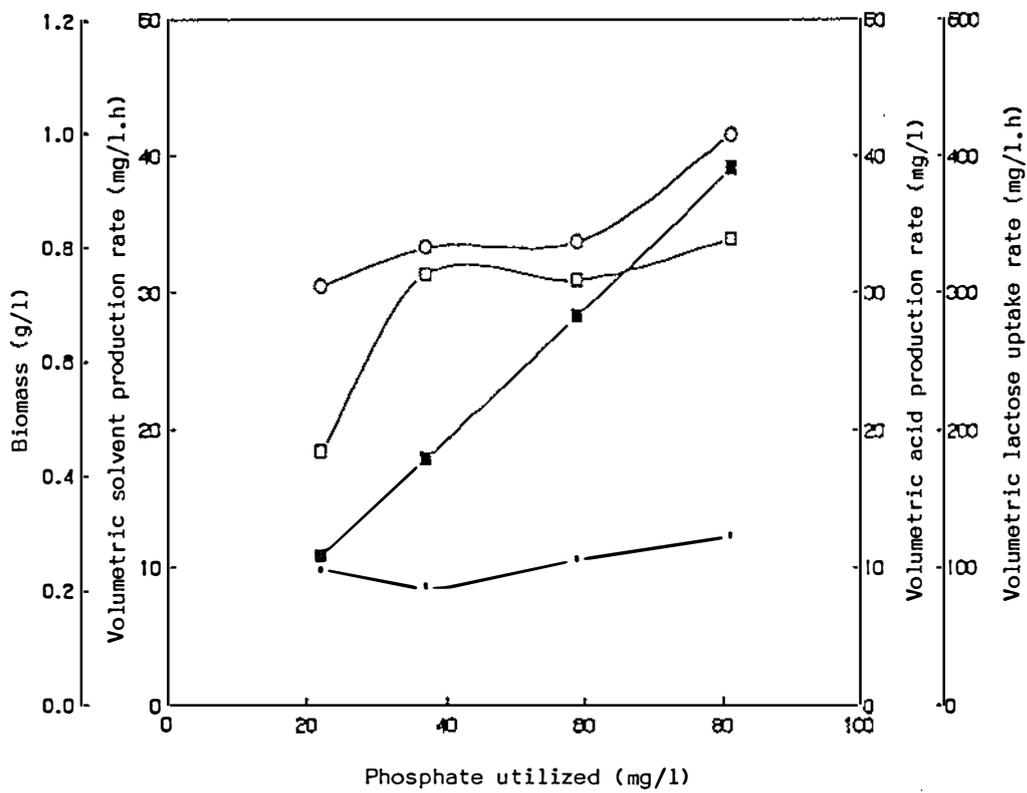


Figure 4.3

Plot of volumetric production rate (mg/l.h) versus potassium phosphate utilized (mg/l) in continuous culture at pH 4.5 and $D = 0.056 \text{ h}^{-1}$. Symbols as for Fig. 4.1.

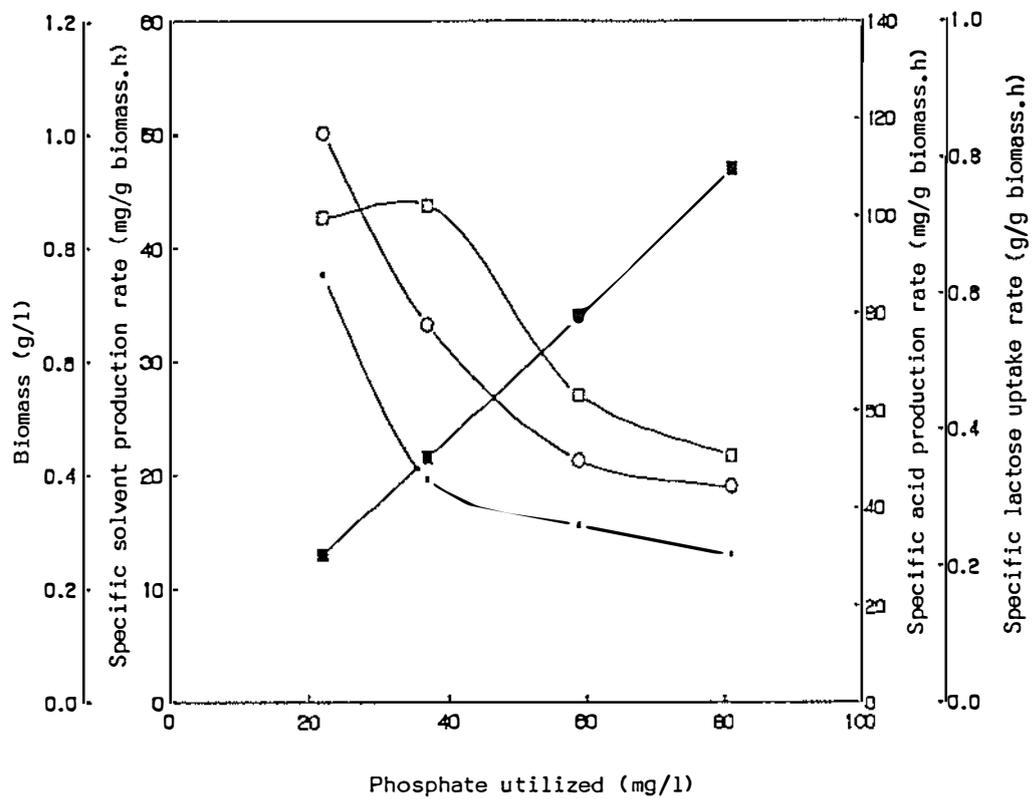


Figure 4.4

Plot of specific production rate (mg/g biomass.h) versus potassium phosphate utilized (mg/l) in continuous culture at pH 4.5 and $D = 0.056 \text{ h}^{-1}$. Symbols as for Fig. 4.1.

4.2.3 Discussion

The objective of this work was to assess the effect of varying cell density on the specific solvent productivity of *C. acetobutylicum* P262. The reason for the study was to gather fundamental information which can be used in the development of novel fermentation technologies which often rely for their effectiveness on the achievement of high cell densities. Chemostat culture was used as the experimental technique rather than turbidostat culture because of its precise control of biomass concentration.

The overall results concerning volumetric productivities of solvents and acids and lactose uptake under nitrogen limitation (Fig. 4.1) displayed the same behaviour as those results under phosphate limitation (Fig. 4.3) in response to increases in biomass concentrations (cell densities); i.e. increases in cell density resulted in increased volumetric productivities of solvents, acids and lactose uptake. However, in term of specific productivities, the results demonstrated an inverse relationship between cell densities and the appropriate uptake and production rates (Fig. 4.2 and 4.4).

In contrast to this present work, Soni *et al.* (1987) and Stephens *et al.* (1985) reported a direct relationship between the limiting nutrient concentration and the specific production rate in a nitrogen-limited chemostat. It has also been stated by Stephens *et al.* (1985) that under phosphate-limited conditions the maintenance of a suitably high biomass concentration is a prerequisite for solvent production. Furthermore, Gottschal and Morris (1982) reported that in turbidostat culture low cell densities favoured acidogenesis, while high cell densities were required for solventogenesis. This result appeared to be also true of chemostat cultures of the same strain according to the results of Stephens *et al.* (1985). When the biomass concentration in ammonium-limited chemostat cultures was varied, reasonably good yields of solvents were produced only at high cell densities. The specific rates of acid production decreased and solvent

production increased with increasing cell density. However, it is unfortunate that in this report no evidence was provided for nutrient limitation, and examination of feed-medium composition casts doubt on such claims, since the concentration of ammonium chloride used was in the range of 0.24 to 1.2 g/l. Nevertheless, the general trend of results was still in contradiction to the present work.

Similar results to the present work with regard to specific production rates were reported by Bahl and Gottschalk (1984) in a phosphate-limited chemostat. Although, their data suggested that solventogenesis is favoured at high cell densities, calculation of specific production rates from the data provided indicates that the reverse may be true. i.e. as the concentration of the limiting nutrient, and thus biomass, increased, the specific rate of solvent production decreased. Further, the results of this work were in good agreement with the results reported by McNeil and Kristiansen (1987), who provided good evidence for both nitrogen- and phosphate-limitation, with regard to specific rate of solvent production and the limiting nutrient concentration. Thus, there is evidence of an inverse relationship between the biomass concentration and the specific rate of solvent production in chemostat culture.

