UNIVERSITY OF CAPE TOWN

A REASSESSMENT OF THE PRODUCTION OF ACETONE AND BUTANOL BY *Clostridium acetobutylicum* IN CONTINUOUS CULTURE

by

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CERTIFICATION BY SUPERVISORS

During the course of this study, the following articles have been published or accepted for publication:

1) Clarke, K. G. and Hansford, G. S., "Production of acetone and butanol by Clostridium acetobutylicum in a product limited chemostat", Chem. Eng. Commun., 45, 75-81 (1986).

2) Clarke, K. G., Hansford, G. S. and Jones, D. T., "The nature and significance of oscillatory behaviour during solvent production by Clostridium acetobutylicum in continuous culture", Biotechnol. Bioeng., in press.

In terms of paragraph 9 of "General Rules for the Degree of Ph.D", we, as supervisors of the candidate, K. G. Clarke, certify that we approve of the incorporation in this thesis of material that has been published or accepted for publication.

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SUMMARY

The production of acetone and butanol by Clostridium acetobutylicum P 262 was studied in continuous culture under conditions where the nutrients were present in excess of the requirements and the cell growth was limited by the products formed during the fermentation. This system differs from most continuous culture systems used to obtain solvent production where the limitation of a specific nutrient was utilised to limit the cell growth.

To facilitate the study of product limited continuous culture, a semi-defined medium was developed. In this medium, the residual broth concentrations of carbon, nitrogen and phosphate, analysed at a dilution rate of 0,2 hr^{-1}, were 9,6 gl^{-1}, 2,4 gl^{-1} and 0,4 gl^{-1} respectively, with corresponding percentage utilisations of 55%, 7% and 33%. This suggests that these nutrients were supplied in the medium in concentrations which would not limit the cell growth during continuous culture. It was assumed that, under these conditions, the specific growth rate was limited by the product concentration(s). Continuous culture was conducted in this medium under strict anaerobic conditions at 34°C and a pH of 5,0. A maximum solvent concentration of 12 gl^{-1} was attained at the lowest dilution rate investigated, namely 0,05 hr^{-1}, and the average yield attained over the dilution rates from 0,05 hr^{-1} to 0,35 hr^{-1} inclusive, approximated 0,3. These results strongly suggest that a nutrient limitation is not a fundamental requirement for solvent induction or for the attainment of high solvent concentrations and yields.

During these product inhibited continuous cultures, oscillations in the concentrations of cells, glucose and

products were observed when the dilution rate and all other operating conditions were maintained constant. The oscillations in these parameters were monitored at a constant dilution rate of 0,10 hr^{-1} at a pH of 5,0 for 6 volume changes and related to the accompanying variations in the cell morphology. During the periods when acid production predominated, a large proportion of the cells occurred as short, granulose negative rods, whereas during the periods when solvent production predominated, the proportion of granulose positive, elongating rods increased. At a pH of 5,2, sporulating cells rather than elongating rods were observed to accompany solvent production. The shift in growth pattern from active division to elongation or sporulation, resulted in shifts in the specific rates of growth and division about the dilution rate with values of 0,102 $\pm$ 0,044 hr^{-1} and 0,099 $\pm$ 0,095 hr^{-1} respectively. High specific rates were associated with high acid concentrations and low specific rates with high solvent concentrations. Similar oscillations in all the parameters were also observed over 7 volume changes at the higher dilution rate of 0,25 hr^{-1}, although the amplitude of the oscillations was significantly decreased. A conceptual model has been proposed to explain the nature of this oscillatory behaviour. The model suggests that the oscillations are a consequence of the intrinsic nature of the fermentation to shift from a predominance of fast growing, acid producing cells to a predominance of slow growing, solvent producing cells and, therefore, are an inherent characteristic of the continuous solvent fermentation, irrespective of the factor limiting the cell growth.

The possible causes of the shift in cell growth from a high specific growth rate during acid production to a low specific growth rate during solvent production were

investigated. The growth patterns during batch culture of the solventogenic P 262 strain were compared with those of a mutant strain, which produced only acids in significant amounts. During the culture of this mutant strain, the accumulation of acid end products resulted in the inhibition of cell growth. The specific growth rate was first decreased when the acid level reached 4,3 gl^{-1} or 8,6 gl^{-1} at a pH of 5,0 or 6,0 respectively. Corresponding internal concentrations of the dissociated acid were estimated at 13-26 gl^{-1} (depending on the assumed pH gradient across the cell membrane), irrespective of the culture pH. During the parallel cultures of the P 262 strain, the initial decrease in the specific growth rate occurred at an acid concentration of 1,6 gl^{-1} or 5,1 gl^{-1} at a pH of 5,0 or 6,0 respectively. These acid levels corresponded to internal concentrations of dissociated acid of 5-10 gl^{-1} or 8-15 gl^{-1} at a pH of 5,0 or 6,0 respectively (estimated over the same range of assumed pH gradients). These results suggest that acid accumulation is not the cause of the decrease in the specific growth rate during a normal solventogenic fermentation. In addition, since solvent production, in the P 262 strain, occurred prior to the decrease in the specific growth rate at a low culture pH, the data also show that the decrease in ATP generation accompanying the shift to solvent production is not responsible for the concomitant decrease in the specific growth rate.

NOMENCLATURE

D	Dilution Rate
P	Phosphate
R	Regression coefficient
Y	Yield Coefficient
μ	Specific Rate

Subscripts:

a	accumulation
g	growth
i	inorganic
w	washout
TS/S	total solvents on substrate
TA/S	total acids on substrate

CHAPTER 1

INTRODUCTION

The production of acetone and butanol from carbohydrate by Clostridium acetobutylicum was at one time a major industrial fermentation; the original process being patented by Weizmann in 1915. However, changing economics caused the decline in the fermentative process and by 1960, synthesis from organic feedstocks became the preferred route in most countries. The uncertainty of the availability of oil and the continually escalating oil price since the early 1970's has stimulated renewed interest in the development of an alternate technology based on renewable resources. There is a strong indication that the production of solvents from carbohydrates will become increasingly important in the future (Lenz and Moreira, 1980; Zeikus, 1980; Volesky et al, 1981; Linden and Moreira, 1982; Gibbs, 1983; Marlatt and Datta, 1986). In addition to the value of acetone and butanol as solvents and chemical intermediates (Moreira, 1983; Millis, 1984), butanol also has potential as a blending agent for gasohol and diesohol (Lenz and Moreira, 1980; Arlie et al, 1981; Garcia et al, 1986; Marchal et al, 1985; Marchal et al, 1986).

Although maize and molasses were the traditional raw materials for the production of acetone and butanol by fermentation, a wide variety of other carbohydrates, including wastes, have been used (Table 1.1). The use of a waste carbohydrate as a raw material could make the fermentative process potentially more attractive. The raw material accounts for more than 50% of the manufacturing

TABLE 1.1: Some renewable resources used for the fermentative production of acetone and butanol

Substrate	Reference
waste sulphite liquor	Wiley et al, 1941; Grondal and Berger, 1945
whey	Hanson and Rogers, 1946; Maddox, 1980; Maddox et al, 1981; Welsh and Veliky, 1984; Ennis and Maddox, 1985; Voget et al, 1985b
wood hydrolysate	Sjolander et al, 1938; Leonard et al, 1947; Taha et al, 1973b; Maddox and Murray, 1983; Yu et al, 1984a; Yu et al, 1984b
straw hydrolysate	Soni et al, 1982; Marchal et al, 1984; Marchal et al, 1986
bagasse hydrolysate	Soni et al, 1982
oat hull hydrolysate	Underkofler et al, 1937
maize cob hydrolysate	Langlykke et al, 1948; Nakhmanovich and Shcheblykina, 1959; Nakhmanovich et al, 1961; Nakhmanovich et al, 1963; Taha et al, 1973b
maize husk hydrolysate	Nakhmanovich and Shcheblykina, 1960b
maize stalk hydrolysate	Nakhmanovich, 1957; Nakhmanovich and Shcheblykina, 1960a; Nakhmanovich et al, 1965
maize stalk, sunflower husk plus hemp scutch hydrolysate	Nakhmanovich and Shcheblykina, 1960c
sunflower seed hydrolysate	Nakhmanovich et al, 1965
peat	Forsberg et al, 1986

TABLE 1.1: continued

Substrate	Reference
bran	Logotkin *et al*, 1970; Qadeer *et al*, 1980
apple pomace	Voget *et al*, 1985a
Jerusalem artichokes	Thaysen and Green, 1927; Wendland *et al*, 1941; Fan *et al*, 1983; Hermann *et al*, 1985; Marchal *et al*, 1985
horse chestnuts	Gill, 1919b
Phoenix clactylifera fruit	Hongo and Nagata, 1958b
sweet potato	Tsuchiya, 1932b; Brown and Brinson, 1943; Hongo *et al*, 1959; Taha *et al*, 1973b
potato	Tsuchiya, 1933; Hongo and Nagata, 1958d
sorghum	Pomar,1967
millet	Pomar *et al*, 1965a; Pomar *et al*, 1965b; Mahmoud *et al*, 1974b
tapioca / cassava	Tsuchiya, 1932b; Banzon *et al*, 1941
Jawari flour	Fowler *et al*, 1921
Kaoliang	Tsuchiya, 1932a; Tsuchiya, 1934
wheat	Logotkin and Zaritskii, 1959
rye	Logotkin and Zaritskii, 1959
sugar cane juice	Perdomo, 1958

costs (Ross, 1961; Spivey, 1978). Consequently, the use of a waste raw material would considerably improve the overall economics of the process (Lenz and Moreira, 1980; Maddox et al, 1981; Volesky et al, 1981), although the expense of materials handling might still be considerable (Hildebrandt, 1947).

The use of plant hydrolysates as the raw material has attracted considerable interest. These hydrolysates contain as much as 40% pentoses (Fulmer, 1936). The ability of the solvent producing Clostridia to utilise pentoses almost as efficiently as hexoses, although with a more sluggish fermentation, has ensured the suitability of these hydrolysates as a raw material (Peterson et al, 1924b; Underkofler et al, 1936; Underkofler and Hunter, 1938; Langlykke et al, 1948; Compere and Griffith, 1978; Ounine et al, 1983; Volesky and Szczesny, 1983; Yu and Saddler, 1983; Ounine et al, 1985; Wayman and Yu, 1985; Fond et al, 1986a; Fond et al, 1986b). In addition, C. acetobutylicum could be used as a co-culture with yeast, in a mixed fermentation, to provide a more efficient means of utilising raw materials which contain pentoses as well as hexoses (Maddox, 1982a).

Plant hydrolysates also contain a significant percentage of cellulose and hemicellulose and consequently, the potential of the fermentative process would be further enhanced by the ability of the organism to degrade these compounds. However, the organism lacks the full enzyme complement to completely hydrolyse hemicelluloses (Lemmel, 1986). Also, although both endoglucanase and cellobiase activity have been exhibited in a few strains of C. acetobutlycum (Allcock and Woods, 1981; Lee et al, 1985) and solvent production on cellobiose comparable to that on glucose has been observed (Compere and Griffith, 1978; Mes-Hartree and Saddler, 1982), the organism has not shown the enzymatic activity necessary for the complete

degradation of crystalline cellulose. Some success with the direct conversion of cellulose has however, been achieved in co-cultures with cellulolytic Clostridia (Fond et al, 1983; Petitdemange et al, 1983; Petitdemange et al, 1984; Yu et al, 1985).

During the fermentation, a large quantity of carbon dioxide and hydrogen gas is also produced. This means that some of the raw material is diverted from solvent production to the formation of by-products. Since the cost of maize and molasses is significant, this decreases the economic viability of a process using these traditional raw materials. However, the economics could be improved by the marketing of the by-products. The fermenter off gas has been used for various processes such as methanol production (Moreira et al, 1982) or, after separation, the carbon dioxide has been used for the production of bottled gas (Spivey, 1978; Jones and Woods, 1986) or for the manufacture of dry ice (Ryden, 1958; Spivey, 1978) and the hydrogen for ammonia synthesis (Killeffer, 1927) or for the hydrogenation of vegetable oils (Hastings, 1971). Also, the stillage, which is generated by the downstream solvent recovery, has been used as a source of riboflavin, especially in animal feeds (Hildebrandt, 1947; Beesch, 1953; Ross, 1961; Hastings, 1971; Spivey, 1978). Evaporated and spray dried stillage has also been marketed as a supplementary animal feed in block form (Spivey, 1978).

In addition to the limitation imposed by the high cost of the traditional raw materials, the fermentation route also suffers from a number of other limitations which affect the economic viability of the commercial process. One such limitation is the inhibitory effect of the butanol (Ryden, 1958; Leung and Wang, 1981; Moreira et al, 1981). The butanol inhibition limits the conversion of raw material to solvents, resulting in a low solvent

concentration irrespective of the raw material supplied (Spivey, 1978). The low solvent concentration results in a high downstream processing cost for solvent recovery (Solomons, 1976; Compere et al, 1984; Marlatt and Datta, 1986).

The problem of butanol inhibition has been addressed in two ways, firstly by attempting to increase the butanol tolerance of the strain and secondly, by improving methods of removing butanol from the broth. Limited success with respect to the selection of strains with improved butanol tolerance by adaption (Jerusalimskij, 1958; Lin and Blascheck, 1983) and by mutagenesis (Hermann et al, 1985) has been achieved. Several methods of in situ removal of solvents have been reported (Ennis et al, 1986). In particular, some improvement has been achieved by the removal of solvents from the broth by liquid extraction (Bekhtereva, 1939; Mattiasson et al, 1982; Griffith et al, 1983; Ishii et al, 1985; Taya et al, 1985; Traxler et al, 1985), by adsorption on to activated carbon (Yamazaki and Hongo, 1958; Yamazaki et al, 1958a; Yamazaki et al, 1958b) and silicalite (Maddox, 1982b) and by reverse osmosis (Garcia et al, 1984; Garcia et al, 1986).

Another serious disadvantage of the fermentation route is the low solvent productivity which is, in part, attributable to the low solvent concentration. Increased productivity has been achieved through the use of continuous instead of batch operation (Leung and Wang, 1981; Soni et al, 1987). The use of cell retention techniques has the potential to increase the productivity even further. Two types of cell retention have been used for solvent production with varying degrees of success, namely cell immobilisation (Häggström, 1979; Häggström and Molin, 1980; Häggström and Enfors, 1982; Mattiasson et al, 1982; Förberg et al, 1983; Förberg and Häggström, 1985; Largier et al, 1985) and cell recycle (Garcia et al, 1984;

Afschar et al, 1985; Ferras et al, 1986; Pierrot et al, 1986; Schlote and Gottschalk, 1986).

The use of a multistage continuous culture system has proved to be advantageous over a single-stage system. Bahl et al (1982b) demonstrated that the conversion of glucose to solvents in a two-stage system was superior to that in a single-stage system. This is reminiscent of the early laboratory and pilot plant studies on the production of acetone and butanol in cascade systems (Dyr et al, 1958; Yarovenko, 1964; Hospodka, 1966). The use of the cascade systems stemmed from the opinion that the physiological states of the cells could be separated in successive fermenters such that these states would be equivalent to the consecutive growth phases in batch culture. In this manner, the growth phase could be separated from the solvent producing stage so that the inhibitory effect of the butanol could be avoided during the growth phase (Dyr et al, 1958). Although complete separation of these phases in a multistage system has not been reported, it is probable that multiple fermenter systems are still likely to reduce the inhibitory effect of the butanol by virtue of their closer approximation to plug flow than the single-stage system. It is well known that, in plug flow, the concentrations of the products vary along the length of the reactor, whereas in mixed flow, the products are present in their maximum concentrations, namely those in the outlet stream (Bailey and Ollis, 1977). Thus a fermentation which is subject to product inhibition, would suffer least from the effect of the inhibitory product during pure plug flow. Plug flow is approached as the number of fermenters in series is increased, implying that a fermenter battery would yield a higher conversion than a single fermenter of the same volume operating at the same dilution rate.

It is evident that continuous culture systems, in particular, multistage systems, have much potential for the production of solvents. Furthermore, the modification of these systems to include some means of cell retention and *in situ* solvent removal, is a promising area of development. In view of the importance of continuous culture for the production of acetone and butanol by fermentation, this study has dealt with some fundamental physiological problems associated with solvent production under these conditions.

CHAPTER 2

LITERATURE SURVEY

2.1 HISTORICAL BACKGROUND

The early industrial development and expansion of the concurrent production of acetone and butanol by fermentation has been described by several authors (Gill, 1919a; Gill, 1919b; Nathan, 1919; Speakman, 1919; Killeffer, 1927; Gabriel, 1928; Gabriel and Crawford, 1930; Wynkoop, 1943; Prescott and Dunn, 1949; Mc Cutchan and Hickey, 1954; Ross, 1961; Casida, 1964; Hastings, 1971; Hastings, 1978; Walton and Martin, 1979; Rosenberg, 1980; Jones and Woods, 1986).

The initial incentive to produce the solvents by fermentation dates back to the first decade of this century. Interest in the manufacture of synthetic rubber from butadiene was stimulated and investigation of the fermentative production of butanol, the precursor to butadiene, began.

The outbreak of World War 1 in 1914 caused a change in the emphasis from butanol production to acetone production for the manufacture of cordite and later as a solvent for the nitrocellulose dope used on aircraft. Plants in Britain and, later, Canada and the United States, were opened to meet the rapidly increasing demand for acetone. However, the fermentation produced twice as much butanol as ethanol and during this period, the butanol had very little use and was either stored in large vats or converted to methylethyl ketone as a substitute for acetone.

At the end of the war, however, the demand for acetone decreased suddenly and consequently, the plants closed down. Soon afterwards, it was found that butanol, in the form of butyl acetate, was an ideal solvent for use in the production of nitrocellulosic lacquers for the automobile industry. The plants in the United States were sold to the private sector and reopened and expanded along with the increasing demand for butanol.

In the 1920's and 1930's, fermentation remained the main route to acetone and butanol production world wide. Organisms were developed which could ferment molasses, a waste product from the sugar industry. However, even the use of a cheaper raw material did not eliminate the threat of acetone and butanol production from petrochemical feedstocks. After the second world war, the development of new technology for the synthesis of solvents from petroleum derived precursors caused the economics of the fermentative process to become uncompetitive. By 1960, this route was almost obsolete in Britain and the United States. Recently, renewed interest in the fementation route has been generated due to an increased awareness of the importance of an alternate technolgy based on renewable resources.

2.2 METABOLISM

2.21 Microorganism

2.211 Classification The original name given to the solvent producing organism was Bacillus granulobacter pectinovorum. Later, the generic name of Clostridia was used for these organisms. Mc Coy et al (1926), Weyer and Rettger (1927) and Mc Coy and Mc Clung (1935) defined the characteristics of several strains and proposed that the organism be called Clostridium acetobutylicum. However,

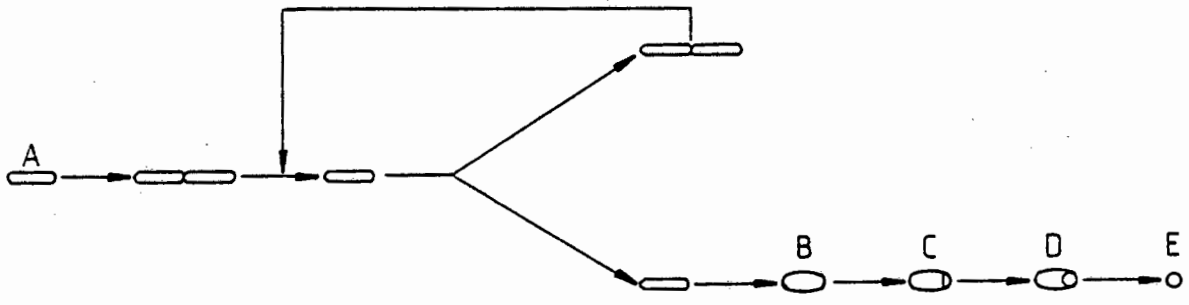
there has been a lack of a systematic approach to the classification of these organisms leading to confusion in the literature. This is particularly apparent in the patent literature in which various specific names have been used (Beesch, 1952; Mc Cutchan and Hickey, 1954; Ross, 1961).

2.212 Morphology Early investigations reported the organism as an actively motile bacillus (Gill, 1919a; Thaysen, 1921). Later however, it was observed that during the fermentation cycle of the organism in batch culture, several morphological variations existed, depending on the environment and age of the culture (Figure 2.1) (Speakman, 1926; Mc Coy et al, 1926; Weyer and Rettger, 1927; Peterson and Fred, 1932; Rubbo et al, 1941; Spivey, 1978; Jones et al, 1982). The exponentially growing cells were characterised by actively dividing, phase dark, motile rods. Later in the fermentation, granulose accumulation was observed. Subsequently, active division ceased and in many strains, the rods converted to the morphologically distinct presporulation stage consisting of phase bright clostridial forms in which forespore septa were present. Although the production of mature forespores did not normally occur in the industrial fermentation (Spivey, 1978), in the appropriate laboratory culture media, mature forespores, which in some cases led to the liberation of free spores, were observed (Long et al, 1983; Long et al, 1984a; Long et al, 1984b).

2.213 Nutritional Requirements Initially, maize mash was used as the raw material for the production of solvents by fermentation. Later however, the high cost of maize resulted in the conversion to the use of molasses as the fermentation substrate of choice.

Several other carbohydrate sources have also been used as raw materials with varying degrees of success (Table

FIGURE 2.1: Morphological development during batch fermentation of C. acetobutylicum (according to Jones et al, 1982). A: rod. B: clostridial form. C: clostridial form showing forespore septum development. D: clostridial form with mature forespore. E: free spore.



1.1). The wide variety of raw materials suitable for the fermentation is due to the ability of the organism to ferment an extensive range of both hexoses and pentoses (Robinson, 1922; Guymon, 1939; Peterson et al, 1924b; Underkofler and Hunter, 1938; Ounine et al, 1983).

The microorganisms were able to obtain all their nitrogenous requirements from the maize mash through proteolytic activity (Peterson et al, 1924a; Fulton et al, 1926; Speakman, 1926). However, molasses and other substrates have, in general, needed to be supplemented with nitrogen. Both organic (Table 2.1) and inorganic nitrogen sources have been used to satisfy the nitrogenous requirements. Some studies have suggested that an organic source was essential (Speakman, 1926; Wilson et al, 1930; Brown et al, 1939; Prescott and Dunn, 1949; Spivey, 1978; Baghlaf et al, 1980) while others have found inorganic nitrogen alone satisfactory (Langlykke et al, 1948; Nakhmanovich et al, 1961) or superior (Qadeer et al, 1980). It is likely that the nitrogenous requirements were to some extent strain dependent. However, this paradox partially arises from the fact that the organism required growth factors which are usually present in an organic nitrogen source and that the defined media which proved unsatisfactory, did not include these growth factors.

Early workers isolated and purified the growth factors. These studies led to the establishment of a requirement for l-asparagine (Tatum et al, 1934; Tatum et al, 1935), biotin (Weizmann and Rosenfeld, 1937; Mc Daniel et al, 1939; Snell and Williams, 1939), p-aminobenzoic acid (Weizmann and Rosenfeld, 1939; Peterson et al, 1940; Woolley et al, 1939; Oxford et al, 1940; Rubbo and Gillespie, 1940; Rubbo et al, 1941; Lampen and Peterson, 1941; Park and Wood, 1942; Lampen and Peterson, 1943; Reyes-Teodoro and Mickelson, 1944; Reyes-Teodoro and Mickelson, 1945) and thiamine (Doi et al, 1958).

TABLE 2.1: Organic nitrogen sources

Nitrogen Source	References
maize meal	Underkofler et al, 1937; Banzon et al, 1941; Wendland et al, 1941
corn gluten meal	Underkofler et al, 1936; Guymon, 1939; Banzon et al, 1941; Nakhmanovich and Shcheblykina, 1959; Nakhmanovich et al, 1960; Nakhmanovich and Yaravenko, 1970
soy bean meal	Tsuchiya, 1933a; Guymon, 1939; Banzon et al, 1941; Wendland et al, 1941; Hongo and Nagata, 1958a; Abou-Zeid et al, 1976; Abou-Zeid and Yassien, 1979; Baghlaf et al, 1980
cotton seed meal	Brown and Brinson, 1943; Taha et al, 1973a; Mahmoud et al, 1974a; Mahmoud et al, 1974b
corn steep liquor	Langlykke et al, 1948; Mahmoud et al, 1974a; Mahmoud et al, 1974b; Spivey, 1978; Abou-Zeid and Yassien, 1979; Baghlaf et al, 1980
recycled slop	Abou-Zeid et al, 1976
yeast or yeast extract	Nakhmanovich et al, 1965; Mahmoud et al, 1974a; Mahmoud et al, 1974b; Abou-Zeid et al, 1978; Abou-Zeid and Yassien, 1979; Baghlaf et al, 1980; Maddox, 1980
peptone	Banzon et al, 1941; Foaud et al, 1976; Abou-Zeid et al, 1978
urea	Fouad et al, 1976; Abou-Zeid et al, 1978
bran mash	Logotkin et al, 1970; Lukina et al, 1972; Mahmoud et al, 1974a; Mahmoud et al, 1974b; Abou-Zeid et al, 1976; Abou-Zeid and Yassien, 1979; Baghlaf et al, 1980
peanut cake	Mahmoud et al, 1974a; Mahmoud et al, 1974b
malt sprouts	Leonard et al, 1947
shrimp powder	Banzon et al, 1941

Most of the early studies on the evaluation of the growth factor requirements used the salt solution proposed by Speakman (1926). A recent study of salt concentrations in a glucose medium containing p-aminobenzoic acid and biotin determined the optimum ranges of salt concentrations required for maximal solvent yields (Monot et al, 1982). The concentrations of Speakman's salts were within these ranges.

From these studies on the nutrient requirements, it is apparent that a defined glucose medium comprising an inorganic nitrogen source, l-asparagine, biotin, p-aminobenzoic acid, thiamine and Speakman's salts should provide all the nutrients required by the organism.

2.22 Biochemistry

2.221 Biochemical Pathway From the earliest batch culture studies, the production of the end products of the fermentation have been demonstrated to occur in two distinct phases (Reilly and Hickenbottom, 1919; Reilly et al, 1920; Speakman, 1920a; Speakman, 1920b). During the first phase, the major end products formed from the sugar were observed to be acetate, butyrate, carbon dioxide and hydrogen. During the second phase, the acids were reassimilated concomitantly with the continued consumption of sugar and converted to acetone and butanol. An increase in carbon dioxide and a decrease in hydrogen evolution was also observed. A small amount of ethanol was usually also formed.

The early workers attempting to elucidate the mechanism leading to the production of the acids and solvents either isolated and identified possible intermediates from active cultures (Stiles et al, 1929; Speakman, 1923; Wilson et al, 1927; Pett and Wynne, 1932; Schmidt et al, 1924) or added suspected intermediates to active cultures or culture

suspensions and observed the resultant change in the fermentation pattern (Neuberg and Arinstein, 1921; Johnson et al, 1933; Blanchard and Mac Donald, 1935; Cohen-Bazire and Cohen, 1949; Cohen-Bazire and Cohen, 1950; Davies, 1942; Bernhauer et al, 1936; Bernhauer and Kürschner, 1935; Cohen and Cohen-Bazire, 1949b; Blanchard and Mac Donald, 1935; Bernhauer et al, 1936). The use of active cultures or culture suspensions, however, resulted in complications caused mainly by the simultaneous catabolism and anabolism of the other metabolites present. Consequently, the experimental findings were in most cases conflicting, and despite the considerable number of investigations, few conclusions could be drawn. The findings of Johnson et al (1933), Peterson and Johnson (1933), Simon (1943), Simon (1947a), Simon (1947b), Cohen and Cohen-Bazire (1948), Cohen and Cohen-Bazire (1949a), Rosenfeld and Simon (1950a) and Rosenfeld and Simon (1950b) did, however, lead to the identification of pyruvate as an intermediate and, therefore, established the role of glycolysis in the biochemical pathway.

The studies which eventually led to the understanding of the biochemical pathway, made use of cell-free extracts or purified enzymes. Once these could be prepared, the individual reactions could be studied in vitro. The first of these studies demonstrated the dehydrogenation and decarboxylation of pyruvate (Koepsell and Johnson, 1942; Koepsell et al, 1944) according to:

$$\text{pyruvate} + P_i = \text{acetyl-P} + CO_2 + H_2 \quad \text{(Eqn 2.1)}$$

and the formation of acetate from acetyl-phosphate (Koepsell et al, 1944) according to:

$$\text{acetyl-P} + \text{ADP} = \text{acetate} + \text{ATP} \quad \text{(Eqn 2.2)}$$

Although a large number of Clostridia have been reported to

synthesize acetate from the carbon dioxide and hydrogen released by Equation 2.1 (Wieringa, 1940; Slade et al, 1942; Ljungdahl, 1969), this has not been shown for C. acetobutylicum.

Much of the mechanism of solvent production was evaluated by analogy with the mechanism of fatty acid synthesis. The synthesis of fatty acids was proposed to occur via a multiple condensation of two carbon atom compounds when the formation of butyrate from the condensation of acetyl-phosphate and acetate was demonstrated (Stadtman and Barker, 1949a; Stadtman and Barker, 1949b; Stadtman and Barker, 1949c) according to:

$$\text{acetyl-P} + \text{acetate} + 2\ H_2 = \text{butyrate} + P_i + H_2O \quad \text{(Eqn 2.3)}$$

As it was known at this time that reduction involved the transfer of two electrons only, at least one intermediate between the condensation product of acetyl-phosphate and acetate, and the butyrate finally formed, was expected. However, none of the proposed intermediates, nor the corresponding phosphorylated compounds, could be identified (Kennedy and Barker, 1951) until the role of CoA had been established.

CoA was isolated from yeast in the form of acetyl-CoA (Lynen et al, 1951) and was shown to be obligatory for the incorporation of inorganic phosphate into acetyl-phosphate (Stadtman et al, 1951a; Stadtman et al, 1951b; Stadtman, 1952a; Stadtman, 1952b) according to:

$$\text{acetyl-CoA} + P_i = \text{acetyl-P} + \text{CoA} \quad \text{(Eqn 2.4)}$$

CoA derivatives of the substrates and substrate analogues were made available through chemical and enzymic methods and used to study the individual reactions using

enzymes purified from microbial and animal sources (Lynen et al, 1952; Lynen and Ochoa, 1953; Goldman, 1954; Stern et al, 1956; Stern and Campillo, 1956; Wakil and Mahler, 1954; Valentine and Wolfe, 1960; Twarog and Wolfe, 1962). These studies elucidated the formation of butyrate from acetyl-CoA according to the following reactions:

1) acetyl-CoA condenses to acetoacetyl-CoA according to:

$$2 \text{ acetyl-CoA} = \text{acetoacetyl-CoA} + \text{CoA} \quad \text{(Eqn 2.5)}$$

2) acetoacetyl-CoA is dehydrogenated to β-hydroxybutyryl-CoA according to:

$$\text{acetoacetyl-CoA} = \beta\text{-hydroxybutyryl-CoA} + 2\,H \quad \text{(Eqn 2.6)}$$

3) β-hydroxybutyryl-CoA is dehydrated to crotonyl-CoA according to:

$$\beta\text{-hydroxybutyryl-CoA} = \text{crotonyl-CoA} + H_2O \quad \text{(Eqn 2.7)}$$

4) crotonyl-CoA is dehydrogenated to butyryl-CoA according to:

$$\text{crotonyl-CoA} = \text{butyryl-CoA} + 2\,H \quad \text{(Eqn 2.8)}$$

5) butyrate is formed from butyryl-CoA in a similar manner to the formation of acetate from acetyl-CoA (Equations 2.4 and 2.2 respectively):

$$\text{butyryl-CoA} + P_i = \text{butyryl-P} + \text{CoA} \quad \text{(Eqn 2.9)}$$

$$\text{butyryl-P} + \text{ADP} = \text{butyrate} + \text{ATP} \quad \text{(Eqn 2.10)}$$

The acetate and butyrate thus formed (Equations 2.2 and 2.10 respectively) are reassimilated and converted to acetone and butanol. Although early studies have revealed

that butyrate was taken up and, almost quantitatively, converted to butanol (Wood *et al*, 1945), the actual mechanism of the uptake of acids has only recently been elucidated (Andersch *et al*, 1983; Hartmanis *et al*, 1984). Acid reassimilation has been suggested to occur via the transfer of CoA from acetoacetyl-CoA to either acetate or butyrate to form acetoacetate and the acid thioester according to:

acetoacetyl-CoA + acetate (or butyrate) = acetoacetate + acetyl-CoA (or butyryl-CoA) (Eqn 2.11)

The decarboxylation of acetoacetate has been shown to result in the formation of acetone (Davies, 1943) according to:

acetoacetate = acetone + CO_2 (Eqn 2.12)

and the dehydrogenation of the thioesters in the formation of the corresponding alcohols via the appropriate aldehyde (Burton and Stadtman, 1953) according to:

acetyl-CoA + 2 H = acetaldehyde + CoA (Eqn 2.13)

acetaldehyde + 2 H = ethanol (Eqn 2.14)

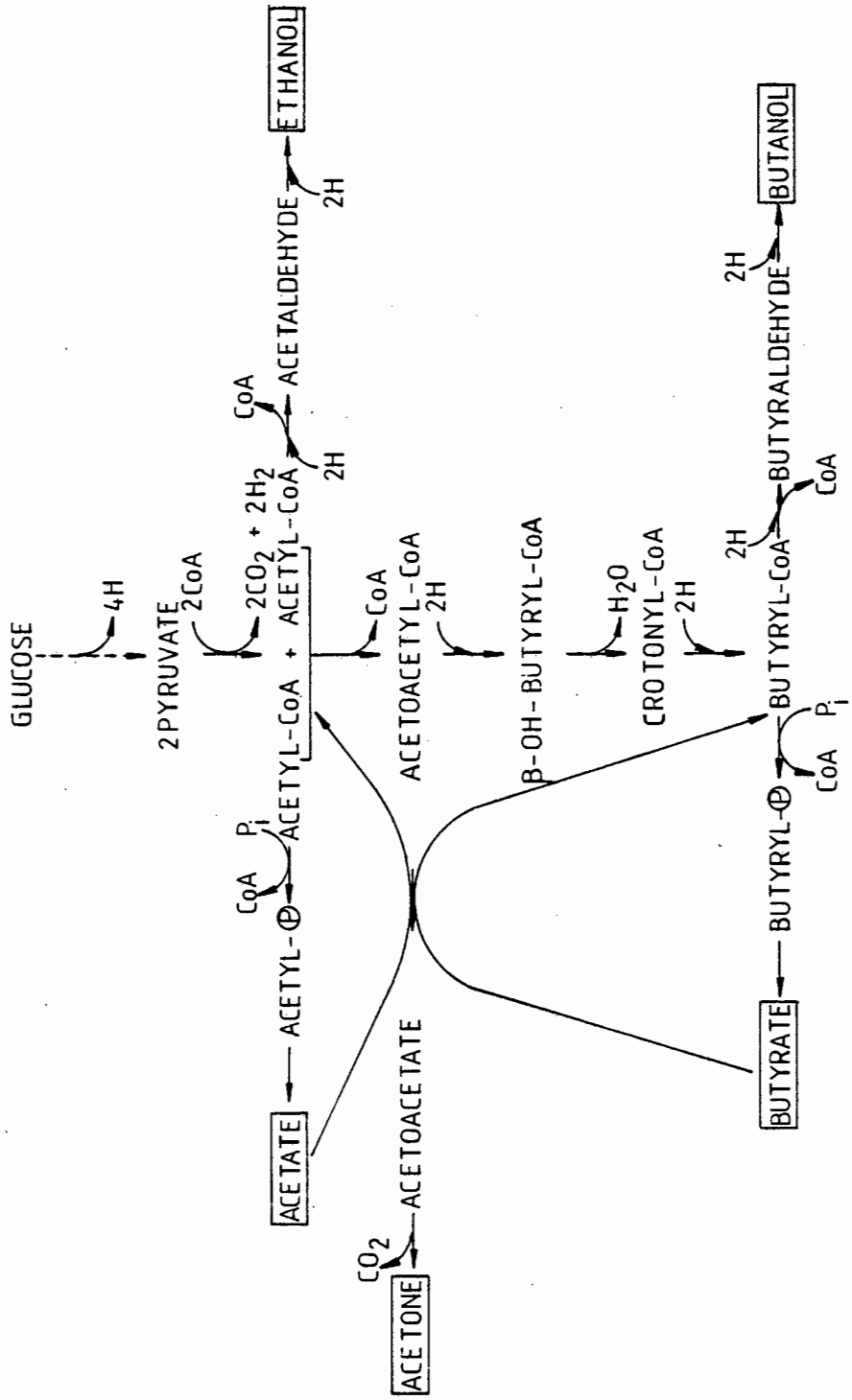
butyryl-CoA + 2 H = butyraldehyde + CoA (Eqn 2.15)

butyraldehyde + 2 H = butanol (Eqn 2.16)

The individual reactions taking place in the mechanism of solvent production have, therefore, been completely elucidated. These are summarised in Figure 2.2.

