INTENSIFICATION OF THE ACETONE : BUTANOL : ETHANOL FERMENTATION USING WHEY PERMEATE AND *CLOSTRIDIUM ACETOBUTYLICUM* :

A Preliminary Study

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ABSTRACT

The use of whey permeate as the fermentation substrate for the production of acetone:butanol:ethanol (solvents), using C. acetobutylicum P262 was studied. Initial experiments were conducted in a batch mode using sulphuric acid casein whey permeate medium, in an attempt to optimize the culture conditions for maximal extent of lactose utilization and solvents production. A high initial lactose concentration (65-75 g/l) in combination with a culture pH maintained in the region pH 5.4 to 5.6 were the most favourable conditions for solvent production. An inverse relationship between the lactose utilization rate and solvents yield was observed. Solvent productivities were only 60% however, of that achievable with this strain of organism on an industrial scale using a molasses medium, but comparable productivities were obtained using a semi-synthetic medium containing glucose. Hydrolysed-lactose sulphuric acid casein whey permeate medium was investigated as a medium for solvent production. Glucose and galactose were utilized simultaneously, although glucose was used preferentially. Only a small increase in solvents productivity was obtained compared with that obtained using non-hydrolysed permeate.

Experiments were performed in continuous culture using cheese whey permeate medium and alginate-immobilized cells. Significantly greater solvent productivities were obtained, compared with those achieved using free cells in batch culture. Fermentations were operated for over 650 hours with no detectable loss in fermentation performance. The extent of lactose utilization was low, however (less than 40%), and attempts to increase this by the use of pH regulation or a two-stage process were unsuccessful. This fermentation process was described as a biomass volume process (volumetric fraction of alginate beads in the reactor), where the lactose utilization and hence the solvents production, was defined by an inhibitory concentration of butanol, approximately 5 g/l.

An alternative continuous fermentation process using free cells and cheese whey permeate medium was investigated. External cell recycle using cross-flow microfiltration (CFM) membrane plant to continuously separate cells from the fermentation culture and recycle them back to the fermenter was utilized. Biomass was continuously removed from the fermenter in order to achieve a stable biomass concentration. Stable

solvents production was not achieved under the range of culture conditions investigated; culture degeneration was attributed to the complex interactive morphological cyclic behaviour of the organism. A tubular CFM unit which could be periodically backflushed to maintain the filtrate flux, was found to be the most suitable of those tested.

The integration of in-situ or in-line solvents recovery with batch culture using free cells, and continuous fermentation using cells immobilized by adsorption to bonechar, was investigated in order to remove toxic solvents and so increase the extent of lactose utilization and solvents productivity. A novel process using gas-stripping with an inert gas, and solvents recovery from the vapour phase by condensation using a cold trap, was described. An increase in lactose utilization and solvents productivity was achieved in both fermentation modes compared with control fermentations. The use of adsorbent resins and a molecular sieve for integrated fermentation solvents recovery was also demonstrated. However, the adsorption of medium components may mitigate against the usefulness of such a process option.

The batch refermentation of batch fermentation effluent treated by gas-stripping to remove solvents was investigated. However, solvent production was favoured only when lactose and nutrients were supplemented to concentrations similar to those present originally. Conversely, fermentation medium treated by gas-stripping to remove solvents could be readily refermented to produce solvents when an existing cell population was used, suggesting that this option of an integrated continuous fermentation-product recovery process may be promising for whey permeate solvent production.

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

Page

Page

LIST OF TABLES

Page

Page

ABBREVIATIONS

°C	degrees Celcius
cm	centimetre
g	gram
h	hour
l	litre
m	metre
mg	milligram
min	minute
ml	millilitre
mm	millimetre
rpm	revolutions per minute
µl	microlitre
M	molar

OTHER ABBREVIATIONS

C_A	acetone concentration (g/l)
C_B	butanol concentration (g/l)
C_E	ethanol concentration (g/l)
C_{HAc}	acetic acid concentration (g/l)
C_{HBu}	butyric acid concentration (g/l)
C_L	lactose concentration (g/l)
$C_{B,max}$	maximal butanol concentration (g/l)
C_{so}	feed substrate concentration (g/l)
C_s	effluent substrate concentration (g/l)
C_{xs}	biomass in solid phase (g/l)
C_x	biomass in liquid phase (g/l)
ΔC_s or ΔS	consumed substrate concentration (g/l)
d.w.	dry weight
D or D_t	dilution rate based on the total reactor volume (h^{-1})
$(1-\varepsilon)$	bead fraction of alginate beads in the reactor (l. alginate/l)
$D_t/(1-\varepsilon)$	normalized dilution rate (l/l. alginate h)
ϕ_{vi}	volumetric flow rate in (l/h)
ϕ_{vo}	volumetric flow rate out (l/h)
k	ratio of butanol/acetone (g/g)
P	specific butanol production rate (g. butanol/l. alginate h)

Prod	volumetric fermenter productivity (g/l.h)
r_s	substrate consumption rate (g/l.h)
r_{max}	maximal specific substrate consumption rate (g. substrate/ l. alginate h)
V_t	total working volume (l)
Y	solvents yield based on substrate consumed (g/g) = $(C_A + C_B + C_E)/\Delta S$
Y_{sb}	butanol yield on substrate (g/g)
Y_{ss}	total solvents yield on substrate (g/g)
Y_{sx}	biomass yield factor on substrate (g d.w./g)
μ_{max}	maximum growth rate (h^{-1})
>	greater than
<	less than
% w/v	percentage weight by volume

CHAPTER 1

INTRODUCTION

Since the oil price rise of the early-mid 1970's and the ensuing price uncertainties, there has been a resurgence of interest in the manufacture of fermentation products from renewable resources. One such fermentation process is the acetone:butanol:ethanol (ABE) fermentation. The present demand for these chemicals is met entirely from petrochemical sources, with the fermentation process becoming obsolete due to the rising costs of grain and molasses substrates, the increased costs of effluent disposal and, most importantly, the vast quantities of cheap by-products, propylene and ethylene, produced during petroleum cracking (Gibbs, 1983).

Until very recently, National Chemical Products Ltd, Germiston, South Africa, operated a batch fermentation process for solvents production from a cane molasses substrate. This situation occurred as a result of difficulty in importing oil due to political problems, and the ready availability of cheap cane molasses and cheap coal for raising steam (Spivey, 1978). The overall economics were good despite the considerable difficulties in effluent disposal (Gibbs, 1983). A further key parameter for the operational longevity of the fermentation process was the fact that this plant manufactured a diverse range of by-products other than solvents, including stock foods (Spivey, 1978).

Butanol is currently produced in the United States via the OXO process, involving a catalyzed reaction between propylene, carbon monoxide and hydrogen to yield aldehydes with subsequent hydrogenation to alcohols, whilst acetone is commercially produced as a by-product of phenol via the cumene route and as a derivative of isopropyl alcohol production (Volesky et al, 1981).

The possibility of using ethanol or methanol in petrol or diesel blends as automotive fuels, requiring butanol addition as a tertiary component for phase stabilization, has accentuated much of the renewed interest in this fermentation (Lenz & Moreira, 1980; Cheremisinoff,

1980; Noon, 1981; Miller et al, 1981; Cowley et al, 1982; Guibet & Vandecasteele, 1983) and has been the subject of feasibility studies in New Zealand (N.Z.E.R.D.C., 1982).

The historical and present-day uses of these chemicals and their derivatives are largely confined to the industrial chemicals (e.g. lacquers), solvent, and cosmetic areas (Prescott & Dunn, 1959). More recent uses include use as a co-surfactant/coagent in tertiary oil recovery from existing wells (Compere & Griffith, 1979; Jang et al, 1983).

Until the late 1970's, the only ABE technology that could be described was that obtained during the period 1930-1950. Previously, most ABE fermentations had used complex undefined media (starch or molasses based) in comparatively uncontrolled batch fermentations, making interpretation of growth data in meaningful physiological terms very difficult, with consequently very little being known about the regulation of solvent production. Additionally, translation of data for different media and strains is misleading. Despite the rise in oil prices it is unlikely that the economic viability of the process will be restored worldwide without concomittant process development (Gottschalk & Bahl, 1981; Bu'Lock & Bu'Lock, 1983). Two factors in particular mitigate against the ABE fermentation per se: dilute solvent streams are produced (<20 g/l) due to the toxic nature of the products, such that large quantities of water must be removed in a fractional distillation product recovery process, and the fermentation time (36-48 h) is much longer, hence productivities are lower, than routinely achieved in the ethanolic fermentation. Consequently, recent process intensification research has focussed on the use of continuous reactor systems utilizing free cells, immobilized cells, and external cell recycle, and novel, possibly more cost-efficient, processes for product recovery. Some of the product recovery processes may be integrated with the intensified fermentation processes to further increase the extent of substrate utilization, solvent concentration and productivity. Concurrent fundamental studies into the organism and the fermentation have been performed, thus allowing an insight into the factors controlling the regulation of solvent production. Additionally, mutation and/or recombinant DNA studies have been undertaken to isolate strains with

superior product formation rates and reduced sensitivity to product inhibition.

A critical aspect of the feasibility of any fermentation process is the availability and cost of a suitable fermentation substrate. Traditionally, the ABE fermentation has used either starch or molasses, but neither is particularly cheap or readily available in New Zealand, although starch-based raw materials and possibly wood hydrolysates may be attractive substrates in the future.

One such readily available substrate in New Zealand is whey obtained from the manufacture of cheese and casein in the dairy industry. Protein extraction from whey by ultrafiltration has become a relatively well-established process in New Zealand. The resulting deproteinated whey (permeate) of approximately 5-6% total solids, consists essentially of lactose (80%+ of the solids) with smaller quantities of protein, non-protein nitrogen (vitamins, etc) and mineral salts, and may be a suitable fermentation substrate (Short, 1978; Hobman, 1984). The use of whey permeate for solvent production was demonstrated by Maddox (1980), although Wix & Woodbine, (1958) had earlier mentioned the use of whey as a substrate for this fermentation. Recent, preliminary feasibility studies have been undertaken to demonstrate the process potential (Lenz & Moreira, 1980; Volesky et al, 1981; Maddox et al, 1981; Gapes, 1982; Hobman, 1984; Schoutens & Groot, 1985), however, the process feasibility is likely to be case-specific.

Few definitive studies have been undertaken however, to closely define the fermentation characteristics using whey substrate, let alone the economics of production, or the market potential for the products. The political uncertainties associated with alternative liquid fuels for New Zealand, complicates such a market analysis. At the present time however, the current domestic market for butanol and acetone (assuming a A.B.E. solvents ratio of 3:6:1) could be met by a single large fermentations factory of 1000 m^3 permeate/day operating for 240 days per annum, based on an approximate costing reported by Hobman (1984). This suggests that fermentative solvent production is not a panacea for whey lactose utilization in the New Zealand dairy industry unless some

further large markets, such as use in liquid fuels, are developed. Little information is available in New Zealand on the costs asscciated with effluent disposal from this process or the potential benefit of fermentation by-products. These factors need to be considered in possible conjunction with the concept of a "fermentations factory" having the flexibility to produce a range of fermentative products.

The objective of the work described in this thesis was to examine technological aspects of the ABE fermentation. In particular, the efficacy of intensified continuous fermentation processes for greater fermentative productivity was investigated. A key aspect of this study was the use of whey permeate as the fermentation substrate. The study is placed in the context of technological development as it relates to the dairy industry and is therefore of applied rather than fundamental nature.

In Chapter 4, the ability of two strains of C. acetobutylicum to produce solvents from whey permeate, lactose-hydrolysed whey permeate and various sugars in semi-synthetic medium was investigated in order to compare the individual sugars as substrates for the process and to choose the better strain for further work.

The objective of work described in Chapter 5 was to define the pH conditions for maximum solvent productivity and yield from the fermentation of whey permeate medium in traditional batch fermentation using freely suspended cells.

Intensified continuous fermentation processes utilizing alginate-immobilized cells (Chapter 6) or external cell recycle by cross-flow microfiltration (Chapter 7) were examined. The objective of the work described in each of these chapters was to determine those parameters that control each of the processes in order to achieve long-term stable solvent production, and to assess the relative technological merits of each intensified process.

In Chapter 7, a technological appraisal of different membrane plant configurations was conducted so that subsequent studies could be undertaken using the most suitable configuration.

Finally, Chapter 9 describes the integration of in-situ (gas-stripping) or in-line (absorbent resins or molecular sieve) butanol removal processes with batch or continuous fermentation processes. The objective was to demonstrate an increase in the attainable solvents concentration and overall fermentation productivity as a result of increased sugar consumption following toxic solvents removal.

In conclusion, information obtained in this study will enable the dairy industry to more accurately assess technical aspects of the ABE fermentation for profitable lactose utilization.

CHAPTER 2

PRODUCTION OF ACETONE : BUTANOL : ETHANOL (ABE) BY FERMENTATION

2.1 INTRODUCTION

There is an extensive body of literature on solvents production by fermentation, however, most studies examining fundamental aspects of the fermentation have been published in the past 10-15 years. Some comprehensive reviews of the process and its historical development have been published (e.g. Killifer, 1927; Gabriel, 1928; Beesch, 1952; Steel, 1958; Prescott & Dunn, 1959; Rose, 1961; Ross, 1961; Hastings, 1978; Spivey, 1978; Walton & Martin, 1979; Volesky et al, 1981; Robson & Jones, 1982; Volesky & Szczesny, 1983; Linden & Moreira, 1983; Moreira, 1983; Gibbs, 1983). These authors have adequately covered the work performed over the last 75 years, consequently no attempt will be made here to give a detailed review of the literature. Instead, this chapter will highlight those factors shown to be important in the fermentation process and which relate to this study.

2.2 HISTORY OF THE ABE FERMENTATION

Unlike the ethanol fermentation, the recorded history of the ABE fermentation does not date from antiquity, since it was not until 1862, that Louis Pasteur first discovered that butanol was a direct product of bacterial fermentation. The high cost of natural rubber at the turn of this century was the principal accelerant to develop a commercial process for the manufacture of butanol to produce butadiene for use as a raw material in synthetic rubber manufacture.

In 1910 the firm of Strange and Graham Ltd, in Liverpool, England, employed several researchers, Perkin, Weizmann, Fernbach, Schoen and others, to investigate and develop processes for producing higher alcohols (Gabriel, 1928). Fernbach subsequently isolated a culture that fermented potato starch to produce butanol and acetone. Commercial production began in 1913 based on this process. Meanwhile in 1912, Chaim Weizmann left the company and continued with his own culture isolation work. Nearly two years later, he succeeded in isolating a

strain able to ferment a variety of starches (4% w/v), and which required only simple inorganic salts such as ammonium sulphate and soluble phosphates as nutrients. Starches included corn, maize, wheat, rice and horse chestnuts. Acetone and butanol were produced at a higher yield than achievable by Fernbach's strain, with an acetone yield four times that of previous isolates (Compere & Griffith, 1979; Gibbs, 1983). This bacillus was originally named Bacillus granulobacter pectinovorum but later became known as Clostridium acetobutylicum.

The fermentation process underwent a further rapid commercialization when World War I broke out in 1914, this time to principally produce acetone for use as a gelatinizing agent for nitrocellulose in the manufacture of cordite, used in small-arms ammunition and as a propellant for heavy artillery shells, and airplane wing-dope production. Up until this time, acetone had been produced by wood pyrolysis in a retort, producing pyroligneous acid, this being neutralized with lime to give impure calcium acetate. This was then converted by heat to a mixture of calcium carbonate and acetone. Demand for acetone exceeded supply. Strange and Graham Ltd were contracted by the British Government to supply acetone and in 1916 they substituted Weizmann's process in place of Fernbach's due to its ability to ferment cheaper and more readily available maize starch and because of the higher inherent acetone production. This process became known as the Weizmann process, and fermentation plants were subsequently operated in Canada (2), India (1) and in the United States (2) (Gabriel, 1928).

Large quantities of butanol were stored in the hope that a use for it would be discovered, since initial hopes of synthetic rubber production from butanol were not fruitful. After the war, the acetone demand decreased significantly even though new uses were found (e.g., varnishes, textiles) and many plants were shut down. A use for butanol was subsequently discovered in the manufacture of nitrocellulose lacquers for the automobile industry. Butanol demand increased dramatically and plants were reopened. After expiry of the Weizmann patent in 1936, new plants were opened in the United States, Puerto Rico, and Japan (Walton & Martin, 1979).

Strains able to ferment molasses were isolated (using new isolates and existing culture collections) during the intervening period between

the World Wars. Molasses was later commercially used as the fermentation feedstock due to its lower price and greater availability. This significantly increased the profitability of the process since 6% (w/v) sugar could be fermented in this instance, instead of 3.8% (w/v) starch, giving a 60% increase in plant capacity with no capital expenditure. Further decreases in sterilization costs and labour costs per unit of product were obtained. A similar reduction in steam costs at the first and most expensive stage i.e. the stripping of dilute solvents from the fermentation medium, was obtained (Hastings, 1978). A 48 h batch fermentation was used with the solvents recovered by distillation and fractionation. Solvent yields of up to 37% by weight of the initial fermentable carbohydrate were obtained and acetone:butanol:ethanol produced in the ratio 3:6:1 was obtained in this process (Gibbs, 1983). In Britain, plants used dilute aqueous ammonia for nutrient addition, replacing the combined use of ammonium sulphate and calcium carbonate, and for pH control during the fermentation, at a measurably lower cost (Hastings, 1978). Other sources of fermentable carbohydrate were also examined including wood hydrolysates (Leonard et al, 1947; Volesky et al, 1981) and waste sulfite liquor (Wiley et al, 1941).

The gases given off during the fermentation make up 65-70% by weight of the fermented carbohydrate. Hydrogen found a market in the manufacture of synthetic methanol (Gabriel, 1928) and for hydrogenation of edible oils, whereas carbon dioxide was used for the preparation of compressed gas and dry ice. Hastings, (1978) reports that in some instances, the mixed gases were passed through activated carbon adsorption towers, to recover a small but additional quantity of solvents, prior to separation by scrubbing with water under pressure. Additionally, the spent medium after primary stripping was evaporated to give a protein-rich animal feed supplement containing appreciable amounts of riboflavin together with small quantities of other growth factors suitable for blending into stockfood (Hastings, 1978).

The fermentation process became largely uneconomic in the 1950's, not only due to a supply of comparatively cheap petroleum byproducts for chemical synthesis, but also due to the increased costs of molasses. This cost increase arose due to the competitive buying of molasses on

the open market by the farming industry, particularly in the United States, where molasses was used as a substantial component in cattle feed (Hastings, 1978).

2.3 ORGANISMS

Bacteria of the Clostridium genus carry out a fermentation with end-products such as butyric and acetic acids, ethanol, acetone or isopropanol, butanol, acetoin, hydrogen and carbon dioxide. The range of end-products formed during the fermentation of glucose for various Clostridium sp, is shown in the fermentation balance sheet given in Table 2.1 (Wood, 1961) and has been recently updated by Rogers (1986). All species produce butyric acid (butyrate) and acetic acid (acetate). Butyric acid plus hydrogen and carbon dioxide are the major end products of Clostridium butyricum, Clostridium lactoacidophilum and Clostridium perfringens with the latter species also producing ethanol. These are the so called "butyric" organisms. Clostridium acetobutylicum produces mostly acetone and butanol with smaller amounts of ethanol and acetoin. Clostridium butylicum, renamed Clostridium beijerinckii (George et al, 1983), produces butanol and isopropanol with smaller amounts of ethanol. Both these organisms produce hydrogen and carbon dioxide in addition to acids and are the so called "butylic" organisms. George et al, (1983) revealed that the strains labelled "C. butylicum", such as those listed in the American Type Culture Collection, are actually C. beijerinckii and, therefore, the species is no longer recognized, nor is it considered a synonym for an existing species. C. acetobutylicum can ferment starch, hexoses, or pentoses to solvents whereas C. beijerinckii ferments starch or hexoses to solvents.

Much of the early classification work was done by McCoy et al, (1926, 1930) and is still in a somewhat confused state because of the lack of sufficient data necessary for a thorough taxonomic study (Gottschalk et al, 1981). More recent classification work (Rousseau et al, 1971; Cummins & Johnson, 1971; Magot et al, 1983) should help alleviate this confused state, however, taxonomic studies on several butyric acid-producing clostridia (the umbrella group which includes the butyl organisms), using cell wall composition and DNA homology did not include 'C. butylicum' (C. beijerinckii) (Cummins & Johnson, 1971).

Table 2.1 End-products from the fermentation of glucose by various species of the Clostridium genus (modified from Wood, 1961).

End-Products	Moles/100 moles glucose fermented				
	C. butyricum	C. lactoacidophilum	C. perfringens	C. acetobutylicum	C. butylicum
Butyric acid	76	73	34	4	17
Acetic acid	42	28	60	14	17
Lactic acid			33		
CO_2	188	190	176	221	203
H_2	235	182	214	135	77
Ethanol			26	7	
Butanol				56	58
Acetone				22	
Acetoin				6	
Isopropanol					12
% C recovered	96	91	97	99	96
O/R Balance	0.97	1.16	1.05	1.01	1.06

Two species have been developed for solvent production, namely, Clostridium acetobutylicum McCoy, Fred, Peterson, Hastings, and Clostridium beijerinckii Donker (Smith & Hobbs, 1974). Other species have been found recently which could be potentially useful for the development of butanol and/or acetone production, such as C. aurantibutyricum (Cummins & Johnson, 1971; George et al, 1983) and the C. tetanomorphum group (Nakamura et al, 1979; Gottwald et al, 1984).

2.4 THE FERMENTATION PROCESS

2.4.1 Course of Fermentation

The growth and product fermentation profile in the ABE batch fermentation by C. acetobutylicum characteristically has two distinct phases, corresponding to a two-stage mechanism of product formation (Prescott & Dunn, 1959; Spivey, 1978). The actual values of the various fermentation parameters viz, temperature, initial pH, pH breakpoint value, final pH, products concentration as a function of time, etc, vary with the strain and medium used. Long et al, (1983) demonstrated that there were marked differences among C. acetobutylicum strains in their ability to grow, sporulate and produce solvents in various media, emphasizing the importance of strain differences, and suggesting that it is necessary to determine the optimum conditions for each strain in order to maximize solvent production. The following description of the fermentation profile is specific for C. acetobutylicum P262 on an industrial molasses medium, but is a suitable example of a typical outline of a ABE fermentation profile (Spivey, 1978; Jones et al, 1982).

In the first stage, called the acidogenic phase, active logarithmic growth occurs after a short lag phase, and butyric acid and acetic acid are produced over the period 7-18 h, with a resultant decrease in the medium pH from pH 6.8 to pH 5.1. No increase in cell numbers was observed after 18 h. The second phase, called the solventogenic phase occurs from 18 h through to 36-60 h. Solvents are detected after 18 h when the culture is in the stationary growth phase, and after the culture has reached a pH "breakpoint". Partial acid uptake is observed (Hartmanis et al, 1984) with a commensurate rise in the culture pH as solvent production proceeds. Hydrogen and carbon dioxide gases are

liberated throughout the fermentation with gas evolution being maximal during solvent production. Sugar is utilized throughout the fermentation. The solvents yield by strain P262 is about 30% (solvents produced/weight sugar utilized). However slightly higher yields have been obtained using different strains and media (Beesch, 1952; Yerulshalmi et al, 1983). Solvent productivities vary depending on the strain and medium used, however a value of 0.55 g/l.h (20 g/l solvents in 36 h) has been obtained industrially with strain P262 (Spivey, 1978; Jones et al, 1982).

The medium and conditions used for the conventional batch ABE process are also favourable for the development of lactic acid bacteria whose activity is detrimental to the process. Conventional fermentations are thus run under aseptic conditions, sometimes with a positive head gas pressure. The microorganisms are also subject to bacteriophage attack (Walton & Martin, 1979). This is often minimized by production strain rotation coupled with efficient sterilization techniques and careful stock culture maintenance. Phage-resistant cultures are available (Compere & Griffith, 1979).

2.4.2 Morphological Characteristics

C. acetobutylicum is described as a Gram-positive, anaerobic straight rod measuring 0.6-0.9 by 2.4-4.7 μm (Smith & Hobbs, 1974). The cells can be Gram-variable with age. The vegetative cells are motile with peritrichous flagella, however motility is only observed during certain stages of the fermentation. Division occurs by transverse fission resulting in chains of organisms that break apart into single cells in liquid medium during the early stages of fermentation. Subterminal ovoid spores (approximately 1 μm by 1.5 μm) are formed which usually distend the bacilli.

The morphology of the culture varies with the strain, medium and culture stage. Spivey, (1978) stated that for strain P262, distinct morphological changes can be used as a convenient index for monitoring the progress of the fermentation. Jones et al, (1982) using strain P262, correlated morphological changes of this strain on an industrial molasses medium with growth and physiological changes. Inoculated cells

were elongated rods which developed septa and resulted in the formation of long chains of phase-dark cells with sporadic and sluggish motility. After 6 h, chains broke up and highly motile vegetative rods were released. After 14-18 h, the motility decreased corresponding with a cessation of active growth. Spivey (1978) stated that there is a correlation between motility and good solvent production. If motility was absent during any time of the inoculum development procedures then low solvent concentrations were obtained.

Granulose accumulation was detected in >90% of the cells prior to the pH breakpoint (18 h) and solvent production. Distinctive, swollen, cigar-shaped clostridial forms (pre-sporulation cells) were involved in the conversion of acids to solvents, and the number of clostridial forms was directly related to the solvent concentration. Cells that did not differentiate to clostridial forms underwent degradative changes.

Sporulation mutants which did not form clostridial forms, did not produce solvents, whereas sporulation mutants blocked after the clostridial stage were able to produce normal levels of solvents. This clostridial stage may be specific to strain P262, since Long et al, (1983) did not mention clostridial forms for strains ATCC 824, ATCC 10132, NRRL 527, 592 and 593 when tested on complex glucose medium. Long et al, (1983) tested a sporulation medium for strain P262 for further studies on solvent production and sporulation. The ability to sporulate has long been recognized to be associated with solvent production (Prescott & Dunn, 1959). The ability to produce solvents in continuous culture is lost when there is a loss in the ability to form spores (Gottschal & Morris, 1981a). During the inoculum development procedure, heat shocking and successive subculture have been commonly used to destroy vegetative cells and weaker spores, and to induce spore germination. Different heat shocking procedures have been used (Appendix 6), with 75°C/2 min followed by rapid cooling, being used for strain P262 (Jones et al, 1982; Long et al, 1984a, b; Van der Westhuizen et al, 1982).

2.4.2.1 Maintenance of Cultures

Stock cultures of C. acetobutylicum are usually maintained as spores rather than vegetative cells because the former are not as

sensitive to oxygen or other environmental conditions (Prescott & Dunn, 1959; Hastings, 1978). Gutierrez (1985) has reported that culture degeneration occurs with frequent or periodic transfers, whereas storage of spores in distilled water at either 4°C or -20°C was found to be the most satisfactory technique for conserving both viability and solventogenic ability of the organism. Aspects of inoculum development, a critical aspect for a successful batch fermentation have been reviewed by Gutierrez, (1985).

2.4.3 Biochemistry of the Fermentation

Biochemical pathways depicting known and postulated metabolic steps have been published (e.g. Doelle, 1975; Thauer *et al*, 1977; Lenz & Moreira, 1980; Gottschalk & Bahl, 1981; Volesky *et al*, 1981; Volesky & Szczesny, 1983; Monot *et al*, 1983; Kim *et al*, 1984; Papoutsakis, 1984; Hartmanis & Gatenbeck, 1984; Petitdemange & Gay, 1986; Rogers, 1986). A general schematic representation of the biochemical pathway of glucose fermentation by butyric acid bacteria and the associated enzymes for the production of acids, solvents, hydrogen and carbon dioxide is given in Figure 2.1 (Papoutsakis, 1984). Possible extracellular products are shown in boxes. Not all the reactions shown in Figure 2.1 occur in all butyric acid bacteria, e.g. only *C. beijerinckii* possesses an isopropanol dehydrogenase enzyme (No.17 in Figure 2.1) able to catalyse the irreversible reduction of acetone to produce isopropanol. The biochemical routes for acetoin production (*C. acetobutylicum*) and formate production from pyruvate (see Doelle, 1975) are not shown.

Initially, the metabolism of glucose by *Clostridium sp* begins with its conversion to pyruvate via the EMP glycolytic pathway. Two molecules of ATP and two molecules of NADH + H^+ are produced and used for biosynthesis and as a hydrogen donor respectively, in the further breakdown of pyruvate. Pyruvate is oxidized to acetyl CoA with the release of carbon dioxide and hydrogen coupled with the reduction of ferredoxin. The acetyl CoA plays a central role in the metabolism in the *Clostridium sp* since it serves as a precursor to all the products.

Strict anaerobes such as the *Clostridia* control their high energy ATP production by controlling the flow of electrons in their metabolism

of substrate. The action of the hydrogenase enzyme facilitates the disposal of excess electrons in the form of molecular hydrogen (Dellweg, 1981; Kim & Zeikus, 1985) as shown by reactions 3 and 4 in Figure 2.1. The electrons are transferred to the electron carrier NAD^+, and the reoxidation of these carriers requires another molecule as an electron acceptor. The Clostridia contain a special electron carrier, ferredoxin, an iron-containing co-enzyme with a redox potential of nearly -400 mV, low enough to allow the reduction of protons to molecular hydrogen (Volesky & Szczesny, 1983). The reduced ferredoxin is oxidised by hydrogenase to yield hydrogen. In an iron-deficient environment, there is no hydrogen formation and in the absence of hydrogen evolution there is a shift to lactate production.

The ($NADH + H^+$) - ferredoxin oxidoreductase reaction (enzyme 4 in Figure 2.1) and the hydrogenase reaction (enzyme 3 in Figure 2.1) are reversible. The regulation of the ($NADH + H^+$)- ferredoxin oxidoreductases have been studied with C. acetobutylicum grown on glucose (Petitdemange et al, 1976, 1977). These researchers found that acetyl CoA is an obligatory activator of ($NADH + H^+$) -ferredoxin oxidoreductase activity, and $NADH + H^+$ a competitive inhibitor of ferredoxin - NAD^+ reductase activity. Regulation by acetyl CoA and $NADH + H^+$ allows the enzymes to function correlatively with glyceraldehyde phosphate dehydrogenase in the EMP pathway and thus regulate the amount of NAD^+ and $NADH + H^+$ in the cells, which influence the flow of electrons from ferredoxin to H_2 or butyrate. Thus, in C. acetobutylicum this activity controls electron flow; it functions in the direction of $NADH + H^+$ reduction when butanol is formed, and it functions in the direction of ferredoxin reduction when butyrate and hydrogen gas are formed (Petitdemange et al, 1976). This control is important for subsequent solvent production since $NADH + H^+$ is required during reaction steps 6 and 8 in Figure 2.1.

Considering the biochemical pathway: two acetyl CoA molecules undergo a condensation reaction to form acetoacetyl CoA with acetyl-CoA-acetyl transferase as the catalyzing enzyme (5) liberating CoA. Acetoacetyl CoA is further converted to butyryl CoA via steps 6, 7 and 8. Butyryl CoA can be converted to butyric acid by the catalytic activity of enzymes phosphotransbutyrylase (11) and butyrate kinase (12),

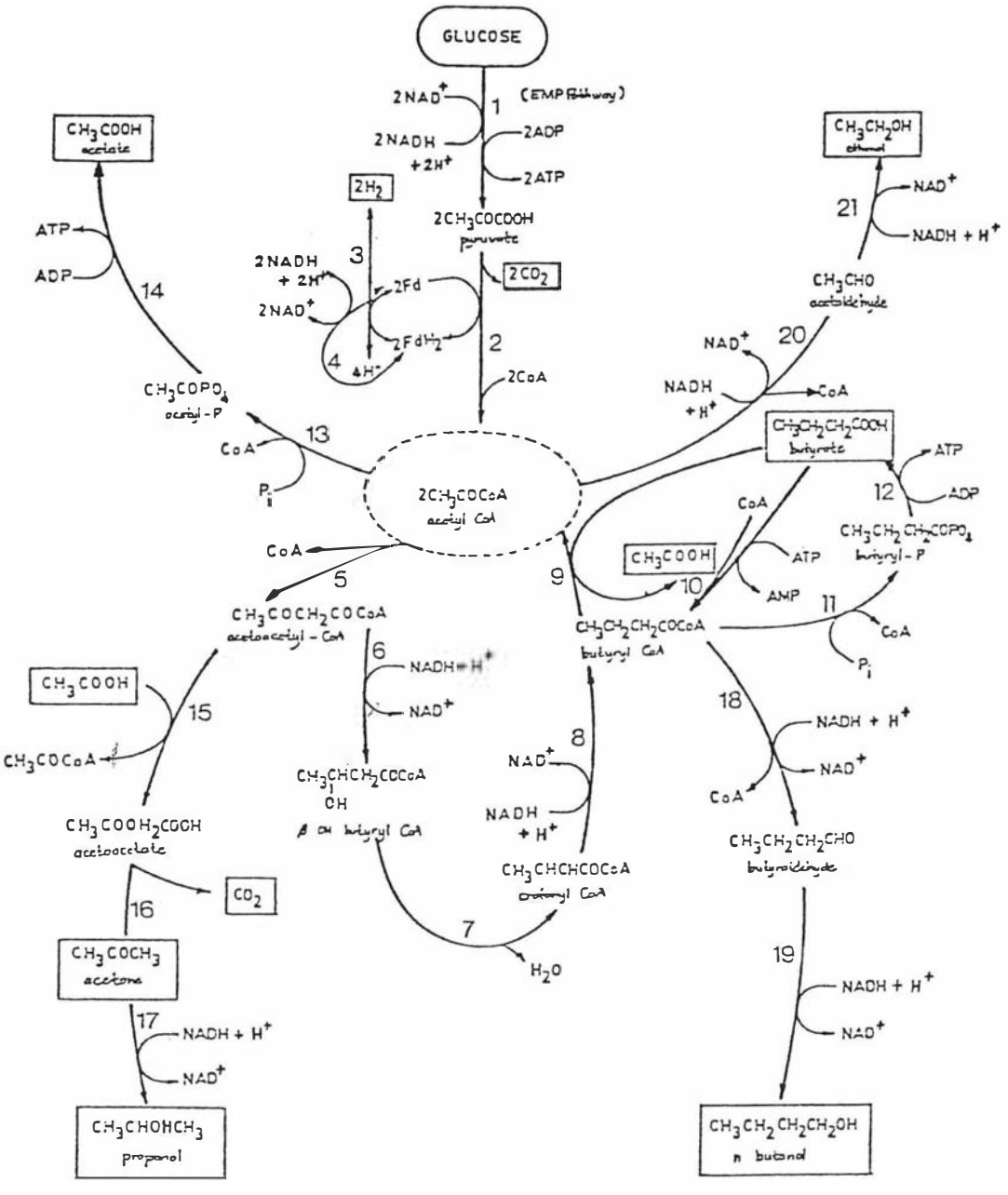


Figure 2.1. Biochemical pathways of glucose fermentation by butyric acid bacteria.

The pathways or enzyme systems catalyzing the reactions shown are as follows: (1) phosphoenolpyruvate phosphotransferase system and the Embden-Meyerhof pathway, (2) pyruvate-ferredoxin oxidoreductase, (3) hydrogenase, (4) NADH-ferredoxin oxidoreductase, (5) acetyl-CoA-acetyl transferase, (6) L(+)-β-hydroxybutyryl-CoA dehydrogenase, (7) L-3-hydroxyacyl-CoA hydrolyase, (8) butyryl-CoA dehydrogenase (9) CoA transferase, (10) butyryl-CoA synthetase (ATP/AMP), (11) phosphotransbutyrylase, (12) butyrate kinase, (13) phosphotransacetylase, (14) acetate kinase, (15) CoA transferase, (16) acetoacetate decarboxylase, (17) isopropanol dehydrogenase, (18) butyraldehyde dehydrogenase, (19) butanol dehydrogenase, (20) acetaldehyde dehydrogenase, and (21) ethanol dehydrogenase.

in C. acetobutylicum (Andersch et al, 1983). The CoA transferase reaction (9) has not been definitely established in butyric acid bacteria (Gottschalk & Bahl, 1981) or in C. acetobutylicum (Andersch et al, 1983). As the pH drops due to acid formation, acetoacetyl CoA is diverted from the normal cyclic mechanism and is utilized via a transferase system for the production of acetoacetate catalysed by the enzyme CoA transferase (15). Acetoacetate is then decarboxylated in an irreversible step to produce acetone catalyzed by the enzyme acetoacetate decarboxylase (16). Whereas this enzyme has been studied extensively, it is not known how acetoacetate is formed from acetoacetyl-CoA (Gottschalk & Bahl, 1981).

The diversion of the original cyclic system to form acetone stops further butyric acid formation. This also causes the elimination of two electron accepting reactions which generate NAD^+, namely the reduction of acetoacetyl CoA to β-OH-butyryl CoA catalyzed by the enzyme L(+)-β-hydroxybutyryl-CoA dehydrogenase(6), and the reduction of crotonyl CoA to butyryl CoA catalyzed by the enzyme butyryl-CoA dehydrogenase (8), and consequently the Clostridia must find alternative reducing sequences for NAD^+ regeneration. The only alternative reaction of this type available to the bacteria is to reverse the last step of the cycle, butyryl CoA to acetyl CoA, and reducing the butyric acid already accumulated to butanol through three consecutive reactions where butyryl CoA and butyraldehyde are formed as intermediate compounds (steps 9, 18 and 19 in Figure 2.1). The acetic acid formed in this reversal (9) is used for the generation of acetyl CoA in the acetone production.

If there is a deficiency in the amount of acetyl CoA available, then it is possible that butyryl CoA can be formed by an alternative mechanism whereby ATP and CoA are required (10) (Doelle, 1975). Also there is nothing known about the cyclic regulation at the point where butyryl CoA is no longer converted to butyryl phosphate (11) but is reduced to butyraldehyde (Gottschalk & Bahl, 1981). These authors postulate that the activity of the phosphotransbutyrylase is somehow adjusted to the metabolic fluxes for butyrate or butanol production.

The production of ethanol occurs normally together with the acetone-butanol formation by C. acetobutylicum. Ethanol production

branches off from acetyl CoA where the enzyme acetaldehyde dehydrogenase catalyzes the formation of acetaldehyde with CoA release and NADH + H^+ being oxidized to NAD^+ (20). The acetaldehyde is then reduced to ethanol catalyzed by the enzyme ethanol dehydrogenase (21) with a further molecule of NADH + H^+ oxidized to NAD^+. Hydrogen and carbon dioxide are produced throughout the fermentation in a ratio dependent on the species used (Rogers, 1986).

A number of researchers have examined the intermediary enzymes involved in acid and solvent formation (Andersch et al, 1983; George & Chen, 1983; Hartmanis & Gatenbeck, 1984; Hartmanis et al, 1984). Andersch et al, (1983) determined the levels of enzymes involved in either solvent (pH 4.3) or acid (pH 6.0) formation using C. acetobutylicum DSM 1732 fermenting a complex phosphate-limited glucose medium in a chemostat at 33-37°C and D = 0.03 h^{-1}. They found that the highest activities of phosphotransacetylase (13), acetate kinase (14), phosphotransbutyrylase (11) and butyrate kinase (12) were found in cells carrying out an acetate-butyrate fermentation, with these enzymes present in solvent-producing cells at a level of 10-50% compared to acid producing cells. These data are in accordance with the findings of Bahl et al, (1982a) who demonstrated that a change back to acid production in continuous cultures occurs instantaneously if cells are brought into a neutral medium, i.e. the pH is raised.

Butyraldehyde (18) and butanol dehydrogenase (19) were found in small amounts (0.4 and 0.6 U/mg protein respectively) exclusively in solvent-producing cells. Hydrogenase (3) activity was present in approximately the same amounts in both cell types, however in solvent-producing cells it was present only after a 10-15 min lag period. It is possible that the hydrogenase was inactive at pH 4.3 and became active under conditions of the assay since in-vitro measurements of activity demonstrated no activity below pH 6.

Hartmanis & Gatenbeck, (1984) determined enzymes involved in solvent production using C. acetobutylicum ATCC 824 and a semi-synthetic medium containing glucose. These workers made similar observations to those of Andersch et al, (1983) concerning the level of enzymes during the acid and solvent phases. They suggested that the

phosphotransbutyrylase enzyme (11), by adjusting the metabolic flux to either butyric or butanol, plays a major role in the regulatory mechanism that causes the switch from acid to solvent production as originally postulated by Gottschalk & Bahl, 1981). In summary, no definitive results have been obtained from this work that would facilitate the manipulation of enzyme synthesis to maximize solvent production.

2.4.4 Regulation of Solvent Production

Solvent regulation, that is the distinctive metabolic transition from acid production to solvents production involves the re-routing of both carbon and electron flow.

Factors which have been implicated include the culture pH (Bahl et al, 1982a; Andersch et al, 1982; Monot et al, 1983; Nisiho et al, 1983; George & Chen, 1983), where a critical low pH value (in the region pH 4-5) is achieved prior to solvent production, the concentration of acetic and butyric acids (Martin et al, 1983; Monot et al, 1984; Hartmanis et al, 1984; Fond et al, 1985), nitrogen availability (Roos et al, 1985) and the residual sugar concentration (Long et al, 1984a). Addition of acids has been found to induce solventogenesis and alter the final distribution ratio of solvents (e.g. Gottschal & Morris 1981b; Martin et al, 1983; George & Chen, 1983). Examination of reports describing chemostat cultures suggests that high growth rates are associated with acidogenesis, while solventogenesis occurs only at low growth rates (Bahl et al, 1982b; Monot & Engasser, 1983a). This is in general agreement with the concept that in batch cultures, solventogenesis is associated with a lowering of the growth rate.

Many reports have been published concerning the role of culture pH and the concentration of acetic and butyric acids in the medium, but some of these reports are conflicting. Monot et al, (1983) concluded that the regulation of C. acetobutylicum metabolism was mediated through the undissociated butyric acid concentration. At a critical undissociated butyric acid concentration of 0.25 g/l, cellular growth was inhibited with butanol production being induced at an undissociated butyric acid concentration of 1.5 g/l. Long et al, (1984a) also

suggested that inhibition of growth by these undissociated acids plays a role in the transition from acidogenesis to solventogenesis.

In contrast, other workers (e.g. George & Chen, 1983; Gottwald & Gottschalk, 1985; Terracciano & Kashket, 1986; Huang et al, 1986) have suggested that the undissociated butyric acid, because of its inert nature, does not play a role per se, rather that it is the elevated acids concentration within the cells (at low culture pH); a response of the cell to maintain a constant pH gradient. George & Chen,(1983) claim that the interference of the regulation of the internal pH and magnitude of the transmembrane proton motive force due to the accumulation of acids, may explain the slow down of general metabolism as a consequence of some feedback enzyme inhibition. Hartmanis et al, (1984) have suggested that acetic and butyric acid formed in the acidogenic phase are taken up and activated by their respective CoA thiolesters via an acetoacetyl-CoA : acetate (butyrate) - transferase. Papoutsakis,(1984) has also suggested active ATP-coupled acids uptake. Roos et al, (1985) however, suggests that there is no minimum butyric acid concentration necessary for solvent production, and that solvent production is controlled by the availability of nitrogen and the demand and availability of ATP.

There have been several reports describing the effects of agitation and head-space pressure on solvent production in batch culture. Spivey (1978) mentioned that during the commercial process a positive pressure is maintained, and this has subsequently been demonstrated to be beneficial to solvent production compared to operating at atmospheric pressure (Maddox et al, 1981; Doremus et al, 1985). The reason for the effect is believed to be linked to the retention in solution of hydrogen (and re-uptake?). Agitation during fermentation has been found to be detrimental to solvent production (Welsh & Veliky, 1984, 1986; Yerushalmi & Volesky, 1985), possibly because of stripping of hydrogen from solution. Yerushalmi et al, (1985) performed experiments using exogenous hydrogen gas to achieve high head-space pressures of hydrogen, and demonstrated increased yields of butanol and ethanol, but not acetone. Kim & Zeikus, (1985) demonstrated that the initiation of solventogenesis by C. acetobutylicum was directly related to a decrease in hydrogen production caused by the regulation of hydrogenase activity.

However further studies are required to relate this to exact biochemical mechanisms.

Several investigators have demonstrated that flushing carbon monoxide into a fermentation enhances solvent production (Kim et al, 1984; Datta & Zeikus, 1985; Meyer et al, 1986). This effect is believed to be one of the carbon monoxide inhibiting the enzyme hydrogenase which is responsible for hydrogen production and, thus, loss of reducing power from the fermentation. Following inhibition, reducing power is retained in the form of enhanced levels of reduced ferredoxin, thus making available larger amounts of $NAD(P)H_2$ for solvent production. Overall, the effects of carbon monoxide, head-space pressure and agitation are similar in that they influence the retention in the fermentation of reducing power needed for solvent production.

In summary, the regulatory mechanisms involved in the "triggering" of solvent production are complex and interrelated, and are largely unelucidated. In some instances, conclusions are conflicting, particularly in the role of acid concentration and culture pH. An understanding of the regulatory mechanism will have importance for future process development and strain improvement.

Further research is required in the area of monitoring enzyme activities at key branching points of carbon flow, at butyryl-CoA and acetoacetyl-CoA or those associated with the regulation of production or interconversion of reduction energy. This will better define the physiological changes in response to specific treatments.

2.5 ENVIRONMENTAL FACTORS AFFECTING SOLVENT PRODUCTION

2.5.1 Oxygen Sensitivity and Eh Requirement

C. acetobutylicum requires anaerobic conditions for growth, with optimal growth occurring at a negative culture redox potential, Eh, in the range -250 mV to -400 mV. Its reductive metabolism leads to the creation and maintenance of this low Eh value. Studies by O'Brien and Morris, (1971) revealed that the dissolved O_2 concentration rather than the Eh affects growth and metabolism of C. acetobutylicum. The rates of growth, glucose consumption and production of acetate, butyrate and

pyruvate remained normal in glucose and casein hydrolysate basal medium at very high Eh values but low dissolved 0_2 concentration. Growth ceased when the culture was made aerobic with 40 µM dissolved 0_2 but the same high Eh value.

2.5.2 Temperature and pH Requirement

C. acetobutylicum is a mesophilic bacterium with an optimum growth temperature of 37°C (Buchanan & Gibbins, 1974). The optimal pH for growth is 6.5, while solvent production is favoured in the region pH 4.5-5.5 (Prescott & Dunn, 1959; Spivey, 1978).

2.5.3 Carbohydrate Sources

Butyric acid-forming Clostridia species are able to utilize a very wide range of hexose and pentose sugars for solvents production. These range from complex polysaccharides (e.g. hemicellulose, cellulose) and mixed sugars (e.g. wood hydrolysates) to simple monosaccharides (e.g. glucose, fructose, xylose, arabinose, ribose) and disaccharides (e.g. sucrose, lactose). Nutrient requirements and fermentation parameters vary according to the substrate and Clostridia species used. Molasses and corn mash have traditionally been the most common carbohydrate raw materials. Research interest has turned to alternative (waste) streams as possible cheaper raw materials. These have included (apart from whey) sulphite waste liquor (Wiley et al, 1941) wood hydrolysates (Leonard et al, 1947; Mes-Hartree & Saddler, 1982; Maddox & Murray, 1983; Yu et al, 1984a, b; Volesky & Szczesny 1983; Compere et al, 1985), agricultural wastes (Ounine et al, 1983), molasses (Beesch, 1952; Spivey, 1978; Sekar, 1981; Fouad et al, 1982), potato starch (Beesch, 1952; Abou-Zeid et al, 1976), cornbroth extract (Lin & Blaschek, 1983), bagasse and rice straw (Soni et al, 1982), cellulose (Hayashida et al, 1983), cellulose using cocultures of mesophilic cellulolytic Clostridium and glycolytic Clostridium (Petitdemange et al, 1983), lignocellulose (Marchal et al, 1986) and steam treated peat (Forsberg et al, 1986).

2.5.4 Nitrogen and Other Nutrient Requirements

Biotin and p-aminobenzoic acid are necessary for the growth of some acetone-butanol organisms (Lampen & Peterson, 1941). These requirements

may be satisfied by the use of yeast extract or other raw materials containing the essential vitamins (Prescott & Dunn, 1959). Commercial nitrogen sources (e.g. corn steep liquor) supply degraded nitrogen e.g. polypeptide and amino acids. Natural organic nitrogen sources are considered the best (Baghlaf et al, 1980). The ratio of carbohydrate to nitrogen affects the solvent yield with a ratio of 5-10:1 being considered optimal (Prescott & Dunn, 1959). Monot et al, (1982) found that Mg^{2+} and K^{+} promoted growth and solvent production, respectively. Baghlaf et al, (1980) reported that on a complex medium, K_2HPO_4 is essential with an optimum concentration of 1-2 g/l.

2.5.5 Product Inhibition

The attainable products concentration in the ABE fermentation is limited by product inhibition, with butanol being the most inhibitory product (Van der Westhuizen et al, 1982; Gapes et al, 1982; Herrero, 1983; Linden & Moreira, 1983; Ingram, 1986). Typically, the maximum total solvent production concentration is 20 g/l (Spivey, 1978; Moreira et al, 1981); the starting concentration of fermentable carbohydrate is thus limited to approximately 60 g/l, assuming a solvents yield of 30%. This leads to large, capital intensive plant and high energy demands in product recovery by conventional distillation, and represents a major technological constraint on the ABE fermentation.

Biological and engineering approaches have been used to minimize the problem. Only recently have fundamental biological studies been undertaken, and the inhibitory effect of the fermentation products on cell growth rate and the kinetics of product formation have been investigated by a number of researchers (e.g. Leung & Wang, 1981; Gapes et al, 1982; Anon, 1982; Costa & Moreira, 1983; Moreira, 1983; Ounine et al, 1985). The effects of product inhibition involving fundamental mechanistic studies (Wang et al, 1979; Moreira et al, 1981; Vollherbst-Schneck et al, 1984; Bowles & Ellefson, 1985) and cell morphological studies (Van der Westhuizen et al, 1982) have also been undertaken. Other attempts to minimize the toxicity problem have centred on mutation, adaption and selection methods for tolerant strains (e.g. Wang et al, 1979; Allcock et al, 1981; Jones et al, 1982; Lin & Blaschek, 1983; Hermann et al, 1983, 1985; Largier et al, 1985) and

modification of the cytoplasmic membrane (Anon, 1982). Genetic engineering approaches also have considerable potential for strain improvement (Rogers, 1986).

Engineering approaches have focussed on the integration of continuous in-situ or in-line solvents recovery processes with batch or continuous fermentation processes (e.g. Wang et al, 1979; Groot et al, 1984a; Larsson et al, 1984; Levy, 1986). A closer examination of the literature using this approach is given in Appendix 6.

Both acids and solvents are inhibitory to the anaerobic Clostridia, although the inhibitory "target sites" may be different (Herrero, 1983). At certain concentrations of acids and solvents, cell growth stops, but some sugar continues to be degraded by the cells, whereas at higher product concentrations microbial activity ceases (Herrero, 1983). When in contact with alcohols and neutral forms of organic acids, equilibration occurs within the lipid milieu of the cell membrane, and these species can interact with cell structures, altering membrane-bound enzyme activities and the conformation of 'key functional' systems, or interfere with microbial regulatory processes (Herrero, 1983).

Costa & Moreira, (1983) found that there was a threshold concentration of products formed before growth inhibition occurred using C. acetobutylicum ATCC 824. This was followed by a linear decrease in growth rate with an increase in product concentration. This threshold concentration varied between products with butyric acid, butanol and acetic acid being approximately ten times more inhibitory towards cell growth than acetone and ethanol. A synergism amongst the fermentation products was observed indicating that the total products profile and concentration are important in determining the actual toxic effect on the growth rate. Added solvents were also considered less toxic than those produced fermentatively, (as also found by Novak et al, (1981) for ethanol inhibition of yeast), and may possibly be explained by the different product concentrations within and outside the cell.

Leung & Wang, (1981) have determined the inhibitory concentrations of the various fermentation products on the growth of C. acetobutylicum ATCC 824. These are reproduced in Table 2.2.

These authors concluded that acetone, ethanol, acetate or butyrate alone in the fermentations did not contribute significantly to the inhibition of growth. Either butanol or butanol together with butyrate in the fermentation broth during batch or continuous fermentation have detrimental effects on further microbial growth.

Costa and Moreira, (1983) found however, that the acetic and butyric acid concentrations causing 50% inhibition of growth, were half those reported by Leung & Wang, (1981) and attributed this to the different methods used to stabilize the pH of the fermentation medium during the acid challenge.

Van der Westhuizen et al, (1982) found that the vegetative cell growth rate of strain P262 in molasses fermentation medium was progressively reduced by butanol concentrations between 4-8 g/l and growth was prevented by 10 g/l butanol. Solvent-producing clostridial forms could withstand a higher butanol concentration, however an increase in degeneration occurred proportionally to the increase in butanol concentration, except at high concentrations (26-35 g/l) where the opposite effect was observed.

Ounine et al, (1985) demonstrated that butanol had an effect on the sugar uptake rate of C. acetobutylicum ATCC 824. These workers found that this strain, when grown on xylose-containing medium, is more strongly inhibited by solvent production than when grown on glucose-containing medium. This may be explained by the different sugar transport mechanisms (membrane associated) and the relative disruptive effects of butanol on the cell membrane.

Incorporation of the butanol into the cytoplasmic membrane disrupts, due to the hydrophobicity of the butanol, the membrane lipid bilayer increasing the membrane fluidity (Anon, 1982).

This bilayer consists of stabilized layers of glyceride esters of long-chain fatty esters. These molecules feature hydrophilic ends attached to the strongly hydrophobic hydrocarbon tails of the fatty acid components. These materials are aligned into bilayers of molecular thickness where only the hydrophilic ends of the assembled molecules are exposed to the surrounding aqueous medium. Alcohols are attracted to

Table 2.2 Inhibition by end-products on the growth of *C. acetobutylicum* ATCC 824 on glucose

Product	Conc. attained during fermentation (g/l)		Conc. at which growth was inhibited by 50% (g/l)
	Batch	Continuous	
Ethanol	2	1	60
Acetone	7	5	40
Acetate	3	2	15
Butyrate	3	2	14
Butanol	12	10	13
Butyrate & Butanol	15	12	15

these films because they have similar polar-non-polar imbalance along their length. As the carbon chain length increases, so does the hydrophobicity, hence the reason why butanol is more toxic than ethanol.

Vollherbst-Schneck et al, (1984) state that in response to the membrane-perturbing physical effect of butanol, cellular fatty acid synthesis in C. acetobutylicum ATCC 824 is shifted to higher saturated chain to unsaturated chain ratio, and that these changes play a role in determining the toxic effects of butanol. Modifying the cytoplasmic membrane composition by incorporation of supplemented fatty acids (oleate, elaidate) from the growth medium into the cell membrane of C. acetobutylicum ATCC 824 in order to adjust the membrane fluidity, and increase cell tolerance to higher butanol concentrations, has been reported (Anon, 1982; Linden & Moreira, 1983). Butanol tolerance (up to 22 g/l) was extended up to threefold from the control.

Bowles & Ellefson, (1985) state that the effects of butanol on strain ATCC 824 are complex, inhibiting several interrelated membrane processes. Butanol destroys the ability of the cell to maintain its internal pH, lowers the intracellular ATP concentration and inhibits substrate uptake at butanol concentrations high enough to inhibit growth. The magnitude of the butanol inhibitory effect is a function of substrate type, and culture conditions, e.g. pH and/or presence of acids. The fact that these workers found that an alteration in the cell internal pH occurred with this species is interesting, since Herrero et al, (1985) showed that in ethanol-inhibited Clostridium thermocellum, a build-up of phosphorylated sugars occurred suggesting inhibition of membrane-bound glycolytic enzymes, whilst the internal cell pH and ATP concentrations were unchanged.

Hutkins & Kashket,(1986) found that butanol exhibited a chaotropic effect that resulted in the leakage of intracellular metabolites, but butanol up to 20 g/l did not affect the phosphoenolpyruvate-dependent phosphotransferase system for glucose uptake.

Organic acids are also thought to disrupt the cell internal pH regulatory mechanism in anaerobic Clostridia (Herrero, 1983). The intracellular pH is more alkaline by approximately one pH unit compared

with the extracellular pH. Anaerobes generate ATP by sugar oxidation and use it for: (i) synthetic reactions for growth and, (ii) membrane energization via the H^+ translocating ATPase where the metabolic energy in ATP is invested in a different form of usable energy; the proton motive force.

The physiological role of the H^+-translocating ATPase in anaerobic bacteria is the generation of the transmembrane pH gradient (interior alkaline) and of an electrical potential difference (interior negative), both required (the proton motive force) for various types of transport processes and biosynthetic reactions (Riebeling & Jungerman, 1976).

Organic acids act as uncouplers and are thought to allow protons to enter the cell from the medium and thus counteract the proton pump mechanism. Unionized acid species are thought to partition in the cell membrane and the internal and external concentrations equilibrate. Since the internal pH is higher than the outside, i.e. lower internal H^+ activity, the dissociation equilibrium will proceed inside the cell resulting in a nett anion efflux, leaving behind a H^+ ion, resulting in a lowering of the ΔpH. This altered ionic environment may also inhibit enzyme kinetics. Maximum inhibition is expected at the pKa of the acid (e.g. pH 4.8 for butyric acid) and it is postulated that when the internal pH is reduced, the cell attempts to restore it by utilizing ATP via the membrane bound ATPase for membrane energization. This is to restore the proton pump with the consequence that there is less ATP for biosynthesis, i.e. acid inhibition causes an increase in cell maintenance energy requirements (Herrero, 1983). The function of the ATPase is not to "buffer" the cell at a constant intracellular pH but to form and maintain a pH gradient essential for growth.

The acid end-product inhibition mechanism in *C. thermocellum* has been examined (Herrero *et al*, 1985). The inhibitory potency of any given organic acid was found to be a function of: the partition coefficient of the acid in the undissociated form, the pKa, the ΔpH across the membrane, acid concentration and the ΔpH required by the cell for proper function. Such an inhibitory potency is likely to be different for ABE producing *Clostridia sp*.

2.6 THE USE OF WHEY FOR THE ABE FERMENTATION

Wix & Woodbine, (1958) cite literature describing the use of whey in the 1930's-1940's for solvent and/or riboflavin production by Clostridium sp. More recently, the use of various Clostridium sp and whey types for solvent production, has been described for conventional batch fermentation using free cells, and continuous fermentation using alginate-immobilized cells (e.g. Schoutens et al, 1985). A brief summary of the fermentation medium, strain, culture conditions and fermentation parameters obtained for batch fermentation studies is given in Table 2.3, whilst data for the continuous fermentation studies is presented later in this chapter. Generally, whilst whey was a suitable substrate for the growth of Clostridium sp, solvents concentration, productivity, and the extent of lactose utilization were lower than reported for industrial substrates (Beesch, 1952; Spivey, 1978). Other workers (e.g. Abou-Zeid et al, 1976; Compere & Griffith, 1979, 1984) found that lactose was generally a poorer substrate compared to glucose for solvent production, using synthetic media.

Maddox, (1980) fermented sulphuric acid casein whey permeate using C. acetobutylicum NCIB 2951. The permeate containing 53 g/l lactose was supplemented with 5 g/l yeast extract and adjusted to pH 6.5 using aqueous ammonia prior to autoclaving. A butanol concentration of 15 g/l was obtained after a 5 day incubation at 30°C. The ratio of ABE solvents obtained was 1:10:1. If the yeast extract was not added, then a poorer butanol concentration of 13 g/l was obtained after a 7 day incubation.

Moreira, (1983) makes reference to work using C. acetobutylicum (strain unidentified) fermenting cheese whey to solvents, where the butanol:acetone ratio obtained, was 12:1 to 20:1, compared with 3:1 obtained on a glucose-containing medium. Details of the work are lacking however. The shift in this ratio was attributed to a combination of unspecified factors. More recently, Bahl et al, (1986) reported a butanol:acetone ratio of approximately 100:1, obtained using various C. acetobutylicum strains and an unspecified whey type, treated by heating and centrifugation to remove the whey proteins. A butanol:acetone ratio of 2:1 was obtained on complex medium containing

Table 2.3 Summary of the literature describing solvents production by batch fermentation using whey media.

Substrate	Strain	Temp (°C)	pH (initial)	Duration (h)	Solvents (g/l)	RATIO A:B:E	Prod (g/l.h)	Y (g/g)	Lactose Utilization (%)	Ref
Sulphuric whey permeate + 5 g/l yeast extract + NH_4^+	C. acetobutylicum NCIB 2951	30	5.3	120	17.0	1:10:1	0.14	0.52	62	1
Sulphuric whey permeate + 5 g/l yeast extract + NH_4^+	C. acetobutylicum NCIB 2951	30	-	48	11.3	1.5:8:0.5	0.24[a]	-	-	2,3
Sulphuric whey permeate + 5 g/l yeast extract	C. butylicum NRRL-592	30	-	120	11.2	2.2:7.6:0.2	0.09	0.43	68	4
Cheese whey permeate + 13 g/l yeast extract paste	C. beijerinckii LMD 27.6	30	5.2	100	5.0	2:8:0[b]	0.05	0.29	43	5
Lactalbumin serum + 10 g/l yeast extract + NH_4^+	C. acetobutylicum NCIB 2951	32	6.5	111	8.4 (butanol)	-	0.09[c]	-	-	6
Acid whey, pH 6.0 using NaOH	C. acetobutylicum ATCC 824	37	5.9	120	9.2	1:13:4.4	0.08	-	50	7
Cheese whey + 5 g/l yeast extract	C. acetobutylicum ATCC 4259	37			9.4	1.7:8.3:0	0.10	0.27	-	8
	C. acetobutylicum NRRL-596	30	5.9-6.1	96	7.8	2.3:7.7:0	0.08	0.35	-	8
	C. butylicum NRRL-592	30			7.2	2.4:7.6:0	0.08	0.32	-	8

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 Hobman, (1984)

7 Welsh & Veliky, (1984)

8 Voget et al, (1985)

Notes:

a 10 l fermentation with evolved gas used to maintain a headspace pressure of 105 kPa

b isopropanol produced instead of acetone

c estimated from other data given in reference

glucose. The difference in the solvent ratios obtained on these two media, was attributed to the more favourable growth conditions in the whey medium, and in particular, the presence of lactic acid, and a growth limiting iron supply at an instrinsically optimum concentration favouring butanol production.

Welsh & Veliky, (1984) describe the batch fermentation of acid cheese whey using C. acetobutylicum ATCC 824. They obtained 9.2 g/l total solvents, 50.5% lactose utilization and a solvents yield of 23.5%, when sterilized (121°C/20 min), pH adjusted whey (pH 6.0) was fermented at 37°C for 120 h in an unagitated fermenter. Whey sterilization by autoclaving prior to fermentation and the effect of agitation (100 rpm) were shown to be important variables with respect to the solvent ratio produced and the yield obtained. Generally, agitation was detrimental to solvent production with both unsterilized and sterilized whey. These workers confirmed the detrimental effect of agitation (100 rpm) on solvent production using the same strain and semi-synthetic medium containing lactose (Welsh & Veliky, 1986).

Gapes et al, (1982) used the same organism and conditions as used by Maddox, (1980) when conducting larger scale batch experiments. They found that the maintenance of a positive head-space during a batch fermentation was necessary to obtain 1.7 g/l, 9.0 g/l and 0.6 g/l of the ABE products respectively after a 2 day incubation. A pressure rise up to 105 kPa occurred, due to carbon dioxide and hydrogen gas release. When the pressure was not maintained the production rates were much lower.

Schoutens et al, (1984) investigated the IBE (isopropanol:butanol: ethanol) fermentation using whey permeate with C. beijerinckii LMD 27.6 in batch cultures. Synthetic media containing glucose, galactose, lactose and mixtures of glucose and galactose were also investigated. Variations in medium composition, carbohydrate concentration, inoculum pretreatment, pH, and incubation temperature were investigated. Heat shocked spore suspensions were used as the inoculum. Two series of batch experiments were done, the first on a one litre scale where the pH was regulated and the second on a 200-ml scale where no pH regulation was done.

From the first series of experiments, the butanol concentration produced from whey permeate, and the amount of carbohydrate (lactose) fermented, was greater at 30°C than at 37°C under comparable conditions. Variations in the media pH, yeast extract concentration and inoculum pretreatment did not lead to butanol production at 37°C. Butanol and isopropanol concentrations were 4.0 g/l and 1.0 g/l respectively, in 50 g/l whey permeate supplemented with 13 g/l yeast extract (initial pH 5.2), fermented at 30°C for 100 h. More products were produced as the carbohydrate concentration was increased but poorer percentage utilization resulted.

The overall product yields (g butanol + g isopropanol/g carbohydrate utilized) were comparable between whey permeate and glucose medium but the batch reactor mean productivity (g/l.h), including the lag phase, was two to three times lower for permeate (lactose) than for glucose. The lag phase was longer and substrate conversion rate slower for the permeate fermentations.

In the second series of experiments, mixtures of glucose and galactose were used to investigate the possibility of fermenting lactose-hydrolysed permeate.

Whilst galactose alone could be used for solvent production, in mixtures of glucose and galactose the two sugars were used simultaneously with a preference shown for glucose.

2.7 CONTINUOUS FERMENTATION USING FREE CELLS

In an effort to improve fermentation productivities with the objective of reducing fermentation costs, continuous production of solvents has received attention. Continuous production of solvents in serially connected fermenters has been reported (Wheeler & Goodale, 1932) and has been operated commercially in the Soviet Union (Yarovenko, 1962; Hospodka, 1966). Dyr et al, (1958) describe the use of a 5-stage system where continuous solvents production was possible for 18 days. The productivity was three times higher when compared to batch fermentations, however solvent yields were low due to high acid production in the initial stages. Yarovenko, (1962) reported a 20% productivity improvement during continuous fermentation using a serial

cascade of 11 pilot scale fermenters over a 90 h period. Hospodka, (1966) reported that a commercial plant in Dokshukin was equipped with three batteries of 7 or 8 fermenters (220 or 270 m^3) fermenting a mixture of molasses, flour and a pentose-containing hydrolysate. The duration of the continuous cycle was 40 to 90 h and was limited by the spread of infection (Lactobacillus). Unfortunately, the exact fermentation conditions and performance for each of these continuous processes were not reported.

More recently, continuous fermentation studies (chemostat and continuous flow) have been undertaken to determine a process with improved productivity, feasible for possible commercial exploitation, and to examine the following fundamental areas of the fermentation itself:

(1) The regulatory biochemical mechanism and kinetics of solvent production.

(2) The effect of different nutrients and metabolites on microbial physiology and activity, since at steady-state a constant environment is obtained, e.g. the interaction of culture pH, temperature, substrate and nitrogen concentrations, acid intermediates, dilution rate (D), etc.

(3) Maximum conversion yields, attainable solvent concentrations, productivity and the duration of continuous operation. The duration of operation is important because repeated transfer of growing cultures, reducing the subsequent solventogenic culture performance (degeneration), has been reported (Kutzenok & Aschner, 1952; Finn & Nowrey, 1955) and may occur in continuous solventogenic cultures over extended operational periods.