There are two possible explanations for the observed effect. First, each individual cell in the population may perform equally less well as the total biomass concentration increases, perhaps due to the strong selective pressure imposed on the microbial population via competition for a limiting nutrient. Secondly, the population within the reactor may not be homogeneous, i.e. not all of the cells are actively solventogenic, and the proportions of the different cell types may vary with the biomass concentration. This latter hypothesis has been postulated previously by Stephens *et al.* (1985), Clarke *et al.* (1988), and Ennis and Maddox, (1989), and has been supported by microscopical examination of the cultures observed by Ennis and Maddox (1989). It appears that three types of cells are present. These are, first, actively growing, vegetative, acidogenic cells; secondly, non-growing, clostridial, solventogenic forms; thirdly, inert spores.

The exact determinants governing the interconversions of those cell types are not known, but it is now well-established that both sporulation and solventogenesis are strongly influenced by the balance of nutrients, and this can also influence the total biomass concentration (Long *et al.*, 1984b). In the present work, microscopical examination of the cultures was not performed, but it can be postulated that the observed decrease in specific solvent productivity with increasing biomass concentration is due to a shift in the proportions of different cell types caused by changes in the nutrient balance. The apparently contradictory results described by Gottschal and Morris (1982), Stephens *et al.* (1985) and Soni *et al.* (1987) are probably caused by the same effect. The problem remains, however, of the exact nature of the determinants responsible for interconversion of the cell types.

4.2.4 Conclusion

The effect of biomass concentration (cell density) on solventogenesis has been studied in continuous culture under conditions of nitrogen and phosphate limitation. Solvent production is possible in nitrogen- and phosphate-limited chemostat cultures, but specific solvent productivity decreases with increasing biomass. It is suggested that this may be due to the cell population not being homogeneous, and that a change in the nutrient *balance* leads to a change in the relative proportions of acidogenic, solventogenic and inert cells (spores).

These results will require consideration in the development of novel fermentation technologies, where high cell densities are achieved, and nutrient limitation may occur.

4.3 Continuous Solvent Production from Sulphuric Acid Casein Whey Permeate

4.3.1 Introduction

The purpose of the work described in this section was to investigate the production of solvents in continuous culture using whey permeate as the substrate. Despite there being many reports of batch fermentation using this substrate, and of immobilized cells in continuous culture, there appear to be no reports on continuous culture using free cells in a CSTR. Information on such a system will be useful in the development of novel fermentation technologies.

In single-stage continuous culture in a CSTR, solvent production is known to be favoured at low dilution rates while acid production is predominant at high dilution rates (Bahl *et al.*, 1982b; Monot and Engasser, 1983b). Thus, one objective of the present work was to investigate the effect of dilution rate using a substrate of whey permeate. In addition, the results obtained would indicate a 'baseline' value for productivity in continuous culture using whey permeate as substrate.

A second objective of this section was to investigate the requirement for yeast extract as a nutrient supplement for whey permeate in continuous culture. Results in batch fermentation (Section 3.4) have indicated that whey permeate without any supplementation does not allow strong cell growth or solvent production. Hence, knowledge of the minimum level of supplementation required in continuous culture would be useful. In addition, when using a novel fermentation technology, it may be desirable to restrict the amount of cell growth after a desired biomass concentration has been achieved. This could possibly be achieved by restriction of the yeast extract supplementation. Hence, knowledge of the effect of this on solvent production would be essential information.

4.3.2 Results

4.3.2.1 Effect of Dilution Rate

Experiments were performed to investigate the effect of dilution rate on the performance of the reactor. Influent *pH* value was 4.5 and yeast extract concentration was at 5 g/l. The results are summarized in Figure 4.5. An increase in dilution rate over the range 0.03 - 0.15 h⁻¹ resulted in increased acid (acetic + butyric acid) productivity. A maximum solvent productivity of 77.17 mg/l.h was observed at a dilution rate of 0.056 h⁻¹ representing a solvent yield of 0.10 g/g lactose utilized (lactose utilization 27.2%). This corresponded to concentrations in the effluent of acetone, 0.48 g/l; butanol, 0.85 g/l; and ethanol, 0.04 g/l. The solvent yield and lactose utilization also displayed maxima at the same dilution rate. At dilution rates higher than 0.056 h⁻¹, acid productivity steadily increased, whereas solvent productivity, solvent yield and lactose utilization decreased. Therefore, an increase in dilution rate resulted in acid production at the expense of solvent production.

In addition to increased acid productivity, operation of the reactor at high dilution rates (over the range studied) resulted in increased concentrations of residual lactose in the effluent stream. Hence, the dilution rate of 0.056 h⁻¹ appeared to be the optimum operational condition for the CSTR performance.

4.3.2.2 Effect of Supplementary Yeast Extract Concentration

Experiments were performed to determine the requirement for yeast extract supplementation of the whey permeate feed medium in a CSTR. Culture *pH* was maintained at 4.5, and dilution rate at 0.056 h⁻¹. Initially, the reactor was operated with a yeast extract concentration of 5 g/l. After a steady-state condition was attained, the yeast extract concentration was reduced in increments, allowing a steady-state to be achieved at each stage which usually took place after three residence times.

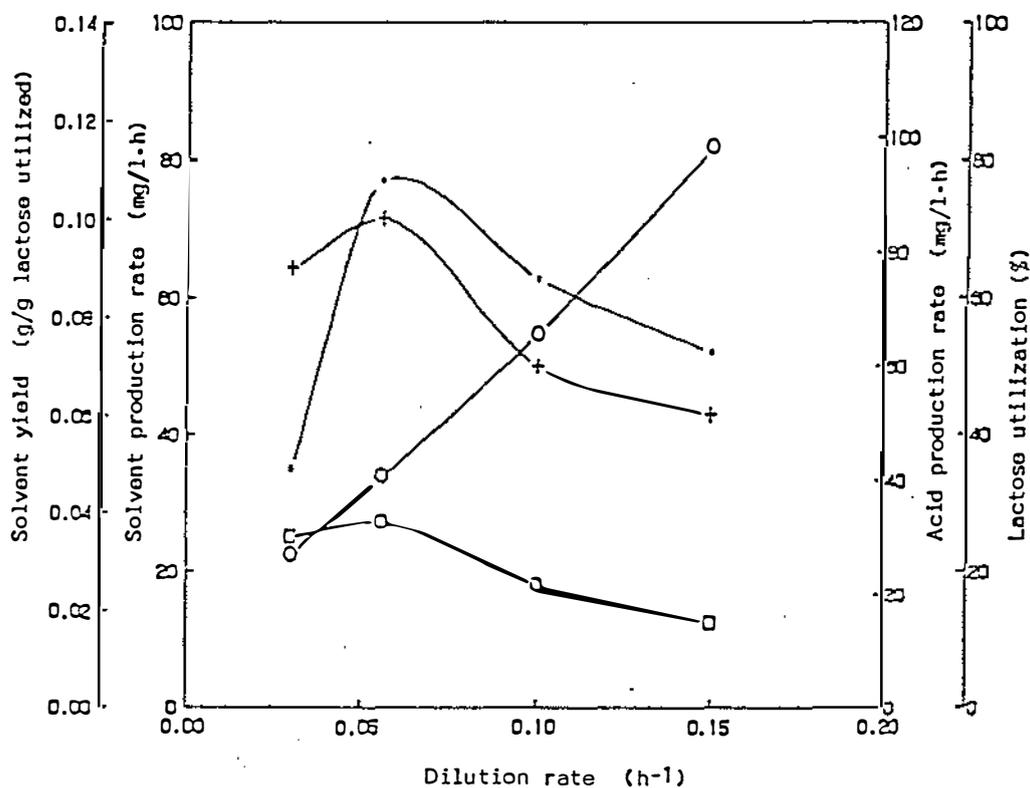


Figure 4.5

Effect of dilution rate on reactor performance using whey permeate as substrate at 34°C and pH 4.5: (•) solvent production rate; (○) acid production rate; (□) lactose utilization; (+) solvent yield.