2.222 Stoichiometry and Yields The relative proportions of the acids and solvents produced will depend on the physiological state of the organism, resulting in

FIGURE 2.2: Biochemical pathway



more than one possible stoichiometric relationship. By considering only the desired end products, namely acetone and butanol, however, Reilly and Hickenbottom (1919) and Leung and Wang (1981) suggested that the following overall stoichiometric equation can be written: :

$$3 \text{ glucose} = 2 \text{ butanol} + \text{acetone} + 7\ CO_2 + 4\ H_2 + H_2O \quad \text{(Eqn 2.17)}$$

The equation predicts that the molar ratio of butanol to acetone is 2 to 1. This ratio has been found in practice (Spivey, 1978), thus supporting the validity of the equation. Freiberg (1925) suggested an alternate reaction but this does not agree as well with the experimental data.

Authors have attempted to calculate the maximum theoretical yield of solvents which would, in general, be attained at the end of a batch fermentation. Values varying from 0,38 to 0,40 have been reported (Leung and Wang, 1981; Yerushalmi et al, 1983). During industrial batch fermentations, a yield of 0,30 was usually obtained (Spivey, 1978). The actual yield obtained, therefore, is relatively close to that of the theoretical maximum reported.

2.223 Energy and Electron Balances It is evident from the studies on the biochemical pathway that the formation of acetate generates 4,0 moles of ATP per mole of glucose and the formation of butyrate, 3,0 moles of ATP per mole of glucose (Figure 2.3). Thauer et al (1977) reported that the overall energy output during acid production averages at 3,3 moles of ATP per mole of glucose.

The electron flow is mediated through the electron carriers, $NAD(P)^+$ and ferrodoxin (Doelle, 1975; Gottschalk, 1979). Although some of the NADH generated from glycolysis is re-oxidised by the transfer of the

electrons to ferrodoxin, followed by the evolution of molecular hydrogen (Jones and Woods, 1986), the excess NADH has to be regenerated by the formation of butyrate (Figure 2.3). Therefore, although the formation of acetate is the most energetically favourable pathway, some butyrate production is necessary in order to satisfy the electron balance.

The electrons released by the phosphoroclastic cleavage of pyruvate to acetyl-CoA are transferred directly to ferrodoxin. During acid production, the ferrodoxin is regenerated by hydrogenase and hydrogen gas is evolved (Figure 2.3). During solvent production, however, the electrons released by the regeneration of ferrodoxin are diverted from molecular hydrogen to pyridine nucleotides (Kim and Zeikus, 1984; Kim et al, 1984; Datta and Zeikus, 1985), resulting in an increase in the reducing power in the form of NAD(P)H (Figure 2.4). The production of butanol provides the additional electron sink required for $NAD(P)^+$ regeneration (Figure 2.4).

The transition to solvent production, therefore, provides an alternate mechanism for the elimination of the electrons released by the phosphoroclastic reaction. However, during solvent production the net energy generated decreases from 3,3 moles of ATP per mole of glucose to that generated by glycolysis alone, namely 2,0 moles of ATP per mole of glucose (Figure 2.4). The electron balance during solvent production is, therefore, satisfied at the expense of the energy generation.

2.23 Metabolic Regulation

2.231 The Metabolic Transition The metabolic transition leading to the production of acetone and butanol has been demonstrated to occur once the synthesis of the enzymes which catalyse the terminal steps in the solvent

FIGURE 2.3: Electron flow during acid production

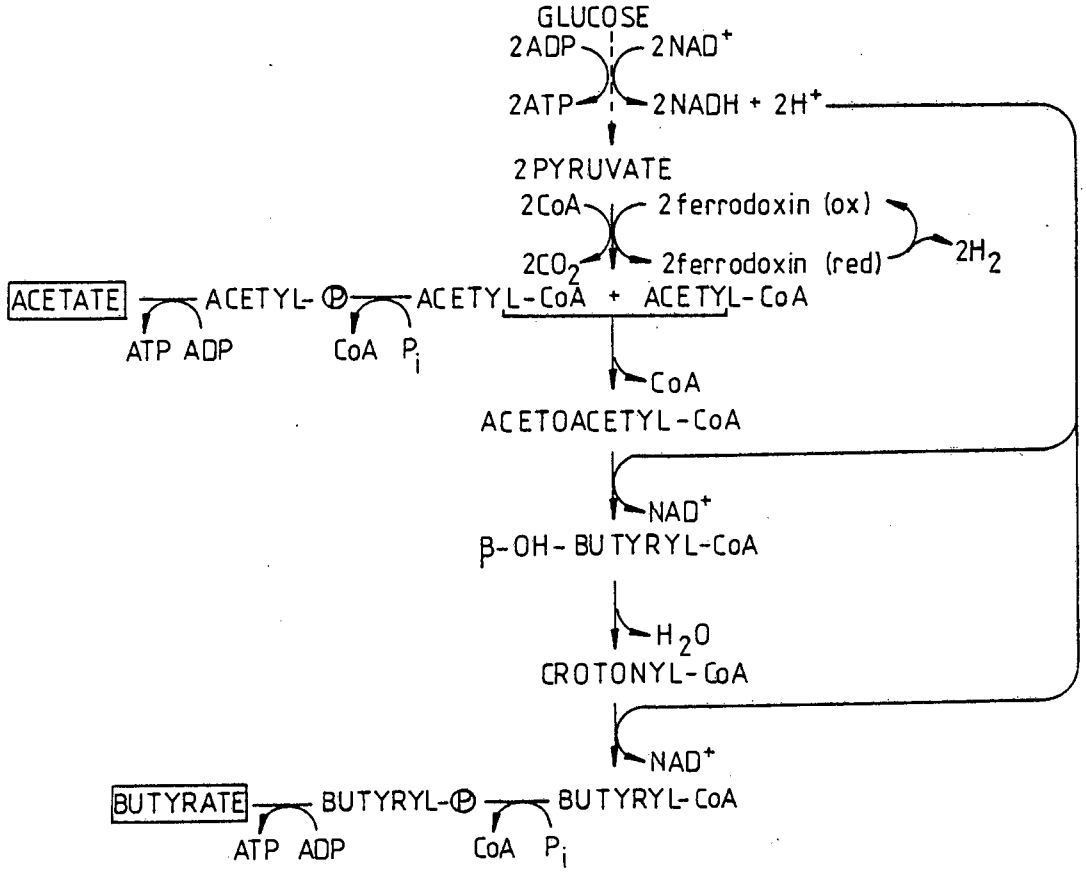
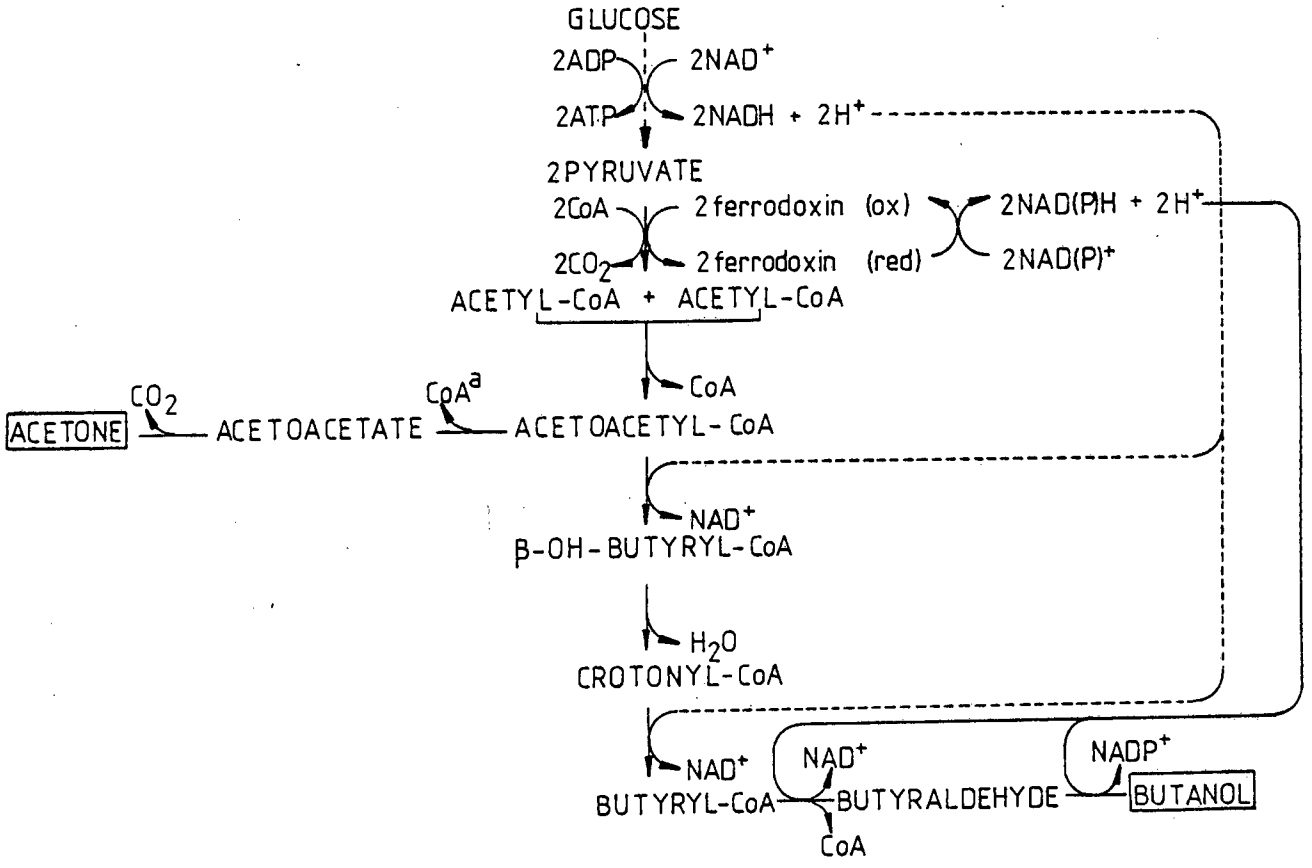


FIGURE 2.4: Electron flow during solvent production (ethanol production assumed negligable)



a: to ACETATE / BUTYRATE

producing pathways, namely acetoacetyl-CoA: acetate (butyrate) CoA-transferase, acetoacetate decarboxylase, butyraldehyde dehydrogenase and butanol dehydrogenase, is induced or accelerated (Andersch et al, 1983; George and Chen, 1983; Hartmanis et al, 1984; Ballongue et al, 1985). However, the mechanism of the regulation controlling this transition has not yet been conclusively established. In order to understand this regulation, several studies have been conducted to elucidate the role of factors which possibly influence the onset of solvent production, namely the reducing power, pH, acid concentration and specific nutrient limitations.

2.232 Factors Influencing the Metabolic Transition

The elucidation of the electron balance in the biochemical pathway has shown that the formation of butanol provides additional steps for the elimination of reducing power, suggesting that an increase in the reducing power would favour butanol formation. This has been supported by several studies. Datta and Zeikus (1985), Kim and Zeikus (1984), Kim et al (1984), Meyer et al (1985) and Meyer et al (1986) increased the reducing power in the organism by inhibiting hydrogen gas production through carbon monoxide sparging and observed enhanced solvent production. Doremus et al (1985), Welsh and Veliky (1986), Yerushalmi and Volesky (1985), Yerushalmi et al (1985) increased the reduction of the medium through an increased total pressure or decreased agitation and similarly observed enhanced solvent production. However, although an increase in the reducing power clearly favoured solvent formation, it has not been conclusively shown to result in the induction of solvent production.

The elucidation of the individual reactions in the biochemical pathway has shown that acids are reassimilated during acetone formation, suggesting that the transition to solvent production may be a response to an unfavourable

environment caused by a high level of acids and/or a low pH (George and Chen, 1983; Hartmanis et al, 1984). The requirement for a low pH value has been supported by the studies which reported that solvents were absent during batch culture at a pH of 6,0 (Nishio et al, 1983; Monot et al, 1984) while solvent production was initiated at a pH of 5,5 or less (Spivey, 1978; Gottschal and Morris, 1981a; Leung and Wang, 1981; Jones et al, 1982; Long et al, 1984b). A requirement for a low pH value has also been reported during continuous cultures operated under several different environmental conditions (Andersch et al, 1982; Bahl et al, 1982a; Bahl et al, 1982b; Bahl and Gottschalk, 1984). However, the attainment of a low pH alone was not an invariable requirement for solvent induction and depended on the culture conditions and the particular strain used. Solvents have been produced at a pH above 6,0 during batch cultures of C. butylicum (George and Chen, 1983), C. tetanomorphum (Gottwald et al, 1984) and C. acetobutylicum (Fouad et al, 1976; Holt et al, 1984).

The requirement for a high acid level is supported by the batch studies which have shown that the addition of acetate and butyrate to the fermentation broth enhanced solvent production (Nakhmanovich and Shcheblykina, 1960a; Gottschal and Morris, 1981a; Yu and Saddler, 1983; Martin et al, 1983; Long et al, 1984b; Fond et al, 1985; Häggström and Molin, 1980; George and Chen, 1983; Holt et al, 1984). Similarly, during continuous culture, an increase in the butyrate concentration, brought about directly by the addition of butyrate to the nutrient feed (Bahl et al, 1982a) or indirectly by the increase of the glucose concentration in the nutrient feed (Monot et al, 1983), has also been shown to enhance solvent production. Furthermore, Gottschal and Morris (1981a) demonstrated that while the addition of acetate and butyrate favoured solvent induction, the addition of a non-metabolisable acid had no effect, suggesting that the acetate and butyrate had a more

specific effect on solvent production than the mere lowering of the pH value.

Monot et al (1983), Monot and Engasser (1983c) and Monot et al (1984) observed that, in their studies, solvent induction occurred at a specific undissociated butyrate concentration during batch cultures, irrespective of the culture pH. They suggested, therefore, that the effect of pH and acid concentration could be related to an essential role of undissociated butyrate. Other studies, however, have been unable to confirm this finding (George and Chen, 1983; Holt et al, 1984). Furthermore, Gottwald and Gottschalk (1985) suggested that as the undissociated acid is a biochemically inert compound, it is unlikely to directly influence the induction of solvents. Recent studies have indicated that elevated intracellular acid concentrations result in solvent induction (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985; Huang et al, 1985). Gottwald and Gottschalk (1985) suggested furthermore, that elevated intracellular butyrate concentrations and as a result, elevated intracellular concentrations of butyryl-phosphate and butyryl-CoA which cause a correspondingly decreased pool of phosphate and CoA, is the basis of the triggering mechanism of solvent induction.

In addition to the role of the reducing power, pH and acid concentration in solvent production, specific nutrient limitations have also been suggested to influence solvent production. This suggestion was prompted by the observation that during continuous culture in which different nutrient limitations were imposed, solvent production was not invariably induced and maintained. (These studies have been discussed in detail in the following section.)

2.3 CONTINUOUS CULTURE STUDIES

2.31 The Role of a Nutrient Limitation

The renewed interest in the fermentative production of acetone and butanol motivated the investigation of continuous culture techniques for this purpose. Since the usual practice of operating a continuous culture is under conditions where the cell growth is restricted by a specific nutrient limitation, one or other nutrient limitation was, initially, arbitrarily imposed on the culture. The absence of solvent production in some of these studies led to the idea that the induction and maintenance of solvent production during continuous culture required an appropriate nutrient limitation and consequently, several studies were conducted to determine which specific nutrient limitations would promote solvent production. In a number of these studies, continuous culture techniques were explicitly used for this purpose because they may provide an environment in which a steady state is reached and the concentrations of the cells, nutrients and products are constant (Cooney, 1979). Such an environment would enable a rigorous evaluation of the effect of a single specific nutrient on solvent production. However, when considering all the studies of the production of acetone and butanol during nutrient limited continuous culture, it is evident that several of these studies have drawn apparently conflicting conclusions from the results obtained.

The studies which have examined solvent production under nitrogen limitation have generated the most controversy. Monot and Engasser (1983a) reported successful solvent production over a wide range of dilution rates under nitrogen limitation. At the lowest dilution rate investigated, namely 0,038 hr^{-1}, a maximum solvent concentration and yield of 8 gl^{-1} and 0,31, respectively,

were attained. These authors concluded from their results that nitrogen limitation may promote solvent production. Monot and Engasser (1983c) suggested, furthermore, that excess nitrogen inhibits the production of solvents. Similarly, Roos et al (1985) reported that solvents were produced under nitrogen limitation at a dilution rate of 0,16 hr^{-1} and concluded from their results that nitrogen availability controls solvent production.

Contrary to these studies, Gottschal and Morris (1981b) reported an absence of solvent production under nitrogen limitation at a dilution rate of 0,077 hr^{-1} and Andersch et al (1982) reported negligible solvent production under nitrogen limitation at dilution rates from 0,066 hr^{-1} to 0,217 hr^{-1} inclusive. The results of these studies may be interpreted to mean that nitrogen limitation is not conducive to solvent production.

The paradoxical results obtained from the continuous culture studies under nitrogen limitation may be explained by the difference in the pH and feed glucose concentrations used. In the studies in which no solvents were produced, the pH was maintained at 5,7 or 6,0, whereas in those studies in which solvents were produced, the pH was maintained at 4,5 or 5,0. Andersch et al (1982), Bahl et al (1982a), Bahl et al (1982b) and Bahl and Gottschalk (1984) demonstrated that a low pH was beneficial for solvent production during continuous culture. This suggests that the absence of solvent production observed under nitrogen limitation may be attributable to the relatively high pH values used. In addition, in one of the studies reporting an absence of solvent production, a feed glucose concentration of 2,7 gl^{-1} was used (Gottschal and Morris, 1981b). This was low in comparison with those of 18,0 gl^{-1} and 45,5 gl^{-1} used in the studies which reported solvent production. The findings of Monot et al (1983) suggested that a feed glucose concentration of 2,7

gl^{-1} would be too low to yield an inducing level of butyrate. This suggests that the absence of solvent production could, in addition to the high pH, be attributable to the low feed glucose concentration.

It is apparent, therefore, that provided that the pH was maintained at a low value and sufficient glucose was supplied in the feed, solvents could be produced under nitrogen limiting conditions. This is corroborated by the study of Stephens et al (1985). Basing their medium on that used by Gottschal and Morris (1981b), who reported an absence of solvent production with a pH of 5,7 and a feed glucose concentration of 2,7 gl^{-1}, they showed that with a decrease in the pH to 5,5 and an increase in the feed glucose concentration to 30 gl^{-1}, transient solvent production was obtained under nitrogen limiting conditions at a dilution rate of 0,06 hr^{-1}.

Low solvent concentrations and yields have, however, been reported under nitrogen limiting conditions even though both the pH and feed glucose concentration used, namely 5,2 and 54,0 gl^{-1} respectively, have previously been suggested to be suitable for solvent production (Andersch et al, 1982). However, solvent production was only investigated at a dilution rate of 0,217 hr^{-1} in this study and, as it is evident from the literature that high solvent concentrations were attained only at the lower dilution rates, the low solvent concentrations reported in this study are a likely consequence of the relatively high dilution rate used and, therefore, do not invalidate the suggestion that provided the pH or the feed glucose concentration do not preclude solvent production, solvents will be produced under nitrogen limiting conditions.

In the studies in which solvent production was examined under carbon limiting conditions, similar apparently conflicting results have been reported. Leung and Wang

(1981) reported that solvents were produced in significant amounts when the residual glucose concentration was negligible at low dilution rates. At the lowest dilution rate investigated, namely 0,10 hr^{-1}, a maximum solvent concentration and yield of 16 gl^{-1} and 0,32, respectively, were attained. These authors hypothesised that solvent production is subject to catabolite regulation and that the high concentration of solvents at the low dilution rates was caused by the derepression and activation of enzyme activities due to the slow flow of carbon through the metabolism.

Gottschal and Morris (1981b), on the contrary, reported an absence of solvent production under carbon limiting conditions at the dilution rates of 0,045 hr^{-1} and 0,079 hr^{-1}. Negligible solvent production was also reported by Bahl *et al* (1982a) under carbon limitation at a dilution rate of 0,133 hr^{-1}. Similarly, Roos *et al* (1985) found that acids were produced almost exclusively under carbon limitation at a dilution rate of 0,18 hr^{-1}. Roos *et al* (1985) suggested, furthermore, that solvents were not produced under carbon limitation because solvents would not be produced if the culture was limited by ATP.

The conflicting opinions in the literature, with respect to solvent production under carbon limitation, may once again be explained in terms of the pH value and/or the feed glucose concentration. In the study in which solvents were produced, the culture was maintained at a pH of 5,0 and a feed glucose concentration of 50 gl^{-1} was used. When considering the studies in which solvent production was absent it is evident that in the first study (Gottschal and Morris, 1981b) a pH of 5,7 or 6,5 and a glucose feed concentration of 2,7 gl^{-1} was used, suggesting that the absence of solvent production was due to these operating conditions. Using the basic medium from this study, Stephens *et al* (1985) decreased the pH to 5,5 and increased

the feed glucose concentration to 25 gl^{-1} and observed transient solvent production at a dilution rate of 0,062 hr^{-1}, thus supporting this suggestion. Although the pH was low in the other two studies which reported an absence of solvents (Bahl *et al*, 1982a; Roos *et al*, 1985), namely 4,3 and 4,5 respectively, low feed glucose concentrations of 3,4 gl^{-1} and 9,0 gl^{-1} respectively, were used, suggesting that in these studies, the absence of solvent production may have been due to the inability of the glucose concentration to yield inducing butyrate levels. This is supported by the finding that if butyrate was added to the feed containing 3,4 gl^{-1} glucose, a good solvent yield was attained (Bahl *et al*, 1982a).

There is also some uncertainty as to whether solvents were produced under magnesium limitation. Bahl and Gottschalk (1984) demonstrated that solvents were not produced under magnesium limitation at a pH of 4,3 and a dilution rate of 0,059 hr^{-1} when using a glucose feed concentration of 20 gl^{-1}. According to the data from the studies using other nutrient limitations, the pH and glucose feed concentrations used in this study should not have prevented solvent production, suggesting that magnesium limitation is not conducive to solvent production. However, Stephens *et al* (1985) reported solvent production under magnesium limitation using the same glucose feed concentration of 20 gl^{-1}, albeit transient. The reason for this discrepancy is not immediately clear from the results of these studies.

The studies of solvent production under phosphate and sulphate limitation have all shown that solvents were produced under these conditions. Bahl *et al* (1982b) observed solvent production at a pH of 4,3 and a feed glucose concentration of 20 gl^{-1} under phosphate limitation at several dilution rates. At the lowest dilution rate investigated, namely 0,025 hr^{-1}, a maximum

solvent concentration and yield of 14 gl^{-1} and 0,34 respectively, were attained. Gottwald and Gottschalk (1985) suggested that phosphate limitation enhances solvent production because it contributes to a decreased intracellular phosphate pool and decreased intracellular phosphate levels may be partially responsible for the triggering mechanism which leads to solvent production.

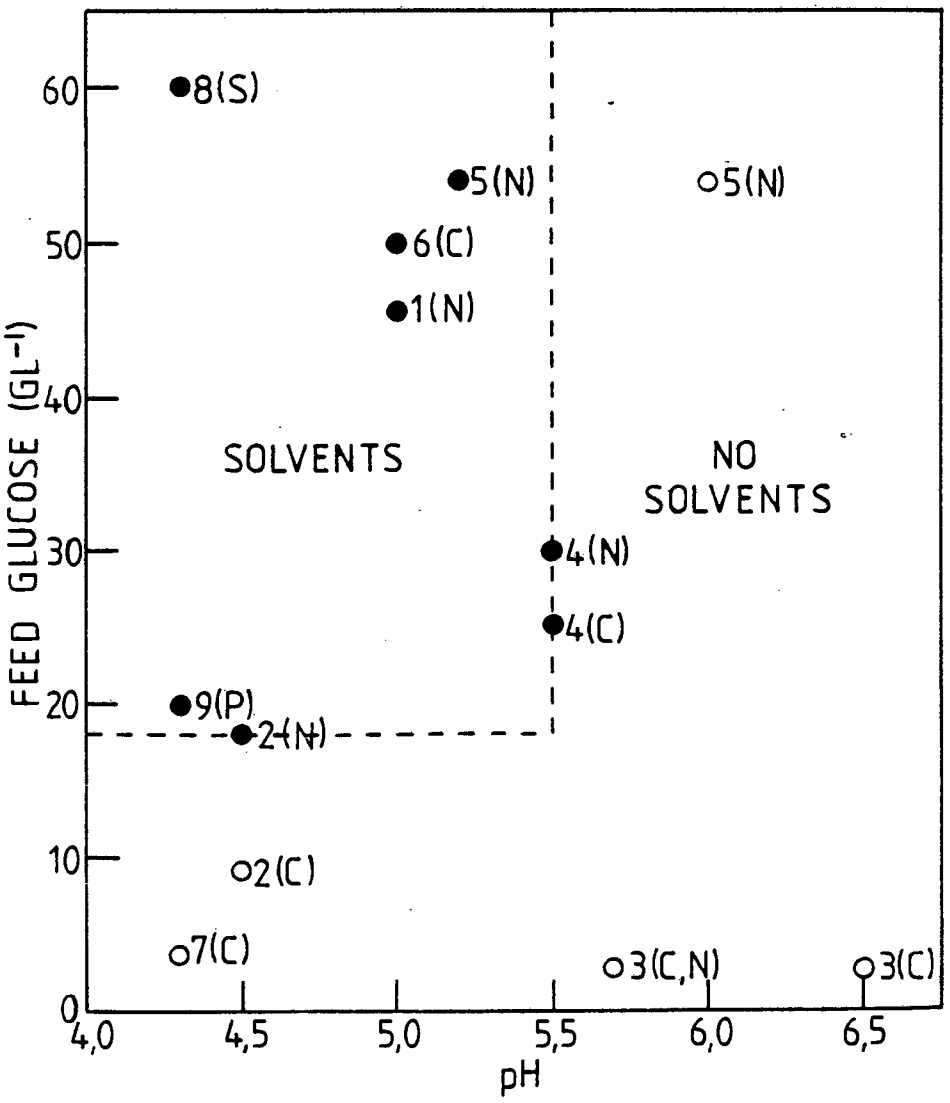
Similarly, good solvent production was observed under sulphate limitation at a pH of 4,3 and a feed glucose concentration of 60 gl^{-1} (Bahl and Gottschalk, 1984). At the lowest dilution rate investigated of approximately 0,03 hr^{-1}, a maximum solvent concentration and yield of 8 gl^{-1} and 0,27 respectively was estimated from the graphical results presented. These authors proposed that sulphate limitation is conducive to solvent production because it influences the evolution of hydrogen such that excess reducing power is formed. Solvent formation is known to provide an additional means of eliminating the reducing power in the cell (Kim and Zeikus, 1984; Kim et al, 1984; Datta and Zeikus, 1985).

A careful consideration of the results from the studies of solvent production under several specific nutrient limitations, indicates that solvents have been produced under nitrogen, carbon, phosphate and sulphate limitation when the culture pH was maintained at or below 5,5 and the feed glucose concentration was at least 18 gl^{-1}, irrespective of the nutrient limitation imposed (Figure 2.5). Several of these studies have interpreted this to mean that more than one nutrient limitation promotes solvent production. However, while it is valid to conclude from these studies that solvents could be produced under any of these specific limitations, it has not been conclusively shown whether a limitation of any of these nutrients plays a key role in solvent production. Consequently, an alternate possibility exists, namely that

FIGURE 2.5: Feed glucose concentrations and pH values of the nutrient limited continuous cultures (relevant nutrient limitation in brackets) according to:

1)Monot and Engasser (1983a)
2)Roos et al (1985)
3)Gottschal and Morris (1981b)
4)Stephens et al (1985)
5)Andersch et al (1982)
6)Leung and Wang (1981)
7)Bahl et al (1982a)
8)Bahl and Gottschalk (1984)
9)Bahl et al (1982b)

Key: ● = solvents produced
○ = no solvents produced



solvent production is independent of a requirement for any nutrient limitation and is dependent solely on some other factor(s) common to all the successful fermentations. Such a factor could be a high internal butyrate concentration.

2.32 Product Limited Operation

Although nutrient limited continuous culture for the production of solvents has received considerable attention in the literature, other fermentation systems can be used where the nutrients are present in excess and the cell growth is limited by some other factor. In one such system, the cell growth is restricted by the concentration of an inhibitory metabolic product (Levenspiel, 1980; Hoppe and Hansford, 1982). This is known as product inhibited (or limited) continuous culture.

One of the more well known fermentations which has been conducted under product inhibition in continuous culture is the ethanol fermentation (Bazua and Wilke, 1977; Ghose and Tyagi, 1979; Hoppe and Hansford, 1982). However, any fermentation in which the cell growth rate is subject to inhibition by a metabolic product, could be carried out under conditions of product limitation provided that the essential nutrients were present in excess and the culture was not inhibited by any other operating conditions, for example, the presence of oxygen during culture of an obligate anaerobe. The cell growth rate in the acetone-butanol fermentation is subject to product inhibition by acetate (Costa and Moreira, 1983), butyrate (Costa and Moreira, 1983; Monot et al, 1983; Monot et al, 1984) and by butanol (Leung and Wang, 1981; Moreira et al, 1981; Van der Westhuizen et al, 1982; Costa and Moreira, 1983; Monot et al, 1983; Vollherbst-Schneck et al, 1984; Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985; Ounine et al, 1985). This suggests that the cell growth during continuous culture in the absence of a nutrient

limitation, would be limited by the inhibitory metabolic product(s).

It is important to note that the advantage which may be gained for physiological studies from steady state operation in which the concentration of cells, nutrients and products are constant, would apply equally to nutrient limited and product limited continuous culture. Classical continuous culture theory is based on the assumption that the cell growth is a function of the concentration of a single limiting nutrient alone (Herbert et al, 1956). The concentration of this nutrient is adjusted by the system until the specific growth rate of the organism equals the imposed dilution rate, at which stage the rate of cell growth equals the rate of cell washout, there is no tendency to change and the system remains in a steady state. This theory has been extended to take account of the fermentation systems in which the cell growth is a function of the concentration of a metabolic product (Levenspiel, 1980; Hoppe and Hansford, 1982). In this system, the concentration of the inhibitory product changes until the specific growth rate of the organism equals the imposed dilution rate. At this stage there is again no tendency to change and the system remains in a steady state.

2.33 Reported Difficulties in Attaining Steady States in the Acetone-Butanol Fermentation

The continuous culture studies on solvent production have, in general, used the traditional steady state concepts in the analysis of the continuous culture data. However, it is known that all continuous cultures do not necessarily adjust to a steady state. Both damped and continuing oscillations have been observed during the continuous culture of several different organisms under constant operating conditions (Harrison and Topiwala, 1974).

In particular, unsteady behaviour has been reported during continuous production of acetone and butanol. Bahl et al (1982b), who investigated continuous solvent production by C. acetobutylicum DSM 1731 under phosphate limitation, reported that steady conditions could not be achieved at dilution rates less than 0,025 hr^{-1}. When examining solvent production by C. acetobutylicum ATCC 824 under glucose limitation at a dilution rate of 0,18 hr^{-1} and under nitrogen limitation at a dilution rate of 0,16 hr^{-1}, Roos et al (1985) found that steady state could not be achieved. Stephens et al (1985), investigating continuous solvent production by C. acetobutylicum NCIB 8025 under conditions of glucose, nitrogen, phosphate and magnesium limitation and in a pH-auxostat at dilution rates ranging from 0,061 hr^{-1} to 0,24 hr^{-1}, observed cyclic variations in the product concentrations. Monot and Engasser (1983a), who investigated the effect of nitrogen limitation during continuous solvent production by C. acetobutylicum ATCC 824, did not report unsteady behaviour. However, such behaviour may be inferred from the qualification of their steady states achieved over the dilution rates from 0,036 hr^{-1} to 0,2 hr^{-1} as "approximate". Fick et al (1985) also reported steady state operation at dilution rates ranging from 0,05 hr^{-1} to 0,1 hr^{-1} during continuous solvent production by C. acetobutylicum ATCC 824. (The limiting conditions were not explicitly stated.) However, some variation is evident in their data and it is not clear that steady states were achieved. From these studies reported in the literature, it may be concluded that unsteady behaviour during continuous solvent production has been experienced with several different bacterial strains and limiting conditions over a range of dilution rates. It is also evident that the unsteady behaviour was manifest as cyclic changes in the product concentrations.

In some studies of continuous solvent production, a physiological deterioration of the culture was observed (Gottschal and Morris, 1981b; Stephens et al, 1985). This deterioration was similar to that observed after repeated subculturing (Prescott and Dunn, 1949; Kutzenok and Aschner, 1952; Finn and Nowrey, 1958; Gapes et al, 1983; Hartmanis et al, 1986). However, it is unlikely that the inability to achieve a steady state was a consequence of a physiological deterioration as Stephens et al (1985) observed oscillatory behaviour during continuous solvent production in both the absence and the presence of a physiological deterioration of the culture.

2.4 THESIS OBJECTIVES

The initial objective was to investigate the hypothesis that solvent induction and the attainment of high solvent concentrations and yields during continuous culture is independent of a requirement for any nutrient limitation. To meet this objective, solvent production was evaluated during continuous culture over a wide range of dilution rates in the absence of a nutrient limitation, that is, under product limiting conditions. These experiments have been described in Chapter 4.

During the course of these experiments, oscillations in the concentrations of cells, glucose and products were observed, similar to the cyclic behaviour reported in the literature. In view of the importance of maintaining constant fermentation parameters during continuous culture, a further objective of this study was to investigate the nature and the significance of this oscillatory behaviour. These experiments have been detailed in Chapter 5.

As the work progressed, it became apparent that the relationship between the specific growth rate of the

organism and the induction of solvent production was an important characteristic of the oscillatory behaviour. The final objective of this study was, therefore, to investigate the association between the specific growth rate and solvent induction. This has been discussed in Chapter 6.

CHAPTER 3

MATERIALS AND METHODS

3.1 MICROORGANISM

3.11 Bacterial strains

The *Clostridium acetobutylicum* P 262 strain used in this study is an industrial strain obtained from National Chemical Products, Germiston, South Africa. This strain differs from the type culture strain ATCC 824 in that it exhibits a distinct presporulation, phase bright, swollen clostridial form which contains large amounts of granulose and which is associated with solvent production during batch culture (Jones *et al*, 1982; Long *et al*, 1983; Long *et al*, 1984b). This strain was maintained as a spore suspension in sterile distilled water and stored at 4°C. The P 262 strain was used for all the experiments except when specified to the contrary in the text.

A mutant strain of P 262 which was unable to produce clostridial forms or to produce solvents during batch culture was supplied by the Department of Microbiology, University of Cape Town, South Africa. This mutant has been described previously as the cls^- mutant by Jones *et al* (1982) and Long *et al* (1984a). The mutant was maintained as a freeze dried culture or in beef liver medium at room temperature.

3.12 Preparation of the Inoculum

The P 262 inoculum was developed in two stages. Spore suspension (0,5 ml) was heat shocked at 70°C to 75°C in

a water bath for 2 minutes, followed by rapid cooling in ice for 1 minute. A 5 to 10 µl aliquot of the heat shocked spore suspension was pipetted into 10 ml of sterile, anaerobic complex medium (Appendix A) in a Hungate tube. The culture was incubated at 37°C until the cells were near the end of the exponential phase (about 10 hours). The culture in the Hungate tube was then transferred to 90 ml of sterile, anaerobic semi-defined medium (Appendix A) in the inoculum flask to make 100 ml total volume. The flask was incubated at 34°C until the cells were again growing in the exponential phase (about 3 hours). Preparation of the inoculum was carried out in an anaerobic glove box (Forma Scientific Inc., Marietta, Ohio) under an atmosphere of 75% (v/v) nitrogen, 20% (v/v) carbon dioxide and 5% (v/v) hydrogen.