(4) The isolation of improved strains (Meinecke et al, 1984; Leeme & Frankiewicz, 1983).

Several investigators have used chemostat cultures with various types of nutrient limitation, for example nitrogen limitation (Gottschal & Morris, 1981a; Andersch et al, 1982; Monot & Engasser, 1983a; Jobses & Roels, 1983; Stephens et al, 1985), glucose limitation (Bahl et al,

1982a; Jobses & Roels, 1983; Stephens et al, 1985; Roos et al, 1985; Monot et al, 1983) and phosphate limitation (Bahl et al, 1982b; Stephens et al, 1985).

A summary of the fermentation and performance of various nutrient limited chemostats is given in Table 2.4. Direct comparison of results is difficult and results are often conflicting due to differences in the strains used, medium composition and fermentation conditions (Roos et al, 1985). Generally, in glucose and nitrogen-limited chemostats, steady-states were difficult to obtain and the direct application of these findings to commercial processes is limited.

Bahl et al, (1982b) investigated the continuous production of acetone and butanol by C. acetobutylicum DSM 1731 using a two-stage phosphate-limited chemostat. In the first stage (D = 0.125 h^{-1}, pH 4.3, 37°C) cells grew actively, producing solvents and acids, and in the second stage (D = 0.04 h^{-1}, 33°C) since phosphate was almost exhausted and growth no longer possible, solvents production was facilitated utilizing the remaining substrate. The low dilution rate (0.03 - 0.04 h^{-1}) in Stage II, necessary to ensure maximal substrate utilization and hence high solvent concentrations, limits the overall attainable solvents productivity to the order of that obtainable in conventional batch fermentations. Additionally, the cost of removal of excess phosphate by precipitation from commercially-used media needs to be considered. Whilst in principle this operation is promising (Anon, 1982), the economics remain to be demonstrated and may be strain, and hence carbohydrate specific since Stephens et al, (1985) were unable to reproduce these results using C. acetobutylicum NCIB 8052 (= ATCC 824). Meinecke et al, (1984) subsequently demonstrated that an asporogenous mutant strain of C. acetobutylicum was selected during chemostat operation under phosphate limitation (Bahl et al, 1982b), since if sporulation is connected with the onset of solvent production, then theoretically a steady-state condition could not be obtained. Bahl & Gottschalk, (1984) also reported that continuous solvents production is possible under sulphate limitation but not magnesium limitation.

Marlat & Datta (1986) describe a combined continuous-batch process for solvents production from corn. An improved asporogenic C.

Table 2.4 Summary of results from studies investigating continuous production of solvents in chemostat or continuous flow fermentation processes

Fermentation process - description	Substrate/ concentration (g/l)	Organism	Temp (°C)	pH	Dilution rate (h^{-1})	[Solvents] (g/l)	Prod.* (g/l.h)	Yield (g/g)	Comments	Ref.
Nutrient Limitation										
Nitrogen (15.15mM NH_4)	Glucose/54	*C.acetobutylicum* DSM 1731	37	5.2	0.217	4—5	0.95	—	Acids produced at pH 6.0, but solvents produced in the pH range 4.3—5.4. Nitrogen limitation chemostat considered unsuitable for commercial operation.	1
Nitrogen (0.18 g/l ammonium acetate)	Glucose/45.5	*C.acetobutylicum* ATCC 824	35	5.0	0.038	8	0.31	0.29	Solvent production obtained at low dilution rates.	2
Nitrogen & glucose (0.4 g/l ammonium chloride)	Glucose/2.7	*C.acetobutylicum* NCIB 8052	35	5.7	—	v.low	—	—	No solvent production in glucose & nitrogen limited chemostat over the pH range 5.0—6.5.	3
Nitrogen or glucose (1.98mM nitrogen or 1.8 g/l glucose)	Glucose	*C.beijerinckii* LMD27.6	37	4.5	0.075	<1	0.06	0.14	No solvent production under glucose limitation, acid production favoured. Solvent production under nitrogen limitation is ultimately lost.	4
Phosphate (0.74mM) (Two-stage chemostat)	Glucose/54	*C.acetobutylicum* DSM 1731	Stage I 37 Stage II 33	4.3	Stage I 0.125 Stage II 0.03	18.2	0.55	0.34	Successful chemostat production of solvents using phosphate as the growth limiting nutrient. Almost complete substrate utilization achieved by use of Stage II. Duration of continuous operation not reported.	5
Nitrogen, glucose, phosphate, magnesium Turbidostat & pH auxostat operation	Glucose/various	*C.acetobutylicum* NCIB 8052	35	5.0—5.5	—	—	—	—	Long term solvent production (*i.e.* >150—250 h) was not obtained under ammonium, glucose phosphate or magnesium limitation. pH auxostat and turbidostat cultures (nutrient excess and high biomass) were used for continuous solvent production up to 500 h, but a cyclic acid/solvent production pattern was observed.	6
Nitrogen or glucose	Glucose/various	*C.acetobutylicum* ATCC 824	37	controlled	—	—	—	—	Acids predominant under glucose limitation. Solvents produced under nitrogen limitation - availability of nitrogen affected by culture pH and controls solvent production.	7
Substrate excess										
Synthetic medium	Glucose	*C.acetobutylicum* ATCC 824	35	4.8	0.03	12	0.40	0.30	Complete glucose utilization at $D = 0.03\ h^{-1}$. Biomass = 2.0 g l^{-1} at this dilution rate. As D is increased, the residual glucose increases and biomass and solvent concentrations decrease.	8
Synthetic medium	Glucose/50—100	*C.acetobutylicum* ATCC 824	37	5.0	0.22	11.4	2.5	0.32	Biomass of 4.5—5 g l^{-1} obtained at $D < 0.2\ h^{-1}$. Maximum specific solvent production obtained at $D = 0.22\ h^{-1}$. End products vary as D varies.	9

Table 2.4 (continued) Summary of results from studies investigating continuous production of solvents in chemostat or continuous flow fermentation processes

Fermentation process - description	Substrate/ concentration (g/l)	Organism	Temp (°C)	pH	Dilution rate (h^{-1})	[Solvents] (g/l)	Prod.* (g/l.h)	Yield (g/g)	Comments	Ref.
Synthetic medium	Glucose/40	*C.acetobutylicum* ATCC 824	35	4.8	0.06	13	0.78	0.33	A butanol concentation of > 9 g l^{-1} must not be reached otherwise a rapid decline in fermentation performance results. This determines the D and substrate concentration that can be used. Biomass = 5 g l^{-1} at D = 0.05 h^{-1}. Operation for 1400 h reported.	10
Turbidostat	Glucose/20	*C.acetobutylicum* NCIB 8052	35	5.0	varies	3.7 at 0.96g-l^{-1} biomass	—	—	Turbidostat facilitates cell density control *i.e.* growth rate. At a cell density of <0.5 g l^{-1} acids predominated, and at a cell density of 0.96 g l^{-1} solvents predominated.	11

*Productivity

References

1 Andersch et al, (1982)
2 Monot & Engasser, (1983a)
3 Gottschal & Morris, (1981a)
4 Jobses & Roels, (1983)
5 Bahl et al, (1982b)
6 Stephens et al, (1985)
7 Roos et al, (1985)
8 Monot & Engasser, (1983b)
9 Leung & Wang, (1981)
10 Fick et al, (1985)
11 Gottschal & Morris, (1982)

acetobutylicum strain (parent strain C. acetobutylicum ATCC 4259) is used. A dilution rate of 0.05 h^{-1} is used in the continuous fermenters followed by a 30 h batch holding period to maximize solvent production. The process effluent contains 23 g/l solvents. A solvent productivity of 1.3-1.5 g/l.h is claimed in this combined continuous-batch process compared with the batch process solvent productivity of 0.7-0.9 g/l.h using the parent strain. The duration of continuous operation was not reported.

Recently, continuous fermentation studies using synthetic medium (nitrogen and/or glucose excess) have been reported (Table 2.4). Similar solvent concentrations have been obtained using the same organism and similar glucose concentrations (Monot & Engasser, 1983b; Leung & Wang, 1981; Fick et al, 1985). However, Leung & Wang, (1981) obtained a higher solvent productivity (higher dilution rate) which can be attributed to a superior biomass concentration. Complete substrate utilization and improved solvents productivity compared with those obtained in batch fermentations were reported. Generally, high solvent concentrations are obtainable at low values of D (<0.05 h^{-1}). Whilst at higher D values an improved productivity may be obtained, this is at the sacrifice of reduced substrate utilization and solvent concentration, the latter being particularly important since greatly increased downstream processing costs result.

2.8 CONTINUOUS FERMENTATION USING IMMOBILIZED CELLS

2.8.1 Introduction

Immobilization of whole microbial cells (sometimes called biocatalysts) involves a technique that confines cells within a reactor system, permitting their easy and hopefully economic re-use. A very wide range of cell immobilization methods (paralleling those used for enzymes) are available. A plethora of literature reviews, covering the methodology, applications and definitions of types of immobilized cells, are available and more recent notable examples include: Vojtisek & Jirku, (1983); Bucke, (1983); Klein, (1984); Rosevear, (1984); Lilly, (1986). The advantages of using immobilized cells in fermentation processes can include the following:

(1) An increase in cell density per unit reactor volume can be achieved, facilitating increased productivities.

(2) Continuous processing for extended periods is possible.

(3) Cell wash-out does not occur at dilution rates greater than the maximum specific growth rate. However, operation at dilution rates greater than the maximum specific growth rate of contaminating species (non-immobilized) may make the use of unsterilized feed possible.

(4) Growing or non-growing cells can be used. In the latter instance, higher product yields may be possible with cell metabolism rejuvenated at periodic intervals by feeding a nutrient medium.

(5) It is possible that modified intrinsic behaviour can be used for greater specific productivity.

The disadvantages of using immobilized cells in fermentation processes can include the following:

(1) Immobilization may result in some undesirable loss of enzyme or metabolic activity, hence, the choice of immobilization method is critical.

(2) Increased diffusional resistance due to the immobilization carrier may occur, i.e. some cells may not have substrate access. Additionally there may be a build up of inhibitory product.

(3) There may be difficulty in maintaining cells in the optimal physiological state for product synthesis.

(4) Mutation and/or loss of activity may occur with time.

Studies into the use of biocatalyst-based fermentations also include an examination of reaction engineering parameters (Karel et al, 1985), mass transfer effects (Radovich, 1985) and reactor design (Rosevear, 1984; Schoutens, 1986).

2.8.2 Cell Immobilization Methods

The scheme depicted in Figure 2.2 is a simple classification based on the principal mechanism involved. Whole cell immobilization is not a novel concept, rather a refinement of an existing phenomenon observed in nature. Under natural conditions certain microbes have the capacity to grow as films, facilitated by ionic attractive adsorption or excretion of an exopolysaccharide, on a wide variety of biotic and abiotic supports (e.g. sand grains, teeth, intestinal villi, mineral and metal surfaces). In certain classical fermentations and waste water treatment operations e.g. the Schuetzenbach "Quick" vinegar and trickling filter processes, respectively, the microbes are purposely provided with a surface for growth and hence process enhancement (Jack & Zajic, 1977; Messing, 1980).

2.8.2.1 Bonding

Adsorption is a simple, mild process. Various natural or synthetic particles can be used as carriers for microbial cell attachment (Kolot, 198la,b). Cell binding and possible detachment at some later stage, is affected by electrostatic interaction between the cell wall and the carrier, solution pH, ionic strength, competing minerals and fluid flow (Jack & Zajic, 1979). A novel application of this process is the use of Biomass Support Particles (BSP) as carriers for microbial attachment (e.g. Atkinson et al, 1980, 1982; Black et al, 1984). Two types of BSP are favoured, either knitted mesh stainless steel spheres or polyester foams of variable size, used in a fluidized bed fermenter and in a circulating bed fermenter respectively (Black et al, 1984). Much of this work has been concerned with yeast.

In covalent bonding, cells are mixed with chemically preactivated or modified carrier particles containing chemical groups having an affinity towards groups on the cell surface. Alternatively, physical sorption of cells on a carrier may be followed by covalent cross-linking e.g. using gluteraldehyde. Compared with adsorption, covalent coupling is a severe technique with losses in cell viability resulting from the coupling agent action making it unsuitable for fermentation processes (Bucke, 1983). Coupling agents that have been investigated include:

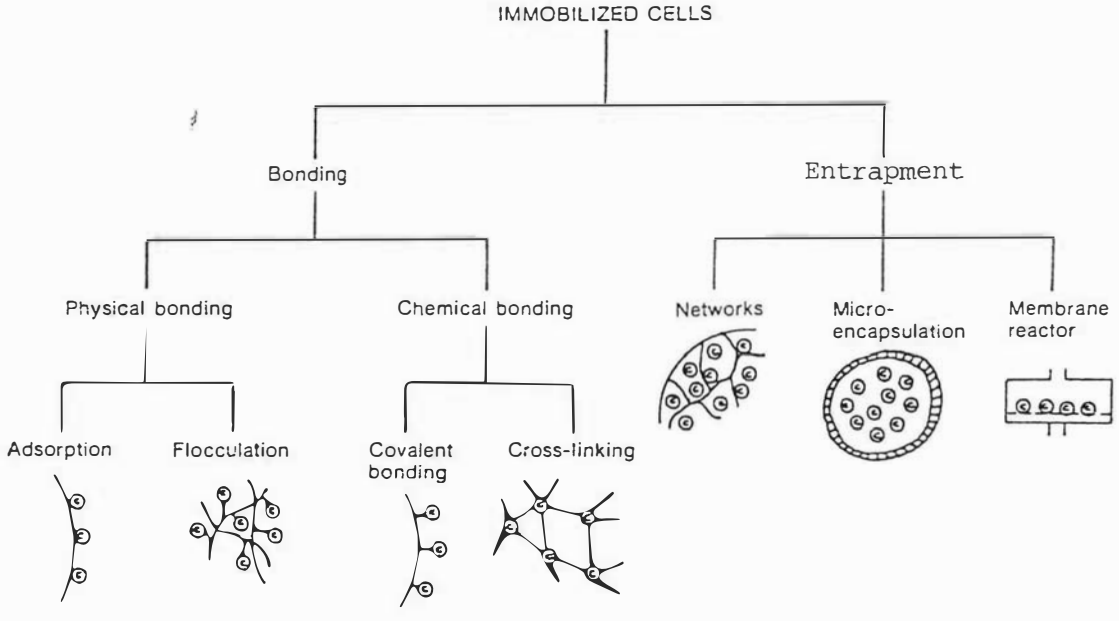


Figure 2.2. Methods for whole cell immobilization.

isocyanate, amino-silane, gluteraldehyde and carbodiimide (Kolot, 1981a).

2.8.2.2 Entrapment

Entrapment has been the most widely studied method for cell immobilization. Microbial cells are physico-mechanically entrapped directly into polymeric porous networks or matrices. The technique used varies with the selection of the polymer, its properties (e.g. solubility) and the mechanism of immobilization. The resulting biocatalyst can vary in shape, size and density. Usually spherical particles (2-5 mm diameter) are used although sheets can be prepared. The following matrices have been investigated: agar, alginate, carrageenan, cellulose and its derivatives, chitosan, collagen, gelatin, epoxy resins, nylon prepolymers (photocrosslinkable and urethane), polyacrylamide and polyester.

Using water-insoluble polymers involves mixing a cell paste with a water-insoluble polymer which is solubilized in organic solvent, followed by precipitation of the mixture in another organic solvent, water or in air. Cell microencapsulation is an example of this technique but the resulting biocatalysts are probably too fragile to be used industrially (Cheetham, 1980).

Collagen is a natural hydrophilic fibrous protein that swells in water. Cells can be entrapped within a collagen network by the formation of multiple ionic interactions, hydrogen bonds and Van der Waals interactions between the cell and the collagen which collectively form a stable network (Venkatsubramanian, 1980). Thin membrane sheets can be prepared and can be strengthened using a gluteraldehyde crosslinking treatment.

The use of polyacrylamide for cell entrapment in gels, was the first entrapment method studied intensively (Durand & Navarro, 1978; Chibata et al, 1979; Rosevear, 1982). An aqueous solution of acrylamide monomers and cells is prepared. The acrylamide is then polymerized by a free-radical process in which linear chains of polyacrylamide are built. A bifunctional reagent (e.g. N-N-methylenebisacrylamide) which has unsaturated bonds susceptible to inclusion in the polymer, effects

cross-links between the polymer chains. Factors affecting gel preparation include: the content of acrylamide, the ratio of cells to acrylamide and acrylamide to bifunctional cross-linking agent. Polymerization must be carried out at temperatures of 0 to 10°C to minimize enzyme inactivation. This method is severely disadvantaged due to the toxic effect of the acrylamide and the polymerization activator on enzyme activity and cell viability in the resulting biocatalyst.

Carrageenan is a polysaccharide isolated from seaweed, consisting of β-D-galactose sulphate and 3,6 anhydro-α-galactose units. It will form a gel under mild conditions with the addition of gel-inducing agents such as Ca^{2+}, Mg^{2+}, Fe^{3+}, K^{+}, NH_4^{+} ions or amines, and forms a suitable polymeric matrix for cell immobilization (Tosa et al, 1979; Wada et al, 1979). The method is simple and does not interfere with microbial metabolism. Typically K-carrageenan (0.5-2.0 g/l) is dissolved in physiological saline and warmed to 37 to 50°C. A microbial suspension is also warmed to this temperature and mixed in. The mixture is cooled and contacted with an aqueous solution containing the gel inducing agent. The gel particle can be cross-linked and hardened with agents such as gluteraldehyde, tannin, hexamethylenediamine, isothiocyanate and carboiimide (Klein & Wagner, 1978).

2.8.3 Comparison of Cell Immobilization Methods

A simplified comparison of adsorption and entrapment cell immobilization methods is given in Table 2.5. Entrapment and adsorption have been the most popular methods reported, are simple, comparatively cheap, non-toxic, with the former method facilitating long-term stable operation, and are considered to be of greatest technological interest (Vorlop & Klein, 1983; Klein, 1984; Rosevear, 1984). Vojtisek & Jirku, (1983) have presented various criteria by which biocatalysts can be evaluated and these include biochemical, physico-chemical, technological and economic criteria.

2.8.4 Cell Entrapment in Alginate Gel

The use of alginate for cell immobilization is probably the most well documented entrapment method. Alginic acid is a copolymer of β-D-mannuronic (M) and α-L-guluronic (G) acids joined by 1-4 linkages and composed of M blocks, G blocks and MG blocks (Vorlop & Klein, 1983).

Table 2.5 A summary of the advantages and disadvantages of some microbial cell immobilization methods

Method	Advantages	Disadvantages
Bonding		
(a) Adsorption	Mild, easy, viable cells result. Wide range of carriers available.	Temporary immobilization. Excessive cell growth may cause reactor blocking.
(b) Covalent	Permanent. Wide range of carriers available.	Harsh technique. Some or total loss of cell viability. May not be suitable for growing cells.
Entrapment		
(a) Polyacrylamide and other artificial polymers.	Wide range of gel properties can be obtained. Cell division possible within the beads.	Essential enzymes may be destroyed by the monomer and polymerization activator. Hazardous chemicals use. Limited cell loading.
(b) K-carrageenan	Food grade material. Cell division occurs readily. Extended periods of cell activity obtained. Gel can be easily reversed.	Crude carrageenan unacceptable. Need to use K-carrageenan since λ form reduces gel strength and increases solution viscosity. Difficulty in scaling-up production.
(c) Alginate	Food grade material. Cell division occurs readily. Extended periods of cell activity obtained, gel can be easily reversed, large scale production demonstrated. Fundamental studies available.	Gels are destroyed by chelating agents.
(d) Cellulose acetate	Extended periods of cell activity obtained.	Cells contact organic solvents, limited possibility of cell division.
(e) Collagen	Useful in membrane form.	Cross-linking needed for strengthening. May be toxic.

$$-(M - M)_n-(G - G)_n-(M - G)_n-$$

The acids are extracted from brown seaweeds. The ratio of the two acids varies between different seaweed species, with alginic acids from Laminaria sp having a high α-L-guluronic acid content, whilst those from Macrocystis and Ascophyllum sp have a high β-D-mannuronic acid content. Gels of alginic acids are formed when divalent ions (e.g. calcium ions) crosslink α-L-guluronic acid units of different molecules, hence alginic acids from Laminaria sp are preferred for cell entrapment unless some gel property other than strength e.g. pore size, is required (Bucke, 1983).

Typically, cell preparations (vegetative cells or spores) are stirred into solutions of sodium alginate (20-80 g/l) and the mixture is extruded drop-wise into a solution containing multivalent counterions such as Ca^{2+}, Co^{2+}, Ba^{2+}, Al^{3+}, where beads are formed (0.1 to 4 mm in diameter, varying with the extrusion nozzle dimensions, etc) by the mechanism of ionotropic gelation. The beads can be hardened by holding for a period to ensure complete ion exchange.

Alginate beads are unstable in media containing phosphate ions or other calcium chelating agents since they disrupt the gel by destabilizing the bound calcium. The use of added calcium ions (usually as $CaCl_2$) to the culture medium has been shown to be effective in preserving bead integrity, and at a concentration of 5 g/l (as $CaCl_2.2H_2O$) to be non-toxic to various solvent-producing Clostridium sp (Krouwel, 1982; Largier et al, 1985). Strontium and barium alginates are more stable to chelating agents and have been used successfully to entrap cells (Paul & Vignais, 1980). Aluminium alginate has also been successfully used (Klein & Wagner, 1978). Alginate gels can be coated with cationic polymers such as polyethyleneimine, polypropyleneimine and polyvinylamine to stabilize them against destruction by chelating agents (Birnbaum et al, 1981; Veliky & Williams, 1981).

The characterization, stability, utility and application of alginate biocatalysts has been fully documented by a number of authors (Kierstan & Bucke, 1977; Buchholz, 1979; Cheetham, 1980; Klein & Wagner, 1978; Birnbaum et al, 1981; Veliky & Williams, 1981; Bucke, 1983; Vorlop

& Klein, 1983; Klein et al, 1983; Klein, 1984; Tanaka et al, 1984; and Hannoun & Stephanopoulos, 1986). Manipulation of the alginate source, and choice of the polymer/counter-ion systems and their concentrations, can give biocatalysts of different spherical size and porosity, or in the form of flat or spiral-wound sheets (Rosevear, 1984). Klein (1984) has stated that high cell loading is possible in calcium alginate beads e.g. up to 30% wet biomass on a weight basis, and claims that partial air drying of the bead can lead to cell densities of up to 100%, calculated on a basis of initially immobilized wet weight.

Alginate-immobilized yeasts have been successfully used on a pilot scale for ethanol production using fluidized column reactors of 4 m^3 total volume (Nagashima et al, 1983, 1984). Alginate entrapment was preferred over carrageenan entrapment despite comparable fermentation activity, due to the former method's ease of large scale preparation and operation (Nagashima et al, 1983). Large scale production of alginate biocatalysts using a resonance nozzle is described by Tramper, (1985) and Hulst et al, (1985).

The use of alginate biocatalysts for the fermentative production of ethanol (e.g. Luong & Tseng, 1984; Linko et al, 1983; Klein & Kressdorf, 1983; Marwaha & Kennedy, 1984; Linko et al, 1981; Lee et al, 1983) lactic acid (e.g. Stenroos et al, 1982), citric acid (e.g. Vaija et al, 1982) and 2,3-butanediol (Lee & Maddox, 1986) has been described.

2.8.5 Continuous Solvents Production using Biocatalysts

Immobilization of vegetative cells and spores (subsequently germinated) of C. acetobutylicum ATCC 824 in calcium alginate, for continuous solvent production using a glucose medium has been described (Haggstrom & Molin, 1980; Haggstrom & Enfors, 1982; Forberg et al, 1983). Both spherical beads and a spiral-wound flat sheet have been used. The use of non-growing cells was investigated; the consideration here is that a higher product yield could be obtained since minimal substrate would be utilized for biomass production.

Pulse-wise nutrient dosing was demonstrated to be a suitable means of preserving cell productivity. However, low solvent concentrations (approximately 2-3 g/l) were obtained. Forberg & Haggstrom, (1985)

described the adsorption of C. acetobutylicum ATCC 824 to beechwood shavings for continuous solvents production (non-growing active state, nutrient dosing technique) from a glucose medium. Operational times in excess of 700 h were reported with a maximum productivity of 1.2 g/l.h and a solvent yield of 0.30-0.32 g/g.

Continuous solvent production from glucose medium using alginate immobilized spores (subsequently germinated) of C. beijerinckii LMD 27.6 has been demonstrated using a packed bed and continuous stirred tank reactor (Krouwel et al, 1983a, b). Productivity improvements of 6 times, at 100% sugar utilization, and 16 times, at 80% sugar utilization, were obtained with these biocatalysts when compared with batch fermentations. More recently a comparative study of a fluidized bed reactor and gas lift loop reactor for continuous solvents production using alginate entrapped Clostridium sp has been reported (Schoutens, 1986). The use of alginate biocatalysts for solvent production using whey permeate is also documented (Schoutens et al, 1985).

The fermentation conditions and performance of immobilized cells for continuous solvents production from various studies is summarized in Table 2.6. Generally, significant productivity increases have been obtained but substrate utilization and solvents concentration are low. Butanol toxicity has been shown to limit the substrate utilization, and has been shown to be more severe in whey permeate-based fermentations than glucose-based fermentations (Schoutens & Kossen, 1986). Recently, Frick & Schugerl, (1986) demonstrated that an alginate immobilization matrix was more suitable for continuous solvents production from glucose-containing medium and C. acetobutylicum ATCC 824, than a carrageenan or chitosan matrix.

Significantly higher solvent concentrations have been reported using an immobilized C. acetobutylicum P262 mutant fermenting a molasses medium (Largier et al, 1985). From such studies it is clear that strain improvement will play an increasingly important role in the development of a fermentation process. Mutation and/or recombinant DNA studies will hopefully lead to isolation of strains with superior product formation rates and reduced sensitivity to product inhibition. Alternatively the development of processes for in-situ butanol recovery (e.g.

Table 2.6 Comparison of productivities of various immobilized cell processes for continuous solvents production from synthetic and technical media

Substrate	Organism	Immobilization method	Reactor	Temp. (°C)	Dilution rate (h^{-1})	Solvent concentration (g/l)	Prod. (g/l.h)	Yield (g/g)	Comments	Ref.
Glucose (synthetic medium)	*C.acetobutylicum* ATCC 824	Alginate entrapment	Batch	35	—	3.69 (butanol	—	0.17 (butanol)	Similar fermentation patterns to free cells	1
		Alginate entrapment	PB	35	0.055	2.05 (butanol)	—	—	Immobilized cells - non-growth system. Beads treated with 1 g l^{-1} gluteraldehyde to improve their mechanical strength.	2
		Alginate entrapment	Cast on wire netting. Concentric cylinder reactor	37	0.4	1.5—1.8	0.7 (butanol)	0.20 (butanol)	Non-growing viable cells. Intermittant nutrient dosing used to maintain constant production. Biomass yield reduced and butanol yield coefficient increased from 0.11 - 0.20 with this technique compared with growth conditions.	3
		Adhesion	Adhesion to beechwood shavings arranged as parallel sheets	37	0.18	5—6	1.1—1.5	0.30—0.32	Non-growth conditions. Intermittant nutrient dosing. Low cell leakage. 1000 h operation.	4
	C.beijerinckii LMD 27.6	Alginate entrapment of spores (beads—2mm)	PB	37	various	7—8 (maximum)	0.6-0.8	0.30—0.34	Fermentation activity coupled to growth. Immobilized spore germination using 50% ethanol treatment. Packed bed reactor unsuitable due to poor degassing, channelling, pH gradients.	5,6
		Alginate entrapment of spores (beads—2mm)	CSTR $(1-\epsilon) = 0.36$	37	0.72	—	4.0	0.37	80% substrate conversion at $D = 0.72$. Biocatalyst reuse demonstrated. Mathematical model developed and tested.	7
		Alginate entrapment of spores (beads—2mm)	CSTR/FCR $(1-\epsilon)$ up to 0.5	37	various	6—7 (maximum)	various	0.22 (butanol)	Kinetic model developed describing solvents productivity as a function of $(1-\epsilon)$ and D.	8
		Alginate entrapment of spores (beads—2mm)	CSTR + Pervaporation solvents recovery $(1-\epsilon) = 0.2$	37	—	—	1.0—1.7	—	Glucose conversion increased by 65—70%, productivity increase.	9
	C.acetobutylicum ATCC 824	Entrapment in K-carrageenan	—	—	—	—	5.2	—	450 h continuous operation reported. Details lacking.	10
Sucrose (molasses)	*C.acetobutylicum* P262	Alginate entrapment of clostridial stage cells	FCR	34	0.42	15	3.02	0.44—0.65	Early sporulation-deficient mutant used. A five-fold increase in solvent concentration compared with wild type cells.	11
Lactose (whey permeate)	*C.beijerinckii* LMD27.6	Alginate entrapment of spores (beads—2mm)	CSTR	30	0.23	2—3	1.0 at D = 0.7	—	Fermentation start-up at $D < 0.1\ h^{-1}$ is critical. No solvents production at 37°C. 5 g l^{-1} $CaCl_2.2H_2O$, used to maintain bead integrity has no effect on solventogenic performance.	12

Table 2.6 (continued) Comparison of productivities of various immobilized cell processes for continuous solvents production from synthetic and technical media

Substrate	Organism	Immobilization method	Reactor	Temp. (°C)	Dilution rate (h^{-1})	Solvent concentration (g/l)	Prod. (g/l.h)	Yield (g/g)	Comments	Ref.
	Clostridium sp DSM 2152	Alginate entrapment of spores (beads—2mm)	CSTR	30	various	5—6 at D = 0.1	various	0.20 (butanol)	Kinetic model verification with this strain on glucose and whey permeate. Butanol more inhibitory with lactose than glucose substrate. Fermentations of 1000—1800 h reported.	13

Notes:
D = dilution rate.
PB = Packed Bed Reactor.
CSTR = Continuous Stirred Tank Reactor.
FCR = Fluidised Column Reactor.
$(1-\epsilon)$ = bead fraction in the reactor.
Prod.: volumetric productivity ([solvents] x dilution rate).

Yield = g total solvents/ g substrate utilised.

References

1 Haggstrom & Molin, (1980)
2 Haggstrom & Enfors, (1982)
3 Forberg et al, (1983)
4 Forberg & Haggstrom, (1985)
5 Krouwel et al, (1980)
6 Krouwel et al, (1983a)
7 Krouwel et al, (1983b)
8 Schoutens et al, (1986)
9 Groot et al, (1984a)
10 Anon, (1981)
11 Largier et al, (1985)
12 Schoutens et al, (1985)
13 Schoutens & Kossen, (1986)

pervaporation) may increase solvent productivity by reducing butanol inhibition and increasing substrate utilization (Groot et al, 1984a,b).

2.8.6 Kinetic Model for Solvents Production using Alginate Biocatalysts

A simple model has been developed to describe the continuous production of butanol and isopropanol from glucose using calcium alginate immobilized cells of Clostridium beijerinckii LMD 27.6 (Schoutens et al, 1986). Linear inhibition kinetics were used to describe the effect of butanol on the substrate consumption rate. A homogeneous biomass distribution in the alginate beads (2 mm diameter) was assumed. The biocatalyst effectiveness factor (η), i.e. the ratio between the actual conversion rate and the conversion rate that would occur if no diffusional limitation in the carrier material was present, was found to be equal to 1, under the range of experimental conditions used for the model determination. The efficacy of the model has been demonstrated on glucose, lactose and cheese whey permeate media using Clostridium species DSM 2152 (Schoutens & Kossen, 1986). Model kinetic parameters for this strain were determined from steady-state product concentration and residual substrate concentration data in experiments where the dilution rate (D_t) was varied at a given bead fraction in the reactor ($1-\varepsilon$). These model parameters enable the prediction of fermentation productivities at values of D_t and ($1-\varepsilon$) over a greater range than used in the experimental work. A summary of the model equations follows, based on the simplified schematic diagram in Figure 2.3.

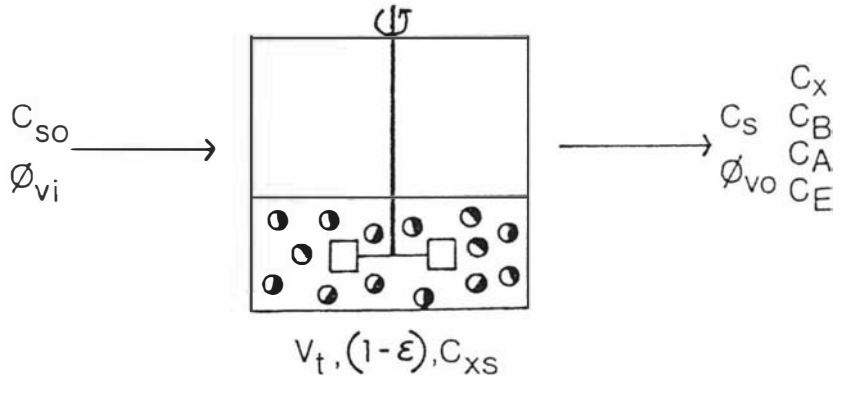


Figure 2.3. A schematic diagram of the immobilized cell system showing those measurable parameters necessary for the kinetic model parameter estimation.

where

C_{so} = feed substrate concentration (g/l)
C_s = effluent substrate concentration (g/l)
C_{xs} = biomass in solid phase (g/l)
ϕ_{vi} = volumetric flowrate in (l/h)
ϕ_{vo} = volumetric flowrate out (l/h)
$(1-\varepsilon)$= bead fraction of alginate beads in the reactor (l.alginate/l)
C_x = biomass in liquid phase (g/l)
C_B = butanol concentration (g/l)
C_A = acetone concentration (g/l)
C_E = ethanol concentration (g/l)

Fermenter substrate mass balance:

$$\frac{\phi_{vi}\ C_{so}}{V_t} = \frac{\phi_{vo}\ C_s}{V_t} + r_s\ (1-\varepsilon)$$

substrate in = substrate out + substrate consumed

where $r_s = r_{max}\left\{1 - \frac{C_B}{C_{B,max}}\right\}$ (Linear inhibition kinetics)

and $r_{max} = \frac{\mu_{max}\ C_{xs}}{Y_{sx}}$

where r_s = substrate consumption rate (g/l.h)
r_{max} = maximal specific substrate consumption rate (g.substrate/l.alginate h)
μ_{max} = maximum specific growth rate (h^{-1})
Y_{sx} = biomass yield factor on substrate (g.d.w/g)
$C_{B,max}$= maximal butanol concentration (g/l)
V_t = total working volume (l)

The following model equations were determined:

$$\frac{D_t\ (\Delta C_s)}{(1-\varepsilon)} = r_{max}\left\{1 - \frac{C_B}{C_{B,max}}\right\} \qquad (1)$$

$$C_B = Y_{sb} (\Delta C_s) \quad (2)$$

and $$C_A = \frac{C_B}{k} \quad (3)$$

where D_t = dilution rate based on the total reactor volume (h^{-1})
ΔC_s = consumed substrate concentration (g/l)
Y_{sb} = butanol yield factor on substrate (g/g)
k = ratio of butanol/acetone (g/g)

Rearranging equations (1) - (3) gives the following equations which enable determination of the model parameters r_{max} and $C_{B,max}$.

$$\frac{1}{P} = \frac{1}{Y_{sb} r_{max}} + \frac{1}{C_{B,max}} \frac{(1-\varepsilon)}{D_t} \quad (4)$$

where $$P = \frac{Y_{sb} D_t \Delta C_s}{(1-\varepsilon)} \quad (5)$$

where P = specific butanol production rate (g.butanol/l.alginate h)

$\frac{D_t}{(1-\varepsilon)}$ = normalized total dilution rate (l/l.alginate h)

A reciprocal plot of P versus $D_t/(1-\varepsilon)$ gives a straight line, of intercept $\frac{1}{Y_{sb} r_{max}}$ and slope $\frac{1}{C_{B,max}}$

Hence r_{max} and $C_{B,max}$ can be determined.

Rearranging equation (1) gives the following:

$$\Delta C_s = \frac{r_{max} C_{B,max}}{\left(\frac{D_t}{(1-\varepsilon)}\right) C_{B,max} + r_{max} Y_{sb}} \quad (6)$$

From equation (6), the steady-state consumed substrate concentration can be calculated for any value of D_t and $(1-\varepsilon)$, and from equations (2) and (3) steady-state butanol and acetone concentrations respectively, can be determined.

2.9 CONTINUOUS FERMENTATION BY FREE CELLS USING EXTERNAL CELL RECYCLE BY CROSS-FLOW MICROFILTRATION

2.9.1 Introduction

Traditional solid-liquid separation processes used for downstream cell recovery include centrifugation, gravity settling and induced flocculation/aggregation. The use of membranes in biotechnological processes has been described only recently, but is a fast growing area of research (Michaels, 1980; Strathmann, 1985; Mulder & Smolders, 1986). Applications include the sterilization of fermentation media, concentration of fermentation products by membrane dewatering, the recovery of extracellular products and the harvesting and purification of cells. Novel solid-liquid separation technologies for fermentation processes, including electrocoagulation, electrophoresis and dielectrophoresis, magnetism, ultrasonics and novel micro-flotation, have been recently reviewed (Bowden, 1985).

One area of membrane process application receiving increased research attention, is their use in "intensified" fermentation processes. Microbial cells can be recovered from a fermentation broth using membranes and recycled back to the fermenter in order to increase the biomass concentration and the fermenter productivity. Cells are retained within a closed loop, permitting higher dilution rates to be used than in conventional continuous free-cell fermentations where the dilution rate must be less than the maximum growth rate (Gabler, 1984; Ripperger & Schulz, 1986). This membrane process is called tangential flow filtration or more commonly cross-flow microfiltration (CFM). The application of recycle processes to fermentation processes in general have been recently reviewed (Hamer, 1982).

The potential advantages of a continuous fermentation cell recycle by CFM process can be summarized as follows: productive cells are returned to the fermenter for reuse; greater biomass concentrations are achievable due to the availability of fresh nutrients in the feed medium, facilitating improved volumetric productivities; and inhibitory products can be removed from the cell-free filtrate stream, possibly more easily than when cells are present, prior to its return (partial) to the

fermenter, giving rise to even greater productivities. Potential disadvantages may include: the need for expensive membrane plant; membrane fouling, reducing the operational period of continuous fermentation; the increase in biomass concentration may not give proportional increases in productivity due to diffusitivity limitations or retarded growth or metabolic activity; and the process may be complex and difficult to operate for extended periods. The long-term effect of continuous contact of microbial cells with high product concentrations has yet to be determined. Ideally membranes should be steam-sterilizable to ensure long-term fermentation operation.

2.9.2 Principles of Cross-Flow Microfiltration

Cross-flow microfiltration using either ultrafiltration or microporous membranes is a pressure driven solid-liquid separation process. Fermentation broth flows tangentially across the membrane surface with cells (i.e. larger molecular size species than the membrane pore size) being swept away from the membrane surface, and cell-free filtrate (containing fermentation products and unutilized substrate components) permeating through the membrane (Strathmann, 1985). The shear and lifting forces produced by this cross-flow minimizes the formation of a layer of separated cells at the membrane surface - a phenomenon known as concentration polarization (Ripperger & Schulz, 1986) such that a high filtrate rate (flux) can be maintained over a long period. Microporous membranes are more "open" than ultrafiltration membranes, and symmetric microporous polymer membranes typically have a pore size range of 0.05 μm to 10 μm compared with assymmetric (two-layered) microporous polymer ultrafiltration membranes where membranes of a pore size range of 1 nm to 50 nm are available (Strathmann, 1985). The actual membrane pore size would have a narrow pore size distribution to ensure quantitative cell retention (Ripperger & Schulz, 1986).

Backflushing is a means of prolonging the filtrate flux by reducing the effects of concentration polarization and can be used periodically, so long as it is compatible with the membrane configuration and membrane life. The direction of filtration during backflushing is reversed, e.g. when membranes are in capillary or tubular form, the filtrate can flow

from either the inside to the outside in normal operation or in the reverse direction during backflushing operations. During backflushing, which normally lasts for a short period of a few seconds and at pressures compatible with membrane strength, any layered cells or particles on or within the membrane structure are dislodged and swept away.

The following parameters will influence cross-flow microfiltration: cell suspension characteristics, (e.g. size and morphology of the microorganism, solute concentration, viscosity and rheological characteristics), pH, membrane characteristics, (e.g. pore size, pore distribution homogeneity, membrane material and structure, surface physico/chemical behaviour, thickness), and operational conditions, (e.g. module geometry, tangential velocity, pressure and temperature).

A wide range of new membrane types (wide pH and temperature tolerance), some of which are autoclavable and having wide chemical species compatibility, are now available. Hollow fibre, capillary, tubular and flat sheet membrane configurations are suitable for CFM applications (Strathmann, 1985).

Capital and operating costs are proportional to the membrane area, hence average flux performance data as a function of cell concentration is a major factor determining the economic feasibility of the application of CFM to continuous free-cell fermentation processes. Such data are most likely to be case specific, relating to the rheological properties (relationship between viscosity and cell concentration) of a given cell-medium suspension and the hydrodynamic properties of the CFM plant.

A simple mathematical analysis of a continuous fermentation - cell recycle system (Appendix 1) has shown that for steady state metabolite production, a constant biomass concentration must be maintained. In order to achieve this, cells must be 'bled' from the reactor at a rate equal to the growth rate ($\mu = \alpha D$, where αD is a fraction of the overall dilution rate).

2.9.3 Application of CFM to Continuous Fermentation Processes

A number of continuous fermentation processes employing CFM for cell recycle have been published. A similar application of membrane technology involves the immobilization of cells within a membrane plant (e.g. hollow fibres) giving rise to improved productivities (e.g. Vick Roy et al, 1983; Inloes et al, 1983), but such processes will not be reviewed here.

Continuous ethanol production using CFM for cell recycle has been described using different CFM configurations, including the use of hollow fibres (Charley et al, 1983; Cheryan & Mehaia, 1983, 1984; Nishizawa et al, 1983; Khorakiwala et al, 1985) capillary (Lee et al, 1980), plate and frame (Rogers et al, 1980, 1982; Terrell et al, 1984; Janssens et al, 1984) and tubular (Damiano et al, 1985) systems. Considerable productivity increases were obtained on both glucose-containing media and lactose (whey permeate) media compared with batch fermentation productivities. Most workers, however, did not report the duration of the continuous experiments and steady-state productivity values were obtained after a small number of residence times (e.g. 5 residence times). Generally, performance data on the CFM units are sparse and little consideration has been given to the possibility of process scale-up.

Continuous solvents production from glucose-containing medium has been more recently described (Afschar et al, 1985; Vijjeswarapu et al, 1985; Pierrot et al, 1986; Schlote & Gottschalk, 1986; Ferras et al, 1986). A proposed pilot plant study for continuous solvents production from saccharified starch, involving external cell recycle by capillary cross-flow microfiltration has been published but few details have been presented (Ripperger & Schulz, 1986). In a comprehensive piece of work, Afschar et al, (1985) obtained significant productivity increases using a capillary (95 X 1.8 mm diameter capillaries, 0.20 μ pore size) CFM device in a continuous fermentation using C. acetobutylicum ATCC 824 fermenting a complex glucose medium. Turbidostat control of the fermenter cell concentration was used. The effect of cell concentration (2,4,8 g.d.w/l) and dilution rate (range 0-0.8 h^{-1}), on the specific solvent productivity, product concentration and residual glucose

concentration were examined. The maximum specific solvent productivity (0.65 g/g.d.w.h) occurred at a butanol concentration of 3-4 g/l after which a decrease occurred. In order to prevent degeneration effects, i.e. a solvent concentration decrease and acids concentration increase after prolonged exposure to a high solvent concentration (details lacking), a multi-stage fermentation system was successfully used. In the first stage, the solvent and biomass concentrations were kept low in order to provide product cells to the later stages (2 - 5 stages) operated at higher biomass concentrations. A two-stage fermentation process proved optimal and a solvent productivity of 2.3 g/l.h was achieved at an effluent solvent concentration of 15 g/l. The biomass concentrations were 2.38 g.d.w/l and 10.88 g.d.w/l in Stage 1 and Stage 2 respectively. An increase in dilution rate gave a higher solvent productivity but at a lower solvent concentration. No performance data for the CFM were supplied although the unit was capable of being backflushed.

Pierrot et al, (1986) also used strain ATCC 824 and a synthetic medium containing glucose. An Amicon hollow fibre CFM device (250 fibres, 0.5 mm ID x 22 cm long) of 550 cm^2 surface area was used. A total effluent solvent concentration of 16 g/l was obtained at a cell concentration of 20 g/l (higher than used by Afschar et al, 1985) and at an overall dilution rate of 0.3 h^{-1}, in an experiment of 150 h duration. A biomass bleed dilution rate of 0.027 h^{-1} was used to maintain a steady biomass concentration. An increase in the biomass bleed dilution rate to 0.065 h^{-1} and the overall dilution rate to 0.5 h^{-1} gave a lower solvent concentration of 13 g/l but a higher productivity of 6.5 g/l.h.

Schlote & Gottschalk, (1986) have described the continuous production of solvents using a phosphate-limited chemostat with external biomass recycle using a CFM device (Sartorius, Plate and Frame). A synthetic medium containing glucose was used, and the fermentation pH was controlled at a value of pH 4.4 at 37°C. Solvent concentrations of 16 and 22 g/l and productivities of 6.5 and 2.2 g/l.h respectively, were obtained. Biomass concentrations of 13.1 g.d.w/l and 23.3 g.d.w/l were obtained at overall dilution rates of 0.1 h^{-1} and 0.4 h^{-1}, respectively.

Ferras et al, (1986) used a mineral tubular (Carbosep M1) membrane device (internal diameter 6 mm, 0.0226 m^2 membrane area, recirculation

velocity between 3-7 m/s) for continuous solvent production from a semi-synthetic medium containing glucose using strain ATCC 824 at 35°C and at pH 5.4. A maximum biomass concentration of 125 g.d.w/l was obtained (complete biomass retention) and an average solvent productivity of 4.5 g/l.h was obtained. At these high biomass concentrations, severe membrane plugging occurred after 400-500 h operation, and oscillations in solventogenic and acidogenic fermentation phases were observed throughout the fermentation. Total cell counts of approximately 1 to 5 x 10^{11}/ml were obtained after 400 h operation but no estimate of the fraction of actively producing cells was made.

In summary, available literature on the application of CFM to continuous solvent fermentations using free cells has demonstrated the ability of C. acetobutylicum ATCC 824 to produce solvents from glucose media at considerably higher productivities than obtained in batch fermentations. Performance data for the various CFM devices is sparse and extraction of flux data for estimating membrane requirements for scale-up purposes is difficult. Possibly this is due to the non-optimal operation of the CFM devices, where the simple objective was to obtain a long-term filtrate flux greater than the feed rate. Additionally, membrane cleaning regimes have not always been published. Very little information has been published in these reports concerning the morphology of the organism or the growth characteristics in concentrated suspension. No culture rheological information is supplied.

2.10 PRODUCT RECOVERY

2.10.1 Recovery by Distillation

Distillation is the traditional method for acetone:butanol:ethanol recovery from dilute fermentation broth (Othmer, 1936; UK Patent 1949; Spivey, 1978; Lenz & Moreira, 1980; Strobel & Bader, 1981). A description of the commercial distillative recovery processes used in the 1950's, is given by Lenz & Moreira, (1980) and Strobel & Bader (1981), and the following is based on these reports. Fermentation broth is fed to beer stills, and an approximate 40:60 (% w/w) solvent/water stream is removed overhead. Beer still slops are removed from the

bottom and used to preheat the beer still feed prior to being concentrated by evaporation, then spray dried to produce an animal feed. The mixed solvents/water stream from the beer still flows to a batch column which separates acetone (low boiler) and the ethanol/water azeotrope (sidestream, intermediate boiler) from a butanol/water tail stream. Acetone and ethanol/water are sent directly to separate product storage or for further distillative purification as required. The butanol/water stream is sent to two additional columns. Butanol recovery is made easier by the fact that butanol and water form two layers (immiscible) at a 0.45 butanol mole fraction (Strobel & Bader, 1981). The butanol/water azeotrope from the top of the first butanol column enters a decanter, from which the butanol rich phase is separated and fed into a second column from which high purity butanol (99.5 mole %) is recovered, with the denser water-rich phase being recycled to the first column.

2.10.2 Alternative Product Recovery Methods

Over the past decade, conventional distillation process innovations, including vapour recompression and multiple effect distillation, and novel, more energy-efficient processes have been developed, predominantly for ethanol recovery from fermentation broths. Rising energy costs and the concern for a favourable nett energy balance (energy content of useful products less energy input into all production stages) for fuel ethanol production, has accentuated much of this research effort (Hartline, 1979; Maiorella et al, 1981; Essien & Pyle, 1983). The novel non-distillation alternatives include the use of membranes (reverse osmosis, dialysis and pervaporation), adsorbents (polymeric resins, molecular sieves), liquid-liquid extraction and chemical recovery, and a technical description of these processes, a review of the literature describing their use, and an assessment of their potential for the ABE fermentation is given in Appendix 6. The use of salts to favourably alter the ethanol:water equilibrium has also been proposed for improved distillative recovery (Schmitt & Vogelpohl, 1983) and has been considered for the ABE fermentation (Card & Farrell, 1982; Donaldson, 1984).

Comparatively fewer studies have been undertaken for the ABE fermentation, but much of what has been learnt with the ethanolic studies can be used to determine future possibilities for the ABE fermentation.

The alternative product recovery processes may be:

(a) as 'stand-alone' processes as direct competitors for distillation recovery processes.

(b) as adjunct units where the alternative process replaces distillation over part of the concentration range.

(c) as suitable, in some instances, for continuous and selective in-situ or in-line toxic product recovery to further improve intensified fermentation processes (Kosaric et al, 1983; Maiorella et al, 1984; Roffler et al, 1985). Further downstream recovery will be required for complete recovery but its capacity or energy usage could be reduced. Integrated fermentation - product recovery processes may offer the potential of fermenting more concentrated substrates.

Not all of the product recovery techniques examined in the literature are suitable for in-situ recovery, not only from a technological standpoint but also for reasons of process simplicity, economics, product purity and safety.

Commercialization of alternative processes (a) is unlikely in the medium term, but alternative processes (b) and (c) look promising for future application in either the improvement of existing plant economics (b), or in the construction of new plant (b and c). A number of integrated continuous ethanol fermentation processes are now commercially available (Guidoboni, 1984).

Maiorella et al, (1984) in examining the total manufacturing costs for eleven alternative yeast-based ethanol fermentations, found that those utilizing in-situ ethanol recovery produced the lowest cost ethanol. Essentially, this resulted from the fermentation of more concentrated feedstock and the attainment of high cell biomass, resulting in significantly improved volumetric productivities.

In summary, the integration of continuous fermentation with in-line product recovery processes, to further intensify the ABE process by reducing product inhibition effects, appears technologically promising. Pilot-plant/semi-commercial studies are necessary before the adoption of these integrated processes takes place. Those separation processes that remove water (e.g. reverse osmosis or pervaporation) prior to distillation, are most likely to be adopted in the near future. Preliminary feasibility studies conducted using such integrated processes (continuous fermentation using alginate-immobilized cells coupled with in-situ and downstream solvents recovery by pervaporation) appear promising (Schoutens & Groot, 1985).

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Microbiological Media

Spray-dried cheddar cheese whey permeate was obtained from the Dutch Institute of Dairy Research (Ede, The Netherlands) or the New Zealand Dairy Research Institute (Palmerston North, New Zealand). Sulphuric acid casein whey permeate (liquid or spray-dried powder) was obtained from the New Zealand Dairy Research Institute. The preparation of this permeate is described by Matthews et al, (1978). 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.

Hydrolysis of the lactose in whey permeate was conducted using a ß-D-galactosidase (lactase) enzyme. Maxilact LX5000 or Sumylact L enzyme (0.5 g/l) was added to cheese whey permeate or sulphuric acid casein whey permeate respectively, and held at 4°C for 72 h.

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 with aqueous ammonia prior to autoclaving.

Media used for the various fermentation experiments are given in Tables 3.1 to 3.5 inclusive. The typical composition of cheddar cheese whey permeate and sulphuric acid casein whey permeate is given in Table 3.6.

Table 3.1 Semi-synthetic medium used for the culture screening batch fermentation experiments

Component	Concentration (g/l)
Sugar[a]	50.0
Yeast extract	5.0
Ammonium acetate	2.0
NaCl	1.0
Cysteine hydrochloride H_2O	0.5
KH_2PO_4	0.75
K_2HPO_4	0.75
$MgSO_4$	0.20
$MnSO_4.7H_2O$	0.01
$FeSO_4.7H_2O$	0.01

[a] lactose, glucose or galactose. For a glucose/galactose mixture, 25 g/l of each sugar was used.

Table 3.2 Sulphuric acid casein whey permeate medium for batch fermentation experiments

Component	Concentration (g/l)
Sulphuric acid whey permeate	55.0[a]
Yeast extract	5.0

Medium was adjusted to pH 6.5 using aqueous ammonia prior to autoclaving.

[a] spray-dried permeate. Liquid permeate was used as received (approx 40 g/l lactose).

Table 3.3 Cheese whey permeate medium for continuous fermentations using immobilized cells or external cell recycle[a]

Component	Concentration (g/l)
Cheese whey permeate	60.0
Yeast extract	5.0
$CaCl_2.2H_2O$	5.0

[a] Medium for cell recycle did not contain $CaCl_2.2H_2O$

Table 3.4 Semi-synthetic medium for continuous fermentations using immobilized cells

Component	Concentration (g/l)
Sugar	50.0
Yeast extract	5.0
$CaCl_2.2H_2O$	5.0

Table 3.5 Semi-synthetic medium for continuous fermentations using external cell recycle

Component	Concentration[a] (g/l)
Lactose	65.0
Yeast extract	6.0
Other components as in Table 3.1	

[a] The sugar and yeast extract concentrations for semi-synthetic medium containing glucose were 70 g/l and 5 g/l respectively.

Table 3.6 Typical composition of cheddar cheese whey permeate and sulphuric acid casein whey permeate

		Cheddar Cheese Whey Permeate[a]	Sulphuric Acid Casein Whey Permeate[b,c]
Total solids	(g/kg)	64.1	56.4
Total nitrogen	(g/kg)	0.47	0.37
Non-protein nitrogen	(g/kg)	0.36	0.32
Ash	(g/kg)	5.4	7.9
Lactose (monohydrate)	(g/kg)	58.0	46.0
Ash/Total solids	(%)	8.4	14.0
Lactose/Total solids	(%)	90.5	82.0
Calcium	(g/kg)	0.5	1.1
Sodium	(g/kg)	0.6	0.5
Potassium	(g/kg)	1.8	1.2
Phosphate (total)	(g/kg)	1.2	1.9
Chloride	(g/kg)	1.5	0.9
Sulphate	(g/kg)	-	2.2
pH		5.5-5.8	4.5-4.7[c]

a derived from Hobman (1984)
b derived from Short (1978)
c derived from Ennis & Higgins (1981), Higgins & Short (1980).

3.1.2 Chemicals

All chemicals used for fermentation medium and analytical work were of analytical grade. Their sources were:

- Ajax Chemicals (Sydney, Australia)
  butanol, ethanol

- Alginate Industries (Croydon, England)
  sodium alginate (manucol LD)

- Baker Chemicals (Deventer, The Netherlands)
  calcium chloride dihydrate; butyric acid

- BDH Chemicals Ltd (Palmerston North, New Zealand)
  ammonium hydroxide; ammonium acetate; acetone; acetic acid; butanol; butyric acid; calcium acetate; dipotassium hydrogen phosphate; cysteine hydrochloride; ethanol; ferrous sulphate; formalin; galactose; glucose; lactose; magnesium sulphate; manganese sulphate; methanol; potassium dihydrogen phosphate; sodium chloride; silicone antifoam

- Bevaloid Ltd (Levin, New Zealand)
  antifoam 5901

- Gist Brocades nv Ltd (Delft, The Netherlands)
  Maxilact LX5000 lactase

- Lactose Company Ltd (Kapuni, New Zealand)
  lactose

- May and Baker (Dagenham, England)
  ammonium hydroxide

- Riedel - De Haen AG (Hannover, West Germany)
  formic acid

- Shin Nihon Chemical Co Ltd (Anjyo, Japan)
  Sumylact L lactase

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

- Swift Consolidated (NZ) Ltd (Wellington, New Zealand)
Dow-Corning antifoam A.F. emulsion (food grade)

3.1.3 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.).

Amberlite® adsorbent resins XAD-2, XAD-4, XAD-7 and XAD-16 were obtained from Rohm and Haas (Philadelphia, PA, U.S.A.) via Rohm and Haas NZ Ltd (Auckland, New Zealand). The resins were washed twice in methanol:deionized water, 1:1, to remove resin preservatives and kept at 4°C. The resins were washed with deionized water immediately prior to use. The ratio of deionized water:resin on a volumetric basis was 4:1 for small scale screening experiments and 10:1 for experiments where the resin was used in a packed column. XAD-2 is a crosslinked polystyrene copolymer hydrophobic adsorbent; XAD-4 is a crosslinked polystyrene copolymer non-ionic hydrophobic adsorbent; XAD-7 is an acrylic ester polymeric adsorbent; XAD-16, resin type unknown, development phase.

Silicalite, a porous zeolite analogue, was obtained as a white coloured powder from Dr D Bibby, Chemistry Division, Department of Scientific and Industrial Research (Petone, New Zealand). The silicalite was used as supplied.

3.1.4 Organisms

Clostridium acetobutylicum ATCC 824 was purchased as a freeze-dried specimen from the American Type Culture Collection (Rockville, Maryland, U.S.A.). It was reconstituted (without heat shocking) into 20 ml Cooked Meat Medium supplemented with glucose (10 g/l) and incubated at 30°C in an anaerobic jar for 48 h. Spore stocks were prepared from this vegetative culture as follows:

(a) Culture was streaked onto slopes of Reinforced Clostridial Agar and incubated in an anaerobic jar at 30°C for 96 h.

(b) The sporulated culture was scraped off the slopes into sterile distilled water and aseptically dispensed into sterile tubes (2ml aliquots). This spore stock was stored at 4°C.

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

Fresh spore stock of this organism was prepared as follows:

(a) A vegetative culture was prepared by heat-shocking 0.1 ml of the original spore stock in 20 ml Cooked Meat Medium supplemented with glucose (10 g/l) at 75°C for 2 min, followed by rapid cooling in an ice-water bath for 1.5 min. This culture was incubated at 30°C in an anaerobic jar for 72 h until vigorous gassing was observed.

(b) This culture was streaked onto slopes of sulphuric acid casein whey permeate agar and incubated at 30°C in an anaerobic jar.

(c) The growing culture was regularly inspected for spore formation, using Bartholomew and Mittwer's spore staining method using malachite green (Harrigan and McCance, 1966). After 42 days incubation, the highly sporulated culture was scraped off into sterile distilled water and aseptically dispensed as 2 ml aliquots into sterile Kimax tubes. The spore count by haemocytometer was approximately 1.7×10^8 spores/ml. This stock spore suspension was stored at 4°C and used for inoculum preparation throughout the duration of this study.

3.2 STERILIZATION PROCEDURES

3.2.1 Media Sterilization

All microbiological media were sterilized by autoclaving at 121°C for 15 minutes. All fermentation media of volumes less than 10 litres were sterilized by autoclaving at 121°C for 15 minutes, and for volumes greater than 10 litres at 121°C for 20 minutes.

All media for inoculum preparation and batch fermentations were autoclaved immediately prior to use. Media components for continuous fermentation using immobilized cells were sterilized separately (121°C/15 minutes) and mixed together at ambient temperature.

3.2.2 Equipment Sterilization

Glass wool gas filters and glass pipettes were sterilized in a hot air oven at 160°C for 3 hours.

pH electrodes for insertion into fermenters (85°C) were sterilized in 2% (v/v) formalin solution for 2 h or 50% (v/v) ethanol solution for 18 h, and rinsed with hot sterile distilled water immediately before use.

3.3 CLEANING OF GLASSWARE

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

3.4 ANAEROBIC INCUBATION

Cultures for anaerobic incubation were placed in either Baird and Tatlock (Baird and Tatlock Ltd, Chadwell Heath, Essex, U.K.) or BBL (BBL Microbiology Systems, Cockeysville, Maryland, U.S.A.) anaerobic jars.

An anaerobic atmosphere 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 check on the development of anaerobic conditions in the jar. The palladium catalyst contained in wire gauze support was rejuvenated by direct flaming until red hot immediately prior to use.

A Forma anaerobic cabinet (Forma Scientific Inc, Marietta, Ohio, U.S.A.) was used for experiments described in Chapter 10. An atmosphere of carbon dioxide gas was used to achieve anaerobic conditions.

3.5 ANALYTICAL METHODS

3.5.1 pH Measurement

Routine pH measurements were made with a Metrohm pH meter E520 (Metrohm A.G., Herisau, Switzerland) which was calibrated with pH 4.0 and pH 7.0 buffers immediately prior to use.

3.5.2 Determination of Biomass Dry Weight

3.5.2.1 Free Cell Cultures

A known volume of fermentation culture (usually 3 ml) was centrifuged at about 5500 x g for 15 min using a Clandon Centrifuge (Clandon Scientific Ltd, Aldershot, England, Model T52.1). The supernatant liquid was discarded, while the pellet was washed in 6 ml of distilled water, re-centrifuged, and then dried at 100°C for 48 h.

3.5.2.2 Immobilized Cells

Alginate beads (150-200 ml) were suspended in 1 litre of 0.2M citrate-phosphate buffer, pH 6.0. Approximately 15 ml of 5% (v/v) formaldehyde was added to this suspension.

The suspension was stirred using a magnetic stirrer for 4-6 weeks, maintaining the pH value at pH 6.0$\pm$0.2. Microscopic inspection of the suspension, and inspection of centrifuged suspension pellets, for evidence of undissolved alginate crystals, was conducted regularly. When the alginate had dissolved, the suspension was centrifuged (5000 x g, 20 min) and the pellet was dried at 110°C for 48 h. The dry weight of biomass in unused beads was subtracted from that in used beads to give the biomass concentration in the beads due to growth.

3.5.3 Total Cell Count

The total cell count was performed immediately after sampling using a standard haemocytometer (Assistant, West Germany) under 400X magnification. Samples were diluted either 10X or 50X using sterile

peptone water (5 g/l) prior to counting. Only vegetative cells were counted.

3.5.4 Determination of Colony Forming Units

Colony forming units (CFU) were determined by pour plating in Reinforced Clostridial Agar. Dilutions were prepared using peptone water (5 g/l). The agar plates were incubated in an anaerobic jar at 30°C for 72 h, or at 34°C for 48 h.

3.5.5 Analysis of Solvents and Acids

Solvents and acids analysis was carried out by Gas Chromatography (GC). Various methodologies/instrumentation were used for different sections of work. Method I was used for work described in Chapters 4 and 5, Method II for Chapter 6, and Method III for Chapters 6, 7, 9 and 10. All analyses were performed on samples that had been centrifuged to remove cells and particulate matter.

METHOD I

A Varian Aerograph GC (Varian Instruments Ltd, California, U.S.A., Model 1400) was used with a flame ionization detector system. A 2m x 0.32 cm ID glass column of Chromosorb 101 (mesh size 100/120, acetone treated) was used at a carrier gas (nitrogen) flowrate of 40 ml/min. The column was operated by increasing the temperature from 120°C to 180°C at a rate of 10°C/min and holding at this temperature for a further 9 min. The injector and detector temperatures were 200°C.

Sample product quantitation was accomplished using a Hewlett-Packard Integrator (Model 3392A) to measure peak areas. An internal standard of iso-butyric acid (10 g/l) in 20% (v/v) formic acid (Riedel-de Haen) was used.

Response factors for each sample component to the internal standard were determined by injection of a standard solution (containing internal standard at 10 g/l) containing (g/l), butanol, 10; acetone, 4; ethanol, 4; butyric acid, 10; and acetic acid, 10. Mean values from duplicate analyses were used for programming the integrator.

A sample volume of 2 µl plus 2 µl of 10% (v/v) formic acid (Riedel-de Haen) was injected into the column. After every 6 sample injections the column was cleaned of any adsorbed products (acids) by flushing (repeated injections) the column with 10 µl of 10% (v/v) formic acid and holding at 180°C for 10 min.

METHOD II

A Packard GC (Packard Becker, Groningen, The Netherlands, Model 437) was used with a flame ionization detector. A 2m x 0.18 cm ID stainless steel column of Chromosorb 101 (mesh size 100/120) was used at a carrier gas (nitrogen) flowrate of 25 ml/min. The column was operated by increasing the temperature from 135°C (1 min hold) to 185°C at a rate of 10°C/min and holding at this temperature for a further 3 min. The detector and injector temperatures were 210°C and 200°C respectively.

Sample product quantitation was achieved using a Hewlett-Packard Integrator (Model HP3390A, peak area measurement) and Packard Autosampler (Packard Becker, Model LS607). Samples were acidified (approximately pH 2.0) with 50% H_2SO_4 prior to injection of a 2 µl sample volume (precise volume measurement). Analyses were conducted in duplicate and mean values reported.

A standard solution containing (g/l), butanol, 3; acetone, 2; ethanol, 2; butyric acid, 3; and acetic acid, 3; was used to calculate product response factors. Every eight sample injections, a standard was injected (duplicate analyses) and the product response factors obtained were used to recalibrate the integrator.

METHOD III

A Shimadzu GC (Shimadzu Corporation, Kyoto, Japan, Model GC-8APF) was used with a flame ionization detector. A 1m x 0.15 cm column containing Porapak Q was used at a carrier gas (nitrogen) flowrate of 60 ml/min at a column temperature of 200°C. The detector and injector temperatures were 220°C.

Sample product quantitation was achieved by peak height measurement using an internal standard in the sample and comparison with

a parallel standard of a similar known solvents and acids composition. An internal standard of sec-butanol (50 g/l) in 20% (v/v) orthophosphoric acid was used. The standard solution contained (g/l), butanol, 5; acetone, 2; ethanol, 2; butyric acid, 2; and acetic acid, 2. Internal standard (0.1 ml) was added to 1 ml of either sample or standard. In some instances, samples were diluted with deionized water prior to analysis, so that peak heights were similar to those obtained for the standard solution.

For the parallel standard a response factor, F_S, was calculated for each solvent and acid component.

$$F_S = \left(\frac{IS}{P}\right)_H \Big/ \left(\frac{IS}{P}\right)_C$$

where:

$\left(\frac{IS}{P}\right)_H$, is the average of the ratio of internal standard: solvent or acid peak heights 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 concentration of the solvents and acids in the sample.

$$C = IS_C * \left(\frac{C}{IS}\right) * F_S$$

where: C, is the concentration of a solvent or acid in the sample (g/l)

IS_C, is the concentration of the internal standard in the sample

$\left(\frac{C}{IS}\right)$, is the ratio of the product component : internal standard peak heights from the sample chromatogram

F_S, is the response factor for the sample component.

A single injection (2 µl) was performed for each sample.