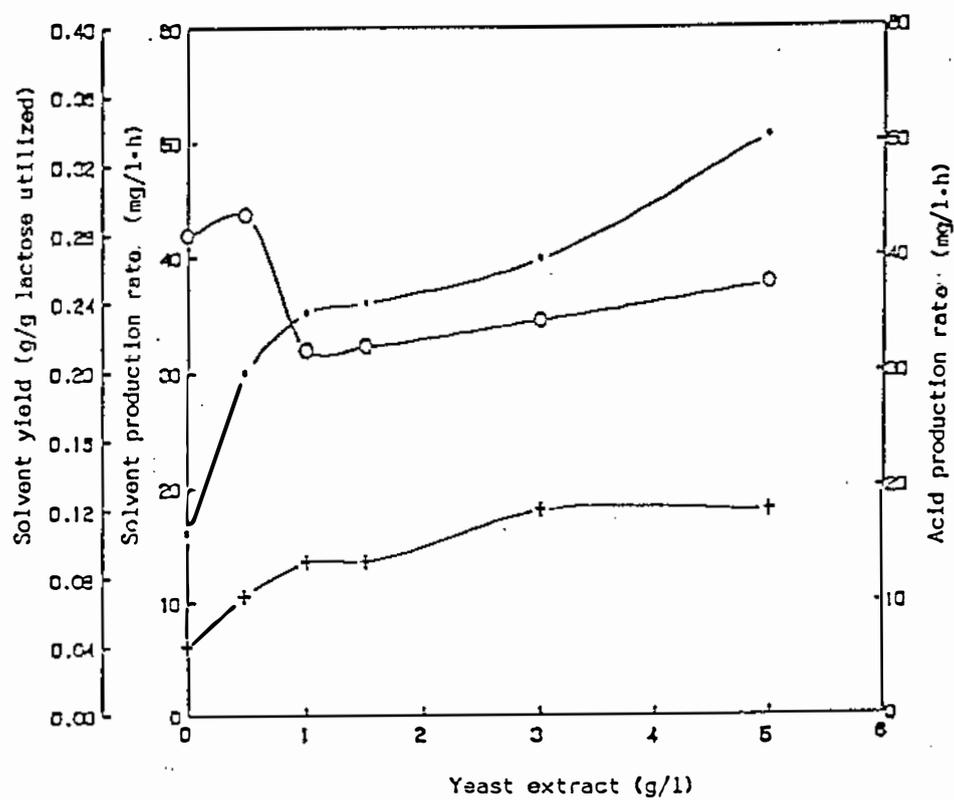


Figure 4.6

Effect of yeast extract concentration on reactor performance using whey permeate as substrate at 34°C, pH 4.5 and $D = 0.056 \text{ h}^{-1}$; (•) solvent production rate; (○) acid production rate; (+) solvent yield.

Figure 4.6 shows the effect of yeast extract concentration on the solvent and acid productivities and the solvent yield. At a concentration of 1 g/l or above, these parameters showed little variation except at a concentration of 5 g/l where a somewhat higher value for solvent productivity was observed. However, below a yeast extract concentration of 1 g/l, a marked reduction in solvent productivity, accompanied by an increase in acid productivity, was observed. The solvent yield displayed similar behaviour to solvent productivity in response to a yeast extract concentration below 1 g/l.

When no yeast extract was present in the whey permeate, a steady state was maintained for over five residence times. The observed solvent production was relatively poor and this fermentation condition favoured acid production rather than solvent production. This weak but positive solvent production in the absence of supplementary yeast extract was similar to the result observed in batch culture (Section 3.4.2 and Table 3.4). Thus it is possible to maintain a continuous culture on unsupplemented whey permeate. In batch culture without yeast extract supplementation (Run V, Table 3.5), there was no growth and solvent production. This is in apparent contrast to the results in CSTR and the batch fermentation reported in Table 3.4. The reason for this contradiction is not certain, but it can be concluded that unsupplemented whey permeate contains *marginally* sufficient nutrients to support growth.

4.3.3 Discussion

The objective of this work was to examine the effect of dilution rate on solvent productivity, and to investigate the requirement for yeast extract supplementation.

In terms of the dilution rate, solvent productivity and yield decreased with increasing dilution rate, over the range studied. In contrast, acid productivity increased with increasing dilution rate. This trend is in agreement with other reports (Bahl *et al.*, 1982b; Monot and Engasser, 1983b; Fick *et al.*, 1985; Qureshi

and Maddox, 1987), and is probably due to the presence of differing proportions of vegetative acidogenic cells and clostridial, solventogenic forms as the dilution rate varies (Clarke *et al.*, 1988). The former are favoured at high dilution rates, while the latter are favoured at low dilution rates. It follows from this that a single-stage continuous fermentation process, using freely-suspended cells in a CSTR, is unlikely to provide much improvement over a batch reactor in terms of solvent productivity.

For the study into yeast extract concentration, a dilution rate of 0.056 h^{-1} was used. The results show that solvent production was favoured when a minimum yeast extract supplementation of 1 g/l was used. Below this concentration, reductions in solvent productivity and yield were observed, while acid production increased. Similar results have been observed by Qureshi and Maddox (1987) when using immobilized cells in a packed-bed reactor.

4.3.4 Conclusion

The optimum dilution rate and the requirement for supplementary yeast extract for continuous solvent production from whey permeate using free cells in a CSTR have been demonstrated. Solventogenesis is favoured at lower dilution rate, while acidogenesis is favoured at higher dilution rate.

A yeast extract supplementation of less than 1 g/l favours acid production rather than solvent production, while solvent production is observed at a concentration of 1 g/l or above.

It is unlikely that high solvent productivities can be attained using whey permeate in a single stage continuous culture system with freely-suspended cells. Thus, novel fermentation technologies require investigation.

CHAPTER 5

CONTINUOUS SOLVENT PRODUCTION IN A PACKED BED

REACTOR USING CELLS OF *C. ACETOBUTYLICUM* P262

IMMOBILIZED BY ADSORPTION ONTO BONECHAR

5.1 Introduction

An extensive amount of work has examined the use of continuous culture systems for the production of acetone and butanol using *C. acetobutylicum*. Many of these systems have been based on immobilization of the cells, either by entrapment or by adsorption. Cell immobilization is a technique that allows high cell densities to be achieved, resulting in increased fermentation productivities compared to the corresponding system with freely-suspended cells. In addition, high dilution rates can be used with little washout of cells from the supports.

A variety of different support materials have been used for cell immobilization, and subsequent continuous operation, to achieve high reactor productivities with long term stability. Carbohydrates, e.g. alginate, have been used to entrap *C. acetobutylicum* cells (Häggström and Molin, 1980; Häggström and Enfors, 1982; and Häggström, 1985; Ennis *et al.*, 1986), while solid supports such as beechwood shavings (Förberg and Häggström, 1985), charcoal (Bahadur and Saroj, 1960) and bonechar (Qureshi and Maddox, 1987) have been used for adsorptive cell attachment.

Immobilization by adsorption onto bonechar (as used in sugar refining), represents a cheap, simple and mild immobilization method, which involves adsorbing the cells onto the surface of the support (i.e. bonechar) by passing a growing culture onto a sterile bed of the material (Qureshi and Maddox, 1987). In contrast to immobilization by entrapment, the use of thin layers of adsorbed cells improves the mass transfer characteristics of the system. *C. acetobutylicum* is particularly suited to immobilization by adsorption owing to the polysaccharide material produced by the organism, allowing the cells to adhere to the bonechar, and to each other, extremely tightly (Maddox, 1988).

Multilayer adsorbed cells could, however, result in a substantial cell leakage from the system, since they are in direct contact with the surrounding environment, and hence subject to any forces of shear or attrition which may result from the relative motion of particles and fluid. It is therefore likely that some cells will become detached and enter the bulk fluid phase (Webb, 1989). In this regard, the use of non-growing (but viable) cells may reduce this disadvantage.

Another disadvantage of multilayer adsorbed cells is possible blocking of the reactor due to excessive biomass growth, leading to operational problems such as channelling. This could shorten the useful life of the reactor.

Hence the purpose of the work described in this chapter, was to investigate the effect of restricted cell growth, by means of a nutrient limitation, on the production of solvents in defined medium and in sulphuric acid casein whey permeate during continuous fermentation in a packed-bed reactor (PBR) using cells immobilized by adsorption onto bonechar. Further, it is fundamental to fermentation studies that techniques be developed to maximize the performance and stability of the reactor.

5.2 Effect of Growth-Limiting Nutrient Concentration on Continuous Solvent Production in Defined Medium

5.2.1 Introduction

Immobilized viable cells can be either growing or non-growing. Immobilized non-growing cells are characterized by their ability to utilize their metabolic capacity more efficiently, since the same cells are used for a longer time period. Part of the substrate which otherwise would have been used for biomass production is utilized for product formation, resulting in a higher overall yield of products.

It has been demonstrated by Häggström (1981) and Häggström and Molin (1980) that immobilized non-growing cells of *C. acetobutylicum* can be used for the production of solvents and that a continuous process is feasible. However, immobilized non-growing cells will lose activity with time, and it has been observed that a rapid initial loss of activity occurs when the cultures are fed with a glucose-based medium lacking in other nutrients in order to maintain the organisms in an active but non-growing state. Förberg *et al.* (1983) suggested that the nutrient supply should be sufficient to enable the organisms to restore essential cell constituents, but not rich enough for reproduction to proceed.

The objective of the work described in this section was to develop a packed bed reactor based on immobilized cells for solvent production from defined medium, and then to investigate the effect of a nutrient-limited medium on subsequent solvent production. The nutrients investigated were phosphate, ammonium ions and iron. Lactose was used as the carbon source because of the potential use of whey permeate (Table 2.5) as a commercial substrate for the ABE fermentation process (Maddox, 1980).