The cls^- mutant inoculum was prepared in a similar manner except that the vegetative cells were inoculated directly into the complex medium in the Hungate tube.

3.2 CULTURE MEDIA

3.21 Media Preparation

A complex medium was used for the first stage of the inoculum and for the preparation of solid medium. The medium comprised the buffered basal growth medium of O'Brien and Morris (1971), modified by the addition of yeast extract. Cysteine hydrochloride and resazurin were also added (Appendix A).

The medium was prepared by steaming for 15 to 20 minutes to drive off the oxygen. Aliquots of 10 ml were poured into Hungate tubes and a hydrogen and carbon dioxide mixture was bubbled into the medium before the tubes were capped.

Solid medium was prepared by solidifying the complex medium (without indicator) by the addition of 1,5% (w/v) agar. The cysteine hydrochloride and sodium bicarbonate were added as stock solutions only after the medium had been autoclaved. Sterile medium was poured hot into sterile petridishes and the prepared plates were cooled in the anaerobic glove box.

A semi-defined medium was used for the second stage of the inoculum and for all batch and continuous fermenter experiments. The medium comprised glucose, salts, vitamins and yeast extract (Difco). Cysteine hydrochloride and resazurin were also added (Appendix A).

The medium for the inoculum contained 10 gl^{-1} glucose. Medium (90 ml) was poured into screw cap flasks fitted with a bottom drain for connection to the fermenter. The pH was adjusted to 6,3 with 10% (w/v) sodium bicarbonate.

The medium for use in the fermenter, during both batch and continuous operation, contained 50 gl^{-1} glucose and was made up to a final volume of 20 litres. The final pH of the prepared medium after autoclaving was 5,7. No adjustment to the pH was made. The glucose concentration was analysed after autoclaving as a small amount was invariably caramelised during the long sterilisation time.

3.22 Media Sterilisation

The complex medium in the sealed Hungate tubes and the complex medium containing agar were autoclaved at 121°C and 105 kPa for 20 minutes. No special precautions were taken to prevent medium degradation as the sterilisation time was short.

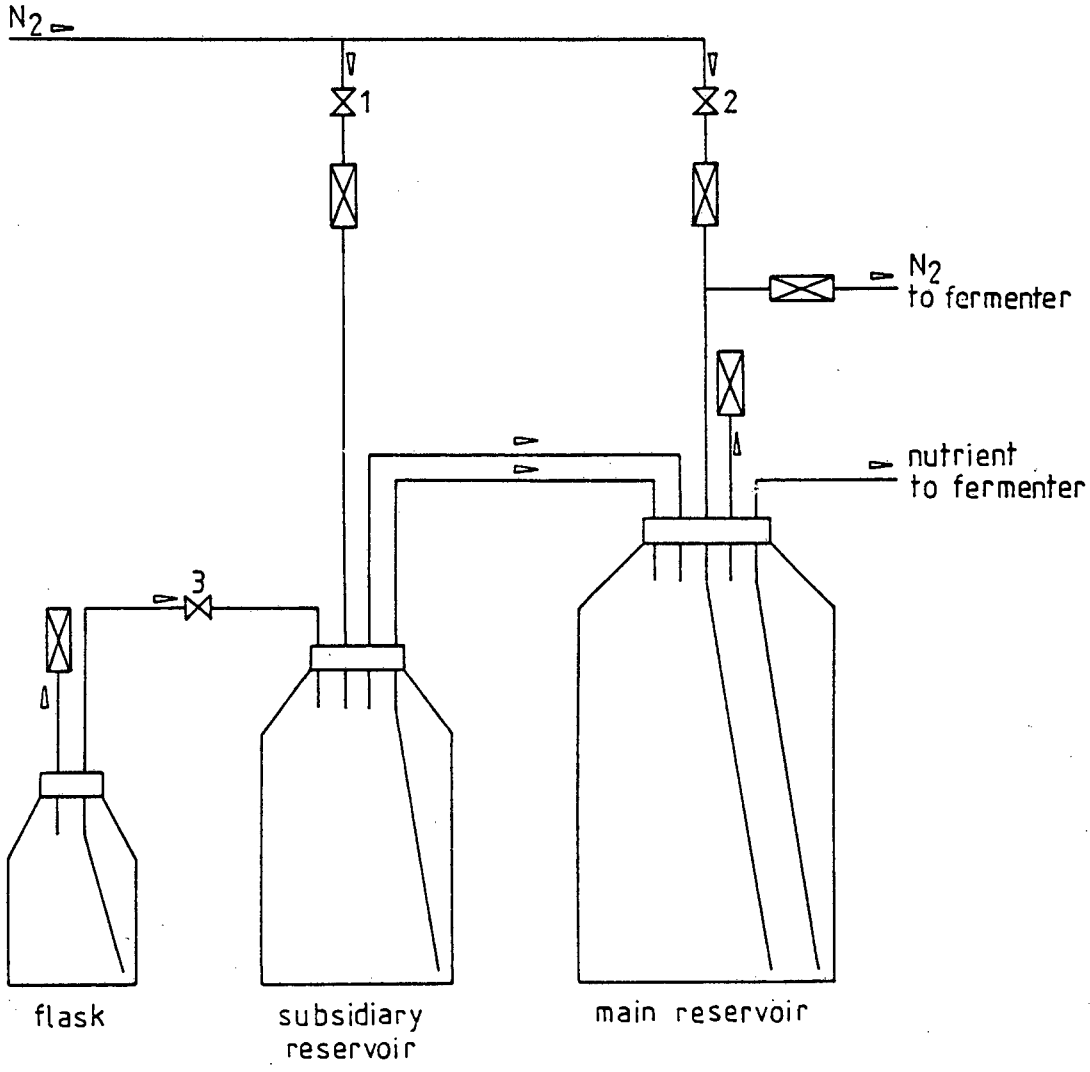
The semi-defined inoculum medium was autoclaved in the screw cap flasks at 121°C and 105 kPa for 20 minutes. The tube provided for connection to the fermenter was plugged with a deep bed filter containing non-absorbant cotton wool and covered with aluminium foil during autoclaving. Again, no special precautions were taken to prevent medium degradation. The medium was allowed to cool in the anaerobic box.

The 20 litre volumes of medium made for use in the fermenter required a sterilisation time of 1,5 hours at 121°C and 105 kPa. The long sterilisation time necessitated the following precautions to minimise medium degradation during autoclaving:

1) the carbon and nitrogen sources were separated to prevent loss due to the Maillard reaction,
2) the magnesium and phosphate sources were separated to minimise loss by reaction and precipitation and
3) the heat labile compounds were autoclaved separately in a small volume for 15 minutes at 121°C and 105 kPa and subsequently added aseptically to the larger volume of medium.

These precautions required the separation of the medium into three portions before autoclaving (Appendix A). The medium reservoirs are shown in Figure 3.1. Each reservoir contained cysteine hydrochloride and resazurin in amounts proportional to the liquid volume. The medium in the main and subsidiary reservoirs was autoclaved for 1,5 hours at 121°C and 105 kPa. All tubes which were immersed in the liquid were clamped and all tubes for subsequent connection were plugged with deep bed filters, as described previously, before autoclaving. Each reservoir was provided with a vent through a deep bed filter to allow for pressure release during autoclaving.

FIGURE 3.1: Schematic diagram of the medium preparation



The reservoirs were removed while still hot from the autoclave as soon as atmospheric pressure had been reached and connected immediately to the supply of purified nitrogen at valves 1 and 2. Valve 1 was opened and the nitrogen flow rate adjusted to 30 to 40 ml min^{-1}. The nitrogen was sterilised by passage through a 0,22 µm Millipore filter, held in a steam sterilisable Sartorius filter holder, and was first passed into the subsidiary reservoir and subsequently, into the main reservoir from where it was vented to the atmosphere. The reservoirs were allowed to cool under the nitrogen blanket to ambient temperature after which the nitrogen flow rate was reduced to 10 ml min^{-1}.

The medium containing the heat labile compounds in the flask was then autoclaved. The flask was removed from the autoclave as soon as atmospheric pressure had been reached and immediately connected to the subsidiary reservoir at valve 3. The hot, reduced medium was pumped from the flask into the cool anaerobic medium in the subsidiary reservoir. This method ensured rapid cooling of the heat labile compounds. Valve 3 was shut as soon as the flask was empty, to prevent the ingress of air.

The medium in the subsidiary reservoir was then pumped into the medium in the main reservoir under the nitrogen blanket. When the transfer of the medium from the subsidiary reservior was complete, valve 2 was opened and valve 1 closed to divert the nitrogen flow directly to the main reservoir and the vent from the main reservoir was clamped. The nitrogen inlet was extended to the bottom of this reservoir so that the flow rate of the nutrient feed during continuous culture would not vary with the liquid level in the reservoir. A bleed on the nitrogen inlet was provided for the diversion of excess nitrogen not required for the replacement of liquid volume removed from the

reservoir. The medium was mixed and was then ready for use.

All tubing was made of viton (Watson Marlow, Falmouth, Cornwall, England) to minimise diffusion of oxygen into the system. The tubing was connected to stainless steel tubes fitted into rubber bungs sealing the top of the pyrex aspirators.

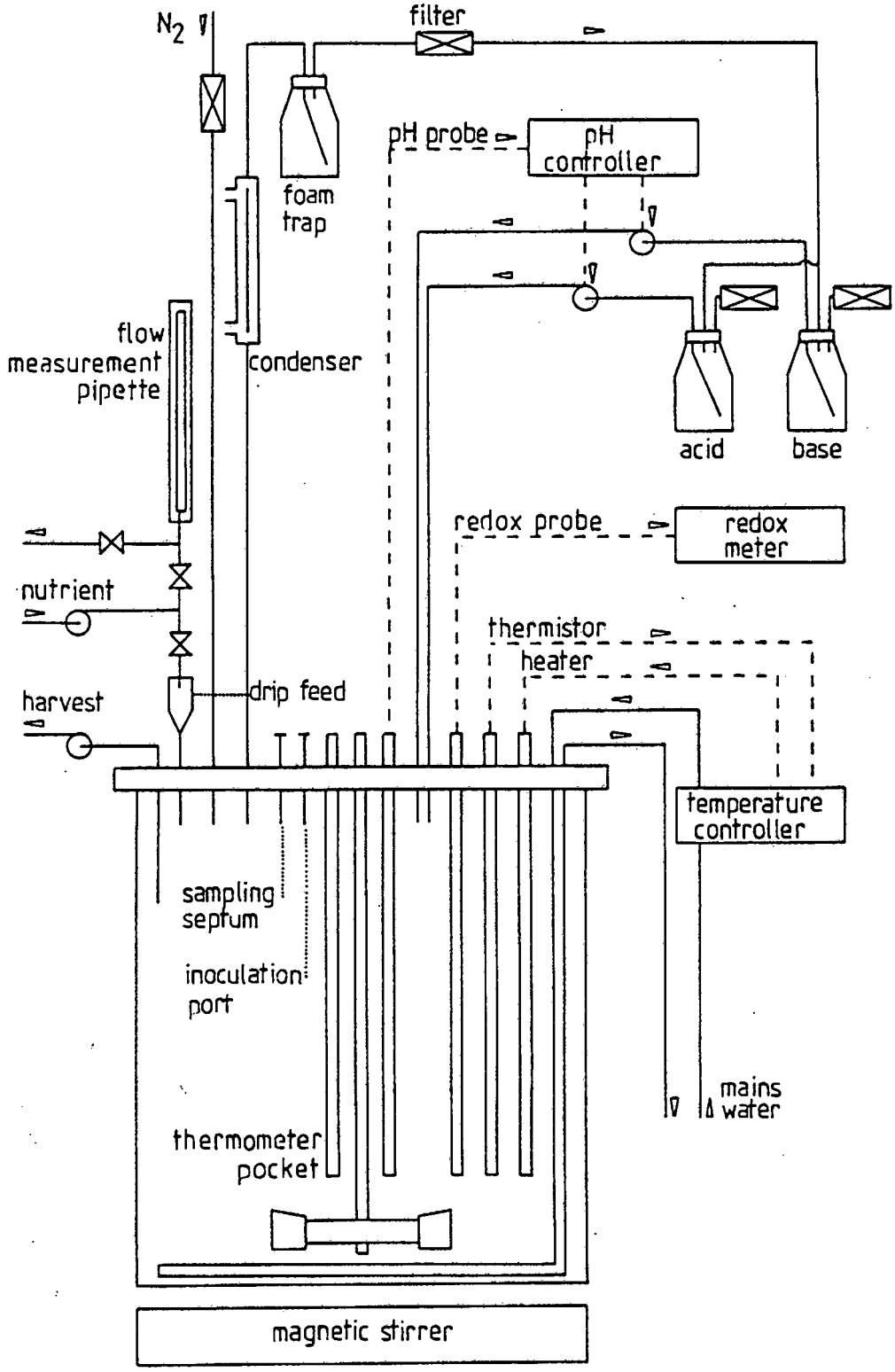
3.3 EXPERIMENTAL SYSTEM

3.31 Fermenter and Associated Equipment

Experiments were conducted in a 1 litre Gallenkamp modular fermenter (Figure 3.2). The fermenter incorporated the following modules:

1) a 1 litre glass, flanged, Quickfit culture vessel with a stainless steel multiple port head plate;
2) a temperature controller comprising a thermistor probe which actuated either a heater element or a solenoid valve which controlled the flow of cold mains water through an immersion coil; a thermometer pocket was provided so that the fermentation temperature could be checked with a thermometer;
3) a pH controller comprising a steam sterilisable, Ingold pH probe (type 425-60-K9) and a pH meter and set point controller which actuated separate peristaltic pumps delivering acid or base from their respective reservoirs;
4) a magnetic stirrer with continuous speed control acting on a four blade turbine;
5) a combined feed/harvest pump comprising two concentrically mounted peristaltic pumps with continuous speed control; the feed line assembly was arranged so that flow could be diverted to a mounted pipette in

FIGURE 3.2: Schematic diagram of the fermenter



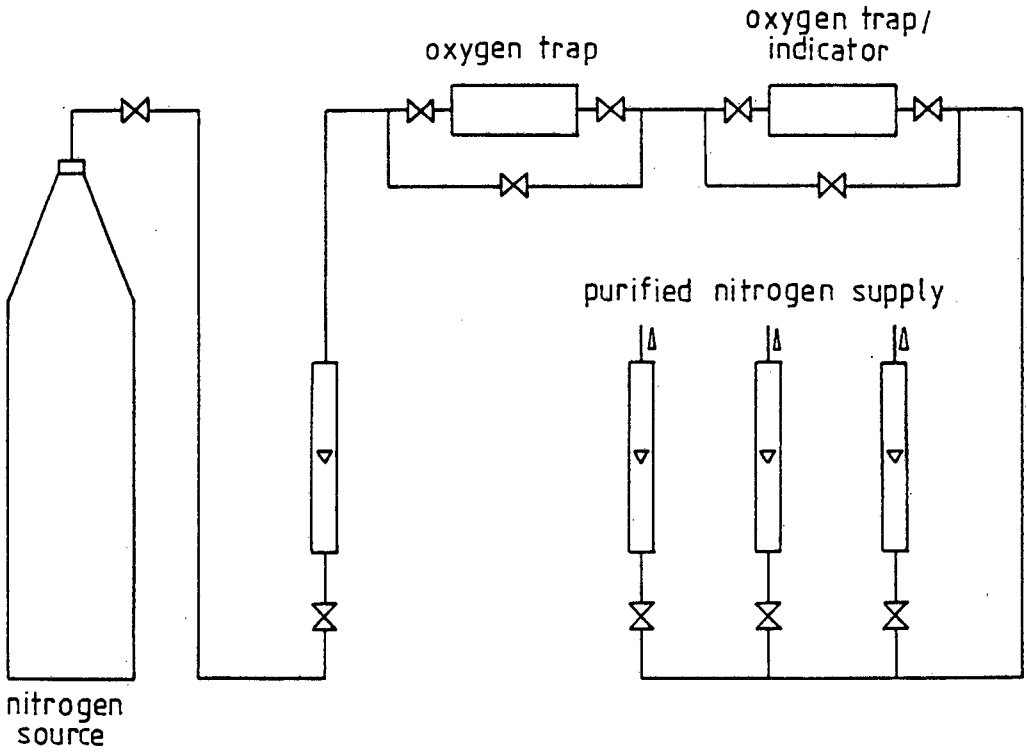
order to determine the flow rate of the nutrient feed and a drain was provided to remove residual nutrient in the pipette; a drip feed was connected at the feed inlet to prevent back growth down the feed line.

A steam sterilisable, combined, platinum, Ingold redox electrode with a silver/silver chloride reference (type Pt 4865-35-K9) connected to a Metrohm pH/volt meter (Model E520) was added to the fermenter modules in order to measure the redox potential of the broth.

Purified nitrogen was used to maintain anaerobiosis. The nitrogen was fed to the fermenter from the bleed on the nitrogen inlet connected to the feed reservoir. The nitrogen passed through a 0,22 µm Millipore filter before entering the fermenter and passed out, together with the fermenter off gas, through a condenser (to reduce the loss of water vapour and entrained solvents), a foam trap and a 0,22 µm Millipore filter to the acid and base reservoirs from where the gases were vented to the atmosphere. Thus during start up and operation, the medium in the reservoir, the broth in the fermenter and the pH control solutions were continuously under anaerobic conditions. All tubing was made of viton.

The nitrogen was purified to contain less that 0,1 ppm oxygen by passing high purity nitrogen with an oxygen content of less than 10 ppm through two oxygen traps (Alltech Associates, Deerfield, Illinois, U.S.A.) in series (Figure 3.3). The second trap contained an indicator, sensitive to the presence of oxygen, which was positioned such that oxygen could be detected before the capacity of the trap was full. As both traps were fitted with a bypass, the first trap could be removed for regeneration while the second trap remained in operation and, once the regenerated trap had been replaced, the second trap could be similarly removed. This arrangement allowed the

FIGURE 3.3: Schematic diagram of the nitrogen purification system



experiments to continue without interruption. Three outlets for the purified nitrogen were provided. The flow rate of each outlet was regulated using a rotameter, calibrated for 4 to 40 ml min^{-1} air, fitted with a needle valve (GEC-Elliot Process Instruments Ltd, Croyden, U.K.). The gas purification assembly was connected with quarter inch copper piping.

3.32 Fermenter Sterilisation

The fermenter was prepared for sterilisation as follows. Prior to insertion into the fermenter, the pH probe was calibrated with a pH 4,0 and a pH 7,0 buffer. The output of the redox probe was similarly checked in a Ingold redox buffer (No 9881) poised at 220 mV at 25°C for the silver/silver chloride reference; the redox probe was found to be accurate during all the experiments. The fermenter was completely assembled, including the calibrated pH probe and redox probe, the flow measurement pipette, the condenser, the foam trap, the acid and base reservoirs and all the necessary filters. Tubing provided for connections was plugged with deep bed filters as described previously. Distilled water was poured into the fermenter so that the porous plugs of the pH and redox probes were fully immersed. The acid and base reservoirs were filled with 189 ml and 170 ml of distilled water respectively, so that when 11 ml of concentrated sulphuric acid and 30 ml of 25% (w/v) ammonia were added to make a total volume of 200 ml after autoclaving, the required strengths of 2 N of both the acid and base would be obtained. The concentrated acid and base solutions were not autoclaved prior to addition as they were assumed to be sterile by virtue of their high concentrations. A 1 ml aliquot of an aqueous silicone emulsion (30% w/v) was added to 100 ml of distilled water in the foam trap. The complete fermenter assembly was placed in an aluminium bracket and autoclaved for 1 hour at 121°C and 105 kPa.

After autoclaving, the fermenter assembly was allowed to cool. The nitrogen bleed from the main medium reservoir was connected to the nitrogen inlet of the fermenter. The distilled water was pumped out and the fermenter flushed with nitrogen. The feed line from the main medium reservoir was connected to the feed assembly on the fermenter and 800 ml of medium was pumped in under the nitrogen blanket. A small sample was withdrawn and the pH was measured with an independent external pH meter. The pH meter in the pH control module was adjusted, if necessary, to read the same pH. Another sample was withdrawn to analyse the glucose concentration of the feed. Finally, the concentrated acid and base solutions were added to the respective reservoirs as required, the magnetic stirrer set to approximately 50 rpm and the temperature adjusted to 34°C. The fermenter was then ready for inoculation.

3.33 Fermenter Operation

The inoculum flask was connected aseptically to a port in the head plate of the fermenter and the 100 ml inoculum was pumped into the sterile, anaerobic medium in the fermenter. During operation, the pH, temperature and dilution rate (if appropriate) were continually checked. The pH values were always checked using the independent, external pH meter. A sampling septum was provided for the withdrawal of samples with sterile syringes. The septum was set in a holder so that it could be covered with 70% ethanol while not in use. Approximately 7 to 10 ml of broth was withdrawn per sample. This allowed 9 to 13 samples to be taken during batch cultures, which had a working volume of 900 ml, without depleting the volume by more than 10% over the course of the experiment. Sample size during continuous operation was not critical and could be increased. The samples were processed immediately. The contents of the syringe were gently released into a Mc Cartney bottle and cooled to ambient temperature under

running water without shaking. A portion of the sample was centrifuged at 6500 rpm for 15 minutes in a refrigerated Beckman centrifuge (model TJ6) while the pH, percentage transmission, cell counts and, in some samples, the true cell dry mass, was determined using the remaining portion. The supernatant from the centrifuged sample was used for the analysis of glucose, acids and solvents and, if applicable, nitrogen and phosphate.

Although antifoam had been added to the medium (Appendix A), it was occasionally necessary to add more antifoam to the batch culture. A sterile aqueous emulsion of silicone (10 gl^{-1}) was added with a sterile syringe through the sampling septum as required.

Continuous culture was initiated after 20 hours of batch culture had elapsed. By this time solvent production was well advanced, most (90-100%) of the glucose had been utilised and a large proportion (approximately 80%) of the cells had stopped dividing and produced forespore septa. The system was switched to continuous operation by feeding nutrient medium initially at a dilution rate of 0,10 hr^{-1} and adjusting the pH to 5,0. The feed pump was set to the required flow rate as determined with the use of a flow measurement pipette and a stop watch. The harvest tube was positioned at a liquid level such that the volume of the broth below this level was 800 ml. A constant working volume of 800 ml was maintained during operation by fitting the harvest pump with tubing of a larger bore than that of the feed pump, thus ensuring a withdrawal rate greater than that of the feed rate.

During continuous culture the pH was maintained at 5,0 unless otherwise stated in the text. The dilution rate was subsequently adjusted to obtain the desired value.

3.4 ANALYSES

3.41 Biomass and Cell Counts

3.411 Cell Dry Mass The cell dry mass was determined in the following manner. A 0,45 µm Millipore filter was dried at 80°C for 48 hours, cooled in a desiccator and weighed to an accuracy of 10^{-4} g. A sample of known volume was filtered by vacuum and washed twice with an equal volume of distilled water. The filter, plus retained solids, was dried, cooled and weighed as before. The difference in mass gave the true cell dry mass of the sample. These values are designated cell dry mass^{m} in the text to indicate the measured or true cell dry mass.

The percentage transmission of several samples from continuous cultures, in the absence of sporulation, at dilution rates from 0,05 hr^{-1} to 0,35 hr^{-1} inclusive, diluted 1:100 with distilled water to obtain a reading of greater than 70% transmission, was read relative to a blank of distilled water in a Varian spectrophotometer (model superscan 3) at 650 nm. The true cell dry mass of the samples was also determined as described previously, and the following calibration curve was evaluated:

cell dry mass = (0,14 - 0,0014. % transmission).dilution

The data fitted this curve with a regression coefficient of 0,9266. The estimated cell dry mass could, therefore, be calculated from a percentage transmission using this equation, provided that the samples were sufficiently diluted so that the percentage transmission was greater than 70%. Samples were analysed in triplicate and the results agreed to within 1% transmission.

The estimation of the cell dry mass suffers from the disadvantage that it assumes constant optical properties.

This assumption is likely to be invalid during sporulation and, therefore, inaccuracies may have occurred during batch fermentations in which sporulation was prevalent. The measurement of the true dry mass would, however, have required sample volumes greater than those which could be removed from the batch fermentation without decreasing the volume by more than 10% during the experiment. An increase in the fermenter volume would have required a corresponding increase in the volume of the nutrient feed for the continuous culture experiments and the experimental constraints made this impractical.

3.412 Total Cell Count The total cell count was determined with the use of a Thoma bacterial counting chamber (Weber Scientific International, England) and a Nikon microscope fitted with phase contrast optics.

3.413 Differential Cell Counts Differential cell counts of sporulating cells were determined. Sporulating cells all accumulate granulose to a large extent and, therefore, the presence of forespore development could be easily detected during microscopic observation by staining the cells with Gram's iodine. Wet mounts of the stained cells were made and the percentage of the total cells in which forespore septa and mature forespores were present, was determined using the Nikon microscope, fitted with bright field optics. The percentage of phase bright free spores was determined using phase contrast optics.

The differential counts of rod lengths and the presence of division septa were determined by examining stained cells or unstained cells using phase contrast optics. The percentage of short, medium and long rods was determined relative to the total number of cells. The rod length was qualitatively assessed in terms of unit lengths, where 1 unit length is approximately equal to 3 μm, the average length of the shortest rods. (The total percentage of all

the morphological forms, however, did not always add up to exactly 100% due to a small rounding off error.)

The presence of granulose accumulation in the rods was determined by staining with Gram's iodine. The percentage of rods showing the absence or presence of granulose was determined relative to the total number of rods, using bright field optics.

Several fields (5 to 10) were evaluated for each differential cell count.

3.414 Viable Cell Count The viable cell counts were determined by making serial dilutions of the sample; each dilution was spread onto solid complex medium and incubated at 37°C for 2 days. The colonies on the plates were counted and the number of colony forming units evaluated. The total cell count on the same sample was evaluated as described previously so that the percentage of colony forming units could be calculated. All plating was carried out in the anaerobic glove box.

3.42 Glucose

Glucose was measured enzymatically with a Beckman glucose analyser (model 2). A 10 µm aliquot of sample supernatant, suitably diluted with distilled water, was injected into the reaction cup of the analyser where it was mixed with 1 ml of oxygen saturated glucose oxidase. The instrument related the maximum rate of oxygen utilisation to the glucose concentration in the sample via a calibration point. A standard solution of 1,5 gl^{-1} glucose was used for calibration and the sample supernatants were diluted to contain approximately the same value before being analysed. Samples were analysed in triplicate and agreed to within 0,002 gl^{-1}.

3.43 Acids and Solvents

Acids and solvents were analysed using a Varian gas chromatograph (series 1400) fitted with a flame ionisation detector. A 200 cm x 3 mm ID glass column was packed with 100/120 mesh chromasorb W-AW coated with 10% diethylene glycol adipate. The chromatograph was connected to a Varian integrator (model CDS 401) and samples were analysed directly using the internal standard method of calibration. The internal standard was prepared in saturated benzoic acid and contained 2,5% (w/v) n-propanol and 0,8% (w/v) sulphuric acid. The sulphuric acid ensured that all the acetate and butyrate were in the acid form. A 1 ml aliquot of sample supernatant was diluted with 0,25 ml of internal standard; 1 µl of diluted supernatant was then injected into the column of the chromatograph. Samples were analysed at least twice to agree to within 0,1 gl^{-1} or to within 2% of the mean value.

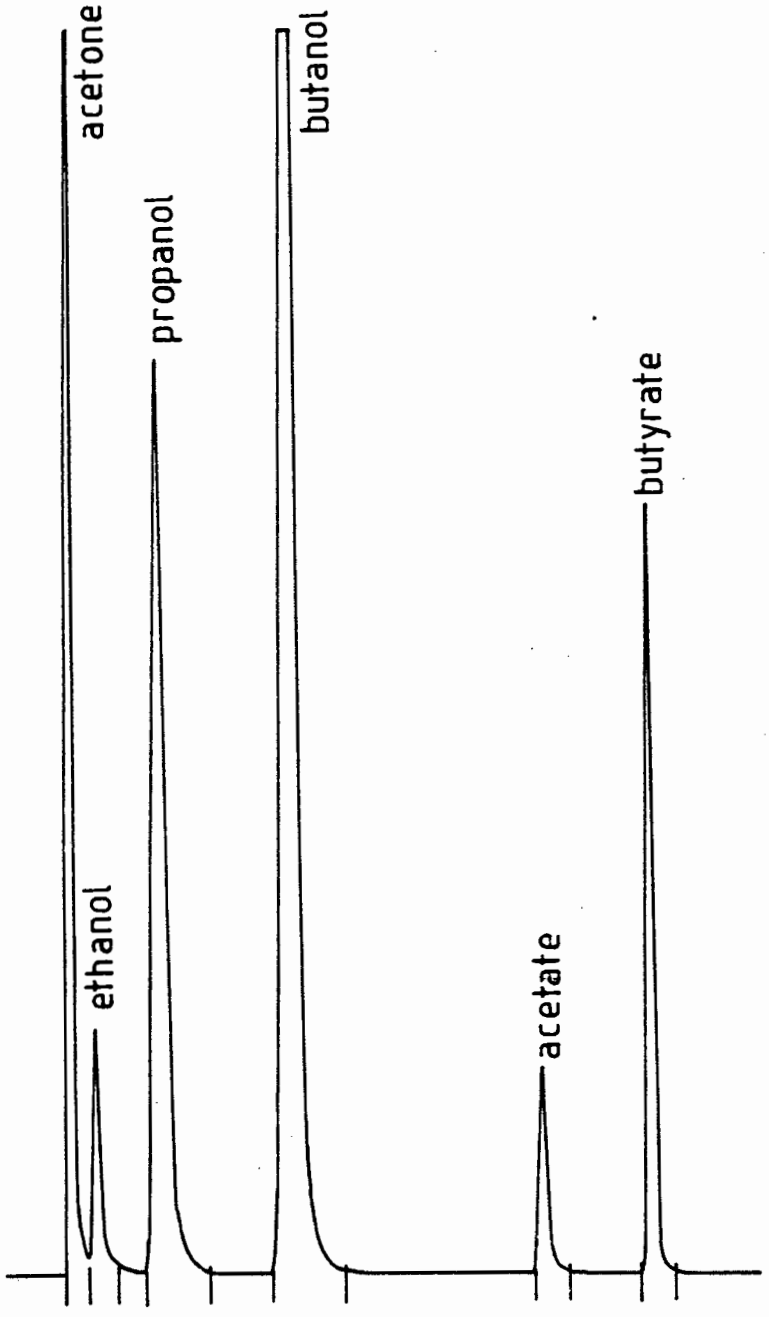
Efficient separation of the components was achieved (Figure 3.4) using the following operating conditions:

1) injector temperature = 200°C
2) detector temperature = 250°C
3) column temperature = 75°C held for 3 minutes, then increased at 20°C min^{-1} up to 180°C
4) air flow rate = 300 ml min^{-1}
5) hydrogen flow rate = 30 ml min^{-1}
6) carrier gas (nitrogen) flow rate = 30 ml min^{-1}

Under these conditions, the retention times for acetone, ethanol, butanol, acetate and butyrate were 1,0, 1,6, 2,4, 4,3, 7,6 and 9,0 minutes respectively.

The chromatograph was calibrated using a standard sample solution of known concentrations. This solution was made up in saturated benzoic acid and contained all the expected

FIGURE 3.4: Chromatogram of the metabolic products



acids and solvents plus the internal standard and sulphuric acid in similar concentrations to those which would, in general, be found in a prepared sample. The concentrations of the components in the sample were accurately determined to 0,001 gl^{-1}.

Both the internal standard solution and the standard sample solution were stored in Mc Cartney bottles at 4°C.

3.44 Nitrogen

Total nitrogen was analysed using the standard Kjeldahl method (Franson, 1985). Appropriately diluted samples were analysed in duplicate. In each case the results were within 0,7% of the mean value.

3.45 Phosphate

Phosphate was analysed using the vandomolydo-phosphoric acid calorimetrical method (Franson, 1985). Appropriately diluted samples were analysed in duplicate. In each case the results were within 0,6% of the mean value.

3.46 Contamination

Contamination was regularly checked by means of microscopic examination. In addition, contamination was checked by spreading culture broth onto prepared nutrient agar plates and incubating the plates at 32°C for 4 days.

CHAPTER 4

SOLVENT PRODUCTION DURING PRODUCT INHIBITED CONTINUOUS CULTURE

Numerous studies in the literature have examined solvent production during continuous culture under conditions where the cell growth is restricted by a specific nutrient. Although the results of these studies are not strictly comparable as the dilution rate, pH and feed glucose concentration were not always similar, it is apparent that provided the pH was maintained at or below 5,5 and the feed glucose concentration was at least 18 gl^{-1}, significant solvent production would be obtained at a low dilution rate under nitrogen, carbon, phosphate and sulphate limitation. From this, it is proposed that a nutrient limitation *per se* is not an essential requirement for solvent induction or for the attainment of high solvent concentrations and yields, but that it is dependent on some other factor(s) present during all the continuous cultures in which solvents were produced.

The major objective of the work described in this chapter was, therefore, to establish whether a nutrient limitation *per se* plays a fundamental role in the onset and maintenance of solvent production during continuous culture. In order to do this, solvent production was examined during continuous culture in which the nutrients were supplied in excess of the requirements for cell growth. In this manner, it was possible to evaluate solvent concentrations and yields in the absence of a nutrient limitation.

To meet this objective, it was necessary to first develop a medium which would provide nutrients in concentrations such that they would not be depleted to a limiting level before the inhibitory metabolic product(s) had reached a concentration which would limit the cell growth, that is, to develop a medium which would be suitable for studying product inhibited continuous culture. The media reported in the literature, which were used for the nutrient limited continuous culture studies, have provided a basis from which to develop a suitable medium.

Continuous culture studies were then conducted over a wide range of dilution rates using this medium and solvent concentrations and yields were evaluated at each dilution rate. During the course of these experiments, oscillatory behaviour was observed, especially at the lower dilution rates. However, in order to evaluate the ability of the organism to produce solvents under these conditions, it was convenient to use mean values of the fermentation parameters obtained from the cyclic behaviour.

4.1 RESULTS

4.11 Formulation of the Medium

A potentially suitable medium was formulated by using the media reported for continuous culture studies under nitrogen, carbon and phosphate limited conditions as a basis. All the essential nutrients were added in approximately the maximum concentrations reported in these media. The formulated medium, together with the media which were used as a basis, are detailed in Table 4.1.