3.5.6 Analysis of Sugars

Quantitative analysis of lactose monohydrate, glucose, galactose and glucose/galactose sugar mixtures was performed using High Performance Liquid Chromatography (HPLC). Two different methods were used for different sections of work. Method II was used for some work described in Chapter 6, while Method I was used for the remainder of the work.

METHOD I

A Waters Associates liquid chromatograph (Model ALC/GPC 244) equipped with a solvent delivery system (Model 6000A) and septumless injector (Model U6K) was used (Waters Associates Inc., Milford, Massachusetts, U.S.A.).

A Sugar-PAK1 Carbohydrate column (30 cm x 0.65 cm ID, P/N 85188, Waters Associates) was used for the analysis. The detector was a differential refractometer (Model R401, Waters Associates). The response was recorded on a CR600 twin-pen, flat-bed chart recorder (J.J. Lloyd Instruments Ltd, Southhampden, England).

Analyses were conducted using a solvent system of 20 mg/l calcium acetate solution prepared using deionized water. The column was operated at 90°C and at a solvent flowrate of 0.5 ml/min. The solvent (mobile phase) was filtered and degassed prior to use, using a 0.45 µm filter (Millipore Corporation, Bedford, Massachusetts, U.S.A.).

Samples were centrifuged (3000 x g for 10 min) and the supernatant liquid was recovered and diluted using deionized water to give a sugar concentration in the region 3 g/l to 5 g/l. The liquid was then filtered through a 0.45 µm membrane using a Swinney Filter Kit (Millipore Corporation).

The detector response was linear over the range 0 to 5 g/l using a 50 µl injection volume. Quantitation was performed by peak height

measurement and reference to a standard curve.

METHOD II

A Waters Associates liquid chromatograph (Model ALC/GPC 244) equipped with a solvent delivery system (Model 6000A) and a Wisp Autoinjector (Model 710-B) was used (Waters Associates).

A BIO-RAD HPX-87C column was used for the analysis. The detector was a differential refractometer (Model R401, Waters Associates). The column temperature was 85°C and a solvent of double-distilled water at a flowrate of 0.6 ml/min was used. The sample injection volume was 15 µl and the average result of duplicate injections was reported. A Hewlett-Packard Integrator (Model HP 3390A) was used to measure peak areas and compare sample peak areas to those of the appropriate sugar of known concentration.

3.6 CELL IMMOBILIZATION

3.6.1 Spore Production for Immobilization

Stock spore suspension (0.1 ml or 0.3 ml) was added to freshly autoclaved cheese whey permeate medium (25 ml) (cheese whey permeate, 60 g/l, and yeast extract, 5 g/l, initial pH 6.5), heat-shocked at 75°C for 2 min, then cooled in ice water for 1.5 min prior to incubation in an anaerobic jar at 30°C. After 24-30 h incubation, this motile culture was used to inoculate fresh medium (100 ml) which was incubated for 18-24 h in an anaerobic jar at 30°C, prior to adding a 1% (v/v) inoculum of this culture to the fermenter.

A 20S Biolafitte fermenter apparatus (Biolafitte, Poissy, France) of 18-litre working volume was used. Medium (as above) was indirectly sterilized to 90°C, then directly sterilized with live steam and held at 115°C for 20 min. After cooling to 30°C, oxygen-free nitrogen gas was bubbled through the medium and the fermenter was inoculated. The nitrogen flow was stopped at the first sign of gassing due to bacterial growth in the medium. The fermenter agitator speed was maintained at 80 rpm during the fermentation.

Samples were withdrawn at regular intervals for evidence of spore formation by microscopic examination. Spores were harvested after 120-144 h fermentation (>50% spore formation) using a Dijkstra Labofuge 15000 continuous centrifuge. The culture was cooled to 15°C prior to spore recovery, while the centrifuge bowl was sterilized with live steam for 15 min prior to use.

Spore paste was recovered from the centrifuge bowl and refrigerated overnight (4°C) prior to immobilization.

3.6.2 Immobilization in Calcium Alginate

Immobilization was carried out under non-aseptic conditions in an aerobic environment.

Wet spore paste (20 g or 60 g) was suspended in 1 litre of deionized water. Sodium alginate (80 g) was added slowly over a 60 min period. A high speed turbine mixer was used to provide good mixing and to minimize lump formation. The viscous alginate spore suspension was then passed through a fine mesh powder sieve screen to ensure that any undissolved lumps of >0.5 mm were removed.

The suspension was pumped through a device containing 6 hypodermic needles in parallel, operated with a concentric air flow, into a bath containing 20 g/l $CaCl_2.2H_2O$ solution (Figure 3.1). The bath was continuously stirred using a magnetic stirrer.

Calcium alginate beads of 1.5-2.0 mm diameter were formed almost instantaneously. The 20 g/l $CaCl_2.2H_2O$ solution was periodically replaced to ensure an excess of calcium ions for the sodium/calcium ion exchange reaction to take place. The beads were kept in this solution for approximately 20 h at 4°C under mixing using a magnetic stirrer, prior to transferring to a 5 g/l $CaCl_2.2H_2O$ solution for storage at 4°C in a sealed container.

Close control of the bead size and size distribution was achieved by various process adjustments during operation of the immobilization equipment (Figure 3.1). The viscosity of the sodium alginate spore suspension was occasionally reduced by dilution using deionized water

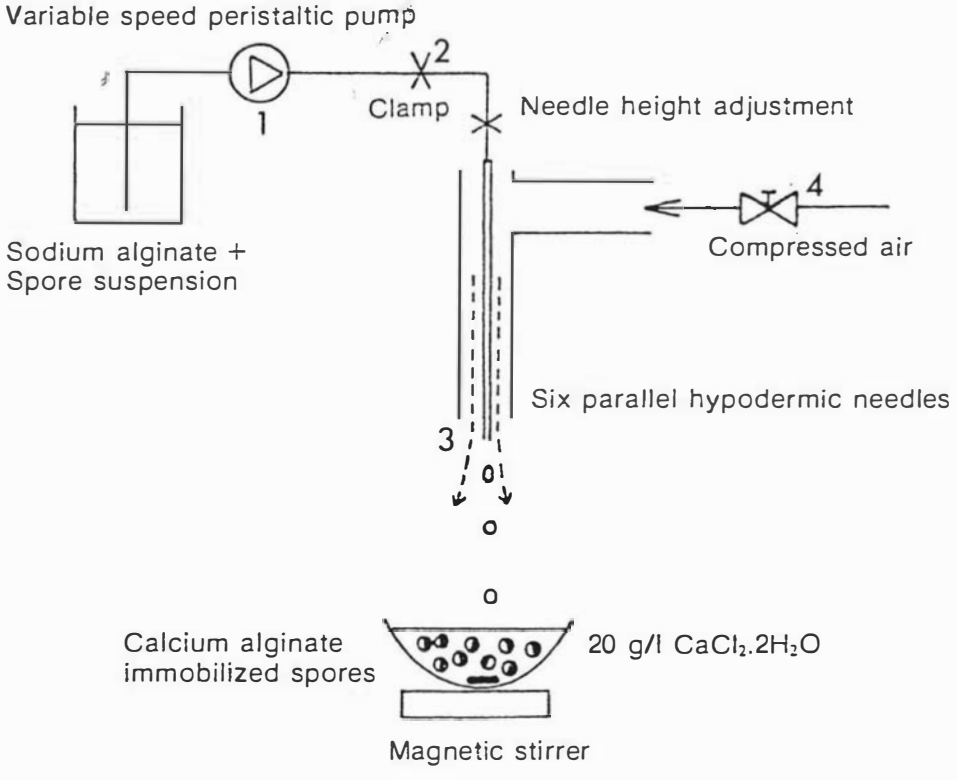


Figure 3.1. A schematic diagram of the immobilization equipment.

(<5% dilution). Additionally, adjustments in the suspension flowrate (1), individual immobilization needle flowrates (2), needle height adjustment, that is, the distance between the end of the needle and the concentric air flow tube (3), and the air flowrate (4), were made initially on a trial-and-error basis in order to achieve steady-state operating conditions.

3.7 FERMENTATION CULTURE CONDITIONS

3.7.1 100-ml Scale 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 (Section 3.1.4) 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 cooling in an ice-water bath for 1.5 min (omitted for strain ATCC 824) and incubated at 30°C until vigorous gassing occurred.

For experiments using semi-synthetic medium, 1 ml of the revived culture was then transferred to 20 ml of medium (Table 3.1) containing the appropriate sugar, and incubated at 30°C for 24 h or 48 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 30°C for 48 h. One ml of this culture was then transferred to 20 ml of whey permeate and incubated at 30°C for 24 h prior to transferring a 5 ml inoculum to 100 ml of whey permeate.

For experiments using lactose-hydrolysed sulphuric acid casein whey permeate, 1 ml of the revived culture was transferred to 20 ml of Cooked Meat Medium supplemented with glucose (5 g/l) and galactose (5 g/l) and incubated at 30°C for 48 h. One ml of this culture was then transferred to 20 ml of hydrolysed whey permeate and incubated at 30°C for 24 h prior to transferring a 5 ml inoculum to 100 ml of hydrolysed whey permeate.

Samples (5 ml) were withdrawn from the fermentations at 24 h intervals using a sterile pipette. Prior to sampling, the screw cap on the bottle was tightened and the bottle was inverted three times to obtain a homogeneous sample.

3.7.2 Preparation of Inoculum for Batch Fermentation Cultures

Spore stock of C. acetobutylicum P262 (0.1 ml) was transferred to 20 ml of Cooked Meat Medium supplemented with lactose (10 g/l) and heat shocked at 75°C for 2 min, then cooled in an ice-water bath for 1.5 min prior to incubation at 34°C in an anaerobic jar. After 18-24 h incubation, vigorous gassing and a highly motile culture were observed. This culture was used to inoculate sulphuric acid whey permeate medium (Table 3.2), either 20 ml or 100 ml depending on the experiment, and incubated at 34°C. The inoculum ratio was varied between 0.1 to 1.0% (v/v) so that the most highly motile culture could be selected by microscopic examination and used as the inoculum for the final fermentation (0.5-1.0% (v/v) inoculum).

3.7.3 Batch Fermentation Culture

3.7.3.1 2-litre Fermentation Apparatus

The basic fermenter used 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 1.6 litre working volume.

Continuous pH measurement and one way control of pH were performed by an Automatic Mini pH Control System (New Brunswick Scientific Co.) consisting of a pH 40 Controller module, a XpH 42 Pump module and a pH 40 Recorder module. The culture pH was measured by a EIL 33 1070 030 toughened glass electrode and an EIL 33 1320 210 laboratory sealed reference electrode (Electronic Instruments Ltd, Richmond, Surrey, England). Where necessary, ammonia solution (1M or 3M) was used for pH control. A permanent record of culture pH could be printed on the strip chart recorder module.

3.7.3.2 7-litre Fermentation Apparatus

The fermentation apparatus was constructed in the Biotechnology Department, Massey University. The fermenter vessel used was a 7-litre pyrex glass vessel (New Brunswick Scientific Co.) with a working volume of 5 litres, and was provided with a stainless steel head containing parts for the insertion of the pH probes.

Continuous pH measurement and one way control of pH were performed using pH electrodes (Section 3.7.3.1) connected to a Horizon pH Controller (Ecology Co., Oak Park Avenue, Chicago, Illinois, U.S.A., Model 5997-20). The pH controller was connected to a Honeywell Versaprint multipoint chart recorder (Amiens, France). Where necessary, ammonia solution (3M or 5M) was used for pH control and was delivered using a Masterflex peristaltic pump (Cole Palmer Instrument Co., Chicago, Illinois, U.S.A.).

3.7.3.3 Batch Fermenter Operation

The fermenter vessel (2 l or 7 l) 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 swept across the medium surface. Gas sweeping was continued until good gassing due to microbial growth was observed.

When the fermentation culture was at 34°C, the medium pH was adjusted as required using ammonia solution, and then inoculated with motile cells prepared as described in Section 3.7.2. Agitation (50 rpm) was used to gently mix the vessel contents at this time. The culture was unagitated, except periodically at 100 rpm for approximately 1 min prior to taking samples and during times of high ammonia solution demand. A thermostated water bath was placed underneath the fermenter base to ensure this portion of the fermenter remained at 34°C.

Sampling was achieved by using the oxygen-free nitrogen gas to pressurize the culture vessel and, by blocking the gas outlet, force sample out. Culture was initially withdrawn into a measuring cylinder

to clear the sample line prior to taking a 5 ml sample. When operating with pH control, the total volume of culture removed during sampling and the volume of ammonia solution added were recorded at the time of sampling.

Antifoam emulsion (10% v/v, Dow-Corning Antifoam AF) was manually added as required to suppress foaming in the culture.

Prior to each fermentation, the pH electrodes were calibrated using pH 4.0 and pH 7.0 buffer solutions. After each fermentation the electrodes were washed and soaked in 0.1M HCl containing pepsin for 2-4 h to remove contaminating proteins from the electrode. When not in use, the probes were stored in pH 7.0 buffer solution. The pH value of fermentation samples was independently measured (Section 3.5.1) in order to check the fermenter pH measurement, and any discrepancies were corrected.

3.7.4 Continuous Fermentation Using Immobilized Cells

3.7.4.1 Continuous Stirred Tank Reactor (CSTR) Apparatus

The experimental apparatus described in the following pertains to that used at Massey University. A very similar experimental apparatus was used (initially) at the Delft University of Technology and is described in Appendix 9.

The fermenter used was a Multigen F2000 Benchtop culture apparatus (New Brunswick Scientific Co.), equipped with a 2 litre pyrex glass vessel of 0.6 litre working volume. The vessel used a polyethylene-polypropylene head containing holes for probe insertion. Figure 3.2 shows a schematic diagram of the fermenter unit and its ancillary equipment.

The fermenter contents were agitated at 180-200 rpm by an indirect magnetic coupling, using a 4-bladed impeller mounted 3 cm above the base of the vessel. This agitation speed was the minimum required to provide complete bead movement and prevent settling.

Temperature control was by means of a heating element. A thermometer was used as a visual 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 (Ecology Co.). A Masterflex peristaltic pump (Cole Palmer Co.) was used for delivery of 5% (v/v) ammonia solution to the fermenter vessel.

Feed medium was continuously fed to the top of the fermenter using a Masterflex peristaltic pump. The fermenter contents level was controlled using a fixed level teflon filter with 7 x 1 mm slits, connected to a constantly running Masterflex peristaltic pump. Fermentation samples were periodically removed via this exit line.

The feed medium was kept anaerobic by either sparging or sweeping the medium surface with oxygen-free nitrogen gas. The fermenter was kept anaerobic during the start-up phase by flushing nitrogen gas across the culture surface. Silicone antifoam (10% (v/v) solution) was manually added as required to suppress foaming.

The fermentation start-up procedure was as follows: The empty fermenter vessel was sterilized (121°C, 15 min), cooled, and filled with alginate beads suspended in ethanol:water, 1:1, and stirred at 200 rpm for 30 min. This treatment was used to deactivate any contaminants introduced during the non-aseptic immobilization procedure (Section 3.6.2) and to activate spore germination (Krouwel et al, 1981). The pH probe was inserted into the fermenter head during this treatment.

The ethanol:water solution was pumped from the fermenter and the alginate beads were then washed three times with approximately 0.6 l of sterile 5 g/l $CaCl_2.2H_2O$ solution and three times with sterile cheese whey permeate fermentation medium (Table 3.3). A 2.5 cm x 0.5 cm teflon filter with multiple 0.50 mm slits, situated at the base of the fermenter vessel, was used to effect liquid entry and removal without bead removal.

The fermenter was filled (0.6 litre) with cheese whey permeate. The pH value of the fermenter contents was adjusted to pH 5.8-6.0 using ammonia solution and controlled at this value for 22-26 h. The dilution rate during this time was maintained at less than or equal to $0.1h^{-1}$. Spore germination, as observed by gas release from the beads, and/or

floating beads (<1%) occurred after this time. The pH control was stopped and the dilution rate was increased to 0.15 - 0.20 h^{-1} as appropriate. The concentration of fermentation products was closely monitored in order to determine the procedure for increasing the dilution rate.

3.7.4.2 Fluidized Column Reactor (FCR) Apparatus

The vertical fluidized column reactor apparatus is schematically depicted in Figure 3.3. The unjacketed column was 42 cm high, and 4 cm in diameter for 36 cm of its height, widening to 5.5 cm for the remaining 6 cm. The total working volume of the reactor was 0.45 l.

A porous plate containing evenly distributed apertures of 0.5 mm diameter was situated 6 cm above the column base and supported the alginate beads. Liquid mixing and particle fluidization was facilitated by recirculating medium using a peristaltic pump (Watson-Marlow Limited, Falmouth, Cornwall, England. Type 603 UR 165). The column was inclined at a 10° angle from vertical to enable good degassing and medium mixing to occur.

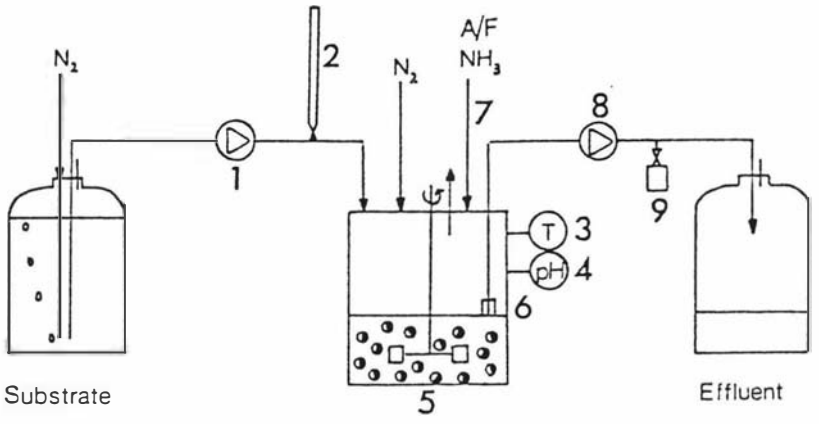
Feed medium was continuously fed to the top of the column using a Masterflex peristaltic pump. Effluent outflow was controlled via a fixed level teflon filter as described in the previous section.

Temperature control was by means of a thermostated circulating water bath and a heating coil wrapped around the column.

Silicone antifoam (10% (v/v) solution) was added to the top of the column via a peristaltic pump using a timer mechanism to control the interval between each dose and the duration of dosing.

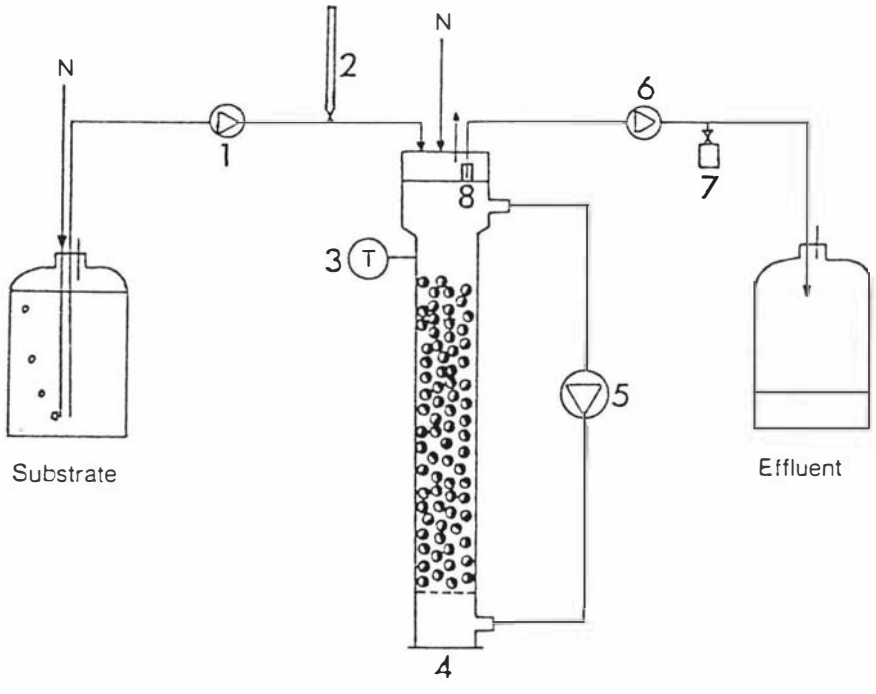
The feed medium was kept anaerobic by sparging with oxygen-free nitrogen gas. The fermenter was kept anaerobic during the start-up phase by flushing oxygen-free nitrogen gas across the culture surface.

The fermentation start-up procedure was identical to that described for the CSTR in Section 3.7.4.1, except that semi-synthetic medium containing glucose (Table 3.4) was used on the feed medium. pH



1 Feed pump
2 Flow measuring device
3 Temperature control
4 pH control/monitoring
5 2.0 L fermenter
6 Fixed outlet filter
7 pH control/antifoam addition
8 Exit pump
9 Sampling device

Figure 3.2. A schematic diagram of the continuous stirred tank reactor and ancillary equipment used with immobilized cells.



1 Feed pump
2 Flow measuring device
3 Temperature control - heating tape
4 Column reactor
5 Recirculation pump
6 Exit pump
7 Sampling device
8 Fixed outlet filter

Figure 3.3. A schematic diagram of the fluidized column reactor and ancillary equipment used with immobilized cells.

control during start-up was not used. The initial dilution rate was approximately $<0.1 h^{-1}$ and was increased after 24 h as necessary. Spore germination as evidenced by gassing occurred after this time.

3.7.4.3 Determination of Alginate Bead Fraction

The fraction of alginate beads in the reactor ($1-\varepsilon$) was determined by measurement of the total and liquid volumes. Beads were placed into a fine mesh "fish net" and gently squeezed by hand to remove entrained moisture. Compression was not so severe as to alter the bead shape or integrity. The beads were then placed into a measuring cylinder containing a known volume of distilled water. The total volume minus the known initial volume equals the bead volume.

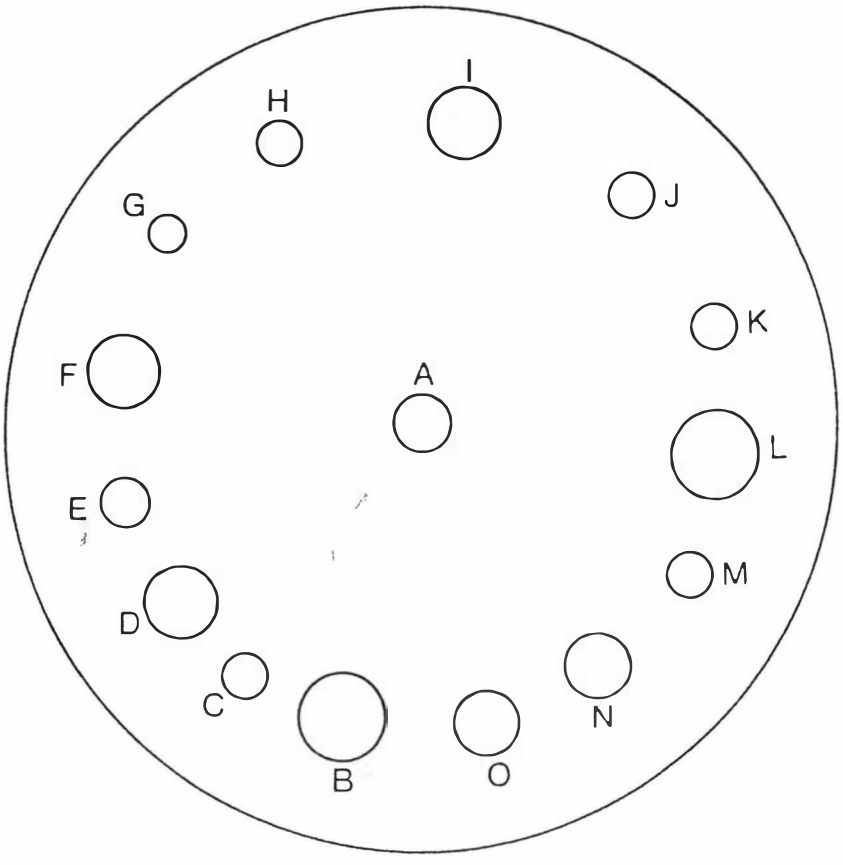
3.7.5 Continuous Fermentation Using Free Cells with External Cell Recycle by Cross-Flow Microfiltration (CFM)

3.7.5.1 Plate and Frame CFM Apparatus

A Multigen F2000 Benchtop culture apparatus was used (Section 3.7.4.1) with a 2 litre vessel of 0.4 l working volume. Figure 3.4 gives a diagram of the fermenter head, and Figure 3.5 and Figure 3.6 show a schematic diagram and a photo respectively, of the fermenter cell recycle apparatus plus its ancillary equipment.

A Horizon pH Controller was used for pH control in conjunction with a Masterflex peristaltic pump for ammonia solution delivery and a Kent combination pH electrode. A heating element was used to control the fermenter temperature. After sterilizing, the fermenter was cooled using a thermostated waterbath under a nitrogen atmosphere. The fermenter was inoculated (1% v/v) and maintained at the desired temperature in the water bath for 5 hours prior to connecting it to the fermenter apparatus. Agitation was maintained at 50 rpm and was provided by a single 6-bladed disc turbine mounted 3 cm above the vessel base. A culture volume of 1.2 l was used for the initial batch fermentation phase.

The Plate and Frame CFM system was a Pellicon® cassette system (Millipore Corporation, Bedford, Massachusetts, U.S.A., Part No. XX42



A Agitator shaft
B Conductivity level probe/Inoculation port
C Thermometer
D Culture feed line to CFM unit
E Antifoam inlet
F Filtrate return line from CFM unit
G Thermocouple/Temperature control
H Ammonia solution inlet
I Culture (concentrate) return from CFM unit
J Medium-feed inlet
K N_2gas-outlet
L pH probe
M N_2 gas-inlet
N Heating element
O Biomass removal

Figure 3.4. The cell recycle (CFM) fermenter head.

00060 high volume cell) obtained via Smith-Biolab Ltd (Auckland, New Zealand). The unit was constructed of acrylic.

The high volume cell employs a plate and frame design which manifolds incoming fluids, distributing them in tangential-flow paths across parallel layers of membrane. A recirculation flowrate of 3 L/minute/m^2 is recommended for the Millipore membrane cassettes to minimize polarization or filter fouling (Operation Manual OM 029, Millipore Corporation). No back pressure was applied to the system. The initial retentate to filtrate flow ratio was approximately 3:1 using distilled water and a clean membrane.

Two cross-flow microfiltration microporous membrane cassette systems were independently used in the Pellicon system:

(a) Durapore cassette module (Millipore Corporation) of 0.5 m^2 membrane area and 0.45 μm pore size. A coarse retentate screen (HVLP 000C5) was used.

(b) Ultrasart II cassette system (Sartorius GmbH, Gottingen, Federal Republic of Germany, obtained via Medic DDS Ltd, Lower Hutt, New Zealand), of 0.7 m^2 membrane area and 0.2 μm pore size.

A Procon® positive displacement rotary vane pump (Metone, Victoria, Australia) connected to a fixed speed AC motor (1425 rpm) was used to feed the Pellicon CFM system. This pump had a flowrate of approximately 4.5 l/min against a 69 kPa back pressure. Black polypropylene tubing was used to connect the fermenter to the rotary vane pump and Pellicon CFM system.

After a batch fermentation for 24-28 h, the fermenter was aseptically connected to the CFM device via quickfit glass connectors. The fermenter volume was reduced by flushing out the CFM unit to displace the hold-up volume, prior to connecting the two units. Oxygen-free nitrogen gas was swept across the culture surface during continuous operation.

Feed medium supply and fermenter culture removal from the fermenter were accomplished using Masterflex peristaltic pumps.

Legend

1 Medium reservoir
2 Medium feed pump
3 Medium feed flow measurement
4 Nitrogen gas supply
5 Gas filter
6 Nitrogen gas regulator
7 Biomass effluent container
8 Biomass removal pump
9 Fermenter
10 CFM recirculation pump
11 Millipore Plate and Frame CFM unit
12 Conductivity probe
13 Conductivity level control
14 Effluent pump (filtrate)
15 Effluent container
16 Filtrate holdup/overflow
17 pH controller
18 pH recorder
19 Ammonia solution pump
20 Ammonia solution reservoir

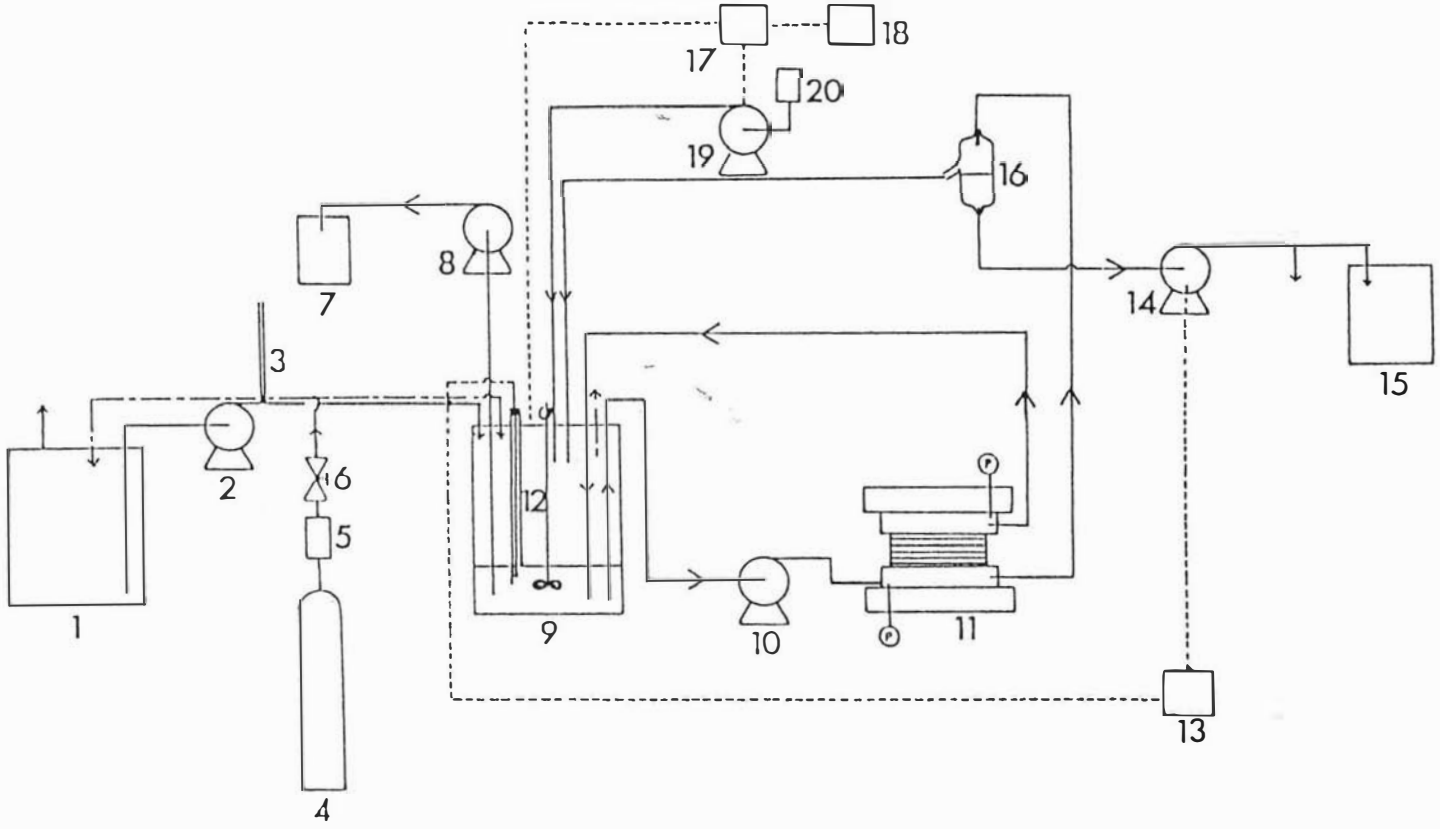


Figure 3.5. A schematic diagram of the fermenter and ancillary equipment used for continuous fermentation using external cell recycle with a Millipore® Plate and Frame cross-flow microfiltration (CFM) unit.

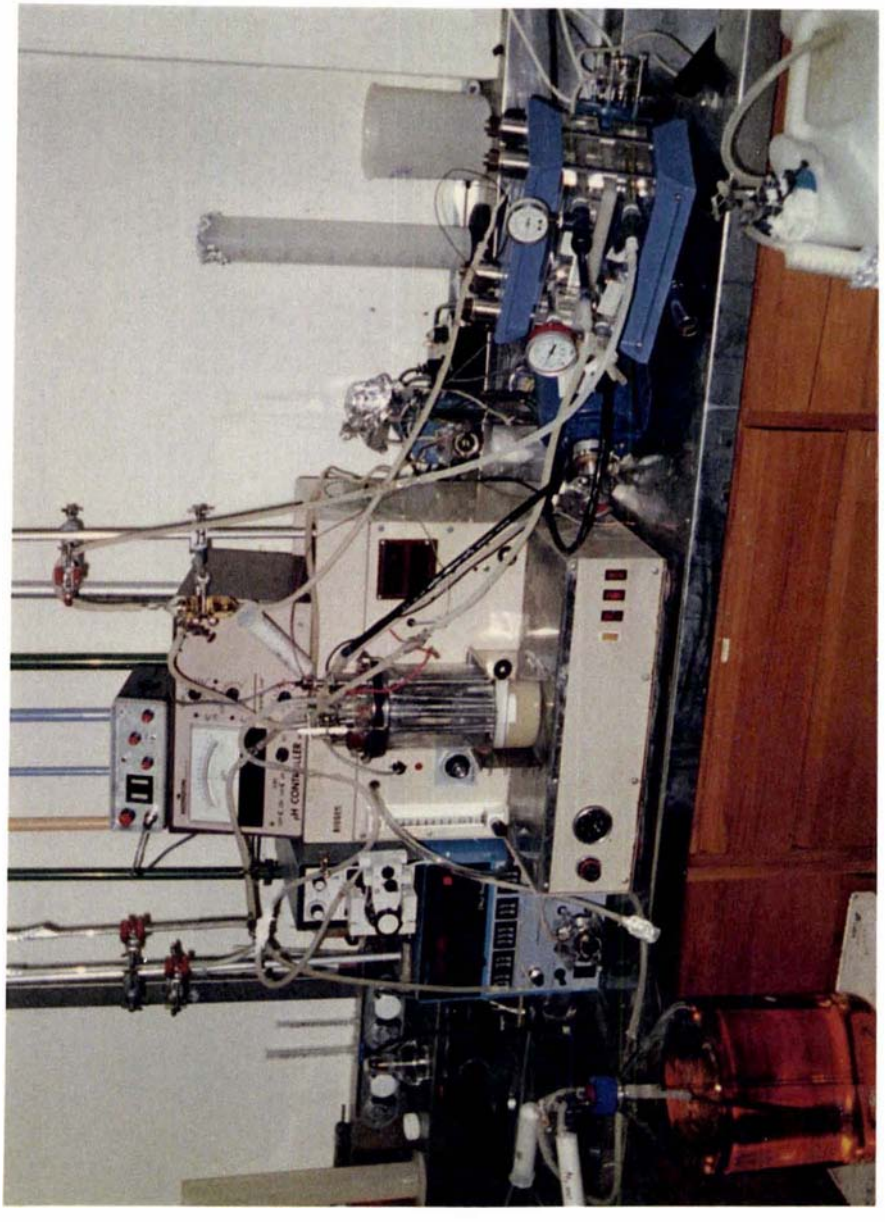


Figure 3.6. A photo of the fermenter - Plate and Frame CFM apparatus.

A three-contact conductivity probe plus conductivity level controller apparatus were used to control the culture level in the fermenter (Appendix 2). The culture volume was maintained constant in the apparatus by maintaining the sum of the culture (biomass) removal rate and filtrate removal rate equal to the medium feed rate (overall volumetric dilution rate). Filtrate was removed from the fermentation system using a peristaltic pump connected to the conductivity level controller apparatus and using a 50 ml reservoir with overflow arm to return filtrate back to the fermenter. Hence, the overall dilution rate could be varied up to the maximum capacity of the filter device. Samples of the fermenter culture for biomass determination or microscopic examination were taken from the concentrate return line to the fermenter via a bleed valve.

The microporous membrane cassette systems were flushed with distilled water after use. They were then cleaned by recirculating 0.1M sodium hydroxide solution for 10 minutes. This step was repeated 2-3 times. Membranes were stored in 0.1M sodium hydroxide between use (Operation Manual OM 029, Millpore Corporation).

3.7.5.2 Hollow Fibre CFM Apparatus

A Multigen F2000 Benchtop culture apparatus was used with a 2 litre vessel of 0.35 l working volume. The fermenter apparatus and ancillary equipment was the same as described in Section 3.7.5.1 except for the CFM membrane system and the CFM recirculation pump.

An Amicon Diaflo® Hollow Fibre Cartridge (Type H1MP01-43) fitted in a DH2 hollow fibre cartridge holder equipped with an inlet pressure gauge was obtained from Amicon Corporation (Danvers, Massachusetts, U.S.A.) through N.Z. Medical and Scientific Ltd (Auckland, New Zealand). The CFM cartridge (0.03 m^2 membrane area) contained 55 fibres of 1.1 mm diameter constructed of polysulfone with a pore size of 0.1 μm. The hollow fibre cartridge had a maximum inlet operating pressure of 205 kPa. A backpressure valve on the DH2 cartridge holder outlet was used to control the back pressure. The recommended recirculation rate was 0.6-1.8 l/min. (Amicon Publication No. 1-116C).

A peristaltic pump (Watson-Marlow Ltd, Falmouth, Cornwall, England, Type 603 UR 165) was used to circulate the fermenter culture to the CFM apparatus. Norprene tubing (Norton Company, Worcester, Massachusetts, U.S.A.) was used in this peristaltic pump.

The apparatus was operated as described in Section 3.7.5.1.

3.7.5.3 Tubular CFM Apparatus

The fermenter apparatus used is described in Section 3.7.3.2. The fermenter vessel was a 7 litre pyrex glass vessel (New Brunswick Scientific Co.) of 1.1-1.2 litre working volume. Figure 3.4 gives a diagram of the fermenter head with the exception that temperature control was provided using internal coils rather than a heating element. Agitation was maintained at 50 rpm and was provided by a single 4 flat bladed impeller (8 cm diameter) situated 6 cm above the base of the fermenter. Agitation was stopped when cell recycle was commenced. A schematic diagram and photo of the fermenter cell recycle apparatus plus ancillary equipment are depicted in Figures 3.7 and 3.8 respectively.

A Horizon pH Controller was used for pH control in conjunction with a Masterflex peristaltic pump for ammonia solution delivery and a Kent combination pH electrode. A Versaprint recorder was used to continuously record fermenter temperature and a Sekonic recorder to continuously record the culture pH.

Antifoam solution (10% (v/v) Bevaloid 5901) was manually or automatically added via a peristaltic pump using an electronic timer (Biotechnology Department, Massey University) to control the duration of, and interval between additions.

Variable speed peristaltic pumps (Masterflex) were used for feed medium metering, fermenter culture removal from the fermenter, and filtrate removal from the system.

A Norton Ceraflo® assymetric ceramic microfilter cartridge (0.1 m^2 membrane area) was obtained from the Norton Company (Worcester, Massachusetts, U.S.A.) via the Amicon Corporation (Danvers, Massachusetts, U.S.A.) through N.Z. Medical and Scientific Ltd

(Auckland, New Zealand). The CFM cartridge contained 28 tubes of 0.28 cm diameter constructed of sintered alumina with a surface pore size of 0.45 μm. This membrane cartridge could be steam sterilized and operated at temperatures up to 750°C and pressures up to 1550 kPa. The maximum flowrate was 50 litres/minute.

The membrane cartridge was constructed of 316 l stainless steel and equipped with standard sanitary clamp fittings to connect the cartridge to the feed pump. Teflon fittings were used on the filtrate outlets.

An Amicon Lab-Scale variable speed motor (Amicon Corporation, Model DC 10LA) fitted with an Albin gear pump unit (Albin Ltd, Georgia, U.S.A., Type RBS-4-83150) was used to feed the CFM cartridge. This pump had a maximum flowrate capacity of 30 litres/min at 170 kPa and a maximum discharge pressure of 310 kPa. The CFM feed and return lines were placed below the fermenter culture level.

The pressure differential across the membrane was maintained at 14-17 kPa. A ball valve on the concentrate outlet was used to provide backpressure for filtrate flow. Flow through the CFM cartridge was maintained as high as practically possible for continuous operation of the apparatus.

A three-contact conductivity probe plus conductivity level controller apparatus (Appendix 2) was used to control the culture level in the fermenter operated as described in Section 3.7.5.1.

Initially the fermentation was operated in batch mode (4 litre culture volume) under pH control at a value of pH 5.4-5.6. At the commencement of cell recycle the fermenter was connected to the cell recycle system and the hold up volume of the unit displaced to waste. Medium feed was commenced. Oxygen-free nitrogen gas was swept across the culture surface during continuous operation. Culture removal was commenced at a later time.

During continuous operation the Ceraflo membranes were "cleaned" by a technique known as backflushing. This membrane self-cleaning mode of operation is designed to reduce membrane fouling and facilitate long

term operation. This was controlled using an electronic sequence timer (Biotechnology Department, Massey University). A separate electronic timer controlled the interval between backflush operations. Once activated, an energised open solenoid valve (Honeywell Inc, Skinner Valve Division, Connecticut, U.S.A.) was closed, blocking the filtrate flow and allowing the pressure to rise in the filtrate side of the membrane cartridge and equilibrate at the transmembrane pressure (average of the inlet and outlet pressure). Concurrently the feed medium pump was stopped. After allowing the pressure to increase on the filtrate side a second solenoid valve was opened (0.5 - 2 seconds) and a nitrogen gas purge (140-205 kPa) was used to force filtrate in the filtrate line back into the membrane cartridge. This valve was then shut and after a 10 second delay the other solenoid valve reopened to allow filtrate to flow back to the fermenter. After a further delay sequence ($1_{\frac{1}{4}}$ min - 5 min) the feed medium pump was restarted. This delay enabled the membrane cartridge to remove by filtration that volume of filtrate forced by backflushing back into the concentrate side of the membrane and hence into the fermenter.

A portable fan placed in front of the pump, was used to assist fermenter temperature control by removing heat input from the CFM pumping operation.

The membrane cartridge was cleaned by the following procedure:

1. Cold water flush/5 min
2. 2.0% (w/v) sodium hydroxide/60°C/30 min recirculation
3. Hot water flush/60°C/5 min
4. 0.5% (w/v) nitric acid/60°C/30 min recirculation
5. Cold water flush/5 min

and stored in distilled water prior to use. The cartridge and lines were left in 0.5% (w/v) sodium hydroxide solution overnight prior to use, then flushed with distilled water prior to connection with the fermenter.

Legend

1 Effluent container
2 Sampling
3 Effluent pump (filtrate)
4 Filtrate holdup/overflow
5 Conductivity level control
6 Conductivity probe
7 Ceraflo Tubular CFM unit
8 CFM recirculation pump
9 Pressure tube for backflush
10 Solenoid valve : nitrogen gas pulse
11 Solenoid valve : filtrate pressure buildup
12 Electronic sequence control
13 Electronic timer
14 CFM backpressure valve
15 Fermenter
16 Medium feed flow measurement
17 Medium feed pump
18 Medium reservoir
19 Antifoam reservoir
20 Antifoam pump
21 Antifoam electronic timer
22 Nitrogen gas supply
23 Gas filter
24 Ammonia solution reservoir
25 Ammonia solution pump
26 pH controller
27 pH recorder
28 Temperature recorder
29 Nitrogen gas regulator
30 Biomass removal pump
31 Biomass effluent container

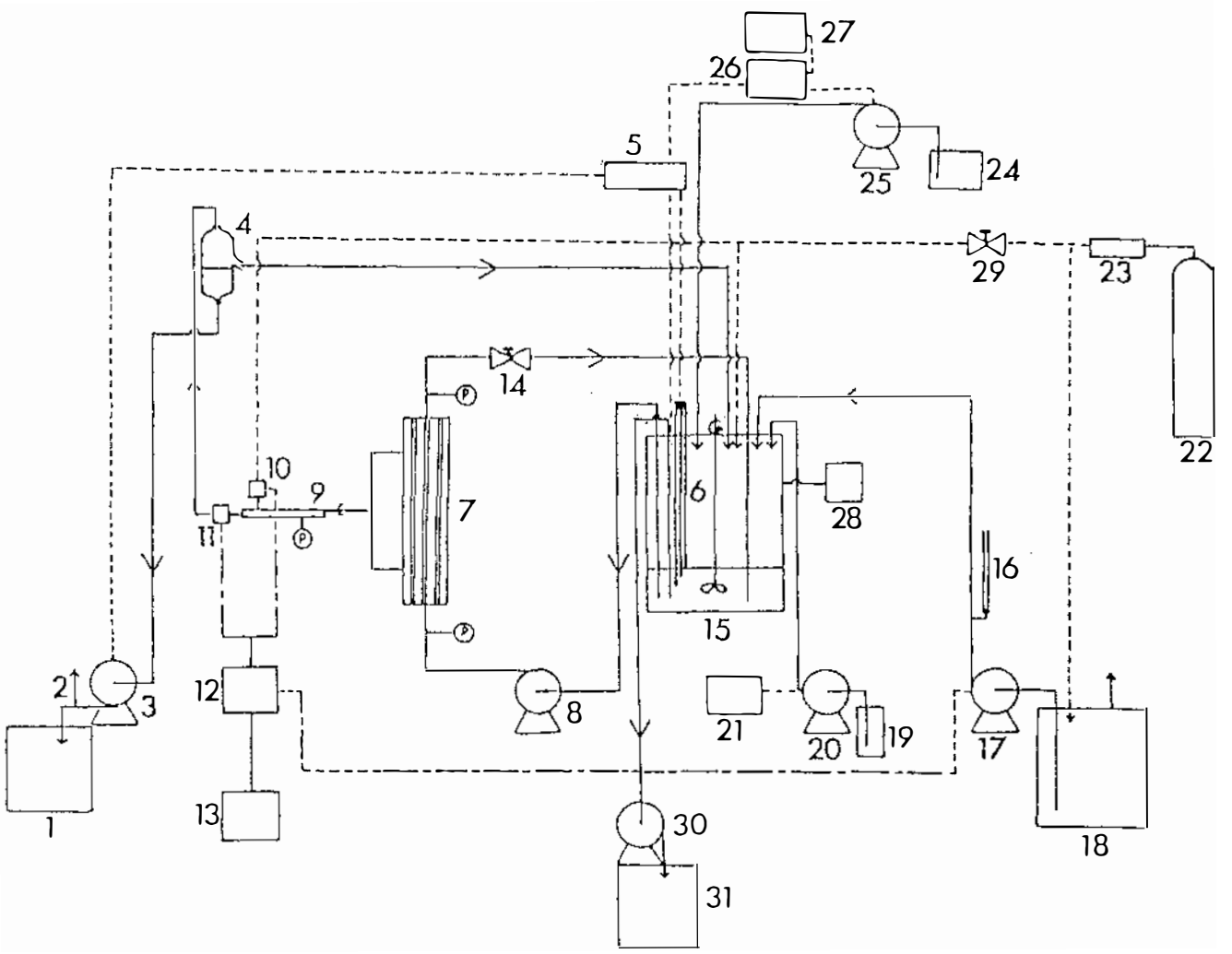


Figure 3.7. A schematic diagram of the fermenter and ancillary equipment used for continuous fermentation using external cell recycle with a Ceraflo® Tubular cross-flow microfiltration unit.

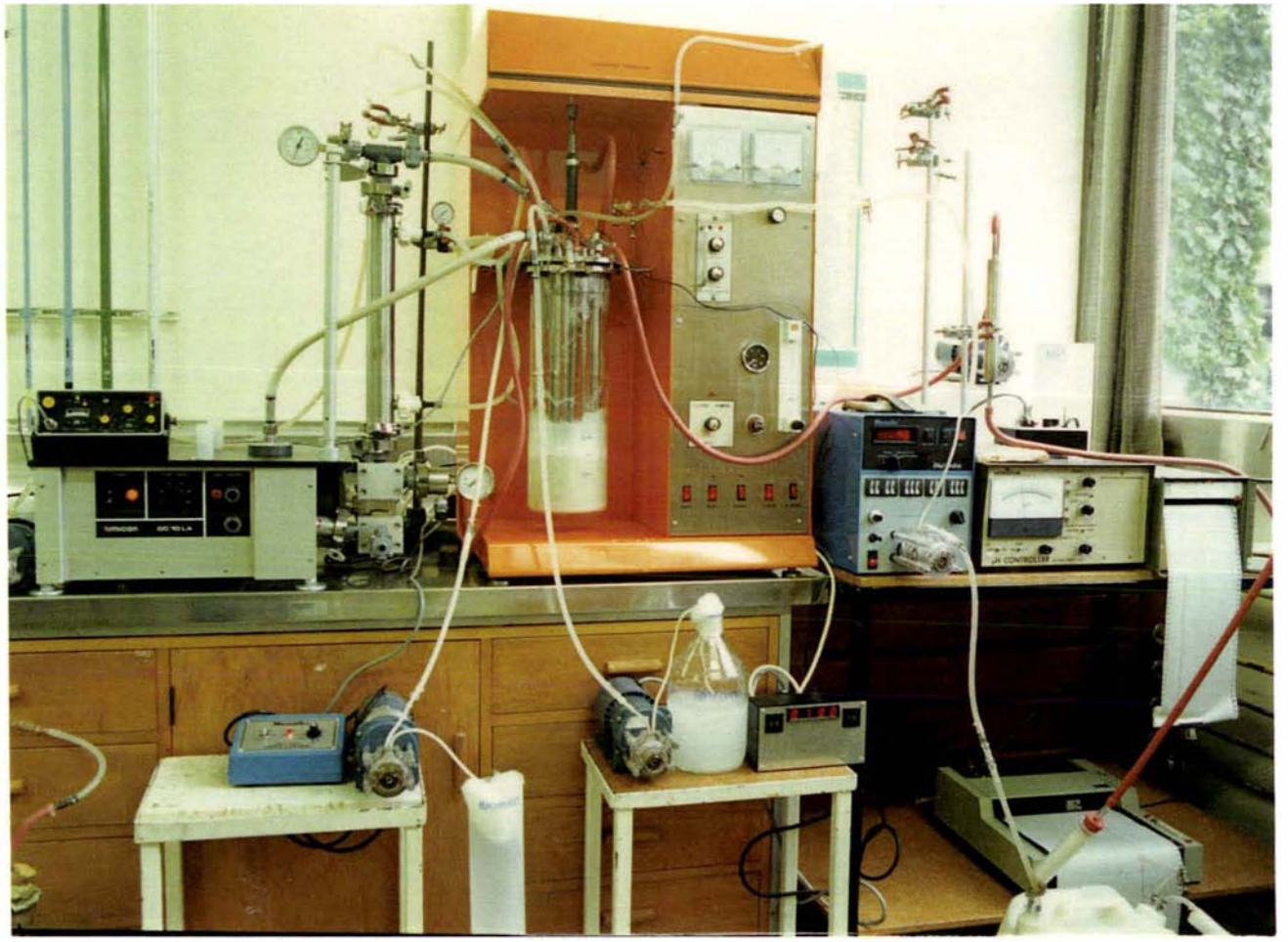


Figure 3.8 A photo of the fermenter - Tubular CFM apparatus.

3.7.6 In-Situ Gas-Stripping/Condensation for Solvents Recovery

The Multigen F2000 Benchtop culture apparatus (New Brunswick Scientific Co.) using a 2 litre capacity pyrex glass vessel of 1.2 litre working volume with a polyethylene-polypropylene head, was used. Figure 3.9 shows a schematic diagram of the experimental apparatus.

Continuous pH measurement and one way control of pH were performed using an Automatic Mini pH Control system as described in Section 3.7.3.1. A Honeywell Versaprint multipoint chart recorder was used to continuously record the culture pH and temperature.

Agitation was maintained at 50 rpm and was provided by an impeller assembly of two, 6-bladed disc-turbine impellers mounted at 3 cm above the vessel base and 3 cm below the liquid surface. At the commencement of gas-stripping, oxygen-free nitrogen gas was introduced into the fermenter via the agitation shaft through 8 sparge holes (0.5 mm diameter) at its base. Exit gas was passed from the fermenter to the condenser apparatus (10 litre) containing a cold finger, prior to being recirculated to the fermenter using a twin head Masterflex peristaltic pump. The cold finger (30 cm x 6 cm ID) contained solid carbon dioxide (approximate sublimation temperature = -60°C) which was periodically replaced. Tubing associated with the nitrogen-gas recirculation loop had a wall thickness of 2.5 mm to minimize oxygen diffusion or diffusivitive gas losses.

Nitrogen gas inlet/outlet bubble meters were used to ensure that a positive nitrogen flow operated in the recycle system and that no air was drawn in.

At the completion of the experiment, the condenser apparatus was disassembled and the frozen condensate was scraped off, or allowed to thaw from the cold finger and was analyzed.

Antifoam emulsion (10% w/v, Dow Corning) was added to the culture using an automatic foam control system, and was used to suppress the severe foaming generated by the in-situ gas-stripping. When foam contacted the stainless steel sensor, the completed foam controller circuit (Design Electronics, Palmerston North, New Zealand) activated a

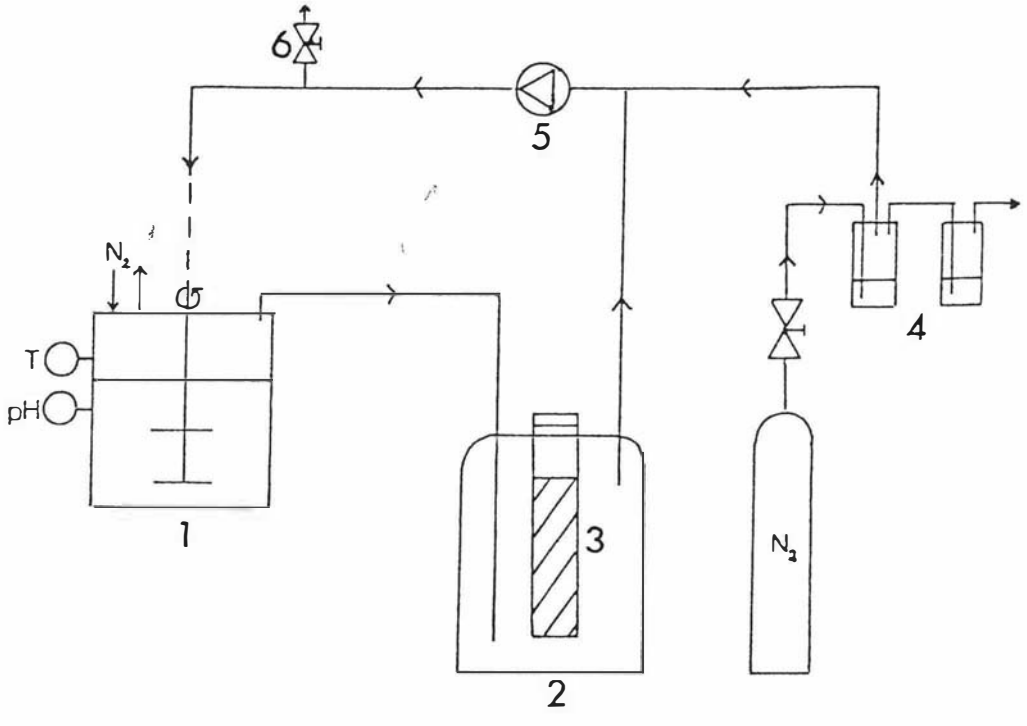


Figure 3.9. A schematic diagram of the integrated batch fermenter - gas stripping/ condensation apparatus. 1 fermenter; 2 condenser; 3 cold finger; 4 gas inlet/outlet; 5 gas recycle pump; 6 gas exit.

Masterflex peristaltic pump to add a small dose of sterile antifoam solution to the fermenter. Foam collapse caused the circuit to be broken and the pump to stop. To avoid excessive antifoam addition, the foam controller power input was activated 1 minute out of every 15 minutes by an electronic timing device (Biotechnology Department, Massey University). Therefore two conditions had to be met to facilitate antifoam addition, first, the foam had to contact the sensor and second, the controller had to be activated by the electronic timer.

3.8 BATCH FERMENTATION DILUTION CORRECTION CALCULATION

In some batch fermentations, pH-control was used. The total culture volume removed during sampling, and the pH correctant solution volume added, were measured with time. These data were used to determine correction factors which were used to adjust analytical data for the calculation of fermentation parameters, so as to enable an equitable comparison with parameters obtained from batch fermentation experiments where no pH-control was used. The following equations were used:

Let: t = time
V = volume of culture (in fermenter) at time t_i
s = absolute substrate/product concentration
N = volume of pH correctant solution (ammonia) added between t_{i-1} and t_i
v = volume of culture (sampling) removed at t_i

$$S_i = \frac{V}{V_i} \times s \qquad (1)$$

$$S_i = \frac{V_{i-1} + N - v}{V_{i-1} + N} * S_{i-1} \qquad (2)$$

$$S = \frac{S_{i-1}}{V_{i-1} + N} \qquad (3)$$

adjust answers by the ratio $\frac{S_{initial}}{S_{measured}}$

3.9 DISCUSSION OF METHODS

During GC analysis: Method I, used for work described in Chapters 4 and 5, some difficulty was experienced in obtaining good separation of the acetic acid (sample) and formic acid peaks (internal standard). Despite the use of various integrator programme options to minimize this effect (operating the GC under optimal conditions for other sample product determination), the errors associated with acetic acid measurement were estimated to be 10-15%. The error associated with the measurement of other sample products using Method I was estimated to be 5%.

In GC Method II, errors were estimated to be less than 2% for acetone and butanol, and 3-5% for ethanol, butyric acid and acetic acid analyses. The use of an autosampler enabling frequent standard analyses, and integrator calculated response factors facilitated small measurement errors.

In GC Method III, errors were estimated to be 2-3% for acetone and butanol, and 5% for ethanol, butyric acid and acetic acid analyses.

The errors associated with the HPLC analysis of sugars (Section 3.5.6) were estimated to be 3-5% for Method I and 2% for Method II.

The preparation of alginate beads as described in Sections 3.6.1 and 3.6.2 was carried out at the Delft University of Technology, Delft, The Netherlands. These beads were imported to New Zealand and work continued at Massey University. No differences in fermentation performance were detected between similar experiments conducted at the two sites, hence data have been treated integrally.

The inoculum development procedure described in Section 3.7.2 was a key aspect for the successful operation of a batch fermentation experiment. Variations in the inoculum ratio, used in the second step of the inoculum development procedure, were used to account for unexplained variations in the germination rate of heat-shocked spores. The procedure of transferring a motile inoculum (i.e. exponential growth phase cells) to freshly autoclaved and cooled medium (complete operation completed within 2 h), with the medium surface flushed with oxygen-free

nitrogen gas during medium cooling and until visible gassing occurred due to bacterial growth, was rigorously followed. This procedure gave reproducible results characterized by a small fermentation lag time (2-3 h) and good growth.

CHAPTER 4

STRAIN SELECTION AND PRELIMINARY EXPERIMENTS

4.1 INTRODUCTION

At the commencement of this study, only a few literature reports were available describing the use of whey or deproteinated whey (hereafter referred to as permeate) for the production of solvents by batch fermentation (e.g. Maddox, 1980; Gapes et al, 1983). In addition, Compere & Griffith, (1979) had presented some data on the fermentation of lactose in semi-synthetic medium by various strains of C. butylicum and C. acetobutylicum. Subsequently, reports by Schoutens et al, (1984) using cheese whey permeate (C. beijerinckii LMD 27.6) and Welsh & Veliky, (1984) using acid cheese whey (C. acetobutylicum ATCC 824) were published. Generally, these workers reported long fermentation times (80-120 h) resulting in much lower productivities than achievable on industrial media, e.g. molasses, or simple glucose media.

Two strains of C. acetobutylicum dominate the published literature concerning the ABE fermentation in general. These strains are C. acetobutylicum ATCC 824 and C. acetobutylicum P262, the latter organism being an industrial strain used by National Chemical Products, Germiston, South Africa.

The objective of the work described in this chapter was to investigate the ability of these two strains to produce solvents from sulphuric acid casein whey permeate, lactose-hydrolysed sulphuric acid casein whey permeate, and various sugars in semi-synthetic medium. The experiments using the semi-synthetic medium were conducted to compare the individual sugars as substrates for the process, with the possibility of using lactose-hydrolysed permeate as substrate.

Initial screening experiments were conducted on a 100-ml scale at both 30°C and 37°C. The experimental procedure was as described in Section 3.7.1. Samples were taken daily over a 7 day period, for solvents, acids and sugars analysis, and pH determination. A subsequent series of experiments was conducted in a fermenter (5 l) at 34°C, using the experimental procedure described in Section 3.7.3.2, without any pH control.

4.2 RESULTS AND DISCUSSION

4.2.1 100-ml Scale Batch Fermentation

The results for the production of solvents from whey permeate, semi-synthetic medium (ssm) containing lactose, ssm + glucose, ssm + galactose, lactose-hydrolysed whey permeate and ssm + glucose/galactose are summarized in Tables 4.1 to 4.6 respectively.

The fermentation time, t_f, is the time including any lag-phase, when the butanol concentration was 90% of the maximum concentration observed. This was determined from a time course profile of each fermentation. The final pH value, concentration data, sugar utilized, percentage sugar utilization, yield and mean reactor productivity values were determined at this time t_f. The mean reactor productivity was calculated as: total solvent concentration/t_f (g/l.h). The maximum observed butanol production rate and maximum observed sugar utilization rate values, were obtained from the time course profile by taking tangents from the butanol production and sugar utilization curves respectively.

Strains ATCC 824 and P262 both produced solvents from whey permeate at 30°C and 37°C (Table 4.1). Strain P262 produced higher solvent concentrations than ATCC 824, typically 9.5 g/l at 30°C and 7.7 g/l at 37°C, after 39.2 h and 33.6 h respectively. A mean reactor productivity of 0.24 g/l.h at 30°C was obtained with strain P262 compared with the highest value of 0.08 g/l.h obtained at 37°C using strain ATCC 824. This particular result for strain ATCC 824 is very similar to that reported by Welsh & Veliky, (1984). In each instance, similar cell concentrations were obtained after 24 h fermentation,

Table 4.1 Production of solvents from sulphuric acid casein whey permeate by C. acetobutylicum ATCC 824 and C. acetobutylicum P262

	C. acetobutylicum ATCC 824		C. acetobutylicum P262	
	Temperature (°C)			
	30	37	30	37
Initial pH	5.30	5.30	5.35	5.35
Final pH	4.45	4.50	4.70	4.85
Fermentation time, t_f (h)	96.0	52.0	39.2	33.6
Butanol (g/l)	3.20	3.40	7.00	6.00
Acetone (g/l)	0.65	0.85	2.5	1.60
Ethanol (g/l)	nd	0.15	nd	0.10
Acetate (g/l)	2.30	2.20	0.90	1.00
Butyrate (g/l)	3.20	1.95	nd	nd
Ratio of solvents (A:B:E)	1.7:8.3:0	1.9:7.7:0.4	2.6:7.4:0	2.1:7.8:0.1
Sugar utilized (g/l)	20.0	21.0	22.4	20.9
Sugar utilization (%)	54.8	57.5	55.4	51.7
Yield at time t_f (g/g)	0.19	0.21	0.42	0.36
Yield at 168 h (g/g)	0.19	0.22	0.38	0.36
Mean reactor productivity (g/l.h)	0.04	0.08	0.24	0.23
Maximum observed butanol production rate (g/l.h) x 10^{-2}	12.9	11.7	16.8	23.2
Maximum observed sugar utilization rate (g/l.h) x 10^{-1}	7.1	6.7	7.4	8.6
Cell number after 24 h (no./ml)	3.2×10^8	4.9×10^8	6.3×10^8	4.0×10^8

nd = none detected

indicating that the specific solvent production rates for strain P262 are superior to strain ATCC 824 on this substrate. Lower yields were obtained using strain ATCC 824 than using strain P262. Slightly greater than 50% lactose utilization was observed in each fermentation.

Both strains produced solvents from ssm + lactose (Table 4.2). Strain ATCC 824 demonstrated acid production (and low solvent yield) at 30°C, while solvent production was observed at 37°C (8.85 g/l). These data show that this strain can utilize lactose for solvent production and that whey permeate is a poor medium compared to the ssm + lactose. The fermentation at 37°C was very slow however; a low mean reactor productivity was observed. For strain P262 on this medium, solvent production was favoured at 30°C compared with 37°C (Table 4.2). However, longer fermentation times and hence lower mean reactor productivities were obtained compared with whey permeate (Table 4.1). In addition, lower cell numbers were obtained compared to those on whey permeate.

High solvent concentrations were obtained on ssm + glucose for both strains (Table 4.3), confirming their solventogenic capability on this sugar. Considerably higher sugar utilization was obtained for both strains compared to that obtained on whey permeate (Table 4.1) or ssm + lactose (Table 4.2). Solvent concentrations and yields at 30°C were similar for both strains, with the yields being close to that theoretically predicted. A temperature of 30°C was more favourable than 37°C for both strains. Strain ATCC 824 produced 15.2 g/l solvents in 88 h. The fermentation time in this instance included a 36 h lag period (data not shown). Recalculating the mean reactor productivity using a fermentation time of 52 h gives a value of 0.29 g/l.h. The mean reactor productivity obtained at 30°C by strain P262 (0.22 g/l.h) was similar to that obtained on whey permeate.