5.2.2 Results

The start-up procedure for experiments using defined medium is described in Section 2.6.4. The culture *pH* of the feed medium was adjusted before autoclaving to *pH* 6.5 using 4M KOH. A dilution rate of 0.15 h^{-1} was used, while the temperature of the reactor was maintained at 34°C using a thermostatically controlled water jacket. These parameters were kept constant in all experiments.

Initially, each reactor was operated 'batchwise' for 18 h, after which it was supplied with the full medium (all medium components in excess; Table 2.5) until a thin cell layer developed on the bonechar. The experiment then commenced. After a steady state condition was established the influent medium composition was changed as appropriate.

Samples were taken at least once a day and concentrations of suspended (non-adsorbed) cells, products and lactose in the effluent stream were determined by methods described in Chapter 2. The adsorbed biomass was not, however, determined since this would have involved the sacrifice of an entire reactor. Steady state was defined as constant concentrations of solvents, acids and lactose in the reactor effluent over a period of at least three residence times. After each steady-state was established, the concentration in the feed medium of the nutrient under study was changed, and the reactor was further operated until a new steady-state was achieved. All other influent nutrient concentrations were maintained constant.

5.2.2.1 Effect of Ammonium (Nitrogen) Ion Concentration on Solvent Production in a Packed Bed Reactor

A packed bed reactor of immobilized cells was established to determine the effect of different levels of influent nitrogen on solvent production. The steady-state results are summarized in Table 5.1, while Figure 5.1 illustrates the progress of the reactor performance.

Table 5.1 Fermentation parameters on the effect of ammonium concentration on the performance of PBR.

Influent NH ₄ (g/l)	Effluent NH ₄ (g/l)	Solvent productivity (g/l.h)	Acid productivity (g/l.h)	Lactose uptake rate (g/l.h)	Solvent Yield (g/g)	Non-absorbed cell mass(g/l)	PO ₄ in Effluent (g/l) ^a	Fe in Effluent (g/l) ^b
3.0	2.40	0.42	0.31	3.13	0.13	0.22	2.04	0.024
2.0	1.47	0.38	0.19	2.94	0.13	0.11	1.20	0.035
3.0	1.93	0.49	0.17	3.21	0.15	0.14	1.26	0.030
1.0	0.66	0.17	0.34	1.49	0.11	0.11	0.93	0.027
0.5	0.25	0.11	0.28	1.25	0.09	0.10	0.80	0.025
0.2	0	0.08	0.23	0.97	0.08	0.11	0.96	0.030
0	0	0	0.35	0.88	0.08	0.10	0.98	0.027

^a Influent concentration, 0.40 g/l as K₂HPO₄

^b Influent concentration, 0.02 g/l as FeSO₄

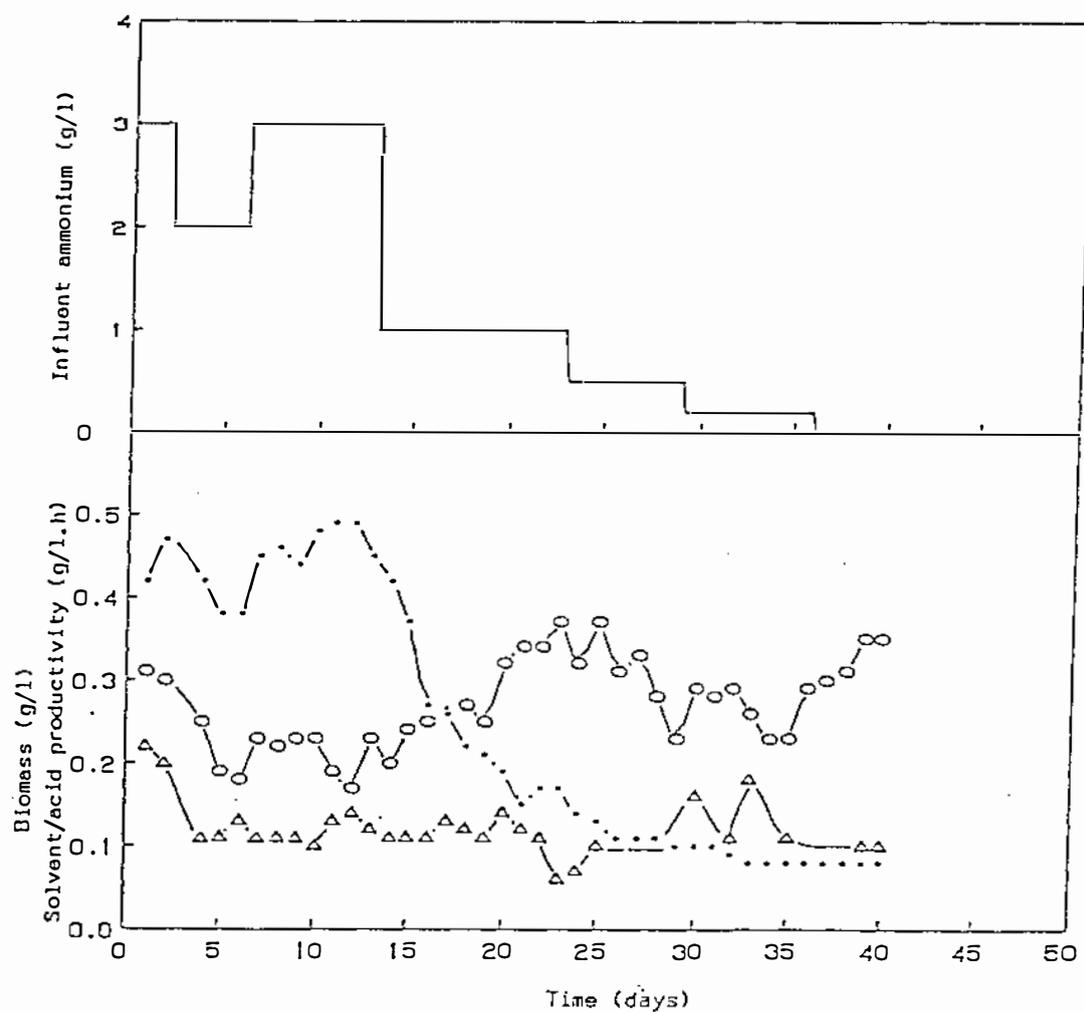


Figure 5.1 Effect of influent ammonium ion concentration on the continuous fermentation profile of a packed bed reactor. $D = 0.15 \text{ h}^{-1}$: (\bullet) Total solvent productivity; (\circ) Total acid productivity; (Δ) Biomass.

The first steady-state condition was observed during the late period of reactor stabilization with nutrient-excess medium (ammonium sulphate 3 g/l). This is shown in Fig. 5.1 as zero time. Immediately after this first steady-state, the ammonium sulphate concentration in the feed medium was adjusted to 2 g/l. By day 5 (10.8 residence times), the reactor had reached another steady-state condition, represented by decreases in solvent and acid productivity. Next, an attempt was made to restore the productivities by returning the ammonium sulphate concentration to 3 g/l. By Day 11, the solvent productivity had largely been restored.

The data show that during this period of initial operation, all nutrients were in excess, as judged by their concentrations in the effluent stream.

The ammonium ion concentration was now reduced in increments, allowing a steady-state to be achieved at each stage. The results in Table 5.1 and Figure 5.1 demonstrate that a decrease in the influent concentration from 3.0 g/l (at Day 13) to 1.0 g/l resulted in a reduction in the solvent yield of 0.04. In addition there was a marked decrease in solvent productivity, accompanied by an increase in acid productivity. Uptake of ammonium ions was also reduced, but there was still an excess of all nutrients. Further decreases in the influent ammonium sulphate concentration led to corresponding decreases in solvent production while acid production remained relatively unchanged. At an ammonium sulphate influent concentration of 0.2 g/l, no ammonium ions were detected in the effluent, showing that a nutrient limitation had been achieved. Solvent productivity was poor, although acid productivity and the biomass concentration in the effluent stream remained relatively high.

Throughout this Run, the phosphate and iron concentrations in the effluent were in excess of those in the influent.

5.2.2.2 Effect of Phosphate Ion Concentration on Solvent Production in a Packed Bed Reactor

A packed bed reactor similar to that described above was established to determine the effect of different concentrations of phosphate ion on solvent production in the system. The steady state results are summarized in Table 5.2, while Figure 5.2 illustrates the progress^{of} the reactor performance.

The initial steady state condition was reached by the end period of reactor stabilization where all nutrients were supplied in excess (0.4 g/l dipotassium hydrogen phosphate), and from which the zero time was counted. After the first steady state had been reached, the concentration of phosphate in the feed medium was reduced to 0.19 g/l at Day 1. During the next 14 days there was a steady decrease in the solvent productivity with no real steady-state being attained. The acid productivity initially showed an increase, but then decreased, while the non-adsorbed cell concentration also showed some fluctuation. Despite the decrease in solvent productivity, the solvent yield remained relatively constant.