TABLE 4.1: Medium compositions for continuous culture studies

limiting nutrient (gl^{-1})	carbon	carbon	carbon/ nitrogen	nitrogen	nitrogen	phosphate	none
glucose	50,0	3,4	2,7[b]	54,0	45,0	54,0	50,0
$(NH_4)_2SO_4$				2,0		2,0	11,0
NH_4Cl			0,4[b]				
NH_4CH_3COO					1,0		
yeast extract	15,0	6,0					
peptone		10,0					
KH_2PO_4	0,75	2,5	1,0	1,0	0,5	0,1	1,0
K_2HPO_4	0,75		1,0				1,0
$K_2HPO_4.3H_2O$		4,0		1,0	0,5		
$MgSO_4.7H_2O$	0,2	0,246	0,4	0,1	0,2	0,1	0,4
$MnSO_4.H_2O$							0,01
$MnSO_4.3H_2O$	0,01						
$MnSO_4.4H_2O$				0,015		0,015	
$FeSO_4.7H_2O$	0,01		0,01	0,015	0,01	0,015	0,015
NaCl	1,0[a]			0,01		0,01	0,01
asparagine	0,5						0,5
biotin			10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-4}
p-aminobenzoic acid			10^{-4}	2.10^{-3}	10^{-3}	2.10^{-3}	2.10^{-3}
thiamine HCl				2.10^{-3}		2.10^{-3}	2.10^{-3}
$Na_2MoO_4.2H_2O$				0,01		0,01	
$CaCl_2.6H_2O$				0,01		0,01	
$Na_2S_2O_4$		0,035				0,035	
Na dithionite				0,035			
cysteine HCl	0,5						0,5
resazurin	0,003	0,001		0,001		0,001	0,003
reference	Leung and Wang (1981)	Bahl et al (1982a)	Gottschal and Morris (1981b)	Andersch et al (1982)	Monot and Engasser (1983a)	Bahl et al (1982b)	Formulated medium

a: misprint?
b: authors do not indicate change in medium for nitrogen or carbon limitation

The formulated medium contained a glucose concentration of 50 gl^{-1} and an ammonium sulphate concentration of 11 gl^{-1}. This concentration of ammonium sulphate represented a nitrogen concentration which was slightly (10%) higher than the maximum reported in these literature studies. Ammonium acetate was specifically not added to avoid complications involving a possible stimulative effect of the acetate on solvent induction. Organic nitrogen sources were also avoided to ensure a defined medium. (In order to calculate the nitrogen content of the media reported in the literature which contained organic nitrogen sources, it was assumed that the yeast extract contained 10% nitrogen and the peptone, 15% nitrogen. The contribution made by the addition of asparagine was neglected.) The other components necessary for good growth, namely the growth factors (l-asparagine, biotin, p-aminobenzoic acid and thiamine) and Speakman's salts, which have been discussed in detail previously in the literature survey (Section 2.213), were added in the medium in approximately the maximum concentrations reported in these studies. Also, as the organism is inhibited by oxygen (Knaysi and Dutky, 1936; Morris, 1970; Morris and O'Brien, 1971; O'Brien and Morris, 1971), cysteine, which is an effective reducing agent (Hall, 1929), and resazurin, which acts as a redox indicator (Jacob, 1970), were added to ensure anaerobiosis.

4.12 Modification of the Formulated Medium

An inoculum culture was prepared in the formulated medium. When the culture was near the end of the exponential phase, it was used to inoculate a batch fermenter containing the same medium. However, after some sluggish growth, the culture soon lysed. Several attempts were made with the same result.

In order to investigate the inability of the medium to sustain cell viability, several inoculum cultures were prepared at different initial pH values and the progress of the cultures was qualitatively assessed by the microscopic examination of the increase in cell numbers, the motility and the morphological changes (Table 4.2). It was observed that a large proportion of the rods were actively dividing and motile and there was a significant increase in the cell numbers by 6 hours. However, it was clear that no conversion of these rods to the clostridial form took place. Instead cell lysis occurred and by 49 hours, only degraded rods and cell debris were observed. During the course of these experiments, the pH remained above 5,0 in all of the flasks (not shown), suggesting that the cell lysis was not a consequence of an inhibitory pH level.

The defined medium was then modified by the addition of 10 gl^{-1} yeast extract (Difco) and the ammonium sulphate was reduced to 6,1 gl^{-1} from 11,0 gl^{-1} so as to maintain the same total nitrogen concentration. The progress of the inoculum cultures in the modified, semi-defined medium was monitored as before (Table 4.2). The effect of the substitution of the yeast extract was dramatic. Cell lysis did not occur, instead all cultures showed a normal pattern of cell differentiation which led to the development of forespores.

Furthermore, the use of an actively dividing motile culture from one of these flasks as an inoculum for a fermenter containing the semi-defined medium resulted in a successful batch fermentation. (This has been described in Section 4.131).

4.13 Evaluation of the Modified Medium

4.131 Batch Culture The fermentation profiles of the batch culture in the semi-defined medium are shown in

TABLE 4.2: Growth, motility and morphological changes of C. acetobutylicum P 262 in defined and semi-defined media

Time (hrs)	Parameter	Formulated Medium				Modified medium			
		pH 6,3	pH 6,5	pH 6,8	pH 7,0	pH 6,3	pH 6,5	pH 6,8	pH 7,0
4	Growth	+	+	+++	+	++	+++	++	++
	Motility	++	+	+	+	++	++	+	+
	Morphology	R	R	R	R	R	R	R	R
6	Growth	++	++	++	+	+++	+++	+	+
	Motility	++	+	-	-	+++	++	-	-
	Morphology	R	R	R	R	R/C	R/C	R	R
28	Growth	++	++	++	+	++	++	++	++
	Motility	+	+	+	+	-	-	-	-
	Morphology	R	R	R	R	C/F/S	C/F/S	C/F/S	C/F/S
49	Growth	-	-	-	-	-	-	-	-
	Motility	-	-	-	-	-	-	-	-
	Morphology	R	R	R	R	C/F/S	C/F/S	C/F/S	C/F/S

Key			
Growth/motility		Morphology	
none	-	rods	R
slight	+	clostridials	C
moderate	++	forespores	F
good	+++	free spores	S

Figure 4.1. The culture pH was maintained above 5,0. The typical two phase behaviour of the solvent fermentation was observed. During the initial acidogenic phase, the cells grew exponentially with a specific growth rate of 0,45 hr^{-1} (regression coefficient = 0,9997). No solvents were produced. Solventogenesis was initiated 6 hours after the start of growth and was characterised by the consumption of acids, increased glucose consumption and the onset of solvent production. A maximum solvent level of 15 gl^{-1} was achieved at 20 hours; this corresponded to a solvent yield of 0,30. The end of solvent production coincided with glucose exhaustion and cell lysis.

Similar morphological changes to those previously reported for this strain during batch culture (Jones et al, 1982) were also observed in this medium. At the start of exponential growth, the rods were phase dark and actively dividing (Plate 4.1A). The conversion of the phase dark rods to phase bright clostridial forms was observed at the transition from acid to solvent production (Plate 4.1B). Forespore septum formation (Plate 4.1C) and phase bright mature forespores (Plate 4.1D) were observed later in the fermentation.

4.132 Continuous Culture In order to establish whether this medium supplied the nutrients in excess during continuous culture it was necessary to measure residual nutrient concentrations in the broth. For this purpose, a dilution rate of 0,2 hr^{-1} was arbitrarily chosen. The culture was operated initially at a dilution rate of 0,1 hr^{-1} until sporulating forms and free spores were no longer observed. The dilution rate was then increased in steps to 0,2 hr^{-1}. The percentage transmission and glucose concentration at this dilution rate were measured over a period of time until 3 samples, at least 1 volume change apart, yielded a standard deviation of less than 1% and 1,5 gl^{-1}, respectively. The concentrations of

FIGURE 4.1: Growth and physiological changes in C. acetobutylicum P 262 during batch culture at pH above 5,0. A: ○, cell dry mass; , glucose; ◇, total solvents; ◆, total acids. B: ○,butanol; ●, acetone; □, ethanol; ◇, butyrate; ◆, acetate.

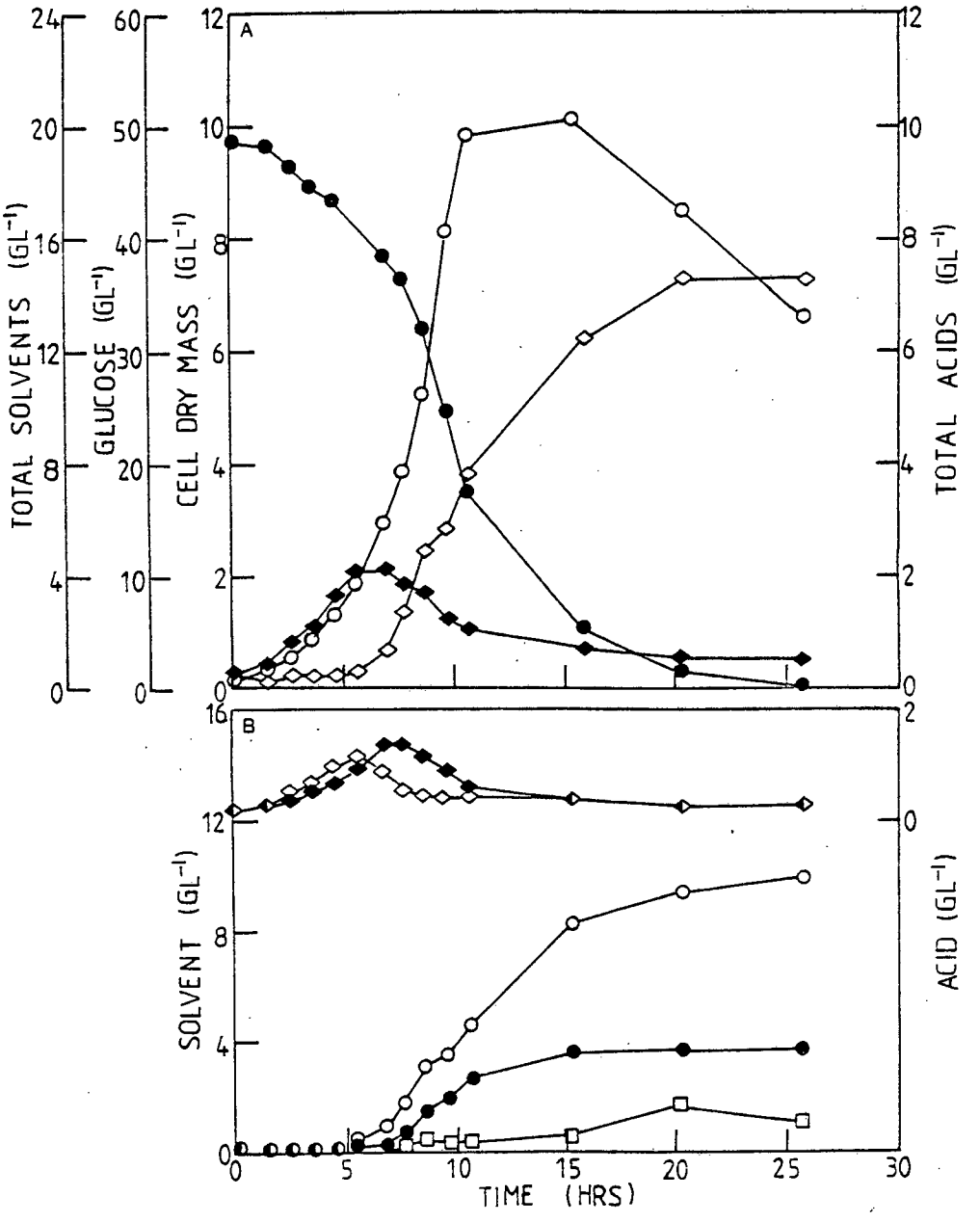
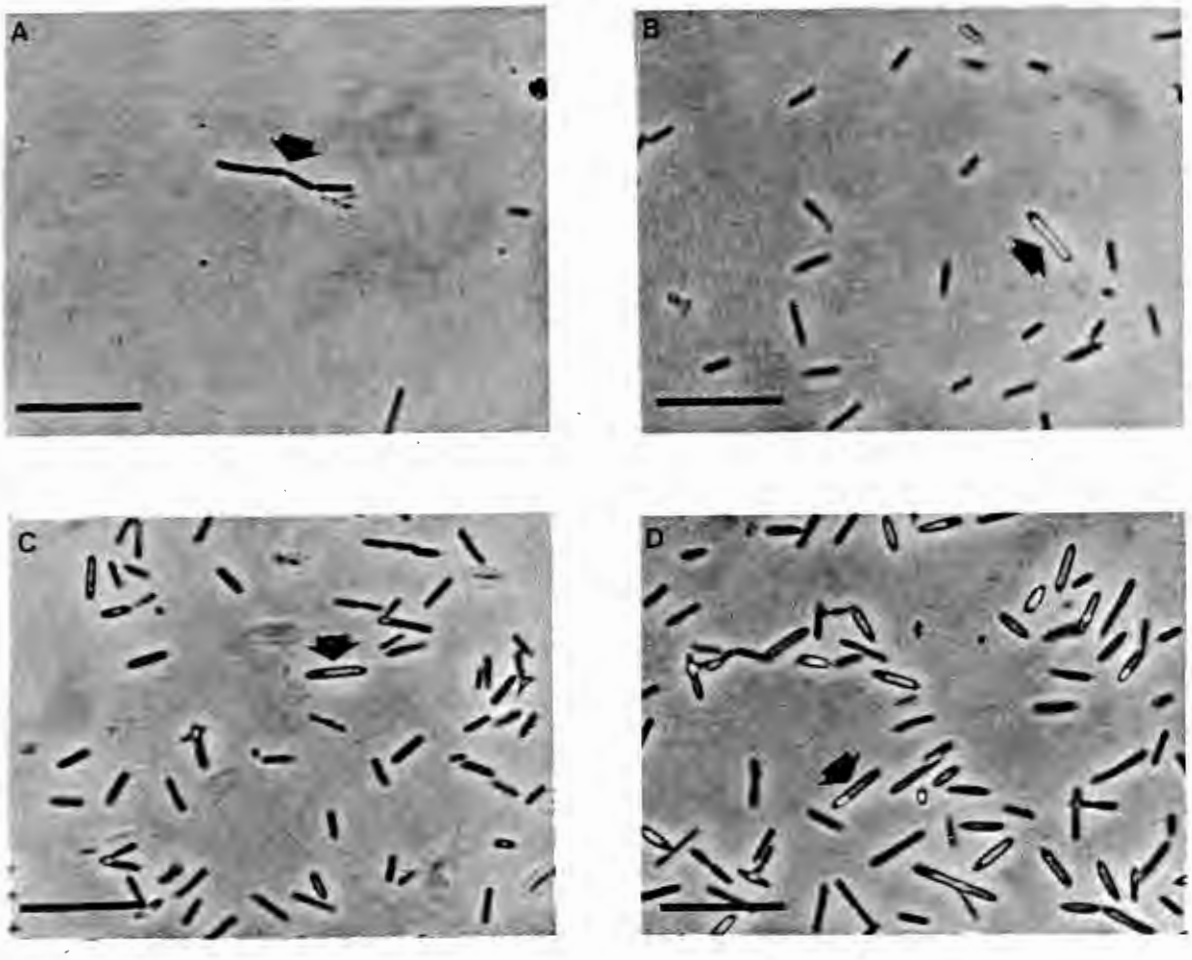


PLATE 4.1: Photomicrographs of morphological changes in C. acetobutylicum P 262 during batch culture at pH above 5,0. A: actively dividing vegetative rod (0,1 hours); B: clostridial form (5,6 hours); C: clostridial form after forespore septum formation (7,6 hours); D: clostridial form with mature forespore (10,6 hours). Bars: 10 µm.



carbon, nitrogen and phosphate were then measured in the broth. The concentrations of these nutrients in the nutrient feed were also measured and compared with those in the broth (Table 4.3).

From these analyses, it is evident that less than 55% of the carbon, nitrogen and phosphate sources were utilised at this dilution rate, resulting in relatively high residual concentrations of these nutrients in the broth.

TABLE 4.3: Nutrient concentrations in feed and broth at a dilution rate of 0,2 hr^{-1}

	carbon	nitrogen	phosphate
Feed concentration (gl^{-1})	21,3	2,56	0,61
Broth concentration (gl^{-1})	9,6	2,39	0,41
Utilisation (%)	55	7	33

4.14 Accumulation of Data from Continuous Cultures

Continuous cultures were operated in the modified medium at dilution rates from 0,05 hr^{-1} to 0,35 hr^{-1} inclusive. In all cases, the culture was initially operated at a dilution rate of 0,1 hr^{-1} until no more sporulating forms or free spores were observed. The dilution rate was then adjusted in steps to the required value (if appropriate). At this stage, the time was arbitrarily set to zero hours and the fermentation parameters were monitored from this time.

During continuous culture at the lowest dilution rates of 0,05 hr^{-1} and 0,10 hr^{-1}, the glucose concentration and percentage transmission still exhibited pronounced unsteady behaviour after 680 hours or 34 volume changes (Figure 4.2) and 270 hours or 27 volume changes (Figure 4.3) had taken place respectively. The analyses of these parameters from several samples, taken at least one volume change apart over the last 10 volume changes in each case, showed a large variation. The percentage transmission readings showed a standard deviation of 1,6% at 0,05 hr^{-1} and 2,9% at 0,10 hr^{-1} and the glucose concentration showed a standard deviation of 3,1 gl^{-1} at 0,05 hr^{-1} and 1,8 gl^{-1} at 0,10 hr^{-1}.

A more detailed analysis of the fermentation products, analysed over the last 10 volume changes at the dilution rate of 0,05 hr^{-1}, is shown in Figure 4.4. It is evident that the cell, acid and solvent concentrations also showed considerable variation. Similar variations were observed at a dilution rate of 0,10 hr^{-1} (not shown). Consequently, the parameter values obtained from continuous operation at the dilution rates of 0.05 hr^{-1} and 0,10 hr^{-1} were mean values obtained from averaging the data measured over 10 volume changes.

During continuous culture at the relatively higher dilution rates of 0,15 hr^{-1} to 0,35 hr^{-1} inclusive, the variation of the glucose concentration and percentage transmission was less pronounced (Figures 4.5 to 4.9). After several volume changes had taken place the standard deviation of the percentage transmission and glucose concentration was less than 1% and 1,1 gl^{-1} respectively at each dilution rate (Table 4.4). Consequently, the mean parameter values at these dilution rates were obtained from averaging the data measured over only 3 to 4 volume changes. The broken limes in the relevant figures (Figures 4.5 to 4.9) indicate the time at which the standard

FIGURE 4.2: Unsteady behaviour during continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,05 hr^{-1}. ○ , percentage transmission; ● , glucose.

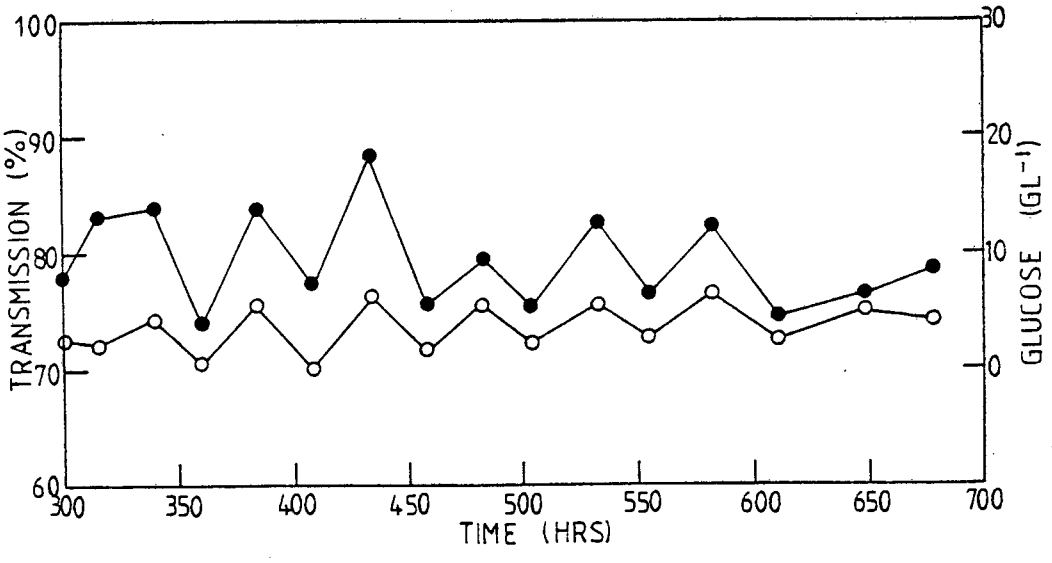


FIGURE 4.3: Unsteady behaviour during continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}. ○ ,percentage transmission; ● , glucose.

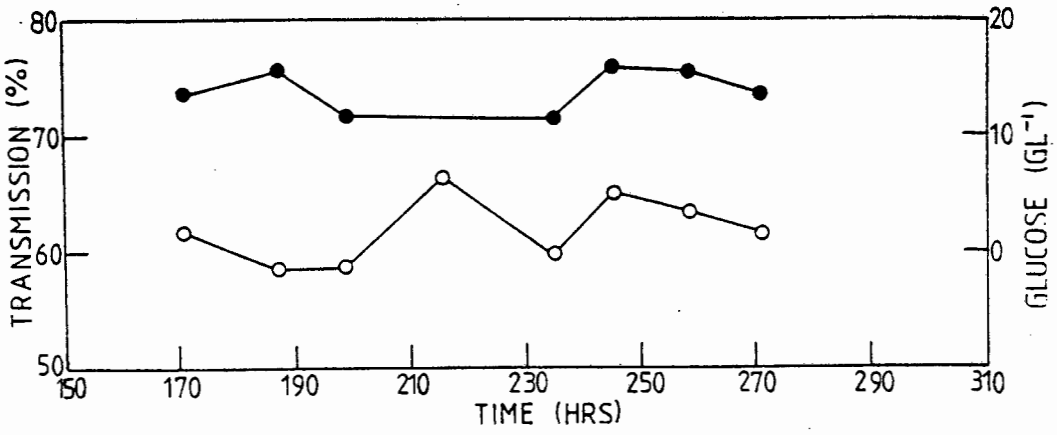


FIGURE 4.4: Fermentation products over the last ten volume changes during continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,05 hr^{-1}. A: ○ , total solvents; ● , total acids; ◇ , cell dry mass. B: ○ , butanol; , acetone; □ , ethanol; ◇ , butyrate; ◆ , acetate. m = measured or true cell dry mass

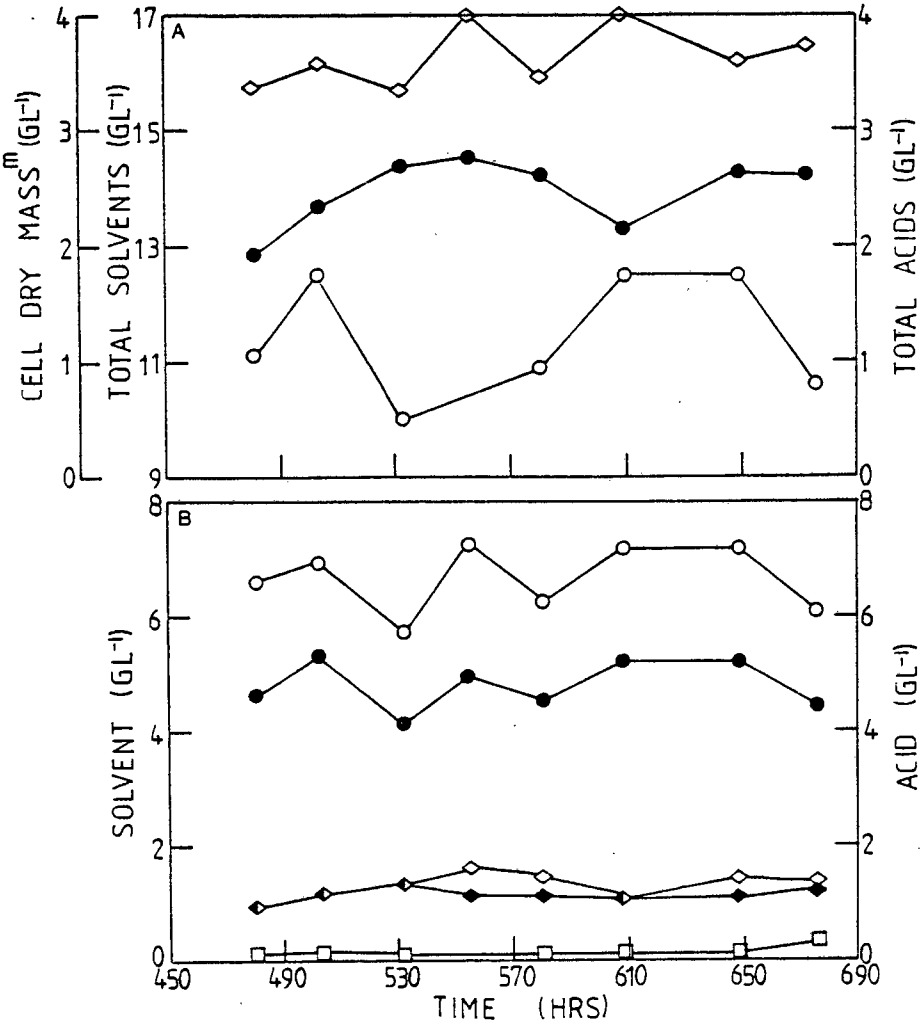


FIGURE 4.5: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,15 hr^{-1}. ○, percentage transmission; ●, glucose.

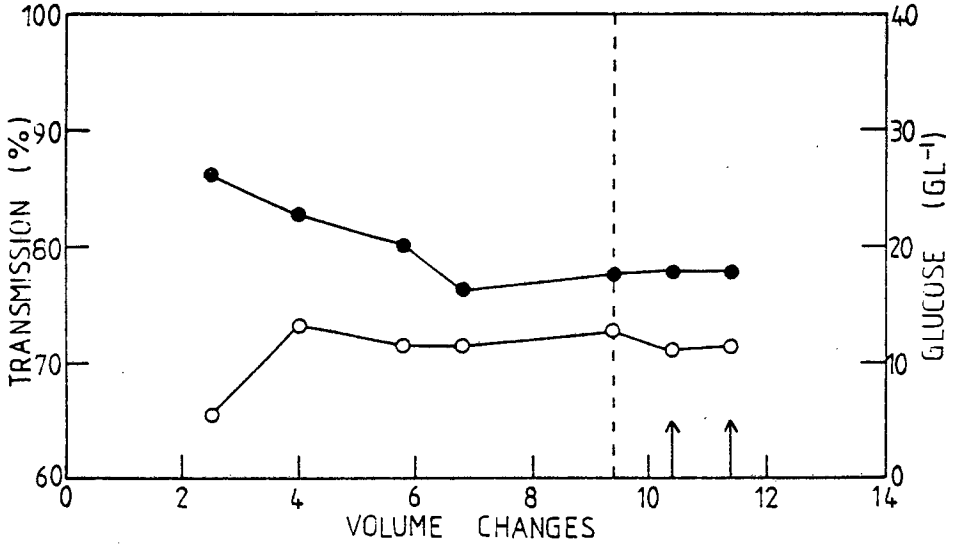


FIGURE 4.6: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,20 hr^{-1}. ○, percentage transmission; ●, glucose.

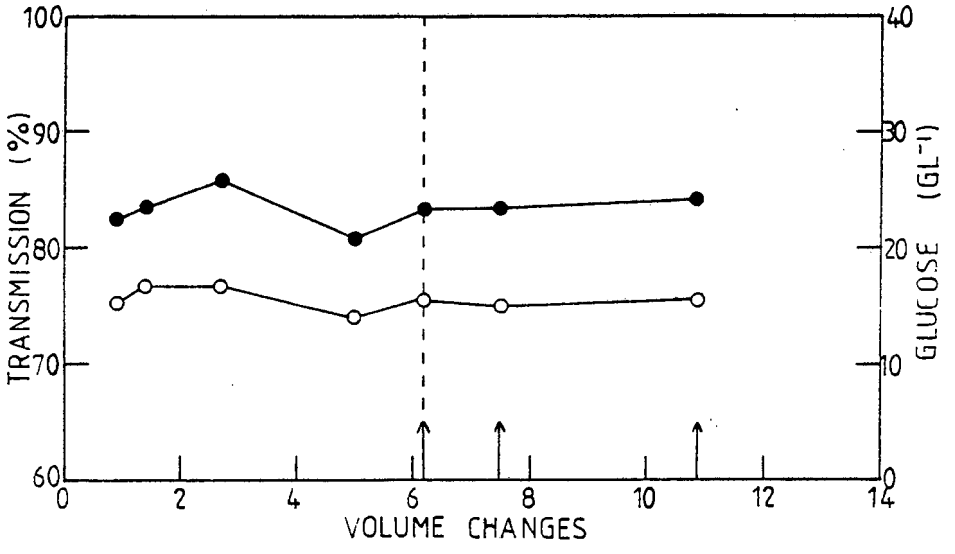


FIGURE 4.7: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,25 hr^{-1}. ○, percentage transmission; ●, glucose.

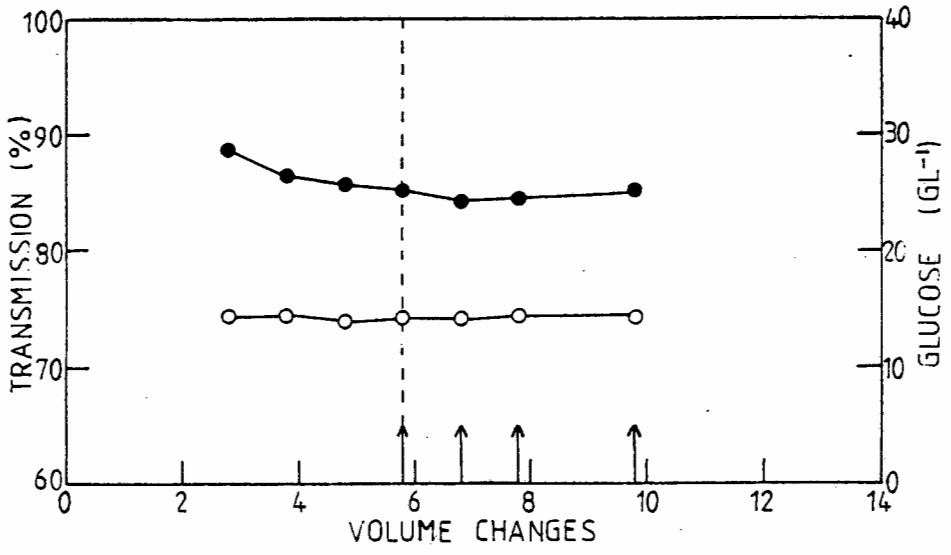


FIGURE 4.8: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,30 hr^{-1}. ○, percentage transmission; ●, glucose.

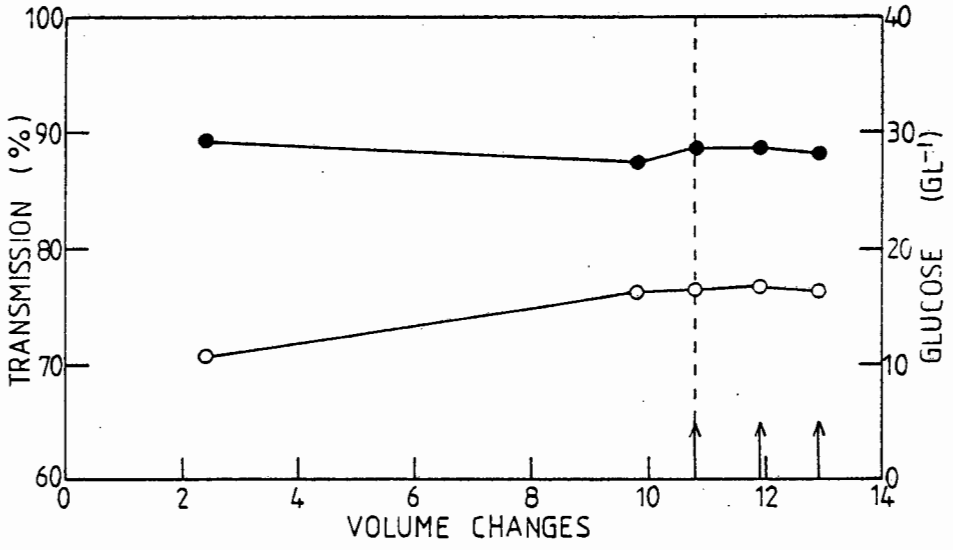
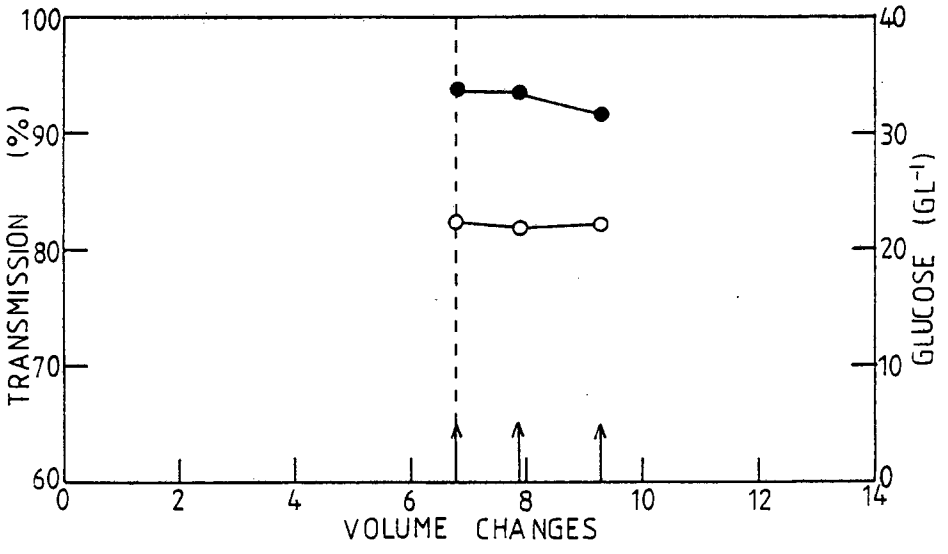


FIGURE 4.9: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,35 hr^{-1}. ○, percentage transmission; ●, glucose.



deviation of the parameters had decreased to these limits and the arrows indicate the times at which the parameter values were analysed for the calculation of the mean values.

TABLE 4.4: Parameter variation during continuous culture at dilution rates of 0,15 hr^{-1} to 0,35 hr^{-1} inclusive

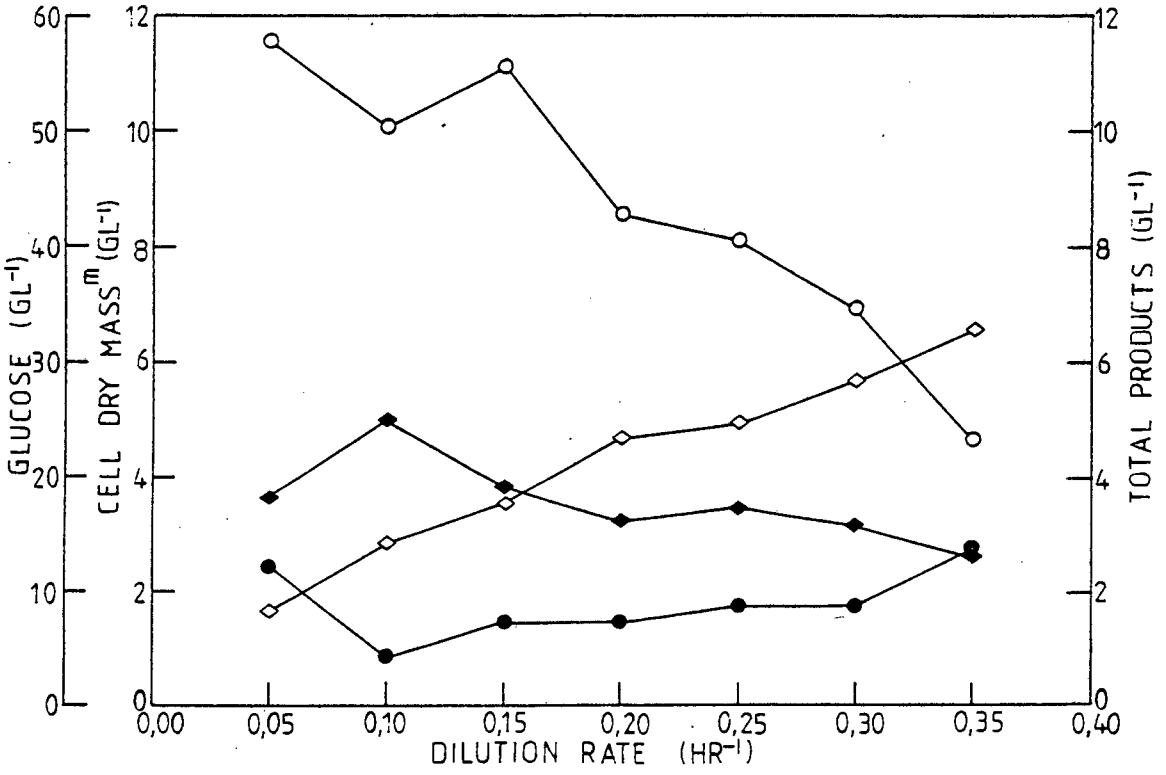
Dilution rate (hr^{-1})	Standard deviation	
	transmission (%)	glucose(gl^{-1})
0,15	1,0	0,1
0,20	0,3	0,5
0,25	0,2	0,5
0,30	0,3	0,3
0,35	0,2	1,1

In order to check the reproducibility of these mean values, duplicated continuous cultures were conducted. A dilution rate of 0,2 hr^{-1} was arbitrarily chosen. The mean product concentrations, obtained once the standard deviation of the percentage transmission and glucose concentration was less than 1% and 1,5 gl^{-1} respectively, are detailed in Table 4.5. The duplicated mean concentrations are within 0,1 gl^{-1} of each other. It should be noted that the analytical method used to measure the product concentrations was only accurate to within 0,1 gl^{-1} (Section 3.43).