Data in Table 4.4 show that galactose is a very poor substrate for solvent production by strain ATCC 824, confirming the observation of Mes-Hartree and Saddler, (1982). Predominantly acids were produced at both 30°C and 37°C. Conversely, strain P262 was capable of utilizing galactose for solvent production. Solvent concentrations, yields and mean reactor productivities were very similar to those obtained on ssm +

Table 4.2 Production of solvents from semi-synthetic medium containing lactose by C. acetobutylicum ATCC 824 and C. acetobutylicum P262

	C. acetobutylicum ATCC 824		C. acetobutylicum P262	
	Temperature (°C)			
	30	37	30	37
Initial pH	5.50	5.50	5.70	5.70
Final pH	4.28	4.75	5.25	5.00
Fermentation time, t_f (h)	96.0	162.0	96.0	96.0
Butanol (g/l)	0.60	6.50	6.70	1.00
Acetone (g/l)	0.20	1.90	2.60	0.50
Ethanol (g/l)	0.10	0.45	0.15	nd
Acetate (g/l)	4.75	1.90	1.40	2.00
Butyrate (g/l)	4.40	1.70	0.50	2.25
Ratio of solvents (A:B:E)	2.2:6.7:0.1	2.2:7.3:0.5	2.8:7.1:0.1	3.3:6.7:0
Sugar utilized (g/l)	15.0	34.5	24.5	5.5
Sugar utilization (%)	31.3	71.9	51.9	10.8
Yield at time t_f (g/g)	0.06	0.26	0.38	0.27
Yield at 168 hr (g/g)	0.06	0.26	0.36	0.28
Mean reactor productivity (g/l.h)	0.01	0.05	0.10	0.02
Maximum observed butanol production rate (g/l.h) x 10^{-2}	1.2	8.6	12.8	1.2
Maximum observed sugar utilization rate (g/l.h) x 10^{-1}	3.9	4.3	4.1	1.0
Cell number after 24 hr (no./ml)	3.3×10^8	8.2×10^8	1.0×10^8	1.7×10^8

nd = none detected

Table 4.3 Production of solvents from semi-synthetic medium containing glucose by C. acetobutylicum ATCC 824 and C. acetobutylicum P262

	C. acetobutylicum ATCC 824		C. acetobutylicum P262	
	Temperature (°C)			
	30	37	30	37
Initial pH	5.40	5.35	5.55	5.55
Final pH	4.45	4.15	4.90	5.15
Fermentation time, t_f (h)	88.0	39.0	58.4	41.6
Butanol (g/l)	10.50	3.10	9.00	3.80
Acetone (g/l)	3.40	1.20	3.40	2.60
Ethanol (g/l)	1.30	0.10	0.25	0.15
Acetate (g/l)	0.90	3.60	0.75	1.60
Butyrate (g/l)	nd	1.60	nd	nd
Ratio of solvents (A:B:E)	2.2:6.9:0.9	2.8:7.0:0.2	2.7:7.1:0.2	4.0:5.8:0.2
Sugar utilized (g/l)	50.0	18.0	40.0	18.0
Sugar utilization (%)	100.0	36.0	80.0	36.0
Yield at time t_f (g/g)	0.30	0.24	0.32	0.36
Yield at 168 h (g/g)	0.36	0.22	0.24	0.32
Mean reactor productivity (g/l.h)	0.17	0.11	0.22	0.16
Maximum observed butanol production rate (g/l.h) x 10^{-2}	24.2	10.0	14.6	13.2
Maximum observed sugar utilization rate (g/l.h) x 10^{-1}	7.2	7.6	9.9	7.1
Cell number after 24 h (no./ml)	7.3×10^8	1.2×10^9	8.5×10^8	5.4×10^8

nd = none detected

Table 4.4 Production of solvents from semi-synthetic medium containing galactose by *C. acetobutylicum* ATCC 824 and *C. acetobutylicum* P262

	C. acetobutylicum ATCC 824		*C. acetobutylicum* P262	
	Temperature (°C)			
	30	37	30	37
Initial pH	5.30	5.35	5.58	5.58
Final pH	4.25	4.40	5.20	5.68
Fermentation time, t_f (h)	96.0	101.0	42.4	28.0
Butanol (g/l)	0.40	1.60	7.10	6.20
Acetone (g/l)	0.20	0.90	2.70	2.00
Ethanol (g/l)	0.05	0.25	0.20	0.15
Acetate (g/l)	4.70	4.00	1.10	1.30
Butyrate (g/l)	4.55	2.75	nd	nd
Ratio of solvents (A:B:E)	3.1:6.1:0.1	3.3:5.8:0.9	2.7:7.1:0.2	2.4:7.4:0.2
Sugar utilized (g/l)	7.5	12.0	32.6	27.6
Sugar utilization (%)	15.0	24.0	64.4	54.5
Yield at time t_f (g/g)	0.09	0.23	0.31	0.30
Yield at 168 h (g/g)	0.10	0.19	0.33	0.30
Mean reactor productivity (g/l.h)	0.01	0.03	0.24	0.30
Maximum observed butanol production rate (g/l.h) x 10^{-2}	-	3.3	26.4	23.9
Maximum observed sugar utilization rate (g/l.h) x 10^{-1}	1.5	2.6	11.8	10.7
Cell number after 24 h (no./ml)	3.0×10^8	6.9×10^8	1.1×10^9	4.8×10^8

nd = none detected

glucose. Both of the monosaccharide substrates were more favourable substrates than lactose (Table 4.2) for strain P262. However, no significant increase in mean reactor productivity was observed compared with whey permeate (Table 4.1).

The experiments using lactose-hydrolysed whey permeate (Table 4.5) and an equal mixture of glucose and galactose in semi-synthetic medium (Table 4.6) confirm the finding that for strain ATCC 824, galactose is a poor substrate for solvent production. Solvents were produced from the glucose portion in both media. This strain produced very low solvent concentrations from lactose-hydrolysed whey permeate although higher concentrations were produced from the ssm + glucose/galactose. This result confirms that the former medium is a poor substrate for this strain. Cell numbers were comparable for each substrate. Strain P262 utilized both sugars simultaneously but glucose was used preferentially to galactose in both media (Tables 4.5 & 4.6). Similar yields and mean reactor productivity values were obtained on lactose-hydrolysed whey permeate (Table 4.5) and whey permeate (Table 4.1) suggesting that there is no real advantage in the hydrolysis of whey permeate prior to fermentation using strain P262. Preferential uptake of glucose over galactose has also been demonstrated using C. beijerinckii (Schoutens et al, 1984).

4.2.2 5-Litre Scale Batch Fermentation

A time course profile for the fermentation of sulphuric acid casein whey permeate at 34°C is given in Figure 4.1 for strain ATCC 824 and Figure 4.2 for strain P262. Results are summarised in Table 4.7.

For strain ATCC 824, solvent production commenced after 18 h (Figure 4.1). Acid production peaked after 25 h (4.85 g/l total acids) but acid uptake during the solventogenic phase was low. Only a small increase in culture pH was observed at this time.

For strain P262, solvent production commenced after 11 h (Figure 4.2) and proceeded at a faster rate than with strain ATCC 824 (Table 4.7). Acid production was lower (maximum 2.6 g/l total acids after 13 h) than occurred with strain ATCC 824 and a greater acid uptake plus a greater increase in culture pH occurred.

Table 4.5 Production of solvents from lactose-hydrolysed sulphuric acid casein whey permeate by *C. acetobutylicum* ATCC 824 and *C. acetobutylicum* P262

	C. acetobutylicum ATCC 824		*C. acetobutylicum* P262	
	Temperature (°C)			
	30	37	30	37
Initial pH	5.55	5.55	5.58	5.58
Final pH	4.05	4.15	4.75	5.05
Fermentation time, t_f (h)	78.0	88.0	62.4	16.8
Butanol (g/l)	0.90	1.00	5.10	5.70
Acetone (g/l)	nd	0.10	1.80	1.50
Ethanol (g/l)	nd	nd	0.20	0.20
Acetate (g/l)	2.50	2.35	1.20	1.40
Butyrate (g/l)	3.10	2.85	nd	nd
Ratio of solvents (A:B:E)	-	0.9:9.1:0	2.5:7.2:0.3	2.0:7.7:0.3
Sugar utilized (g/l)	11.4[a]	13.9[a]	17.9[b]	18.4[c]
Sugar utilization (%)	24.1	29.4	47.0	48.3
Yield at time t_f (g/g)	0.08	0.08	0.39	0.40
Yield at 168 h (g/g)	0.08	0.07	0.38	0.40
Mean reactor productivity (g/l.h)	0.01	0.01	0.11	0.44
Maximum observed butanol production rate (g/l.h) x 10^{-2}	3.3	2.3	12.2	28.6
Maximum observed glucose utilization rate (g/l.h) x 10^{-1}	3.4	5.2	6.4	8.3
Maximum observed galactose utilization rate (g/l.h) x 10^{-1}	-	-	1.0	1.9
Cell number after 24 h (no./ml)	1.7×10^8	6.8×10^8	7.4×10^8	5.7×10^8

a no galactose used
b 14.3 g/l glucose, 3.6 g/l galactose
c 15.0 g/l glucose, 3.4 g/l galactose

nd = none detected

Table 4.6 Production of solvents from semi-synthetic medium containing glucose and galactose by C. acetobutylicum ATCC 824 and C. acetobutylicum P262

	C. acetobutylicum ATCC 824		C. acetobutylicum P262	
	Temperature (°C)			
	30	37	30	37
Initial pH	5.25	5.25	5.58	5.50
Final pH	4.55	4.15	5.30	4.98
Fermentation time, t_f (h)	144.0	40.0	48.0	120
Butanol (g/l)	5.00	3.40	6.90	2.90
Acetone (g/l)	1.90	1.00	2.90	1.80
Ethanol (g/l)	0.20	0.15	0.30	0.10
Acetate (g/l)	4.05	4.20	1.20	2.50
Butyrate (g/l)	1.50	1.40	nd	0.80
Ratio of solvents (A:B:E)	2.7:7.0:0.3	2.2:7.5:0.3	2.9:6.8:0.3	3.8:6.0:0.2
Sugar utilized (g/l)	25.0[a]	25.0[a]	28.0[b]	15.2[c]
Sugar utilization (%)	50.0	50.0	57.1	31.0
Yield at time t_f (g/g)	0.28	0.18	0.36	0.32
Yield at 168 h (g/g)	0.31	0.21	0.36	0.32
Mean reactor productivity (g/l.h)	0.05	0.11	0.21	0.04
Maximum observed butanol production rate (g/l.h) x 10^{-2}	7.5	36.3	15.0	3.5
Maximum observed glucose utilization rate (g/l.h) x 10^{-1}	5.2	10.2	6.7	3.9
Maximum observed galactose utilization rate (g/l.h) x 10^{-1}	-	-	1.0	0.7
Cell number after 24 h (no./ml)	4.9×10^8	1.1×10^9	4.7×10^8	4.1×10^8

[a] no galactose used
[b] 24.0 g/l glucose, 4.0 g/l galactose
[c] 14.0 g/l glucose, 1.2 g/l galactose

nd = none detected

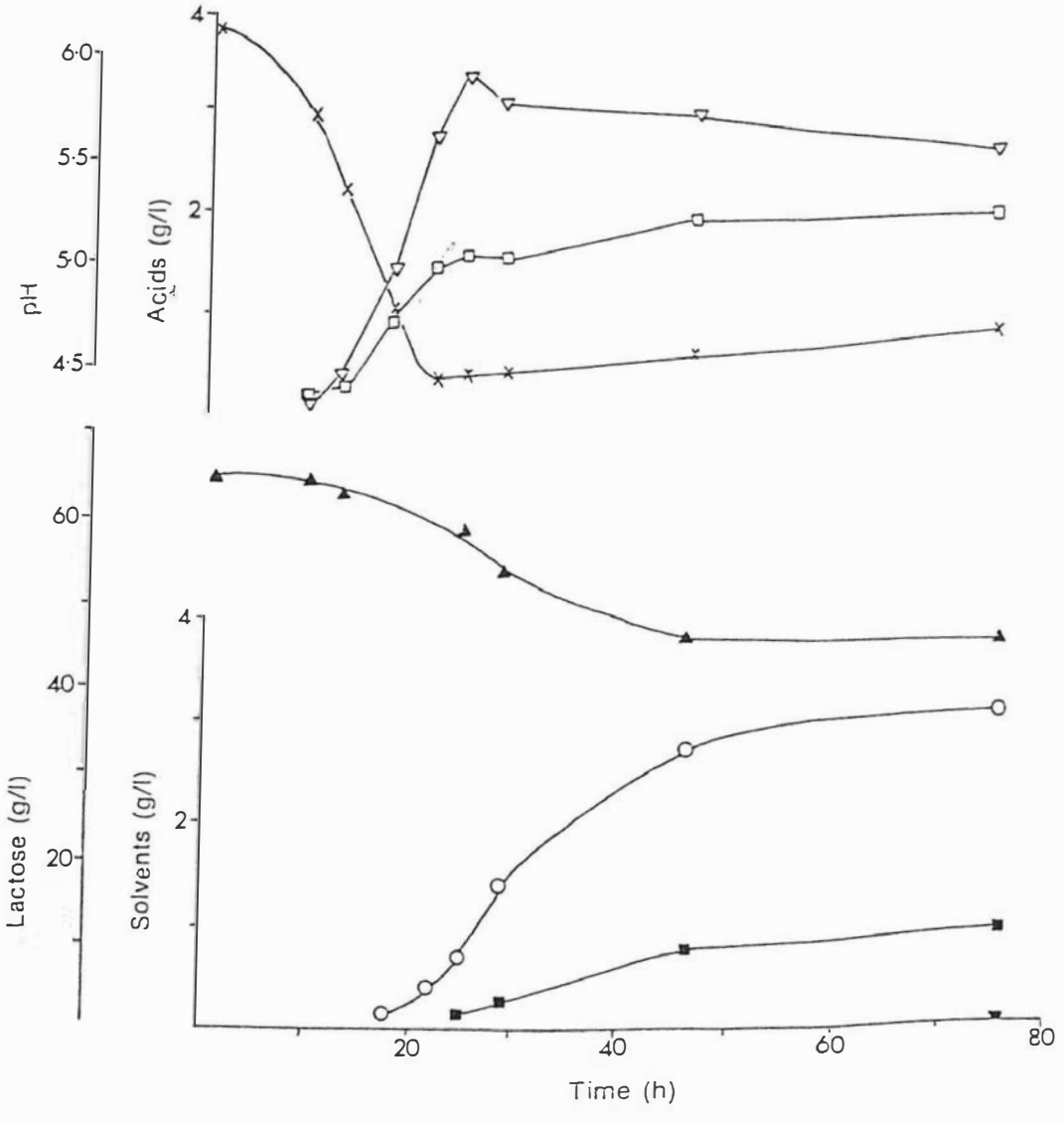


Figure 4.1. Batch fermentation profile at 34°C, for *C. acetobutylicum* ATCC 824 using sulphuric acid casein whey permeate with no pH control: (○) butanol; (■) acetone; (▼) ethanol; (▽) butyric acid; (□) acetic acid; (x) pH; (▲) lactose.

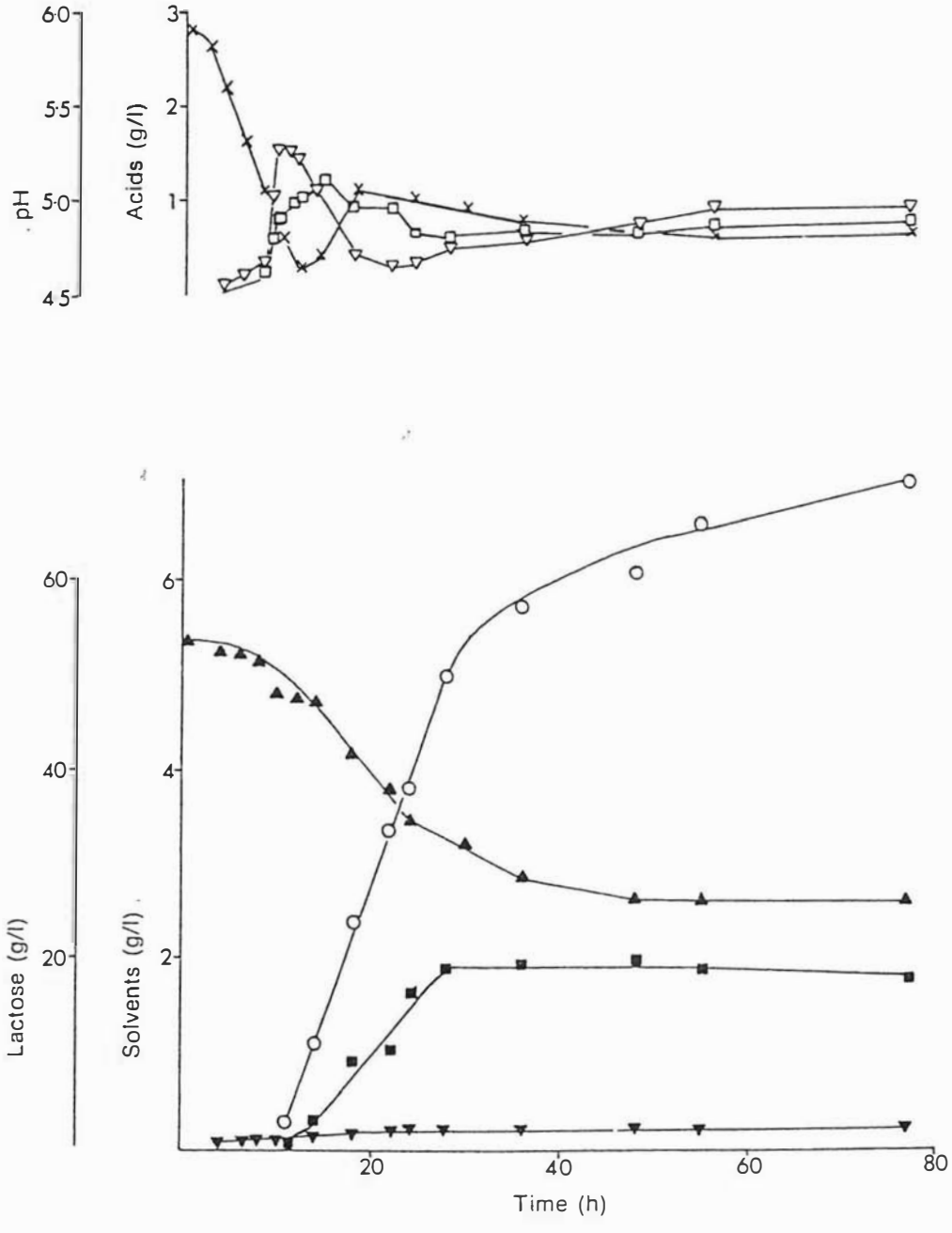


Figure 4.2. Batch fermentation profile at 34°C, for *C. acetobutylicum* P262 using sulphuric acid casein whey permeate with no pH control. Symbols as for Fig. 4.1

Table 4.7 Production of solvents at 34°C, from sulphuric acid casein whey permeate by C. acetobutylicum ATCC 824 and C. acetobutylicum P262 in a 5-litre batch fermentation with no pH control

	C. acetobutylicum ATCC 824	C. acetobutylicum P262
Initial pH	6.15	5.90
Final pH	4.50	4.80
Fermentation time, t_f (h)	46.0	46.0
Butanol (g/l)	2.72	6.00
Acetone (g/l)	0.74	1.95
Ethanol (g/l)	0.40	0.25
Acetate (g/l)	1.90	1.10
Butyrate (g/l)	2.94	1.30
Ratio of solvents (A:B:E)	1.9:7.1:1.0	2.4:7.3:0.3
Sugar utilized (g/l)	19.2	24.7
% Sugar utilization	29.8	46.2
Yield at time t_f (g/g)	0.21	0.33
Mean reactor productivity (g/l.h)	0.084	0.18
Maximum observed butanol production rate (g/l.h) x 10^{-2}	18.0	27.5
Maximum observed sugar utilization rate (g/l.h) x 10^{-1}	14.5	11.5

The yield and mean reactor productivity parameters obtained from these fermentations at 34°C (Table 4.7) agree well with those given in Table 4.1 for those fermentations conducted on a 100-ml bottle scale.

4.3 CONCLUSIONS

Although the experiments described in this chapter were not performed under ideal "controlled" conditions, the following general conclusions can be made:

1. C. acetobutylicum ATCC 824 was able to produce solvents from lactose (whey permeate and ssm) and glucose but galactose was a poor substrate for solvent production.

2. C. acetobutylicum P262 was able to produce solvents from lactose (whey permeate and ssm), glucose and galactose sugars. In mixtures of glucose and galactose both sugars were used simultaneously but a preferential uptake for glucose was demonstrated. There appears no advantage in hydrolysis of lactose prior to fermentation.

3. The optimum fermentation temperature varied for each substrate type and medium composition.

4. Higher mean reactor productivities were obtained on whey permeate using strain P262 than using strain ATCC 824.

5. Incomplete lactose utilization (approximately 50%) was observed for both strain P262 and strain ATCC 824 on whey permeate.

Based on the results of this simple experiment, C. acetobutylicum P262 was chosen for further study. This particular strain shows superior performance on whey permeate to that reported for other strains (Table 4.8). However, the results obtained are still inferior to those reported for an industrial molasses medium (Spivey, 1978).

Table 4.8 Comparison of mean reactor productivities in batch fermentation for different strains of *Clostridia* using whole whey or whey permeate substrates

Substrate	Strain	Mean Reactor Productivity (g/l.h)	Reference
Whey permeate	*C. acetobutylicum* NCIB 2951	0.14	Maddox, (1980)
Whey permeate	*C. butylicum* NRRL B-592	0.09	Gapes *et al*, (1983)
Whey permeate	*C. beijerinckii* LMD 27.6	0.05	Schoutens *et al*, (1984)
Acid whey	*C. acetobutylicum* ATCC 824	0.08	Welsh & Veliky, (1984)
Whey permeate	*C. acetobutylicum* P262	0.24	This work
Molasses	?	0.55	Spivey, (1978)

CHAPTER 5

PRODUCTION OF SOLVENTS BY BATCH FERMENTATION FROM SULPHURIC ACID CASEIN WHEY PERMEATE USING C. ACETOBUTYLICUM P262

5.1 INTRODUCTION

In the previous chapter it was reported that C. acetobutylicum P262 could produce solvents from sulphuric acid casein whey permeate in high yields and with a productivity superior to any previously reported in the literature on this type of substrate. The extent of lactose utilization was poor, however (approximately 50%).

This chapter presents data from a series of batch fermentation experiments (2 l or 7 l scale, Sections 3.7.3.1 - 3.7.3.3) which were undertaken in an attempt to define the conditions for maximum solvent production, productivity and solvent yield from the fermentation of sulphuric acid casein whey permeate medium (Table 3.2). Principally, the pH value of the culture was investigated for its effects on the fermentation process. Time course fermentation profiles were determined for each experiment in order to more closely follow the kinetics of the fermentation process. A fermentation temperature of 34°C was selected for this work since this temperature has been used on an industrial scale with this organism (Spivey, 1978). Results from this work are described in Section 5.2.1.

Attempts to correlate microbial morphological changes with the fermentation kinetics were undertaken, and these observations are given in Section 5.3.

In Section 5.4, batch fermentation experiments are reported for lactose-hydrolysed sulphuric acid casein whey permeate and semi-synthetic medium containing glucose, using the optimal fermentation conditions determined in Section 5.2. These experiments were conducted in order to more closely examine the use of these substrates and to re-examine the preliminary data reported in Chapter 4.

5.2 OPTIMIZATION OF BATCH FERMENTATION CULTURE CONDITIONS

5.2.1 Results

The results for all experiments are summarised in Table 5.1 and time course profiles for each fermentation are shown in Figures 5.1 - 5.10. Concentration data have been corrected for dilution effects (Section 3.8) in those experiments where pH control was used. The dilution effects varied between 5% to 15%. Parameters given in Table 5.1 have been derived using these data.

The initial experiment, Run I, was performed without any initial medium pH adjustment prior to autoclaving, or pH control during the fermentation. Figure 5.1 shows that the culture pH decreased from pH 5.0 to 4.25 during the acidogenic phase (0-18 h), then increased to pH 4.4 during the solventogenic phase. Partial uptake of butyric acid, but not acetic acid, occurred during this phase. Total solvent production after 41 h (Table 5.1) was 8.45 g/l, representing a yield of 0.41 g/g based on lactose utilized. The overall solvent productivity was 0.21 g/l.h. Thus, these fermentation conditions favoured a high solvent yield and productivity, but the total lactose utilization of 50% was poor. Since strain P262 is known to be capable of producing a much higher solvent concentration (approximately 16-20 g/l) on synthetic medium (Long et al, 1984a) and industrial molasses medium (Barber et al, 1979) it was reasonable to suggest that the low culture pH value was responsible, at least in part, for the low extent of lactose utilization. Run II (Figure 5.2) was carried out at an initial value of pH 5.65, and this subsequently fell to pH 4.65 (14h) before rising to pH 4.9. In comparison with Run I, an improved lactose utilization rate was observed but the solvent yield was lower and was accompanied by a higher concentration of acids. No butyric acid uptake and very low acetic acid uptake occurred. In both Run I and Run II, lactose was used throughout the entire fermentation period, although at a slower rate during the solventogenic phase in Run I (pH 4.0) than in Run II (pH 4.8).

Run III (Figure 5.3) was performed under similar conditions to Run II except that the culture pH value was controlled to prevent it falling below pH 5.2, and Run IV (Figure 5.4) was performed at a value not below

Table 5.1 Summary of fermentation parameters for all experiments[a] used in Section 5.2

	Run No.									
	I	II	III	IV	V	VI	VII	VIII	IX	X
μ_{max}	0.31	0.56	0.38	0.41	0.45	0.55	0.46	0.47	0.55	0.55
initial pH	5.0	5.65	5.65	5.8	5.9	6.2	5.55	5.55	6.1	6.4
fermentation time, h	41	42	29	51	-	54	48	24	50	48
final pH	4.4	4.9	5.3	6.0	4.6	5.2	5.55	6.0	5.8	5.9
butanol, g/l	6.6	5.8	6.5	2.4	0	1.1	1.6	3.4	8.1	10.0
acetone, g/l	1.7	1.6	0.5	0.4	0	0.1	0.2	1.1	2.9	3.6
ethanol, g/l	0.2	0.3	0.3	0.5	0.3[b]	0.2	0.2	0.2	0.3	0.3
acetic acid, g/l	1.6	1.6	4.3	5.0	5.9[b]	4.7	1.4	2.9	3.4	3.3
butyric acid, g/l	0.5	3.2	9.9	13.3	11.8[b]	11.0	8.5	3.6	1.1	1.8
lactose utilized, g/l	21	23	29	33	28	27	19	16	29	49
yield, g.solvents/g lactose utilized	0.41	0.33	0.25	0.10	0.01	0.05	0.10	0.29	0.39	0.28
overall fermenter productivity, g.solvent/l.h	0.21	0.18	0.25	0.06	-	0.03	0.04	0.19	0.22	0.29
maximum observed butanol production rate g/l.h	0.37	0.25	0.51	0.25	-	-	0.22	0.24	0.49	0.28
maximum observed lactose utilization rate, g/l.h	0.90	1.6	2.7	2.9	7.1	3.3	0.60	1.0	1.1	1.2

a The fermentation time is that taken to achieve a butanol concentration 90% of that achieved after 60 h. The concentration of solvents and acids are those present at this fermentation time.

b These concentrations are those observed after 60 h of fermentation.

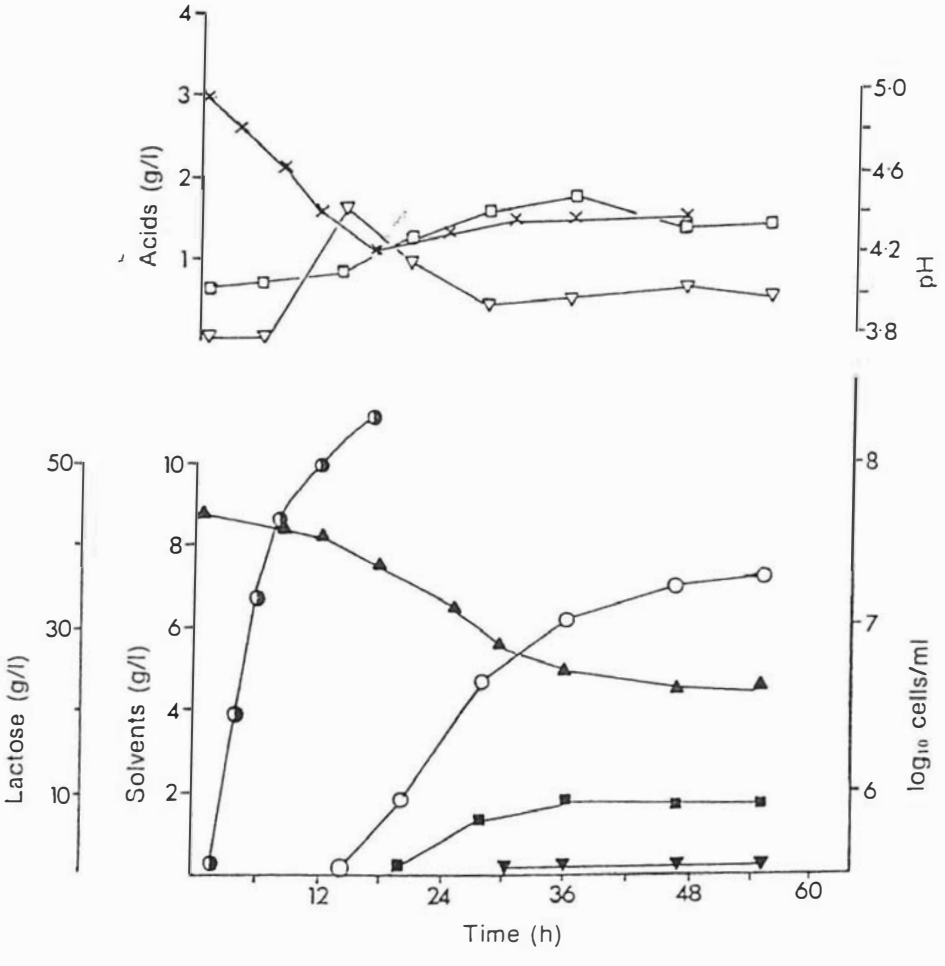


Figure 5.1. Batch fermentation profile of Run I. Initial pH 5.0, no pH control: (○) butanol; (■) acetone; (▼) ethanol; (▽) butyric acid; (□) acetic acid; (×) pH; (▲) lactose; (◑) $\log_{10}$ cells/ml.

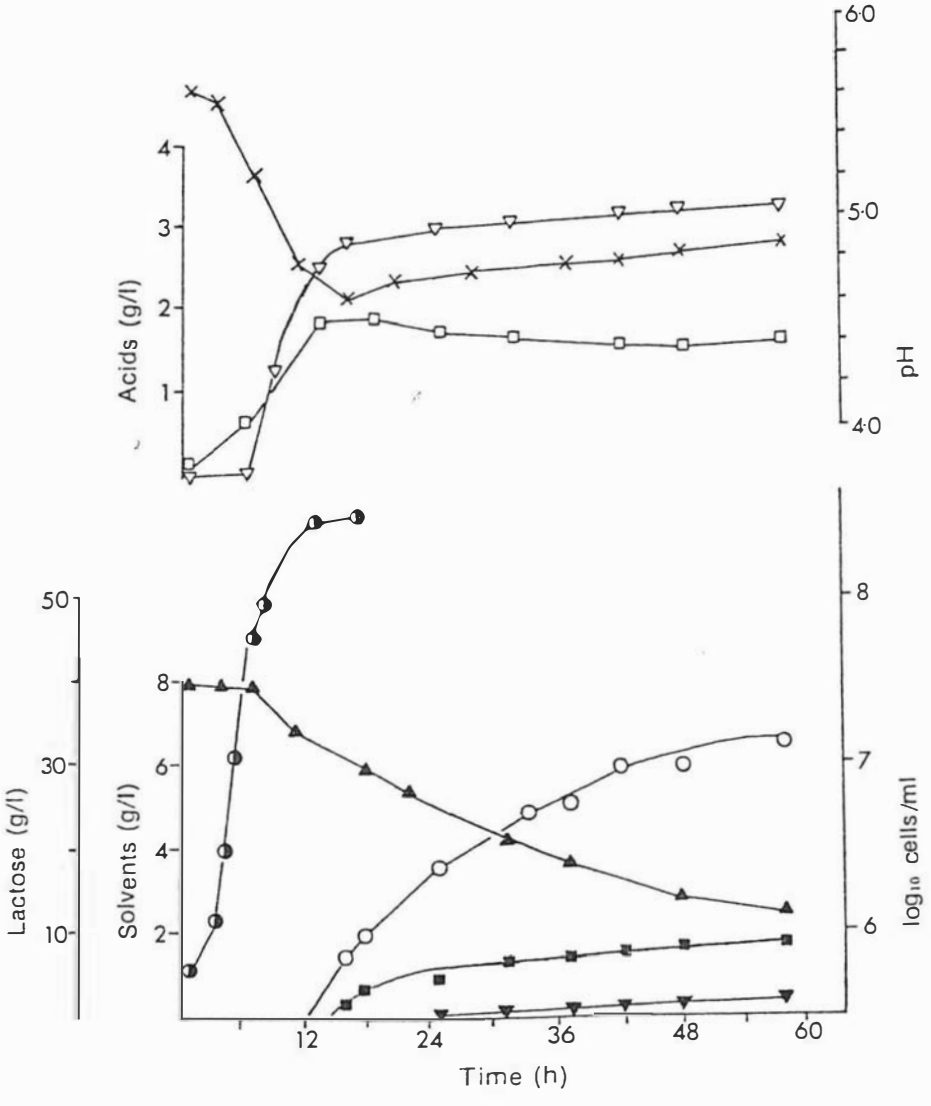


Figure 5.2. Batch fermentation profile of Run II. Initial pH 5.65, no pH control. Symbols as for Fig. 5.1.

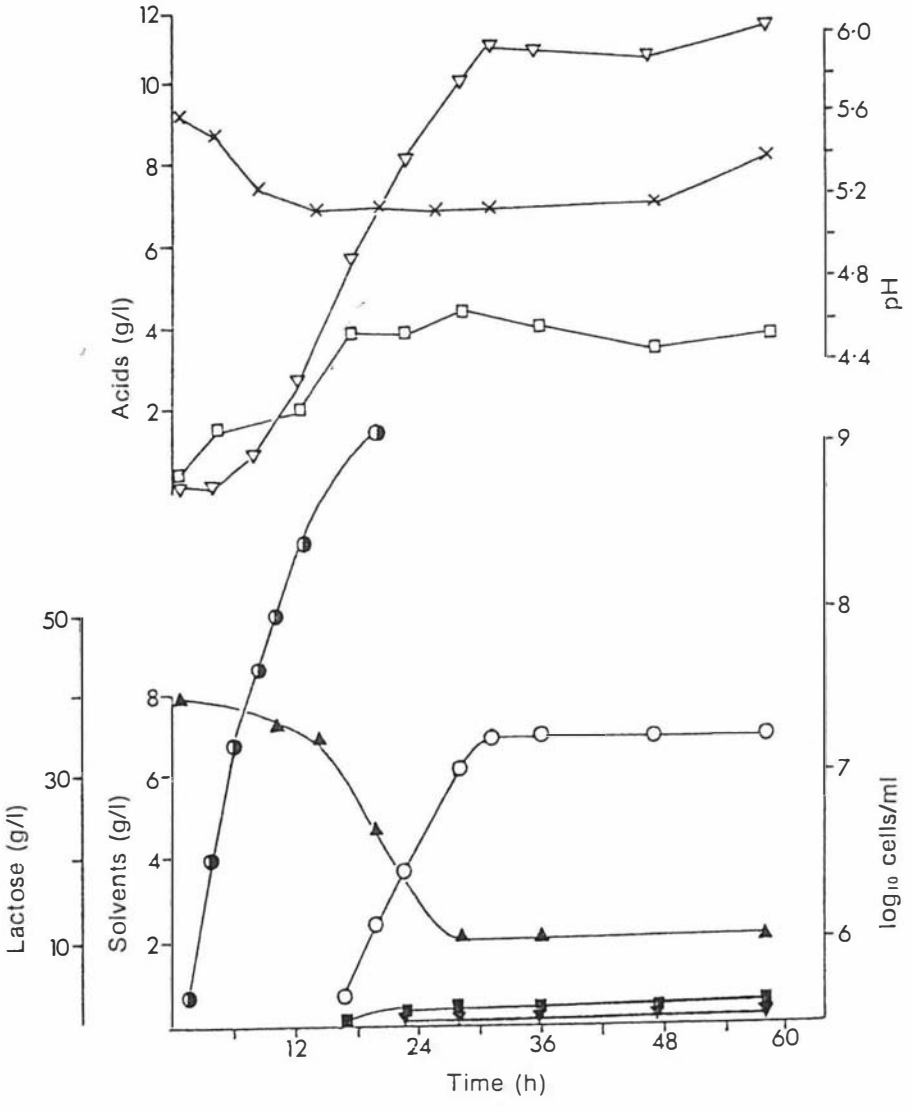


Figure 5.3. Batch fermentation profile of Run III. Initial pH 5.65, controlled to not less than pH 5.2. Symbols as for Fig. 5.1

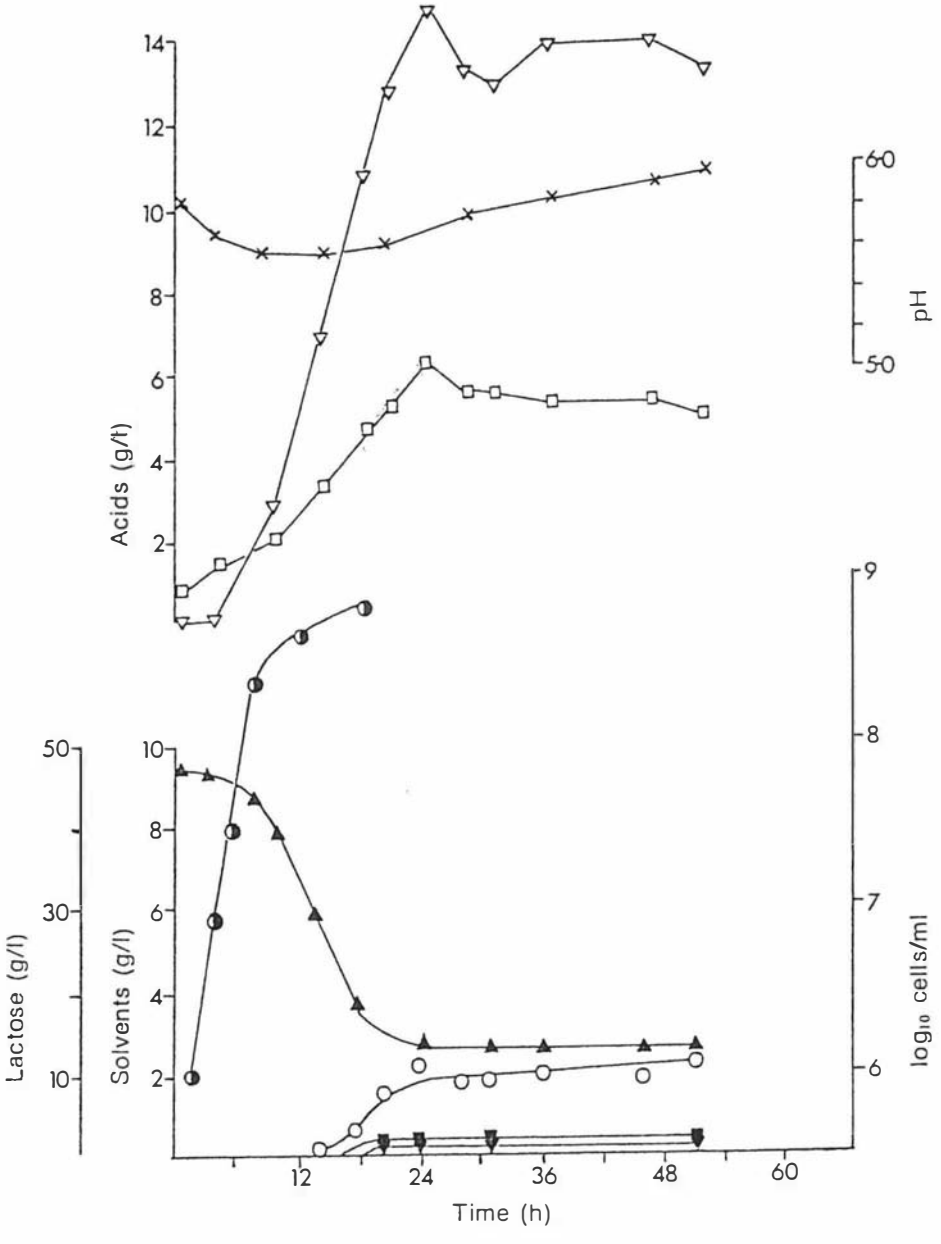


Figure 5.4. Batch fermentation profile of Run IV. Initial pH 5.8, controlled to not less than pH 5.6. Symbols as for Fig. 5.1.

pH 5.6. Good solvent production was obtained in Run III in conjunction with higher acid production than in Run I or Run II, whilst a high acid production and very little solvent production was obtained in Run IV.

Together, Runs I to IV demonstrate that improved lactose utilization rates are associated with increasing pH values, but solvent yields are progressively reduced. The latter effect is clearly related to increased acid production at increased pH values in Runs III and IV. Interestingly, the highest overall solvent productivity and maximum observed butanol production rate were observed in Run III (0.25 g/l.h & 0.51 g/l.h respectively), where the pH value was in the range pH 5.2 - 5.5.

Since the above experiments had shown that increased lactose utilization rates were obtained at higher pH values, further experiments were performed where the initial pH value was maintained at pH 6.0, and was subsequently decreased to a value more conducive to solvent production as observed in Runs I and II. In Run V (Figure 5.5) the initial pH value was held for the first 16 h of fermentation, and was decreased to pH 4.5 over a 60 min period by the addition of HCl. However, the decreased pH value resulted in cessation of lactose utilization. The concentration of acids remained approximately constant and little solvent production was observed. Since it was possible that the pH adjustment had been made too late in the fermentation, and too abruptly, Run VI (Figure 5.6) was performed where the initial value of pH 6.0 was maintained for only 11h, and was then allowed to fall naturally. In this way, a value of pH 5.4 was attained after approximately 15 h of fermentation. Again, however, lactose utilization slowed considerably after the pH decrease and little solvent production was observed.

Consequently, two further experiments were conducted in an attempt to increase solvent production while maintaining a high lactose utilization rate. The technique used was to achieve solvent production at a low pH value, followed by a controlled shift upwards of the pH value. In both Run VII (Figure 5.7) and Run VIII (Figure 5.8) the pH was initially allowed to fall naturally from pH 5.55 in order to achieve low acid production as observed in Runs I and II. After 17h

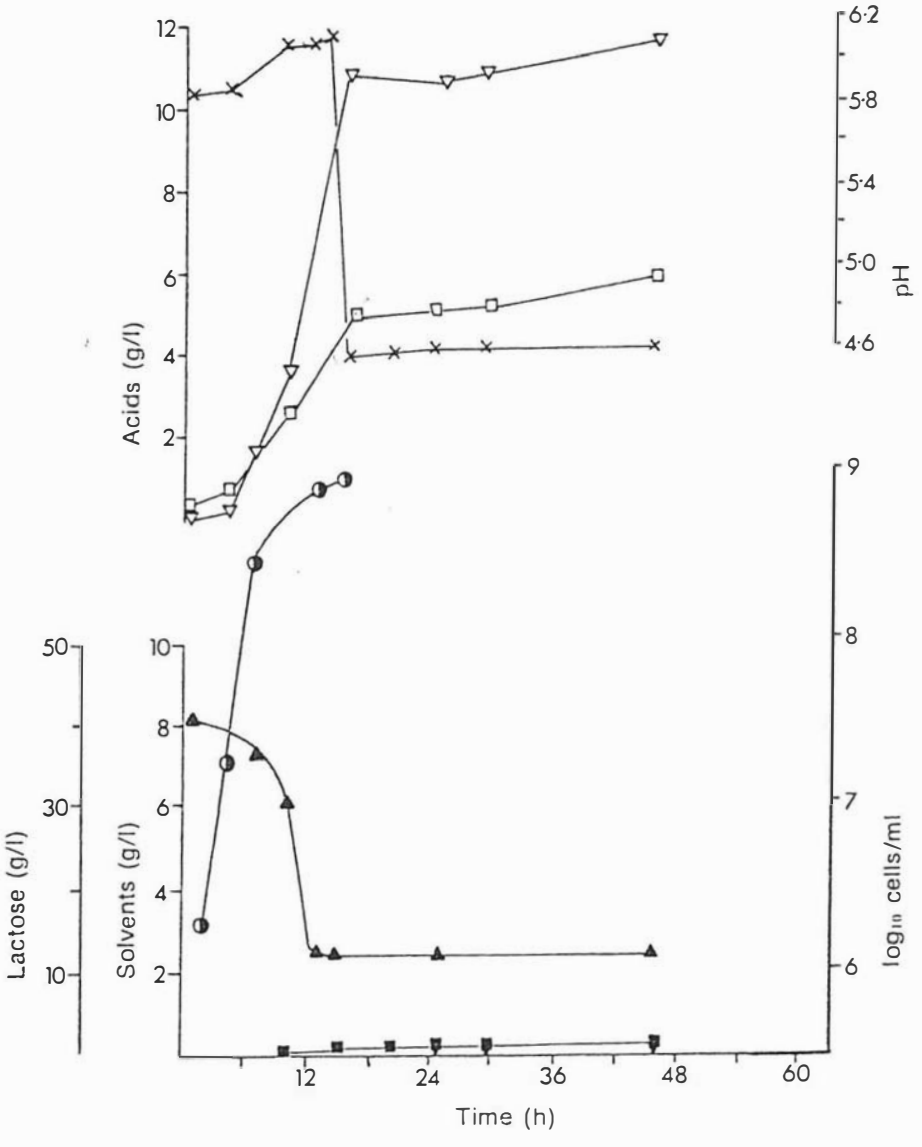


Figure 5.5. Batch fermentation profile of Run V. Initial pH 5.9, controlled at not less than pH 6.0 for 16 h, then adjusted to pH 4.5. Symbols as for Fig. 5.1.

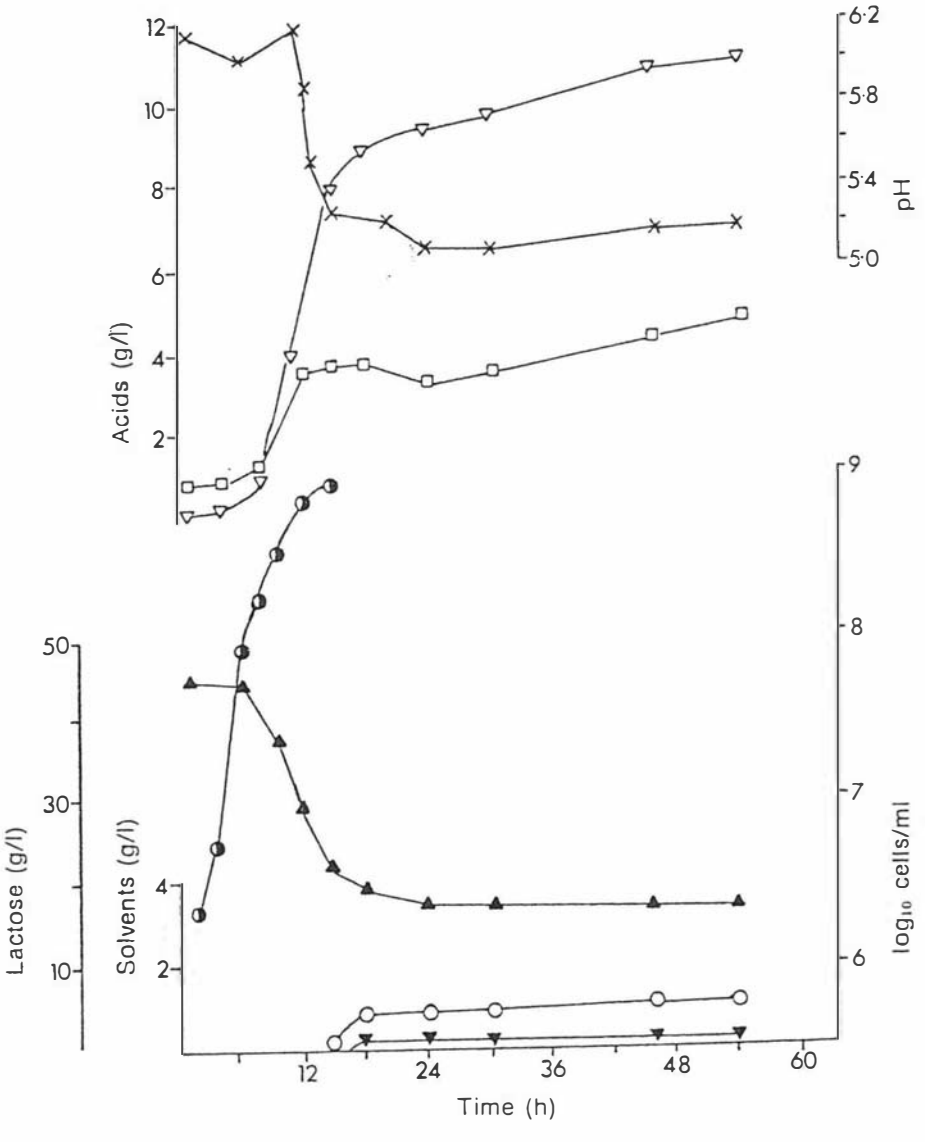


Figure 5.6. Batch fermentation profile of Run VI. Initial pH 6.2, controlled at not less than pH 6.0 for 11 h. Symbols as for Fig. 5.1.

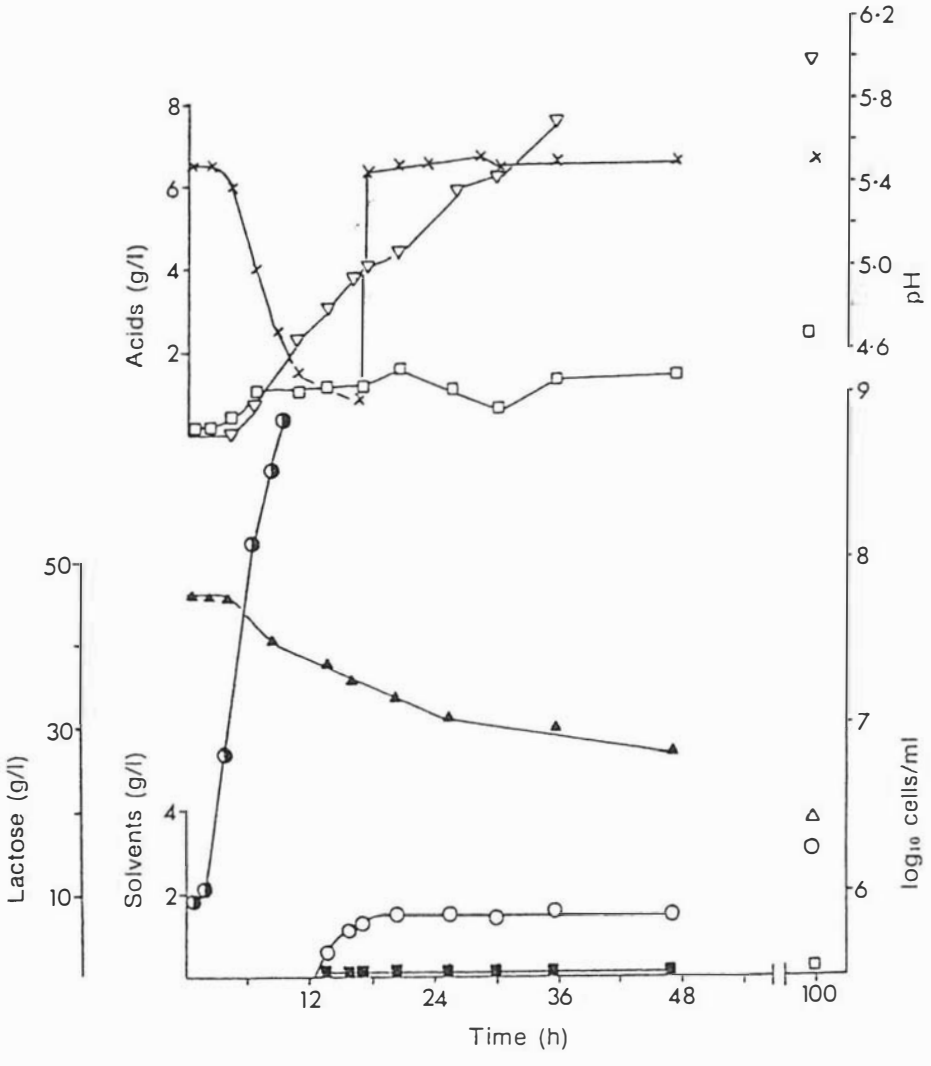


Figure 5.7. Batch fermentation profile of Run VII. Initial pH 5.55, uncontrolled initially for 17 h, then adjusted to pH 5.5. Symbols as for Fig. 5.1.

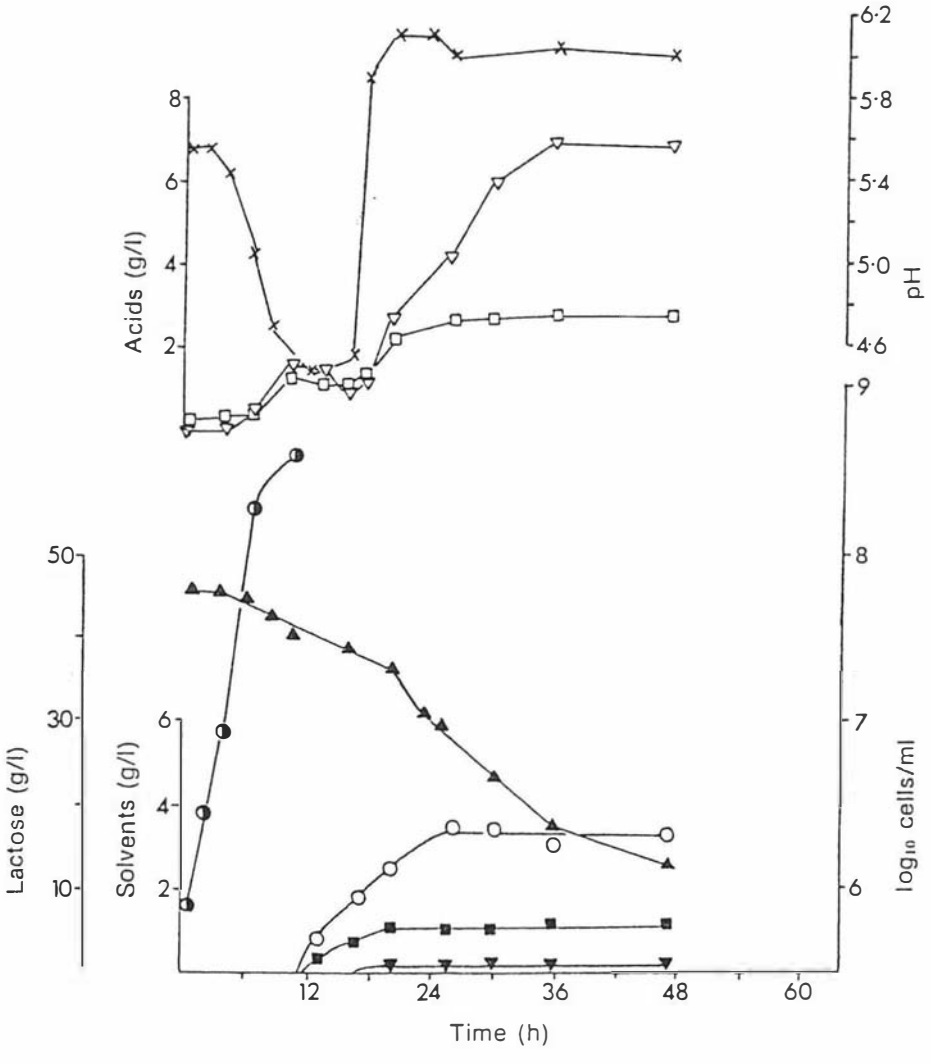


Figure 5.8. Batch fermentation profile of Run VIII. Initial pH 5.55, uncontrolled initially for 17 h, then adjusted to pH 6.0. Symbols as for Fig. 5.1.

fermentation the cultures had values of pH 4.4 and solvent production had commenced. The culture pH was then adjusted using aqueous ammonia to pH 5.5 (Run VII) or pH 6.0 (Run VIII). An increase in acid production resulted in both instances, with an increase in the lactose utilization rate occurring in Run VIII after the pH adjustment. Solvent production was not favoured.

Overall, the above experiments involving manipulation of the pH value of the culture had failed to achieve the desired aim of improved solvent productivities in association with high yields and high lactose utilization. Although improved lactose utilization could be achieved by operating at higher pH values, this was invariably associated with increased production of acids. Thus, on the assumption that acid production is directly related to lactose utilization rate, and only indirectly to the pH value of the culture, a method was sought to decrease the lactose utilization rate while maintaining the pH value in the range pH 5.2 to pH 5.5. Run III had shown this pH value to be conducive to the solvent production rate. The method selected to achieve this aim was to increase the initial concentration of lactose in the whey permeate, on the basis that it would increase the osmotic pressure of the growth medium and place some stress on the organism.

Run IX (Figure 5.9) was performed by supplementing the whey permeate with lactose to a final concentration of 63 g/l. The initial pH value was adjusted to pH 6.1, and was controlled at this value for 11 h after which it was allowed to fall naturally. The value decreased to pH 5.4 (16 h) before increasing to pH 5.8 (29 h). The results (Table 5.1) show that in comparison with Runs V and VI, the lactose utilization rate was decreased considerably. Additionally, lactose utilization occurred throughout the entire fermentation period, as observed in Runs I and II. Acid production was much reduced while solvent yield (0.39 g/g) and productivity (0.22 g/l.h) were increased. After 97h of fermentation, concentrations of 12.2 g/l, 4.2 g/l and 0.3 g/l were observed for butanol, acetone and ethanol respectively. Lactose utilization was 72% of that supplied after 97 h of fermentation.

Finally, a similar experiment (Run X, Figure 5.10) was performed where the initial pH value was pH 6.4, and this was allowed to fall

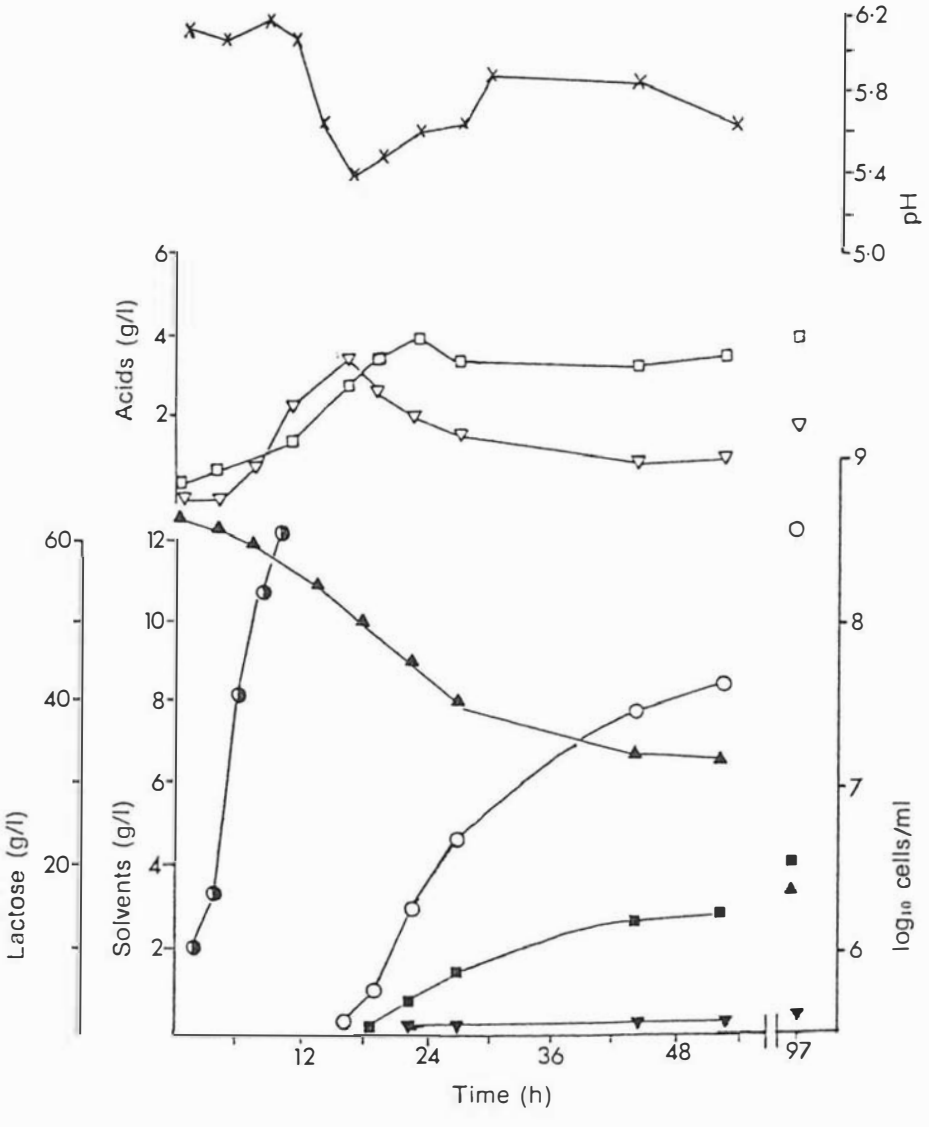


Figure 5.9. Batch fermentation profile of Run IX. Initial lactose concentration adjusted to 63 g/l. Initial pH 6.1, controlled at not less than pH 6.1 for 11 h. Symbols as for Fig. 5.1.

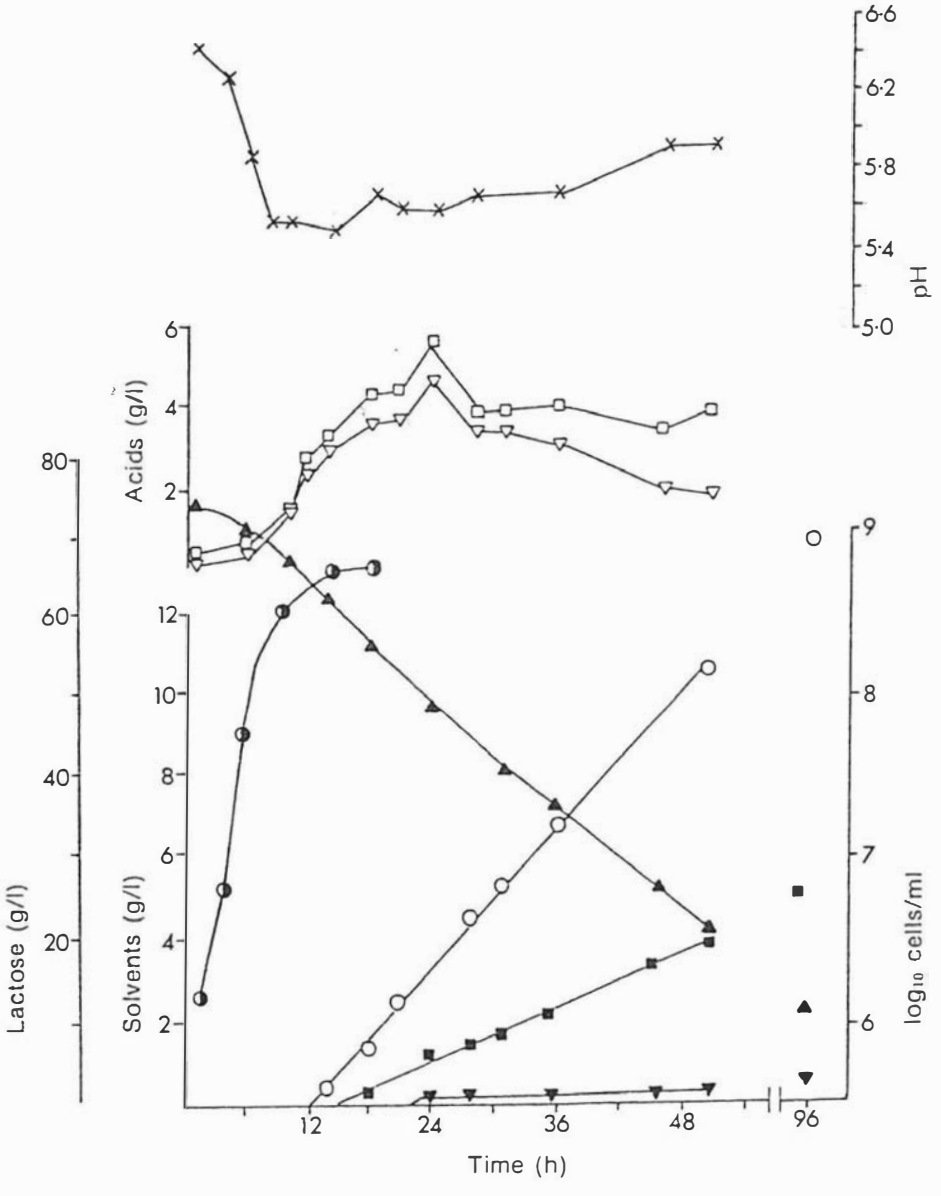


Figure 5.10. Batch fermentation profile of Run X. Initial lactose concentration adjusted to 75 g/l. Initial pH 6.4, controlled at not less than pH 5.6. Symbols as for Fig. 5.1.

naturally to pH 5.6 (8 h), below which it was prevented from falling. The initial lactose concentration was adjusted to 75 g/l. The pH increased to pH 5.85 after 46 h of fermentation. Again, the data show that even at this relatively high pH value the presence of additional lactose resulted in a decreased lactose utilization rate, comparable with that reported for Run IX, but which persisted throughout the fermentation, while high solvent productivity, concentration and yield were achieved. In both Runs IX and X, acid production occurred over a 24 h period, comparatively longer than in Runs I and II, whilst solvent production commenced at the same time (14 h) in each of these runs. After 96 h of fermentation, concentrations of 13.0 g/l, 4.5 g/l and 0.3 g/l were observed for butanol, acetone and ethanol respectively, and lactose utilization was 86% of that supplied.

5.2.2 Discussion

The objective of this work was to define the optimum culture conditions for maximum solvent production, productivity and solvent yield from the batch fermentation of sulphuric acid casein whey permeate. Similar studies have been performed by other workers using different substrates (predominantly glucose based) and strains of C. acetobutylicum. A systematic investigation using whey permeate (lactose) as the substrate has not previously been reported in the literature and such a study has been successfully completed in this work.

Factors which have been implicated in the control of solvent production include the culture pH, concentration of acetic and butyric acids (Gottschal & Morris, 1981b; Martin et al, 1983; Monot et al, 1983, 1984; Gottwald & Gottschalk, 1985), and the residual sugar concentration at the commencement of the solventogenic phase (Long et al, 1984a). The interactive effects of these factors remains largely unelucidated and the relative magnitude of the effect of each factor is likely to be strain and substrate specific.

Long et al, (1984a) using strain P262 with a synthetic medium containing glucose, state that a threshold total acid concentration in excess of 4 g/l is associated with the inhibition of cell division, the

conversion of vegetative rods into distinctive solventogenic clostridial forms, and the triggering of solvent production. At this point in the fermentation no nutrients are limiting. Further, they stated that the culture pH value is important, and that initiation of the solventogenic phase occurred only within a narrow pH range with a decrease in cell viability occurring at low pH values (actual values unstated) in poorly buffered medium. This may be related to the concentration of undissociated acid as suggested by Monot et al, (1983). These authors have reported that solvent production commences at an undissociated butyric acid concentration between 1.6 g/l and 1.9 g/l in the medium. Inhibition of growth by these undissociated acids has been suggested by these authors as playing a key role in the transition from acidogenesis to solventogenesis. If the concentration is less, as can occur at high pH values, little solvent is produced. Further, Holt et al, (1984) have reported the stimulation of solvent production at pH 7 by the addition of acetate and butyrate (100 mM each) to the culture, thus demonstrating that solvent production can occur at neutral pH values. Possibly, this effect was mediated through an increased undissociated acid concentration. In the same study, it was reported that at neutral pH values an increased initial sugar concentration enhanced solvent production, but no data were provided regarding sugar utilization rates. In a similar context, Long et al, (1984a) have stated that the initiation of solventogenesis requires a residual sugar concentration in excess of 10 g/l. These authors also stated that maximum solvent production occurred at 3 g/l diammonium hydrogen phosphate. This is equivalent to 0.64 g nitrogen/l, hence the use of yeast extract at 5 g/l in these fermentations, equivalent to approximately 0.5 g nitrogen/l ensured that no fermentation in this work was nitrogen limited (data not presented).