At Day 15, the system was again supplied with the full feed medium (K_2HPO_4 0.4 g/l). The decline in solvent production was arrested and production became reasonably steady, accompanied by a sharp decrease in acid production. The solvent yield and non-adsorbed cell concentration were reasonably steady. Hence, this fermentation condition appeared to support solventogenesis.

During this entire operational period, it was noticed that the phosphate concentration in the reactor effluent exceeded that in the influent. All other nutrients were also present in the effluent stream. The feed medium was now adjusted to contain no inorganic phosphate (Day 22). A steady state condition was attained by Day 32. The solvent productivity was now lower than previously, while the acid productivity showed a marked increase. However, there were still excesses of phosphate, iron and nitrogen in the effluent stream, indicating that there was no limiting nutrient in this system.

Table 5.2 Fermentation parameters on the effect of phosphate concentration on the performance of PBR.

Influent PO ₄ (g/l)	Effluent PO ₄ (g/l)	Solvent productivity (g/l.h)	Acid productivity (g/l.h)	Lactose uptake rate (g/l.h)	Solvent Yield (g/g)	Non-absorbed cell mass(g/l)	NH ₄ in Effluent (g/l) ^a	Fe in Effluent (g/l) ^b
0.40	1.35	0.49	0.15	2.31	0.21	0.25	2.16	0.030
0.19	1.32	0.19	0.30	1.01	0.19	0.29	1.95	0.030
0.40	1.45	0.23	0.12	1.36	0.17	0.30	1.65	0.016
0	0.92	0.13	0.34	1.50	0.09	0.21	2.29	0.025

^a Influent concentration, 3.0 g/l as (NH₄)₂SO₄

^b Influent concentration, 0.02 g/l as FeSO₄

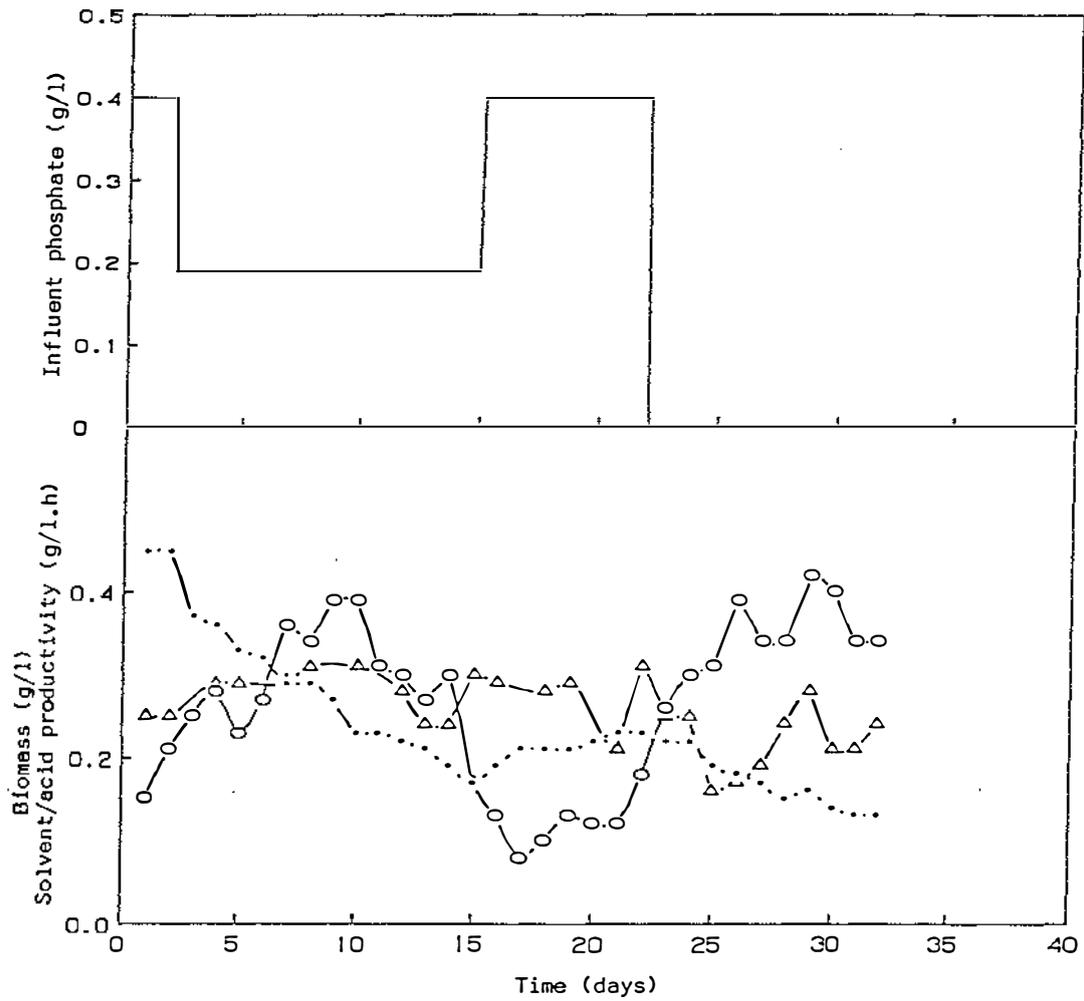


Figure 5.2

Effect of influent phosphate ion concentration on the continuous fermentation profile of a packed bed reactor. $D = 0.15 \text{ h}^{-1}$. Symbols as for Fig. 5.1

5.2.2.3 Effect of Iron Concentration on Solvent Production in a Packed Bed Reactor

In a similar experiment to those described above, the effect of the ferrous sulphate concentration on reactor performance was investigated. The steady state results are summarized in Table 5.3, while Figure 5.3 illustrates the progress of the reactor performance.

The initial steady state condition was attained by the late period of reactor stabilization where all nutrients were supplied in excess (0.03 g/l ferrous sulphate) and from which the zero time was counted. After the first steady-state had been reached, the concentration of iron in the feed medium was reduced to 0.001 g/l on Day 1. During the next 10 days, there was a progressive decrease in solvent productivity accompanied by an increase and then fluctuations in acid production. The non-adsorbed cell concentration showed a gradual decline, and no real steady-state was attained. During this period, the iron concentration in the effluent exceeded that in the influent.

At Day 11 the system was again fed with a complete nutrient medium in an attempt to restore the solvent productivity. At steady-state (Day 17), no significant improvement in solvent yield or productivity had been achieved. In fact, the fermentation condition continued to favour acidogenesis.

Finally, a feed medium without ferrous sulphate was fed to the reactor (Day 18). A steady state condition was reached at Day 25, but there was no improvement in terms of solvent productivity or yield.

Analysis of the effluent revealed the presence of iron, phosphate and ammonium ions, indicating no limitation of these nutrients in this system.

A control reactor was operated with the full medium (3 g/l NH_4SO_4 , 0.4 g/l K_2HPO_4 and 0.02 g/l FeSO_4) and this remained solventogenic over an operating period of 25 days at $D = 0.15 \text{ h}^{-1}$.

Table 5.3 Fermentation parameters on the effect of iron concentration on the performance of PBR.

Influent Fe (g/l)	Effluent Fe (g/l)	Solvent productivity (g/l.h)	Acid productivity (g/l.h)	Lactose uptake rate (g/l.h)	Solvent Yield (g/g)	Non-absorbed cell mass(g/l)	NH ₄ in Effluent (g/l) ^a	PO ₄ in Effluent (g/l) ^b
0.03	0.02	0.21	0.13	1.45	0.15	0.40	2.18	1.90
0.001	0.03	0.10	0.24	1.13	0.09	0.27	2.38	2.05
0.03	0.02	0.10	0.38	1.53	0.07	0.17	2.40	1.36
0	0.03	0.06	0.43	0.93	0.06	0.11	2.59	1.39