4.15 Kinetics of the Continuous Fermentation

The mean substrate and product concentrations (obtained as described previously) at each dilution rate are shown in Figure 4.10. The cell concentration curve, as measured by

FIGURE 4.10: Influence of the dilution rate on the substrate and product concentrations during continuous fermentation of C. acetobutylicum P 262. ◇, glucose; ◆, cell dry mass^{m}; ○, total solvents; ●, total acids.
m = measured or true cell dry mass.



the mean true cell dry mass, decreased at low dilution rates indicating an energy requirement for cell maintenance but, in general, decreased with increasing dilution rate from a maximum of 5 gl^{-1} at 0,1 hr^{-1}.

TABLE 4.5: Reproducibility of the mean product concentrations at a dilution rate of 0,2 hr^{-1}

	Product Concentrations (gl^{-1})				
	acetone	butanol	ethanol	acetate	butyrate
Experiment 1	3,494	4,971	0,133	0,896	0,566
Experiment 2	3,414	4,874	0,191	0,889	0,536
Difference	0,080	0,097	0,058	0,007	0,030

The mean residual glucose concentration increased with increasing dilution rate. At the dilution rate of 0,05 hr^{-1}, 84% of the glucose was utilised resulting in a mean residual glucose concentration of 8 gl^{-1}. The glucose utilisation decreased to 36% at the dilution rate of 0,35 hr^{-1} and a correspondingly higher mean residual glucose concentration of 33 gl^{-1} was present in the broth.

The maximum mean total solvent concentration of 12 gl^{-1} was obtained at the lowest dilution rate investigated, namely 0,05 hr^{-1}, and thereafter decreased with increasing dilution rate to 5 gl^{-1} at 0,35 hr^{-1}. The mean total acid concentration showed less of a dependence on the dilution rate but increased slightly from the dilution rate of 0,1 hr^{-1} with increasing dilution rate although it remained below 3 gl^{-1}.

A more detailed analysis of the solvents showed that the mean butanol concentration decreased from a maximum of 7 gl^{-1} at 0,05 hr^{-1} to 3 gl^{-1} at 0,35 hr^{-1} (Figure 4.11). The mean acetone concentrations decreased in the same way from 5 gl^{-1} at 0,05 hr^{-1} to 2 gl^{-1} at 0,35 hr^{-1}. The ethanol was present in small amounts of a mean value less than 0,3 gl^{-1} at all the dilution rates investigated. The variation of the acetate was similar to that of the butyrate. Neither acid exceeded a mean concentration of 1,5 gl^{-1} at any of the dilution rates.

From the data, the overall mean yield of total solvents, based on glucose consumption, over the range of dilution rates investigated, was calculated as 0,28. Although some variation is evident, the regression coefficient of 0,98 indicates that the fit of these data to a straight line is reasonable (Figure 4.12).

4.2 DISCUSSION

The defined medium, which was formulated from the carbon, nitrogen and phosphate limited media reported in the literature, contained in addition to glucose and inorganic nitrogen, all the components that have been reported necessary for good growth, namely l-asparagine, biotin, p-aminobenzoic acid, thiamine and Speakman's salts. However, initial attempts to maintain cell viability in this medium met with limited success. This strain apparently had an increased or additional requirement for one or more growth factors present in yeast extract and, therefore, this defined medium could not be used. Consequently, it was necessary to modify the medium by the addition of yeast extract. The use of a medium, in which all the individual constituents are not defined, tends to complicate the interpretation of the data. To minimise

FIGURE 4.11: Influence of the dilution rate on the individual products during continuous fermentation of C. acetobutylicum P 262. ○, butanol; ● , acetone; □ , ethanol; ◇, butyrate; ◆, acetate.

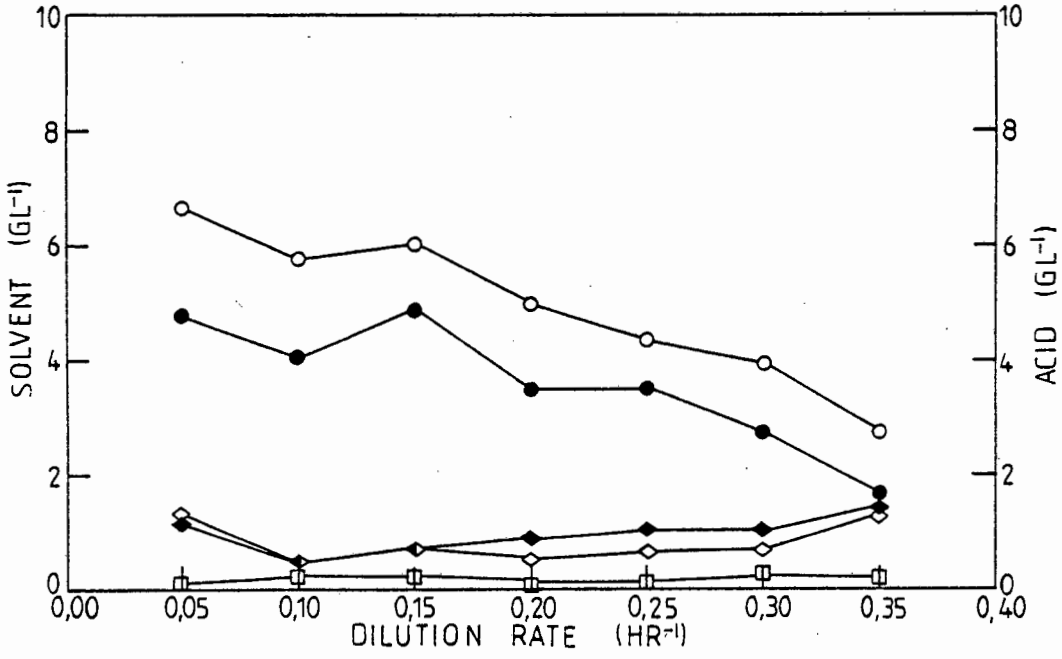
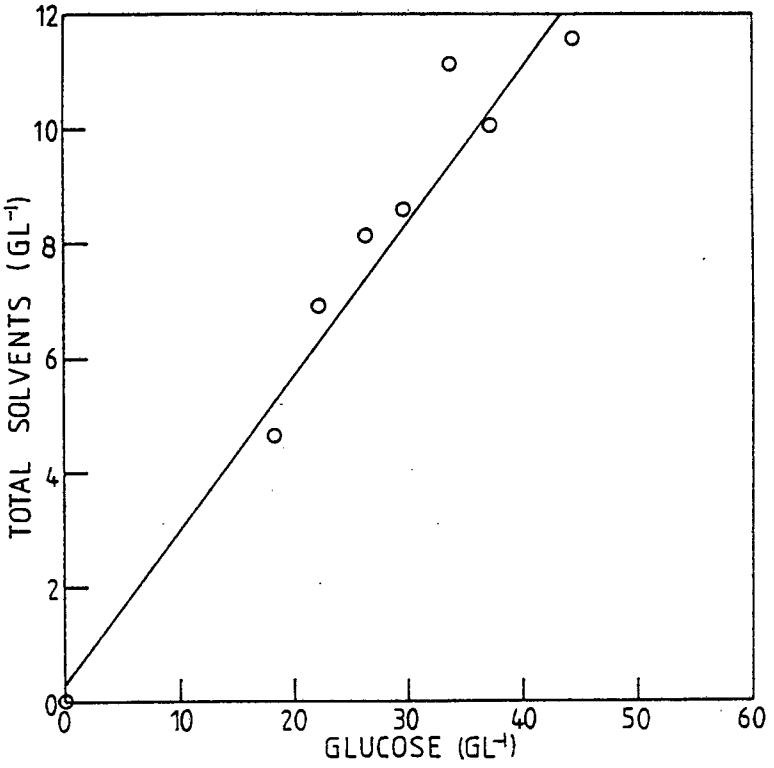


FIGURE 4.12: Total solvents produced as a function of glucose utilised during continuous fermentation of C. acetobutylicum P262 over the range of dilution rates from 0,05hr^{-1} to 0,35hr^{-1}.



this disadvantage, it was ensured that the same brand of yeast extract was used throughout the experiments.

Cell viability was maintained in the modified medium. Moreover, the medium supported good growth during batch culture. The maximum specific growth rate of 0,45 hr^{-1} attained compares well with that of 0,46 hr^{-1} attained with this strain in a complex molasses medium (Jones et al, 1982).

During continuous culture in the modified medium at a dilution rate of 0,2 hr^{-1}, the percentage utilisation of the carbon (55%), nitrogen (7%) and phosphate (33%) sources were relatively low, suggesting that these nutrients did not limit the cell growth. An excess of carbon and nitrogen is also indicated by the relatively high residual concentrations of these nutrients in the broth at this dilution rate. At the same dilution rate, Leung and Wang (1981) reported a residual carbon concentration of 2 gl^{-1} under carbon limitation and Monot and Engasser (1983a) reported a residual nitrogen concentration of 0,05 gl^{-1} under nitrogen limitation. The residual concentrations of carbon and nitrogen in this study were approximately 5 times these values. Although Bahl et al (1982b) did not report similar figures for phosphate limitation, a comparison of their feed concentrations with those used in this study reveals that the phosphate to carbon ratio and the phosphate to nitrogen ratio were more than 22 and 3 times higher in this study, respectively. This supports the suggestion that phosphate was also not limiting.

As the other nutrients supplied in this medium were added in concentrations that were not limiting in the media reported in the literature, it was assumed that they would similarly not be limiting in this medium and therefore, their residual concentrations in the broth were not analysed. Nevertheless, it is interesting to note that the

sulphate concentration used in this study was more than 150 times that used during the study of solvent production under sulphate limitation (Bahl and Gottschalk, 1984).

The evaluation of the medium developed for this study suggests that all the nutrients were supplied in excess of the requirements for cell growth. It may be inferred that under these conditions, the cell growth was limited by the metabolic product(s) produced by the fermentation, that is, the continuous culture was conducted under product inhibiting (or limiting) conditions. Consequently, this medium provided a definitive means for the examination of solvent production during continuous culture in the absence of a nutrient limitation.

During continuous culture over a wide range of dilution rates, variations in the fermentation parameters were observed, especially at low dilution rates. The parameters at each dilution rate, therefore, represent mean values obtained during the unsteady behaviour. However, these mean values were calculated from data analysed over numerous volume changes and, furthermore, the reproducibility of these mean values was found to be within the limits of the error of the analyses. Consequently, these mean values provided a reasonable assessment of solvent production during continuous culture in the absence of a nutrient limitation.

The maximum mean solvent concentration was attained at the lowest dilution rate investigated and decreased with increasing dilution rate. This trend is similar to that found in all the nutrient limited continuous culture studies reported. Furthermore, the maximum mean concentration of 12 gl^{-1} solvents achieved at the dilution rate of 0,05 hr^{-1} in the absence of a nutrient limitation, compares favourably with those attained under nutrient limited growth. Leung and Wang (1981) produced 16

gl^{-1} at 0,1 hr^{-1} (the lowest dilution rate they investigated) under carbon limitation and 7 gl^{-1}, 12 gl^{-1} and 7 gl^{-1} were estimated from the data at a dilution rate of 0,05 hr^{-1} under nitrogen limitation (Monot and Engasser, 1983a), phosphate limitation (Bahl et al, 1982b) and sulphate limitation (Bahl and Gottschalk, 1984) respectively. In addition, the average total solvent yield of 0,28 obtained in the absence of a nutrient limitation, compares well with the total solvent yield of approximately 0,3 obtained under nutrient limitation in these literature studies.

From these results, it is clear that appreciable solvent concentrations and yields were attained in a rich medium containing an excess of carbon, nitrogen and phosphate. This strongly suggests that solvent production is independent of a requirement for a nutrient limitation per se and, therefore, is influenced by some other factor(s) (Clarke and Hansford, 1986). The absence of a requirement of a glucose or a nitrogen limitation for solvent production is supported by the batch studies of Long et al (1984b) in which no solvents were produced when growth was limited by these nutrients rather than by end products.

A major consequence of the finding that a nutrient limitation is not essential for solvent production, is that maximal solvent concentrations and, therefore, productivity, is likely to be achieved under conditions of nutrient excess. Under these conditions, the cell growth is not limited by a nutrient and, therefore, maximum cell concentrations (resulting in maximum solvent concentrations) could be attained. This suggestion is supported by the results of Monot and Engasser (1983b) who increased the feed nitrogen concentration to a nitrogen limited continuous culture and observed an increase in the solvent concentration. Monot et al (1983) also obtained maximal conversion yields of glucose into solvents when the

culture was supplied with a high glucose feed concentration and residual nitrogen was measured in the broth.

CHAPTER 5

NATURE AND SIGNIFICANCE OF OSCILLATORY BEHAVIOUR DURING CONTINUOUS CULTURE

During the studies of solvent production in continuous culture which were described in Chapter 4, oscillatory behaviour in the fermentation parameters was observed. The oscillations were experienced at all the dilution rates investigated, namely 0,05 hr^{-1}, 0,10 hr^{-1}, 0,15 hr^{-1}, 0,20 hr^{-1}, 0,25 hr^{-1}, 0,30 hr^{-1} and 0,35 hr^{-1}, but were most pronounced at the lower dilution rates of 0,05 hr^{-1} and 0,10 hr^{-1}. The unsteady behaviour was apparently similar to that reported by several other continuous culture studies in the literature.

It was, therefore, the objective of this chapter to investigate the nature of the observed oscillations. To meet this objective, experiments were conducted to document the oscillatory behaviour in the growth, physiology and morphology during continuous operation under constant operating conditions. Dilution rates of 0,10 hr^{-1} and 0,25 hr^{-1} were chosen for the detailed investigation since at these dilution rates, the magnitude of the variation in the parameters was typical of that observed at the low (0,05 hr^{-1} and 0,10 hr^{-1}) and high (0,15 hr^{-1} to 0,35 hr^{-1}) dilution rates respectively. Experiments were also conducted to establish that the organism remained genetically stable during periods of prolonged continuous operation in which oscillatory behaviour was observed. The documented information was finally drawn together into a conceptual model to elucidate the nature of the oscillatory behaviour.

5.1 RESULTS

5.11 Oscillations During Continuous Culture

5.111 Start Up The changes in growth, physiology and morphology immediately following the initiation of continuous culture were monitored for a period of 7,7 volume changes at a dilution rate of 0,1 hr^{-1} (Figure 5.1). The time at which continuous operation was initiated was set to 0 hours. The switch to continuous culture was associated with the washout of the non-dividing, sporulating cells present at the end of the batch culture so that at 37 hours the cell concentration had decreased by more than 90%. Cell washout was associated with a decrease in the product concentrations and an increase in the glucose concentration. Between 37 hours and 44 hours both the cell concentration and the concentration of acids began to increase. Renewed solvent production was first detected at 44 hours and was associated with increased glucose consumption and a reduction in the concentration of acid end products. Between 50 hours and 53 hours washout of cells was again observed, followed by a decrease in the solvent concentration and glucose consumption. By 62 hours the cell concentration had decreased by approximately 80%. This second cycle of cell washout was similar to that observed after the initiation of continuous operation but was less extensive. Between 62 hours and 71 hours the acid concentration and cell numbers increased again. This was followed by increases in solvent concentration and acid and glucose consumption at 71 hours.

The accumulation of granulose, the formation of forespore septa and mature forespores and the liberation of free spores were also monitored throughout this period (Figure 5.2). Photomicrographs showing the different morphological forms are shown in Plate 5.1. During the

FIGURE 5.1: Growth and physiological changes in C. acetobutylicum P 262 during fermentation after the switch over from batch to continuous operation at a dilution rate of 0,1 hr^{-1}.
A: ○ , total cell count; ● , cell dry mass. B: ◇ , glucose; ○ , total solvents; ● , total acids. C: ○ , butanol; ● , acetone; □ , ethanol; ◇, butyrate; ◆, acetate.

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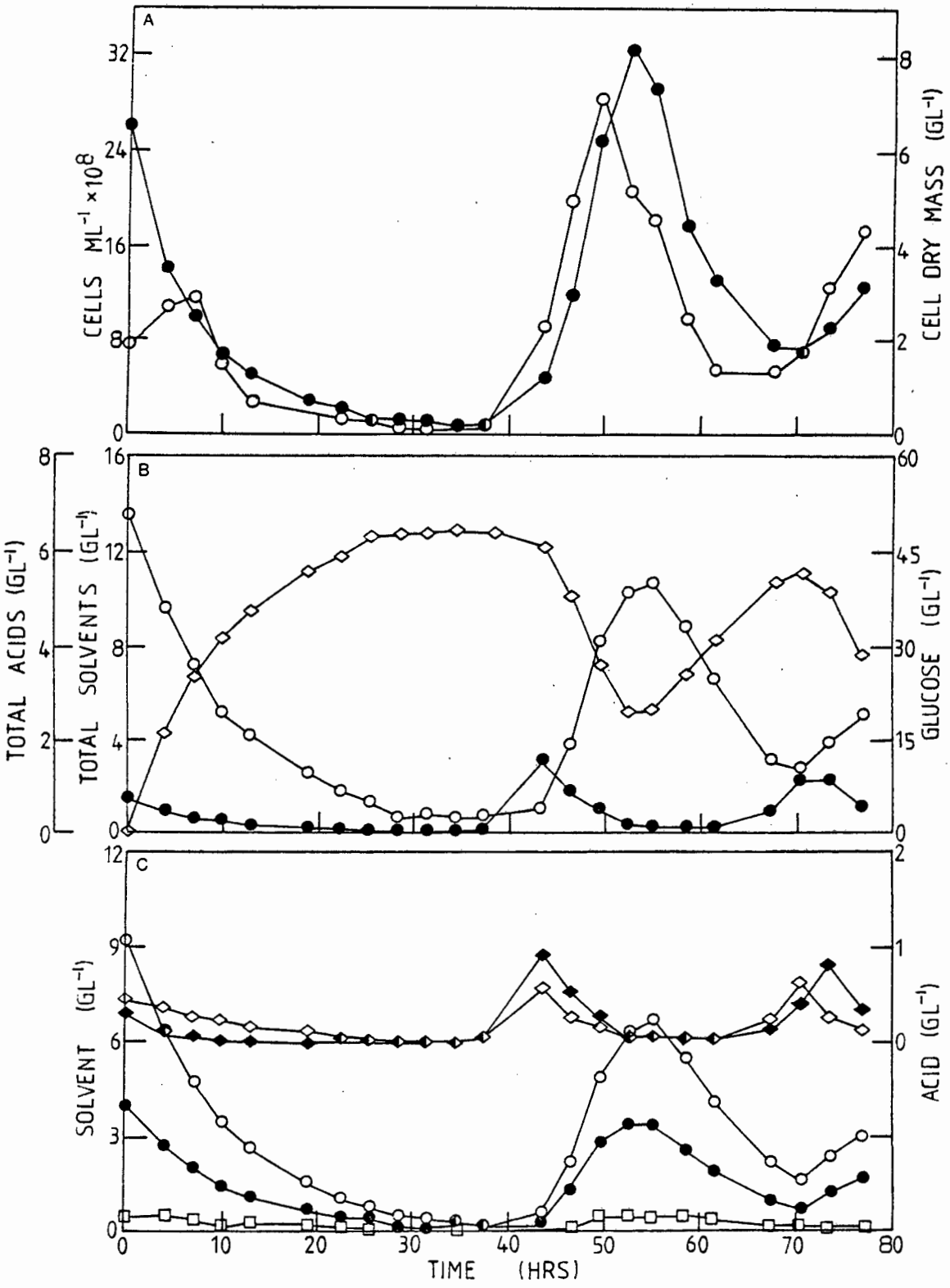

A
B
C
CELLS ML⁻¹ ×10⁸
CELL DRY MASS (GL⁻¹)
TOTAL ACIDS (GL⁻¹)
TOTAL SOLVENTS (GL⁻¹)
GLUCOSE (GL⁻¹)
SOLVENT (GL⁻¹)
ACID (GL⁻¹)
TIME (HRS)

FIGURE 5.2: Morphological and cytological changes in C. acetobutylicum P 262 during fermentation after the switch over from batch to continuous operation at a dilution rate of 0,1 hr^{-1}.
A: ○ , granulose positive rod count; , granulose negative rod count. B: ◇, forespore septum count; ○ , mature forespore count; ●, free spore count. C: percentage of total cells with: ◇ , forespore septa; ○ , mature forespores; ● , free spores.

(please see over)

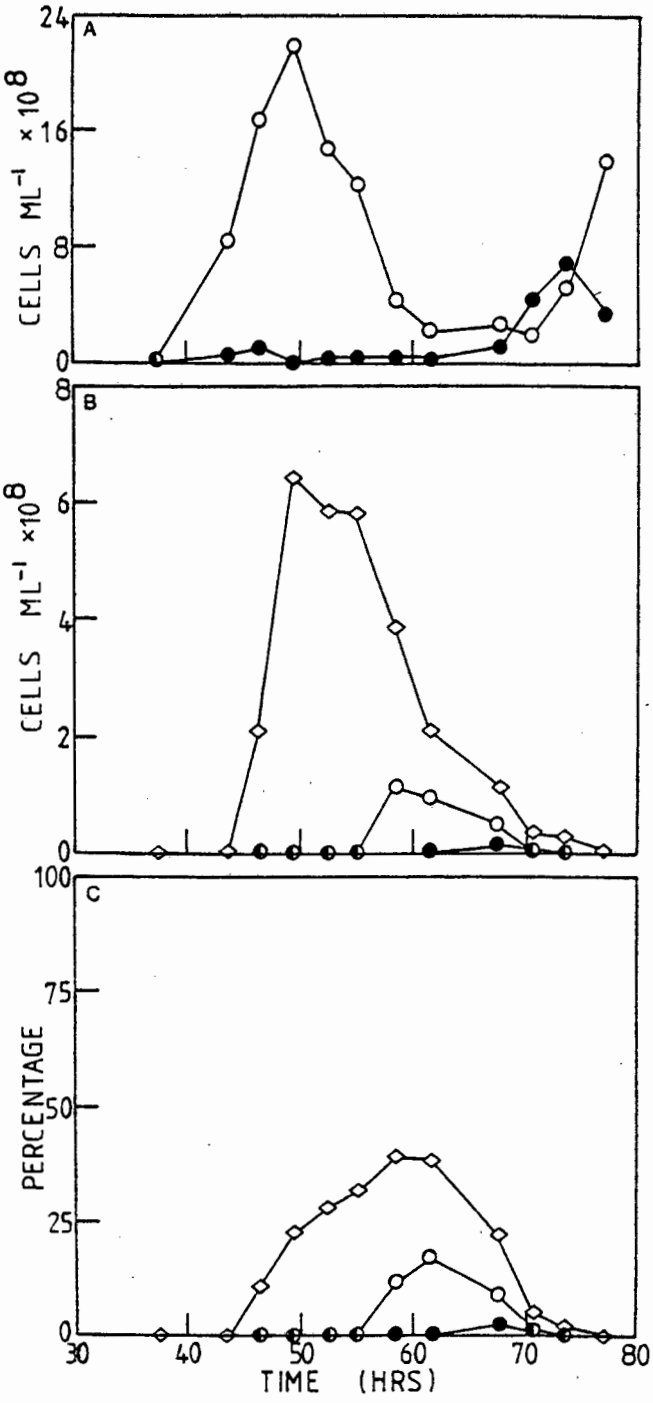
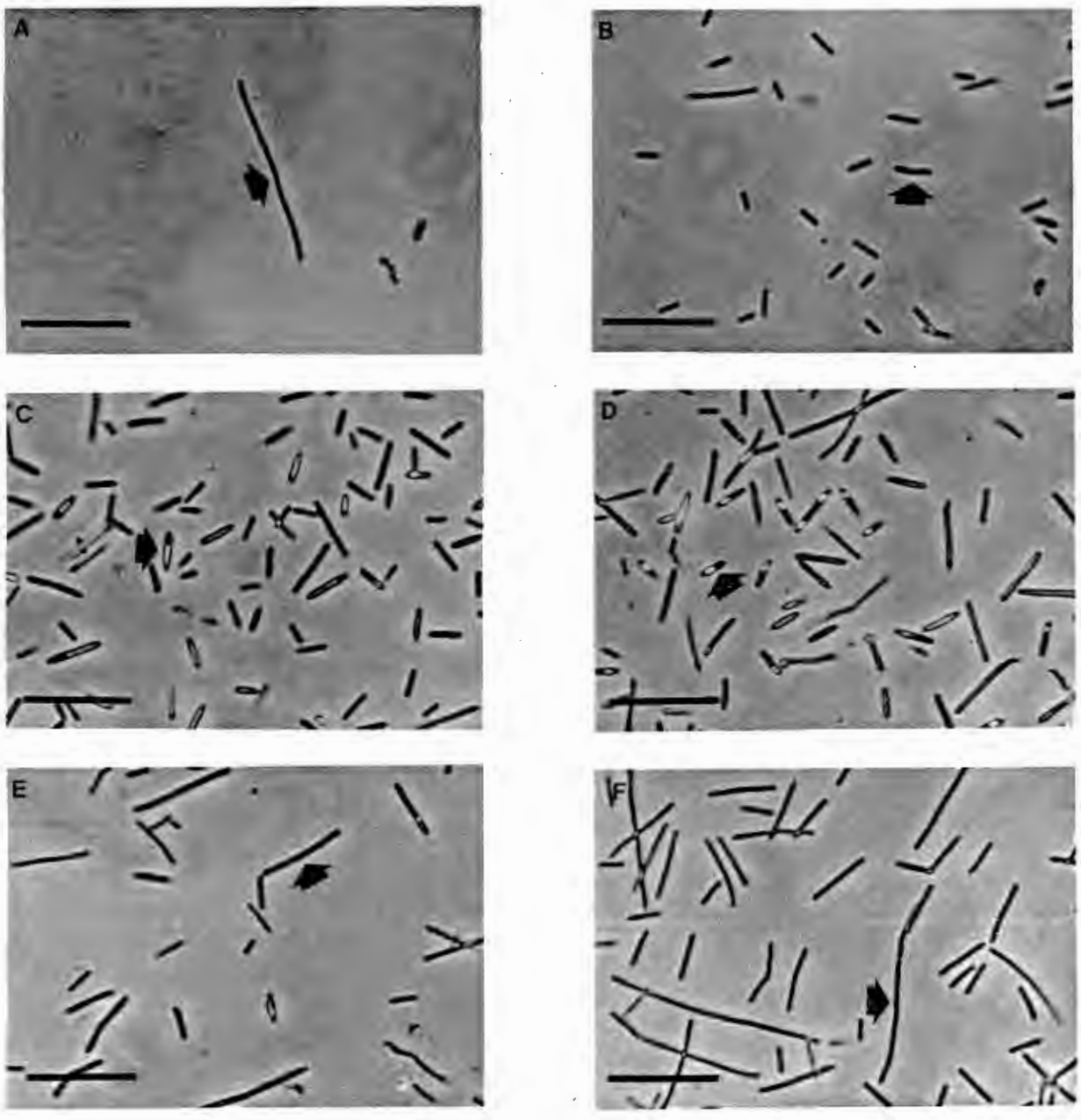

A
B
C
CELLS ML⁻¹ ×10⁸
CELLS ML⁻¹ ×10⁸
PERCENTAGE
TIME (HRS)
24
16
8
0
8
6
4
2
0
100
75
50
25
0
30
40
50
60
70
80

PLATE 5.1: Photomicrographs of morphological changes in C. acetobutylicum P 262 during fermentation after the switch over from batch to continuous operation at a dilution rate of 0,1 hr^{-1}. A: phase dark long rod (37 hours); B: phase dark actively dividing rod (44 hours); C: phase bright clostridial form with phase dark forespore (50 hours); D: phase dark clostridial form with phase bright mature forespore (62 hours); E: phase dark elongated rod (71 hours); F: phase dark elongated rod (84 hours).
Bars: 10 µm.



initial washout of cells by 37 hours the cell population comprised mainly cell debris and spores; the colony forming units had decreased to less than 2% from the 16% which were present at the end of the batch culture. Granulose negative rods were infrequently observed. During acid production between 37 hours and 44 hours, there was a rapid increase in the number of vegetative rods (Figure 5.2A). At 44 hours, when the solvent concentration began to increase, granulose accumulation could be detected in over 90% of these rods (not shown). The increase in solvent concentration coincided with the reduction in cell division and the onset of forespore septa development (Figures 5.2B and 5.2C). Between 44 hours and 50 hours, the rate of cell division exceeded the combined rates of forespore septum formation and cell washout, hence the number of rods increased (Figure 5.2A). At 50 hours however, the rate of cell division was less than the combined rates of washout and sporulation. Consequently, the number of rods began to decrease. As fewer rods were available in which forespore development could occur, the number of cells containing forespore septa decreased simultaneously (Figure 5.2B) although their percentage continued to increase to a maximum of 40% (Figure 5.2C). The decrease of cells with forespore septa was also due in part to the formation of mature forespores (17% maximum) and the liberation of free spores (3% maximum) (Figure 5.2C). The washout of the sporulating forms and free spores was complete by 77 hours. A second increase in vegetative rods (Figure 5.2A) coincided with the second increase in acid concentration between 62 hours and 71 hours and a rapid increase in granulose accumulation coincided with the increase in solvent concentration at 71 hours. However, the development of forespore septa was not observed during the continuation of continuous operation. Instead, during the periods of solvent production, rod elongation was observed. The morphological distinction between these two

forms associated with solvent production is shown in Plate 5.2.

5.112 Extended Culture at a Low Dilution Rate In a separate experiment, the changes in growth, physiology and morphology, subsequent to the complete washout of sporulating cells and free spores, were monitored for a period of 5,7 volume changes at a dilution rate of 0,1 hr^{-1} (Figure 5.3). The time from which the culture was monitored was arbitrarily chosen and set to 0 hours. An increase in the solvent concentration from 0 hours resulted in an increase in glucose and acid consumption as well as a decrease in the total cell number. This decrease in cell number was followed by a decrease in the solvent concentration and glucose consumption. Between 15 hours and 24 hours the cell number and acid concentration increased. At 24 hours the solvent concentration again began to increase as did the glucose and acid consumption. The cycle was then repeated. A straight line fit through these cycles shows either a positive or a negative trend. Hence, at these times the mean values of the parameters were still varying.

The periods during which the production of acids and solvents predominated were mirrored in the product yields (Figure 5.4). The maximum solvent yield of 0,31 and the maximum acid yield of 0,03 were related to the maximum solvent concentration at 9 hours and maximum acid concentration at 24 hours respectively.

The corresponding morphological and cytological changes were also monitored during this period (Figure 5.5). During the initial increase in solvent concentration the granulose positive rods predominated. However, after the increase in acid concentration at 15 hours the number of granulose negative rods increased above those showing granulose accumulation. Also during the first 15 hours, the

PLATE 5.2: Photomicrographs of iodine stained cells of C. acetobutylicum P 262 showing the development of the actively dividing rod into distinct morphological forms associated with solvent production. A: actively dividing rod showing granulose accumulation. B: clostidial form showing granulose accumulation and unstained forespore. C: clostidial form showing granulose accumulation and unstained mature forespore. D, E: elongated rods, some showing granulose accumulation.

Bars: 1 µm.

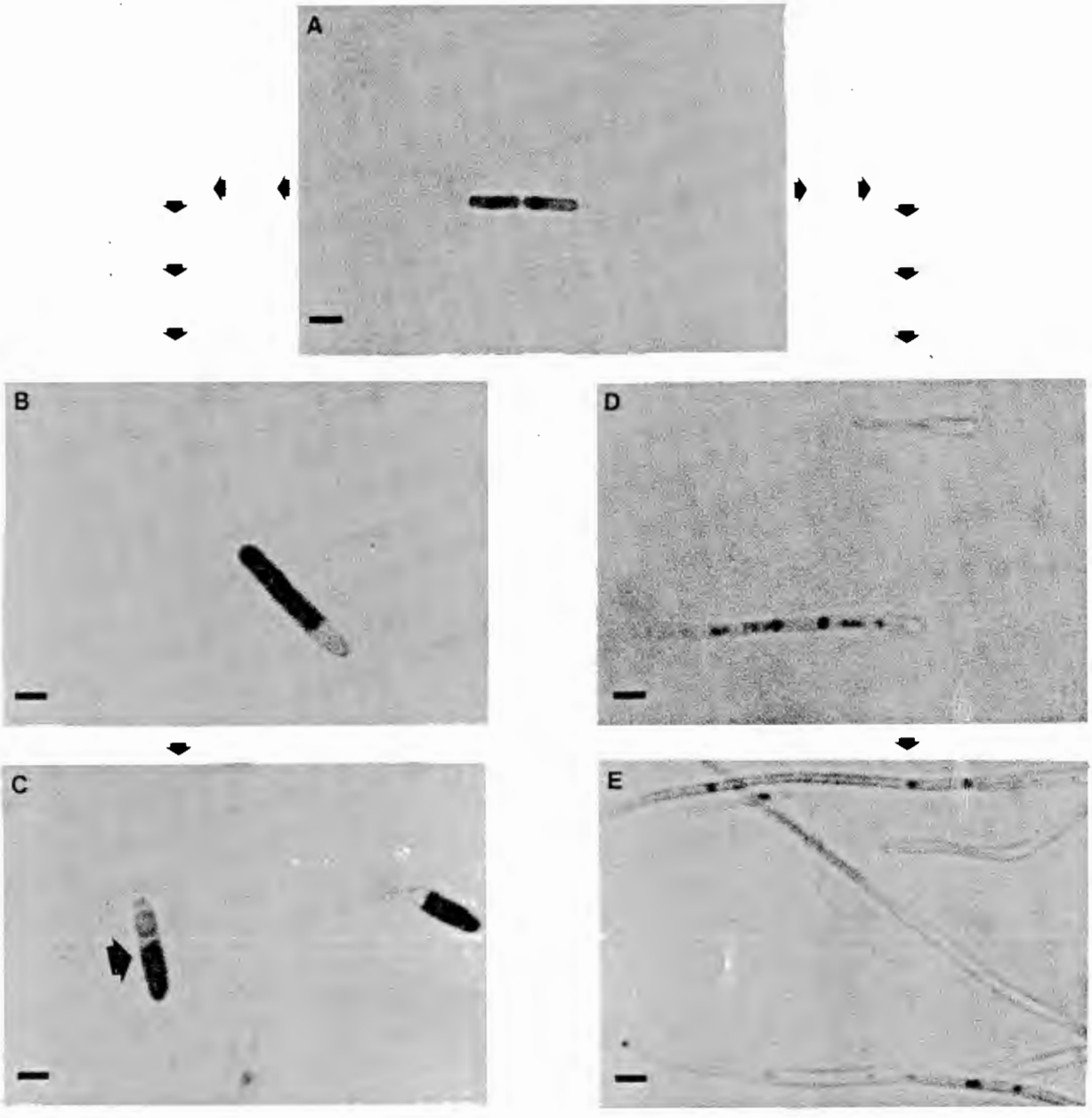


FIGURE 5.3: Growth and physiological changes in C. acetobutylicum P 262 during extended continuous fermentation at a dilution rate of 0,1 hr^{-1}. A: ○, cell dry mass; ●, total cell count. B: ◇, glucose; ○, total solvents; , total acids. C: ○, butanol; ●, acetone; □, ethanol; ◇, butyrate; ◆, acetate.