The results of the present study show that when using whey permeate without additional lactose, higher culture pH values resulted in improved lactose utilization rates and acid production, but solvent production diminished. However, when extra lactose was present, solvent production occurred at these higher pH values (pH 5.4-5.6), while the lactose utilization rate and acid production were decreased. The observations on the effect of culture pH at constant substrate

concentration (Runs I to IV) are in general agreement with those reported by Monot et al, (1984), albeit using a different strain of organism (ATCC 824) with a probable different optimum pH range for solventogenesis, on a glucose (50 g/l) containing synthetic medium.

In earlier work, Monot et al, (1982) using strain ATCC 824 and varying the glucose concentration, found that increasing the initial glucose concentration over the range 5 g/l to 80 g/l resulted in an increase in solvent production. At high sugar concentrations, lower acid concentrations resulted with significant acid uptake during solventogenesis, whilst at lower sugar concentrations, e.g. 20 g/l, acids were not taken up. This poor acid uptake occurred in Run II of the present work (high initial pH, no pH control, initial lactose = 40 g/l) whereas in Runs IX and X (high initial pH, pH control 5.4 - 5.6, initial lactose = 60 - 70 g/l), acid uptake during the solventogenic phase was observed. Welsh and Veliky, (1986) also using strain ATCC 824, reported similar results using a semi-synthetic medium containing lactose. At lactose concentrations of 10 g/l and 30 g/l, acids predominated, whilst at 50 g/l lactose, solvents predominated. No pH control was used during this work. Long et al, (1984a) also found that the glucose concentration affected the solventogenic performance of strain P262 with a minimal glucose concentration of 40 g/l required for good solvent production.

The pH values chosen for experiments involving pH regulation, were based on those reported by South African workers using strain P262 on different substrates. Long et al, (1984a), using a defined minimal medium containing glucose (60 g/l), reported an initial pH value of 6.8 falling to pH 5.8 before increasing to pH 6.0. Jones et al, (1982), using an industrial molasses medium (approximately 65 g/l sucrose), reported a different pH profile with an initial pH value of 6.0 - 6.8, falling to pH 5.2 before increasing to pH 5.6. These data suggested an optimum pH for solventogenesis in the range pH 5.2-5.8 for this strain.

The pH value prior to autoclaving of the medium used in the present study, affected the extent of minerals precipitation during autoclaving, the resulting initial pH value, and the buffering capacity

of the medium. In medium where the pH had not been adjusted prior to autoclaving, the value was pH 4.8, increasing slightly to pH 5.0 (Run I) after autoclaving, with very little precipitation occurring. Adjusting the medium pH to a value of 6.5 with ammonia prior to autoclaving resulted in a heavily precipitated medium (calcium salts) with a post-autoclave pH value of 5.3 - 5.5. This medium had a poor buffering capacity as evidenced by the comparatively rapid pH decrease in Run III (Figure 5.3), faster than that reported by Spivey, (1978) using this strain on industrial molasses medium.

The ability to produce solvents at high pH values (pH 5.5) from a hydrolysed Jerusalem artichoke substrate (approximately 70 g/l;glucose and fructose ratio 1:4) using C. acetobutylicum IFP904 has been demonstrated recently by Marchal et al, (1985). Consideration of the culture pH conditions closely parallels the present work, and two pH control programmes were considered optimal for solvent production.

The first programme involved a controlled linear pH decrease from initial pH 6.4 to pH 5.4 over a 15h period, used to limit acid production while providing optimum growth conditions prior to obtaining a culture pH of 5.4 for the solventogenic phase. A second and improved pH control scheme involved allowing a natural pH drop from pH 6.3 to pH 5.0 over a 5 h period, then a pH adjustment back to pH 6.5 followed by a natural fall to pH 5.4. The total acids concentration in this scheme reached a maximum of 7 g/l after 15 h fermentation, compared with approximately 4.5 g/l after 12 h in the former instance. The first pH control programme is similar to that reported in Runs IX and X of the present work, suggesting that a series of experiments using a controlled linear pH decrease may provide an improved set of operating conditions. The second pH control programme suggests that in Runs VII and VIII, acid production may have been reduced and solvent production enhanced if the pH value had been allowed to fall naturally again as opposed to being controlled at the adjusted value.

The data from all the Runs were analyzed to determine the concentrations of undissociated acids and residual lactose in the cultures at the time of initiation of solventogenesis (Table 5.2). For Run V, where little solventogenesis occurred, the concentrations were

taken at the time when initiation might have been expected to occur. In some experiments where little solventogenesis occurred (Runs IV, V and VI) the residual lactose concentration was 20 g/l or less, a factor considered to be important for solventogenesis (Long et al, 1984a). The low solvent production and immediate cessation of fermentation activity observed in Run V, resulting from the downward pH adjustment, may be attributed to the sudden shift in the ratio of undissociated to dissociated acids with the former species increasing to 6.5 g/l and 2.8 g/l of butyric and acetic acids respectively. These undissociated acids forms could be toxic to the microorganism, as described by Monot et al, (1984). Alternatively, the sudden alteration of the pH gradient across the cell membrane altering the internal acid concentration, may have caused a halt in metabolic activity.

From the data in Table 5.2, there appears to be little relationship between solventogenesis and the undissociated acid concentration in the medium. In fact, for Runs IX and X, where strong solventogenesis occurred, the undissociated acids concentrations were the lowest observed in the study, and less than the critical concentration proposed by Monot et al, (1983). Thus, these data suggest that the requirement of a minimal undissociated butyric acid for the inducement of solventogenesis is doubtful, a point also made by Roos et al, (1985), Gottwald and Gottschalk, (1985) and Huang et al, (1985). Further, in Runs IX and X the ratios of total butyrate to total acetate were the lowest observed, and this relationship with strong solventogenesis agrees with the findings of Roos et al, (1985). Gottwald & Gottschalk (1985) suggest that it is the elevated butyrate and acetate concentration in the cells, due to the maintenance of a positive pH gradient (i.e. internal pH is higher than external pH by approximately 1 unit) that is important in the triggering of solventogenesis. This internal concentration is therefore directly related to the external concentration and external pH. As a consequence of the high internal butyrate concentration, elevated concentrations of butyryl phosphate and butyryl coenzyme A, and hence low concentrations of phosphate and coenzyme A, are the basis of the triggering mechanism for solvent production (Gottwald & Gottschalk, 1985).

The total acid concentration at the commencement of solventogenesis in Run IX of 6.4 g/l and in Run X of 6.2 g/l are similar

Table 5.2 Concentrations of residual lactose and undissociated acids at the time of onset of solvent production

Run No.	Time (h)	pH	Total Butyrate (g/l)	Butyric Acid[a] (g/l)	Total Acetate (g/l)	Acetic Acid[a] (g/l)	Total Butyrate / Total Acetate	Residual Lactose (g/l)
I	14	4.3	1.6	1.2	0.7	0.5	2.29	40
II	16	4.7	2.5	1.4	1.8	1.0	1.39	33
III	17	5.2	5.6	1.9	3.8	1.0	1.47	30
IV	16	5.6	10.0	1.4	4.0	0.5	2.50	20
V	18[b]	4.6	10.6	6.5	4.8	2.8	2.21	12
VI	15	5.3	7.8	1.9	3.8	0.9	2.05	20
VII	13	4.4	2.5	1.8	1.2	0.8	2.08	38
VIII	14	4.4	1.6	1.1	1.1	0.8	1.45	40
IX	18	5.4	3.6	0.7	2.8	0.5	1.28	50
X	15	5.6	3.0	0.4	3.2	0.4	0.93	60

[a] pk values of 4.8 and 4.75 were used for butyric acid and acetic acid, respectively.

[b] since little solvent production occurred in this Run, this figure was selected as described in the text.

to those concentrations reported by Long et al, (1984a) as being necessary for strong solvent production from glucose. The decline in the growth rate at this time (Figures 5.9 and 5.10) also corresponds with these acid concentrations suggesting the toxic nature of these acids to cell division. In Run I however, the total acid concentration of 2.3 g/l at the commencement of solventogenesis was much lower, but the pH value was also lower. The pH gradient would be increased at this lower pH value and hence the internal concentration may have reached a concentration sufficient to trigger solventogenesis. The exact relationship between the external acid concentration and external pH for this particular strain, however, cannot be fully elucidated from this work. In Runs I and II where the pH value was low (4.0-4.4), total cell numbers at this time were lower than achieved in Runs operated at higher pH values. This finding supports the statement of Long et al, (1984a) that low pH values adversely affect cell viability. This may, in fact, be related to the form of organic nitrogen and its availability to the cell as proposed by Roos et al, (1985).

Although a high residual lactose concentration can be directly related to the initial concentration present, the effect of the latter on the lactose utilization rate is interesting. The decreased lactose utilization rates observed at elevated lactose concentrations and pH values had little effect on growth rates (Table 5.1) or final cell numbers, but led to decreased acid production and increased solvent yields. Figure 5.11 shows a plot of solvent yield versus maximum observed lactose utilization rate for all the Runs, and clearly shows a relationship. This relationship may possibly be explained as some form of catabolite repression. The maximum observed rate of lactose utilization used in Figure 5.11 was invariably measured during the acidogenic phase (late exponential growth phase), suggesting that the specific lactose utilization rate could possibly be substituted for this measurement. When the specific lactose utilization rate is high during the acidogenic phase, it is possible that some form of catabolite repression occurs suppressing those enzymes responsible for toxic solvent production. Leung & Wang, (1981) hypothesized that the production of acids and solvents is under catabolite regulation. At low growth rates (low specific lactose utilization rate) during solvents

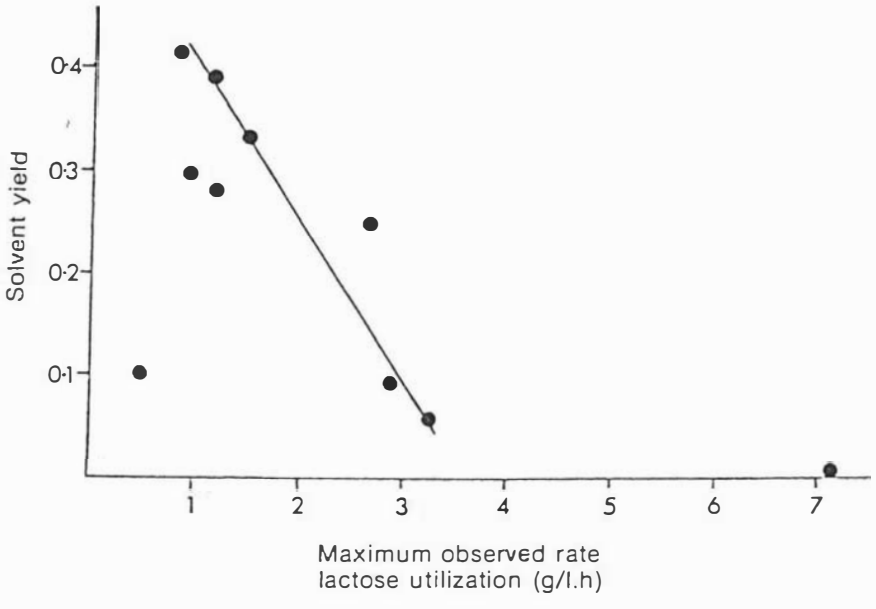


Figure 5.11. Plot of solvent yield (g/g lactose utilized) versus maximum observed lactose utilization rate, g/l.h, for Runs I to X.

production, the enzymes' activities responsible are high due to the slow flow of carbon through the metabolic pathway. These workers state that this may be caused by the derepression and activation of enzyme activities due to the low intracellular concentrations of catabolites. At higher growth rates (high specific lactose utilization rate) during acids production, the converse is true. Solvent enzyme activities are low due to increasing intracellular catabolite concentrations. Catabolite repression is observed in the formation of most secondary metabolites e.g. penicillin. Antibiotics which are toxic to the producing cell, are usually only produced when one or more nutrients are approaching exhaustion giving rise to a lower specific sugar utilization rate, such that the suppression of enzymes responsible for antibiotic production is released. At a low specific lactose utilization rate, repression of those enzymes responsible for solvent production may not occur, favouring solvent production and high solvent yields, however, further work is required to test this hypothesis.

5.2.3 Conclusions

High solvent yields are associated with conditions which minimize the lactose utilization rate. Such conditions can be achieved by operating the fermentation at low pH values, but this is not conducive to complete sugar utilization. Thus, it is preferable to operate the process at higher pH values and to use an elevated lactose concentration to minimize the utilization rate. It is possible that the sugar utilization rate is indirectly the controlling factor in solventogenesis and may provide a unifying concept to explain the control of solventogenesis. Conditions have been described whereby high concentrations of acetic and butyric acids may be achieved. The concept of operating the batch fermentation in two stages, whereby a high concentration of acids is produced in the first stage and the acids are subsequently reduced to solvents in the second stage, does not seem to be a practical proposition.

5.3 MORPHOLOGICAL CHANGES IN C. ACETOBUTYLICUM P262 DURING BATCH FERMENTATION

5.3.1 Introduction

C. acetobutylicum P262 demonstrates various morphological and cytological changes during the batch fermentation of an industrial molasses fermentation medium (Spivey, 1978; Jones et al, 1982). These changes have been related to growth and physiological changes and can be used as an index to measure the progress of a batch fermentation.

During the batch fermentation optimization study described in the previous section, regular microscopic examination of the cultures was undertaken, both for direct cell count determinations, and also to identify and profile these changes on this particular substrate. The objective was not to quantitate such changes but to verify published findings as a possible means of explaining solventogenic performance.

5.4.2 Results and Discussion

In all experiments (Runs I to IX), the fermenter was inoculated with highly motile cells, prepared as described in Section 3.7.2. In some instances, motile chains of 4-6 cells long plus motile rods were inoculated. These motile cells were "running" in straight lines. The size of the inoculum used was chosen to give an initial cell population of approximately 10^6 cells/ml, a number stated by Jones et al, (1982) to result in a very short lag period of less than 3 h on a molasses medium.

In some Runs, motility was observed during the first two hours, but after this time cells became non-motile. In all Runs, growth as evidenced by gassing and a very slight increase in cell numbers occurred after 2 h. Exponential growth invariably occurred over the period 2-14 h with the late exponential phase occurring between 14-18 h, after which no detectable growth occurred. Granulose accumulation as evidenced by the appearance of circular refractile bodies within the vegetative rods was occasionally present.

Interestingly, the observed μ_{max} values (Table 5.1) were higher than reported by Jones et al, (1982) on an industrial molasses medium

(μ_{max} = 0.27h^{-1}). Cell numbers reached approximately 5 x 10^8 cells/ml in Runs I and II, and 7 x 10^8 to 1 x 10^9 cells/ml in Runs III to X inclusive. These final cell numbers were very similar to those reported by Jones et al, (1982) and, together with the μ_{max} data, demonstrate that sulphuric acid casein whey permeate, supplemented with 5 g/l yeast extract, is as good a medium for cell growth as the industrial molasses fermentation medium.

Invariably, during the period 4-6 h, long chains were observed, varying in length from 6 to 10 rods. Motility commenced after approximately 6 h and increased until 70-90% of the chains and rods were motile by 8-10 h. Chains started to break up (6-8 h) as the extent of motility increased. After 12 h, motility became sluggish, with cells displaying a more random tumbling motion. At this time, clostridial forms were observed and their number increased from this point. In Run VI, however, greater than 90% motility was still observed after 15 h. The number of clostridial forms was greater in fermentations where good solvent production occurred than in those where solvent production was poor.

In Runs I and II, clostridial forms were approximately 40-60% of the cells observed after 30 h fermentation, with the remainder being vegetative rods. Forespores were observed after 30h in solvent-producing fermentations, with numbers increasing slowly, although a correlation between spore production and solvent production was not made. In Runs IX and X which were strongly solventogenic, greater than 90% of the cells were in the clostridial form when observed after 97 h and 96 h respectively, with a large unquantified extent of sporulation apparent, including release of some spores.

In summary, these observations made during the batch fermentation optimization study using sulphuric acid casein whey permeate, closely parallel those made and quantitated by Jones et al, (1982) and can be used as an index for the performance of such a fermentation process. It is possible that in those fermentations where the pH value decreased naturally to less than pH 5 and was then subsequently increased (Runs VII and VIII), vegetative rods were preferentially selected for acid production (faster growth rate), and clostridial stage development was retarded.

5.4 BATCH FERMENTATION OF LACTOSE-HYDROLYSED PERMEATE AND SEMI-SYNTHETIC MEDIUM CONTAINING GLUCOSE

5.4.1 Introduction

Preliminary data obtained from the 100-ml scale fermentation screening experiments (Chapter 4) indicated that lactose-hydrolysis prior to fermentation did not convey any advantage to the fermentation process. This was mainly due to incomplete utilization of the galactose moiety, although this same work demonstrated that galactose on its own is a good substrate for solvent production. However, the culture conditions used in this preliminary work were not optimal since the culture pH was not regulated during the fermentation process.

This section presents work from batch fermenter experiments carried out on a 5 l scale (Section 3.7.3.2) which were undertaken to examine the fermentation of lactose-hydrolysed sulphuric acid casein whey permeate, and semi-synthetic medium containing glucose, carried out under more favourable culture conditions.

5.4.2 Results

The results for all experiments are summarized in Table 5.3 and the time course profiles are given in Figures 5.12 to 5.14 inclusive.

Run XI was performed using semi-synthetic medium containing glucose (70 g/l) (Table 3.1). Figure 5.12 shows that the initial culture pH was 6.1 and pH control was used to ensure that the value did not decrease below pH 5.4 during the fermentation. The pH breakpoint occurred after 12 h and the pH increased to pH 6.15-6.20 after 30 h. A total solvent production of 18.6 g/l was achieved after 28 h fermentation and the fermentation was essentially complete after 30 h since very little further solvent production occurred. Data have not been corrected for dilution effects due to ammonia addition. The glucose utilization was 87.2% of that supplied with a solvent yield of 0.34 g/g. Microscopic examination of the culture revealed an estimated >90% clostridial form cells at 25 h, and an estimated >90% sporulation had occurred after 52 h fermentation. The acidogenic phase in this Run, although occurring earlier, is similar to Runs IX and X using whey

Table 5.3 Summary of fermentation parameters for all experiments in Section 5.4.2

	Run No.[a] XI	XII	XIII
initial pH	6.10	5.50	5.60
final pH	6.05	4.80	5.40
fermentation time, (h)[b]	28.0	37.0	35.0
butanol, g/l	13.0	5.60	8.65
acetone, g/l	4.8	1.85	3.20
ethanol, g/l	0.80	0.20	0.35
butyric acid, g/l	1.20	0.15	0.70
acetic acid, g/l	1.55	0.45	0.65
sugar utilized, g/l	61.5	24.0[c]	39.4[d]
sugar utilized, %	87.2	47.5	55.3
yield, g solvents/g sugar utilized	0.34	0.32	0.31[e]
overall fermenter productivity, g.solvent/l.h	0.66	0.21	0.35
maximum observed butanol production rate, g/l.h	1.13	0.48	0.63
maximum observed sugar utilization rate, g/l.h	2.47	3.9*	1.76*
		0.9#	0.35#

* Glucose

\# Galactose

[a] Full details are given in the text

[b] The fermentation time is that taken to achieve a butanol concentration 90% of that achieved after 60 h. The solvent and acid concentrations are those present at this time. For Run XIII, concentration data have been corrected for dilution effects occurring during pH control

[c] 18.0 g/l glucose, 6.0 g/l galactose

[d] 30.4 g/l glucose, 9.0 g/l galactose

[e] This calculation corrects for dilution effects due to pH control

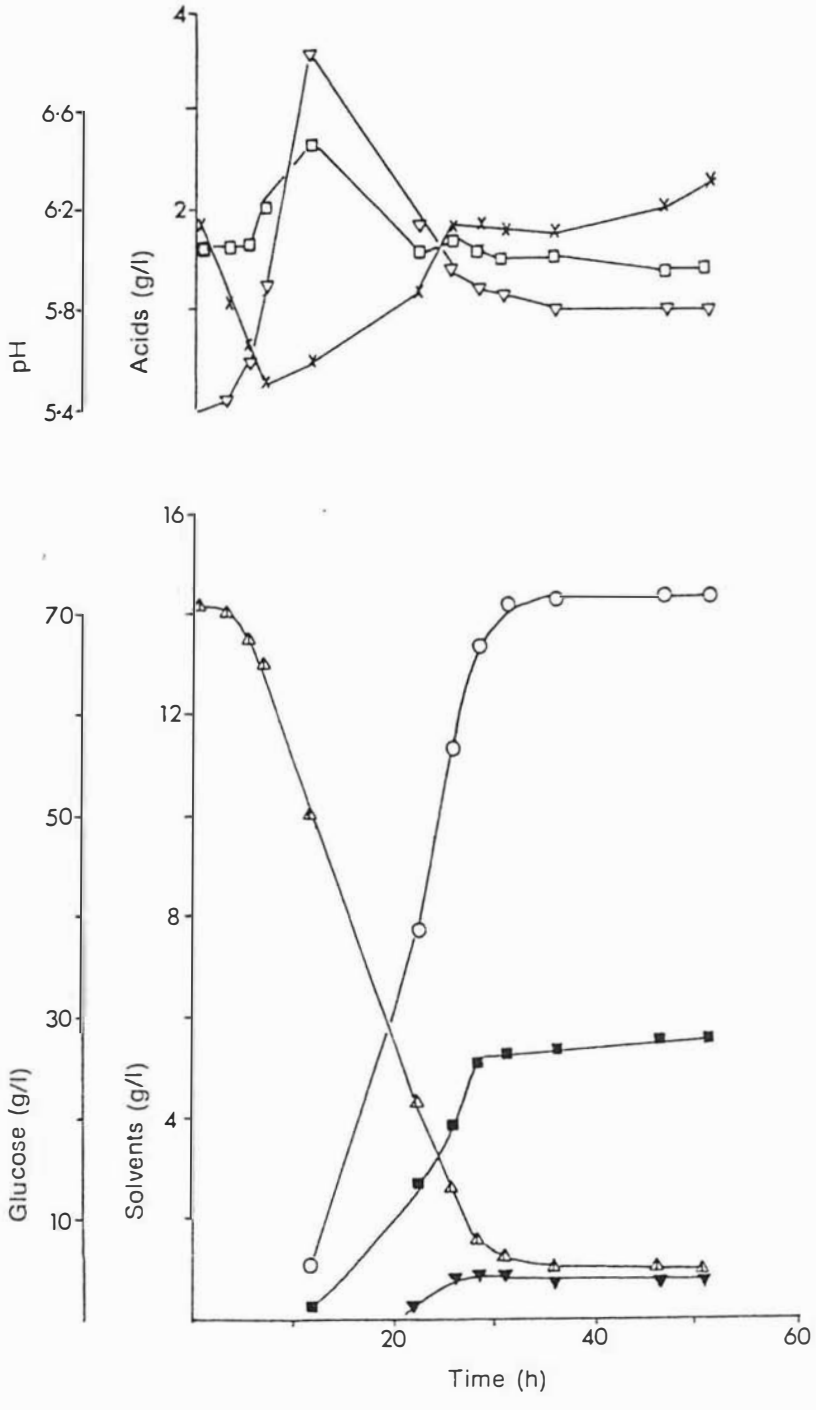


Figure 5.12. Batch fermentation profile of Run X1 (semi-synthetic medium containing glucose) pH controlled at not less than pH 5.4. Symbols as for Fig. 5.1; (▲) glucose.

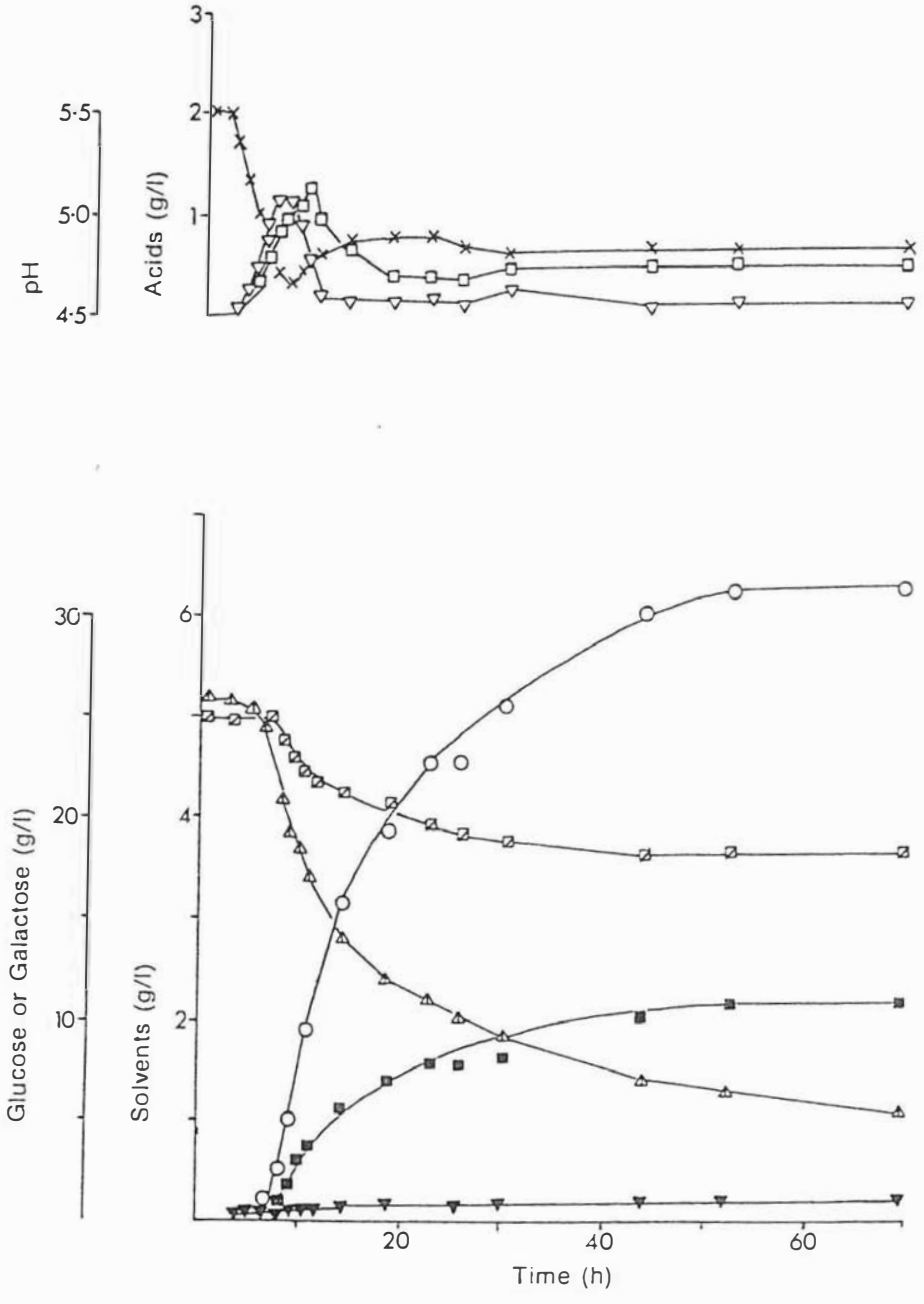


Figure 5.13. Batch fermentation profile of Run XII (lactose-hydrolysed whey permeate, no pH control). Symbols as for Fig. 5.1; (⧄) galactose; (▲) glucose.

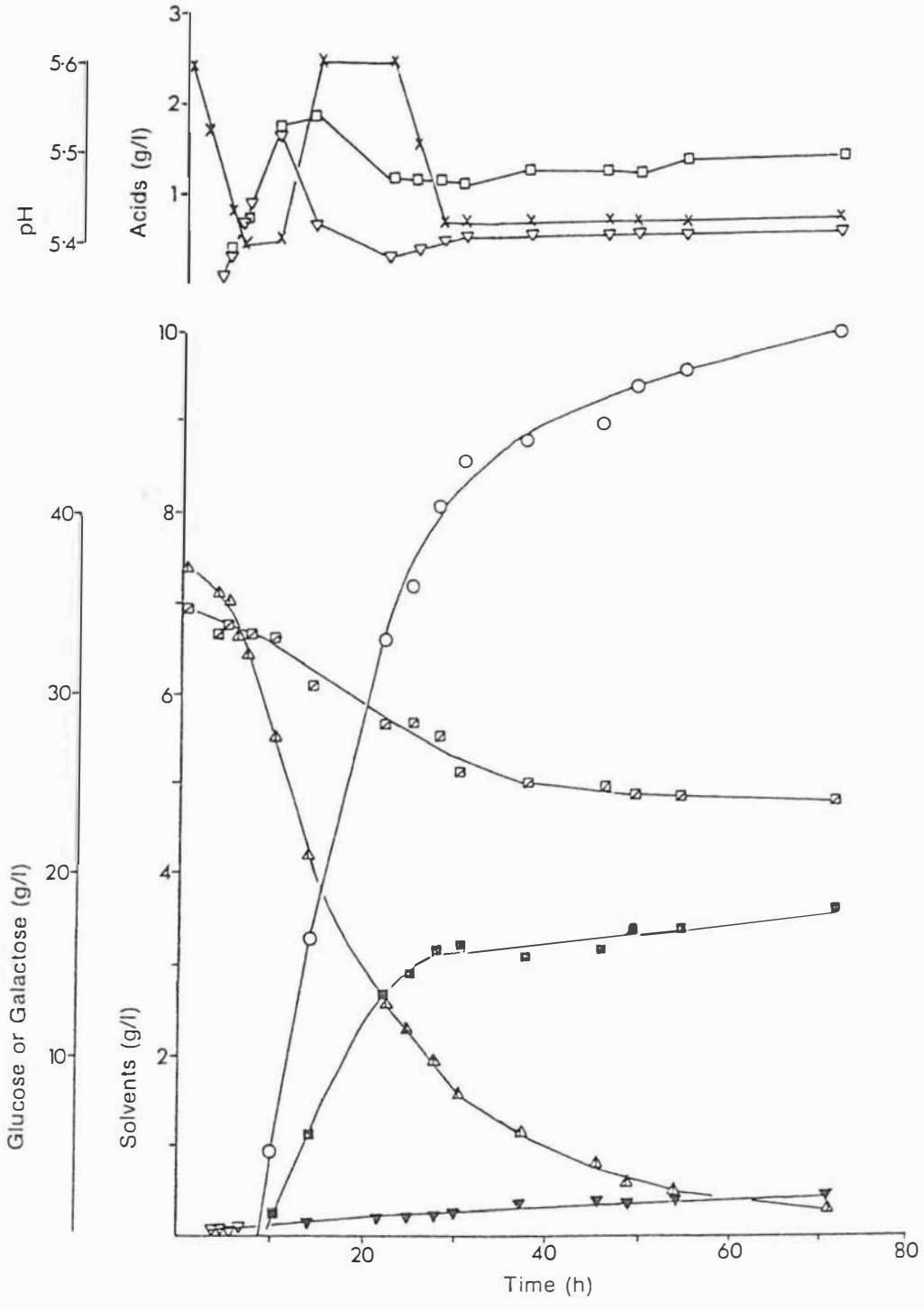


Figure 5.14. Batch fermentation profile of Run XIII (lactose-hydrolysed whey permeate, pH control to not less than pH 5.4). Symbols as for Fig. 5.1; (⊠) galactose; (◬) glucose..

permeate performed under comparable conditions. The total butyrate and undissociated butyric acid concentrations at the commencement of solventogenesis (12 h) were 3.5 g/l and 0.48 g/l respectively and are very similar to the values recorded in Runs IX and X.

Both the maximum observed butanol production rate and glucose utilization rate were significantly greater in this Run compared with Runs IX and X, indicating the preference that this organism demonstrates for glucose over lactose.

Run XII was performed using lactose-hydrolysed permeate with no pH control (Figure 5.13). The permeate was supplemented with 10 g/l of lactose prior to enzymic hydrolysis (Section 3.1.1). Although solvent production commenced slightly earlier, and the maximum observed sugar utilization and butanol production rates were greater, the use of lactose-hydrolysed permeate resulted in a similar yield and productivity to those observed in a comparable fermentation using non-hydrolysed permeate (Run II, Figure 5.2). The glucose and galactose were utilized simultaneously, but the former at a greater rate.

A further batch fermentation (Run XIII) was performed using lactose-hydrolysed permeate, where the culture pH was not allowed to fall below pH 5.4. Prior to enzymic hydrolysis, the permeate was supplemented with additional lactose (25 g/l). The fermentation profile (Figure 5.14) shows data which have been adjusted for the dilution effects resulting from pH control (11.5%). A total solvent concentration of 12.2 g/l was achieved after 35 h of fermentation, while the overall fermenter productivity and yield were 0.35 g/l.h and 0.31 g/g respectively. These parameter values are slightly higher than reported for the comparable run using non-hydrolysed whey permeate (Run X, Table 5.1).

5.4.3 Discussion

The objective of this work was to examine the effects of lactose hydrolysis on the batch fermentation of sulphuric acid casein whey permeate. Preliminary observations that lactose-hydrolysis offers little benefit in terms of fermentation productivity and yield (Chapter 4) are confirmed by the results presented here. Whilst some improvement

in these parameters is obtained, galactose is poorly utilized in the presence of glucose such that no overall increase in sugar utilization is obtained. Batch fermentation parameters obtained on semi-synthetic medium containing glucose (Run XI), confirm the optimum fermentation culture conditions; viz, pH 5.4 for solventogenesis, high residual glucose (50 g/l) concentration and an acid concentration of approximately 6 g/l required for strong solventogenesis on this substrate, stated by Long et al, (1984a). In fact, the performance of strain P262 on this medium, is superior to that reported by these workers using their media.

CHAPTER 6

CONTINUOUS SOLVENTS PRODUCTION USING C. ACETOBUTYLICUM P262 IMMOBILIZED IN CALCIUM ALGINATE GEL

6.1 INTRODUCTION

Most studies investigating the use of whey or whey permeate as fermentation substrates for solvents production have utilized traditional batch fermentation technology using free cells. The full potential of the process, however, may not be realized unless some newer fermentation technologies are applied as a means of improving reactor productivities. One such "intensified" fermentation technology is the use of immobilized cells (biocatalysts) for continuous processing. The principal advantage of using this technique is that high biomass concentrations can be achieved facilitating high reactor productivities and continuous stable processing.

Hence the purpose of the work described in this chapter, was to investigate the production of solvents from cheese whey permeate by continuous fermentation using cells of C. acetobutylicum P262 immobilized in alginate gel. Various parameters known to influence the fermentation, e.g. temperature, pH, substrate concentration, butyrate and butanol concentrations were assessed for their effects on the fermentation process. Additionally, data were obtained for substitution into a published simple kinetic model in order to verify experimental data.

6.2 RESULTS

6.2.1 Fermentation Start-Up

The start-up procedure for experiments using cheese whey permeate medium (Section 3.7.4.1) was a critical step for the successful operation of a continuous solventogenic fermentation using alginate immobilized cells. The pH control procedure (pH 5.8-6.0 for 22-26 h at a dilution rate (D_t approximately equal to 0.1 h^{-1})) was necessary to

ensure good spore germination and subsequent biomass accumulation. After 22-26 h, the total acids concentration was approximately 0.5 - 1.0 g/l. Some floating beads (<5% of total) and gas bubble release from the beads were observed. At this time, the pH control was ceased and the D_t was increased to 0.15 - 0.20 h^{-1}, resulting in a pH drop (pH 4.3 - 4.4 within 16-24 h) and a further increase in the total acids concentration to 1-2 g/l. The ratio of butyrate to acetate was typically 2:1. Solvent production commenced at this time. Subsequently, the acid concentration decreased to approximately 0.5 - 0.8 g/l of each acid, whilst solvent production increased. The D_t was further increased e.g. to 0.3 h^{-1}, to prevent the build-up of toxic solvents and to facilitate rapid biomass accumulation. The D_t was subsequently decreased from 0.3 h^{-1} if performance at lower D_t values was to be determined.

If the D_t was not increased immediately after germination, or the pH control procedure was operated for too long, a high acid concentration (approximately 3 g/l) resulted. This was accompanied by a very high proportion (25-50%) of floating beads that were fractured by gas release. It was not possible to successfully operate these fermentations at subsequent low acid concentrations (<1 g/l) or to accumulate bead biomass to expected levels.

All fermentations were started at 30°C because spore germination at 34°C or 37°C proceeded too quickly. Thus, these higher temperatures led to high acid production rates and bead damage due to gas release, that were difficult to control. When whey permeate was used without initial pH adjustment, poor germination, biomass accumulation and solvent production resulted.

Start-up of continuous fermentations using semi-synthetic medium containing glucose (medium pH 5.8-6.0) was more straightforward. Spores germinated in <24 h and pH control was not necessary. The total acid concentration was much lower than with cheese whey permeate medium after 24 h and solvent production commenced earlier and at a faster rate.

Solvent-producing fermentations started-up on semi-synthetic medium containing glucose did not always continue to perform adequately when switched to cheese whey permeate medium. Considerable variability

was observed in the ability of the culture to utilize lactose after initially using glucose. More often than not, these fermentations failed.

During the fermentation, the spherical or slightly "pear-shaped" alginate beads became irregular in shape as the biomass accumulated within the bead. Portions of white coloured biomass were clearly visible. Alginate beads at the end of a fermentation were fragile and could be easily fractured by gentle squeezing. In contrast, unused beads were stronger and more elastic.

6.2.2 The Effect of Dilution Rate (D_t) and Bead Fraction in the Reactor ($1-\varepsilon$)

In the first series of experiments, undertaken at 30°C, the D_t and bead fraction in the reactor ($1-\varepsilon$) were varied, while the pH was not controlled. The steady - state product concentrations, lactose utilization, solvents yield and productivity data are summarized in Table 6.1. Steady-state product concentration data were averaged over at least 10 residence times. Variations of $\pm$ 10% were obtained. Runs I-VII were conducted using a continuous stirred tank reactor (Section 3.7.4.1) and Run VIII using a fluidized column reactor (Section 3.7.4.2). The solvents yield based on lactose utilized ranged from 0.26 to 0.32 g/g, and the maximum volumetric productivity (1.79 g/l.h) was obtained in Run VIII. The pH of the cultures at steady state varied between 4.25 and 4.35 and was generally independent of D_t. Fermentations were routinely operated for 400-650 h with no apparent loss in performance. The maximum extent of lactose utilization was 20.5 g/l, representing 40% of the available lactose, and was observed in Run VI at $D_t = 0.16\ h^{-1}$. The dry weight cell biomass in the alginate beads varied between 130 to 180 g.d.w biomass/l. alginate, depending on the Run.

6.2.3 Effect of Temperature

The effect of temperature was investigated in two further experiments. Figure 6.1 depicts a time course profile of Run IX (pH 4.1-4.4, $(1-\varepsilon) = 0.20$, $D_t = 0.60\ h^{-1}$) where the temperature was

Table 6.1 Steady-state data obtained on cheese whey permeate medium at 30°C using alginate immobilized beads of C. acetobutylicum P262 [as a function of D_t and $(1-\varepsilon)$]

Run No.	$(1-\varepsilon)$	D_t (h^{-1})	C_B (g/l)	C_A (g/l)	C_E (g/l)	C_{HBu} (g/l)	C_{HAc} (g/l)	ΔS (g/l)	Y (g/g)	Prod (g/l.h)
I	0.13	0.10	3.55	1.49	trace	0.44	0.55	17.7	0.28	0.50
		0.20	2.60	0.78	trace	0.39	0.57	13.0	0.26	0.67
II	0.22	0.30	3.20	1.05	trace	0.45	0.54	15.7	0.27	1.28
III	0.25	0.29	3.25	1.25	0.07	0.38	0.49	17.6	0.26	1.33
IV	0.25	0.30	3.40	1.15	trace	0.36	0.45	16.4	0.28	1.37
V	0.25	0.31	3.15	1.34	0.11	0.39	0.46	17.2	0.27	1.43
VI	0.34	0.16	4.25	1.40	0.08	0.49	0.47	20.5	0.28	0.91
		0.25	3.50	1.30	0.06	0.49	0.54	17.5	0.28	1.22
		0.41	2.80	0.93	0.06	0.61	0.65	11.8	0.32	1.55
VII	0.38	0.23	3.95	1.59	0.09	0.33	0.48	18.9	0.30	1.30
VIII	0.42	0.405	3.45	0.97	trace	0.37	0.52	16.5	0.27	1.79

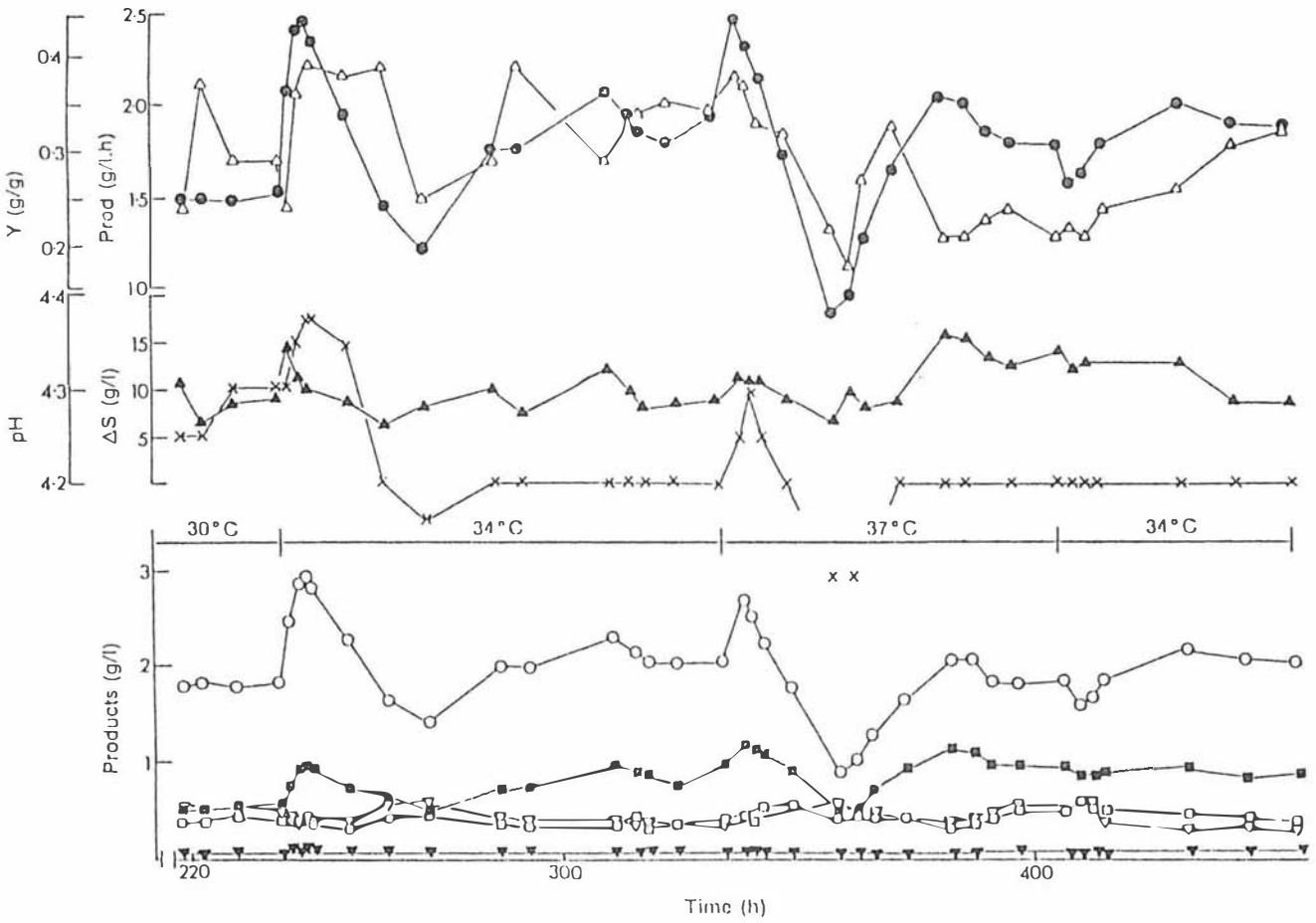


Figure 6.1. The effect of temperature on a continuous fermentation using immobilized *C. acetobutylicum* ($(1-\varepsilon) = 0.20$; $D_c = 0.60\ h^{-1}$). Run IX: (○) butanol; (■) acetone; (▼) ethanol; (▽) butyric acid; (□) acetic acid; (×) pH; (▲) substrate utilization; (●) productivity; (△) yield.

increased stepwise from 30°C to 34°C, then 34°C to 37°C, then decreased from 37°C to 34°C. With each temperature increase, there was a sharp initial increase in culture pH, solvents concentration, lactose utilization, yield and productivity. This was immediately followed by a slower drop in the value in these parameters followed by a small increase prior to a steady-state being achieved. A slight increase in acids concentration corresponded with the temporary fall in solvents concentration. Steady-state productivity, yield and the butanol : acetone ratio data from this Run are summarized in Table 6.2.

The highest yield and productivity data were obtained at 34°C, although the productivity data obtained at 37°C were within the typical 10% fluctuation in solvents concentration experienced in Runs I to VIII. The yield at 37°C was inferior to that obtained at 30°C or 34°C, attributable to a higher extent of lactose utilization rather than to a decrease in solvent production. The ratio of butanol to acetone (Table 6.2) decreased over the temperature range studied. This occurred mainly due to an increase in acetone production, with the butanol concentration increasing slightly at 34°C compared with that obtained at 30°C or 37°C. The fermentation culture was not deleteriously affected by the temperature changes, since comparable productivity data were obtained after the temperature had been decreased from 37°C to 34°C after 410 h operation, to that obtained previously at 34°C.

A further experiment (Run X) was conducted at 34°C to determine the effect of D_t at constant $(1-\varepsilon)$. Steady-state data are given in Table 6.3. The maximum volumetric productivity (3.79 g/l.h) was obtained at a D_t of 1.39 h^{-1}. The ratio of butanol:acetone decreased slightly as the D_t was increased.

6.2.4 Kinetic Model Parameter Estimation

The steady-state solvents concentration data as a function of D_t and $(1-\varepsilon)$ in Table 6.1 (30°C) and Table 6.3 (34°C) were substituted into a published kinetic model (Section 2.8.6).

A double reciprocal plot of P (specific butanol production rate (g butanol/(l.alginate h)), calculated from $D_t \Delta SY_{sb}/(1-\varepsilon)$ versus $D_t/(1-\varepsilon)$

Table 6.2 Steady-state data obtained on cheese whey permeate medium at different temperatures using alginate immobilized beads of C. acetobutylicum P262 (D_t = 0.60 h^{-1}, (1-ε) = 0.20, pH 4.1-4.4) Run IX

Temperature (°C)	Productivity (g/l.h)	Yield (g/g)	Solvent Ratio (Butanol:Acetone)
30	1.49	0.29	3.2:1.0
34	1.87	0.34	2.4:1.0
37	1.77	0.22	1.9:1.0

Steady-state product concentration data were averaged over at least 10 residence times. Productivity, yield and solvent ratio parameters were determined from the average values.

Table 6.3 Steady-state data obtained on cheese whey permeate medium at 34°C using alginate immobilized beads of C. acetobutylicum P262 (D_t was varied, $(1-\varepsilon) = 0.25$, pH 4.2-4.4) Run X

D_t (h^{-1})	C_B (g/l)	C_A (g/l)	C_E (g/l)	C_{HBu} (g/l)	C_{HAc} (g/l)	ΔS (g/l)	Y (g/g)	Prod (g/l.h)
0.65	2.68	1.18	0.08	0.64	0.48	12.7	0.31	2.56
0.92	2.50	1.15	0.08	0.40	0.49	12.0	0.31	3.43
1.39	1.80	0.85	0.08	0.49	0.48	9.3	0.29	3.79

(normalized total dilution rate (1/l.alginate h)) yields a straight line with a slope of ($1/C_{B,max}$) and a y intercept of ($1/Y_{sb}r_{max}$), and was prepared for data at 30°C and 34°C. Figure 6.2 is a double reciprocal plot of these data. A line (correlation coefficient = 0.95) with a slope of 0.197 and an intercept = 0.108 was obtained for 30°C data, and a line (correlation coefficient = 0.97) with a slope of 0.213 and an intercept = 0.058 was obtained for 34°C data. Using a value for Y_{sb} = 0.20, (average of all data used for preparation of Tables 6.1 and 6.3) gives values of $C_{B,max}$ = 5.08 g/l (30°C) and 4.69 g/l (34°C), and values of r_{max} = 46.3 g.lactose/l.alginate h (30°C) and 86.5 g.lactose/l alginate h (34°C). The advantage of operating at 34°C, therefore, is an increase in the maximal specific substrate consumption rate. These kinetic parameters suggested that an inhibitory concentration of butanol controlled the continuous fermentation process.

6.2.5 Effect of Added Butanol

In order to verify experimentally that an inhibitory concentration of butanol controls the fermentation process, further continuous fermentations were undertaken to determine the inhibitory effects of added butanol at 30°C and 34°C. By increasing the butanol concentration in the feed medium in small increments it was possible to determine that concentration range in which butanol production ceased. Figure 6.3 shows a time course profile for such a continuous fermentation at 30°C (Run XI). Butanol production ceased in the added butanol concentration range 4.02 - 5.62 g added butanol/l (30°C, pH = 4.3, $(1-\varepsilon)$ = 0.24, D_t = 0.30 h^{-1}). Acetone production decreased as the added butanol concentration was increased but acetone was still produced (0.3 - 0.5 g/l) even at 7.22 g added butanol/l. For the continuous fermentation at 34°C (Run XII, 34°C, pH = 4.35, $(1-\varepsilon)$ = 0.20, D_t = 0.62 h^{-1}) at a steady state butanol concentration of 2.8 g/l, the produced butanol decreased to 0.8 g/l when 4.5 g/l added butanol was fed to the fermentation. Thus, the results from Runs XI and XII generally agree with the maximum inhibitory butanol concentration determined from the kinetic model.

In several unrelated fermentations at either 30°C or 34°C, it was observed that when the butanol concentration reached 4.5 - 5.0 g/l, e.g. at low D_t values, there was a subsequent decrease in the steady-state

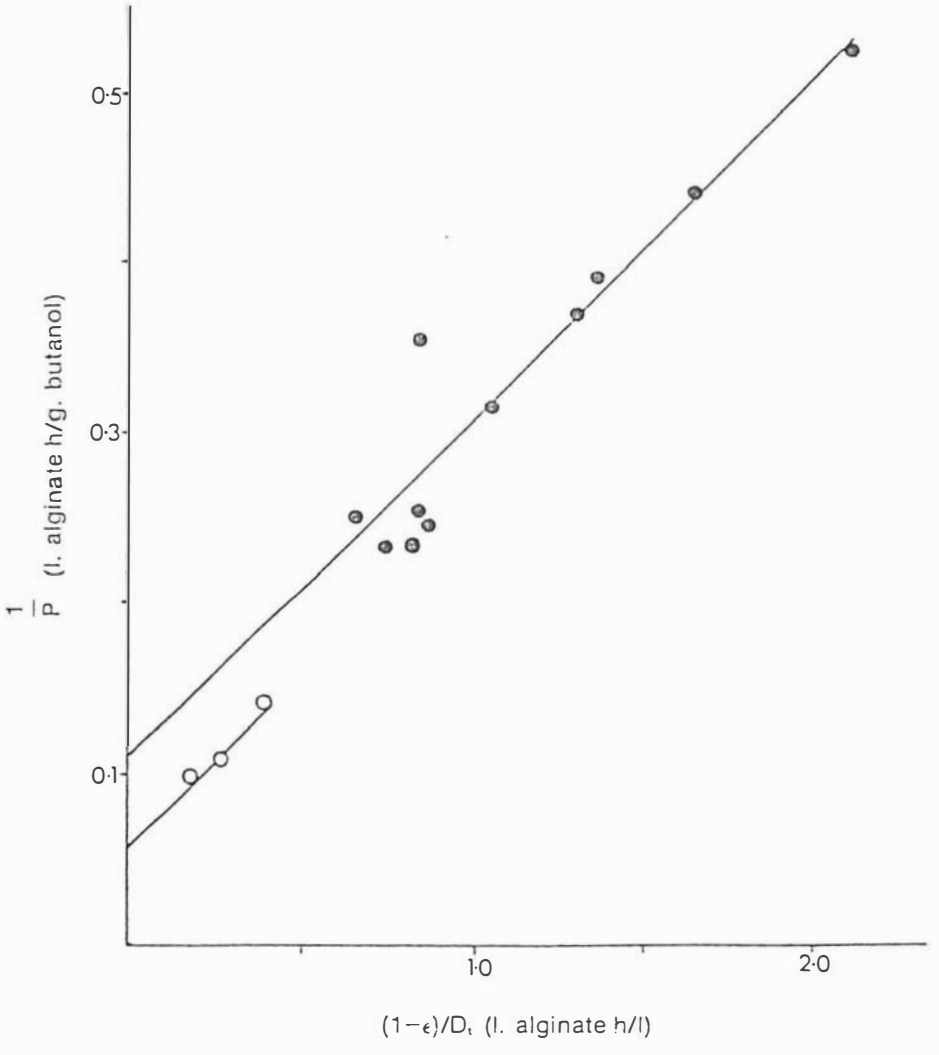


Figure 6.2. Double reciprocal plot of P vs $D_t/(1-\epsilon)$ using data derived from Tables 6.1 and 6.3 for cheese whey permeate medium: (●) 30°C; (○) 34°C.

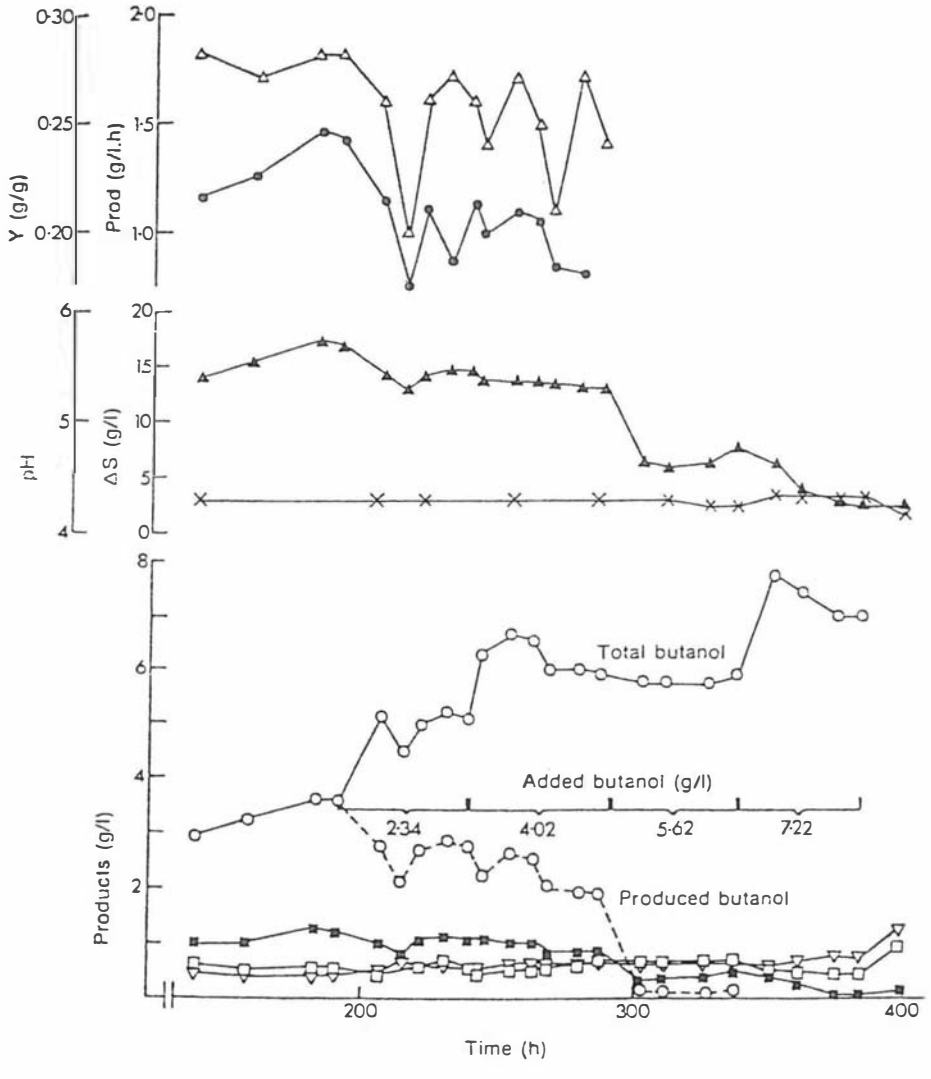


Figure 6.3. The effect of added butanol on a continuous fermentation using immobilized *C. acetobutylicum* at 30°C ($(1-\epsilon) = 0.24$, $D_i = 0.30\ h^{-1}$). Run XI. Symbols as for Fig. 6.1; (○–○) produced butanol.

solvents (butanol) concentration at this D_t value, and in some instances there were lower than expected concentrations at subsequently higher D_t values.

6.2.6 Effect of pH

Incomplete lactose utilization was observed in Runs I to X, possibly due to the low operational pH value, which was in the region pH 4.2-4.4 at steady-state. Previously it has been shown (Chapter 5) that the extent of lactose utilization and the butanol production rate during a batch fermentation using sulphuric acid casein whey permeate supplemented with lactose to a final lactose concentration of 65-75 g/l, were favoured by operating in the region pH 5.2-5.6. Thus, experiments were conducted to investigate the effect of elevated pH, with the objective of improving the extent of lactose utilization and hence solvent production.

A fermentation was performed at 30°C (Run XIII, $(1-\varepsilon) = 0.25$, $D_t = 0.30\ h^{-1}$) where the pH was regulated at higher values using an ammonia solution. Figure 6.4 shows that, as the pH was increased from 4.25 (at steady-state) to 4.8, 5.4 and then 6.0, the acids concentration increased whereas the solvents concentration decreased, with a corresponding decrease in solvents yield and productivity. An increase in gas production was also observed as the pH increased. Any increase in lactose utilization was not easily determined. The 'switch-over' to acids production as the pH was increased was reversible, since a recommencement of solvent production occurred when the pH control was terminated. Steady-state solvent production was monitored for a further 350 h after stopping the pH control.

A further experiment was performed at 30°C (Run XIV, $(1-\varepsilon) = 0.25$, $D_t = 0.40\ h^{-1}$) using a feed medium of cheese whey permeate supplemented with 20 g/l of lactose. In this instance, the extent of lactose utilization was increased from 17.5 g/l to approximately 20 g/l as the pH was increased from pH 4.2 to 4.9, and from approximately 20 g/l to approximately 25 g/l as the pH was increased from pH 4.9 to 5.6 (Figure 6.5). This was accompanied however, by an increase in the acids concentration and a corresponding decrease in both the solvents

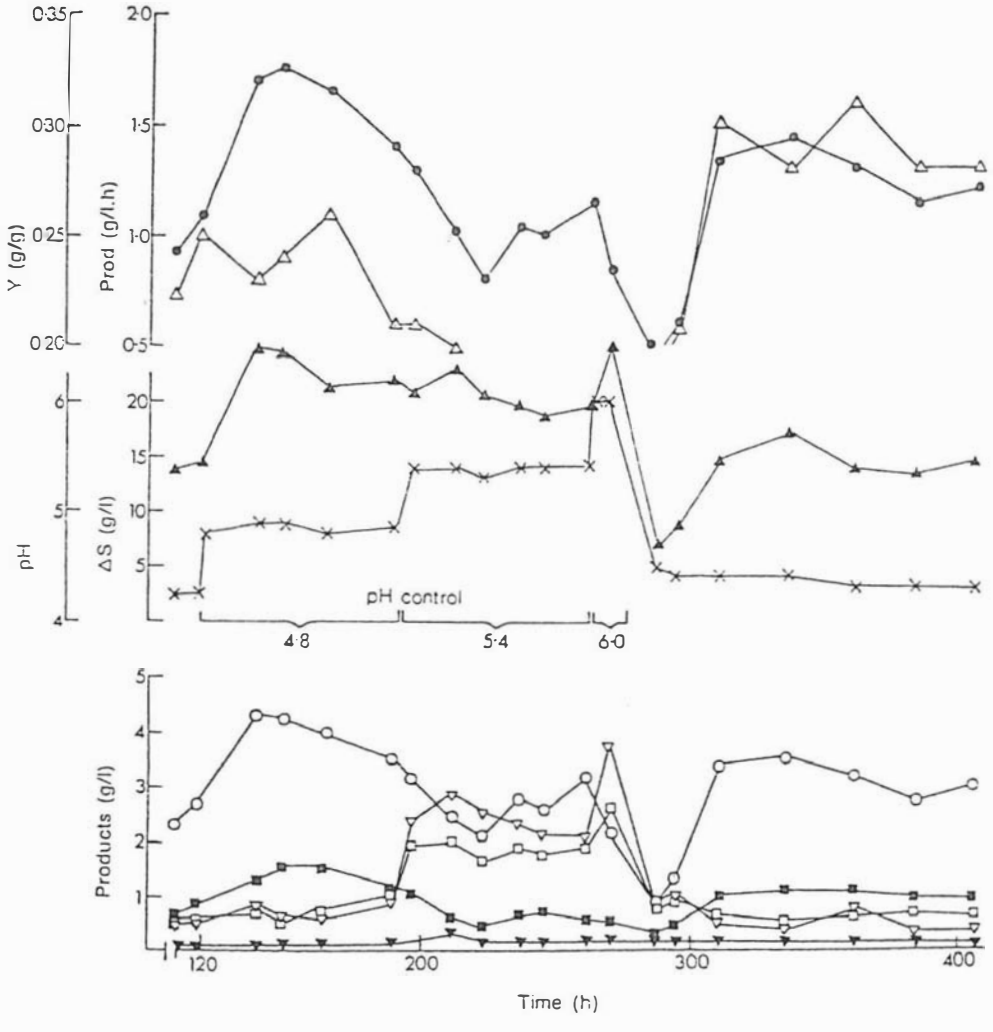


Figure 6.4. The effect of pH control on a continuous fermentation using immobilized *C. acetobutylicum* at 30°C ($(1-\epsilon) = 0.25$, $D_t = 0.30$ h^{-1}). Run XIII. Symbols as for Fig. 6.1.

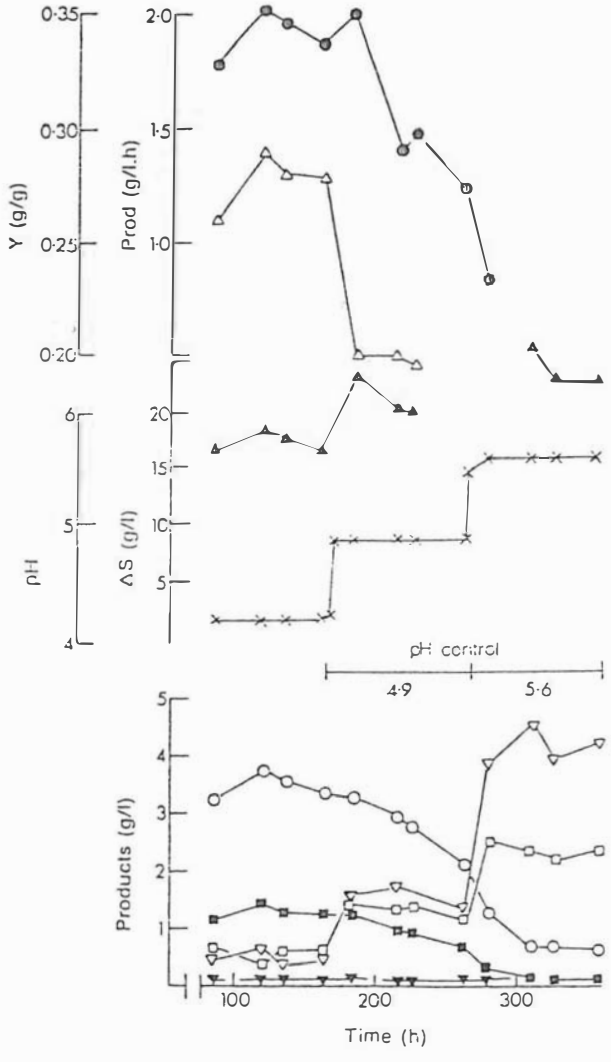


Figure 6.5. The effect of pH control on a continuous fermentation using immobilized *C. acetobutylicum* at 30°C using cheese whey permeate medium supplemented with 20 g/l lactose. ($(1-\epsilon)$ = 0.25, D_i = 0.40 h^{-1} or 0.44 h^{-1}). Run XIV. Symbols as for Fig. 6.1

concentration and yield. A total acid concentration of approximately 6.5 g/l was obtained at a culture pH of 5.6.

6.2.7 Effect of Added Butyrate

A series of continuous fermentations was conducted to determine the effect of butyrate concentration on solvent production. Runs XIII and XIV, utilizing pH control to operate in the region pH 4.8 - 5.6, had demonstrated that high acid concentrations could be continuously produced and that acids were non-toxic to cells at a total concentration of 4.5 g/l at pH 5.4 (Figure 6.4).

In Run XV at steady-state (30°C, pH = 4.35, $(1-\varepsilon) = 0.25$, $D_t = 0.29\ h^{-1}$), sodium butyrate was added to the feed medium at a concentration of 1.87 g/l (Figure 6.6). A small reduction in lactose utilization resulted, with a corresponding increase in solvents yield from 0.27 to 0.33 g/g; that is, butyrate was used in preference to lactose for solvent production. The steady-state butyrate concentration was 0.6 g/l. Increasing the sodium butyrate concentration in the feed medium to 3.23 g/l was inhibitory at this low pH value, and after 40 h the fermentation activity declined rapidly. Prior to this, however, the solvents yield had increased to 0.39 g/g. A continuous fermentation (Run XVI) performed at 34°C under similar conditions to Run XV (30°C) produced similar results. Increasing the temperature to 37°C during this run with 1.91 g/l sodium butyrate in the feed medium, resulted in a rapid decrease in fermentation activity. In a further continuous fermentation at 30°C (Run XVII, $(1-\varepsilon) = 0.25$, $D_t = 0.31\ h^{-1}$), the culture pH was controlled at pH 5.0 or pH 5.5 and sodium butyrate was added to the feed medium at a concentration of 1.73 g/l. No detectable increase in lactose utilization was observed. Additional acids production occurred at these pH values as observed in Runs XIII and XIV, operated at elevated pH values.