^a Influent concentration, 3.0 g/l as (NH₄)₂SO₄

^b Influent concentration, 0.40 g/l as K₂HPO₄

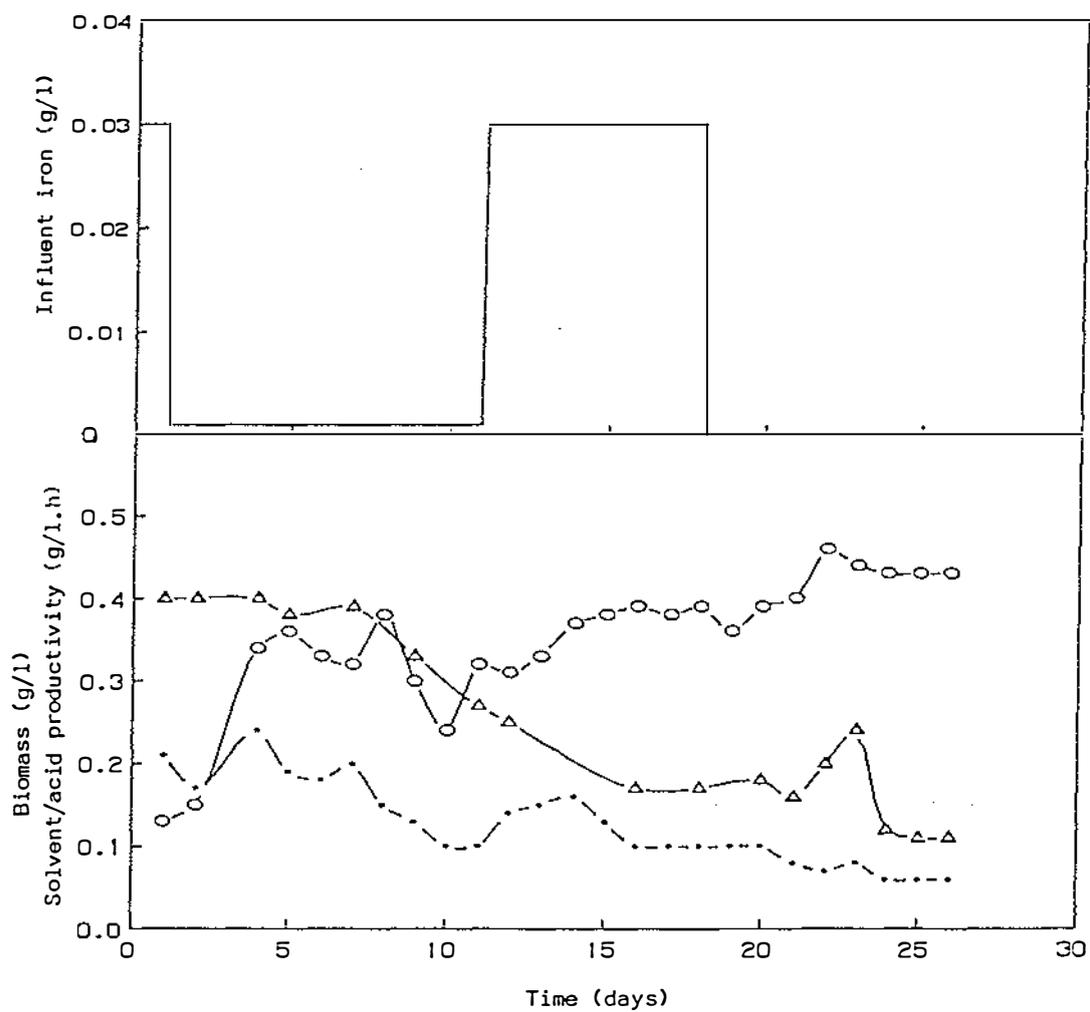


Figure 5.3 Effect of influent iron concentration on the continuous fermentation profile of a packed bed reactor. $D = 0.15 \text{ h}^{-1}$. Symbols as for Fig. 5.1

5.2.3 Discussion

The objective of this series of experiments was to develop a packed bed reactor using cells immobilized by adsorption onto bone char, and to investigate the effect of the nutrient composition of a defined medium on solvent production and operational stability of the reactor. As shown previously (Chapter 3) the nutrient composition of the medium can have a profound effect on solvent production. In addition, it is important when operating a packed bed reactor of this type, that the adsorbed biomass concentration is restricted, otherwise problems of blockage and channelling may occur, shortening the life of the reactor.

Three nutrients were selected for investigation. It was hoped that limitation of these could be used to restrict biomass growth, and to study solvent production under these conditions. However, it proved impossible to limit the supply of either phosphate or iron. When these nutrients were absent from the feed medium, they were still detected in the reactor effluent stream. This is explained on the basis of leaching of phosphate, and possibly iron, from the bone char (manufactured from animal bones), and the leaching of iron from a metal sieve used in the reactor. Nevertheless, the data obtained still allow insight into the operation of this type of reactor.

In the experiments where the influent nitrogen concentration was varied, solvent productivity was at a maximum at the highest influent nitrogen concentration. This appears to be in contrast to experiments in batch fermentation (Chapter 3) where an excess of nitrogen was detrimental to solvent production. A possible explanation for the difference is that it is the *balance* of nutrients which is important, i.e. it is not so much the excess nitrogen concentration that is important, as the ratio of nitrogen to phosphate. If these two nutrients are *balanced*, then solventogenesis might be favoured even at quite high excess concentrations of both nutrients. Another explanation involves the difference between the packed bed reactor and a batch fermentation with freely suspended

cells. In the former, plug flow is assumed. Hence the concentration of nutrients varies throughout the reactor. Optimization of the system can then be done only on an empirical basis.

The results with phosphate and iron also signified that solvent production was favoured at high, rather than low, concentrations of these nutrients.

Thus, overall, the results from the present work support the concept that in a packed bed reactor of this type, using a defined medium, solvent production is favoured by high influent nutrient concentrations. Unfortunately, this condition would allow continued biomass growth, and potential blocking of the reactor. Using bonechar as the support material it is not possible to restrict biomass growth by limitation of phosphate or iron. Hence, the nitrogen nutrient must be used. Time constraints during this work precluded investigation of other nutrients, e.g. vitamins.

Reference to Table 5.1 indicates that it may be possible to operate the reactor under conditions of restricted nitrogen nutrient for short periods of time, followed by provision of excess nitrogen to allow recovery of solvent productivity. Thus, the problem of excess growth, leading to blockage, could be delayed.

5.2.4 Conclusion

The effects of three nutrients i.e. nitrogen, phosphate and iron, on continuous solvent production in a packed bed reactor of bonechar-immobilized cells was investigated. Solvent production was favoured by high concentrations of these nutrients in the influent medium. It was not possible to restrict the supply of phosphate or iron to the reactor, because of leaching. However, conditions where biomass growth was restricted by a restriction in the nitrogen nutrient supply were not conducive to solvent production.

5.3 Effect of Yeast Extract Supplementation on Solvent Production from Whey Permeate

5.3.1 Introduction

It has been shown in Chapter 3 that a successful batch fermentation of whey permeate depends on the presence of supplementary yeast extract. The latter appears to supply certain metal ions, including iron, which are deficient in whey permeate. Nitrogen and phosphate nutrients, and vitamins appear to be present at satisfactory concentrations in whey permeate.

In a commercial fermentation process, supplementary yeast extract represents an additional cost. Hence there is little point in adding more than is required.

In a packed bed reactor, it may be possible to provide a lower concentration of yeast extract than is required for batch fermentation, since the growth requirement is only small. Indeed, it may be beneficial to restrict biomass growth in order to prevent reactor blockage.

Hence the purpose of this section of work was to operate the packed bed reactor using whey permeate as substrate, and to investigate the supplementary yeast extract concentration required for the maintenance of solventogenesis while simultaneously preventing blockage of the reactor. Since the possible limiting nutrient in whey permeate, iron, appears to be very difficult to restrict in the packed bed reactor, it may be possible to operate for long time periods without any supplementation.

5.3.2 Results

The start-up procedure for experiments using sulphuric acid casein whey permeate was described in Section 2.6.4. The culture pH was controlled indirectly by adjusting the pH of feed medium before autoclaving to pH 6.0 using 4M KOH. A dilution rate of 0.15 h^{-1} was used throughout the experiments.