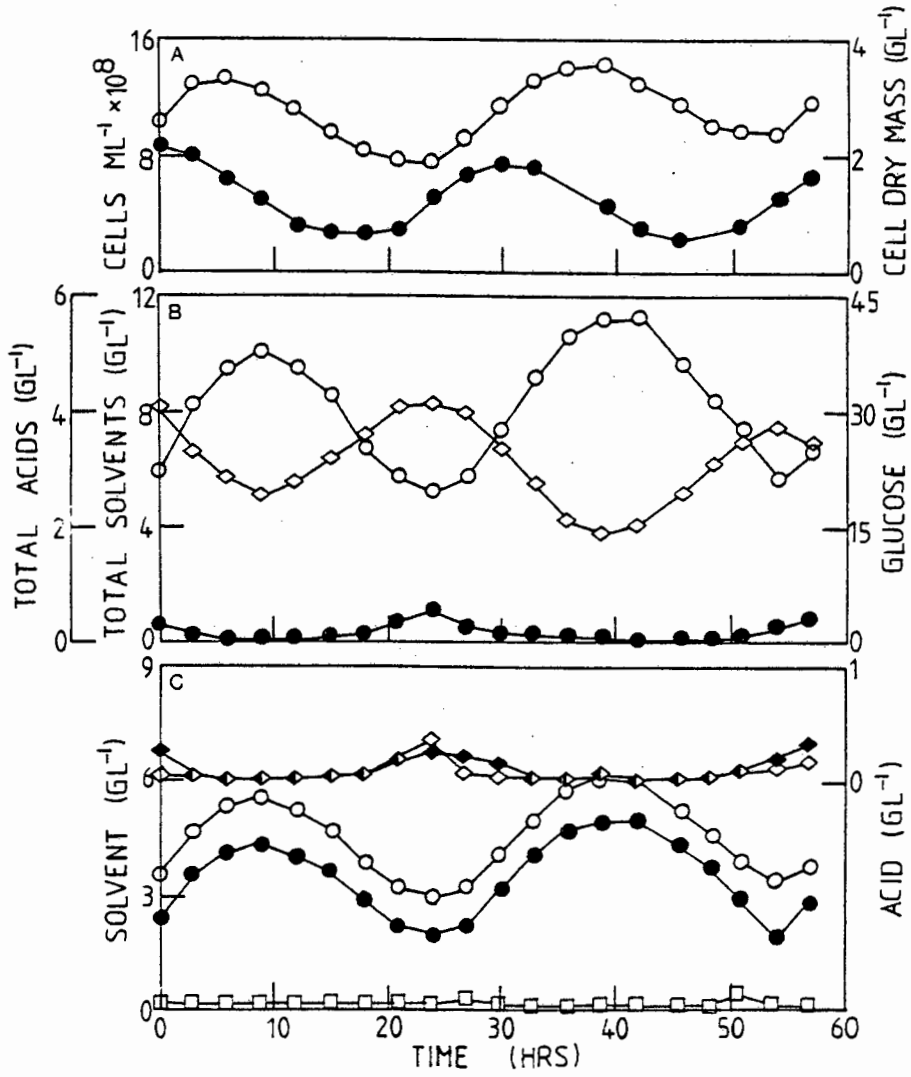


FIGURE 5.4: Product yields during extended continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}. ○ , total solvent yield; ● , total acid yield.

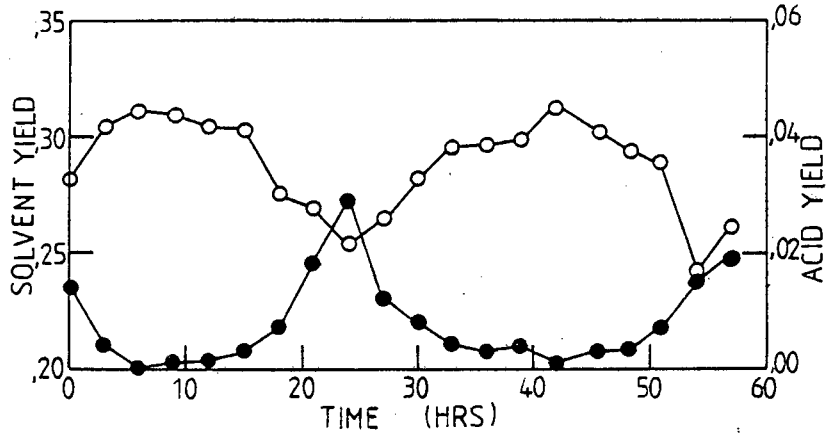


FIGURE 5.5: Morphological and cytological changes in C. acetobutylicum P 262 during extended continuous fermentation at a dilution rate of 0,1 hr^{-1}. A: ○ , granulose positive rods; , granulose negative rods. B: ○ , short (1-2 units) rods; ● , medium (2-6 units) rods; ◇, long (>6 units) rods. C: Percentage of rods which are: ○, short (1-2 units); ●, medium (2-6 units); ◇, long (>6 units).

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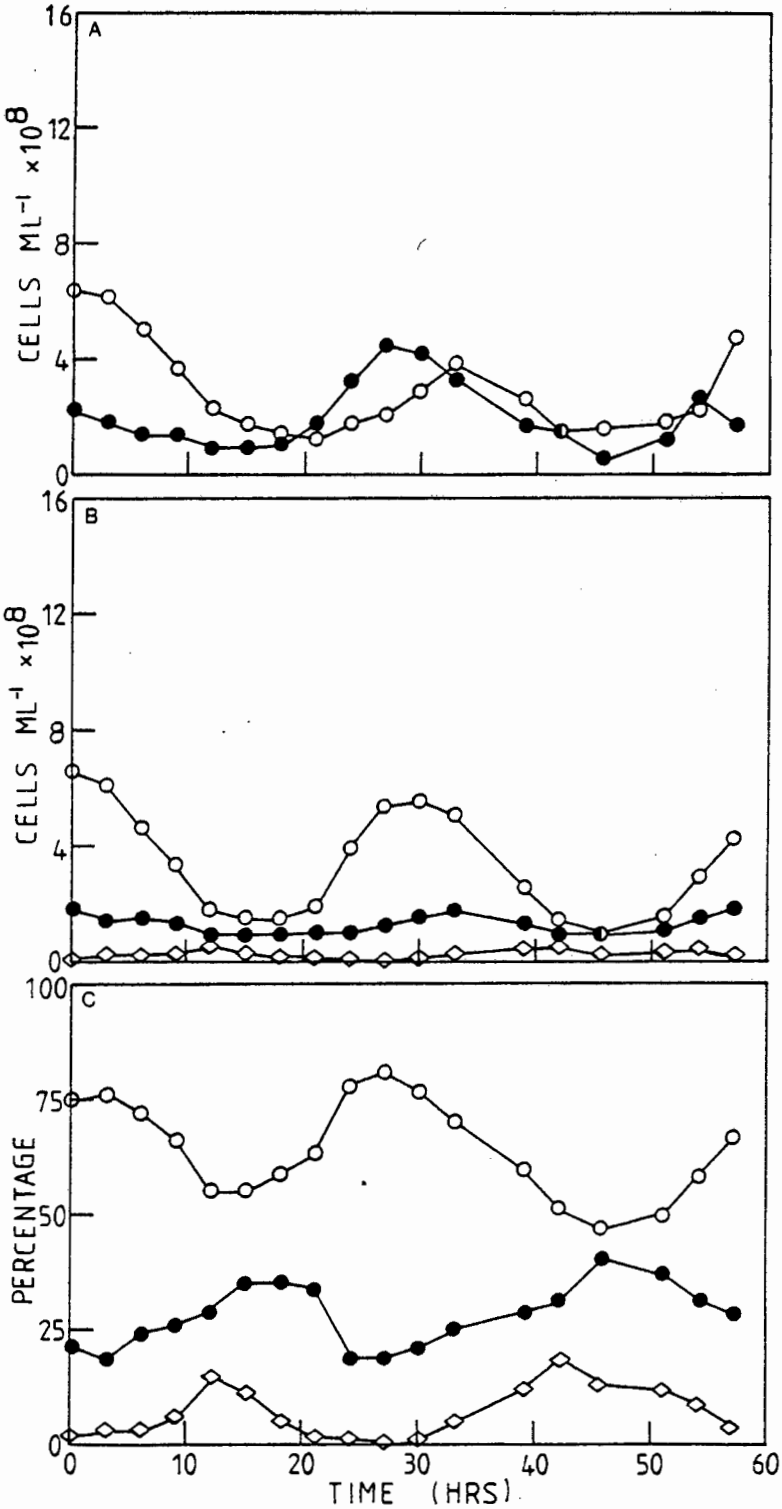

A
B
C
CELLS ML⁻¹ ×10⁸
PERCENTAGE
TIME (HRS)
0
4
8
12
16
25
50
75
100
10
20
30
40
50
60

number and percentage of short rods (1 - 2 unit lengths) decreased while the percentage of medium rods (2 - 6 unit lengths) and long rods (> 6 unit lengths) increased, indicating a decrease in cell division and an increase in cell elongation. This resulted in a rate of cell washout exceeding that of new cell production and a decrease in the total cell number occurred. Conversely, the increase in acid concentration which began at 15 hours, was associated with an increase in the number and percentage of short rods and a concurrent decrease in the percentage of longer rods, indicating an increase in cell division. A corresponding increase in total cell number was observed.

The increase in cell division was confirmed by the microscopic observation of the formation of division septa. The presence of division septa was not observed at the peak of solvent concentration at 9 hours. By 24 hours, at the peak of acid concentration, the percentage of cells showing division septa had increased to 2%. The relationship between cell division and product formation is supported by the coincidence of the maximum overall specific rate of cell division with peak acid concentration and the minimum specific rate of cell division with peak solvent concentration (Figure 5.6). The specific rate of cell division was calculated from the cell numbers as outlined in Appendix B.

The shifting of the pattern of cell growth from predominantly dividing cells to elongating cells resulted in a variation of the overall specific growth rate (Figure 5.7). The specific rate of growth was calculated from the cell dry mass as outlined in Appendix B. An increase in short rods coincided with an increase in specific growth rate and a decrease in short rods coincided with a decrease in this rate. The specific rates of growth and division both oscillated about the dilution rate with values of $0,102 \pm 0,044\ hr^{-1}$ and $0,099 \pm 0,095\ hr^{-1}$ respectively.

FIGURE 5.6: Specific rate of cell division during extended continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}. S = solvent peak. A = acid peak.

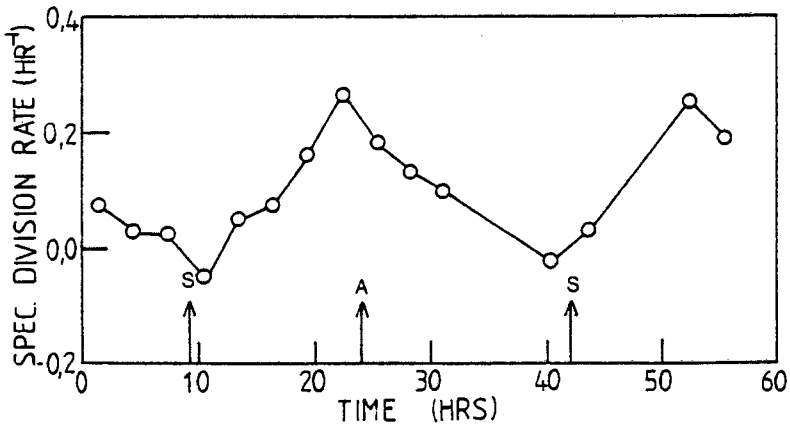
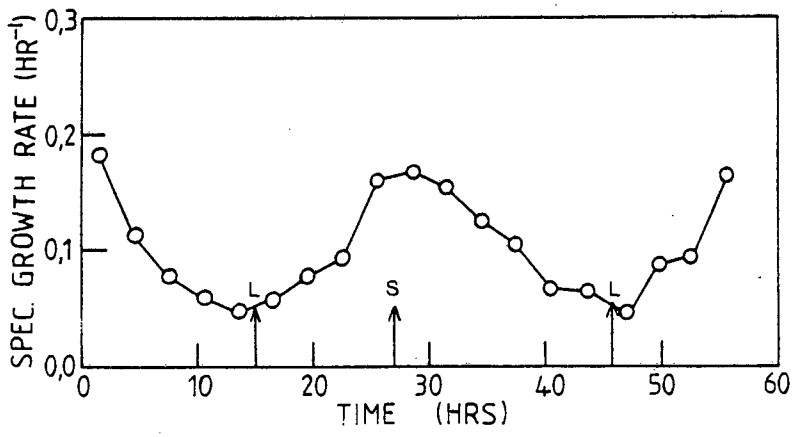


FIGURE 5.7: Specific rate of growth during extended continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}. S = maximum percentage short rods. L = maximum percentage medium plus long rods.



The corresponding changes in the redox potential of the fermentation broth were also monitored (Figure 5.8). The increase in redox potential between 10 hours and 23 hours corresponded with an increase in the specific rate of cell division and concurrent increase in the acid yield. The decrease in the redox potential at 23 hours corresponded with a decrease in the specific rate of cell division and concurrent increase in the solvent yield. The levelling of the redox potential at 30 hours was associated with the decrease in cell numbers at this time caused by the decrease in the specific rate of cell division. The redox potential began to increase again once the specific rate of cell division and acid yield increased.

5.113 Extended Culture at a High Dilution Rate In a similar experiment, the continuous culture was operated at a dilution rate of 0,1 hr^{-1} until all the sporulating forms were washed out. The dilution rate was then increased in steps to 0,25 hr^{-1} and the changes in growth, physiology and morphology were monitored for a period of 7,3 volume changes at this dilution rate (Figure 5.9). The time from which the culture was monitored was arbitrarily chosen and set to 0 hours. The trends observed at this dilution rate were similar to those observed at the lower dilution rate of 0,1 hr^{-1}. The solvent yields of 0,26 to 0,30 attained at this dilution rate (not shown) were also similar to those reported at the lower dilution rate. The oscillation of the parameters at this dilution rate were, however, of a much lower amplitude. This is shown by the lower standard deviation and higher regression coefficient of the product yields and specific growth rate at this higher dilution rate (Table 5.1).

The corresponding morphological and cytological changes were also monitored (Figure 5.10). Again, the trends were similar to those observed at the dilution rate of 0,1 hr^{-1}. The number of rods showing granulose accumulation

FIGURE 5.8: Relationship of redox potential with specific division rate, product yields and cell numbers during extended continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}. A: O, redox potential; ●, specific division rate. B: O, total solvent yield; , total acid yield. C: O, total cell count.

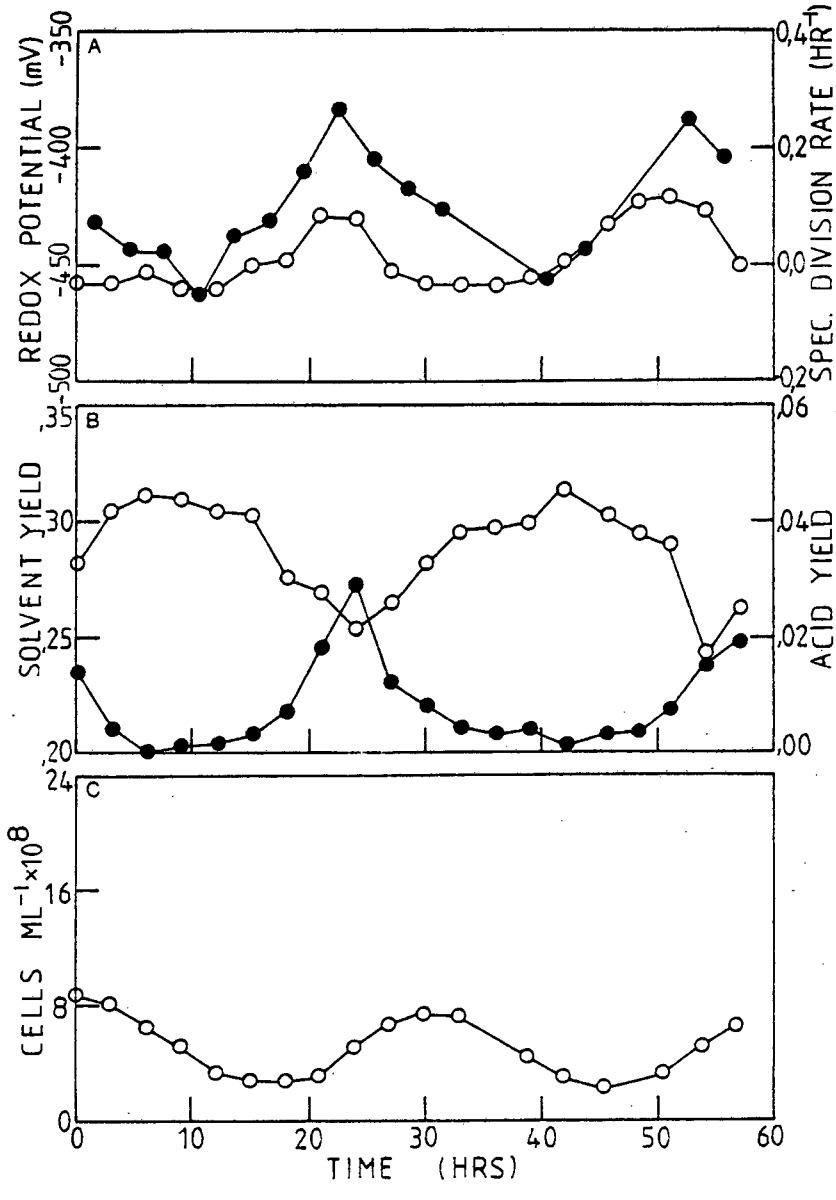


FIGURE 5.9: Growth and physiological changes in C. acetobutylicum P 262 during extended continuous fermentation at a dilution rate of 0,25 hr^{-1}. A: ○, cell dry mass; ●, total cell count. B: ◇, glucose; ○, total solvents; ●, total acids. C: ○, butanol; ●, acetone; □, ethanol; ◇, butyrate; ◆, acetate.

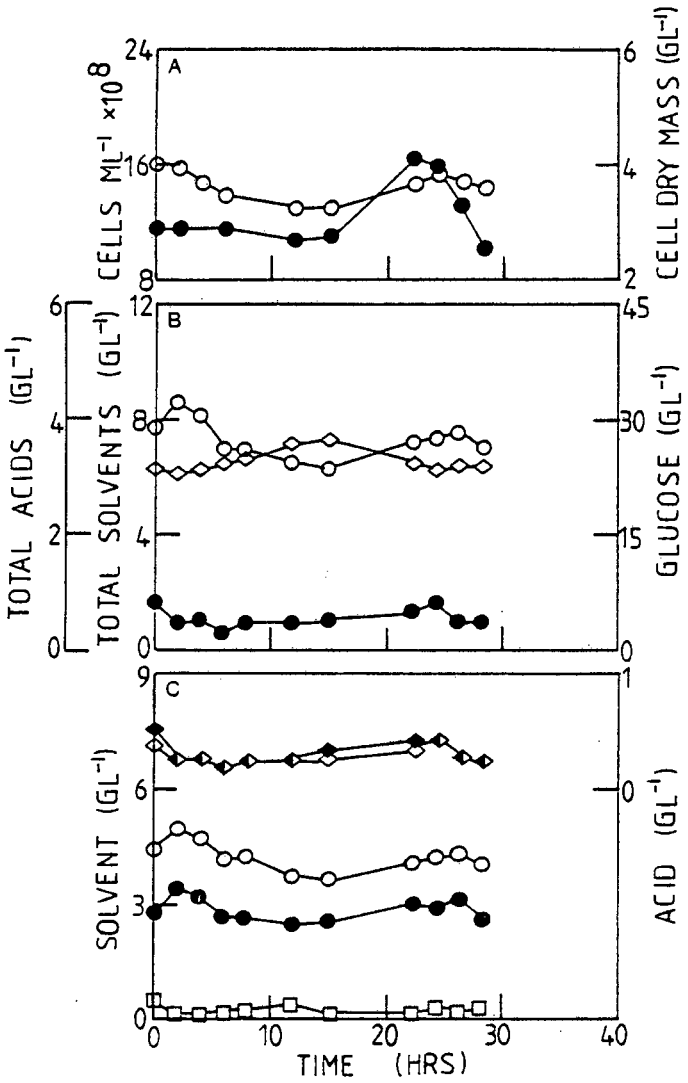


FIGURE 5.10: Morphological and cytological changes in C. acetobutylicum P 262 during extended continuous fermentation at a dilution rate of 0,25 hr^{-1}. A: ○ , granulose positive rods; ● , granulose negative rods. B: ○ , short (1-2 units) rods; ● , medium (2-3 units) rods; ◇ , long (>3 units) rods. C: Percentage of total rods which are: ○ , short (1-2 units); , medium (2-3 units); ◇ , long (>3 units).

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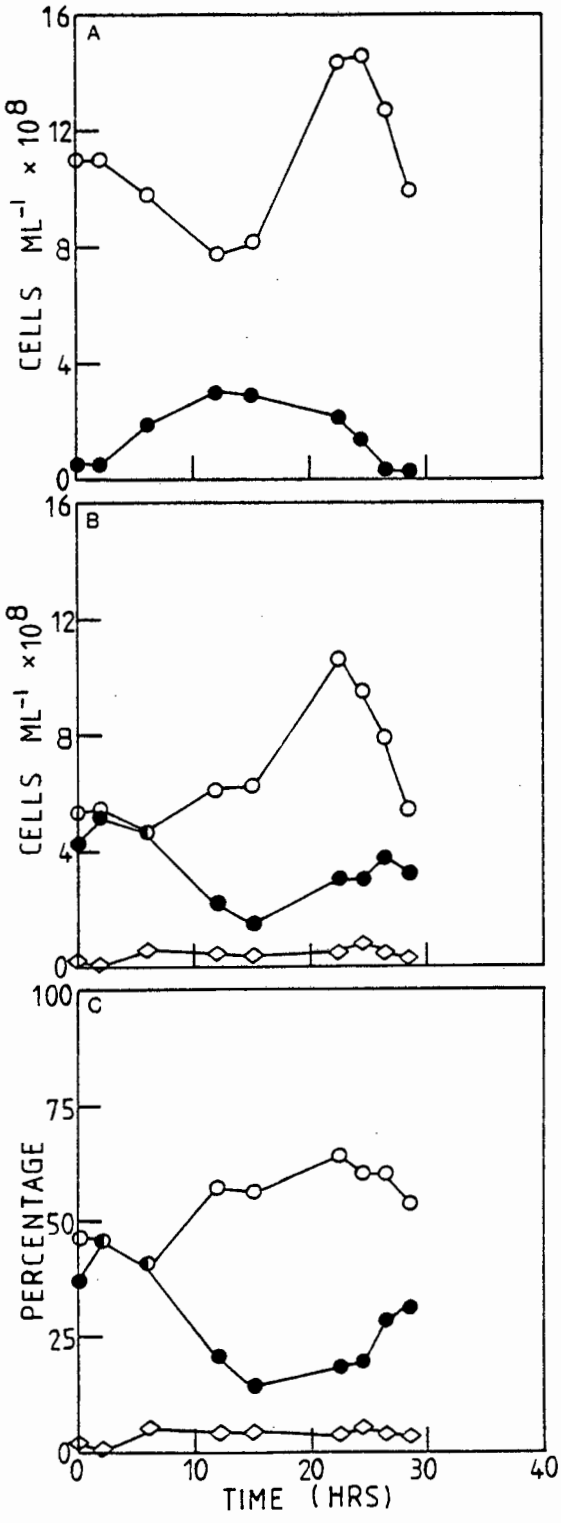
A
B
C
CELLS ML^{-1} x 10^8
CELLS ML^{-1} x10^8
PERCENTAGE
TIME (HRS)
0
4
8
12
16
25
50
75
100
10
20
30
40

was highest at the peaks of solvent concentration and lowest at the trough of solvent concentration. During increasing acid concentration the percentage of short rods (1-2 unit lengths) increased whereas during increasing solvent concentration, the percentage of longer rods (>2 unit lengths) increased. The average rod length observed throughout the fermentation was, however, shorter than that observed at the lower dilution rate and a higher percentage of cells showed division septa (9% - 26%).

TABLE 5.1 Variation of product yields and specific growth rate

D (hr^{-1})	standard deviation (%)			regression coefficient		
	$Y_{TS/S}$	$Y_{TA/S}$	μ_g	$Y_{TS/S}$	$Y_{TA/S}$	μ_g
0,10	7	100	43	0,316	0,138	0,016
0,25	5	27	8	0,491	0,210	0,530

5.12 Genetic Stability During Continuous Culture

During the course of several separate experiments, samples were removed from continuous cultures operated at different dilution rates (0,1 hr^{-1} and 0,25 hr^{-1}) and a pH of 5,0 and plated onto solid culture media. The samples were removed just prior to complete washout of all sporulating forms as well as in the absence of sporulating cells after 5 to 23 volume changes had occurred. The colonies produced after subculture still exhibited normal colonial morphology and patterns of differentiation which resulted in the production of mature spores. Cell morphology was

microscopically indistinguishable from that of the original strain.

In addition, a volume of broth was removed from a continuous culture after more than 10 volume changes had taken place at a dilution rate of 0,25 hr^{-1} and was used as an inoculum for a batch fermentation. The results were similar to those obtained from a batch culture started with a spore inoculum (Table 5.2).

TABLE 5.2: Comparison of growth and final concentrations in batch cultures started with a spore inoculum (A) and an inoculum extracted from a continuous culture (B)

	batch A	batch B
glucose (gl^{-1})	0,2	0,1
solvents (gl^{-1})	14,7	14,6
acids (gl^{-1})	0,5	2,4
μ_g(max) (hr^{-1})	0,45	0,40
$Y_{TS/S}$	0,30	0,28
carbon recovered (%)		
: in acids	1	5
: in solvents	48	45

5.13 Sporulation During Continuous Culture

Sporulation was not detected during extended continuous culture at a pH of 5,0 during any of the dilution rates investigated in this study, namely 0,05 hr^{-1}, 0,10 hr^{-1}, 0,15 hr^{-1}, 0,20 hr^{-1}, 0,25 hr^{-1}, 0,30 hr^{-1} and 0,35 hr^{-1}. The decrease in cell division in all

cases manifested as cell elongation. At the lower dilution rates, a proportionately larger amount of elongation was observed. This is evident in the photomicrographs of the cell morphology during extended continuous culture at several different dilution rates (Plate 5.3).

However, the induction of a step change in the pH of an extended continuous culture at a dilution rate of 0,10 hr^{-1} from 5,0 to 5,2, resulted in the appearance of sporulating cells (Figure 5.11). Again the time from which the culture was monitored was arbitrarily chosen and set to 0 hours.

Four hours after the step change in the pH, the formation of forespore septa was observed. During the following 6 hours, both the total number (Figure 5.11A) and the percentage (Figure 5.11B) of sporulating cells increased. The number of sporulating cells reached a maximum at 38 hours after which a decrease in these cells was observed although their percentage continued to increase until 46 hours. This was similar to the trend observed during the start up period of continuous culture. Mature forespores and free spores were not detected.

The corresponding growth and physiological changes are detailed in Figure 5.12. The pH was increased at the time of minimum cell number, just prior to the resumption of acid production. The transition from acid to solvent production occurred 4 hours later and coincided with the time at which sporulation was first detected. Acid production resumed at 46 hours, occurring simultaneously with the observed decrease in the percentage of sporulating cells.

A detailed examination of the morphology and cytology is shown in Figure 5.13. The pattern of the number and percentage of different rod lengths after the change in pH

PLATE 5.3: Photomicrographs of the phase dark rods of C. acetobutylicum P 262 present during continuous culture at a pH of 5,0. A: Dilution rate = 0,1 hr^{-1}. B: Dilution rate = 0,15 hr^{-1}. C: Dilution rate = 0,20 hr^{-1}. D: Dilution rate = 0,25 hr^{-1}. E: Dilution rate = 0,30 hr^{-1}. F: Dilution rate = 0,35 hr^{-1}. Bars: 10 µm.

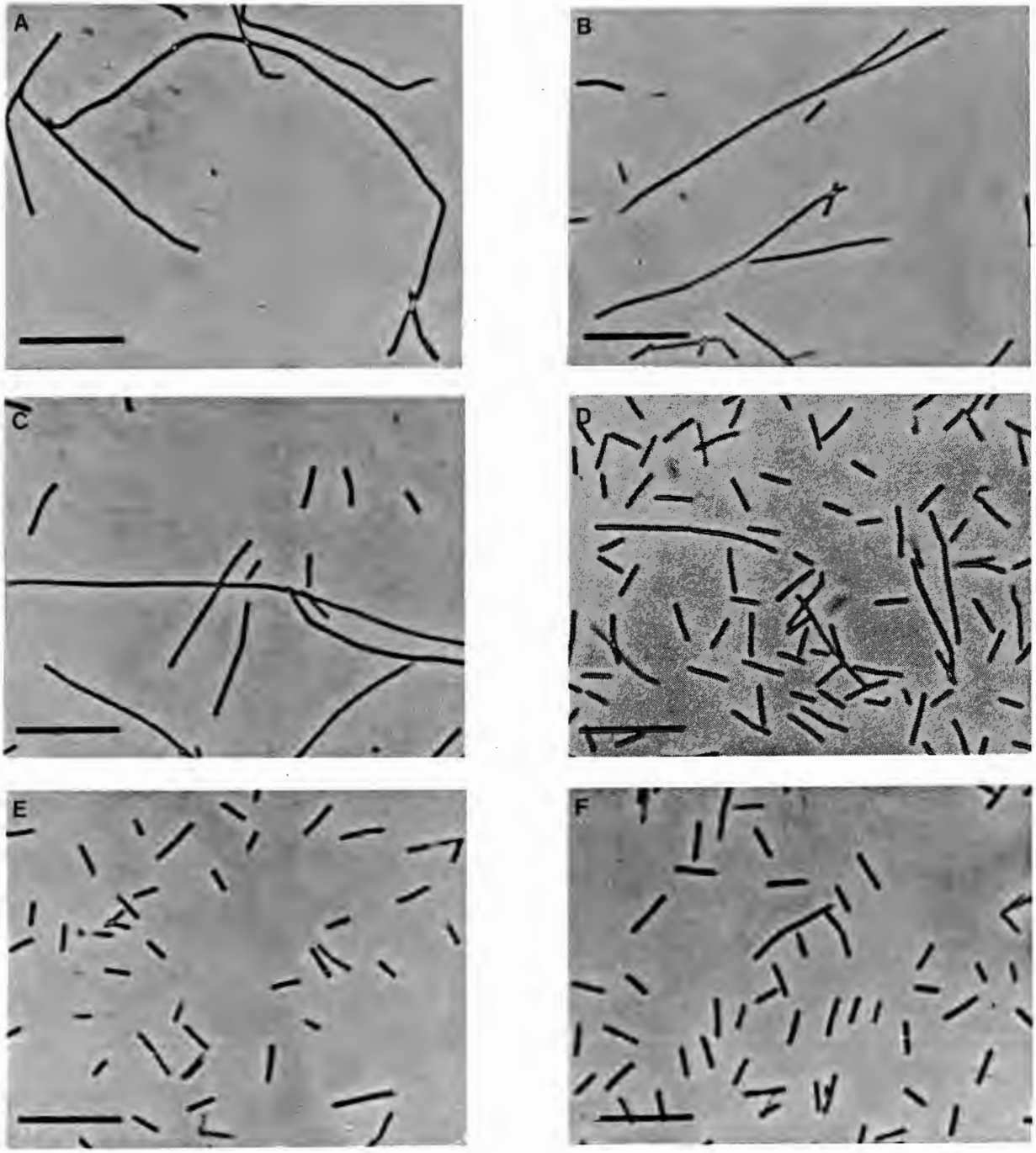

FIGURE 5.11: Morphological changes in C. acetobutylicum P 262 during continuous fermentation at a dilution rate of 0,10 hr^{-1} as the pH is increased by 0,2 units (indicated by arrows). A: Forespore septum count: ○ , total[a] cells; ● , short (1-2 units) cells; ◇ , medium (2-6 units) cells; ◆ , long (>6 units) cells. B: Percentage of cells showing forespore septa: ○ , total[a] cells; ● , short (1-2 units) cells; ◇ , medium (2-6 units) cells; ◆ , long (>6 units) cells.

a = short + medium + long

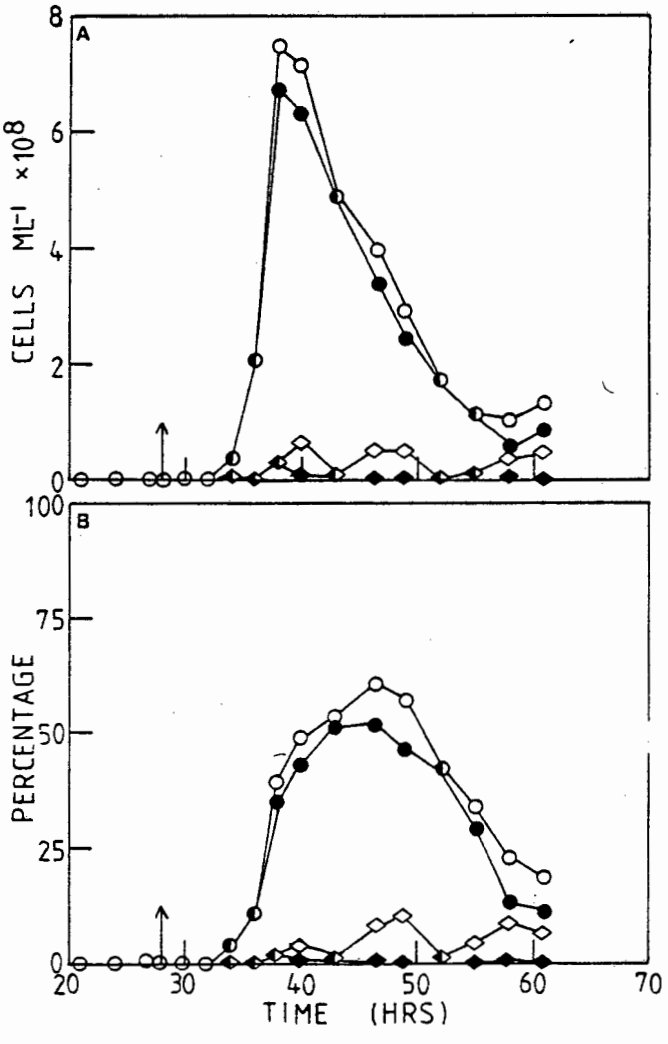

FIGURE 5.12: Growth and physiological changes in C. acetobutylicum P 262 during continuous fermentation at a dilution rate of 0,1 hr^{-1} as the pH is increased by 0,2 units (indicated by arrows). A: ○ , cell dry mass; ● , total cell count. B: ◇ , glucose; ○ , total solvents; ● , total acids. C: ○, butanol; ●, acetone; □, ethanol; ◇, butyrate; ◆, acetate.