Since acid production is possible at elevated pH values (Runs XIII and XIV), and some butyrate uptake was observed at a low pH value (Run XV, Figure 6.6), a possible two stage process was considered, with acids production in Stage I followed by acids reduction to solvents in Stage II, with the objective of increasing overall solvent productivity. In

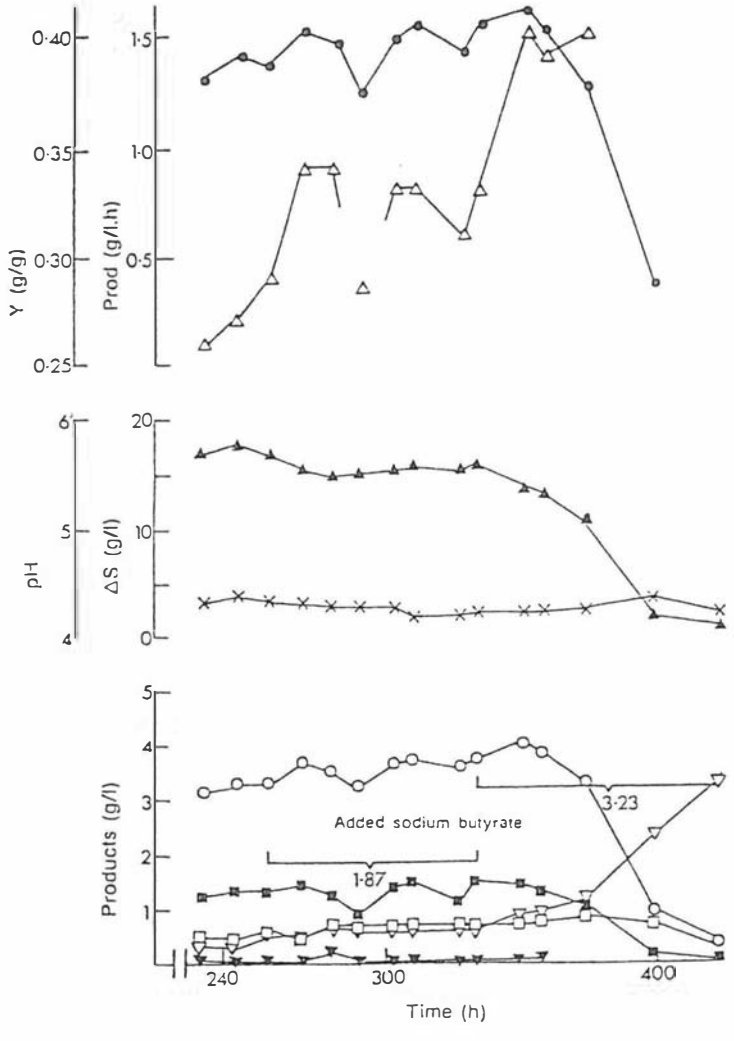


Figure 6.6. The effect of added butyrate (pH 4.6) on a continuous fermentation using immobilized *C. acetobutylicum* at 30 °C ($(1-\epsilon) = 0.25$, $D_i = 0.29\ h^{-1}$). Run XV. Symbols as for Fig. 6.1.

Stage I, the fermentation conditions were controlled (30°C, pH = 5.6, $(1-\varepsilon)$ = 0.24, D_t = 0.32 h^{-1}) so as to produce a product stream containing a butanol concentration of 2.8 g/l plus a concentration of 1.5 g/l for each of butyrate and acetate. Stage II was initially established using whey permeate feed medium with the conditions (30°C, pH = 4.3, $(1-\varepsilon)$ = 0.22, D_t = 0.30 h^{-1}) such that the product stream contained a butanol concentration of 3.2 g/l. Acid production was approximately 0.6 g/l of each acid. Then, over a 30 h period, the product stream from Stage I replaced the whey permeate as the feed medium to Stage II, maintaining a constant value of D_t. However, as soon as Stage II was fed completely by the effluent from Stage I, fermentation ceased. Thus, experiments involving pH manipulation of the culture in a single or two-stage process failed to achieve the desired aim of increasing the extent of lactose utilization and thus solvent productivity.

6.2.8 Effect of Substrate Type

Continuous fermentations using a simple semi-synthetic medium containing glucose (Table 3.4) were conducted at 30°C at various values of D_t and $(1-\varepsilon)$ with the objective of determining model kinetic parameters and comparing these with those obtained for cheese whey permeate medium (Section 6.2.4). The culture pH was not controlled, and varied between pH 4.30-4.40. The steady-state data are summarized in Table 6.4. Yield data was consistent at 0.32 g/g over the D_t range studied. The ratio of butanol to acetone decreased as the dilution rate increased for the Run using a $(1-\varepsilon)$ = 0.25. Consequently, the butanol yield on substrate (Y_{Sb}) changed accordingly. However, an average value of Y_{Sb} = 0.20 was calculated from the steady-state data and this value of Y_{Sb} was used for the model parameter estimation. This was done to be consistent with the approach taken for determination of model parameters for cheese whey permeate medium (Section 6.2.4).

Data taken from Table 6.4 were used for the determination of P, and a double reciprocal plot of P versus $D_t/(1-\varepsilon)$ was prepared (Figure 6.7). A line (correlation coefficient = 0.97) with a slope of 0.161 and an intercept of 0.0917 was obtained. Hence, $C_{B,max}$ = 6.21 g/l and r_{max} = 54.5 g.glucose/l.alginate h.

Table 6.4 Steady-state data[a] obtained on semi-synthetic medium containing glucose at 30°C using alginate immobilized beads of *C. acetobutylicum* P262 (as a function of D_t and $(1-\varepsilon)$

$(1-\varepsilon)$	D_t (h^{-1})	C_B (g/l)	C_A (g/l)	C_E (g/l)	C_{HBu} (g/l)	C_{HAc} (g/l)	ΔS (g/l)	Y (g/g)	Prod (g/l.h)
0.25[b]	0.25	3.85	1.90	0.18	0.31	0.45	18.6	0.32	1.48
	0.38	3.40	2.30	0.15	0.18	0.43	18.4	0.32	2.22
	0.51	2.70	1.70	0.11	0.15	0.43	14.2	0.32	2.30
0.38[c]	0.26	4.90	2.30	0.20	0.16	0.42	23.1	0.32	1.92
	0.41	3.95	1.90	0.16	0.21	0.40	18.2	0.33	2.46

a Data were averaged over at least 10 residence times. Variations of ±10% were obtained

b Obtained using a continuous stirred tank reactor

c Obtained using a fluidized column reactor

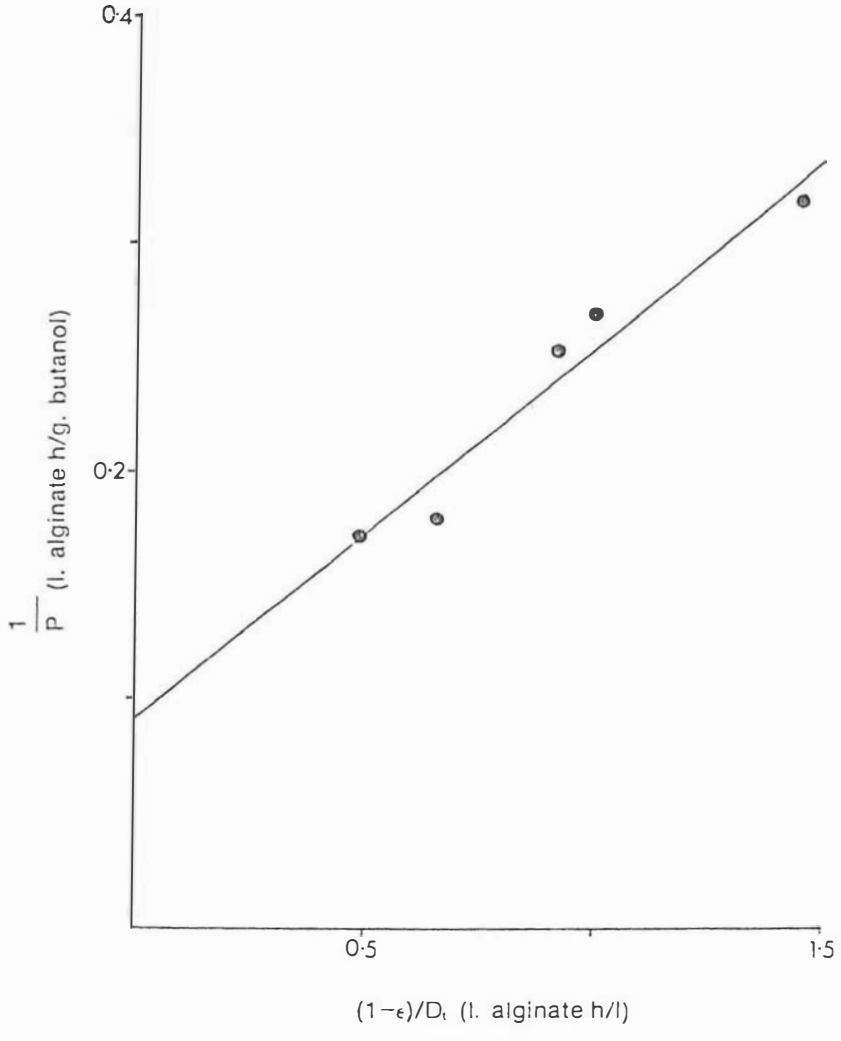


Figure 6.7. Double reciprocal plot of P vs $D_t/(1-\epsilon)$ using data derived from Table 6.4 for semi-synthetic medium containing glucose.

These kinetic parameter values indicate that strain P262 demonstrates a superior specific substrate utilization rate and butanol tolerance when fermenting glucose (semi-synthetic medium) compared with lactose (cheese whey permeate). Hence a further continuous experiment was undertaken to compare the fermentation performance of different substrates (semi-synthetic medium + glucose or galactose, lactose-hydrolysed cheese whey permeate) in order to verify the findings from the kinetic parameter estimations and to further examine the possible use of lactose-hydrolysed cheese whey permeate as a feedstock for continuous culture. Table 6.5 shows the steady-state solvent data obtained. These results show that both glucose and galactose can be used as substrates for solvent production, with glucose giving higher solvent production than galactose. Higher solvent concentrations, and thus productivities, were obtained using hydrolysed permeate compared to non-hydrolysed permeate. Since in hydrolysed permeate, little (<1 g/l) galactose was used, the data indicate that the organism prefers glucose to galactose.

In a further fermentation using semi-synthetic medium containing glucose (30°C, pH 4.40, $(1-\varepsilon) = 0.12$) the D_t value was decreased from 0.23 h^{-1} (no steady-state had been achieved) to 0.05 h^{-1}. The butanol concentration then increased from 3.1 g/l to 6.2 g/l over a 22 h period, but then decreased to 2.6 g/l after a further 22 h and then to 1.41 g/l after a further 24 h. Thus an inhibitory butanol concentration was reached as a result of the decrease in D_t, and this subsequently affected solvent production.

6.2.9 Electron Microscope Examination of Alginate Beads

Electron microscope photographs were taken of an unused alginate bead cross-section (Figure 6.8) and a used alginate bead cross-section (Figure 6.9). The used alginate beads were removed from a fermentation operating at steady-state at 34°C and "fixed" immediately for cutting and subsequent microscopic examination.

A homogeneously distributed mixture (Figure 6.8a) of cells (Figure 6.8b) and spores (Figure 6.8c) was immobilized. Figure 6.9a shows that pockets of biomass were homogeneously distributed throughout the used

Table 6.5 Steady-state data[a] obtained on various media at 30°C using alginate immobilized beads of *C. acetobutylicum* P262 ($(1-\varepsilon) = 0.20$[b])

Substrate Type	D_t (h^{-1})	C_B (g/l)	C_A (g/l)	C_E (g/l)	Y (g/g)
Semi-synthetic + glucose[c]	0.14	4.12	2.58	0.20	0.30
Semi-synthetic + galactose[c]	0.14	3.18	1.58	0.13	0.31
Cheese whey permeate	0.25	2.00	0.49	0.08	0.29
Lactose-hydrolysed cheese whey permeate	0.25	2.67	1.00	0.11	0.31[d]

[a] Steady-state solvent concentration data were averaged over 5 residence times. Variations of ±5% were obtained

[b] Less than 50% bead germination occurred

[c] Medium composition given in Table 3.4

[d] Less than 1 g/l galactose was utilized

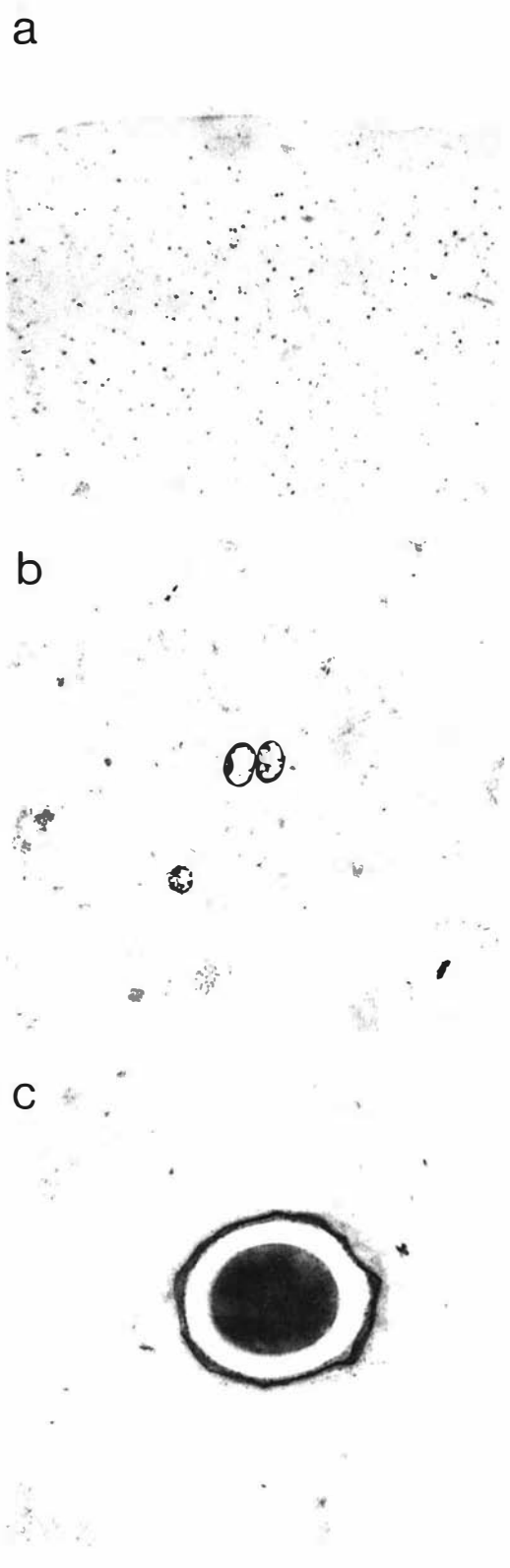


Figure 6.8. Electron microscope photographs of the cross-section of unused calcium alginate beads containing *C. acetobutylicum* P262.

(a) 400× magnification
Dark patches are spores or vegetative cells, or undissolved alginate. The lighter structure represents the alginate.

(b) 5200× magnification
A cross-sectional view of immobilized vegetative cells.

(c) 21200× magnification
A single spore.

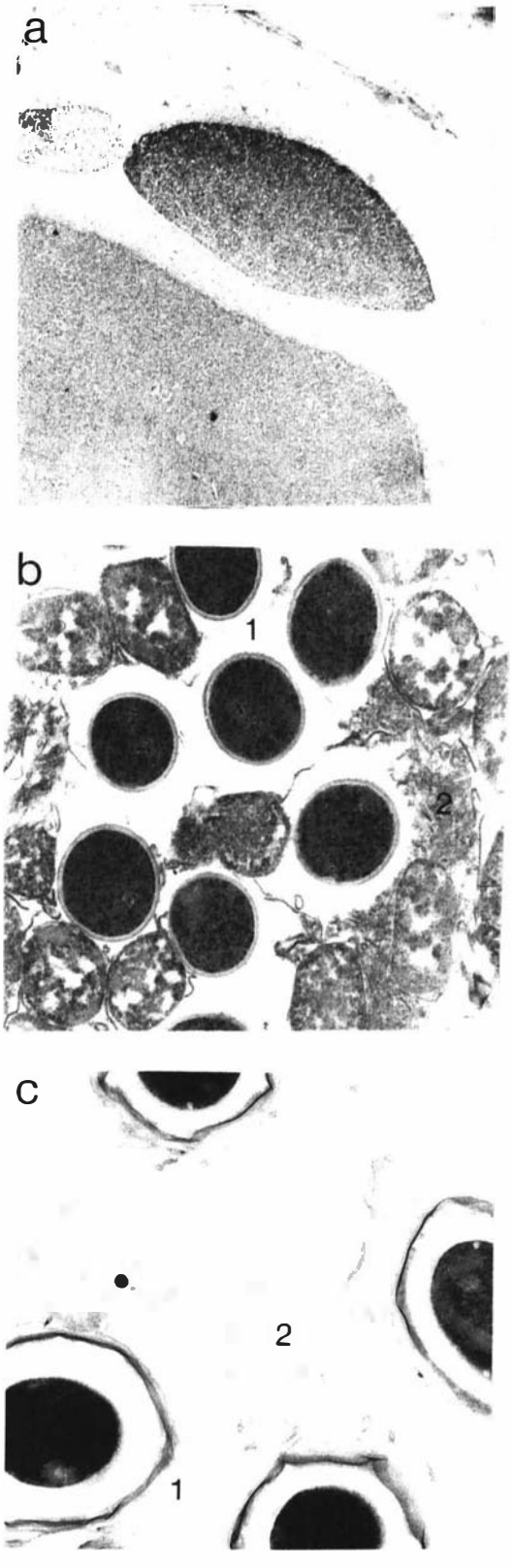


Figure 6.9. Electron microscope photographs of the cross-section of calcium alginate beads removed from a continuous fermentation at 34°C, at steady state

(a) 250× magnification
Dark patches are densely packed areas of biomass.

(b) 15300× magnification
This photograph shows:
1 cross-section of cell
2 long itudinal view of clostridial stage cell

(c) 21200× magnification
This photograph shows:
1 spores
2 cell debris.

calcium alginate beads. These used beads contained a mixture of ungerminated spores, germinating spores, vegetative cells, cell debris, and solvent-producing clostridial stage cells (Figures 6.9b, c). The cell number was greater than the spore number, but the differentiation of cell morphology i.e. vegetative cells as opposed to clostridial stage cells, was not easily determined.

6.3 DISCUSSION

The objective of this work was to investigate and optimize the conditions for long-term continuous stable solvent production from cheese whey permeate medium using alginate-entrapped cells of *C. acetobutylicum* P262.

The initial conditions at the commencement of each fermentation were found to be a critical aspect for a successful experiment. The interaction between culture pH and temperature on the rate of spore germination and vegetative growth in cheese whey permeate medium was not precisely defined in this work. However, commencement of fermentations in the manner described in Section 6.2.1, was more successful than by switching from a simple semi-synthetic medium containing glucose to the cheese whey permeate medium. This contrasts with the findings of Schoutens & Kossen (1986) who routinely commenced fermentations using a simple semi-synthetic medium containing glucose prior to switching to cheese whey permeate medium. This may be strain specific since these workers used alginate-entrapped *C. beijerinckii* LMD 27.6 or *Clostridium sp* DSM 2152.

The fact that elevated pH values (e.g. pH 6) enhance the rate of spore germination was first demonstrated by Krouwel, (1982) using alginate-entrapped *C. beijerinckii* LMD 27.6 and a simple semi-synthetic medium containing glucose.

The results of the first series of experiments conducted at 30°C (Table 6.1) show that solvent productivities obtained using cheese whey permeate medium were 5-6 times greater than that reported in batch fermentations using free cells at 34°C with sulphuric acid casein whey permeate medium (Chapter 5). Even higher solvent productivities were

obtained when operating at 34°C (Table 6.3). Solvent production and lactose utilization varied with dilution rate and bead fraction as shown in Table 6.1. The maximum solvent concentrations (typically 5-6 g/l) were obtained at high values of $(1-\varepsilon)$, and low values of D_t, resulting in productivities of approximately 1.5 to 2 times that typically obtained in a batch fermentation.

Solvent yields obtained at 30°C were typically 0.26 - 0.32 g/g (Table 6.1) and were lower than obtained in some batch fermentations, possibly as a result of additional substrate being utilized for biomass production; excess biomass (typically 1-1.5 g/l) was continuously removed from the fermenter. Solvent yields obtained at 34°C (Tables 6.2 and 6.3) were slightly higher than those obtained at 30°C; however, steady-state variations of $\pm$10% were routinely observed.

Long term stable solvent production has been demonstrated in this work. Experiments were routinely operated for 400-650 h in a CSTR and in one case up to 800 h, with very little detectable decline in system performance. Some bead attrition was observed, but this was minimized in this particular apparatus by maintaining the agitator speed below 200 rpm. The effect of added calcium ions (as $CaCl_2.2H_2O$) in the medium on the fermentation performance was not investigated. Largier *et al*, (1985) using strain P262, found no detectable difference in solvent production in batch fermentations of alginate-entrapped cells with or without added $CaCl_2.2H_2O$ (5 g/l). In the present work, rapid bead disintegration and loss of fermentation performance occurred if calcium ions were omitted from the medium. Schoutens & Kossen (1986) have reported an experiment lasting up to 1800 h in a CSTR with no measurable loss in activity or calcium alginate bead disruption.

Alginate beads could be successfully stored at 4°C in 5 g/l $CaCl_2.2H_2O$ for periods greater than 9 months and used in continuous fermentations with no detected loss in performance.

The use of a CSTR was routinely adopted for all experiments except for Run VIII (Table 6.1) and for some data presented in Table 6.4. This was due to its comparative ease of operation. Gas bubbles escaped unhindered from the liquid phase and concentration gradients were absent

in the well mixed system. In contrast, the FCR was difficult to operate for extended periods due to gas release which resulted in slugs of gas lifting sections of the alginate beads. Bubbles did not coalesce but accumulated in pockets. The column was vibrated in order to facilitate the removal of this accumulated gas. These observations support those of Krouwel et al, (1983a) who stated that a packed bed reactor is unsuitable for this particular fermentation, since high pressure drops and gas formation, leading to stagnant zones and hence clogging and channeling problems, occur. Concentration gradients over the fluidized bed were not estimated since a balancing of production and mixing times was not attempted. The circulation liquid velocity was not measured, but adjusted in order to achieve approximately 50% bed expansion.

Bead fraction volumes ($1-\varepsilon$) in the CSTR of 0.20 to 0.25, were routinely used in experiments after those described in Table 6.1, since easier mixing and less bead settling occurred than in experiments where the ($1-\varepsilon$) value was 0.30 to 0.35.

The ability of alginate-immobilized C. acetobutylicum P262 to continuously produce solvents from whey permeate over the temperature range 30°C to 37°C (Run IX, Figure 6.1) agrees with previously reported results with batch fermentations using free cells (Chapter 4). This observation may be strain-specific since Schoutens et al, (1985) could produce solvents from cheese whey permeate at 30°C but not 37°C during both batch fermentations with free cells and continuous fermentations using alginate-entrapped C. beijerinckii LMD 27.6. That temperature affects the ratio of solvents, yield and solvents concentration (Table 6.2) was also found by Voget et al, (1985) on whey medium using different Clostridium sp. Since the increase in solvents productivity was mainly attributable to an increase in acetone production it is possible that the temperature affects the activity of key enzymes responsible for acetone formation.

The biomass loading in the alginate beads was found to be directly proportional to the rate of increase in the value of D_t (Section 6.2.2). If D_t was increased to 0.3 h^{-1} ($(1-\varepsilon) = 0.25$) within 20 h of spore germination, the biomass concentration in the beads reached 180 g.d.w biomass/l. alginate (e.g. Runs XIII and XVI). If however, D_t was kept

at lower values and not increased to D_t = 0.3 h^{-1} until 60-100 h after germination, then the biomass concentration in several instances (data not shown) reached only 130-150 g.d.w biomass/l. alginate. This effect of D_t on the extent of biomass accumulation may be mediated by the inhibitory butanol concentration on the biomass growth rate. At higher initial values of D_t, and hence lower initial butanol concentrations, biomass is accumulated faster and to a higher extent. The proportion of active biomass was not determined in this study. Since an electron microscopic examination of alginate bead cross-sections revealed a range of cell morphologies (Section 6.2.9), the calculation of specific rates for direct comparison with other fermentation modes could be misleading.

The fermentation kinetic model parameters determined in Section 6.2.4 for cheese whey permeate medium at both 30°C and 34°C enable the calculation of overall reactor productivity as a function of D_t and $(1-\varepsilon)$. Figure 6.10 is a plot of reactor productivity versus D_t for a value of $(1-\varepsilon)$ = 0.45; on alginate bead loading typical of that obtainable using a fluidized bed reactor. The equation

$$\Delta C_s = C_{B,max}\ r_{max} \Big/ \frac{D_t}{(1-\varepsilon)} * C_{B,max} + r_{ax}\ Y_{sb}$$

(Section 2.8.6) was used to calculate ΔC_s for given values of D_t and $(1-\varepsilon)$ using the predetermined values for $C_{B,max}$, r_{max} and Y_{sb}. Solvents productivity was then calculated for each D_t value using the equation:

$$\text{Productivity} = D_t\ \Delta C_s\ Y_{ss}$$

Values for Y_{ss} (total solvents yield on substrate utilized) of 0.30 (30°C) and 0.32 (34°C) were used for this calculation. The curves in Figure 6.10 show greater solvent productivities at values of D_t greater than used in the present study. The improved productivities obtained at 34°C compared with those obtained at 30°C are attributed to the significant increase in the maximal specific substrate consumption rate, since the $C_{B,max}$ was comparable at both temperatures. However, it is clear that high solvent productivities are obtained at the expense of lower substrate consumption.

The kinetic model parameters obtained in this work, are similar to those reported by Schoutens & Kossen, (1986) who used alginate-entrapped

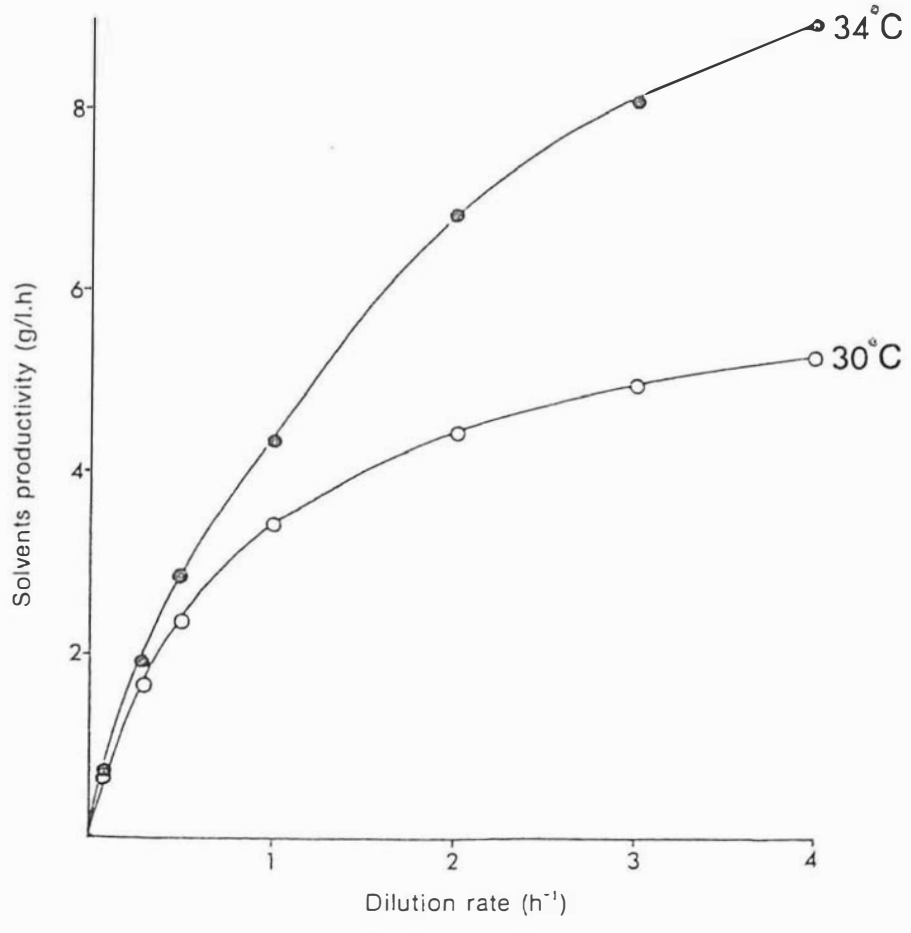


Figure 6.10. The solvents productivity (g/l.h) calculated as a function of the dilution rate for a reactor loading ($1-\epsilon$) of 0.45, using kinetic parameters derived in Section 6.2.4 for cheese whey permeate.

Clostridium sp DSM 2152 at 30°C on cheese whey permeate medium. Values of 4.7 g/l and 55 g lactose/l.alginate h were obtained by these workers, for $C_{B,max}$ and r_{max} respectively. Direct quantitative comparison of these results at 30°C may be misleading since these workers derived their information from a single experiment (constant value of $(1-\varepsilon)$) by varying D_t, in contrast to work presented here, where both D_t and $(1-\varepsilon)$ were varied over a wide range during eight separate experiments (Table 6.1). The fact that the data fit the kinetic model so well, suggests that the observed differences in the biomass content of the beads may be irrelevant.

The continuous fermentation experiments where butanol was added to the feed medium to find that concentration region in which butanol production ceased (Section 6.2.5), generally showed agreement with the values of $C_{B,max}$ predicted from the kinetic model. However, it is interesting that acetone production continued, albeit at a lower level. Acids production did not vary during the experiment, suggesting that some growth was still possible within the alginate beads even at a total butanol concentration of 7-8 g/l. When fresh medium containing no butanol was fed to this fermentation after 400 h (data not shown in Figure 6.3), very low solvent and acid concentrations (<1 g/l total) were detected indicating that some of the original biomass was inactive.

Further investigation of the form of the inhibition kinetics is necessary, either at high D_t or a low $(1-\varepsilon)$ value in order to keep the butanol concentration resulting from the fermentation at a low concentration prior to butanol addition to the feed medium. A plot of substrate consumption rate (g.lactose/l.alginate h) versus the butanol concentration could be constructed, and the form of the inhibition kinetics (e.g. linear) determined. Such a calculation would be approximate only, since it is probable that the internal butanol concentration (produced butanol) is more toxic to the cell than the external butanol concentration as suggested by Novak et al, (1981) for ethanol inhibition of yeast.

Attempts to increase the extent of lactose utilization, and hence solvent productivity, by operating at higher pH values (pH 5.2-5.6) were not successful. An increase in gassing, free cell biomass and acid

concentrations at these pH values can be attributed to an increase in the biomass growth rate. Since electron microscope photographs (Section 6.2.9) have shown that different morphological forms were present in solvent producing alginate beads operating in the region pH 4.2 to 4.6, it is possible that vegetative rods were preferentially selected for, leading to acid production, when the pH was increased. Similar observations were made when adjusting the pH value (pH 4.4 to pH 5.5 or pH 6.0) in the free cell fermentations described in the previous chapter. This contrasts with the findings or Largier et al, (1985) who successfully operated a continuous fermentation using strain P262 entrapped in alginate beads, on a molasses fermentation medium at 34°C, in the pH range of pH 5.4 to 5.6. In this instance, however, an early sporulation deficient (spo A2) mutant was used and was "locked" into the solvent producing clostridial stage. The advantage of using these mutants is reflected in the considerably greater solvent concentrations and hence solvent productivities reported.

The ability of a continuous fermentation to revert to solvent production after the cessation of pH control at higher values (Run XIII, Figure 6.4) supports the postulation that fast-growing acid-producing vegetative cells are preferentially selected at elevated pH, and were washed out of the continuous reactor as the pH was allowed to fall. It can be speculated that those solvent producing clostridial forms in the alginate beads sporulated (or degenerated) as a function of the operating time and the solvent concentration. A dynamic equilibrium would be set up with these sporulating organisms subsequently re-germinating or alternatively the fermentation performance declining after a period of time.

The addition of butyric acid to batch fermentations at low pH values has been observed to induce solvent production by strain ATCC 824 (Gottschal & Morris, 1981b). The role of the undissociated acid concentration, or the total acid concentration inside the cell in response to the maintenance of a positive pH gradient, itself a function of external acid concentration and culture pH, in inhibiting cellular growth and triggering solventogenesis, was discussed in Section 5.2.2.

The inhibitory nature of the external undissociated acid concentration on cellular growth is one possible explanation for the

reduction in fermentation performance (Figure 6.6) when the immobilized cells were exposed at pH 4.3 to a total butyrate concentration of 3.23 g/l, i.e. to an undissociated acid concentration of 2.45 g/l (pKa for butyric acid = 4.8). Conversely, immobilized cells in an acid-producing culture at pH 5.4 (Figure 6.4) tolerated a total butyrate concentration of approximately 2.5 g/l where only 0.5 g/l is in the undissociated form. An alternative explanation is that the increased acids concentration inside the cell at the low pH value, brought about by the increased external acids concentration (butyrate addition), was inhibitory to cellular growth and solvent production. The differentiation between which mechanism is responsible for the reduction in fermentation performance is unclear from this work. The increase in yield associated with butyrate uptake for solvent production at an added butyrate concentration of 1.87 g/l (not toxic at this concentration) agrees with the findings of Martin et al, (1983) who obtained increased solvents yield with added acids to batch fermentation cultures using a synthetic medium containing glucose.

The failure of the two-stage process, where acids (less than 2 g/l butyrate) and solvents were produced in the first stage at high pH value and then passed to a second stage at low pH value for acid reduction to solvents, can possibly be attributed to the synergistic toxicity of butanol and butyric acid, although the latter species was below the inhibitory concentrations found in Run XV. The experimental finding supports the conclusions drawn from the batch fermentation work described in Chapter 5 where such a two-stage process was considered impractical.

The higher values for the kinetic parameters $C_{B,max}$ and r_{max} obtained for a simple semi-synthetic medium containing glucose (Section 6.2.8), compared with those obtained for cheese whey permeate (lactose), are in general agreement with results reported by Schoutens & Kossen (1986) using these media with alginate-entrapped Clostridium sp DSM 2152. The higher butanol tolerance ($C_{B,max}$) exhibited by immobilized strain P262 when fermenting glucose can be related to the substrate transport mechanism. Ounine et al, (1985) found that product inhibition was stronger for xylose in batch fermentations using free cells of C. acetobutylicum ATCC 824, hypothetically due to a membrane bound

substrate transport mechanism that was active in the case of xylose transport but not active for glucose. The higher maximal specific substrate consumption rate obtained on glucose medium compared with that obtained on cheese whey permeate supports the suggestion that glucose is a preferred carbon source to lactose. The increase in solvent productivity when fermenting lactose-hydrolysed cheese whey permeate (Table 6.5) supports this statement. However, the fact that galactose is used only slowly in the presence of glucose supports the earlier conclusions in Chapters 4 and 5 that lactose hydrolysis prior to fermentation conveys no operational advantage to the fermentation process.

The substrate-related effect on the attainable solvents concentration may partly explain the higher solvents concentrations obtained during continuous fermentations using alginate-entrapped C. acetobutylicum P262 on a sucrose-based medium (Largier et al, 1985). It is possible that the sucrose uptake mechanism parallels that for glucose. Additionally, and perhaps more importantly, these workers operated their fermentations with a richer medium and at a higher pH value than reported in the present work. Even if no increase in butanol tolerance were obtained under these improved conditions for work described here, some expected increase in the maximal specific substrate consumption rate should give rise to greater overall solvent productivities.

6.4 SUMMARY

Continuous stable solvents production using alginate-immobilized C. acetobutylicum P262 cells on cheese whey permeate medium has been demonstrated. The fermentation can be essentially described as a biomass volume process ($1-\varepsilon$) in which substrate utilization, and hence solvents productivity, are defined by an inhibitory concentration of butanol.

Significantly greater solvent productivities are obtained, compared with those achieved using free cells in batch fermentations. However, lower extents of lactose utilization were observed. The use of pH regulation and a two-stage process, with the objective of increasing

the substrate utilization, were found to be detrimental to solvent production.

The immobilization of spores in alginate beads is a simple, robust method. Beads were stored at 4°C in 5 g/l $CaCl_2.2H_2O$ for periods greater than 9 months and successfully used in continuous fermentations with no detected loss of performance.

The fermentation start-up procedure was found to be a critical aspect for successful continuous solvents production.

The use of mutant strains able to withstand higher solvent concentrations or the use of in-situ product recovery technologies, and hence the possibility of fermenting even more concentrated substrates, should present significant improvements over the process described in this Chapter.

CHAPTER 7

CONTINUOUS SOLVENTS PRODUCTION BY FREE CELLS OF *C. ACETOBUTYLICUM* P262 USING EXTERNAL CELL RECYCLE BY CROSS-FLOW MICROFILTRATION

7.1 INTRODUCTION

It was demonstrated in the previous chapter, that entrapping cells in calcium alginate beads to effectively increase the reactor biomass concentration, facilitated continuous stable solvents production from cheese whey permeate medium.

Another "intensified" fermentation technology is the use of external cell recycle to increase the reactor biomass concentration. Cross-flow microfiltration (CFM) membrane plant is a technological process that can be used to continuously separate cells from a fermentation culture and to recycle them back to the reactor in order to obtain greater productivities during continuous operation.

The development of CFM membranes in conjunction with the use of existing ultrafiltration plant, has accentuated the interest in the application of this technology to fermentation processes. CFM membrane plant configurations possibly suitable for processing cell-containing fermentation culture include Plate and Frame (Section 3.7.5.1), Hollow Fibre (Section 3.7.5.2) and Tubular (Section 3.7.5.3) devices.

Hence the purpose of the work described in this chapter was to investigate these CFM membrane plant configurations for their suitability for the production of solvents from cheese whey permeate by continuous fermentation with external cell recycle using cells of *C. acetobutylicum* P262. This included the determination of optimal culture conditions. A simple mathematical analysis of the continuous fermentation - cell recycle system was undertaken for the purpose of determining those parameters that control such a fermentation process (Appendix 1).

The figures of the various fermentation time course profiles presented in this chapter do not show acetone, ethanol or acetic acid concentration data. These data were omitted in order to simplify the figures and avoid unnecessary cluttering. Since a technical appraisal of this process was the main objective of the work described here, the "loss" of this detailed information is not considered critical. Point data values are given at appropriate places in the text. Butyric acid was the dominant acid present and was generally present at a 3 to 4 times greater concentration than acetic acid. The ethanol concentration was low and invariably less than 0.4 g/l under all the experimental conditions examined.

7.2 RESULTS

7.2.1 Plate and Frame CFM Apparatus

In the first series of experiments the fermentation start-up conditions were examined. The Millipore 0.45 μm Durapore membrane cassette module was used for this work. Initially, cheese whey permeate medium (Table 3.3) or sulphuric acid casein whey permeate medium (Table 3.2, adjusted to pH 6.5 with aqueous ammonia prior to autoclaving), both supplemented with 10 g/l lactose, were used in the fermenter for the batch fermentation phase carried out using pH control (pH 5.4) at 30°C. Some minerals precipitation occurred in both media as a result of autoclaving, this being more pronounced in the sulphuric acid casein whey permeate medium.

A Masterflex peristaltic pump using twin 018 heads (maximum flowrate) fitted with Norprene tubing (long-life peristaltic tubing, Section 3.7.5.2) was used to circulate the fermenter culture to the CFM unit after the batch fermentation stage (approximately 24-28 h). A recirculation flowrate of 1.5 l/min was used. This was equivalent to a recirculation flow of 3.0 l/min/m^2 membrane area, the minimum flowrate recommended by the membrane manufacturer to minimize polarization (Operation Manual OM 029, Millipore Corporation). A peristaltic pump was chosen since this pump action would present a gentle, low shear stress environment to the cells.

From the commencement of recirculation, the CFM unit backpressure increased within 40 to 60 h, from 68.9 kPa, to 200 kPa to 275 kPa, which resulted in a rapid decline in filtrate flux and in Norprene tube failure on two occasions. The tube-life decreased in proportion to the increase in the backpressure.

It was reasoned that this increase in backpressure occurred as a result of either mineral precipitation in the fermenter (from autoclaving and pH control operation) which rapidly fouled the CFM membrane or, alternatively, of frontal blockage of the inlet membrane distribution face as a result of precipitate and/or cell accumulation. The latter cause seemed reasonable since the cell concentrate distribution channel was <0.5 mm in diameter. Hence in a further experiment, designed to investigate the possibility of the latter phenomenon, the flow direction across the membrane cartridge was reversed in order to dislodge any possible frontal deposits. This did not result in any decrease in the CFM operating pressure. Hence, it was assumed that minerals precipitation causing membrane fouling was occurring, and causing the rapid pressure increase and decrease in filtrate flux.

Experiments were conducted to investigate the use of membrane filtration to remove the mineral precipitate from hot medium (90°C) after autoclaving. Clear medium was produced using filtration; however, the subsequent batch fermentation, using either filtered cheese or sulphuric acid casein whey permeate medium did not perform adequately (pH 5.4, 30°C). A long lag phase (10 h) was observed with lower cell numbers being achieved. This was despite the use of a large, highly motile inoculum in each instance. The media had low buffering capacity which made pH control more difficult.

Consequently, the peristaltic pump was replaced with a fixed speed positive displacement rotary vane pump (Section 3.7.5.1). This pump had a maximum flowrate of approximately 4.5 l/min of distilled water against a 7 kPa backpressure. This flowrate is three times greater than that recommended to minimize polarization. Additionally, a semi-synthetic medium containing lactose (Table 3.5) was used in the fermenter for the batch fermentation phase instead of whey permeate medium. This was

undertaken to obviate the rapid membrane fouling possibly due to minerals precipitation. The use of semi-synthetic medium containing lactose for the initial batch fermentation phase, prior to continuous fermentation using cell recycle and continuous feeding of cheese whey permeate medium, was routinely adapted for experimental work presented in this chapter.

The first series of experiments was undertaken at 30°C at a relatively low value of culture pH, i.e. under similar culture conditions to those described in Section 6.2.2 for alginate-immobilized cell fermentations.

Run I was conducted using the Millipore 0.45 μm Durapore cassette operating the experimental apparatus as described in Section 3.7.5.1. The fermentation time course profile of this Run is given in Figure 7.1. The initial batch fermentation was operated under pH control at pH 5.4 $\pm$ 0.1. Cell recycle and continuous operation were commenced after 27 h, and an initial low overall dilution rate of 0.06 h^{-1} was used. (The overall dilution rate was based on the total volume of the system i.e. volume of fermenter plus CFM device plus all connections divided by the medium flowrate). Semi-synthetic medium containing lactose was used as the feed medium. Clostridial shaped cells were observed at this time. A high initial filtrate flux was observed where the ratio of cell concentrate and filtrate flowrates was estimated to be approximately 3:1. The filtrate flux decreased rapidly however, and after 6-8 h the flow ratio was approximately 10-15:1. There was no biomass removal during this Run.

A high butyric acid concentration (8.4 g/l) was observed after 27 h fermentation and this concentration was reduced, after commencement of cell recycle, at a rate greater than its washout rate, indicating at least in part, that some butyric acid uptake for solvent production was occurring. The pH control was stopped after 51 h fermentation and the overall dilution rate was increased to a value of 0.12 h^{-1}, and then to a value of 0.16 h^{-1} after 92 h. Sporulating cells, clostridial shaped and elongated rod shaped cells were observed after 74 h of fermentation. The culture pH remained steady in the range pH 4.6-4.7. After 124 h of fermentation, predominantly butyric acid (2.4 g/l) was produced with

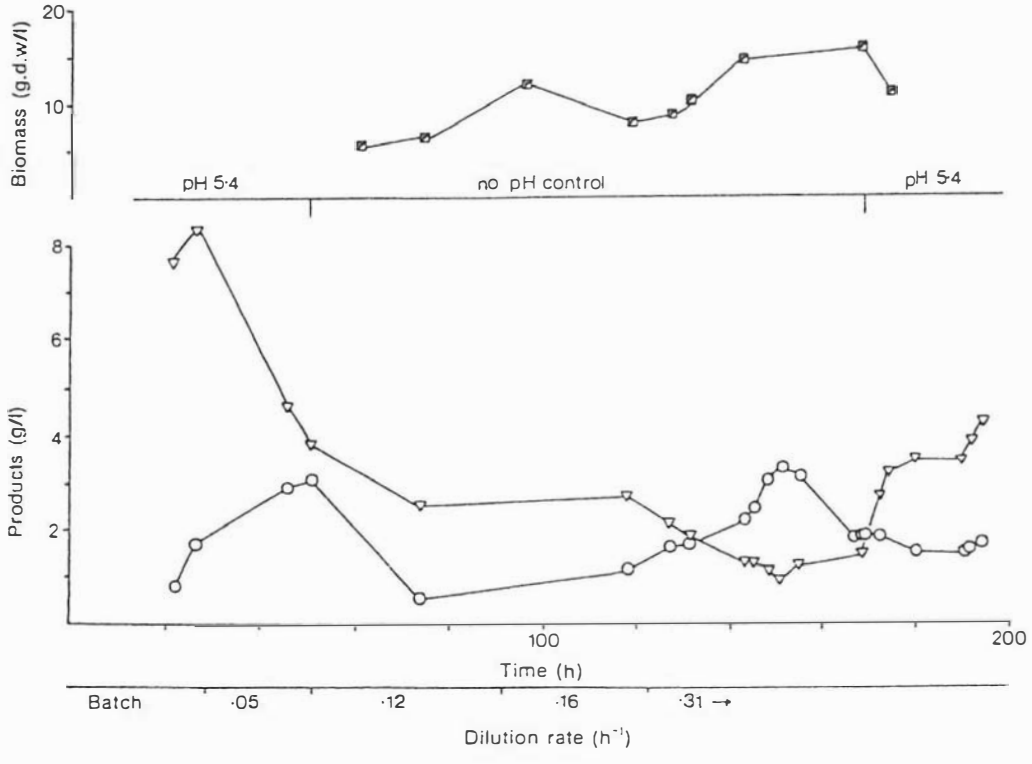


Figure 7.1. Continuous fermentation profile of Run I (complete biomass recycle, 30°C, Plate and Frame CFM apparatus, Durapore cassette): (○) butanol; (▽) butyric acid; (◩) biomass concentration.

only approximately 1 g/l butanol. At this time, the continuous culture was fed with cheese whey permeate medium (Table 3.3) and concurrently, the overall dilution rate was increased to a value of 0.31 h^{-1}. The butyric acid concentration decreased and then remained steady, while the butanol concentration increased to 3.3 g/l (acetone = 1.1 g/l) before decreasing to approximately 1.8 g/l. In an attempt to "rejuvenate" the culture, the pH was adjusted from pH 4.7 to pH 5.4 using 12% w/v ammonia solution. This resulted in a sharp increase in the butyric acid concentration to approximately 3.5-4 g/l whilst the butanol concentration remained steady at approximately 1.5-1.7 g/l. After 174 h of fermentation at pH 4.7, the total cell count (TCC) was approximately 2 x 10^9/ml, a similar number to that obtained in batch fermentation culture (Section 5.2.1). However, the number of colony forming units (CFU) was 1.6 x 10^6/ml. During the fermentation, the biomass concentration increased although some fluctuations were observed. A maximum cell biomass concentration of 16 g.d.w/l was obtained after 168 h of fermentation. Deposition of biomass was observed in the inlet and outlet distribution manifolds. The CFM unit backpressure increased steadily throughout the fermentation increasing to 240 kPa after 194 h. At this time the filtrate flux was less than the feed rate. The unit was shut down and cleaned as described in Section 3.7.5.1. Lactose analyses were not performed on this Run. Steady - state solvent production was not obtained; a maximum instantaneous solvent productivity of 1.44 g/l.h was observed after 156 h.

A further experiment was conducted (Run II) under similar culture pH conditions to those used in Run I. In this instance, however, the continuous biomass removal was investigated in an attempt to control the biomass concentration at a steady-state value (Appendix 1). The biomass removal rate (expressed as a D value, i.e. the biomass removal rate divided by the total system volume) was loosely based on that reported by Pierrot *et al*, (1986) for a continuous culture using cell recycle, for *C. acetobutylicum* ATCC 824 using a semi-synthetic medium containing glucose.

The fermentation time course profile of this Run is given in Figure 7.2. Continuous operation and cell recycle were commenced after

28 h and cheese whey permeate was used at this time. An overall dilution rate of 0.36 h^{-1} was used for the major portion of the experiment, the value of D being increased at a faster rate than in Run I. The biomass removal rate was initially set at a D value of 0.033 h^{-1}. At the commencement of cell recycle the TCC was 1.4 x 10^9/ml and the CFU was 1.2 x 10^8/ml. Both counts steadily increased over the time interval 46 h to 145 h, where the culture pH was controlled at pH 4.8 After 144 h, the TCC was 1.3 x 10^{10}/ml and the CFU count was 2.0 x 10^{12}/ml (biomass = 14 g.d.w/l). After 145 h the pH control was stopped and the culture pH decreased to the region pH 4.4-4.5. The biomass removal rate was increased at this time, despite the lower expected growth rate, in an attempt to stabilize the decline in the filtrate flux by operating at a lower cell concentration. The TCC remained approximately constant (approximately 10^{10}/ml); however, the CFU count decreased significantly to approximately 10^8 - 10^9/ml, despite the biomass concentration remaining in the region of 10-14 g.d.w/l. (The lower CFU count at this pH value agrees with the result obtained in Run I at a comparable pH value indicating that cell viability is reduced at these low pH values). Large fluctuations in the extent of lactose utilization were observed (Figure 7.2), with a lower utilization range being obtained at pH 4.4-4.5. The solvents yield, based on lactose utilized, was variable at pH 4.8 and in the range 0.15-0.27 g/g, whereas at pH 4.4-4.5, the yield varied between 0.29-0.34 g/g. The ratio of butanol to acetone (data not shown) varied but was generally in the ratio 2.5-3.0:1. The ethanol concentration remained in the region 0.2-0.3 g/l.

The highest observed specific butanol and butyric acid production rates at pH 4.8, were 0.067 g/g.d.w.h and 0.085 g/g.d.w.h respectively, at 118 h and 142 h respectively. The specific butanol production rate decreased at pH 4.4-4.5 whilst the specific butyric acid production rate was comparable to that obtained at pH 4.8. The maximum specific lactose utilization rate at pH 4.8 was 0.53 g/g.d.w.h after 127 h decreasing to 0.24 g/g.d.w.h at pH 4.4-4.5, after 199 h. The fermentation was stopped after 221 h as the filtrate flux was lower than the feed rate. The CFM unit backpressure had increased to 240 kPa at this time. Steady-state solvent productivity was not obtained in this Run. The maximum observed instantaneous solvent productivity was 1.58 g/l.h at 128 h.

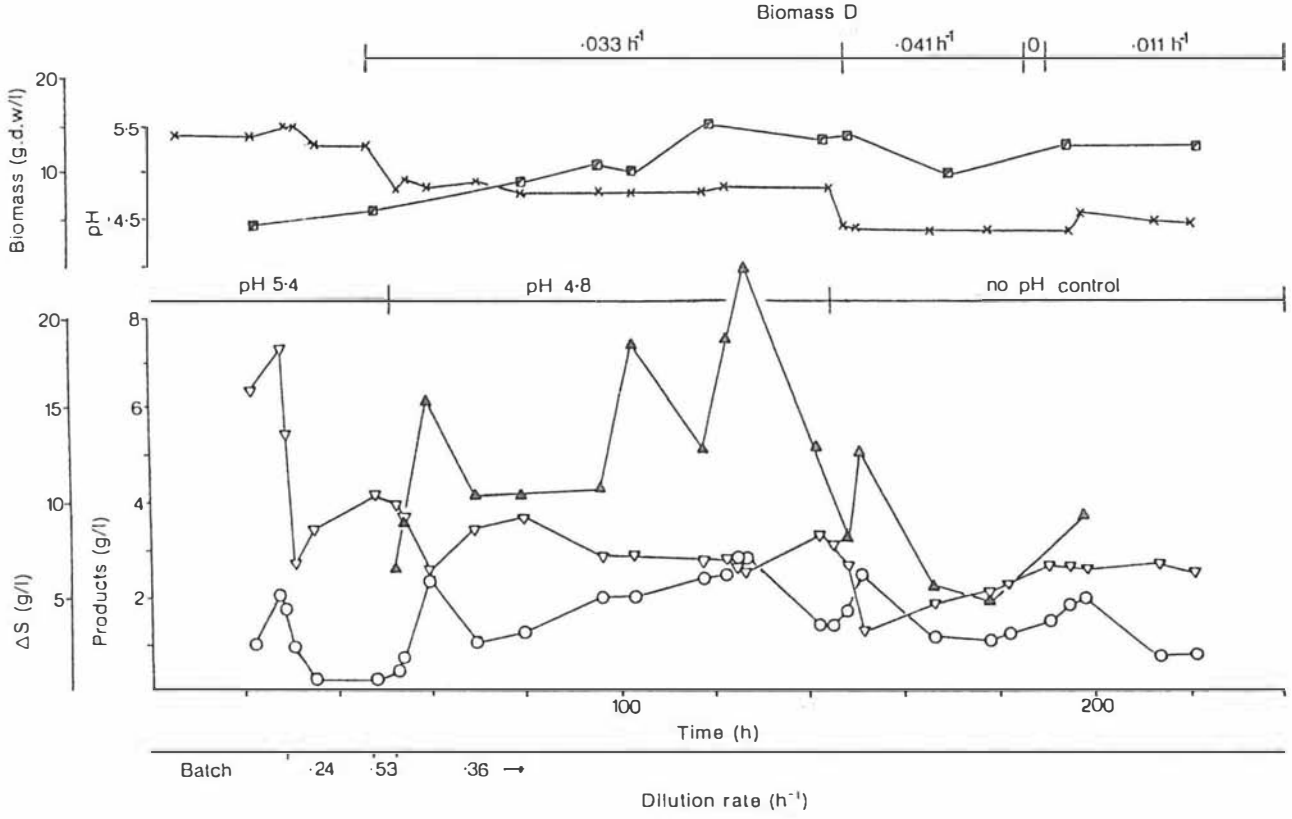


Figure 7.2. Continuous fermentation profile of Run II (biomass removal, 30° C, Plate and Frame CFM apparatus, Durapore cassette): (○) butanol; (▽) butyric acid; (◩) biomass concentration; (▲) substrate utilization; (×) pH.

As a result of the membrane fouling and consequent decrease in filtrate flux, using the Durapore 0.45 μm cartridge during continuous operation, it was decided that this particular system was unsuitable and work was discontinued. This cartridge was replaced with a Sartorius membrane cartridge (Section 3.7.5.1), which has a more 'open' channel design that the Millipore membrane cartridge. A greater tangential flow and lower cell accumulation/concentration polarization would be expected.

Run III was conducted using the Sartorius 0.2 μm Ultrasart II membrane cassette module and was started-up and operated under similar conditions to Run II with no pH control during continuous operation. The fermentation time course profile of this Run is given in Figure 7.3. The culture pH was in the region pH 4.7-4.8, and a biomass removal rate of 0.015-0.020 h^{-1} was used. The highest butanol concentration (4.2 g/l) was observed after 123 h (total solvent = 5.2 g/l) at a dilution rate of 0.31 h^{-1} (instantaneous productivity = 1.61 g/l.h, yield = 0.38 g/g). The biomass concentration was controlled in the region 9-11.5 g.d.w/l throughout the fermentation.

The TCC and CFU counts were 3.2 x 10^{10}/ml and approximately 8 x 10^{12}/ml, respectively, at 120 h. Similar results were obtained at 140 h and are comparable with those obtained in Run II under similar culture conditions. Solvent production peaked within this time interval. The maximum specific butanol and butyric acid production rates observed were 0.12 g/g.d.w.h after 126 h, and 0.14 g/g.d.w.h after 170 h, respectively. The maximum specific lactose utilization rate coincided with the maximum specific butanol production rate and was 0.38 g/g.d.w.h. The solvents concentration and product yield decreased and the butyric acid concentration increased after this time. The overall dilution rate was decreased to a value of 0.21 h^{-1} after 190 h in an attempt to increase the solvents concentration. However, the CFM unit backpressure increased and the filtrate flux decreased steadily throughout the Run, such that the experiment was stopped after 220 h operation. A heterogeneous mixture of solvent-producing clostridial shaped cells, sporulating cells and vegetative rods (very small rods and some long thin cells) were observed throughout the course of the fermentation.

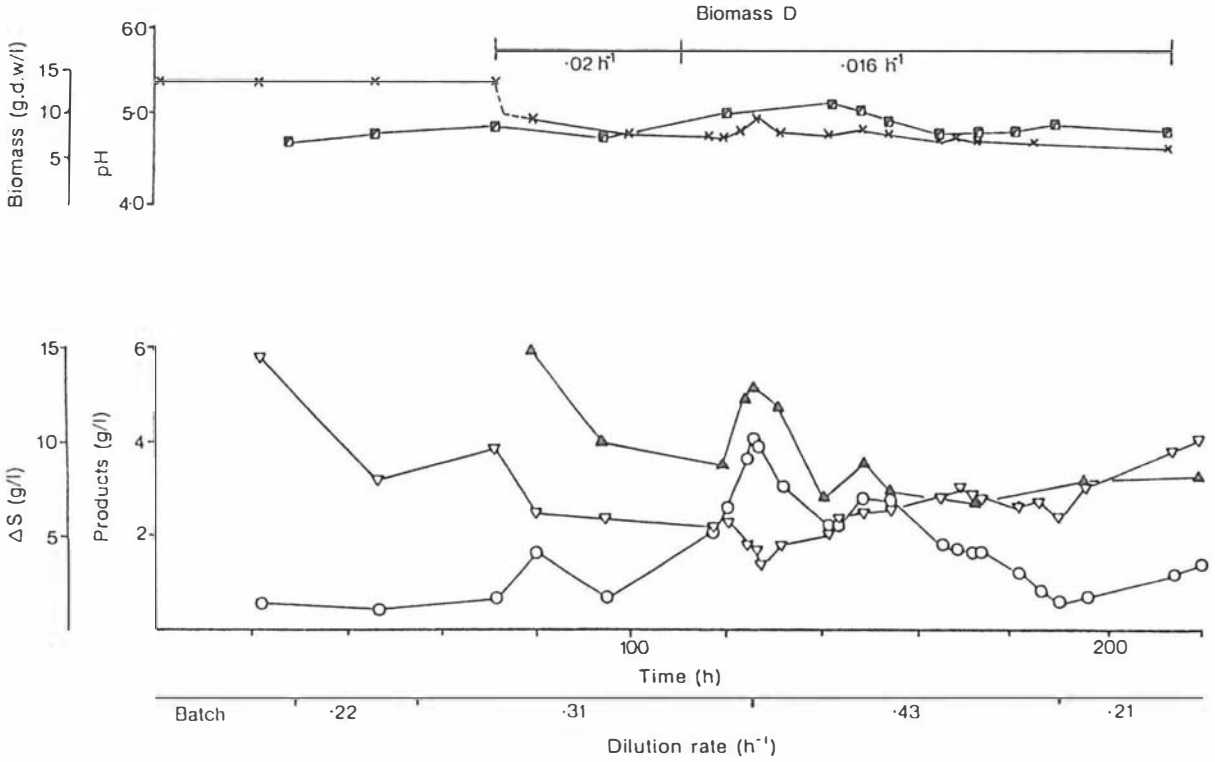


Figure 7.3. Continuous fermentation profile of Run III (30°C, Plate and Frame CFM apparatus, Ultrasart cassette). Symbols as for Fig. 7.2.

Hence, Runs I to III had shown that solvents could be continuously produced in the region pH 4.4-4.8, but this was accompanied by high acid production and after a period of time (approximately 100-120 h) acid production was favoured. After approximately 150-200 h of continuous operation, the extent of membrane "fouling" resulted in a decrease in filtrate flux to an extent where this flowrate was less than the feed medium flowrate (the value of overall dilution rate could not be maintained), and the CFM unit had to be shut down and cleaned.

A further experiment (Run IV) was conducted using a semi-synthetic medium containing glucose (Table 3.5) in order to determine if the 'breakdown' in solvent production observed in Runs I to III, could be attributed to the medium and/or culture conditions, or the equipment itself (e.g. pump shear). Run IV was operated at 34°C and the pH was controlled in the region pH 5.4-5.6, since a batch fermentation conducted under these conditions and using the same medium, gave excellent solvent production (Run XI, Section 5.4.2). The fermentation time course of this Run is given in Figure 7.4. The biomass removal rate was controlled at a value of D = 0.025 h^{-1}; a value chosen based on that given by Pierrot et al, (1986) as used earlier. Cell recycle commenced after 23 h and biomass removal was commenced after 48 h operation. Good solvent production was obtained after 23 h and was accompanied by low butyric and acetic acid concentrations which remained low throughout the fermentation.

Steady-state solvent production was obtained between 54 h and 80 h operation, at an overall dilution rate of 0.28 h^{-1} (7.3 residence times) and an average total solvent concentration of 13.5 g/l (solvent productivity = 3.78 g/l.h), at a biomass concentration of 3.5 g.d.w/l. The specific butanol and butyric acid production rates were 0.62 and 0.01 g/g.d.w.h respectively, throughout this steady state period. The specific glucose utilization rate was 4.24 g/g.d.w.h, with a solvents yield of 0.26, during this same steady-state period. A heterogeneous culture morphology was observed throughout the fermentation, as observed in Run III. Clostridial shaped cells were predominant, but a range of vegetative rod sizes was also observed.

The decrease in solvent concentration and glucose utilization, and increase in biomass concentration observed at 94 h (Figure 7.4) was

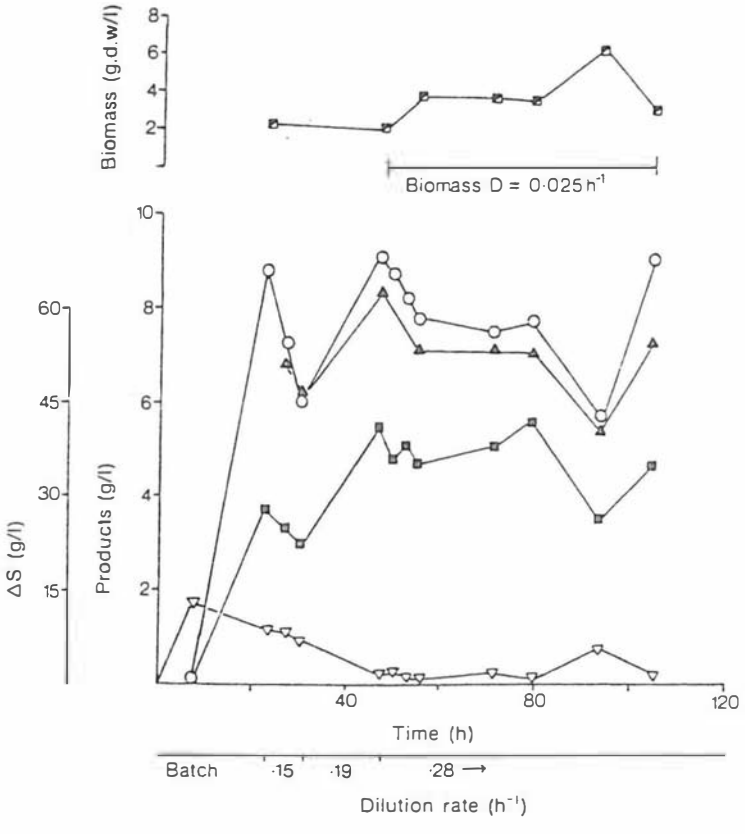


Figure 7.4. Continuous fermentation profile of Run IV (semi-synthetic medium containing glucose, 34°C. Plate and Frame CFM apparatus, Ultrasart cassette). Symbols as for Fig. 7.2; (■) acetone.

attributed to a temporary malfunction in the pH control system resulting in a drop in culture pH to pH 5.1. This situation was corrected and the solvent concentration increased. The system however, had to be shut down after 105 h operation as the filtrate flux had decreased to less than the feed medium flowrate. The CFM unit backpressure was 255 kPa. Increasing the backpressure of the cell concentrate outlet did not result in an increase in filtrate flux. TCC and CFU counts were not determined in this Run.

The results from this Run demonstrated that it was unlikely that the experimental apparatus per se was responsible for the loss of solventogenic performance with time observed in Runs I to III. However, this cannot be a definite conclusion since membrane fouling prevented an experiment of comparative duration.

It was considered that the low operational pH and the heterogeneous culture morphology were the determinant effects. The low filtration performance of these microporous membranes in this particular CFM unit flow configuration, led to the termination of experimental work with this apparatus in favour of more open channel CFM systems. The possibility of backpulse cleaning of the membranes during continuous operation was not investigated.

7.2.2 Hollow Fibre CFM Apparatus

The experimental apparatus was established and operated as described in Section 3.7.5.2. A series of experiments was conducted at 30°C using cheese whey permeate medium (Table 3.3), controlling the culture pH at pH 5.2-5.4. The fermentation time course for Run V is given in Figure 7.5. The biomass removal rate was set to a value of $D = 0.016\ h^{-1}$ as described for Run III (Figure 7.3) and commenced after 56 h fermentation. The feed medium was changed from semi-synthetic medium containing lactose to cheese whey permeate medium after 76 h. A biomass concentration of between 10 and 12 g.d.w/l was obtained at an overall dilution rate of $0.14\ h^{-1}$. The initial batch fermentation profile (high acids, low solvents) was as observed previously (Run I-III), but after the pH was allowed to drop to pH 5.2 and controlled at this value, the butanol concentration reached 4.1 g/l (total solvent

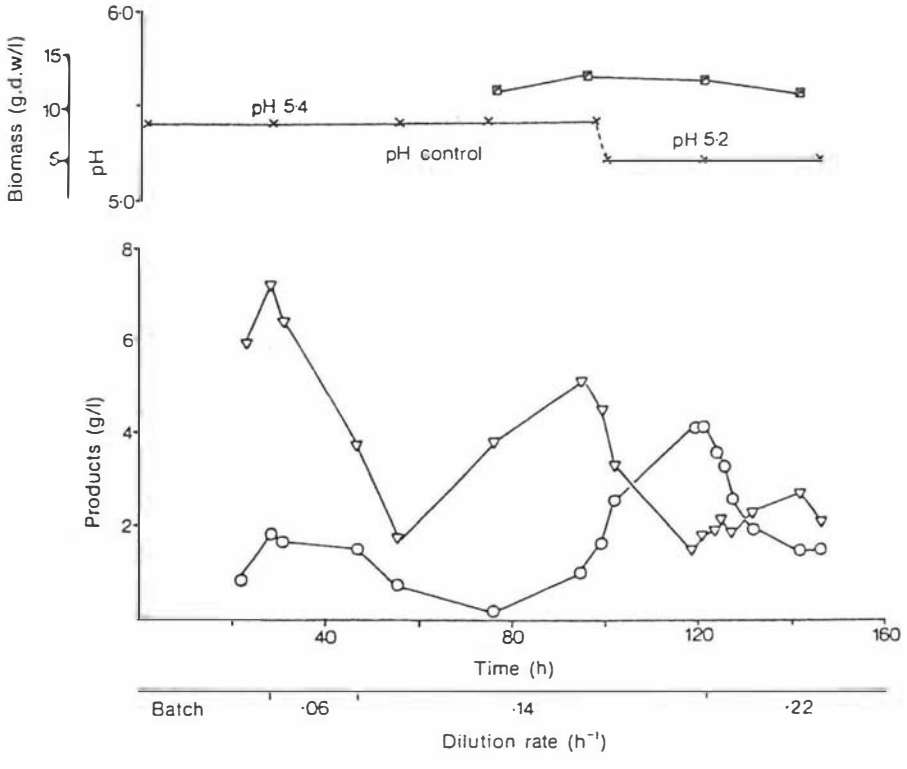


Figure 7.5. Continuous fermentation profile of Run V (30°C, Hollow Fibre CFM apparatus). Symbols as for Fig. 7.1.

concentration = 5.7 g/l). The overall dilution rate was increased at this time to a value of D = 0.22 h^{-1} in an attempt to prevent solvent accumulation to toxic concentrations.