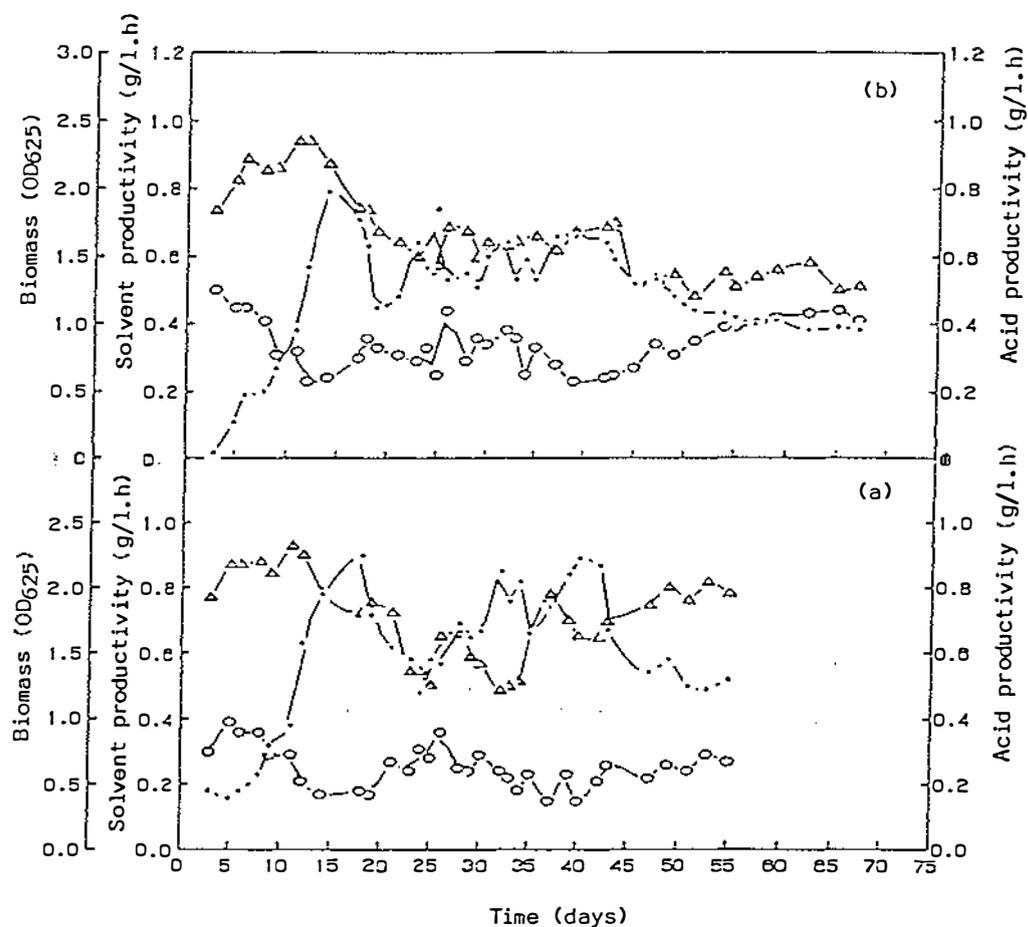


Figure 5.4

Continuous fermentation profile of (a) "control" and (b) "test" packed bed reactors, at 34°C ($D = 0.15 \text{ h}^{-1}$, $\text{pH } 4.5$). Symbols as for Fig. 5.1

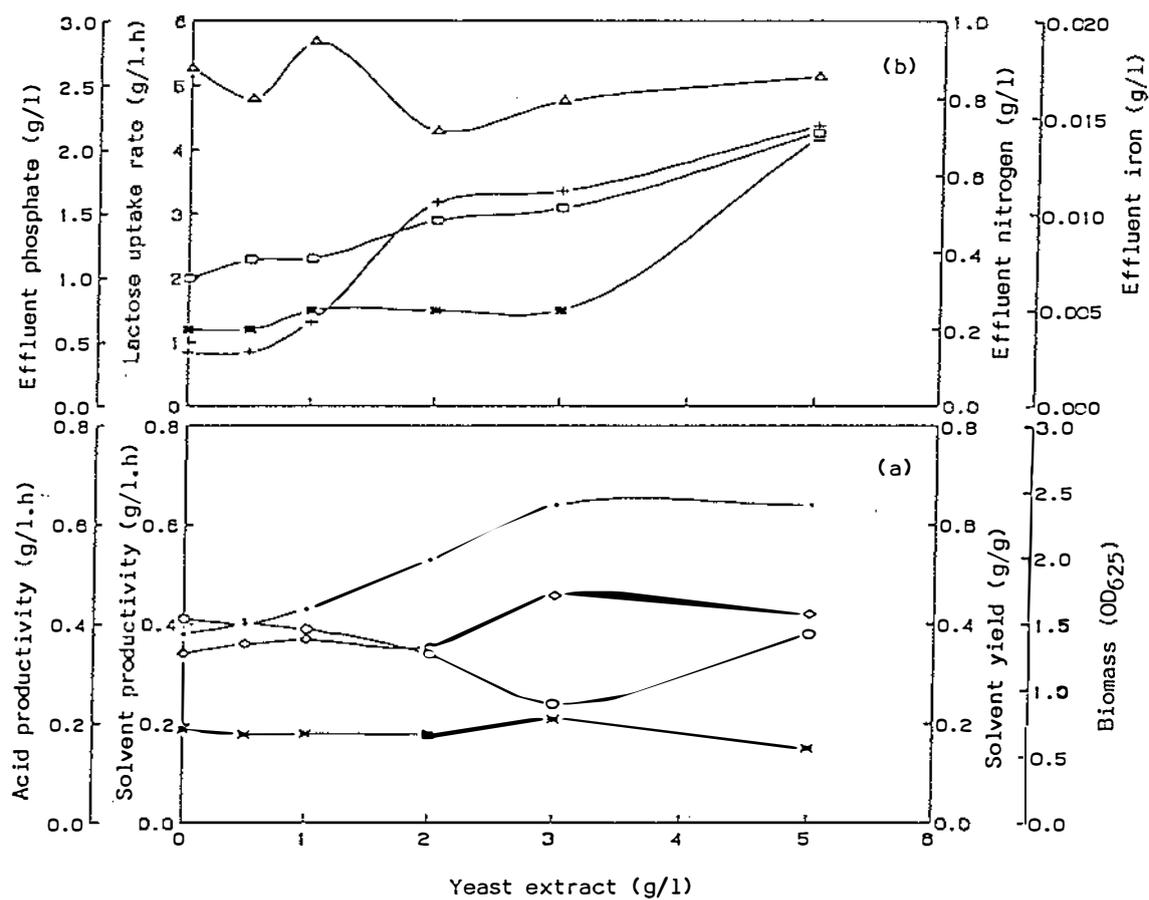


Figure 5.5 Effect of yeast extract concentration on a continuous fermentation using immobilized *C. acetobutylicum* cells at 34°C ($D = 0.15 \text{ h}^{-1}$, pH 4.5): (•) Total solvent productivity; (○) Total acid productivity; (x) Solvent yield; (□) Lactose uptake rate; (+) Effluent nitrogen; (Δ) Effluent phosphate; (◊) Effluent biomass (OD_{625}); (■) Effluent iron

Two reactors were established and fed continuously (after batching for 18 h) with full feed medium, which contained 50 g/l sulphuric acid casein whey permeate and 5 g/l yeast extract. Zero time was taken as the start of the continuous feeding. It then took about 30 days before the biomass had accumulated throughout most of the reactor (reactor stabilization) and a steady state condition was attained. One of the reactors was then used to study the effect of yeast extract concentration by reducing the concentration in increments ('test' reactor), allowing a steady-state to be achieved at each stage. In contrast, the other reactor was continuously fed with whey permeate containing 5 g/l yeast extract for comparison purposes ('control' reactor).

Samples were taken at least once a day or every residence time. Steady state was assumed when samples taken over at least three residence times were constant in effluent lactose and products concentrations. The adsorbed biomass was not determined, since it would have involved the sacrifice of an entire reactor. The progress of reactor performance is illustrated in Figure 5.4 for both of the reactors. The steady-state results for the effect of yeast extract are summarized in Figure 5.5. A photograph of both reactors at the end of fermentation operation is shown in Figure 5.6.

Figure 5.4(a) shows the progress of the reactor which was continuously fed with whey permeate containing 5 g/l yeast extract ('control' reactor). The results in Fig. 5.4(a) demonstrate that as the fermentation progressed, with constant supplementary yeast extract in the whey permeate feed medium, the solvent productivity displayed fluctuations and these corresponded inversely with acid productivity. The fluctuation in solvent productivity was especially pronounced during the stabilization period of the reactor (between Day 3 and Day 32). After Day 32, this fluctuation was less pronounced but was followed by a decreasing trend of solvent production, particularly after Day 42. Acid production, however, displayed less fluctuation, and after Day 32 remained fairly constant. The non-adsorbed biomass displayed a decreasing trend during the first 32 days and then an increasing trend for the rest of fermentation operation. This result probably



Figure 5.6 A photograph of "control" and "test" packed bed reactors at the end of fermentation operation.

indicates that during the stabilization period (the first 32 days), there were progressively more cells becoming adsorbed onto the bonechar, whereas after Day 32 the amount of adsorbed cells became constant, and excess biomass was detached and lost in the effluent stream. This 'control' reactor was in use for only 55 days after which it suffered from severe blockage and channelling problems due to excessive biomass growth, particularly at the reactor base (Fig. 5.6). However, this overall fermentation condition with supplementary yeast extract at 5 g/l favoured solventogenesis over acidogenesis.