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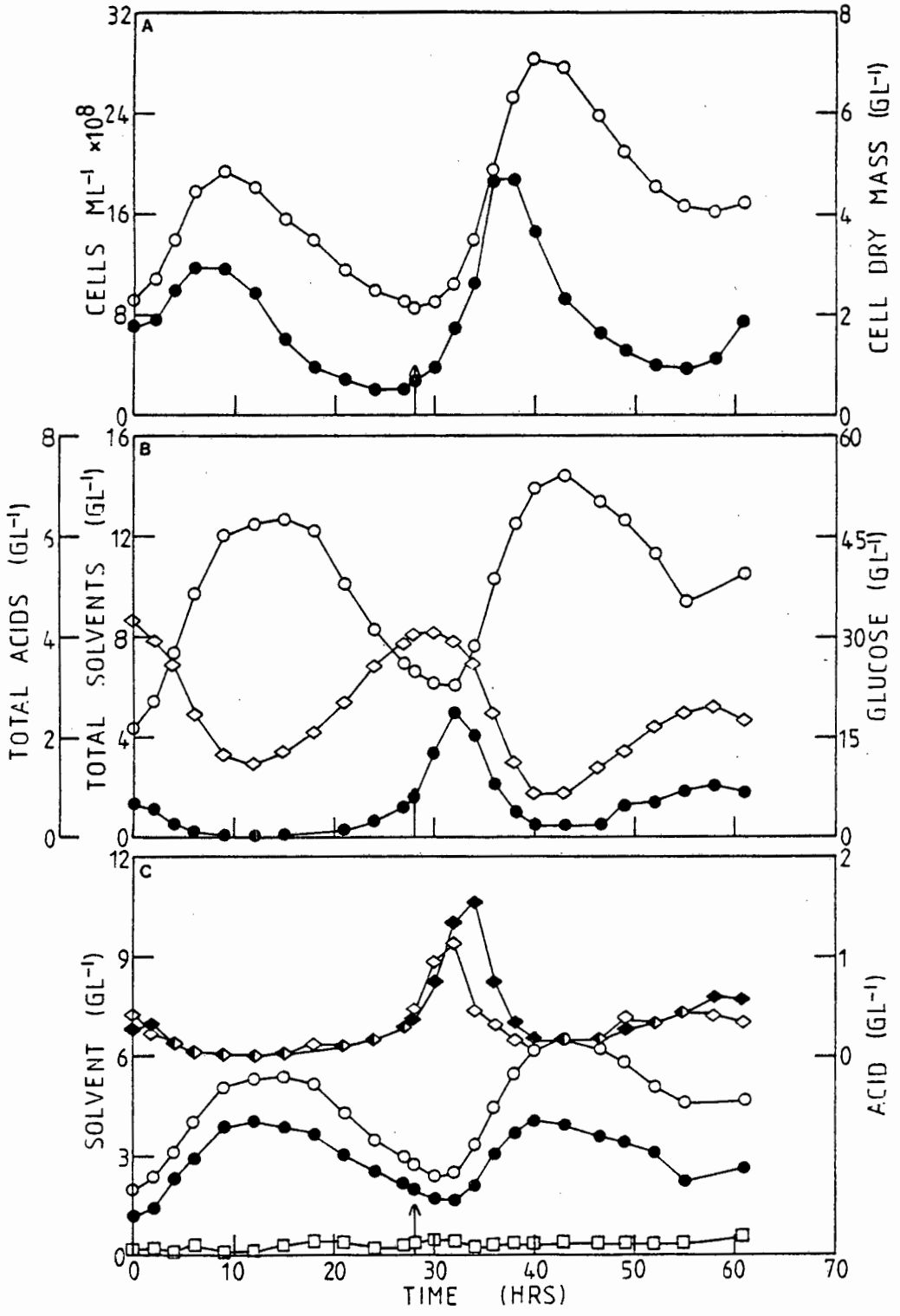
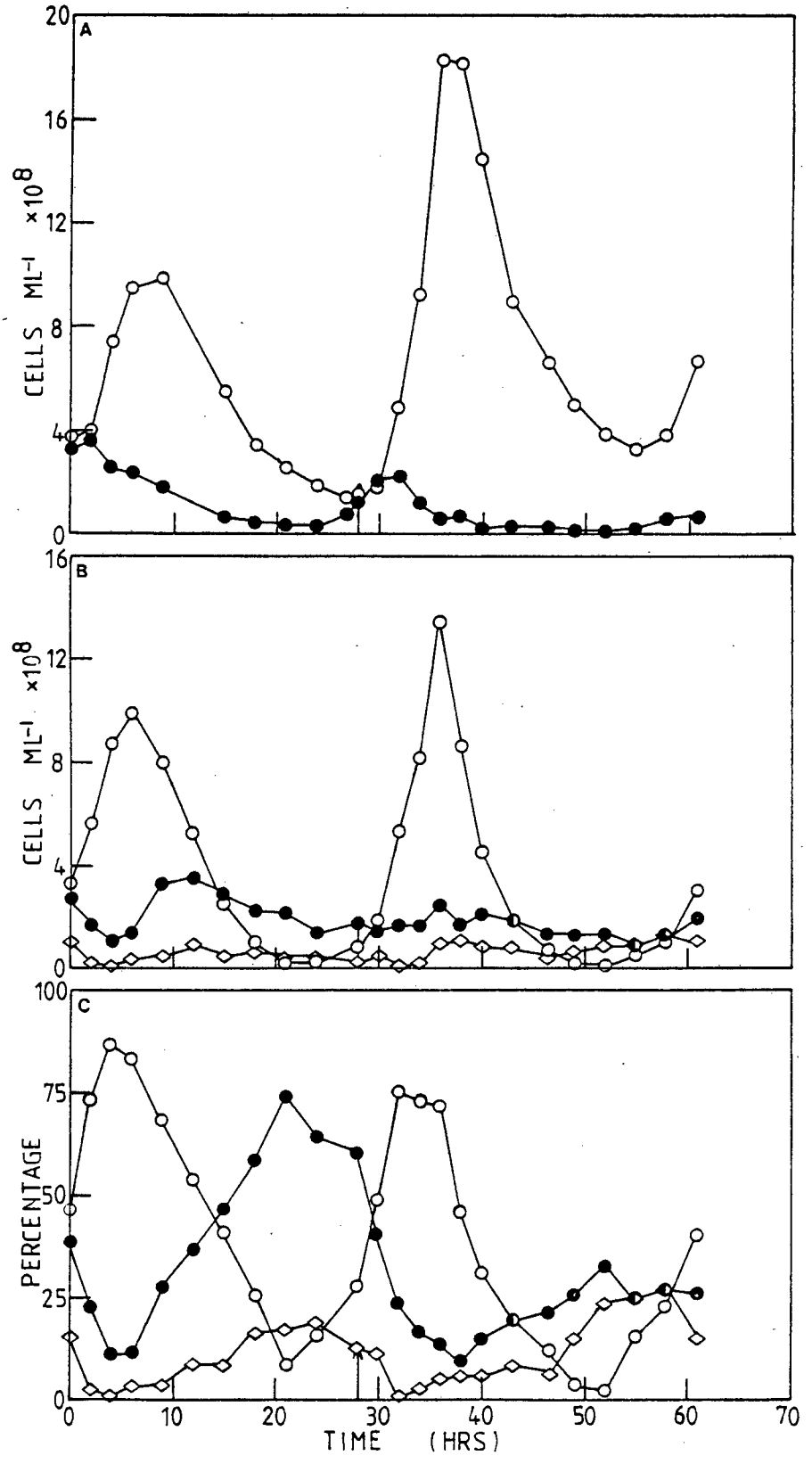

A
B
C
CELLS ML⁻¹ ×10⁸
CELL DRY MASS (GL⁻¹)
TOTAL ACIDS (GL⁻¹)
TOTAL SOLVENTS (GL⁻¹)
GLUCOSE (GL⁻¹)
SOLVENT (GL⁻¹)
ACID (GL⁻¹)
TIME (HRS)
0
10
20
30
40
50
60
70

FIGURE 5.13: Morphological and cytological changes in C. acetobutylicum P 262 during continuous fermentation at a dilution rate of 0,1 hr^{-1} as the pH is increased by 0,2 units (indicated by arrows). A: ○ , granulose positive rods; , granulose negative rods. B: ○ , short (1-2 units) rods; ● , medium (2-6 units) rods; ◇ , long (>6 units) rods. C: Percentage of total rods which are: ○ , short (1-2 units); , medium (2-6 units); ◇ , long (>6 units).

(please see over)


A
B
C
CELLS ML⁻¹ ×10⁸
PERCENTAGE
TIME (HRS)
0
4
8
12
16
20
25
50
75
100
10
20
30
40
50
60
70

was not significantly different from those observed prior to the change. However, granulose accumulation was increased.

For comparison, the effect of pH on sporulation during batch culture was evaluated. Batch cultures were operated with pH control to 5,0 and 6,0 and without pH control, in which the pH decreased to 4,6 (Table 5.3). The onset of sporulation coincided with solvent induction at the lower pH values while at a pH of 6,0, negligible sporulation was observed.

TABLE 5.3: Sporulation during batch culture

pH control	time[a] (hrs)	time[b] (hrs)	maximum solvents (gl^{-1})	maximum sporulation (%)
none	4	4	14,7	84
5	6	6	14,7	80
6	6	6	10,3	1

a: time of solvent induction
b: time of sporulation induction

5.2 DISCUSSION

During product inhibited continuous culture at a constant dilution rate, oscillations were observed. These oscillations were manifest in several parameters, in particular, the concentration of cells, acids and solvents, the variation of the cell division and morphology and the value of the specific rates of growth and division.

The oscillations in the acid and solvent concentrations were accompanied by related oscillations in the specific

growth rate. The increase in the acid concentration was associated with an increase in the specific growth rate and conversely, a decrease in the acid concentration and corresponding increase in the solvent concentration, correlated with a decrease in the specific growth rate.

These oscillations were also accompanied by cyclic changes in the cell morphology and the rate of cell division. During the phase when the acid concentration and the growth rate was increasing, the proportion of cells undergoing division increased and there was an increase in the number of short rods and total cell numbers in the culture. During the phase when the solvent concentration was increasing and the growth rate was decreasing, the proportion of cells undergoing division decreased and the proportion of elongated rods in the culture increased. The total cell numbers of the culture showed a decrease at the end of this phase. Similar elongated rods were observed during batch and continuous cultures of C. thermosaccharolyticum (Hsu and Ordal, 1970; Landuyt et al, 1983). In their studies, the cell elongation was also associated with solvent production.

The decrease in the specific rates of growth and division associated with the shift from acid to solvent production during continuous culture appeared to be analogous to the decrease in the specific growth rate and cell division associated with the shift from acid to solvent production in batch culture (Peterson and Fred, 1932; Davies and Stephenson, 1941; Jones et al, 1982; Long et al, 1984b). These results suggest that two types of cells coexisted throughout the period of continuous culture: short, actively dividing, acid producing cells with a high growth rate and elongating cells with a low growth rate. The acid and solvent producing cells occurred simultaneously but their proportions varied in the population. From this, it is proposed that the following

sequence of events accounts for the observed oscillations during continuous culture (Clarke et al, in press).

1) During acid formation, the rapidly growing, short, dividing rods bring about an increase in the cell numbers and the acid concentration.

2) The elevated acid concentration causes a large number of these cells to switch their metabolism (Gottschal and Morris, 1981a; Monot et al, 1983; Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985; Huang et al, 1985) and morphology and become solvent producing cells. The solvent producing cells reassimilate the acids and convert them to acetone and butanol (Andersch et al, 1983; Hartmanis et al, 1984). This leads to a decrease in the acid concentration and an increase in the solvent concentration.

3) The solvent producing cells have a lower growth rate than the acid producing cells and will be selectively washed out of the system, resulting in a decrease in the cell numbers and solvent concentration and an increase in the glucose concentration.

4) The acid producing cells, having a higher growth rate than the solvent producing cells, are selectively retained in the system. This leads to an increase in the concentration of acid producing cells.

It is possible that the decrease in the solvent concentration and increase in the residual glucose concentration may also cause the solvent producing cells to revert to acid producing cells, thereby contributing to the increase in the concentration of the acid producing cells.

5) The increase in the concentration of acid producing cells results in the increase in the acid concentration and a repeat of the cycle.

The increase in the acid producing cells due to the selective retention of these cells is supported by continuous culture theory which predicts that in a mixed population of cells of different specific growth rates, the cells with the highest specific growth rate will be retained and the slower growing cells may be washed out (Wang et al, 1979). The data do not indicate whether the increase in the acid producing cells also results from a reversion of solvent producing cells to acid producing cells. Andersch et al (1983) demonstrated that the enzymes catalysing the reactions leading to acid production did not disappear completely during solvent production, suggesting that the reversion to acid producing cells is possible. However, even if the reversion does not occur, the selective retention of the acid producing cells will still lead to oscillatory behaviour.

A basic assumption of this conceptual model is that there is a difference in the specific growth rates of the acid and solvent producing cells. Provided that this difference exists, oscillations will be an inherent characteristic of the system related to the continual shift in the proportion of acid and solvent producing cells. Therefore, although this study was conducted under product limiting conditions, the oscillatory behaviour will be independent of the factor limiting the cell growth. This is supported by the observation of oscillatory behaviour during continuous culture under carbon, nitrogen, phosphate and magnesium limiting conditions (Stephens et al, 1985), the inability to achieve a steady state under carbon and nitrogen limiting conditions (Roos et al, 1985) and under phosphate limiting conditions at low dilution rates (Bahl et al, 1982b) and by the description of the steady states achieved under nitrogen limiting conditions as "approximate" (Monot and Engasser, 1983a; Monot et al, 1983). Oscillatory behaviour during continuous culture is not unique to the acetone butanol fermentation. Harrison

and Topiwala (1974) reported that such behaviour is a frequent characteristic of systems in which a feedback regulation causes a shift from a primary to a secondary metabolism.

Intrinsic oscillatory behaviour during continuous solvent production also implies that oscillations will be observed at all dilution rates. From the experimental data, it is evident that oscillations occurred at both a high and a low dilution rate. The data also indicate, however, that the amplitude of the oscillations were greater at the low dilution rate. This is in accordance with Bahl et al (1982b) who observed unsteady behaviour at dilution rates of less than 0,025 hr^{-1} only, while at the higher dilution rates, steady states were apparently achieved.

The conceptual model does not immediately suggest a reason for the greater amplitude of the oscillations at the lower dilution rate. It is possible, however, that while the presence of oscillations is not dependent on product inhibition, the higher concentrations of butanol attained at the lower dilution rates tends to amplify these oscillations. This effect may be observed during nutrient as well as product limiting continuous culture. During nutrient limited continuous culture Leung and Wang (1981) and Bahl et al (1982b) attained butanol concentrations of over 9 gl^{-1} at the lowest dilution rates investigated under carbon and phosphate limitation respectively. Butanol concentrations of 9 to 16 gl^{-1} have been shown to inhibit cell growth (Leung and Wang, 1981; Costa and Moreira, 1983; Monot et al, 1983; Vollherbst-Schneck et al, 1984). This means that although these continuous culture studies (Leung and Wang, 1981; Bahl et al, 1982b) were carried out under nutrient limiting conditions, it is apparent that, at the lowest dilution rates, the butanol concentration may have contributed to the limitation of the

cell growth. Continuous fermentation in which the cell growth is both a function of a limiting nutrient and an inhibitory product concentration has been reported during ethanol production (Hoppe and Hansford, 1982).

From an examination of the literature of continuous acetone-butanol fermentations in which steady states were apparently achieved, it is evident that in these cultures, acids and solvents were produced concurrently at all dilution rates. As acid and solvent production are unlikely to occur simultaneously in the same cell (Andersch et al, 1983; Hartmanis and Gatenbeck, 1984; Hartmanis et al, 1984; Ballongue et al, 1985), this means that both acid and solvent producing cells coexisted even in these cultures. Furthermore, as acetone formation is dependent on the equimolar uptake of acids (Hartmanis et al, 1984; Ballongue et al, 1985), the continued production of acetone implies that acids must be produced and consumed at approximately the same rate, thus confirming that acid and solvent producing cells were present even when the measured acid concentration was low. Under these conditions, it is unlikely that a true steady state would be achieved and it is suggested that in the continuous culture studies which have reported steady states, apparently steady behaviour was observed as the oscillations in the proportions of the acid and solvent producing cells were not pronounced. The experimentally observed steady behaviour, therefore, does not contradict the proposed model.

In addition to these major features, other cyclic variations were also observed. The oscillations in the acid and solvent concentrations were accompanied by changes in the redox potential. An increase in the redox potential was observed during increasing acid concentration and a decrease in the redox potential with increasing solvent concentration. This may be explained in terms of a shift in the electron flow. The redox potential is a measure of

the relative reduction of the broth and, therefore, a change in the redox potential serves to indicate a change in the electron flow. A change in the electron flow from molecular hydrogen to pyridine nucleotides was observed when acid producing cells were converted to solvent producing cells (Kim and Zeikus, 1984; Kim et al, 1984; Datta and Zeikus, 1985). The oscillation of the redox potential is, therefore, further support for the evidence of the continual shift in the proportion of the acid and solvent producing cells.

The decrease in the proportion of cells undergoing division and the increase in cell elongation was also associated with an increase in the proportion of cells containing granulose. This is in accordance with the increase in granulose accumulation observed during the shift from acid to solvent production in batch culture (Jones et al, 1982; Long et al, 1984b) and, therefore, provides further support for the evidence of the oscillation between the proportion of the cell types.

Physiological deterioration of the organism has been noted during some nutrient limited continuous cultures (Gottschal and Morris, 1981b; Stephens et al, 1985). However, during the course of the experiments in this study, no physiological or morphological deterioration of the culture was observed. Although the initiation of forespore formation was not observed under conditions of prolonged continuous culture at a pH of 5,0, these cells were still able to sporulate when subcultured onto appropriate solid culture media, indicating that the cells remained genetically stable with respect to sporulation. In addition, the ability of the culture to produce solvents was shown to be unchanged after prolonged continuous operation. These results confirm that the oscillatory behaviour was not a consequence of a genetic instability.

While during the continuous culture at a pH of 5,0, the decrease in cell division manifested only as cell elongation and no sporulation was observed, an increase of 0,2 pH units to a pH of 5,2, resulted in the initiation of the development of a forespore septum within the cell during the period of solvent production. The concomitant increase in granulose accumulation observed during solvent production at the higher pH compared with that during solvent production at the lower pH, was associated with the increase in sporulating cells. An increase in granulose accumulation with an increase in sporulation has been reported during batch culture (Long et al, 1984b). Both elongating and sporulating cells are forms associated with a decrease in cell division and consequently, a low specific growth rate. Both forms will be similarly washed out of the system and, therefore, the model of the oscillatory behaviour will apply equally to systems in which elongation or sporulation accompanies solvent production.

It is interesting to note that the initiation of sporulation at the onset of solvent production occurred under product inhibiting conditions, suggesting that sporulation is not dependent on a nutrient starvation. This is in accordance with the results of several studies on the sporulation of Clostridia, reviewed by Woods and Jones (1986), which have suggested that a nutrient starvation does not generally lead to sporulation in these organisms, but that sporulation appears to be favoured by conditions which lead to a decrease in growth rate in the presence of substantial energy and carbon source reserves.

The results also indicate that the pH had a pronounced influence on the initiation of sporulation. However, the effect of pH on sporulation during batch culture was different to that observed during continuous culture, suggesting that the pH was not the only factor affecting

sporulation. Continuous culture could provide a useful means to investigate the factors which may initiate sporulation by monitoring the variation in cell morphology, during the shift from acid to solvent production at a constant dilution rate, after inducing a step change in a single operating variable. However, as it was not the objective of this study to investigate the factors which may influence the initiation of sporulation, this aspect of the study was not pursued further.

CHAPTER 6

RELATIONSHIP BETWEEN THE SPECIFIC GROWTH RATE AND SOLVENT PRODUCTION

A decrease in the specific growth rate accompanying the onset of solvent production has been well documented in numerous batch culture studies. These include the early studies where it was reported that the cell numbers (Peterson and Fred, 1932) and the cell dry weight (Davies and Stephenson, 1941) were constant once solvent production was initiated. More recently, Jones *et al* (1982) and Long *et al* (1984b) quantitatively linked the cessation of cell division to the onset of solvent production during batch culture. This association is supported by those studies in which solvent production was demonstrated to occur in non-growing immobilised cells and cells resuspended in buffer (Häggström, 1979; Häggström and Molin, 1980; Förberg *et al*, 1983; Förberg and Häggström, 1985).

The studies presented in Chapter 5 have shown that a decrease in the specific growth rate also accompanies the shift from acid to solvent production during continuous culture. The low specific growth rate of solvent producing cells, relative to acid producing cells coexisting under these conditions, has been suggested to be a major factor contributing to the observed oscillatory behaviour during continuous operation.

The evidence indicating an association between a decrease in the cell growth and a shift to solvent production suggests that these events may be influenced by

some common regulatory factor. The possible factors leading to solvent induction in C. acetobutylicum have been extensively studied and several authors have suggested that solvent induction is promoted by elevated acid concentrations (see Literature Survey). On the other hand, the factors which may be responsible for the accompanying decrease in the specific growth rate in this fermentation have received considerably less attention. There is some evidence, however, to suggest that the acid concentration may exert a repressive influence on the cell growth, parallel to its inducing influence on solvent production. Costa and Moreira (1983) reported a 50% decrease in exponential growth when acetate or butyrate were added in concentrations which are likely to occur during a normal fermentation. Also, Monot et al (1983) and Monot et al (1984) reported a decrease in the specific growth rate at the end of exponential growth during batch culture at a concentrations of undissociated butyrate of 0,25 gl^{-1} and 0,50 gl^{-1} respectively, irrespective of the culture pH, from which they concluded that the cell growth is affected by these concentrations of undissociated butyrate.

In addition to a possible inhibitory effect of the acids on the cell growth, the decrease in the cell growth may be a consequence of the shift to solvent production. This is suggested by the decrease in energy generation accompanying the shift to solvent production. During acid production, 3,3 moles of ATP (Thauer et al, 1977) or 3,5 moles of ATP (Gottschalk and Bahl, 1981) are generated per mole of glucose while during solvent production, only 2,0 moles of ATP are generated per mole of glucose and, therefore, there may not be sufficient energy to maintain the same rate of biosynthesis and cell growth.

Since the decrease in the specific growth rate appears to be a key feature of the oscillatory behaviour during continuous culture, the factors which might affect this

rate were investigated. In this chapter, the examination of the possible roles of acid accumulation and solvent production on cell growth, have been described.

In order to distinguish the effect of acid accumulation on the cell growth, it was advantageous to examine the growth patterns during fermentations in which significant solvent production did not occur. In these fermentations, the acid levels which would result in growth inhibition could be ascertained. Comparison of these levels with the acid concentration at which the cell growth decreases in a normal solventogenic fermentation, would indicate whether the growth was similarly inhibited by acids under these conditions.

The study of the acid inhibition of cell growth in the absence of solvent production was facilitated by the use of a mutant strain which was unable to produce solvents. This mutant, called the cls$^-$ mutant, was derived from the solventogenic P 262 strain. While the P 262 strain exhibited a shift from acid to solvent production, accompanied by the accumulation of granulose and the conversion of the vegetative cell to the clostridial form in which forespores subsequently developed (Jones *et al*, 1982; Long *et al*, 1984b), in the cls$^-$ mutant, both the metabolic events of solvent production and granulose accumulation and the differential events of clostridial formation and sporulation, were absent (Long *et al*, 1984a).

As the factors influencing the specific growth rate have not yet been established in batch culture, it was considered expedient to first examine the factors under these conditions. Moreover, the use of batch culture for these experiments is appropriate because of the time dependent nature of the decrease in the specific growth rate.

6.1 RESULTS

6.11 Measurement of Cell Growth

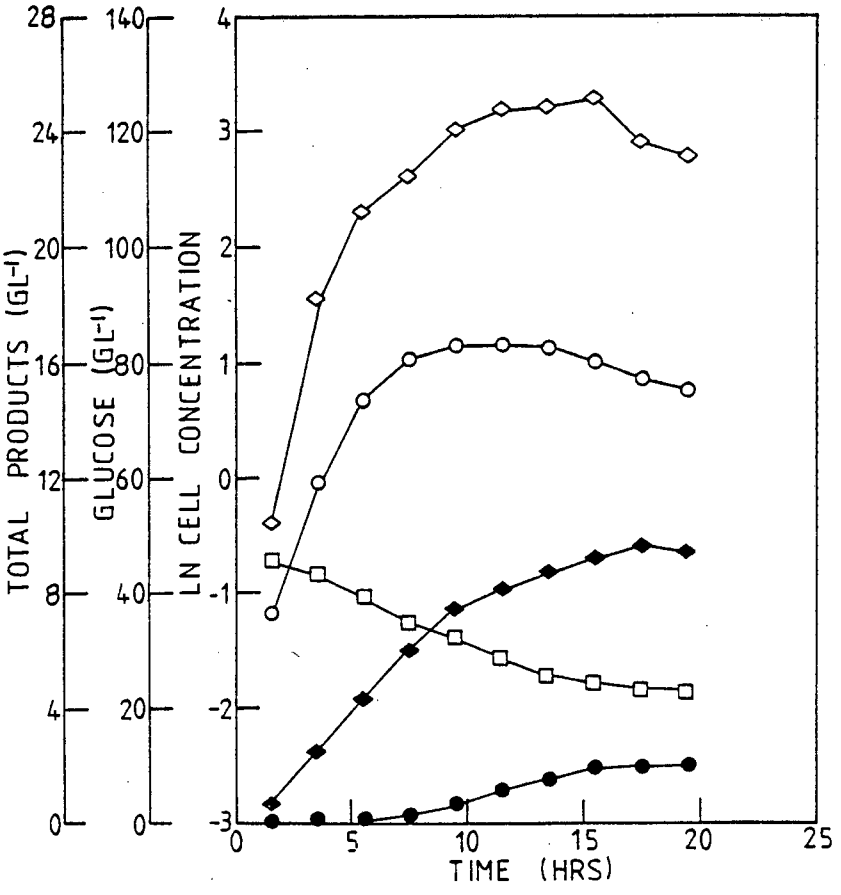
The cell growth was measured in batch cultures of the cls^- mutant strain and the P 262 strain at various pH values. As cell growth may occur by an increase in cell mass and by an increase in cell numbers, both the cell mass and the cell numbers were determined (see Methods and Materials). In these cultures, the cell dry mass was estimated from a standard curve relating the mass to the transmission of light through the culture. Since the standard curve was determined using cultures in which rods only were observed, the estimation may not be accurate during solvent production in the cultures of the P 262 strain at pH values of 5,0 and 4,6 when the formation of the clostridial stage and sporulation normally occurred. However, in all the experiments, the cell dry mass and the cell counts followed parallel trends so that the initial decrease in the specific growth rate was observed at the same time, irrespective of the manner in which the cell concentration was determined. Consequently, either curve could be used to examine the pattern of cell growth during fermentation.

The cell dry mass data were used in each case for the calculation of the maximum specific growth rate.

6.12 Influence of Acid Accumulation on the Cell Growth

6.121 Cell Growth During Acid Fermentation The fermentation-time curves of a batch culture of the cls^- mutant with pH control to 5,0 are shown in Figure 6.1. Initially, the cells grew exponentially with a specific growth rate of 0,46 hr^{-1} ($R = 0,999$), the acid concentration increased and the glucose concentration decreased. When the acid concentration reached 4,3 gl^{-1},

FIGURE 6.1: Growth and physiological changes in C. acetobutylicum cls⁻ during batch fermentation at pH 5.0. ○ , ln(cell dry mass); ◇ , ln(total cell count.10^{-8}); □ , glucose; ● , acetone plus butanol; ◆ , acetate plus butyrate.



a decrease in the specific growth rate was observed. At this stage, only 13% of the glucose had been utilised.

As more glucose was converted to acid, the specific growth rate continued to decrease. At an acid concentration of 8,7 gl^{-1}, further growth ceased. The acid concentration increased marginally to a maximum of 9,7 gl^{-1} during which time cell lysis was observed. A residual glucose concentration of 23,2 gl^{-1} was measured. Solvents were not produced in significant amounts.

The fermentation-time curves of a batch culture of the cls^{-} mutant with pH control to 6,0 are shown in Figure 6.2. During exponential growth, a relatively higher specific growth rate of 0,53 hr^{-1} (R = 0,985) was observed. The decrease in the specific growth rate occurred when the acid concentration reached 8,6 gl^{-1}, at which stage 23% of the glucose had been utilised.

The acid concentration reached 18,5 gl^{-1} before cell growth ceased and increased slightly to a maximum of 20,3 gl^{-1}. However, in this culture, the end of cell growth coincided with glucose exhaustion and cell lysis was only observed after glucose depletion had occurred. Negligible amounts of solvents were produced.

6.122 Cell Growth During Solvent Fermentation The results obtained from a batch culture of the P 262 strain with pH control to 5,0 are shown in Figure 6.3. The specific growth rate of 0,49 hr^{-1} (R = 0,998) was similar to that of 0,46 hr^{-1} observed during the culture of the cls^{-} mutant at the same pH. Also, a similar initial increase in the acid concentration and decrease in the glucose concentration was observed. However, in this fermentation, solvent production was induced when the acid concentration reached 2,3 gl^{-1}. The decrease in the specific growth rate was observed 2 hours later, at an acid

FIGURE 6.2: Growth and physiological changes in C. acetobutylicum cls⁻ during batch fermentation at pH 6.0. ○ , ln(cell dry mass); ◇ , ln(total cell count.10^{-8}); □ , glucose; ● , acetone plus butanol; ◆ , acetate plus butyrate.

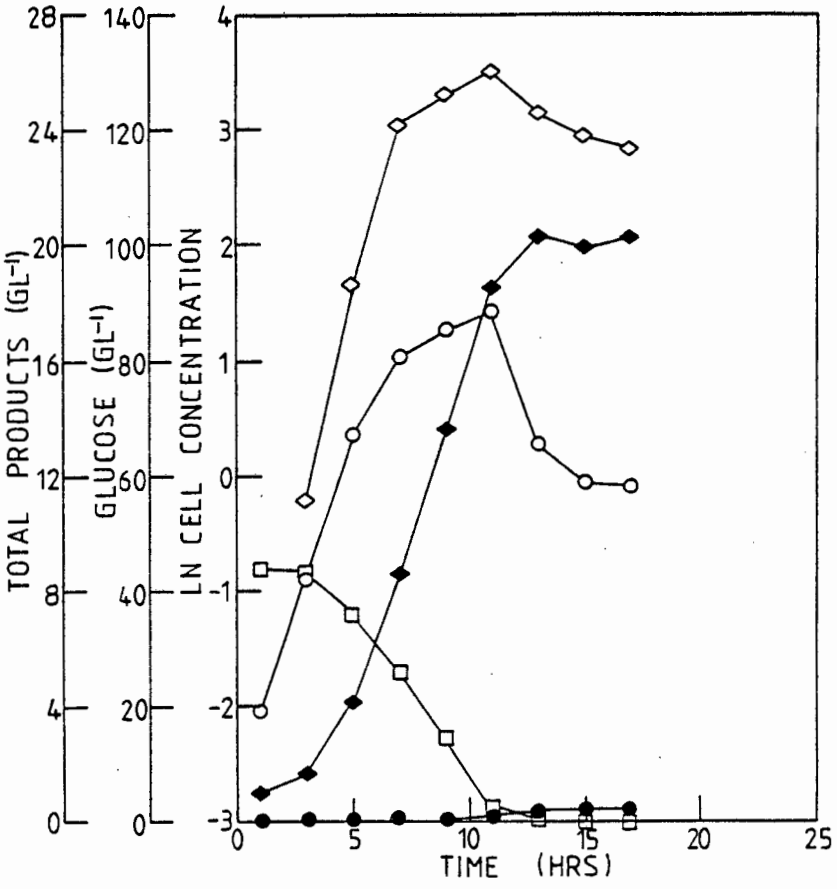
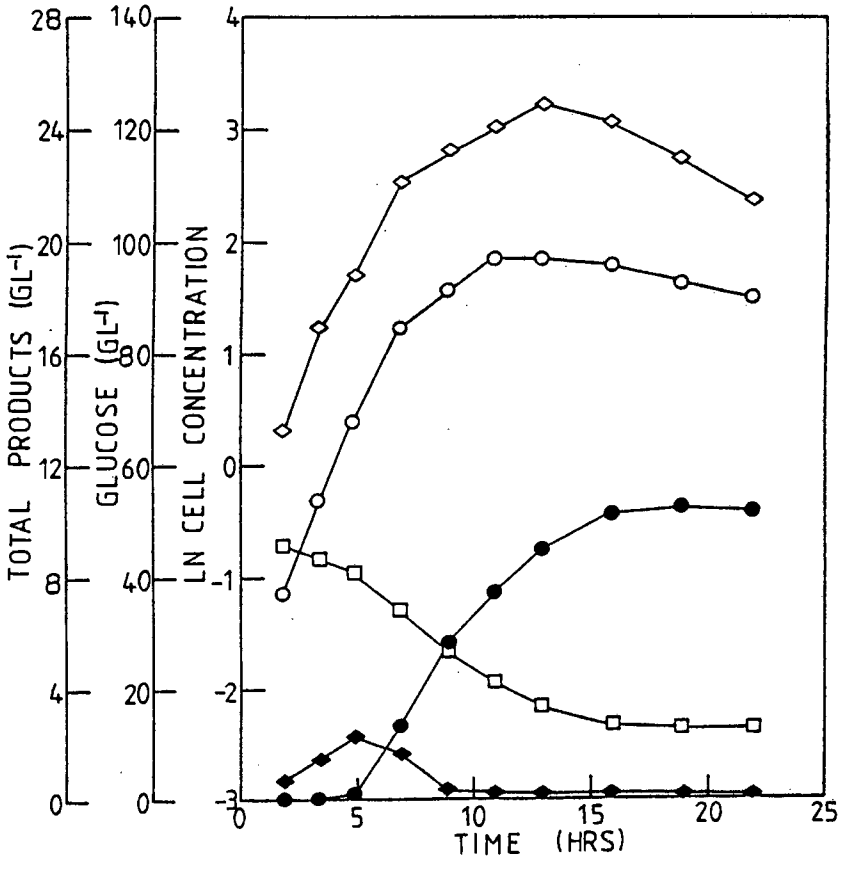


FIGURE 6.3: Growth and physiological changes in C. acetobutylicum P 262 during batch fermentation at pH 5,0. ○ , ln(cell dry mass); ◇ , ln(total cell count.10^{-8}); □ , glucose; ● , acetone plus butanol; ◆ , acetate plus butyrate.



concentration of 1,6 gl^{-1}. By this time 26% of the glucose had been utilised.

The results obtained from a batch culture of the P 262 strain with pH control to 6,0 are shown in Figure 6.4. During exponential growth, a specific growth rate of 0,53 hr^{-1} (R = 0,999) was observed which was equal to that observed during the culture of the cls^{-} mutant at the same pH. Also, an analogous initial increase in the acid concentration and decrease in the glucose concentration was observed. In this culture, solvent production was induced when the acid concentration reached 5,1 gl^{-1}. The decrease in the specific growth rate occurred simultaneously with solvent induction at an acid concentration of 5,1 gl^{-1}. This corresponded to 28% glucose utilisation.

6.123 Estimation of Internal Acid Concentrations

As it has been suggested that the cell is influenced by internal rather than external concentrations, the internal acid concentrations corresponding to the measured external concentrations at the initial decrease in the specific growth rate and at the onset of solvent production were calculated. (The calculations are detailed in Appendix B.)

The undissociated acid equilibrates across the membrane (Kell et al, 1981) and, therefore, it was assumed that the external value was equal to the internal value. These concentrations are listed in Table 6.1. It is apparent that there is no correlation between the undissociated acid concentrations (acetate, butyrate or total) at the end of exponential growth in either culture at different pH values. Similarly, no correlation between the undissociated concentrations at the onset of solvent production in the P 262 cultures at different pH values is observed.

FIGURE 6.4: Growth and physiological changes in C. acetobutylicum P 262 during batch fermentation at pH 6,0. ○, ln(cell dry mass); ◇, ln(total cell count.10^{-8}); □, glucose; ●, acetone plus butanol; ◆, acetate plus butyrate.