To facilitate this increase, the backpressure on the hollow fibre cartridge was increased to 140 kPa. This was accomplished by increasing the recirculation flowrate to 1.2 l/min and, additionally, reducing the return concentrate flowrate by means of an adjustable clamp. Approximately 20 h later, the hollow fibre cartridge failed, with a split (2 mm) occurring in one of the fibres allowing cells into the filtrate side. On two further occasions, the Amicon hollow fibre cartridge similarly failed during cell recycle operating on cheese whey permeate, when the backpressure was 140 kPa (maximum pressure = 205 kPa), and consequently work was discontinued on this CFM unit apparatus.

7.2.3 Tubular CFM Apparatus

An initial experiment (Run VI) was undertaken using a semi-synthetic medium containing glucose (Table 3.5) at 34°C with pH control at pH 5.4-5.6. Figure 7.6 is a time course profile of this fermentation. A maximum solvent concentration of 16.6 g/l solvents was achieved after 46 h (overall dilution rate = 0.14 h^{-1}). This concentration was inhibitory to the culture as evidenced by the sharp decrease in solvent concentration after this time. The solvent concentration then increased again to 16.7 g/l solvents (9.5 g/l butanol) after 80 h prior to decreasing again. The highest biomass concentrations obtained corresponded with the highest solvent concentrations and maximum observed specific butanol production rate (0.12 g/g.d.w.h). The yield values were maximal (0.28 g/g) at this time, decreasing to approximately 0.18-0.22 g/g prior to, and after, the maximum specific activity. A heterogeneous culture was observed in this Run, as observed in the comparable experiment (Run IV, Figure 7.4) using the Plate and Frame CFM system. Some difficulty was experienced in controlling the biomass removal rate and fluctuations gave rise to changes in the biomass concentration.

The duration of the continuous experiment was only 96 h. This was due to the gradual decline in filtrate flux that resulted in a flux less

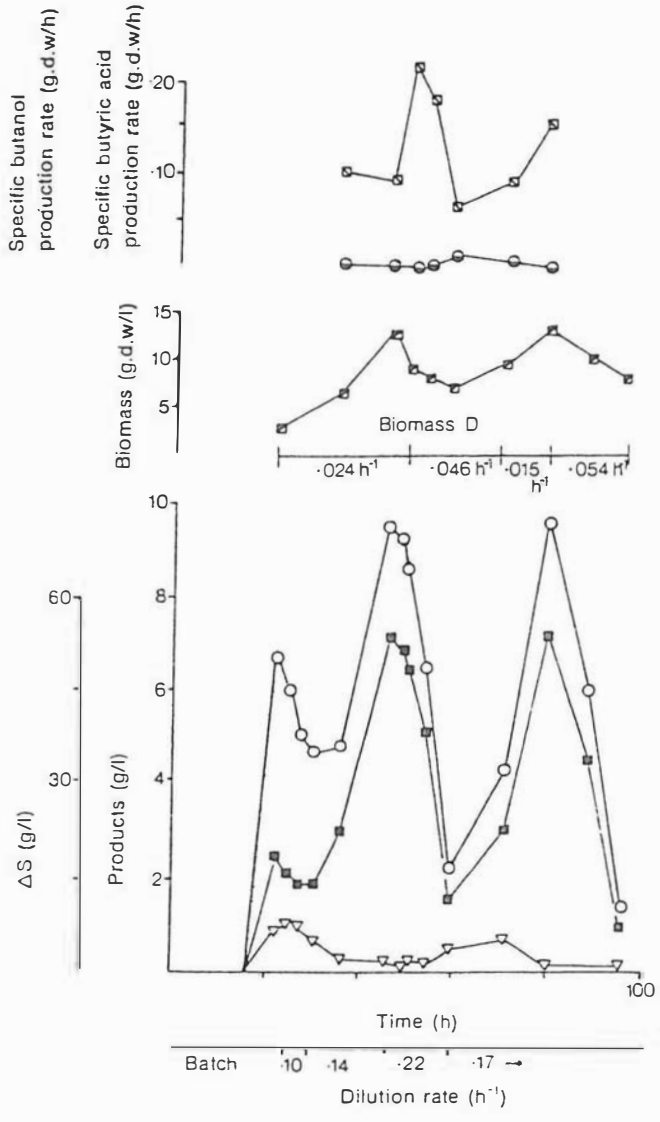


Figure 7.6. Continuous fermentation profile of Run VI (semi-synthetic medium containing glucose, 34 °C, Tubular CFM apparatus): (○) butanol; (● ■) acetone; (▽) butyric acid; (◩) biomass concentration; (▲) substrate utilization; (⧅) specific butanol production rate; (◒) specific butyric acid production rate.

than the medium flowrate to the fermentation. The filtrate flux after 60 h was low and required a decrease in the overall dilution rate to a value of D = 0.17 h^{-1}. The maximum observed solvent productivity at 90 h was 2.84 g/l.h.

In this Run the initial average transmembrane pressure drop ($\Delta\rho$) was 58 kPa ($\Delta\rho$ = inlet + outlet pressure/2). This corresponded to a concentrate recirculation flowrate of approximately 474 l/h (total of concentrate and filtrate flowrate = 581 l/h) as determined previously on clean membranes using distilled water. This in turn corresponded to a tangential velocity of 0.76 m/s. The membranes fouled rapidly under these operating conditions, and in order to match the filtrate flux with the medium feed rate the $\Delta\rho$ was incrementally increased so that a $\Delta\rho$ of 160 kPa was used after 80 h. This pressure is close to the maximum back pressure of the pump unit. As the $\Delta\rho$ value increases, the pump flow capacity decreases and this corresponded to a recirculation flowrate of 120 l/min (tangential velocity = 0.19 m/s), an estimated value obtained as described previously using clean membranes. Consequently, the CFM unit had to be shut down for cleaning and the Run was stopped.

In general, the higher the tangential velocity, the higher the filtrate flux; this arises due to the faster removal of accumulated cells at the membrane surface. Further experiments were therefore conducted at 34°C using cheese whey permeate medium (Table 3.3) with pH control at pH 5.4-5.6, where the $\Delta\rho$ was kept low initially (40 kPa), in order to minimize the possibility of "gel polarization", and the recirculation flowrate was increased to give an initial tangential velocity of 1.6 m/s (approximately 1000 l/h). On two occasions, this resulted in a rapid increase in the culture pH from pH 5.6 to pH 6.0 due to the stripping of fermentation gases from solution, and rapid cell disintegration. The excessive turbulence caused by this high flowrate (total fermenter contents recirculated in approximately 7 seconds) would have made level control impractical even if cells had not been destroyed.

Consequently, for further experiments it was considered that:

(1) The tangential velocity should be kept to less than or equal to 0.76 m/s, to avoid cell destruction.

(2) The initial Δp should be minimized in order to reduce polarization, but sufficiently large so as to provide sufficient filtrate flux (in conjunction with (1) above), so that the initial overall dilution rate could be maintained, prior to its subsequent increase.

The above two limiting conditions were met by the use of an automatic backpulse/flush cleaning system to maintain the filtrate flux over an extended operating period. Thus, the experimental apparatus was established and operated as described in Section 3.7.5.3.

Two further Runs were conducted at 34°C using cheese whey permeate medium with the pH controlled at pH 5.4-5.6. The use of an elevated culture pH value was chosen because in Runs I-III, at lower culture pH values, the culture became progressively acidogenic and this was attributed to the heterogeneous culture morphology. Additionally, good solvent production was obtained at pH 5.4-5.6 in Runs IV and VI using semi-synthetic medium containing glucose.

Figure 7.7 shows the fermentation time course profile of Run VII. The overall dilution rate was increased after a 24 h period of continuous operation to a value of D = 0.24 h^{-1}, and this was maintained throughout the duration of the experiment. A maximum solvent concentration of 9.3 g/l (6 g/l butanol) was observed at 80 h, at a biomass concentration of 12.5 g.d.w/l. At this time, the observed specific butanol production rate (0.11 g/g.d.w.h) was maximal whilst the corresponding specific butyric acid production rate was low (0.03 g/g.d.w.h). A mixture of clostridial shaped cells and rod shaped cells was present, with the former type predominant. The fluctuations in the biomass concentration were due to malfunctions in the biomass removal operation, attributable to the changing rheological characteristics of the culture. The peristaltic pump used for biomass removal was unable to closely control the culture flowrate at 30-50 ml/h, drawing against a 0.5 m head.

At a biomass removal rate of D = 0.018 h^{-1} (between 53 h to 74 h and 108 h to 118 h) biomass accumulated suggesting that the growth rate was higher than 0.018 h^{-1} at this culture pH. The increase in biomass

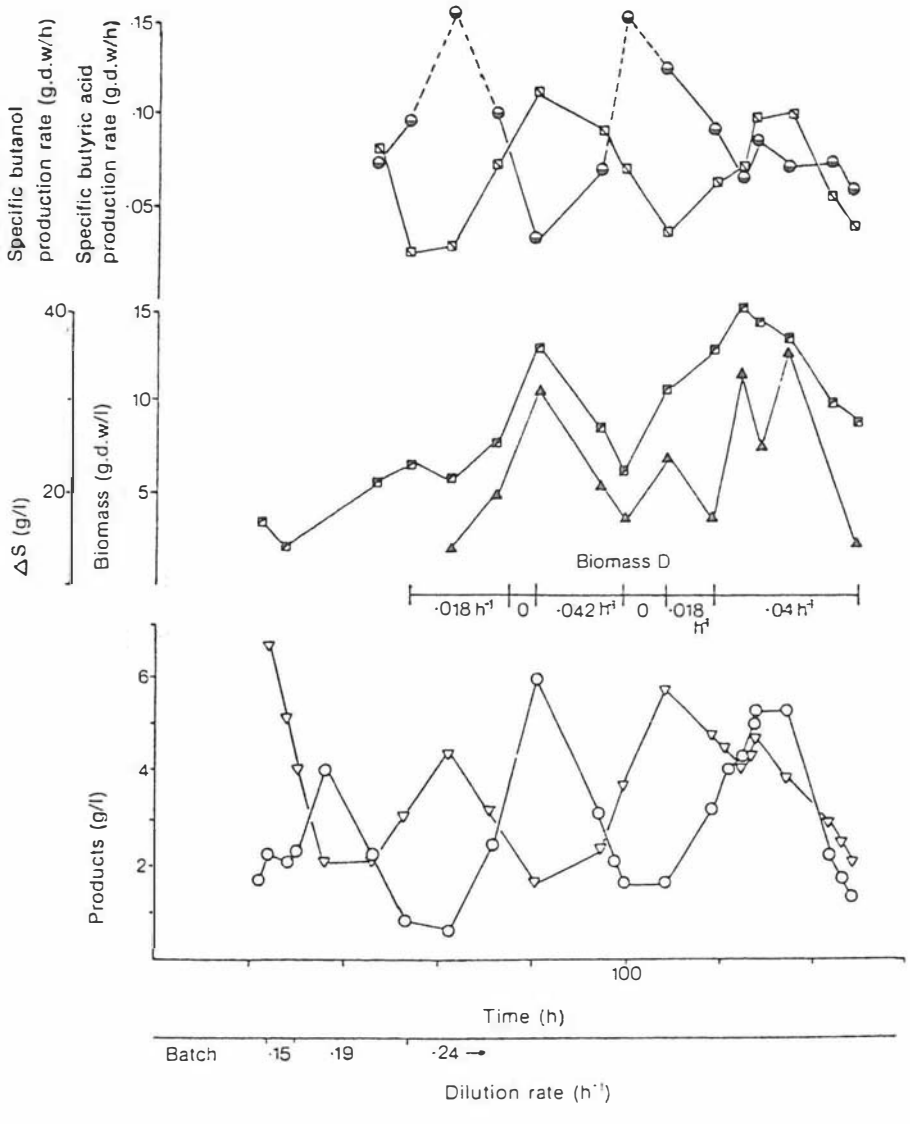


Figure 7.7. Continuous fermentation profile of Run VII (34°C, Tubular CFM apparatus). Symbols as for Fig. 7.6.

concentration corresponded with the maximum observed solvents concentration. A cyclic behavioural pattern was observed; solvent production followed by acid production (Figure 7.7). The specific production rates (g/g.d.w.h) of butanol and butyric acid also followed a cyclic pattern. The maximum observed yield of 0.30 g/g was observed at the time of the maximum observed solvents concentration. Prior to this time and after this time the yield was lower and in the region 0.16-0.26 g/g. Generally the lactose utilization pattern followed the biomass concentration pattern.

The CFU counts after 22 h, 96 h and 124 h operation were 6 x 10^9/ml, 9 x 10^{13}/ml and approximately 1 x 10^{15}/ml, respectively. The TCC at the end of the batch phase was approximately equal to the CFU count (2 x 10^9/ml) but was some two orders of magnitude lower than the CFU count at 96 h and 124 h. The fermentation was stopped after 149 h operation due to the presence of a contaminant. The backpulse/flush system successfully assisted the maintenance of the CFM filtrate flux. The CFM unit was backflushed every 4 h with filtrate for 2s at 140 kPa.

A minor modification was made to the experimental apparatus (Section 3.7.5.3) in an attempt to obtain greater control over the biomass concentration in the fermenter. A biomass recirculation loop equipped with a bleed line was assembled, replacing the peristaltic pump. A further experiment was undertaken (Run VIII) under similar operating conditions (34^{o}C, pH 5.4-5.6) to those described for Run VII and a fermentation time course profile is given in Figure 7.8. The objective in this Run was to allow the biomass to accumulate to a greater concentration than in the previous Run, and to operate at a higher overall dilution rate in order to determine the solvent productivity capacity of this experimental system. Since in Run VII, a biomass removal rate of a value D = 0.018 h^{-1} was shown to be lower than the growth rate under these culture conditions, this value was chosen for this experiment. The overall dilution rate was increased over a 30 h period to a value of D = 0.30 h^{-1} and this was maintained throughout the duration of the experiment. The concentration of fermentative products was monitored regularly so as to ensure that toxic solvent concentrations were not reached. A gradual increase in the biomass concentration was observed, reaching a maximum of 19.5 g.d.w/l

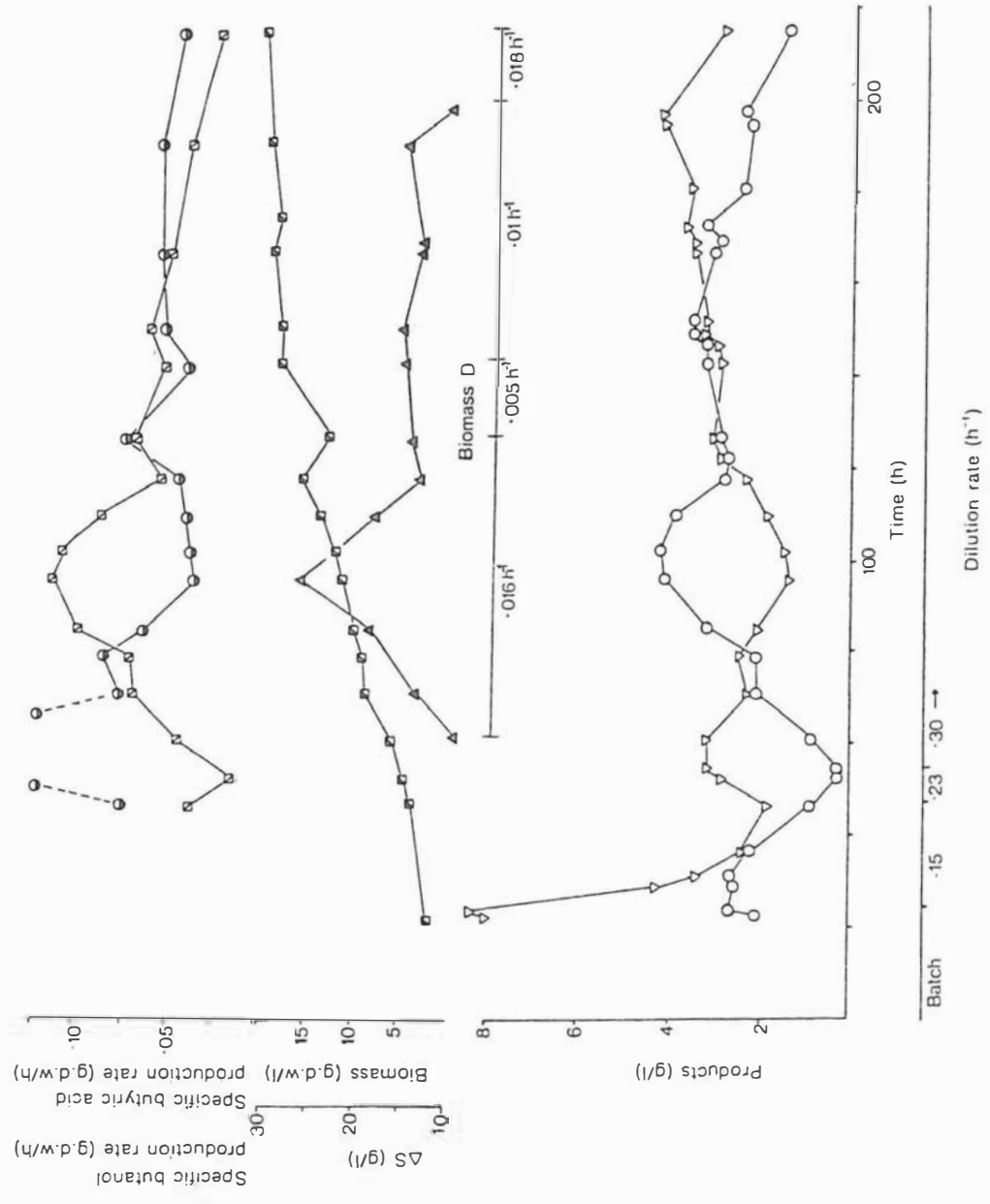


Figure 7.8. Continuous fermentation profile of Run VIII (34°C, Tubular CFM apparatus). Symbols as for Fig. 7.6.

after 215 h operation. Some difficulties in controlling the biomass removal rate were experienced (Figure 7.8) but the fluctuations in the removal were not as pronounced as in the previous Run.

A steady-state solvent production period (±10%) was obtained between 122 h and 173 h (15.3 residence times), and a total solvent concentration of 4.2-4.6 g/l was obtained representing a solvents productivity of approximately 1.32 g/l.h. This was accompanied by a butyric acid concentration of approximately 3 g/l and an acetic acid concentration of approximately 1 g/l (data not shown). After a maximum butanol concentration and observed specific butanol production rate between 85 h and 118 h, both the solvent concentration and the specific butanol production rate decreased whilst the specific butyric acid production rate remained steady. The solvents yield during this steady state solvent production period was 0.31 g/g decreasing to 0.22 g/g after 191 h. Again, a heterogeneous culture morphology was observed throughout the fermentation but no attempt was made to quantitate any particular morphological forms. The Run was stopped after 215 h due to a decline in the fermentation performance.

As in Run VII, the CFM unit was backflushed every 4 h with filtrate for 2s at 140 kPa. The filtrate flux was maintained at a level over the fermentation operating period. A filtrate flux of 5.5 $l/m^2.h$ was obtained at a total biomass concentration of 19.5 g.d.w/l. at the end of the Run.

A further experiment (Run IX, fermentation time course not shown) was undertaken using cheese whey permeate medium, operating under similar conditions as in Runs VII and VIII (34°C, pH 5.4-5.6). In this instance however, the initial batch fermentation phase was conducted using semi-synthetic medium containing glucose (Table 3.5). This was done in an attempt to obtain solventogenic conditions and hence a more homogeneous culture morphology than observed when commencing these fermentations using semi-synthetic medium containing lactose. Continuous operation and cell recycle were commenced after 24 h and cheese whey permeate medium was used at this time at an overall dilution rate of 0.41 h^{-1}. The total solvents and acids concentrations were 9.9 g/l and 2.9 g/l and early and late-stage clostridial cells (>90%) were

observed at this time. After 50 h fermentation, a mixture of clostridial shaped cells, sporulating cells and free spores was observed.

Steady-state solvent production was achieved for approximately 4 residence times at an overall dilution rate of 0.41 h^{-1}, giving 4.90 g/l, 1.94 g/l, 0.29 g/l, 1.50 g/l, and 1.55 g/l of butanol, acetone, ethanol, butyric acid and acetic acid respectively. This was equivalent to a solvent productivity of 2.92 g/l.h with a solvent yield of 0.31 g/g. The biomass concentration was controlled in the region 11.8-12.8 g.d.w/l using a biomass dilution rate of 0.02 h^{-1}. The specific butanol production rate and specific lactose utilization rate were approximately 0.16 and 0.80 g/g.d.w.h, respectively, during this period. After this steady-state, solvent production decreased and acid production increased to 4.9 g/l butyric acid and 1.5 g/l acetic acid (butanol = 2 g/l) after 162 h, and the fermentation was stopped.

7.3 DISCUSSION

The objective of this work was to investigate the conditions for continuous stable solvents production from cheese whey permeate medium, with free cells of C. acetobutylicum P262 using external cell recycle by cross-flow microfiltration (CFM).

In the initial experiments using the Plate and Frame CFM apparatus (Section 7.2.1), the fermentation culture conditions e.g. temperature and pH, were similar to those used in continuous fermentations using alginate-immobilized cells (Chapter 6), that gave stable solvent production. The continuous fermentations (Runs I-III inclusive) operated under these conditions, were characterized by a brief solventogenic period after which the fermentation "broke-down" to an acidogenic state, i.e. culture degeneration occurred. This occurred in Run III despite the fact that in this particular experiment, good control was exercised over the reactor biomass concentration and the overall dilution rate and biomass removal rate. The heterogeneous culture morphology observed at these culture pH values may explain the culture degeneration, that is, fast-growing, acid-producing vegetative rod-shaped cells were progressively selected for under these conditions.

It is conceivable that the semi-synthetic medium containing lactose and the culture conditions used for the initial batch fermentation prior to the commencement of cell recycle and continuous fermentation operation were unsuitable. Since high acids production was observed during the first 20-24 h of fermentation, it is likely that only a low percentage of cells had made the morphological transition to clostridial-shaped solvent-producing cells. The alternative fermentation 'start-up' procedure of concentrating the cells from a batch fermentation prior to continuous feeding, with ongoing cell recycle, was not attempted.

In continuous fermentations (Runs V, VII to IX) operated at higher pH values (pH 5.4-5.6), the culture "broke down" to an acidogenic state, generally after 120-140 h fermentation. This was despite the higher proportion of clostridial-shaped cells observed during the initial period of the continuous fermentation. Cell growth occurred during the fermentation with a consequent increase in the biomass concentration, indicating the presence of vegetative cells. As in Runs I to III, steady-state solvent production was not achieved in these Runs except in Run VIII and briefly in Run IX. The maximum observed specific lactose utilization rate in Run III (low pH) was lower than that observed in Run IX (high pH) indicating the advantage of operating at the higher pH value.

Strain P262 is a highly differentiated species (Jones et al, 1982; Long et al, 1984a). In batch culture, three morphological forms of C. acetobutylicum P262 are observed, and are associated with different stages of the fermentation process (Jones et al, 1982). During acid production, actively growing vegetative rod-shaped acid-producing cells are observed; however, during the solvent production phase, growth ceases and a morphological change is observed with vegetative cells changing to "fattened cigar-shaped" solvent-producing clostridial cells. These cells are then observed to sporulate; these "forms" do not divide or produce acids or solvents (Jones et al, 1982). These morphological changes were observed in batch fermentation of sulphuric acid casein whey permeate medium with strain P262 (Section 5.3).

The same morphological forms and changes as observed in batch culture, were observed in continuous fermentations using free cells of

P262, reported in this chapter. A schematic representation of this morphological cycle is given in Figure 7.9. The rate of conversion or differentiation, of one morphological form to another is designated by a reaction rate 'K'. These non-reversible changes occur as a result of complex interactive "triggering" mechanisms. For example, the morphological change from vegetative cells to clostridial cells at rate 'K', involves the acid concentration (external and internal concentration?, dissociated or undissociated form?), pH, and residual sugar and nitrogen concentrations (Long et al, 1984a).

Conceptually, for the maintenance of continuous stable solvent production, the conditions must be such that the clostridial cells are maintained in a stable non-growing solvent production phase, with vegetative growth, sporulation, and cell lysis inhibited or closely controlled i.e. a balance of culture morphology must be maintained. The degeneration of these continuous fermentations to an acidogenic state i.e. an unbalanced culture morphology, may be due to cells not "triggered" initially or as the result of culture "derepression" where toxic products that are inhibitory to further solvent production are washed out, thus allowing further growth. Additionally, in most Runs on both cheese whey permeate medium and semi-synthetic medium containing glucose, sporulation was observed immediately after the period of solvent production. Ultimately, free spores were observed in the culture prior to vegetative cells being observed, i.e. spore germination had occurred.

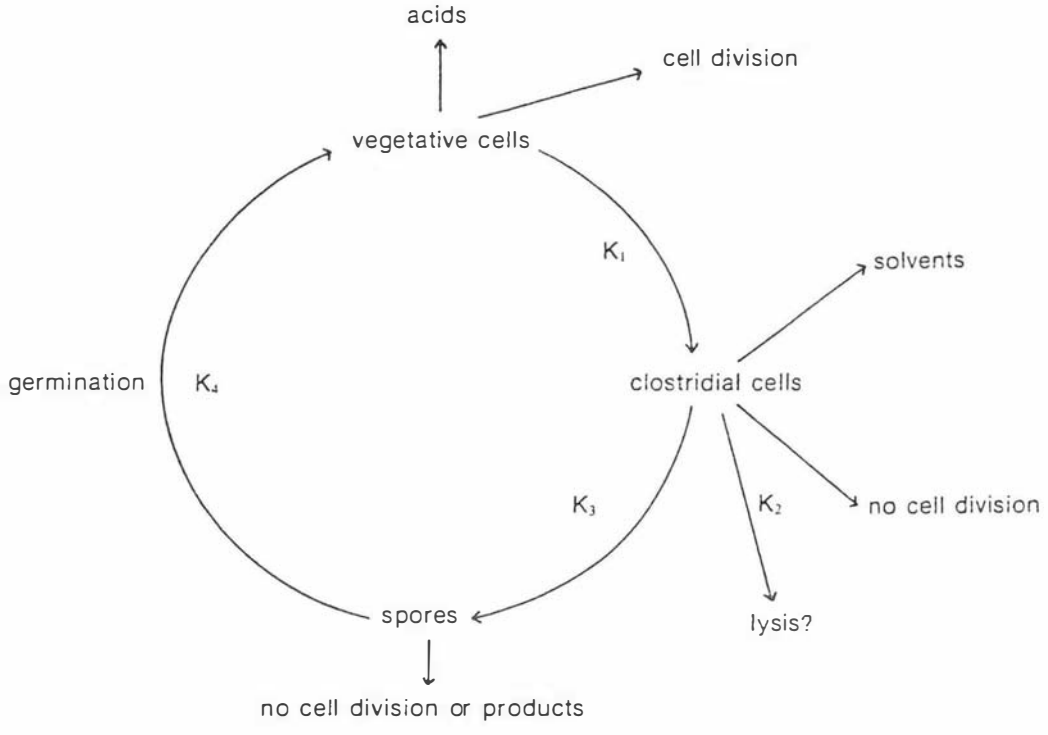
The lowering of the CFU count (at steady TCC) observed when the culture pH was lowered (Runs I and II) agrees with the findings of Long et al, (1984a) who stated that viability was lowered at low pH (value unstated). The discrepancy between the TCC and CFU counts may be explained by two reasons. Firstly, there was great difficulty in counting cells in the presence of microscopic mineral precipitate, and secondly, spores not counted in the TCC determination, may have germinated in the CFU count determination giving higher counts. The CFU determination was more useful as an estimate of overall culture viability.

An attempt was made in Runs II to IX inclusive, to control the biomass concentration, as per Appendix 1, in order to obtain steady-

state solvent production conditions. This was done by removing biomass directly from the reactor on a continuous basis; hence solvent-producing clostridial cells were also removed. For given culture conditions e.g. pH, medium composition, products concentration, overall dilution rate and cell concentration, acid-producing vegetative cells differentiate to solvent-producing clostridial cells at a given rate (K_1). This rate may change under different culture conditions e.g. it may decrease as the solvent concentration increases. It is possible then, that clostridial cells are removed in the biomass bleed, at a rate greater than the differentiation rate that occurs during solvent production (i.e. $\alpha D >> K_1$). Thus a lowering in the solvent production rate occurs, hence the solvents concentration is lowered. This in turn favours growth and an increase in acids production, with these cells in turn differentiating to clostridial forms favouring solvent production.

A cyclic solventogenic pattern can result as depicted in Figure 7.9. If the biomass removal rate were maintained at a rate equal to the differentiation rate K_1, (assuming no sporulation or cell lysis) then theoretically, control over the solvent-producing clostridial population would be possible. Whether this remained stable would depend on the balancing of environmental conditions e.g. avoidance of toxic product concentrations. In this situation however, vegetative cells could still accumulate and the differentiation rate K_1 would be expected to change. Therefore, the maintenance of the growth rate (μ) and spore germination rate, K_4, equal to the differentiation rate of vegetative cells to clostridial cells, K_1, minus the clostridial cell lysis rate, K_2, and sporulation rate, K_3, ($K_4 + \mu = K_1 - K_2 - K_3$) is the controlling index for stable solvent production.

What affects the growth rate (μ) and the differentiation reaction rates (K) of the various morphological forms, is not fully understood. In addition to previously mentioned factors, such effects as mass transfer e.g. diffusivity limitations in concentrated suspension, may occur under some conditions. Some cell loss (clostridial forms, and other types?) was evident in these experiments, as indicated by the turbid supernatant liquid present in culture samples centrifuged for dry weight biomass determination.



K_1 designates the rate of conversion (or differentiation) of vegetative cells to clostridial forms.
K_2 designates the rate of loss of clostridial forms due to lysis.
K_3 designates the rate of sporulation of clostridial forms.
K_4 designates the rate of spore germination to vegetative cells.

Figure 7.9. A schematic diagram of the morphological changes observed during batch and continuous fermentation modes by *C. acetobutylicum* P262 fermenting whey permeate medium.

In conclusion, maintenance of the morphological cycle (Figure 7.9), such that a 'balance' is achieved, facilitating stable solvents production, is very complex and poorly understood. The ease with which such a balance can be maintained may be substrate specific since steady-state conditions were achieved when using medium containing glucose but not when using whey permeate. Possibly, a non-sporulating clostridial forming mutant, as used by Largier et al, (1985) in continuous fermentation using immobilized cells, would 'break' the cycle depicted in Figure 7.9 and possibly simplify the conditions for process control.

Consideration of the technical/operational aspects of the process is given in the following text. If cells were not removed from the fermenter culture and the biomass concentration were allowed to increase in an uncontrolled manner, the culture density and viscosity changes would affect the CFM filtrate flux, and possibly limit the overall dilution rate and volumetric productivity, so that control of the reactor system would not be possible. The filtrate flux achievable directly determines the membrane area required and therefore the plant capital and operating costs. Whilst direct measurements of filtrate flux were not undertaken in this work, the achievable membrane operating cycle (between shut-down and cleaning cycles) was readily determined and is an important determinant of the suitability of membrane type and CFM plant configuration for this type of process.

There was difficulty in controlling the biomass removal rate in all Runs reported in this work, and particularly so for Runs VI to IX inclusive (Tubular CFM apparatus, Section 7.2.3). This was attributed to the difficulty of peristaltic pump operation at the low flowrates of 30-50 ml/h, necessary to obtain biomass removal rates in the range equivalent to D = 0.02 to 0.05 h^{-1}. The pump had to draw against a 0.5 m head. Use of a biomass recirculation loop in Runs VIII and IX did give some improvement in control but was also unsatisfactory.

Fluctuations in culture viscosity were observed during continuous operation. This was attributable to the biomass itself, with the biomass concentration increasing due to growth at a rate faster than the bleed rate, or alternatively, removal of cells at a rate faster than the growth rate e.g. as may occur during solvents production. These observed viscosity changes affected peristaltic pump action with time, so that close control became very difficult. The viscosity changes could also be a result of the production (and subsequent reutilization) of an extracellular polymer (polysaccharide) as observed by Haggstrom & Forberg (1986). These workers used a semi-synthetic medium containing glucose with C. acetobutylicum ATCC 824 in batch fermentation and found that an extracellular polymer was produced during both the acidogenic and solventogenic phases, and that when butanol and butyric acid were produced at the same time, reutilization of previously produced polymer occurred. It was postulated that the polymer is used for the smooth running of the energy metabolism and is a storage product for non-reduced compounds. The polymer is available for when the organism requires excess reducing power, but the conditions for when this is likely to occur were not clearly detailed by these workers. A white viscous substance was occasionally observed during continuous fermentations using immobilized cells of strain P262 (Chapter 6) and it is conceivable that production of this polymer could occur in this strain and during continuous production using cell recycle. If the postulated mechanism regarding the role of this extracellular polymer on the metabolism of strain ATCC 824 has a parallel role in strain P262, then conditions that control its formation and its physiological role need to be determined. Long et al, (1983) found that strain P262 accumulated large numbers of starch storage granules (granulose) associated with the formation of swollen cigar-shaped phase-bright presporulation cells (clostridial cells). Reysenbach et al, (1986) found that the structure of the granulose in strain P262 consisted of a high-molecular-weight polyglucan containing only α(1-4)-linked D-glucopyranose units. Granulose accumulation was detected in cells only at the end of the exponential growth phase. Reysenbach et al, (1986) postulated that granulose may be utilized as a source of carbon and energy during sporulation in Clostridium species. Produced polymer removal during continuous fermentation would result in a lower product

yield. Culture rheological properties determine the achievable filtrate flux, hence polymer production would need to be controlled.

Equipment for in-situ or in-line (via a recycle loop) cell concentration measurement was not available for this work. Direct, non-invasive, physical methods e.g. acoustic, dielectric and laser light scattering techniques, appear most suitable for control over the cell concentration and even possibly measure culture viability in these processes (Clark et al, 1986). Such techniques are sophisticated, problematic and their utility may be case specific (Clark et al, 1986).

Afschar et al, (1985) in their cell recycle studies using C. acetobutylicum, successfully utilized turbidostatic control of the biomass concentration, using a flow-through photometer with calibrated measurements to control the biomass removal rate. Direct application of this control process to the work described here with whey permeate would be more difficult due to the presence of colloidal precipitated mineral matter in the culture that might interfere with the photometer operation.

The steady-state solvent productivity obtained on semi-synthetic medium containing glucose in Run IV using the Plate and Frame CFM apparatus is comparable to those obtained by Afschar et al, (1985) and Pierrot et al, (1985) using glucose-containing medium with strain ATCC 824. The specific butanol production rate obtained in Run IV (0.62 g/g.d.w.h) at a total solvent concentration of 13.5 g/l is superior to those reported by Afschar et al, (1985) and Pierrot et al, (1986). There is some doubt however, about the actual biomass concentration in this Run, since biomass was observed to accumulate in the flow distribution channels in the CFM device, so that culture samples taken from the fermenter vessel were possibly not representative. The volume ratio was approximately 1:1, fermenter : CFM system. The highest specific butanol production rate (0.12 g/g.d.w.h) obtained in Run VI on semi-synthetic medium containing glucose and using the Tubular CFM apparatus is lower than that obtained in Run IV. Although the butanol concentration (9.6 g/l) was inhibitory in this Run, a concentration of approximately 8 g/l was not deleterious to solvent production in Run IV. The specific butanol production rate obtained in Run VI is more

comparable with that reported by Pierrot et al, (1986), who reported a value of 0.13 g/g.d.w.h at a butanol concentration of 8.5 g/l, and by Afschar et al, (1985), where extrapolation of their data suggests a specific solvent production rate of 0.1 g/g.d.w.h at a butanol concentration of 7.5 g/l. These workers reported that the specific solvent production rate is maximal at a butanol concentration of 4 g/l, suggesting that in any further studies of continuous production of solvents by cell recycle using whey permeate medium, an examination of the effects of a lower solvent concentration and higher specific production rate and hence lower biomass concentration and higher filtrate flux etc, could be an area deserving of closer study. The maximum observed specific glucose utilization rate in Run VI (0.76 g/g.d.w.h) is comparable with the value 0.68 g/g.d.w.h reported by Pierrot et al, (1986).

The maximum specific butanol production rates and maximum specific lactose utilization rates obtained in those Runs using cheese whey permeate medium at pH 5.4-5.6 (Runs VII & VIII) were generally lower than those reported on media containing glucose. One exception was the data obtained in Run IX where the steady-state specific butanol production rate and specific lactose utilization rate data are comparable with those obtained on medium containing glucose although at a total solvent concentration half that obtained with the medium containing glucose. These data confirm the findings of Chapter 5 (batch fermentation) and Chapter 6 (continuous fermentation using immobilized cells) that glucose is a preferred substrate to lactose. Much lower acid concentrations were obtained in fermentations performed in this Chapter with glucose-containing medium as observed previously in Chapters 5 and 6.

No attempt was made in this work to characterize the filtrate flux performance of each of the CFM units. This would have involved the examination of a large number of process variables for each CFM configuration/membrane type e.g. transmembrane pressure, tangential velocity, membrane porosity, pore distribution, biomass concentration, and cleaning and sterilization procedures, for their effects on filtrate flux and membrane life with time. Also, it was reasoned that the hydrodynamic properties of the small laboratory scale CFM units are not

necessarily reproduced in a larger manufacturing CFM unit. In these laboratory-scale studies, some "scale-down" was necessary. For example, in the instance of the Tubular CFM apparatus, the Ceraflo CFM 0.1 m^2 membrane area unit was operated at a tangential velocity of approximately 10% of that achievable with this unit, hence considerable flux increases would be expected over that achieved in this work if the unit were operated at a higher tangential velocity. Such operation was not achievable in this particular instance due to the requirements for fermenter vessel level control (excessive turbulence) and the damage done to cells at higher flowrates due to the pump action.

The Millipore Plate and Frame CFM unit equipped with either a 0.5 m^2 Durapore cassette or 0.7 m^2 Ultrasart cassette (Section 3.7.5.1), was unsuitable for the present work. Considerably lower flux values (l/m^2h), despite the higher membrane area, were obtained compared with the Tubular CFM unit. Charley et al, (1983) reported that support screen blockage resulted in frequent shutdowns. Similar observations were made in this study, despite the fact that tangential flows were 3 to 4 times higher than that recommended by the equipment manufacturer and used by Charley et al, (1983). This particular CFM unit may have been severely disadvantaged since backpulse/flushing was not used to dislodge cells or foulant materials lodged within or on the membrane. In contrast, this technique was successfully utilized with the Tubular CFM unit to prolong the filtrate flux. A double retentate screen may also be used in some instances for very viscous material but it is doubtful that such a screen would have given rise to significant performance improvements.

Antifoam (Bevaloid 5901) was added to the fermenter culture (manually or automatically) in order to suppress excessive foaming and aid in the fermenter level control in each of the CFM apparatus tested. Antifoams, which are mostly water-insoluble hydrophobic substances (Kroner et al, 1984), are known to adsorb at the surface of plastic polymeric membranes due to their hydrophobic nature, increasing membrane fouling (Cabral et al, 1985) and reducing the filtrate flux. This foulant effect may be irreversible and progressive, and depend on the membrane type (surface chemistry) and the specific antifoam used. It is possible that the use of this antifoam (esterified poly

(1,2-propanediol)) affected the filtrate flux of the polymeric membranes used in the Plate and Frame CFM unit. However, it is unlikely that this foulant effect occurred in the Tubular CFM unit, due to the inert sintered alumina microfilters used in the Ceraflo® cartridge. The use of antifoam is described by other workers in this field e.g. Charley et al, (1983) and Schlote & Gottschalk (1986). Afschar et al, (1986) stated that antifoam addition was unnecessary in their experimental apparatus and that foam could be destroyed by the recycled culture being sprayed onto the surface of the foam layer. This procedure was unsuccessful in work described here, with the technique actually accentuating the problem; hence the culture recirculation loop was enclosed within the culture. The foaming problem is case specific and depends to some extent on the equipment used e.g. pump type. Although the level control device used (Appendix 2) functioned in the presence of foam, it was observed that the foam, if unsuppressed, became very thick and increased the risk of the conductivity probe being incorrectly set to account for such changes, and hence an out-of-control situation arose.

The failure of the Hollow Fibre CFM unit was surprising and unexplained. Possibly some medium components or fermentation products could have been incompatible with the polysulphone membranes. This explanation appears unlikely since other workers (e.g. Mehaia & Cheryan, 1986) have successfully used similar units with acid whey permeate for ethanol production. Fermentation products of the expected concentrations in this work were not incompatible with the unit according to the manufacturers technical information. It is more likely that pressure fluctuations due to peristaltic pump action, or the use of a faulty inlet pressure gauge, resulted in premature membrane failure. Whilst only one membrane fibre (out of 50) split on each failure occasion and could be repaired by blocking each end of the membrane, such an operation was not undertaken. It was discovered only at the end of the work that whilst the pressure gauge was generally reading correctly, it tended to stick when the pressure was increased or lowered, over the pressure range that was being used in this work. Hence, definite conclusions cannot be drawn from this work regarding the suitability of this CFM configuration for fermentation cell recycle application.

The decline in filtrate flux with time may be attributed not only to the rheological properties and concentration polarization effects of the culture, but also to the intrinsic 'fouling nature' of the fermentation medium *per se* due to membrane-solute interactions. The presence of minerals (ionic or colloidal form) in whey permeate will cause membrane fouling, the nature and extent of which is most likely to be case specific. Calcium complex formation with protein fractions and other ionic species is one of the major contributors to ultrafiltration membrane fouling (Ennis *et al*, 1981), and may also contribute to membrane fouling of microporous membranes. The complete removal of calcium and potassium ions by sodium for calcium ion exchange from sulphuric acid casein whey, gave significant improvements in the flux performance of a pilot-scale batch ultrafiltration process (Ennis *et al*, 1981). A parallel situation may have occurred in work described here, with the possible interaction of cheese whey permeate ionic and nitrogenous species, and yeast extract components (e.g. salts, macromolecules, vitamins) in the medium, with the microporous membrane giving rise to membrane fouling. An alternative and possibly more likely occurrence, is that a rapid accumulation of retained species in conjunction with pore blockage by cells or cell-medium component species, may have formed a "dynamic" secondary membrane impeding the filtrate flow.

No in-line feed medium filtering was used to remove colloidal mineral precipitate which occurred as a result of sterilization by autoclaving. Medium used in this study was turbid and crystalline (?) particles were observed under the microscope. These particles were present despite the fact that the bulk of the feed medium precipitate settled at the bottom of the feed medium reservoir and the medium was drawn off well above this layer. This problem could be partially overcome by membrane sterilization of the feed medium but this was not examined due to the unavailability of suitable equipment. Mineral precipitation would occur however, if the fermentation operated in the region pH 5.4-5.6.

7.4 SUMMARY

The use of external cell recycle using cross-flow microfiltration for continuous solvent production from cheese whey permeate medium using

free cells of C. acetobutylicum P262 has been demonstrated in this preliminary work. Biomass was continually removed from the culture (Appendix 1) in an attempt to control the biomass concentration and hence obtain steady-state solvent production; however, this was not achieved for extended periods (i.e. >5 residence times). Continuous cultures degenerated after 100-140 h fermentation and acid production was favoured in fermentations conducted in the region pH 4.4 to 5.6. This is attributed to the heterogeneous morphological nature of strain P262, such that fast-growing, acid-producing vegetative cells were preferentially selected. Whilst the biomass bleed rate may effect control over the total cell population, the interactive mechanisms that control the morphological differentiation rates (Figure 7.9) is complex and beyond the scope of this work. These experiments confirmed the finding that medium containing glucose is a preferred substrate for solvent production compared with cheese whey permeate (lactose).

Operation of this fermentation process, even at a laboratory scale, was complex and a large number of process variables were identified that could control the process. However, quantitation of their relative effect was also beyond the scope of this work. Suitable processes for in-situ or in-line biomass measurement need to be determined.

Plate and Frame, Hollow Fibre and Tubular CFM units were examined for their suitability for such a process. Despite the fact that these CFM units were examined on a laboratory scale under non-optimal conditions, a number of the technical advantages and disadvantages of each unit were determined and are given in Table 7.1 (This includes technical and specification data supplied by the manufacturer).

A Tubular CFM unit (Ceraflo) was found to be the most suitable of those examined here. This is attributed to the use of a more open channel design, the use of high tangential velocities minimizing concentration polarization, and the ability of this unit to be operated for extended periods assisted by the use of periodic backpulse/flushing.

Considerable further work is required to examine the metabolism of C. acetobutylicum in concentrate suspension, and the factors controlling

solvent production. Predictive kinetic models, and CFM unit performance data are necessary for scale-up calculations and economic assessment. The capabilities of CFM processes have increased significantly in the last few years but the fundamental understanding of microbial kinetics still lags.

Table 7.1 A summary of the advantages/disadvantages of the various CFM units

	Millipore Plate and Frame Unit Membranes		Amicon Hollow Fibre Unit	Norton Ceraflo Tubular Unit
Description of Unit	Durapore	Ultrasart	Diaflo cartridge (H1MP01-43) 55 x 0.011 cm fibres	28 x 0.28cm tubes
Membrane Area/Pore Size	$0.5m^2/0.45\ \mu m$	$0.7m^2/0.2\ \mu m$	$0.03m^2/0.1\mu m$	$0.1m^2/0.45\ \mu m$
Membrane Type	unknown	olefin	polysulphone	sintered alumina (hydrophilic membranes)
Pressure limit	-	unknown	205 kPa	1550 kPa
Temperature limit	50°C	unknown	50°C	750°C
Recirculation flowrate	3 $l/min/m^2$	unknown	0.6-1.8 l/min per cartridge	50 l/min (max)
Membranes Autoclavable	not recommended	yes	yes but not recommended	yes
Cleaning Procedure	simple but not flexible	unknown	simple but not flexible	simple and very flexible
Backpulse/flushing capacity	possible	possible	yes	yes
Membrane inspection capability	very poor	very poor	poor	very good
Advantages	Modular design.		Modular design.	Modular design.

	Small hold-up volume.	Small hold-up volume.	Moderate hold-up volume.
		Backflushing capacity.	Backflushing capacity.
			Chemically robust. Potential to operate at higher cell densities than used in this work.
			Higher flux is possible than obtained in this work.
			No screens, spacers or separators are required to maintain system geometry.
			Long membrane life expected.
Disadvantages	Large initial decline in flux.	Large initial decline in flux.	Expensive?
	Large pressure drops due to membrane support screen blockage.	Low pressure and temperature operating limits.	Membranes are fragile.

Backflushing capacity not demonstrated and limited operating periods achievable. Antifoam may be deleterious to flux performance. Membrane life likely to be limited.	Only chemical sterilization. Antifoam use may be deleterious to flux performance.	Possible damage to cells from pump action if operated at higher recirculation velocities.

CHAPTER 8

A COMPARISON OF IMMOBILIZED CELLS WITH CELL RECYCLE FOR FERMENTATION INTENSIFICATION

The "intensified" fermentation technologies for continuous solvents production from whey permeate, employing alginate-immobilized cells, IMC, (Chapter 6), or external cell-recycle using cross-flow microfiltration, CFM, (Chapter 7), are phenomenologically the same. The objective in both instances, is to increase the reactor biomass in order to obtain greater productivities during continuous operation for extended periods, than is achievable using conventional batch fermentation with free cells.

Although the studies on these technologies reported in the present work were carried out on a laboratory scale, and are rather preliminary, some comparative statements can be made and these are summarized in Table 8.1. The advantages/disadvantages of the processes pertain to the observations made when using the particular apparatus, although some statements are of a more general nature.

The culture characteristics were different in the continuous fermentations using alginate-immobilized cells from in the cell-recycle system. In the latter experiments (Chapter 7), a heterogeneous mixture of cells and spores were removed so as to control the biomass concentration in order to facilitate stable solvent production (Appendix 1). A complex interactive cycle involving various morphological differentiation rates was operative however, such that steady-state solvent production could not be successfully controlled for extended periods. In contrast, in the IMC experiments (Chapter 6) steady-state solvent production was obtained. In this instance the biomass removed during the fermentations was that growing (vegetative cells) within the IMC beads and being "sloughed-off", to be removed in the fermentation effluent. A heterogeneous culture morphology was observed within the alginate beads i.e. it is possible that the same interactive cycle involving morphological differentiation rates was occurring, but it was

contained within the beads such that a selective cell morphological type was removed as opposed to the non-selective cell morphological type removal in the cell-recycle experiments. This apparently permitted a more controlled fermentation system.

In conclusion, this study has shown that continuous solvents production from whey permeate can be achieved using both IMC and CFM "intensified" fermentation technologies. Stable solvents production was achieved in IMC fermentations but not CFM fermentations and this can be explained by the different control mechanism exerted over the biomass concentration. Summarized information presented in Table 8.1 shows that CFM fermentations are intrinsically complex and more difficult to operate and control than IMC fermentations. The development of "tailor-made" membranes, and fundamental studies determining the morphological behaviour (which is probably strain specific) are two important research areas requiring closer study.

Table 8.1 A comparison of continuous fermentation by alginate-immobilized cells and external cell recycle by cross-flow microfiltration for the production of solvents from whey permeate by C. acetobutylicum P262

	Alginate-Immobilized Cells (IMC)	Cell Recycle by Cross-Flow Microfiltration (CFM)
Factors affecting performance	1. Start-up regime.	1. Start-up regime.
	2. pH, temperature, mixing, dilution rate, fraction of alginate beads in reactor, solvents (butanol) concentration.	2. Overall and biomass bleed dilution rates, biomass growth rate.
		3. Complex cyclic morphological behaviour.
		4. Achievable filtrate flux.
		5. Operation of the cross-flow microfiltration unit for extended periods.
Advantages	1. Alginate immobilization of spores is a simple, reliable, and robust method. Immobilized spores can be easily stored for extended periods.	1. High cell densities can theoretically be achieved, the upper limit being constrained by the rheological properties of the culture, pumpability, polarization and fouling problems.
	2. Continuous stable solvents production was achieved.	2. In-line products recovery may be more easily facilitated since cells are not present.
	3. High biomass loadings achieved.	3. No gas hold-up problems, can easily be vented.

	4. High productivities compared with batch fermentation. Higher dilution rates can be achieved.	4. Can operate over the range pH 4.3-5.6
	5. Predictive kinetic models/ reactor design/scale-up methodologies published.	
	6. Contaminant microorganisms can be removed by operating at a dilution rate greater than the growth rate of the contaminant. Use of unsterilized feed may be possible.	
	7. Stages-in-series processes may be operated with solvents removal between stages.	
Disadvantages/ Constraints	1. Butanol inhibition determines the extent of lactose utilization and hence productivity. Concentration obtainable is approximately half that obtained in batch fermentation.	1. Lower solvents concentrations obtained, accompanied by high acids concentrations.
	2. Operation at culture pH value of 1 pH unit lower than optimal in batch culture with free cells, means lower growth and lactose uptake rates (and lower viability?).	2. Butanol inhibition affect, probably as for IMC, although not quantitated.
	3. In-situ/In-line solvents recovery may be more difficult due to the presence of free cells.	3. Continuous operation of CFM units difficult, as prone to fouling, etc.
	4. Diffusional restrictions of substrate to, and product from, active cells; due to alginate carrier and other cells.	4. Heat/mass transfer may be difficult on a large scale.
	5. Loss of activity with time.	5. Contaminating microorganism cannot be removed, plant has to be

shut down. Long term aseptic operation may be difficult.

6. Gas release may be a problem in some reactor types.

6. Stable solvents production related to complex morphological behaviour of strain P262. Kinetic behaviour of this strain in concentrated suspension is unknown. Steady state solvent production not obtained.

7. 'Start-up" conditions determine germination, morphological nature of immobilized culture and operational pH. Excessive gas release at higher pH values gives bead fracture.

7. High capital costs:
- membrane plant
- control?

8. Biomass concentration in the fermentation system is difficult to measure on-line.

9. Cell damage due to pumping?

10. Filtrate flux directly determines achievable system dilution rates. High fluxing, non fouling membrane systems capable of being cleaned during operation (backpulsing) and that can be steam sterilized, are required.

CHAPTER 9

PRODUCT RECOVERY DURING SOLVENT PRODUCTION

9.1 INTRODUCTION

Product inhibition limits the attainable products concentration in the ABE fermentation. Generally, solvents concentrations of 15-20 g/l are obtained with strain P262 during batch fermentation of a molasses fermentation medium, with butanol being the most inhibitory product (Van der Westhuizen et al, 1982). The results obtained in batch fermentation studies using sulphuric acid casein whey permeate medium (Chapter 5) showed that a maximum butanol concentration of 12-13 g/l could be obtained after an extended fermentation period (70-100 h).
Additionally, results obtained in continuous fermentation studies with alginate-immobilized cells (Chapter 6) using cheese whey permeate medium, showed that the inhibitory butanol concentration of approximately 5 g/l, essentially controlled the process. Moreover, this inhibitory concentration was lower in fermentations utilizing lactose (whey permeate) than on substrates containing glucose. This observation was related to the different sugar uptake mechanisms and the different magnitude of effect that butanol exhibits.

In-situ or in-line solvents (butanol) removal processes (continuous or semi-continuous) with subsequent solvents recovery, may be a means of increasing the attainable solvents concentration and productivity as a result of increased sugar consumption, during the fermentation of whey permeate. These solvent removal processes are briefly reviewed in Appendix 6. Three processes (gas-stripping, an adsorbent resin and a molecular sieve) were examined in the work presented here, in a series of preliminary experiments, in order to demonstrate their efficacy for solvent recovery (or removal) during the fermentation of whey permeate medium. The integration of these processes with batch fermentation (sulphuric acid casein whey permeate) and continuous fermentation (cheese whey permeate) using cells immobilized by adsorption onto bonechar, was examined.

9.2 IN-SITU GAS-STRIPPING/CONDENSATION FOR SOLVENTS RECOVERY

9.2.1 Introduction

A novel integrated fermentation-product recovery process is the use of gas-stripping to continuously remove solvents from the fermentation broth with solvent recovery from the vapour phase being achieved by condensation in a cold trap or by the use of a molecular sieve.

The first objective of the present work was to calculate the minimum theoretical gas-stripping usage rate for a given butanol removal rate, and to conduct experiments with a model solvents-water solution in batch mode to test the theory and assumptions. The second objective was to demonstrate the efficacy of using gas-stripping, with solvents recovery by condensation, for continuous solvents recovery during a batch fermentation of sulphuric acid casein whey permeate (Section 3.7.3), and for solvents recovery prior to the refermenting of cheese whey permeate substrate using a continuous fermenter where the bacterial cells were immobilized by adsorption onto bonechar.

9.2.2 Results and Discussion

A calculation (Appendix 3) was undertaken to predict the theoretical stripping-gas usage rate for a butanol removal rate, equal to its production rate of 0.30 g/l.h, and at a butanol concentration of 5 g/l. These values are typical of those obtained during a batch fermentation at 34°C of sulphuric acid casein whey permeate, as described in Chapter 5 (Run IX). The butanol concentration chosen for the calculation is not toxic to cellular growth of strain P262 when producing solvents from a molasses fermentation medium (Van der Westhuizen et al, 1982). Hence, removal of butanol at a rate equal to its production rate should prevent its accumulation to a toxic concentration. This calculation predicted a stripping-gas usage rate of 1.34 l/l.min. This amount of stripping-gas could realistically be sparged through a batch fermentation culture.

An initial series of experiments were conducted at 34°C, using a model fermentation solution and the experimental apparatus described in Section 3.7.6, to determine the actual stripping-gas usage rate, and to

check the gas integrity of the experimental apparatus so that the overall solvent productivity could be determined for an actual fermentation. The model fermentation solution consisted of 5 g/l, 2 g/l and 1 g/l of n-butanol, acetone and ethanol respectively, prepared using distilled water.

A stripping-gas flowrate of 2.7 l/l.min was required to achieve an average butanol removal rate of 0.30 g/l.h over a 12 h period using the experimental apparatus described in Section 3.7.6. Mass balance data for this run are given in Table 9.1, and agree within experimental error. These data show that there were minimal solvent losses due to nitrogen gas leakage.

The selectivity of the butanol removal by gas-stripping/condensation was found to be 19.3 using the equation proposed by Groot et al, (1984b). Acetone and ethanol selectivities were considerably lower. An increase in the butanol selectivity may be possible if the vapours were cooled prior to the condenser. Water vapour in the stripping-gas vapours would be condensed and returned to the fermenter. This treatment would also decrease the condenser heat load. In repeat experiments of this stripping-gas flowrate, using the model fermentation solution, butanol recoveries were typically 90-95% at an average butanol removal rate of 0.30-0.35 g/l.h.

In a further experiment, solvents (at concentrations used in the model fermentation solution) were added directly to an autoclaved sulphuric acid casein whey permeate medium (Table 3.2) containing no bacterial cells. A gas-stripping/condensation experiment at a stripping-gas flowrate of 2.7 l/l.min was conducted at 34°C. An average butanol removal rate of 0.45 g/l.h and a 90% butanol recovery were achieved.

The higher stripping-gas flowrate required in these experiments than theoretically predicted (Appendix 3), was expected, as the assumptions that the butanol concentration remains constant during stripping, and that the stripping-gas is butanol-free (assumptions 5 and 6 respectively, in Appendix 3) were approximations only. The butanol concentration was not maintained constant, hence the instantaneous

Table 9.1 Mass balance data from gas-stripping/condensation recovery of solvents from a model fermentation solution

	Butanol	Acetone	Ethanol	Water
		(grams)		
Initial	6.14	2.27	0.80	1200
Final	2.80	1.24	0.53	1125
Condenser	3.19	0.80	0.29	70
% Recovery	97.6	89.8	105.0	99.5

Table 9.2 Summary of fermentation parameters for a control (no gas-stripping) and gas-stripping/condensation solvents recovery from a batch fermentation using sulphuric acid casein whey permeate medium and C. acetobutylicum P262

	Control[a]	Gas-Stripping/ Condensation Recovery[b]
Fermentation time, h	50	52
Butanol, g/l	8.1	11.0
Acetone, g/l	2.9	3.9
Ethanol, g/l	0.3	0.8
Lactose utilized, g/l	29	58.3
Yield, g.solvents/g.lactose utilized	0.39	0.27
Overall fermenter productivity, g.solvent/l.h	0.22	0.31
Maximum observed lactose utilization rate, g/l.h	1.1	2.3

a Run IX, Chapter 5
b Assumes 100% recovery

removal rate decreased as the butanol was removed. Additionally, the stripping-gas was recycled for purposes of maximizing solvents recovery and was not solvent-free.

In summary, these experiments using model fermentation solutions, successfully demonstrated the principle of in-situ gas-stripping for selective butanol removal with solvents recovery from the vapour phase using a cold trap.

A batch fermentation experiment was conducted using sulphuric acid casein whey permeate medium (Table 3.2) supplemented with 25 g/l of lactose. The medium was adjusted to pH 6.5 prior to autoclaving, and from pH 5.4 to pH 6.0 after autoclaving, using aqueous ammonia. The fermenter was inoculated at 34°C (2% v/v) with a highly motile culture (Section 3.7.2). The pH of the culture was controlled at pH 6.0 $\pm$ 0.1 using 5M ammonia solution for 9.5 h, after which time it was not controlled. After 24 h fermentation, the agitation (50 rpm) was stopped and the gas-stripping/condensation was commenced as described in Section 3.7.6. The butanol concentration after 24 h fermentation was 5.1 g/l. The fermentation profile for this experiment is given in Figure 9.1. The concentration data shown have been corrected for water removal during gas-stripping using the general procedure given in Section 3.8. Parameters derived from this experiment are given in Table 9.2 along with corresponding data from a batch fermentation (Run IX, Chapter 5) conducted under similar conditions to those reported here, but without gas-stripping. Significantly, marked increases in the observed lactose utilization rate and extent of lactose utilization were found in the gas-stripping experiment. A higher solvents productivity was obtained by this integrated process due to in-situ toxic butanol removal. An increase in cell concentration was observed in this experiment but no quantitation was attempted.

A shift to further acid production occurred after 32 h, resulting from a combination of high pH value (5.8) and low residual lactose concentration (23 g/l), which have been shown to favour acid production (Chapter 5). This result also explains the lower yield obtained compared with the control fermentation. Unaccounted solvent losses due to vapour losses (approximately 10%) would also explain the lower yield.

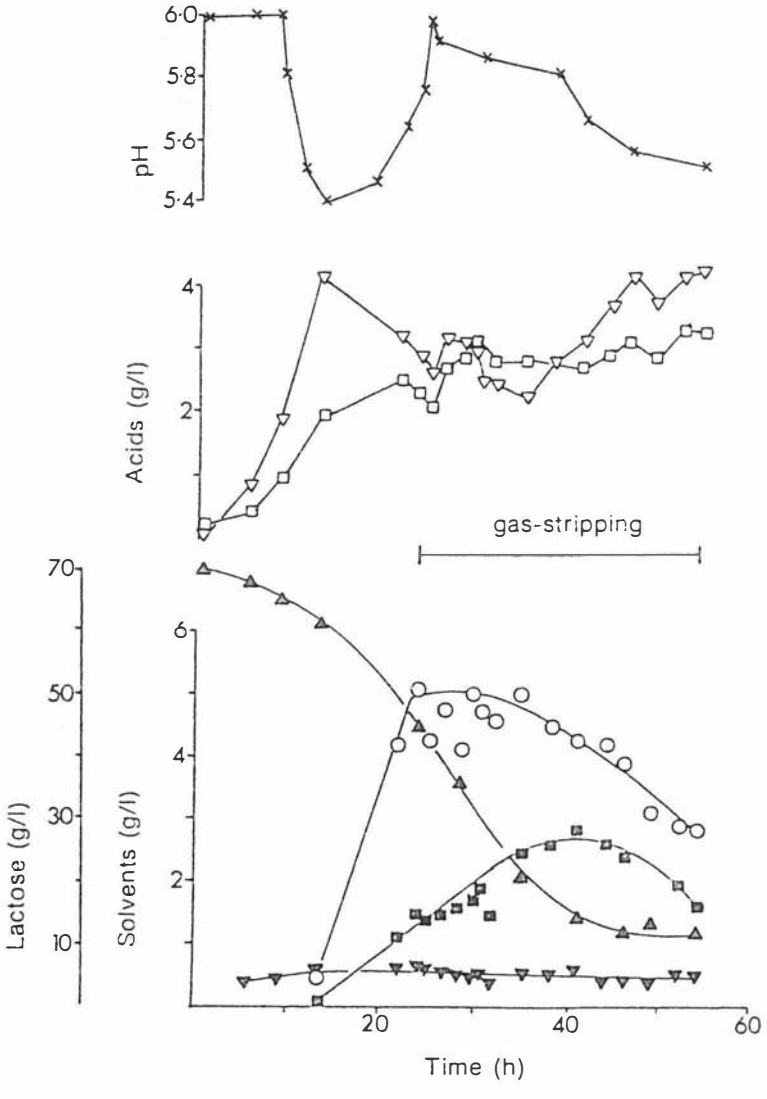


Figure 9.1. Fermentation profile for the gas-stripping/condensation solvents recovery from a batch fermentation using sulphuric acid casein whey permeate medium: (○) butanol; (■) acetone; (▼) ethanol; (▽) butyric acid; (□) acetic acid; (×) pH; (▲) lactose.

This increase in acid production suggests that continuous lactose feeding (plus other nutrients?) during gas-stripping could possibly be used for extended batch operation.

A butanol removal rate of 0.45 g/l.h and a butanol removal selectivity of 23.4 were obtained in this experiment, selectivity being comparable to that obtained in an integrated fermentation-pervaporation process (Groot et al, 1984a). The higher butanol removal rate obtained in this instance, also in the model fermentation solution based on whey permeate, compared to the model fermentation solution in distilled water may be attributed to the presence of salts in the whey permeate that increase the butanol partial pressure (Butler et al, 1933). The effect of antifoam addition (a surfactant) on the butanol selectivity was not determined but in fact may cause a lowering of the butanol selectivity.

The rise in the fermentation culture pH at the commencement of gas-stripping (Figure 9.1) can be attributed to the lowering of dissolved fermentation gases concentration (CO_2+H_2) in the culture. This may be an important consideration since a relationship between the dissolved hydrogen gas concentration in the fermentation culture and the final solvent (butanol) concentration has been demonstrated (Doremus et al, 1985; Yerushalmi et al, 1985). When the dissolved hydrogen concentration is maximized (no agitation, hydrogen head-space pressurization of the fermenter), solvent production is generally enhanced due to a redirection in the electron flow favouring butanol production (Maddox et al, 1981). Conversely, during batch fermentations with increased carbon dioxide head-space pressure (0-690 kPag), lower solvent concentrations and substrate utilization rates were observed (Klei et al, 1984).

The utilization of these gases for methanol synthesis has been proposed (Moreira et al, 1982). Another use may be their recovery and use for solvent recovery by in-situ or in-line stripping with vapour phase solvent recovery. Walsh et al, (1983) proposed the in-situ use of carbon dioxide to continuously strip ethanol from a fermentation with ethanol recovered on activated carbon and finally cellulose. Further investigations are required to determine if such a process option is practical and to examine any effects on the fermentation kinetics.

Integration of this recovery process with continuous fermentation processes based on immobilized cells or cell recycle may give rise to higher fermentation productivities. Such processes would require in-line stripping since large stripping-gas volume usage rates would be impractical for in-situ application. Such an integrated process for ethanol removal, involving immobilized cells of K. fragilis NRRL 2415, has been reported (Dale et al, 1985).

The integration of the gas-stripping solvents removal process with a "stages-in-series" continuous fermentation process was examined in a further set of experiments. The objective was to increase the overall solvents concentration and productivity, and to maximize the extent of lactose utilization by removing toxic volatile solvent products using gas-stripping, prior to reutilizing this medium in a further reactor, i.e. discontinuous fermentation. For the continuous fermentations, cells of strain P262 were immobilized by adsorption onto bonechar and contained within an upflow jacketed packed bed column reactor. The reactor was typically 250 mm x 20 mm, and approximately 90% of the reactor volume was occupied by the bonechar (including voidage). The adsorption and reactor "start-up" procedure have been described by Qureshi & Maddox (1987). Cheese whey permeate medium (Table 3.3, without added $CaCl_2.2H_2O$) was used in the fermentations, conducted at 34°C.