Figure 5.4(b) shows the progress of the reactor performance which was continuously fed with whey permeate containing 5 g/l yeast extract during the first 32 days prior to reducing the concentration in increments in order to study the effect of yeast extract supplementation ('test' reactor). During the first 30 days of continuous fermentation, fluctuations in non-adsorbed biomass, solvent and acid productivity were observed. The first steady-state condition was achieved at Day 32. Immediately after this first steady-state, the yeast extract concentration in the feed medium was then reduced as appropriate.

Figure 5.5(a) shows the effect of yeast extract concentration on solvent (acetone + butanol + ethanol) and acid (acetic + butyric acid) productivities, solvent yield and suspended cells (non-adsorbed cells) in terms of optical density at 625 nm in the effluent. Figure 5.5(b) shows the concentrations of nitrogen, phosphate and ferrous ion concentration in the effluent stream at each corresponding steady state, as well as lactose uptake rate. At a yeast extract concentration of 3 g/l and above, there was no significant difference in solvent productivity. However, the lactose uptake rate was slightly increased, and the acid productivity, solvent yield and effluent cell mass slightly decreased at the higher (5 g/l) yeast extract concentration. As the yeast extract concentrations were decreased below 3 g/l, solvent productivity decreased significantly, accompanied by an increase in acid productivity. At the same time, solvent yield and non-adsorbed cell mass remained constant. In addition, the lactose uptake rate showed a decrease as the concentration of yeast extract was reduced. The

solvent production without supplementary yeast extract in the feed medium was maintained over a period of 8 days, representing 28 residence times, and the experiment was stopped only because of time constraints. Analysis of the effluent stream revealed that at no time was there any limitation in nitrogen, phosphate or iron. In terms of operational stability the 'test' reactor was used in continuous fermentation over a period of 67 days (1600 h), whereas the 'control' reactor was used for only 55 days (1300 h) as it suffered from blockage due to excessive biomass growth, especially at the base (Figure 5.6). The 'test' reactor, however, showed no evidence of blockage.

5.3.3 Discussion

Immobilization by adsorption is a simple technique which involves adsorbing the cells onto the surface of an inert support such as bonechar. This support was chosen because of its low cost and ready availability. Maddox (1988) has pointed out that *C. acetybutylicum* is particularly suited to this technique because of the polysaccharide material which is produced by the cells, causing them to stick to the bonechar, and each other, extremely firmly. Qureshi and Maddox (1987) have successfully applied this method of immobilization to continuous solvent production from whey permeate in packed bed reactor. It has been demonstrated by Qureshi and Maddox (1987) that in terms of solvent concentration and productivity, bonechar immobilization is superior to alginate immobilization, probably due to the greater amount of biomass present in the reactor. However, the system suffered from problems of high gas hold-up and reactor blockage due to excessive biomass growth. On examination of the feed medium used in their study, 5 g/l of yeast extract was used in the whey permeate. Hence the purpose of the present work was to attempt to prevent excessive biomass growth by means of reducing the concentration of the supplementary yeast extract.

The results demonstrate that when yeast extract is included in whey permeate at a concentration of 3 g/l or higher, the reactor produces solvents rather than acids. In contrast, when no yeast extract is added, the fermentation is acidogenic rather than solventogenic. Hence, selection of the appropriate yeast extract supplementation of whey permeate presents a dilemma. At high supplementation the process is solventogenic, but reactor blockage can occur. At low supplementation blockage is delayed considerably, but the process is acidogenic. Even with no supplementation, the reactor effluent contained detectable levels of what appears to be the limiting nutrient in whey permeate, i.e. iron, in addition to ammonium and phosphate ions. This is presumably because of the leaching process referred to earlier in this Chapter.

In general, the results now obtained with whey permeate are similar to those using synthetic medium in this fermentation system, i.e. solventogenesis is favoured at high nutrient concentrations. Thus, the problem still remains of how to prevent reactor blockage while maintaining strong solvent production. Possibly, a nutrient dosing technique, as described by Förberg and Häggström (1985) could be used. Unfortunately, time constraints prevented an investigation of this technique during the present study.

In terms of solvent productivities, a typical value with full medium (5 g/l yeast extract) was 0.85 g/l.h at $D = 0.15 \text{ h}^{-1}$. This compares with the values of 0.06 g/l.h in batch fermentation and 0.05 g/l.h in continuous fermentation in a CSTR. Qureshi and Maddox (1987) have achieved productivity of 6 g/l.h when operating at dilution rates up to 1.0 h^{-1} .

5.3.4 Conclusion

The results in this Section have demonstrated that it is possible to minimize biomass growth in a packed bed reactor based on immobilized cells by reducing the concentration of supplementary yeast extract added to the whey permeate.

Unfortunately, this operating condition leads to acid rather than solvent production. The minimum yeast extract concentration to maintain strong solventogenesis is 3 g/l. However, operation of the reactor with reduced supplementation leads to increased longevity of the reactor.

CHAPTER 6

FINAL DISCUSSION AND CONCLUSIONS

The main aim of this thesis was to investigate the effect of nutrient composition on solvent production and to develop a stable packed bed reactor for continuous solvent production using whey permeate as substrate, by controlling the amount of biomass present in the reactor. Whey permeate was used since it has the potential to be used commercially for the ABE fermentation process. Discussion and conclusion sections have been reported for the work described in each Chapter, so the detailed information contained in those Chapters will not be repeated here.

The industrial strain of *C. acetobutylicum* P262 was used throughout these studies. Initially, a series of batch fermentation experiments was conducted to investigate the effect of the nutrient status of the culture on solvent production in a defined medium as well as in whey permeate. This work appears to be the first instance of such a systematic study being conducted. The nutrient status of the culture has been demonstrated to have a profound effect on cell density and solvent productivity in batch fermentation. For all three nutrients (i.e. nitrogen, phosphate and iron) investigated, it is apparent that the optimum nutrient concentration for solventogenesis is where the concentration is just slightly in excess of that required for growth. A greater excess leads to the fermentation becoming acidogenic.

For whey permeate, the results in batch fermentation (Section 3.4.2) have indicated that whey permeate without any supplementation just *marginally* supports cell growth and solvent production. Addition of vitamins did not improve solvent production, but addition of yeast extract had a marked effect. Therefore the whey permeate was most likely deficient ⁱⁿ iron and possibly other metal ions. It was thought possible that this effect could be utilized as a means of restricting cell growth in an immobilized cell reactor.

The effect of cell density on specific solvent productivity in continuous culture has been demonstrated in Chapter 4. It is possible to achieve solvent production under nitrogen- and phosphate-limited conditions, but the specific solvent productivity decreased with increasing biomass. This inverse relationship was attributed to the complex morphological behaviour of this strain of organism. The exact nature of the determinants responsible for the interconversion of the different cell types remains unknown, but almost certainly includes nutritional factors. This suggests the need for further study to identify those factors which favour the production of the non-growing, clostridial, solventogenic cell types. In this way the advantage of achieving a high cell density in a novel type of reactor could be more fully realized.

In the operation of a packed bed reactor, it is important that the adsorbed biomass concentration is restricted in order that the problems of blockage and channelling are avoided and the life of the reactor can be extended. However, the results in Chapter 5 demonstrated that continuous solvent production in this fermentation mode is favoured by a nutrient excess. Unfortunately, this condition would allow continued biomass growth, thus shortening the longevity of the reactor. Conditions which restrict biomass growth favour acidogenesis rather than solventogenesis. However, the results indicate that it may be possible to operate the reactor under conditions of restricted nitrogen nutrient for short periods of time, followed by provision of excess nitrogen to allow recovery of solvent productivity. This cannot be done with either phosphate or iron nutrients because of leaching of these ions from the system. Nevertheless, with regard to the leached iron, this fact could possibly be applied to whey permeate to make up for the deficiency in this substrate.

The results obtained from the packed bed reactor are in apparent contrast to those from batch fermentation with regard to the optimum nutrient concentration which supports solventogenesis. However, there is a fundamental difference between these two types of reactors. The packed bed reactor assumes

plug flow whereas the batch fermenter is completely mixed. Thus in the former, there is a nutrient gradient throughout the reactor. Furthermore, Qureshi *et al.* (1988) have demonstrated that in the packed bed reactor, less than 5% of the total biomass is actively solventogenic. Hence it appears that data from batch fermentation cannot be applied directly to a packed bed reactor based on immobilized cells, at least with an organism as complex as *C. acetobutylicum*.

When using whey permeate as a substrate in the packed bed reactor, the biomass build-up was controlled by means of limiting the yeast extract concentration of the feed. Unfortunately, this favoured acid rather than solvent production. A minimum yeast extract concentration of 3 g/l was required to maintain strong solventogenesis. Hence it appears that a compromise must be made between solvent production and reactor longevity.

In conclusion this study has illustrated the importance of the nutrient status of the culture medium in determining the extent of solventogenesis, and has stressed that the results obtained from one type of reactor cannot necessarily be applied directly to another.

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