In the first experiment (Run I), effluent was collected from an immobilized cell reactor (Stage I) operating at a dilution rate (D_t, based on total reactor volume) of 0.30 h^{-1}, under steady-state solvent production. The effluent (pH 5.0) was kept at 4-6°C. Steady-state production data for Stage I are given in Table 9.3. This table also includes other steady-state data for Run I.

Volatile solvent products were removed from the Stage I effluent by gas-stripping at 50°C using oxygen-free nitrogen gas. Solvents were vented in the vapour phase i.e. no recovery was attempted. Sterile distilled water was used to replace that water removed during gas-stripping. A significant reduction in the solvents concentration was achieved, with little change in the acids concentration (Table 9.3).

Table 9.3 Steady-state fermentation data for a "stages-in-series" continuous fermentation process with immobilized cells, using gas-stripping for solvents removal between stages (Run I), and no gas-stripping between stages (Control)

	C_E (g/l)	C_A (g/l)	C_B (g/l)	C_{HB} (g/l)	C_{HA} (g/l)	C_L (g/l)	ΔS (g/l)	Y (g/g)	Prod (g/l.h)
Bulk effluent from Stage I $D_t = 0.30\ h^{-1}$	0.48	2.06	5.00	0.52	0.75	26.4	27.4	0.29	2.26
Bulk effluent from Stage I after gas-stripping i.e. Feed to Stage II	0.20	0.05	0.55	0.54	0.70	26.4			
Effluent from Stage II $D_t = 0.13\ h^{-1}$	0.38	1.49	3.68	0.75	0.99	12.9	13.5	0.35	0.62
Effluent from Stage II D_t = 0.20 h-1	0.39	1.02	2.34	0.57	1.08	18.7	7.7	0.38	0.59
Bulk effluent from Stage II (operating at $D_t = 0.13\ h^{-1}$ and 0.20 h^{-1})	0.34	1.39	2.20	1.57	1.39	n.a.			
Bulk effluent from Stage II after gas-stripping i.e. Feed to Stage III	0.33	0.14	0.59	1.69	1.10	14.4			
Effluent from Stage III $D_t = 0.20\ h^{-1}$	0.36	0.50	1.64	2.21	1.41	9.85	4.55	0.32	0.29
Two-stage process, Control									
Effluent from Stage I $D_t = 0.30\ h^{-1}$	0.44	2.76	5.45	0.41	0.85	28.1	24.4	0.35	2.59
Effluent from Stage II $D_t = 0.13\ h^{-1}$	0.44	3.19	6.21	0.69	1.20	23.4	3.7	0.32	0.15

n.a. = not analyzed.

This treated effluent (containing cell material from Stage I) was fed to a second column reactor containing bonechar (Stage II) which had been inoculated with fermentation culture from Stage I. The reactor was maintained under batch conditions (no feed) for 10 h after which the effluent was fed at D_t = 0.13 h^{-1} for 142 h and then D_t = 0.20 h^{-1} for a further 192 h. Steady-state solvent production was obtained (Table 9.3). Effluent from Stage II was collected and gas-stripped as described previously for Stage I effluent, prior to being fed to the established Stage II reactor (Stage III). Further solvent production resulted (Table 9.3).

This Run demonstrated that fermentation effluent from a continuous fermentation using immobilized cells, could be treated by gas-stripping to remove toxic solvents and further fermented so that an overall increase in solvents concentration and productivity, and extent of lactose utilization occurred, compared to that obtained in a two-stage continuous fermentation experiment where no gas-stripping treatment of Stage I effluent was used, i.e. control fermentation. Steady-state data for this experiment are also given in Table 9.3. In Run I, a total of 39.65 g/l lactose was utilized (73.7% of that available) giving a total of 11.9 g/l solvents, calculated using a value of D_t = 0.20 h^{-1} after two refermentations. This represents a solvent yield of 0.30 g/g. In the control fermentation, a total of 28.1 g/l lactose was utilized (53.5% of that available) at a total of 9.8 g/l solvents with a solvent yield of 0.32 g/g.

The build-up of reactor biomass and increase in solvent production in the Stage II reactor (first re-use of gas-stripped treatment medium from Stage I) was slightly slower than when "starting-up" a reactor with fresh cheese whey permeate medium e.g. Stage I. It was reasoned that some growth factors or nutrients were utilized by the biomass in Stage I, and that the lower concentrations may have retarded the subsequent reactor performance. Consequently, two further continuous fermentations were conducted, with the objective to examine the re-use of effluent from an initial reactor (Stage I), operating as described for Run I (Table 9.3), which was then gas-stripped and fed to this same reactor having the established biomass (i.e. Stage II). This treated effluent was unsupplemented prior to re-feeding to the Stage I reactor (Run II).

In a separate instance, treated effluent was supplemented with 2.5 g/l yeast extract and fed to a Stage I reactor (Run III). A summary of steady-state data for these experiments is given in Table 9.4.

Two separate Stage I reactors were operated in parallel, the bulk effluent collected and treated by gas-stripping. The Stage I reactor for Run II was operating at a higher productivity than the Stage I reactor for Run III (Table 9.4). A value of D_t = 0.40 h^{-1} was used in the Stage II reactor for Run II (no supplementation). A significant increase in the solvents concentration and hence productivity was obtained compared with Run I (Table 9.3; operating over two stages), demonstrating that there were sufficient nutrients for the maintenance of solvent production after an initial fermentation, i.e. lower nutrient concentrations are required for continuous solvent production compared with that required for growth. This suggests that it may be possible to operate with a much lower yeast extract concentration in the feed medium to continuous fermentations using immobilized cells or external cell recycle than used in Chapters 6 and 7 respectively. The possibility of refermenting the effluent from Stage II was not examined but could give rise to even higher solvent productivities and increased lactose utilization.

In Run III (supplementation with 2.5 g/l yeast extract), further solvent production was obtained in the Stage II reactor (D_t = 0.40 h^{-1}), however, solvent productivity was lower than in Run II, (higher than in Run I) and was accompanied by increased acid production giving a lower solvent yield (Table 9.4). It is possible that the increased yeast extract concentration in the medium favoured cell growth at the expense of solvent production.

9.2.3 Conclusions

A novel process for in-situ selective butanol recovery from a batch fermentation culture has been described in this preliminary work. The process involves gas-stripping using an inert gas (e.g. oxygen-free nitrogen gas) and recovery of solvents from the vapour phase by condensation using a cold trap. The non-ideality of low mole-fraction butanol solutions and the selectivity for butanol removal by such an

: fermentation data for a two-stage continuous
ı immobilized cells, using gas-stripping for
oval between stages (Runs II and III)

	C_E (g/l)	C_A (g/l)	C_B (g/l)	C_{HB} (g/l)	C_{HA} (g/l)	C_L (g/l)	ΔS (g/l)	Y (g/g)	Prod (g/l.h)
Run II (no supplementation of feed to Stage II)									
Effluent from Stage I $D_t = 0.30\ h^{-1}$	0.32	2.52	5.16	0.61	1.13	28.6	22.6	0.35	2.40
Bulk effluent from Stage I of Runs II and III, prior to gas-stripping	0.37	1.97	4.18	0.97	1.08	31.6			
Bulk effluent after gas-stripping; Feed to Stage II (for Runs II and III)	0.29	trace	0.99	0.95	1.23	31.6			
Effluent from Stage II $D_t = 0.40\ h^{-1}$	0.32	1.54	3.06	1.06	1.02	21.1	10.5	0.35	1.46
Run III (supplementation of feed to Stage II with 2.5 g/l yeast extract)									
Effluent from Stage I $D_t = 0.30\ h^{-1}$	0.33	1.47	4.00	1.41	1.45	33.9	17.3	0.34	1.74
Effluent from Stage II $D_t = 0.40\ h^{-1}$	0.36	0.57	2.88	2.74	1.45	22.7	8.9	0.28	1.01

operation, suggests that butanol has surfactant properties, preferentially moving across the liquid-vapour interface. A significant increase in fermentation performance (e.g. solvent productivity, lactose utilization) has been demonstrated by the integration of this technique with a batch fermentation using sulphuric acid casein whey permeate compared with a control fermentation.

In a series of experiments, gas-stripping was used to remove solvents from the effluent of a continuous fermentation using cheese whey permeate medium (Stage I) and cells of strain P262 immobilized by adsorption onto bonechar. The treated effluent was then fed to a second reactor (Stage II). Increases in the solvents concentration, productivity and extent of lactose utilization were demonstrated compared with a control two-stage process. This preliminary work suggests that the application of in-line solvents recovery in continuous solvent fermentation processes using immobilized cells or external cell recycle may give even greater productivities than demonstrated in Chapters 6 and 7 respectively.

9.3 USE OF AN ADSORBENT RESIN OR MOLECULAR SIEVE FOR SOLVENTS RECOVERY

9.3.1 Introduction

Another integrated fermentation-product recovery process is the use of molecular sieves or polymeric adsorbent resins to recover solvents from the fermentation broth (in-situ or in-line application). Molecular sieves (e.g. silicalite) are porous solid materials that can be used to preferentially adsorb alcohol from alcohol-water mixtures, with heat being used to regenerate the sieves for subsequent re-use, with the volatilized alcohol condensed and recovered (Milestone & Bibby, 1981; Klein & Abraham, 1983). Solvents adsorption from model butanol-water solutions (Milestone & Bibby, 1981) and sulphuric acid casein whey permeate fermentation medium (Maddox, 1982) using silicalite has been demonstrated. These studies however, did not include data for acids adsorption.

Synthetic polymeric resin adsorbents have been evaluated for ethanol recovery from fermentation broths, either in integrated processes or during downstream processing (Pitt *et al*, 1983; Lenckii *et*

al, 1983). Data describing their application to the ABE fermentation are sparse. Their application to continuous solvents recovery has been described for a continuous fermentation process using external cell recycle by cross-flow microfiltration, but details are lacking (Larsson et al, 1984).

The objective of the present work was to examine a series of polymeric adsorbent resins (XAD series) and the molecular sieve, silicalite, for their adsorption capacity/selectivity of components from a series of model fermentation solutions, and to select an adsorbent for further work. In preliminary experiments, the applications of an in-line adsorption process to a batch fermentation process (sulphuric acid casein whey permeate medium) and continuous fermentation process (cheese whey permeate medium) using cells immobilized to bonechar, were examined. No recovery of products from the adsorbent resins or silicalite was attempted.

9.3.2 Results and Discussion

An initial screening experiment was undertaken, at 30°C, using a model fermentation solution to determine the capacity for component removal for each of the 5 adsorbents given in Section 3.1.3. The model fermentation solution consisted of 5 g/l, 2 g/l, 1 g/l, 2 g/l and 2 g/l of n-butanol, acetone, ethanol, butyric acid and acetic acid respectively, in deionized water, pH adjusted to pH 5.0 using 2M NaOH. These product concentrations are typical of those obtained during a batch fermentation (24 h) of sulphuric acid casein whey permeate medium, as described in Chapter 5 (Run IX).

Adsorbents were preweighed into 100 ml conical flasks and 50 ml of model fermentation solution was added. The flasks were stoppered, shaken and then transferred to a Markline shaker and shaken at 100 rpm for 90 min at 30°C. After this treatment, fermentation solution containing no adsorbent, also shaken at 30°C (control), and solution supernatants were analyzed for remaining product concentrations. In order to express the adsorption data on an equivalent basis, approximately 0.5 g of washed resin was dried at 105°C for 36 h to determine the resin dry weight.

Capacity data of the various adsorbents for components in the model fermentation solution are given in Table 9.5 All adsorbents removed each of the model solution components to varying extents, with ethanol and acetic acid generally being the most poorly adsorbed. The preferential adsorption of some components may be related to their concentration, hydrophobicity, molecular size, and shape of the molecule.

High butanol and butyric acid adsorption capacities were obtained with XAD-4, XAD-16, and silicalite. Lower ratios of XAD-4 and XAD-16 : model solution, were used compared with the silicalite, since the filtered resin after washing contained a larger total moisture content (50-70%) than expected. Generally, the specific loading capacity decreased for each model solution component as the ratio of d.w. resin to model solution amount was increased.

Adsorption by silicalite was essentially instantaneous as evidenced by air release from the silicalite as soon as it was added to the solution. The XAD resins became buoyant when added to the model solution.

On the basis of this screening experiment, the adsorbent resins XAD-4 and XAD-16 and the molecular sieve silicalite, were selected for a further series of experiments undertaken at 30°C, using various model fermentation solutions to examine the effect of fermentation medium components, *viz* minerals, lactose and yeast extract, on the adsorption of the fermentation products. The experimental procedure was the same as described previously. The following model fermentation solutions were used:

Solution I:

Spray dried sulphuric acid casein whey permeate was reconstituted at 55 g/l and supplemented with lactose and yeast extract at 10 g/l and 5 g/l respectively. Medium was adjusted to pH 6.5 using aqueous ammonia and autoclaved (121°C/15 min). The hot medium was filtered through Whatman No.1 filter paper, cooled, and butanol, acetone, ethanol, butyric acid and acetic acid added at concentrations of 5 g/l, 2 g/l, 1 g/l, 2 g/l and 2 g/l,

Table 9.5 Adsorption of model fermentation solution components using various adsorbents at 30°C

Adsorbent	mg Resin Used / g Model Solution	mg Solute Adsorbed / g Adsorbent (d.w)					% Removal				
		BuOH	AC_2O	EtOH	HBu	HAc	BuOH	AC_2O	EtOH	HBu	HAc
XAD-2	5	30.0	14.0	0	16.0	4.0	2.9	3.3	0	3.7	0.9
	25	52.4	19.6	1.6	11.6	3.6	25.3	23.3	3.8	13.5	4.1
	50	39.4	5.6	0	8.2	4.0	38.0	13.3	0	19.1	9.0
XAD-4	4.65	170	178.1	25.8	36.6	0	15.3	39.5	11.5	7.9	0
	23.25	103	18.5	6.4	25.0	13.4	46.1	20.5	14.4	26.9	13.9
	46.5	73.9	12.2	0.4	15.3	3.7	66.4	27.1	1.9	33.0	7.7
XAD-7	3.17	63.1	47.3	3.2	34.7	44.1	3.9	7.1	0.9	5.1	6.3
	15.85	41.7	14.5	0	11.4	14.5	12.7	10.9	0	8.4	10.4
	31.70	42.3	8.5	0.3	11.7	2.8	25.9	12.9	0.9	17.2	4.1
XAD-16	2.94	139.5	13.6	0	57.8	78.2	7.9	1.9	0	7.9	10.4
	14.70	132.7	21.1	3.4	31.3	15.7	37.6	14.8	4.8	21.4	10.4
	29.40	97.6	16.7	3.7	19.7	10.5	55.4	23.3	10.6	26.9	13.9
Silicalite	9.91	91.8	23.2	7.1	43.3	37.3	17.6	10.9	6.7	20.0	16.7
	49.55	78.1	7.3	2.0	21.1	6.3	74.7	17.1	9.6	48.8	13.9
	99.1	52.3	17.4	4.0	13.7	4.7	100.0	82.4	38.5	63.3	21.2

respectively. This solution was then adjusted to pH 5.2 with aqueous ammonia.

Solution II:

This solution contained the same product concentrations as in Solution I, prepared in deionized water, and was supplemented with 45 g/l lactose.

Solution III:

This solution was the same as Solution II except that 5 g/l yeast extract was used instead of lactose.

Capacity data of the adsorbents for components in the various model solutions are given in Table 9.6. Similar, although slightly lower, adsorption capacities were obtained for the various components in each of the model solutions (at equivalent ratios of resin : model solution) to those in Table 9.5. Any differences may be due to experimental error, but these results demonstrated that no significant reduction in specific adsorption was observed that could be attributed to the presence of minerals, lactose or yeast extract (Solution I), lactose alone (Solution II) or yeast extract (Solution III). These are preliminary results however, and further experimentation is required. No measurable adsorption of lactose by the resins or silicalite was detected (data not shown). Bul et al, (1985) found no measurable adsorption of glucose by silicalite.

The adsorption capacity values for butanol by silicalite obtained in all these experiments agree closely at equivalent usage rates, with values given by Milestone & Bibby (1981) and Maddox (1982). Lower ethanol adsorption, in the presence of butanol, can be attributed to the lower concentration of the former component since higher capacities can be obtained at higher ethanol concentrations in aqueous ethanol solutions (Klein & Abraham, 1983; Bul et al, 1985). Milestone & Bibby (1981) however, found that no ethanol adsorption occurred in ethanol/n-butanol mixtures, so the low adsorption capacity values reported in Tables 9.5 and 9.6 may be due to experimental errors. These

Table 9.6 Adsorption of components from various model fermentation solutions by Silicalite, XAD-4 and XAD-16 resins at 30°C

Medium	Adsorbent	mg Adsorbent / g. Medium Solution	mg Solute Adsorbed / g Adsorbent (d.w) BuOH	AC_2O	EtOH	HBu	HAc
I.							
Whey permeate	Silicalite	50	77.6	5.8	0	16.4	11.8
		100	47.7	12.2	2.2	13.1	2.4
	XAD-4	47	69.1	7.6	0	12.8	4.0
		94	43.6	8.6	2.6	6.4	2.0
	XAD-16	29.2	93.5	9.2	1.0	31.2	16.4
		58.4	63.0	9.5	1.4	12.5	3.3
II.							
Model solution	Silicalite	50	81.2	7.6	3.8	23.6	9.4
	XAD-4	47	74.5	11.7	6.6	22.3	10.4
	XAD-16	29.2	98.6	11.6	7.2	25.3	14.7
III.							
Model solution	Silicalite	50	75.0	3.4	2.0	21.0	0
	XAD-4	47	67.0	6.6	3.6	15.7	2.1
	XAD-16	24.6	91.0	1.6	1.6	12.6	0

workers also found that acetone appeared to hinder the adsorption of n-butanol in acetone/n-butanol mixtures. The hydrophobicity of silicalite (Milestone & Bibby, 1981; Bul et al, 1985) may explain the preferential adsorption of butanol over acetone and ethanol, although Milestone & Bibby (1981) state that the size and shape of the molecule are also important parameters determining adsorption properties.

Similar adsorption profiles were also obtained for the adsorbent XAD resins (Tables 9.5 and 9.6) as for silicalite. This may be related to the hydrophobic adsorption mechanism demonstrated by these resins, the extent of which varies with the actual resin (Lenckii et al, 1983).

The effect of the presence of cells on adsorption capacity was not determined in this work. The adsorbents may be detrimental to cell metabolism when used for in-situ applications. Lenckii et al, (1983) found that the presence of XAD-2, XAD-7, and silicalite were inhibitory to glucose utilization by Saccharomyces cerevisiae NRRL-Y132. These workers suggested that this inhibition by the adsorbent resin could arise from the leakage of toxins from the resin, adsorption of growth factors and nutrients, or adsorption of cells; and the inhibition by the silicalite could be due to the presence of the alumina binder present in the silica.

On the basis of the preliminary screening experiments described in this work, the adsorbent resin XAD-16 was selected for further work, although the use of this resin in this type of application has not previously been reported in the literature.

The application of in-line adsorption of products by XAD-16 to a batch fermentation using sulphuric acid casein whey permeate medium (Table 3.2, supplemented with 20 g/l lactose) at 34°C, with pH control at pH 5.4-5.6, was examined. The 7-litre fermentation apparatus and ancillary equipment described in Section 3.7.3.2 were used. The fermenter working volume was 5 l initially, and this was decreased to 2 l at the commencement of the in-line adsorption treatment. The fermenter was coupled to a Plate and Frame cross-flow microfiltration (CFM) unit (Pellicon Cassette System; equipped with the Ultrasart II cassette of 0.2 μm pore size, Section 3.7.5.1). Fermentation culture

was circulated, via this CFM device, using a Watson-Marlow 60311 peristaltic pump (Section 3.7.5.2), and back to the fermenter. Cell-free filtrate was passed (flowrate approximately 200 ml/min) to a glass column containing 100 g.d.w. resin, for a period of 60 min (6 recirculation volumes). Samples were removed from this filtrate return line to monitor the extent of product removal.

The fermentation time course profile for this run is given in Figure 9.2. The fermentation was operated in batch mode for 30 h, after which the in-line adsorption process using the CFM unit was used (prior to the accumulation of products at toxic concentrations). This treatment was repeated after 54 h using an equal amount of fresh adsorbent resin, as used initially. No attempt was made to regenerate the adsorbent resin for re-use. In a previous experiment (data not shown) it was found that cell removal prior to in-line adsorption was necessary, due to the retention of cells in the adsorbent column (adsorption or physical retention) with a consequent loss in fermentation performance.

The sharp decrease in the lactose concentration at 30 h, and to a lesser extent at 54 h, was attributed to the dilution of the fermenter culture as a result of flushing the CFM device with sterile distilled water in order to return cells back to the fermenter. The adsorption treatment at 30 h and 54 h resulted in an increase in the culture pH value by 0.3 units; the culture pH value prior to adsorption treatment, was restored by pH adjustment using 5M H_2SO_4.

A total solvent concentration of 14.1 g/l, representing a yield of 0.28 g/g, was obtained after 70 h fermentation. A mass balance calculation was used to correct for dilution effects. These results are comparable with a control fermentation carried out under similar culture conditions but without treatment with adsorbent resin (Chapter 5, Run X). The yield value obtained may be explained by the butyric acid production that occurred immediately after the resin treatment (Figure 9.2); the lower butanol concentration possibly favouring cell division and acid production by vegetative cells.

The specific adsorption capacity of the resin at 54 h for the fermentation products (mg solute adsorbed/g.d.w. adsorbent) was

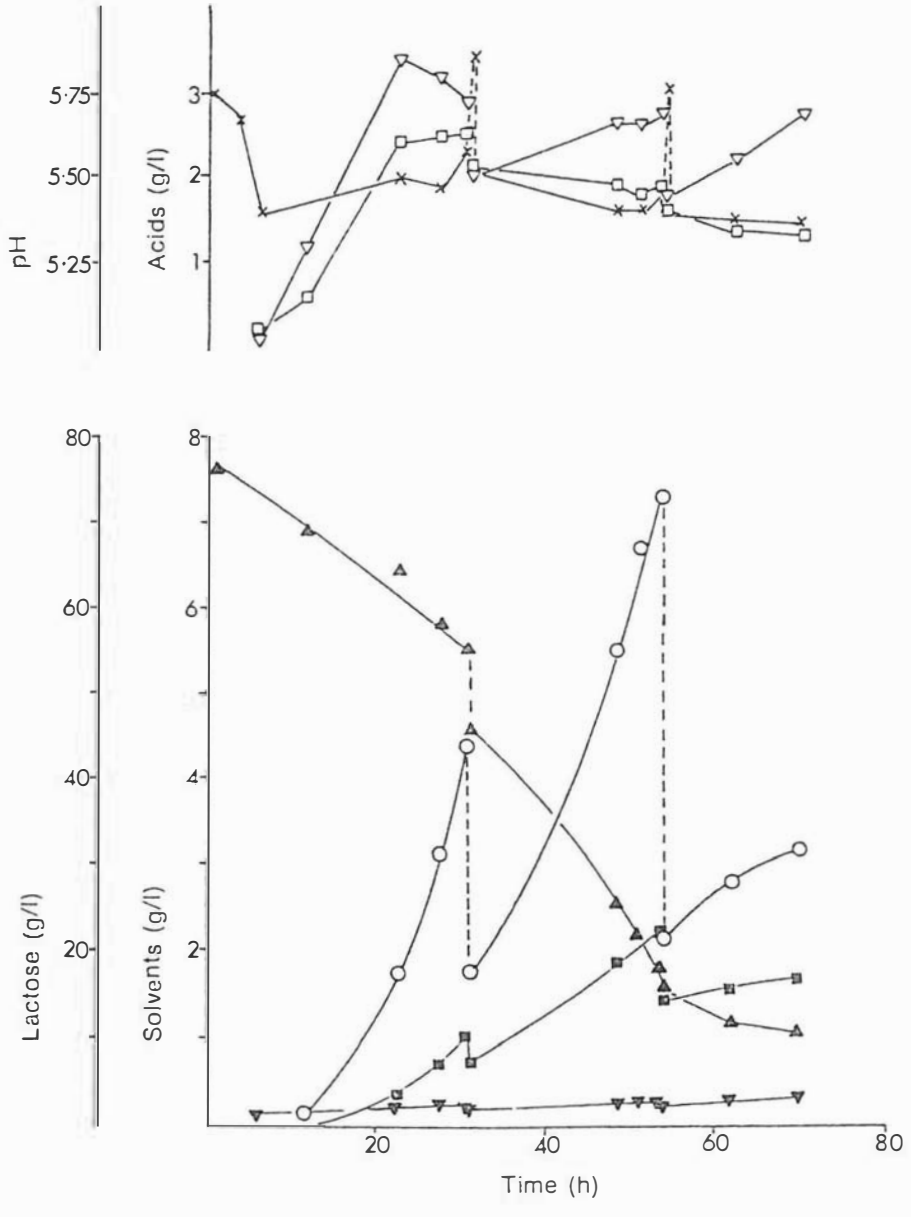


Figure 9.2. Fermentation profile for the adsorption resin (XAD-16) - solvents recovery from a batch fermentation using sulphuric acid casein whey permeate medium. Symbols as for Fig. 9.1.

approximately 86.0, 13.3, 0.7, 15.5, and 5.2 for butanol, acetone, ethanol, butyric acid and acetic acid respectively, at a ratio of 51 mg adsorbent per gram of fermentation solution. This calculation has been corrected for the dilution effects (determined from the change in the lactose concentration, i.e. it was assumed that there was no adsorption of lactose) arising from flushing the CFM unit to return cells to the fermenter. These adsorption capacity results are comparable with those given in Tables 9.5 and 9.6, with the slightly different adsorption profile possibly being due to the presence of different concentrations of fermentation products to those in the model fermentation solutions.

In summary, this batch fermentation experiment demonstrated that in-line solvents removal using an adsorbent resin, is a technically feasible process. Use of a CFM unit prevented direct cell contact with the XAD-16 resin and this process was shown not to be detrimental to batch fermentation performance (Figure 9.2). In this instance, since cells were already present and in a non-growing solventogenic state, the adsorption of any fermentation nutrients etc, would possibly not have the same significant effect as might be observed in a continuous fermentation where the adsorbent when used in an in-situ mode could by its action, interfere directly with cell growth.

The integration of the XAD-16 adsorption resin solvents removal process with a two-stage continuous fermentation process was examined in a further experiment. The objective was to determine if an increase in overall solvents concentration and productivity, plus extent of lactose utilization could be achieved. Cells immobilized by adsorption to bonechar and contained within an upflow column reactor were used, and the columns were operated as described in Section 9.2.2. Cheese whey permeate medium (Table 3.3, without added $CaCl_2.2H_20$) was used in the fermentations, conducted at 34°C.

Effluent was collected from a reactor (Stage I) operating at steady-state solvent production and at a D_t value of 0.3 h^{-1}, and was held at 4-6°C. The steady-state production data for this Stage I, plus other fermentation data pertaining to this experiment, are given in Table 9.7. The effluent was decanted to remove cells and was then treated by passing it through a packed bed containing 77 g.d.w. of fresh

Table 9.7 Steady-state fermentation data for a two-stage continuous fermentation process with immobilized cells, using an adsorbent resin XAD-16 for solvents removal between stages

	C_E (g/l)	C_A (g/l)	C_B (g/l)	C_{HB} (g/l)	C_{HA} (g/l)	C_L (g/l)	ΔS (g/l)	Y (g/g)	Prod (g/l.h)
Bulk effluent from Stage I $D_t = 0.30\ h^{-1}$	0.76	2.86	5.58	0.23	0.78	22.8	31.0	0.30	2.76
Bulk effluent from Stage I, after treatment with XAD-16 resin	0.61	1.04	0.18	0.18	0.58	18.3			
Effluent from Stage II $D_t = 0.13\ h^{-1}$ (no yeast extract)	0.82	1.05	1.77	0.61	0.82	13.3	5.0	0.36	0.24
Bulk effluent from Stage II	1.74	1.06	1.74	0.59	1.24	11.9			
Effluent from Stage II $D_t = 0.13\ h^{-1}$ (3 g/l yeast extract)[a]	1.69	1.33	3.07	1.39	1.32	4.2	7.7	0.20	0.20

[a] A steady-state was obtained only for 2 residence times after which the fermentation degenerated with the total produced acids concentration reaching 1.8 g/l

XAD-16 resin (previously washed with sterile deionized water). Approximately 0.9 l of effluent was treated and predominantly butanol was removed (Table 9.7). The effluent pH increased from pH 5.0 to pH 5.3 during treatment.

This treated effluent was fed to a second column reactor containing bonechar (Stage II) which had been inoculated with fermentation culture from Stage I. The reactor was maintained under batch conditions (no feed) for 10 h after which the effluent was fed at D_t = 0.13 h^{-1}. A steady-state was reached after 120 h fermentation, this time being longer than when starting-up a comparable fermentation using cheese whey permeate medium. Solvent production (Table 9.7) was lower than expected, hence it was reasoned that the resin treatment had removed medium components(?) or these had in fact been used by the cells in Stage I, so that there were insufficient remaining for good growth (slow biomass accumulation) and solvent production. The former reason is the most plausible since in the experiment using gas-stripping, the Stage II reactor was readily operated with treated medium with no yeast extract supplementation. Yeast extract (3 g/l) was added to bulk effluent from the Stage II reactor and further solvent production occurred when fed to the Stage II reactor. Acids production also increased and the fermentation degenerated with a decrease in solvents concentration after a further 120 h. Total produced acids were 1.8 g/l at this time. It is possible that this acidogenic state arose due to the solvents reaching a toxic concentration at this dilution rate and biomass concentration in the reactor, or alternatively, the increased yeast extract concentration promoted cell growth and acids production.

In a further experiment, silicalite was used to treat effluent from a Stage I reactor prior to feeding this treated effluent back to the reactor (Stage II). Steady-state fermentation data are given in Table 9.8. Effluent from Stage I had a higher butyric acid concentration (at comparable yield and productivity) compared to that observed in other fermentations (Tables 9.3, 9.4, 9.7) and is unexplained. Effluent from Stage I was centrifuged to remove cells and silicalite added (60 mg adsorbent/ml effluent supernatant) prior to being removed also by centrifugation. The adsorption capacity (mg solute/g adsorbent) was 75.0, 9.8, 0, 0, and 14.2 for butanol, acetone, ethanol, butyric acid

Table 9.8 Steady-state fermentation data for a two-stage continuous fermentation process with immobilized cells, using the molecular sieve Silicalite for solvents removal between stages

	C_E (g/l)	C_A (g/l)	C_B (g/l)	C_{HB} (g/l)	C_{HA} (g/l)	C_L (g/l)	ΔS (g/l)	Y (g/g)	Prod (g/l.h)
Bulk effluent from Stage I, $D_t = 0.30\ h^{-1}$	0.36	2.33	5.61	1.59	1.18	28.2	24.3[a]	0.34[a]	2.49[a]
Bulk effluent after treatment with Silicalite	0.41	1.74	1.11	1.59	0.33	27.4			
Effluent from Stage II $D_t = 0.40\ h^{-1}$	0.40	2.09	2.66	1.67	1.20	18.1	9.3	0.20	0.76

[a] parameters determined for overall Stage I process

and acetic acid respectively, and data are comparable to that given in Table 9.6 for a model fermentation solution. The fact that no butyric acid adsorption occurred is unexplained.

Further solvent production resulted in the Stage II reactor, however, a low solvent yield was obtained even though significant further acid production did not occur. (Table 9.8). A higher solvent productivity was obtained compared with the experiment using XAD-16 resin, however, a direct comparison is not possible since in this instance a higher dilution rate was used.

9.3.3 Conclusions

The efficacy of using adsorbent resins and the molecular sieve silicalite for the removal of components from various model fermentation solutions has been demonstrated in this preliminary work. A preference, in decreasing order, for butanol, butyric acid and acetic acid was generally obtained for the adsorbents examined, and for the model fermentation solutions tested. No measurable adsorption of lactose was detected for any of the adsorbents.

In a batch fermentation of sulphuric acid casein whey permeate medium, using a CFM unit for providing cell-free filtrate to in-line adsorption (XAD-16 resin), comparable fermentation performance was obtained compared with a control fermentation.

A two-stage continuous fermentation experiment with inter-stage treatment for solvents removal using adsorbent resin, (XAD-16) or a molecular sieve (Silicalite), gave an increase in solvents concentration, productivity and lactose utilization, compared with a two-stage continuous fermentation with no solvents removal between stages (control fermentation). No attempt was made to recover adsorbed products from the adsorbent resin, or to determine its efficacy for adsorption in ongoing treatment cycles; an important estimation for process costing. These solvents removal processes are disadvantaged by the possible adsorption of medium components which affect solvent production, and the poorer selectivity compared with gas-stripping i.e. acids are removed which may result in a lower yield in some instances.

CHAPTER 10

SECONDARY BATCH FERMENTATION OF WHEY PERMEATE FOLLOWING THE REMOVAL OF SOLVENTS

10.1 INTRODUCTION

Generally, products (butanol) inhibition limits the extent of lactose utilization in batch fermentations (Chapter 5) and continuous fermentations, using immobilized cells (Chapter 6) or external cell recycle (Chapter 7), of whey permeate medium containing lactose (50-70 g/l). Incomplete lactose utilization results in a possible loss of valuable fermentation medium, and increased costs. In Chapter 9, it was demonstrated that in-situ (batch fermentation) or in-line (continuous fermentation) solvents (butanol) recovery can be used to increase the extent of lactose utilization, and hence solvents productivity, compared with a control fermentation. An alternative method for increasing the extent of lactose utilization is to recycle the fermentation broth, after downstream product recovery, back to the fermentation process.

The purpose of the work described in this chapter was to investigate the effect of lactose, yeast extract, and butyric/acetic acid concentrations, and medium pH, on lactose utilization and solvents production by batch fermentation of sulphuric acid casein whey permeate, which had been previously fermented and treated by gas-stripping to remove volatile solvent products. Batch fermentation experiments were conducted at 34°C on a 100 ml scale in 120 ml bottles using an anaerobic cabinet (Section 3.4). Motile cells obtained as described in Section 3.7.2 were used as the inoculum (5% v/v) for each fermentation experiment.

10.2 RESULTS AND DISCUSSION

To prepare the medium for the experiments, a batch fermentation using sulphuric acid casein whey permeate medium (Table 3.2), reconstituted in distilled water to a concentration of 60 g/l, was conducted using the 7-litre fermentation apparatus described in Section

3.7.3.2, and operated as described in Section 3.7.3.3. The fermentation was conducted at 34°C, with no pH control or agitation being used. After 90 h fermentation, the culture contained 8.2 g/l, 2.5 g/l, 0.3 g/l, 0.8 g/l and 0.6 g/l, of butanol, acetone, ethanol, butyric acid and acetic acid respectively. The residual lactose was 20 g/l.

The solvents were removed by gas-stripping using oxygen-free nitrogen gas. The fermentation culture was heated to 60°C to increase the solvents removal rate. Distilled water was used to replace that water removed during stripping. The butanol concentration was 0.6 g/l after stripping with no detectable acetone or ethanol. The acids concentration changed little during the stripping. This medium (containing cells) was then stored at -20°C.

In the first experiment, the variables, lactose, yeast extract, butyric/acetic acid concentrations, and medium pH, were investigated, each at two levels (2^4 factorial experiment) for their effects on the batch fermentation characteristics of treated sulphuric acid casein whey permeate medium, prepared as described above. The variables, lactose concentration and medium pH, were chosen because batch fermentation experiments (Chapter 5) had shown the importance of these two parameters on solvent production. The effect of yeast extract concentration was considered important since it was possible that nitrogen and other nutrients taken up by cells in the initial fermentation may not remain at a sufficiently high concentration or in a form readily available to the cells for the second fermentation. Butyric and acetic acid concentration (the ratio of each acid was 1:1) was also investigated, since under some fermentation conditions strong acid production accompanies solvent production. This may be considered a worst case since, for example, in continuous fermentations using immobilized cells, only approximately 1 g/l total acids are produced with culture conditions favouring solvent production.

The variables and levels chosen for this experiment are given in Table 10.1 along with the coded variables used to assist in the statistical analysis of the data. These codes were used in the multivariate linear regression analysis, using the Minitab package (Copyright Pennsylvania State University) on the University main

Table 10.1 Independent variables and levels chosen in determination of various fermentation parameters from the further batch fermentation of fermentation effluent treated by gas-stripping to remove solvents (2^4 experiment)

Variable		Level	
		-1	+1
Lactose concentration (g/l)	X1	15	30
Yeast extract concentration (g/l)	X2	0	2.5
Butyric/Acetic acid concentration (g/l)	X3	0	2.0
pH adjustment	X4	0[a]	6.5[b]

a no adjustment. After autoclaving, the medium pH was in the range pH 4.5-4.9.

b 5M-NaOH used for pH adjustment. After autoclaving the medium pH was in the range pH 5.55-5.65

N.B. The concentrations given in the Table refer to the amounts added to the previously fermented and gas-stripped treated medium. For composition of the latter, see text.

computer, to develop regression equations for the experimental data. A step-wise regression analysis was undertaken. Coefficients having a t-ratio less than that indicating significance at the 5% level were omitted from the model.

The final total solvent concentration (S), final acid concentration (A), and substrate utilized (SU), were determined after 72 h fermentation. The solvent productivity (Prod) and yield (Y), based on solvents produced/lactose utilized, were calculated at this time. Experimental data and the full coded variables for this experiment are given in Appendix 4.

Table 10.2 shows the regression equations of models which fitted the data. Footnotes are used to indicate the coefficients found to be statistically significant using the t-test. The coefficient of determination (R^2) is given as an indication of the accuracy of prediction using the equations. A F-ratio test of R^2 values showed that all the regression models given in Table 10.2 are significant at the 5% level. Where no growth occurred in the fermentation medium, as often observed in the region pH 4.5-4.9 (Appendix 4), an entry of zero was used in the data set. Duplicate experiments were conducted at one set of conditions to allow calculation of a better estimate of experimental error. However, no growth was recorded under the conditions chosen for these replicates.

Generally, solvent production and lactose utilization were low, and in some instances acid production was favoured (Appendix 4). In general, added yeast extract favoured growth and production of solvents and acids. In each equation given in Table 10.2, pH adjustment (X4) was found to be significant at the 0.1% or 0.5% level and was also highly significant as present in a number of interaction terms. In only two instances was growth observed where the pH was unadjusted (Trials No.9 and 14, Appendix 4), otherwise the low pH in combination with the other variables was not conducive to growth. Acid concentration (X3) alone and in interactive terms was found to be significant in all the equations. Generally, solvent production (S), productivity (Prod) and yield (Y) were favoured when no acids were added; addition of acids favoured continued acid production. Lactose concentration (XI) alone

Table 10.2 Linear regression equations describing the relationships between medium composition and various fermentation parameters (2^4 experiment)

S =	$1.53^a + 0.27\ X1^d - 0.52\ X3^a + 1.31\ X4^a - 0.33\ X2 * X4^b - 0.29\ X3 * X4^c$ $R^2 = 0.999$
A =	$2.62^a + 0.31\ X1^a + 0.74\ X3^a + 2.10\ X4^a - 0.22\ X1 * X3^a - 0.22\ X1 * X4^a + 1.26\ X3 * X4^a + 0.31\ X1 * X3 * X4^a$ $R^2 = 0.999$
SU =	$5.54^a + 0.93\ X1^a + 0.38\ X2^d - 1.22\ X3^a + 4.47\ X4^a + 0.33\ X2 * X4^d + 0.84\ X1 * X3 * X4^a$ $R^2 = 0.990$
Prod =	$0.0213^a + 0.0038\ X2^b - 0.0073\ X3^a + 0.0182\ X4^b + 0.0044\ X2 * X4^b$ $R^2 = 0.962$
Y =	$0.166^a - 0.046\ X3^d + 0.114\ X4^a$ $R^2 = 0.759$

Statistical significance of coefficients are indicated thus:

^{a} = 0.1% level; ^{b} = 0.5% level; ^{c} = 1% level; ^{d} = 5% level.

and in interactive terms was found to be significant in equations for (S), (A) and (SU). The best result for solvent production (S) was observed in Run 15 (Appendix 4) under fermentation conditions very similar to those used in Chapter 5 (Run II).

In a further experiment, the variables, lactose, yeast extract and butyric/acetic acid concentrations, were investigated, each at two levels (2^3 factorial experiment), for their effects on the batch fermentation characteristics of treated sulphuric acid casein whey permeate, prepared as described previously. The effect of initial medium pH was not examined; all medium pH values were adjusted to be in the region pH 6.00-6.15 after autoclaving. A total of 10 runs (including two duplicates for a better estimate of error) were conducted. Coded variables and levels are given in Table 10.3. The concentrations of medium constituents were increased compared to those used in the initial experiment (Table 10.1). Experimental data for the same parameters as described previously, and the full coded variables for the experiment are given in Appendix 5. Table 10.4 shows the regression equations which fitted the data, with each equation being determined on data obtained after 72 h fermentation. Each of the coefficients of determinations (R^2) are significant at the 5% level as determined by a F-ratio test.

Growth and solvent production were observed in each run, i.e. solvent production was possible even at high acid concentration at this higher medium pH value. Lactose (X1) and yeast extract (X2) concentrations were found to be significant for equations (S) and (Prod) i.e. the higher the lactose and yeast extract concentrations the higher the solvent concentration (S). Acid concentration (X3) alone and in interactive terms, was found to be significant in equations for (A), (SU) and (Y). Increasing the initial acid concentration (X3) favoured acid production (A) but decreased the substrate utilization (SU). This can be attributed to the increased buffering capacity of medium containing acids so that elevated pH values favouring acid production resulted. The best result for solvent production (S) was observed in Run 8 (Appendix 5) at a high level of lactose and yeast extract, and low acids concentrations. Good agreement was observed in the duplicate experiments (Runs 2, 9 and 10).

Table 10.3 Independent variables and levels chosen in determination of various fermentation parameters from the further batch fermentation of fermentation effluent treated by gas-stripping to remove solvents (2^3 experiment)

Variable		Level	
		-1	+1
Lactose concentration (g/l)	X1	30	45
Yeast extract concentration (g/l)	X2	2.5	5.0
Butyric/Acetic acid concentration (g/l)	X3	2.0	4.0

NOTE:

All fermentation media prior to autoclaving was adjusted to pH 7.0 using 5M-NaOH. Medium pH after autoclaving was in the region pH 6.00 - 6.15.

The concentrations given in the Table refer to the amounts added to the previously fermented and gas-stripped treated medium. For composition of the latter, see text.

Table 10.4 Linear regression equations describing the relationships between medium composition and various fermentation parameters (2^3 experiment)

S	=	$3.45^a + 0.324\ X1^d + 0.493\ X2^d$ $R^2 = 0.661$
A	=	$8.56^a + 1.70\ X3^a + 0.184\ X2 * X3^d + 0.266\ X1 * X2 * X3^d$ $R^2 = 0.984$
SU	=	$14.2^a + 1.39\ X2^b - 0.639\ X1 * X2^d - 1.94\ X1 * X3^b$ $- 2.24\ X1 * X2 * X3^a$ $R^2 = 0.966$
Prod	=	$0.048^a + 0.0045\ X1^d + 0.0068\ X2^c$ $R^2 = 0.662$
Y	=	$0.252^a + 0.0346\ X1 * X2 * X3^d$ $R^2 = 0.421$

Statistical significance of coefficients are as indicated for Table 10.2.

10.3 CONCLUSIONS

This preliminary work has shown that batch fermentation effluent, treated by gas-stripping to selectively remove solvents, can be refermented *per se*. However solvent production is favoured when the lactose and yeast extract concentrations in the medium are adjusted to that similarly used initially, suggesting there is little or no advantage in recycling treated effluent to batch fermentation.

Growth was inhibited at low pH values but favoured at high pH values, with solvent production occurring at a high pH value, even at a high acid concentration (10 g/l). Empirical model equations were obtained from simple factorial experiments and are specific to this work.

The results on the refermentation of fermentation effluent treated by gas-stripping to remove solvents, obtained with an established cell population e.g. immobilized cells (Chapter 9), compared with those reported in this Chapter, may differ, since in this work, if the conditions did not favour growth, then no solvents production occurred. This was not the case when an established cell population was used since it was possible to obtain solvent production at unfavourable growth conditions. Additionally, in this instance, it appears that no nutrient supplement of the treated effluent is necessary. This option, of the two studied for the further utilization of fermentation substrate, appears to be the most promising.

CHAPTER 11

FINAL DISCUSSION AND CONCLUSIONS

The aim of this work was to provide information regarding the use of whey permeate (lactose) as a substrate for the ABE fermentation. This included a preliminary examination of some process technology options for fermentation intensification. Summary or conclusion sections have been reported for work described in each Chapter, and this information will not be repeated here.

The profitable use of whey solids using a range of processes including fermentation, is one aim within the overall objective of the New Zealand Dairy Board to maximize the return to the farmers for their milk. The ABE fermentation is one process option that has been considered, however, at the commencement of this study, comparatively little information was available from the literature describing its use. Feasibility studies based on the published literature were likely to give rise to erroneous conclusions.

Initially, batch fermentation experiments using an industrial strain, C. acetobutylicum P262, were conducted in order to determine the optimal culture conditions for lactose utilization and solvent production, and this appears to be the first instance of such a systematic study being reported in the literature. Whilst the batch fermentation productivities obtained were superior to those previously reported on this type of substrate, they are lower than those reported using other sugars (e.g. medium containing glucose or sucrose). This accentuated the need to examine novel intensified processes for this fermentation using whey permeate medium. Generally, two approaches are possible for ABE fermentation intensification, viz, fermentation technology and microbial/genetic modification studies. In this work, some preliminary aspects of the former approach were examined. These included continuous fermentation processes using immobilized cells and external cell recycle by cross-flow microfiltration (CFM).

Continuous stable solvent production was possible using alginate-immobilized cells of strain P262. Conversely, in studies

undertaken using external cell recycle by CFM, steady-state solvent production was not achieved for significant periods; this breakdown in solventogenesis was attributed to the complex morphological behaviour of this strain, and primarily to the sporulation that occurred during the fermentation. This suggests that future studies should not only examine dense culture fermentation kinetics, but should also attempt to obtain an asporogenic strain that would facilitate extended continuous processing. A technical comparison of these continuous processes is given in Chapter 8.

For the continuous fermentation process using CFM, the relationship between achievable filtrate flux for a given cell concentration determines the fermentation productivity. The filtrate flux determines the membrane area requirement and hence a significant proportion of the capital costs. The use of CFM in a fermentation process of this type has yet to be proven on a pilot-scale. The expected high capital costs and the fact that the plant is likely to be difficult to operate for extended periods (e.g. maintenance of asepsis) suggests that its implementation is unsure or, at best, some time away.

In both of the continuous fermentation processes described in this work, the substrate was not completely utilized, and could at least in part, be directly attributable to the toxic products (butanol) produced. The integration of in-situ or in-line product recovery processes, primarily for butanol recovery, has been shown to improve substrate utilization and consequently solvents productivity in both batch and continuous immobilized cell processes. A novel process based on the use of a gas to selectively strip volatile solvents from a whey permeate fermentation broth, with subsequent recovery using a cold trap, has been reported in this work. No attempt has been made however, to optimize the process, rather a principle has been demonstrated. Future studies need to be undertaken to examine the effects of continuous and simultaneous product recovery on fermentation kinetics, e.g., does the accumulation (partial or complete, depending on the selectivity of the recovery method) of fermentation intermediates and/or salts become toxic to the fermentation?

Although the present work has not developed an industrial process based on whey permeate, the data obtained have provided valuable

information which will be useful to future investigators and enable preliminary feasibility studies to be conducted, since these are undoubtedly case specific.

In conclusion, on-going research undertaken on a broad fundamental and technological front must be maintained if the ABE fermentation is to be recommercialized, and this is summarized in Table 11.1. Not all of the research areas presented in this Table have been examined in this work. The table shows the work described here placed in a wider context of that work which is on-going and that which remains to be done. It is unlikely that any one approach will prove to be the key, rather, a multi-disciplinary approach must be taken to intensify the process.

Table 11.1 Summary of investigative work for the intensification of the ABE fermentation

ABE Fermentation Intensification

Microbiology

(1) Isolation of intrinsically superior strains

(2) Ongoing taxonomic studies

(3) Culture maintenance and inoculum development studies

(4) Mechanism of butanol inhibition

Biochemistry

(1) Regulatory mechanisms controlling the fermentation

(2) Activators/Inhibitors of key enzymes

(3) Correlation studies relating enzymic activity to microbial physiology and culture conditions

Genetics

(1) Strain selection and mutation

(2) Development of strains more resistant to butanol

(3) Broaden the substrate range

Product Recovery

(1) Development of integrated fermentation-product recovery processes for toxic butanol removal

(2) Studies on the long-term inhibitory effects of medium components and/or fermentation intermediates when using selective solvent removal integrated processes

(3) Ongoing studies for cheaper more efficient solvent recovery methods for downstream processing

(4) Generation of suitable product recovery data to enable more accurate costings for process comparisons to be undertaken

Fermentation

(1) Evaluation and development of substrate

(2) Optimization of batch fermentation for a given substrate

(3) Scale-up of processes based on immobilized cells or cell recycle; bioreactor design, kinetics models

(4) Ongoing studies for the profitable use of fermentation by-products

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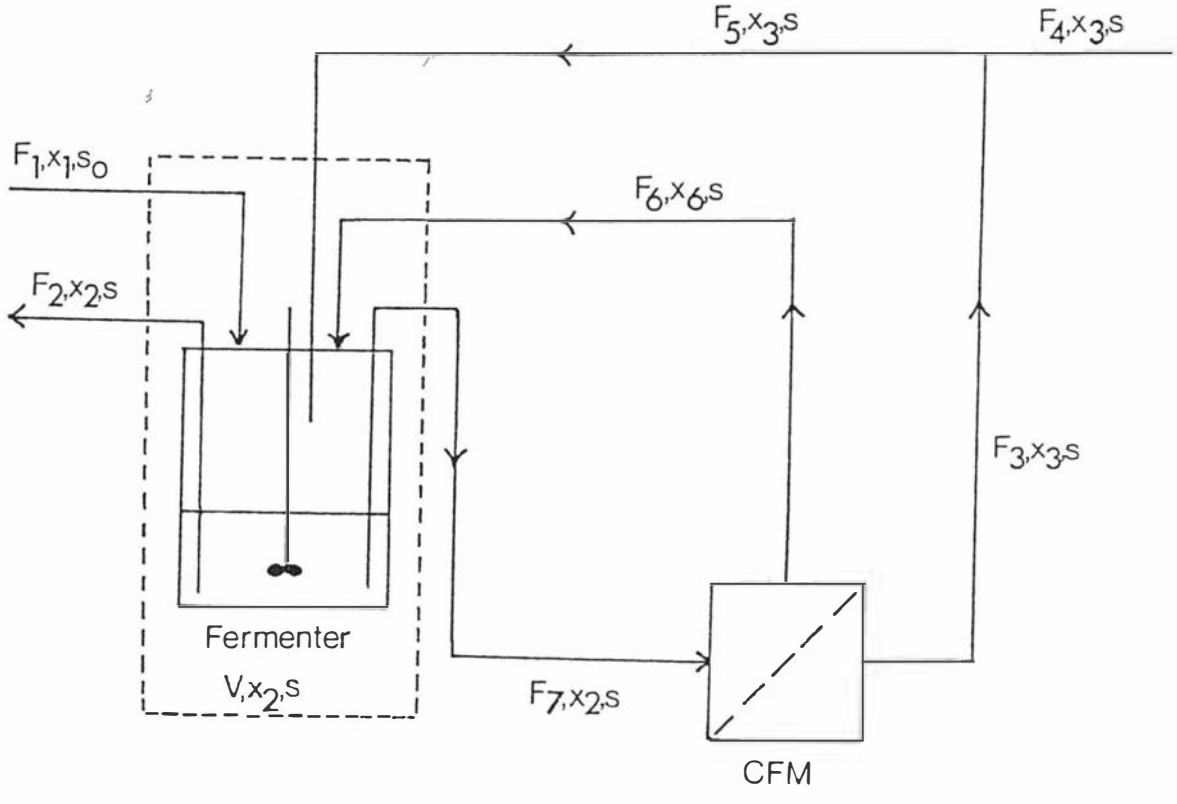
APPENDIX 1

A steady-state mass balance was conducted for the continuous fermentation with external cell recycle, using cross-flow microfiltration (CFM) (Chapter 7), in order to determine those parameters that control the process for stable solvents production.

A schematic diagram of a generalized continuous fermentation - external cell recycle apparatus is given below:



SYMBOLS

F = flowrate

x = biomass concentration

s = substrate concentration

V = total system volume (fermenter and cell recycle device)

SUBSCRIPTS

1 = feed medium

2 = biomass removal

3 = filtrate from cross-flow microfiltration (CFM)

4 = filtrate exit from system

5 = filtrate return to the fermenter

6 = concentrate return to the fermenter

7 = culture feed to CFM

I Volume Balance Equations

(a) Over fermenter

$$\frac{dV}{dt} = (F_1 + F_5 + F_6) - (F_7 - F_2) \qquad (1)$$

rate of volume change = flow IN − flow OUT

(b) Over system

$$\frac{dV}{dt} = F_1 - (F_2 - F_4) \qquad (2)$$

flow IN, flow OUT

$$F_7 = F_6 + F_3 \qquad (3)$$

(volume balance over the CFM device)

$$F_3 = F_5 + F_4 \qquad (4)$$

Substitute equation (4) into equation (3)

$$F_7 = F_6 + F_5 + F_4 \qquad (5)$$

Substitute equation (5) into equation (1)

$$\frac{dV}{dt} = F_1 + F_5 + F_6 - (F_6 + F_5 + F_4) - F_2$$

$$\frac{dV}{dt} = F_1 - F_4 - F_2 \qquad (6)$$

Assume the total system has a constant volume:

$$\frac{dV}{dt} = 0$$

$$F_1 = F_2 + F_4$$

flow IN = flow OUT (7)

II Biomass Balance Equations

Define $D = \frac{F_1}{V}$ = overall system volumetric dilution rate

therefore, from equation (7) $D = \frac{F_2 + F_4}{V}$ (8)

$$\frac{Vdx_2}{dt} = F_1x_1 + F_5x_3 + F_6x_6 - F_7x_2 - F_2x_2 + Vr_x \qquad (9)$$

rate of biomass accumulation = rate of biomass IN - rate of biomass OUT + biomass accumulation due to growth

Know that $x_1 = 0$ i.e. no biomass in the feed

Assume $x_3 = 0$ i.e. no cells in the filtrate from CFM (10)

Assume $F_7x_2 = F_6x_6$ i.e. no accumulation of cells in the CFM device (11)

Substitute (11) into (9) and using (10), equation (9) becomes

$$\frac{Vdx_2}{dt} = Vr_x - F_2x_2 \qquad (12)$$

rate of biomass accumulation = biomass accumulation due to growth - removal of biomass (via bleed)

Divide (12) by V

$$\frac{dx_2}{dt} = r_x - \frac{F_2}{V} x_2 \qquad (13)$$

where $\frac{F_2}{V} = \alpha D$, that is α = fraction of overall dilution rate, for biomass removal

At steady state biomass : $\frac{dx_2}{dt} = 0$

$$r_x = \frac{F_2}{V} x_2 \qquad (14)$$

$$\mu x_2 = \frac{F_2}{V} x_2$$

$$\boxed{\mu = \alpha D \text{ where } \frac{F_2}{V} = \alpha D} \qquad (15)$$

The biomass growth rate = the biomass removal rate at steady state.

at $\mu < \alpha D$ biomass will washout
at $\mu > \alpha D$ biomass will accumulate

μ = f([substrate], [inhibitory products], pH, T, diffusivity, shear, [nutrients])

III Substrate Balance Equations

$$\frac{Vds}{dt} = (F_1 s_0 + F_5 s + F_6 s) - (F_7 s - F_2 s) + V r_s \qquad (16)$$

substrate utilization rate = rate of substrate IN − rate of substrate OUT + rate of substrate conversion to biomass and products

Substitute (5) into (16) and divide by V

$$\frac{ds}{dt} = \frac{F_1}{V} s_o - \frac{F_2 + F_4}{V} s + r_s \qquad (17)$$

Since $F_1 = F_2 + F_4$, (17) becomes

$$\frac{ds}{dt} = D(S_o - S) + r_s$$

Assume at steady-state $\frac{ds}{dt} = 0$

$$D(s_o - s) = -r_s \tag{18}$$

Therefore $D(s_o - s) = \frac{r_x}{Y_{xs}}$ (19)

where Y_{xs} = yield coefficient, biomass on substrate

Rearranging (19)

$$Y_{sx} D(s_o - s) = r_x$$

$$= \mu x_2$$

$$Y_{sx} D(s_o - s) = \frac{F_2}{V} x_2 \tag{20}$$

Rearranging (20) gives

$$x_2 = \frac{Y_{xs} D}{\frac{F_2}{V}} (s_o - s)$$

$$x_2 = \frac{Y_{xs}}{\alpha} (s_o - s)$$

APPENDIX 2

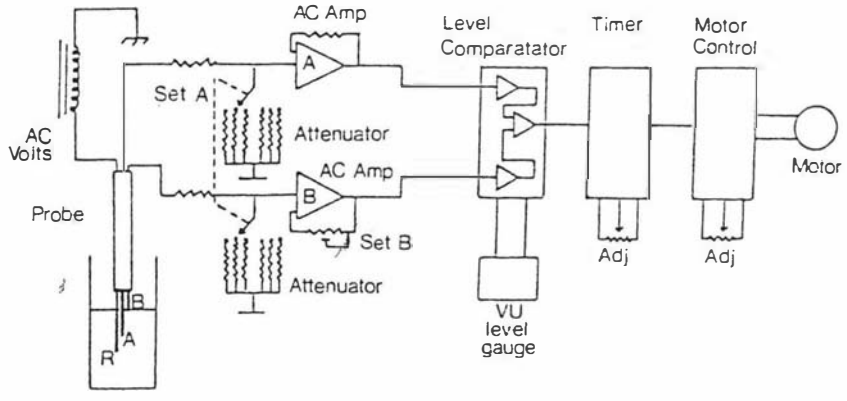
A novel level control device was used to control the culture level (volume) in experiments using cell recycle by cross-flow microfiltration (Section 3.7.5). The level control device was a conductivity probe inserted into the fermenter vessel via the fermenter head, at the commencement of cell recycle.

The probe consisted of three insulated stainless steel rods (1 mm diameter) contained within an outer stainless steel tube (7 mm diameter). The rods were of different length. The rods exposed at the end of the tube came into contact with the culture and were insulated for half their exposed length. The longest rod, fully immersed in the culture, served as a reference. AC voltage at low current was passed through the fermenter culture from this reference rod, to the other two rods designated 'A' and 'B'. Rod 'A' was shorter than the reference but was also fully immersed (insulated and uninsulated portion) in the culture. Rod 'B', the shortest rod, had only part of the uninsulated portion (end of the rod) immersed (2 mm) in the culture.

A schematic diagram of the level control device circuit is given below. The basic circuit consisted of two AC amplifiers. Amplifier 'A' had a fixed gain whilst Amplifier 'B' had an adjustable gain. Both amplifiers were resistor coupled through a six-step attenuator (dual coupled) so that the desired probe current (minimal) could be set for the given culture.

Amplifiers 'A' and 'B' were connected to opposite halves of a fixed level comparator. When amplifier 'B' was adjusted to equal or greater than that of amplifier 'A', the comparator was activated. A signal from the comparator was sent to an adjustable timer so that a small time constant was added to allow for fluctuations in the culture level due to turbulence in the fermenter vessel. A signal from the timer circuit controlled an adjustable DC motor controller. A two-mode motor controller was provided; on/off starting, or alternatively, a 'soft' start (ramp increase and decrease in speed to a desired set point).

Adjustment of amplifier 'B' to that of amplifier 'A' was made when the probe was correctly positioned in the fermenter culture and the culture level was at the required value. The level was automatically controlled even if the conductivity changed (e.g. due to microbial growth or medium/culture composition).



APPENDIX 3

The minimum theoretical stripping-gas usage rate as described in Chapter 9 was calculated as follows:

Raoult's law is given as:

$$P_B = x_B \, P_B^o$$

where P_B = vapour pressure of butanol above a solution of mole fraction x_B (kPa)

x_B = mole fraction of butanol

P_B^o = vapour pressure of butanol (kPa)

If a solution obeys Raoult's Law, the partial vapour pressure of each component is proportional to the molar fraction and is called an ideal solution. If however, the solution is non-ideal, Raoult's Law can be modified by the use of an activity coefficient (γ)

$$P_B = \gamma x_B \, P_B^o$$

Butler *et al*, (1933) demonstrated that binary solutions of water and alcohol were non-ideal and presented activity coefficients to relate partial pressures to various alcohol mole fractions, including butanol-water solutions at 25°C.

The following assumptions were made for the purpose of the calculation

(1) Equilibrium is attained between the stripping gas-bubbles and the butanol in solution

(2) Ideal gas law behaviour

(3) No effect of medium salts or other solvents on the butanol vapour pressure

(4) Operation is at atmospheric pressure

(5) The butanol concentration remains constant

(6) The stripping-gas is butanol-free

Basis of calculation: butanol concentration = 5 g/l
butanol removal rate = 0.3 g/l.h

∴ moles butanol in 1 l solution = $\frac{5}{74.12} = 0.06746$

total no. moles in solution approximately equal to
no. moles water = $\frac{1000}{18} = 55.6$

thus mole fraction butanol in butanol-water solution = $x_B = \frac{0.06746}{55.6}$

$= 1.21 \times 10^{-3}$

vapour pressure butanol at atmospheric pressure and 34°C = P_B^o

$= 1.78 \times 10^3$ Pa
(Perry & Green, 1984)

Butler et al, (1933) reported an activity coefficient value of 42.7 for a 0.01 mole fraction butanol-water solution at 25°C. This mole fraction is two orders of magnitude greater than used in this work, also the temperature is lower than required. Hence, the butanol vapour pressure activity coefficient was calculated by the method of Fieldes (1976) and is shown in the following:

The Non Random Two Liquid Equation (NRTL) was used; the "program correlates binary isobaric vapour-liquid equilibrium data from an input of experimental liquid mole fraction and bubble point measurements", Fieldes (1976). The following derived equation was presented by Fieldes (1976):

$$\ln \gamma = x_2^2 \left\{ \frac{\tau_{21} G_{21}^2}{(x_1 + x_2 G_{21})^2} + \frac{\tau_{12} G_{12}}{(x_2 + x_1 G_{12})^2} \right\}$$

where $\tau_{12} = (g_{12} - g_{22})/RT$

$\tau_{21} = (g_{21} - g_{11})/RT$

$G_{12} = \exp(-\alpha_{12} \tau_{12})$

$G_{21} = \exp(-\alpha_{12} \tau_{21})$

$\alpha_{12} = 0.5$, a preset constant

$g_{12} - g_{22} = 839.55$

$g_{21} - g_{11} = 2099.67$

} adjustable parameters based on interaction energies, data taken from Fig. 11 in Fieldes (1976) - worst case situation since alternative values are given which give a higher value of γ.

$T = 307°K$

$R = 1.9872$ cal/mole °K (universal gas constant)

x_1 = mole fraction n-butanol in water $= \frac{5}{74.12} * \frac{18}{1000}$

$= 0.00121$

$x_2 = 1.0 - x_1$

$= 0.9878$

$$\ln \gamma = 0.9878^2 \left\{ \frac{3.442 * 0.1789^2}{(0.00121 + 0.9878 * 0.1789)^2} + \frac{1.376 * 0.5025}{(0.9878 + 0.00121 * .0525)^2} \right\}$$

$\gamma = 59.5$

This calculated activity coefficient value is of the same order of magnitude as that reported by Butler et al, (1933). The higher value of γ obtained in this calculation is expected, since the mole fraction of n-butanol in water is much lower in this instance.

$P_B = \gamma x_B P_B^o$

$= 59.5 * 1.21 \times 10^{-3} * 1.78 \times 10^3$

$= 128.15$

no. moles butanol in vapour $= y_A = \frac{P_B}{P_T} = \frac{128.15}{1.013 \times 10^5} = 1.265 \times 10^{-3}$

total moles gas in 1 litre $= n = \frac{PV}{RT} = \frac{(1.013 \times 10^5)(10^{-3})}{(8.314)(307)}$

$n = 0.0397$

moles butanol/l. nitrogen $= y_A n$

$= (1.265 \times 10^{-3})(0.0397)$
$= 5.022 \times 10^{-5}$ moles/l
$= 3.72 \times 10^{-3}$ g butanol/l. nitrogen

The amount of nitrogen required to remove 0.3 g/l.h butanol is given by:

$$\frac{0.3 \text{ g/l.h}}{3.72 \times 10^{-3} \text{ g butanol/l. nitrogen}}$$

$$= 80.64 \text{ l. nitrogen/l.h}$$

$$= 1.34 \text{ l. nitrogen/l. min}$$

APPENDIX 4

Trial	X1	X2	X3	X4	S(g/l)	A(g/l)	SU(g/l)	Prod(g/l.h)	Y(g/g)
1	-1	1	1	1	2.46	6.71	8.6	0.034	0.29
2	-1	1	-1	-1	0	0	0	0	0
3	-1	-1	1	-1	0	0	0	0	0
4	-1	-1	-1	1	3.36	2.72	10.2	0.047	0.33
5	-1	-1	-1	-1	0	0	0	0	0
6	-1	1	1	-1	0	0	0	0	0
7	-1	-1	1	1	1.45	6.38	5.9	0.020	0.25
8	-1	1	-1	1	3.79	2.70	12.2	0.053	0.31
9	1	-1	-1	-1	1.11	2.15	4.1	0.015	0.27
10	1	1	1	1	2.91	7.02	10.0	0.040	0.29
11	1	-1	1	-1	0	0	0	0	0
12	1	-1	-1	1	2.86	2.60	11.0	0.040	0.26
13	1	-1	1	1	1.31	6.78	10.1	0.018	0.13
14	1	1	-1	-1	0.67	2.04	4.5	0.009	0.15
15	1	1	-1	1	4.58	2.84	12.1	0.064	0.38
16	1	1	1	-1	0	0	0	0	0

APPENDIX 5

Trial	X1	X2	X3	S(g/l)	A(g/l)	SU(g/l)	Prod(g/l.h)	Y(g/g)
1	-1	-1	1	2.70	10.5	11.3	0.038	0.24
2	-1	1	-1	3.97	6.74	13.2	0.055	0.30
3	-1	-1	-1	3.02	7.08	13.0	0.042	0.23
4	-1	1	1	3.44	10.13	20.2	0.048	0.17
5	1	-1	1	2.80	9.56	13.3	0.039	0.21
6	1	-1	-1	3.30	6.93	13.8	0.046	0.24
7	1	1	1	3.95	10.83	10.7	0.055	0.37
8	1	1	-1	5.04	6.4	19.4	0.070	0.26
9	-1	1	-1	3.36	7.20	12.0	0.047	0.28
10	-1	1	-1	3.24	6.99	11.2	0.045	0.29