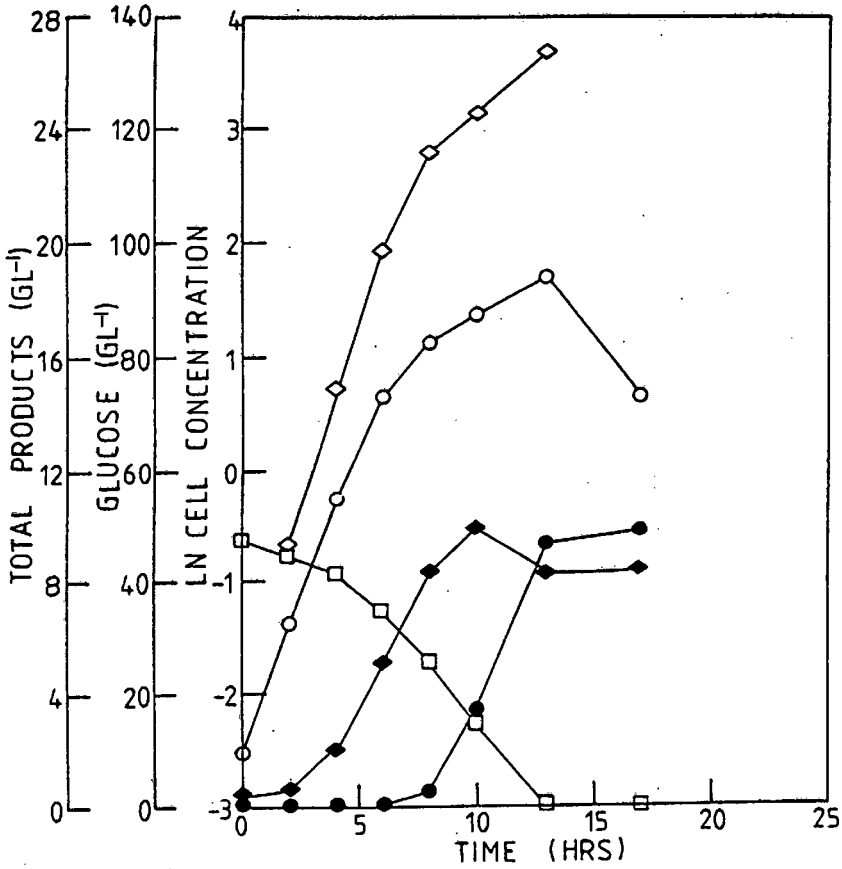


TABLE 6.1: Undissociated acid concentrations

Acid type	Culture pH	Undissociated acid concentration (gl^{-1})		
		cls^{-}(a)	P 262(a)	P 262(b)
acetate	5,0	0,59	0,44	0,35
	6,0	0,17	0,11	0,11
butyrate	5,0	1,06	0,17	0,55
	6,0	0,34	0,20	0,20
total	5,0	1,65	0,61	0,90
	6,0	0,51	0,31	0,31

a: at the end of exponential growth
b: at the onset of solvent production

The internal dissociated acid concentrations will be higher than the external dissociated concentrations as it has been established that these organisms maintain a higher internal pH relative to the external pH (Riebeling et al, 1975; Bowles and Ellefson, 1985; Huang et al, 1985; Gottwald and Gottschalk, 1985). Furthermore, the internal concentration will be proportionately greater than the external concentration as the value of the pH gradient increases (Huang et al, 1986). Therefore, in order to evaluate the internal dissociated acid concentration, it is necessary to ascertain the pH gradient. Although the pH gradient was not measured in this study, a range of possible gradients can be estimated from the values which have been obtained from actual measurements in growing cells of C. acetobutylicum in other studies (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985; Huang et al, 1985). The following pH gradients have been obtained from the graphical results presented in these studies. A pH gradient of 1,0 (Bowles and Ellefson, 1985), 0,9 (Gottwald and Gottschalk, 1985) and 0,7 to 0,9 (Huang et al, 1985) was observed at a culture pH of 5,0. At a pH of 6,0, a

lower pH gradient of 0,2 to 0,4 (Huang et al, 1985) and at least 0,5 (Bowles and Ellefson, 1985), was recorded. (Gottwald and Gottschalk (1985) do not report a value of a pH gradient corresponding to a culture pH of 6,0.) From these studies, it was inferred that the pH gradient at a culture pH of 5,0 may range from 0,7 to 1,0 and at a culture pH of 6,0, from 0,2 to 0,5. These ranges of the pH gradients were used to estimate the internal dissociated acid concentrations at the initial decrease in the specific growth rate and at the onset of solvent production. The relevant estimated internal dissociated acid concentrations are listed in Table 6.2.

In the batch cultures of the cls$^-$ mutant, in which only acids were produced in significant amounts, the internal dissociated acid concentrations at the time of the initial decrease in the specific growth rate were 13-26 gl^{-1}, over the range of possible pH gradients, irrespective of the culture pH. In the solventogenic cultures, the corresponding concentrations were dependent on the culture pH and were only 5-10 gl^{-1} and 8-15 gl^{-1} at pH values of 5,0 and 6,0 respectively, over the same range of assumed pH gradients. In these cultures, the internal dissociated acid concentrations at the time of solvent induction, over this range of assumed pH gradients, were 7-15 gl^{-1}, again irrespective of the culture pH.

The total internal acid concentrations follow the same trends as the internal dissociated acid concentrations because the concentrations of the undissociated acid are relatively insignificant.

6.13 Influence of Solvent Production on the Cell Growth

The cell growth patterns of the solventogenic P 262 strain during batch cultures at different pH values are compared in Figure 6.5. These cultures were conducted at pH control

FIGURE 6.5: Comparison of the growth patterns of C. acetobutylicum P 262 during batch solventogenic fermentations with uncontrolled pH (A), pH control to 5,0 (B) and pH control to 6,0 (C). Broken lines indicate the end of exponential growth. ○ , ln(cell dry mass); ● , acetone plus butanol; ◆ , acetate plus butyrate.

(please see over)

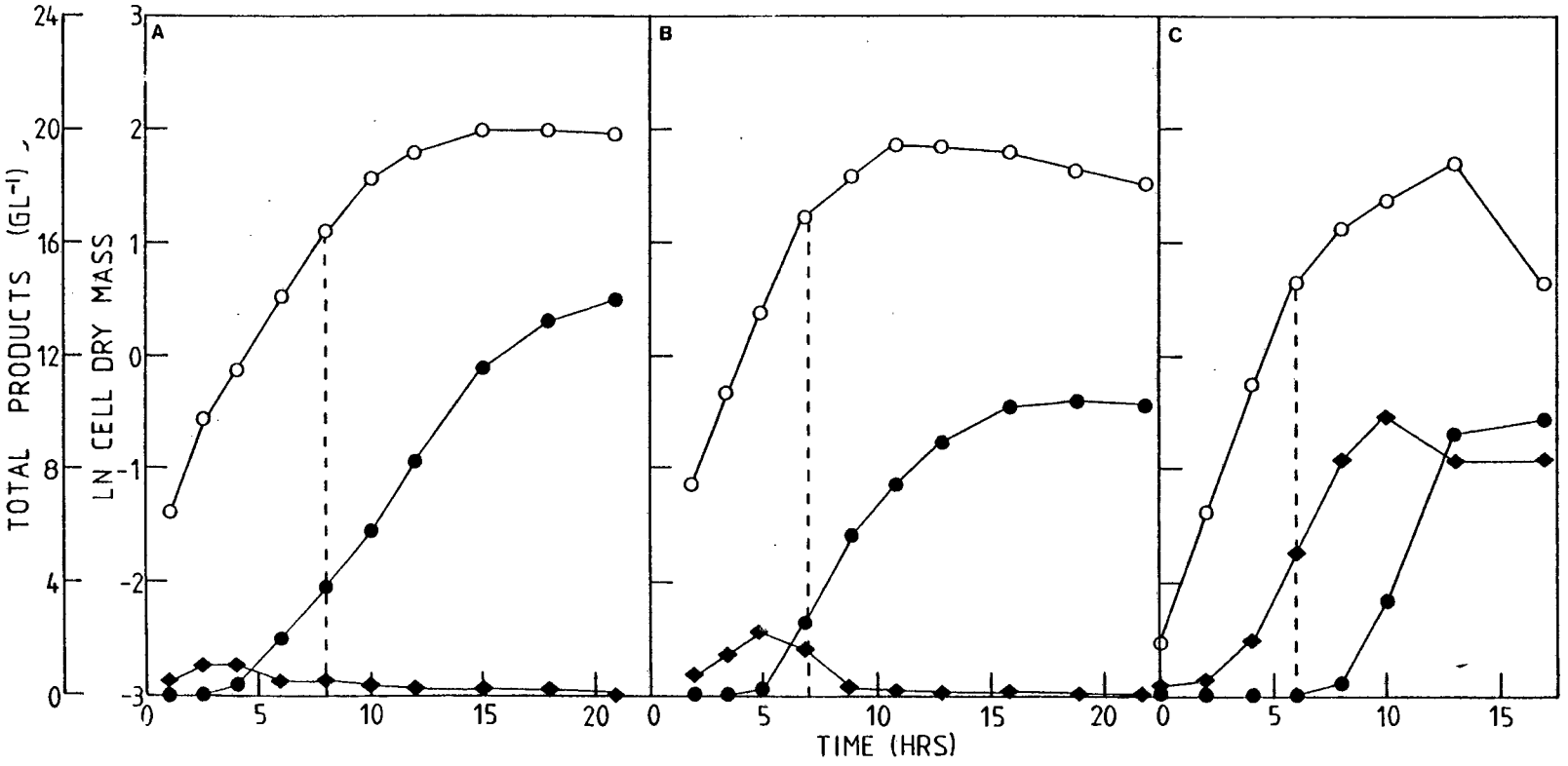

TOTAL PRODUCTS (GL⁻¹)
LN CELL DRY MASS
TIME (HRS)
A
B
C
24
20
16
12
8
4
0
3
2
1
0
-1
-2
-3
0
5
10
15
20

TABLE 6.2: Estimated internal dissociated acid concentrations

Culture pH	Assumed pH gradient	Dissociated acid concentration (gl^{-1})		
		cls^{-}(a)	P 262(a)	P 262(b)
5,0	0,7	13,2	5,1	7,2
	0,8	16,6	6,5	9,1
	0,9	21,0	8,2	11,5
	1,0	26,4	10,3	14,5
6,0	0,2	12,9	7,6	7,6
	0,3	16,2	9,5	9,5
	0,4	20,4	12,0	12,0
	0,5	25,7	15,1	15,1

a: at the end of exponential growth
b: at the onset of solvent production

to 5,0 and 6,0 and without pH control. In the absence of pH control, the culture pH varied throughout the fermentation (not shown) and decreased to 4,6 at its minimum value. While the decrease in the specific growth rate and the onset of solvent production occurred simultaneously at a pH of 6,0, solvent production preceded the decrease in the specific growth rate by 2 and 4 hours at a pH of 5,0 and with no pH control respectively. From these results, it is evident that the onset of solvent production occurred progressively earlier than the decrease in the specific growth rate as the culture pH was decreased and consequently, exponential growth continued into the early stage of solvent production at the lower pH values.

6.2 DISCUSSION

Acid inhibition of the cell growth of acid forming anaerobes is well known (Herrero, 1983). Studies of acid

inhibition of the growth of C. thermocellum (Herrero et al, 1985) and C. thermoaceticum (Baronofsky et al, 1984), suggested that the uncoupling effect of the acids is the most likely cause of growth inhibition in these organisms. The uncoupling effect is a consequence of the relatively greater dissociation of the acids at the higher internal pH which results in a net efflux of the anions from the cell. In order to replenish the internal anion concentration, undissociated acids diffuse into the cell, thereby increasing the internal hydrogen ion concentration. Since Clostridia regulate the internal pH by means of an ATP-dependent extrusion of hydrogen ions (Riebeling and Jungermann, 1976), this influx of hydrogen ions counteracts the effect of the proton pump, necessitating more ATP to maintain the same internal pH. The increased ATP demand at the membrane level means that less ATP will be available for biosynthesis, suggesting that the cell growth rate will decrease (Herrero, 1983). Baronofsky et al (1984) suggested furthermore, that when the cell cannot supply sufficient ATP for hydrogen ion extrusion, the pH gradient will collapse, resulting in the complete inhibition of growth. A decrease in the internal pH of C. acetobutylicum has been observed on the addition of acetate or butyrate to cell suspensions (Huang et al, 1986), suggesting that an inhibitory effect of acid accumulation on the cell growth of this organism could be similarly explained in terms of an uncoupling effect of the acids.

During the batch cultures of the cls^- mutant reported in this study, the decrease in the cell growth was apparently a result of the accumulation of acid end products. At a culture pH of 5,0, solvent production was negligible and a high residual glucose concentration remained at the end of the fermentation, implying that the acid concentration was the major factor contributing to the decrease in the specific growth rate. It may, therefore, be inferred from the data that, at this pH, growth is first

inhibited by acids when the acid concentration reaches 4,3 gl^{-1} and is completely inhibited when the concentration reaches 8,7 gl^{-1}. Similarly, during the fermentation of the cls^{-} mutant at a pH of 6,0, it may be inferred that growth is first inhibited under these conditions when the acid concentration reaches 8,6 gl^{-1}. As the cessation of growth coincided with glucose exhaustion in this fermentation, however, the acid level at which growth is completely inhibited may be even higher than the 18,5 gl^{-1} measured at this time.

Although the external acid concentrations provide a relative measure to indicate whether the acids reach an inhibitory level, the external acid is unlikely to be a critical species relating to the inhibition. In view of the proposed mechanism of the inhibition, the concentrations of the undissociated acids and/or the internal dissociated acids may be expected to relate directly to a decrease in the cell growth due to acid inhibition. However, no evidence of a correlation of the undissociated acid concentrations with growth inhibition could be found. This is contrary to the findings of Monot et al (1983) and Monot et al (1984). On the other hand, the concentrations of internal dissociated acid suggests that a value of 13-26 gl^{-1}, depending on the assumed pH gradient, can be related to the acid inhibition of growth in the acidogenic cultures, irrespective of the culture pH.

During the parallel fermentations of the solventogenic P 262 strain, the initial decrease in the specific growth rate was observed at acid levels of 2,7 gl^{-1} and 3,5 gl^{-1} less than the corresponding levels in the acidogenic cultures, at pH 5,0 and pH 6,0 respectively. Moreover, the decrease in the specific growth rate in these fermentations was observed at corresponding internal concentrations of dissociated acids of 5-10 gl^{-1} and 8-15 gl^{-1} at pH 5,0 and pH 6,0 respectively. These results suggest that the

acid inhibition is not responsible for the decrease in the specific growth rate during a normal solventogenic fermentation.

The internal concentrations of dissociated acid have been suggested to also be an important factor in solvent induction (Gottwald and Gottschalk, 1985; Bowles and Ellefson, 1985). Solvents were induced at concentrations of 7-15 gl^{-1}, over the same range of assumed pH gradients, in the solventogenic cultures, suggesting that this value can be related to solvent induction, irrespective of the culture pH. Since these concentrations are less than 60% of the inhibitory levels, these results indicate that the acids lead to solvent induction before they reach an inhibitory level. This suggests that solvent induction may function to pre-empt an inhibitory effect of the acids on the cell growth.

The absence of a role of the acid concentration in the decrease in the specific growth rate in the solventogenic fermentation suggests that the decrease in the cell growth may, alternatively, be a consequence of the decrease in the ATP generation accompanying solvent production. However, the continued exponential growth into the early stage of solvent production during fermentations at low pH values suggests that the reduced amount of ATP generated was sufficient to maintain the same cell growth and, therefore, was not a significant factor responsible for the decrease in the specific growth rate under these conditions.

The experimental observations presented in this chapter have indicated that the decrease in the specific growth rate may not be directly linked to the onset of solvent production. They do not, however, permit the elucidation of the factor(s) which cause(s) the decrease in the growth rate during normal solventogenic fermentations. As the comparison of the cell growth in the cls^{-} mutant and the

P 262 strain enables the separation of the repressive and inducing effects of the acid, this experimental system could similarly be used to examine other metabolites which may influence growth inhibition. For example, the comparison of ATP levels during acidogenic and solventogenic fermentations may provide valuable data as to the role of ATP limitation in the decrease in the cell growth.

CHAPTER 7

CONCLUSIONS

Continuous culture is well known to be a potentially superior alternative to batch culture for the production of acetone and butanol because the solvent productivity attained during continuous operation can be significantly increased over that attained during batch operation, particularly if cell retention is used to increase the cell concentration. Multistage continuous systems and *in situ* removal of solvents further increases the attractiveness of continuous culture for production purposes.

In addition to the potential advantage of continuous culture techniques for solvent production, these techniques may also have an important role to play in fundamental studies. If a steady state is reached during continuous operation, the concentrations of the fermentation parameters are constant. Under these conditions the effect of a change in a single variable can be related directly to the resultant change in the microbial physiology. Batch culture is less suitable for evaluating the influence of specific variables because the fermentation parameters vary with time, thus complicating the interpretation of the data.

Although the production of solvents during continuous culture has been reported in the literature under a wide range of operating conditions, it is evident that a number of problems have been experienced. One of the main problems is that solvent production is not necessarily induced during continuous culture. In the studies of

solvent production in nutrient limited continuous culture systems, it has generally been suggested that specific nutrient limitations are necessary for the promotion of solvent production during continuous culture. In this study, the interpretation of the results of the nutrient limited continuous culture systems was reassessed. This led to the hypothesis that a nutrient limitation is not a prerequisite for solvent production under these conditions. A major objective of this study was, therefore, to determine the validity of this hypothesis.

For this purpose, it was necessary to examine solvent production during continuous culture in the absence of any nutrient limitation. This was facilitated by the development of a medium which supplied the nutrients in excess of the requirements for cell growth during continuous culture. Under these conditions, it was assumed that the cell growth was limited solely by the accumulation of metabolic product(s) formed during the fermentation.

Continuous culture was conducted under these conditions over a wide range of dilution rates from 0,05 hr^{-1} to 0,35 hr^{-1}. Solvent production was induced over this range of dilution rates and an average solvent yield of 0,28 was attained. At the lowest dilution rate investigated, solvent concentrations of 7 gl^{-1} butanol and 5 gl^{-1} acetone were attained. From these results, it may be concluded that a nutrient limitation is not a prerequisite for the induction or enhancement of solvent production during continuous culture. This means that although good solvent production has been reported in various nutrient limited continuous culture studies, it is likely that in most of these studies, solvent production was not a direct consequence of the particular nutrient limitation imposed.

An important consequence of this finding is that optimal solvent production is likely to be attained during continuous culture under purely product limited conditions. Since the growth is not restricted by the limitation of an essential nutrient under these conditions, the maximum cell concentration would be attained, resulting in maximum solvent concentrations and therefore, maximum solvent productivity. This suggests that a primary requirement for the optimisation of a continuous process is to ensure culture conditions where the essential nutrients are not limiting. In view of the high cost of the raw material, however, the concentration of the nutrients in the feed would need to be adjusted so that while they remained in excess, their residual concentrations in the broth were minimal.

Another problem which has been associated with nutrient limited continuous systems, is the genetic instability of the organism such that the ability to produce solvents decreases progressively during continuous operation. Since the strain of C. acetobutylicum used in this study remained genetically stable with respect to the ability to produce solvents and to sporulate during the continuous culture experiments, this problem was not addressed.

During continuous culture in which the organism remains stable and solvents are produced, it is apparent that a third major problem has been experienced. In the literature it has been observed that, in some cases, the solvent concentration varied significantly at a constant dilution rate. Similarly, during the product inhibited continuous cultures in this study, oscillations in the concentrations of cells, substrate and products were observed even after prolonged continuous operation during which no genetic or physiological deterioration of the organism was detected. The doubts raised by the literature studies and the observations made during the course of this

study with respect to the ability of the culture to maintain the fermentation parameters at constant values, prompted an investigation into the nature of the observed oscillatory behaviour.

This investigation was conducted by monitoring the dynamics of the culture under constant operating conditions. The observations provided evidence to suggest that a heterogeneous population comprising both acid and solvent producing cells was present at a constant dilution rate. Moreover, the acid producing cells were associated with a higher specific growth rate relative to the solvent producing cells. A continual shift in the proportion of these cell types resulted in corresponding oscillations in the specific growth rate and in the fermentation parameters.

It is proposed that the shift in the cell population from a predominance of acid producing cells to a predominance of solvent producing cells resulted from the intrinsic nature of the organism to shift from acid to solvent production. Furthermore, it is proposed that the shift back to a predominance of acid producing cells was caused by the pressure imposed by continuous operation to selectively retain the cells with the higher specific growth rate and possibly also by a reversion of the solvent producing cells to acid producing cells. This suggests that the continual shift in the cell types, and hence the oscillations in the parameters, is an inherent characteristic of the continuous solvent fermentation.

The difference in the specific growth rates of the acid and solvent producing cells is clearly a critical factor leading to the continual shift in the proportion of these cell types. This difference arises from the concomitance of the shift to solvent production and a decrease in the cell growth. Although the shift to solvent production is

widely accepted to be a consequence of acid accumulation, the factors responsible for the accompanying decrease in the specific growth rate have not been established. Since the decrease in the specific growth rate is important in the interpretation of the nature of the oscillations, this study has included an investigation of the factors which might influence the growth rate. This investigation was facilitated by the comparison of the growth patterns during batch culture of the solventogenic P 262 strain with those of a mutant strain which was unable to produce solvents in significant amounts. In the mutant strain, acid accumulation was responsible for the decrease in the specific growth rate. In the solventogenic strain, the decrease in the specific growth rate was observed at acid concentrations lower than the inhibitory level, thus it is unlikely that the acid accumulation was similarly responsible for the decrease in the cell growth under these conditions. Furthermore, solvent production was initiated prior to the decrease in the specific growth rate during solventogenic cultures at low pH values, suggesting that the decrease in the growth rate was also not a consequence of the decrease in ATP generation at solvent induction. The factors which do influence the decrease in the specific growth rate are not, however, immediately clear from these results and the cause of the decrease in the cell growth accompanying solvent induction has yet to be elucidated.

A major implication of inherent oscillatory behaviour during continuous culture is that it precludes the attainment of a true steady state. This seriously limits the value of continuous culture techniques for fundamental physiological studies if traditional steady state concepts are being used to analyse the data, especially under conditions where the amplitude of the oscillations is large. However, it is possible that the oscillatory behaviour could, itself, be used as a basis for physiological studies. Suitable analysis of the data from

the cyclic patterns may provide useful information on the factors which are associated with, or bring about, these changes.

The oscillatory behaviour also has direct consequences for the application of a continuous system for solvent production, particularly since, in this study, the most pronounced oscillatory behaviour was observed when the solvent concentration was highest. The inability of the culture to sustain a constant solvent concentration means that the output from the fermenter would periodically contain low solvent concentrations, resulting in high recovery costs. Moreover, the oscillation in the concentration of the residual substrate would adversely affect the overall conversion because the output would periodically contain a high residual substrate concentration. As the substrate is generally a major cost of the fermentation, the loss of the substrate in this manner is significantly detrimental to the economics of the process. In addition, some of the substrate will be diverted to acid production by virtue of the heterogeneous nature of the cell population, resulting in a further decrease in the conversion of substrate to solvents.

The success of the continuous operation for solvent production, therefore, depends to a large extent on eliminating or minimising these oscillations. In an ideal system, only solvent producing cells would be present. Under these conditions, the population would be inherently stable and the solvent and substrate concentrations in the output would be constant. Two approaches could be followed to obtain such a system. Firstly, genetic manipulation of the organism which succeeded in removing the acid producing pathways is likely to result in the conversion of the substrate directly to butanol with no acid or acetone production and, therefore, a constant butanol output. Secondly, by adding acids to the feed of a continuous

system, a high acid level would be maintained, suggesting that the overall proportion of acid producing cells in the population would be decreased. Under conditions in which the proportion of these cells was negligible, a steady state would presumably be approached at which the acid concentrations in the output would be negligible and the solvent concentrations would be essentially constant.

This suggests that future process developments relating to continuous solvent production are likely to focus on the optimisation of solvent production from nutrient feeds containing acids. As an alternative to adding acids to the feed, the output from a preliminary continuous culture, in which conditions were optimised for acid production alone, could be used as the feed to the cells. (Although the acid producing cells entering in the feed would convert to solvent producing cells under these conditions, they would be unlikely to have a significant effect on the solvent concentrations.) Since the specific growth rate of the solvent producing cells is low, these multistage systems would be improved by the incorporation of cell recycle or cell immobilisation in the second stage. Further improvements to the continuous system may be achieved through the combination of the advances in process technology with those in genetic manipulation, for example, by the immobilisation of a mutant, which exhibited an increased tolerance to butanol, in the solvent producing stage. It is envisaged that such systems will eventually lead to an economically competitive fermentation route for the production of acetone and butanol.

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LIST OF APPENDICES

APPENDIX A

CULTURE MEDIA

COMPLEX BASAL GROWTH MEDIUM COMPOSITION

glucose	10,0 g
casein hydrolysate	4,0 g
yeast extract	4,0 g
$MgSO_4.7H_2O$ stock solution	1,0 ml
$MnSO_4.4H_2O$ stock solution	1,0 ml
$FeSO_4.7H_2O$ stock solution	1,0 ml
p-aminobenzoic acid stock solution	1,0 ml
biotin stock solution	1,0 ml
thiamine HCl stock solution	1,0 ml
$NaHCO_3$ stock solution	10,0 ml
cysteine HCl stock solution	10,0 ml
resazurin stock solution	10,0 ml
distilled water to	1000,0 ml

Stock solutions

$MgSO_4.7H_2O$ stock solution:

$MgSO_4.7H_2O$	20,0 g
distilled water to	100,0 ml

$MnSO_4.4H_2O$ stock solution:

$MnSO_4.4H_2O$	1,0 g
distilled water to	100,0 ml

$FeSO_4.7H_2O$ stock solution:

$FeSO_4.7H_2O$	1,0 g
distilled water to	100,0 ml

Addition of 2 drops of concentrated HCl per 100 ml of stock solution prevents oxidation.

p-aminobenzoic acid stock solution:

p-aminobenzoic acid	0,1 g
distilled water to	100,0 ml

biotin stock solution:

biotin	0,02 g
distilled water to	100,0 ml

solution was then diluted 1:100

thiamine HCl stock solution:

thiamine HCl	0,1 g
distilled water to	100,0 ml

$NaHCO_3$ stock solution:

$NaHCO_3$	10,0 g
distilled water to	100,0 ml

cysteine HCl stock solution:

cysteine HCl	5,0 g
distilled water to	100,0 ml

resazurin stock solution:

resazurin	0,02 g
distilled water to	100,0 ml

All stock solutions were stored at 4^oC.

SEMI-DEFINED MEDIUM COMPOSITION

glucose monohydrate (inoculum)	11,0 g
glucose monohydrate (fermenter)	55,0 g
yeast extract	10,0 g
$(NH_4)_2SO_4$	6,1 g
K_2HPO_4	1,0 g
KH_2PO_4	1,0 g
$MgSO_4.7H_2O$	0,4 g
asparagine	0,5 g
$MnSO_4.H_2O$ stock solution	0,5 ml
$FeSO_4.7H_2O$ stock solution	0,5 ml
NaCl stock solution	0,5 ml
vitamin stock solution	5,0 ml
cysteine HCl stock solution	10,0 ml
resazurin stock solution	0,5 ml
silicone aqueous emulsion (30% w/v)	0,25 ml
distilled water to	1000,0 ml

<u>Stock solutions</u>

$MnSO_4.H_2O$ stock solution:

$MnSO_4.H_2O$	2,0 g
distilled water to	100,0 ml

$FeSO_4.7H_2O$ stock solution:

$FeSO_4.7H_2O$	3,0 g
distilled water to	100,0 ml

Addition of 2 drops of concentrated HCl per 100 ml of stock solution prevents oxidation.

NaCl stock solution:

NaCl	2,0 g
distilled water to	100,0 ml

vitamin stock solution:	
p-aminobenzoic acid	0,04 g
biotin	0,002 g
thiamine HCl	0,04 g
distilled water to	100,0 ml
cysteine HCl stock solution:	
cysteine HCl	5,0 g
distilled water to	100,0 ml
resazurin stock solution:	
resazurin	0,6 g
distilled water to	100,0 ml

All stock solutions were stored at 4°C.

DIVISION OF A 20 LITRE VOLUME OF MEDIUM FOR AUTOCLAVING

Main reservoir (25 litre pyrex aspirator):	
glucose monohydrate	1 100,0g
cysteine HCl stock solution	150,0ml
resazurin stock solution	7,0ml
silicone aqueous emulsion (30% w/v)	5,0ml
distilled water to	16 000,0ml
Subsidiary reservoir (5 litre pyrex aspirator):	
$(NH_4)_2SO_4$	122,0g
yeast extract	200,0g
KH_2PO_4	20,0g
K_2HPO_4	20,0g
$MnSO_4.H_2O$ stock solution	10,0ml
$FeSO_4.7H_2O$ stock solution	10,0ml
NaCl stock solution	10,0ml
cysteine HCl stock solution	40,0ml
resazurin stock solution	2,0ml
distilled water to	3 800,0ml
Flask (250 ml pyrex screw cap flask):	
asparagine	10,0g
vitamin stock solution	100,0ml
$MgSO_4.7H_2O$	8,0g
cysteine HCl stock solution	10,0ml
resazurin stock solution	1,0ml
distilled water to	200,0ml

CALCULATION OF THE UNDISSOCIATED AND INTERNAL DISSOCIATED ACID CONCENTRATIONS

The concentration of the undissociated acid is calculated in the following way.

Let: $[A^T]$ = external total acid concentration
$[HA]$ = external undissociated acid concentration
$[A^-]$ = external dissociated acid concentration

Then: $$[A^T] = [HA] + [A^-] \quad \text{(Eqn 1)}$$

Also, according to the Henderson-Hasselbach equation (Glasstone and Lewis, 1970):

$$pH = pKa + \log [A^-]/[HA] \quad \text{(Eqn 2)}$$

or $$[HA]/[A^-] = 10^{pKa - pH}$$

or $$[HA] = [A^-].10^{pKa - pH}$$

then from Eqn 1: $[HA] = ([A^T] - [HA]).10^{pKa-pH}$

or $$[HA] = \frac{[A^T].10^{pKa - pH}}{1 + 10^{pKa - pH}}$$

These equations apply equally to acetate and butyrate. The pKa of the butyrate used was 4,82 and that of acetate was 4,76.

The external undissociated acid concentration equals the internal undissociated acid concentration because the acid diffuses freely through the membrane in the undissociated form (Kell et al, 1981).

The concentration of the internal dissociated acid is calculated in the following way.

From Eqn 2: $pH_i = pKa + \log [A_i^-]/[HA]$,
where i = internal values

or $[HA]/[A_i^-] = 10^{pKa - pH_i}$

or $[A_i^-] = \frac{[HA]}{10^{pKa - pH_i}}$

The total internal acid concentration can then be calculated fron Eqn 1:

$$[A_i^T] = [A_i^-] + [HA]$$

The data used for the calculation of the concentrations in the text are as follows.

Culture pH	Acid	External concentration (gl^{-1})		
		cls^-(a)	P 262(a)	P 262(b)
5,0	acetate	1,626	1,202	0,969
	butyrate	2,668	0,438	1,380
6,0	acetate	3,109	1,922	1,922
	butyrate	5,512	3,154	3,154

a: at the end of exponential growth

b: at the onset of solvent production

APPENDIX C

EXPERIMENTAL DATA USED IN FIGURES

Figure 4.1: Growth and physiological changes in C. acetobutylicum P 262 during batch culture at pH above 5,0

time (hrs)	cell dry mass (gl^{-1})	glucose (gl^{-1})	total solvents (gl^{-1})	total acids (gl^{-1})	butanol (gl^{-1})	acetone (gl^{-1})	ethanol (gl^{-1})	acetate (gl^{-1})	butyrate (gl^{-1})
0,1	0,18	48,7	0,580	0,259	0,204	0,056	0,320	0,135	0,124
1,6	0,34	48,1	0,349	0,484	0,147	0,069	0,133	0,245	0,239
2,6	0,56	46,4	0,513	0,826	0,123	0,087	0,303	0,320	0,506
3,6	0,86	44,9	0,534	1,135	0,189	0,042	0,303	0,478	0,657
4,6	1,32	43,5	0,416	1,659	0,119	0,083	0,214	0,703	0,956
5,6	1,88		0,706	2,091	0,413	0,152	0,141	0,965	1,126
6,8	2,93	38,6	1,434	2,171	0,944	0,269	0,221	1,352	0,819
7,6	3,85	36,2	2,803	1,863	1,806	0,747	0,250	1,366	0,497
8,6	5,25	32,0	5,026	1,580	3,057	1,522	0,447	1,137	0,443
9,6	8,12	24,6	5,730	1,225	3,571	1,935	0,224	0,823	0,402
10,6	9,84	17,2	7,651	1,025	4,643	2,696	0,312	0,558	0,467
15,8	10,08	5,1	12,514	0,715	8,320	3,581	0,613	0,276	0,439
20,3	8,51	1,5	14,714	0,542	9,345	3,616	1,753	0,278	0,264
26,3	6,58	0,2	14,741	0,545	9,904	3,658	1,179	0,279	0,266

Figure 4.2: Unsteady behaviour during continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,05 hr^{-1}

time (hrs)	transmission (%)	glucose (gl^{-1})
295,5	72,5	8,0
315,5	72,1	13,2
339,8	74,3	14,0
360,0	70,5	4,0
383,8	75,6	13,9
408,0	70,0	7,4
433,8	76,2	18,5
457,3	71,8	5,7
480,8	75,6	9,5
502,8	72,3	5,3
632,3	75,5	12,7
554,3	72,9	6,3
582,0	76,5	12,1
609,8	72,5	4,3
648,8	75,0	6,3
675,8	74,2	8,7

Figure 4.3: Unsteady behaviour during continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}

time (hrs)	transmission (%)	glucose (gl^{-1})
170,8	61,8	13,7
187,3	58,5	15,8
198,8	58,7	11,9
215,8	66,3	
234,5	59,8	11,8
245,3	65,0	16,0
258,3	63,5	15,6
271,3	61,5	13,7

Figure 4.4: Fermentation products over the last ten volume changes during continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,05 hr^{-1}

time (hrs)	cell dry mass^{m} (gl^{-1})	total solvents (gl^{-1})	total acids (gl^{-1})
480,8	3,38	11,069	1,933
502,8	3,58	12,456	2,335
532,3	3,31	9,983	2,696
554,3	4,00		2,768
580,2	3,45	10,920	2,605
609,8	4,10	12,519	2,148
648,8	3,60	12,533	2,627
675,8	3,73	10,622	2,601

time (hrs)	butanol (gl^{-1})	acetone (gl^{-1})	ethanol (gl^{-1})	butyrate (gl^{-1})	acetate (gl^{-1})
480,8	6,297	4,623	0,149	0,930	1,003
502,8	6,933	5,358	0,165	1,207	1,128
532,3	5,732	4,142	0,109	1,373	1,323
554,3	7,261	4,916		1,636	1,132
580,2	6,266	4,542	0,112	1,439	1,166
609,8	7,179	5,189	0,151	1,099	1,049
648,8	7,191	5,200	0,142	1,441	1,186
675,8	6,058	4,257	0,307	1,371	1,230

m: measured or true cell dry mass

Figure 4.5: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,15 hr^{-1}

volume changes	transmission (%)	glucose (gl^{-1})
2,5	65,6	26,1
4,0	73,3	22,9
5,8	71,6	20,1
6,8	71,5	16,2
9,4	73,0	17,8
10,4	71,2	17,9
11,4	71,5	17,8

Figure 4.6: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,20 hr^{-1}

volume changes	transmission (%)	glucose (gl^{-1})
0,9	75,2	22,4
1,4	76,8	23,5
2,7	76,7	25,8
5,0	74,0	20,9
6,2	75,5	23,3
7,5	75,0	23,4
10,9	75,6	24,2

Figure 4.7: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,25 hr^{-1}

volume changes	transmission (%)	glucose (gl^{-1})
2,8	74,4	28,8
3,8	74,5	26,6
4,8	74,0	25,9
5,8	74,1	25,2
6,8	74,1	24,3
7,8	74,4	24,6
9,8	74,3	25,3

Figure 4.8: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,30 hr^{-1}

volume changes	transmission (%)	glucose (gl^{-1})
2,4	70,7	29,2
9,8	76,1	27,4
10,8	76,4	28,6
11,9	76,8	28,6
12,9	76,2	28,1

Figure 4.9: Continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,35 hr^{-1}

volume changes	transmission (%)	glucose (gl^{-1})
6,8	82,2	33,8
7,9	81,9	33,5
9,3	82,3	31,7

Figure 4.10: Influence of the dilution rate on the substrate and product concentrations during continuous fermentation of C. acetobutylicum P 262

dilution rate (hr^{-1})	cell dry mass^{m} (gl^{-1})	glucose (gl^{-1})	total solvents (gl^{-1})	total acids (gl^{-1})
0,05	3,64	8,2	11,555	2,464
0,10	5,10	14,1	10,065	0,849
0,15	3,85	17,9	11,161	1,481
0,20	3,27	23,6	8,598	1,462
0,25	3,50	24,9	8,138	1,730
0,30	3,20	28,4	6,933	1,716
0,35	2,62	33,0	4,625	2,738

m: measured or true cell dry mass

Figure 4.11: Influence of the dilution rate on the individual products during continuous fermentation of C. acetobutylicum P 262

dilution rate (hr^{-1})	butanol (gl^{-1})	acetone (gl^{-1})	ethanol (gl^{-1})	butyrate (gl^{-1})	acetate (gl^{-1})
0,05	6,615	4,778	0,162	1,312	1,152
0,10	5,736	4,062	0,268	0,423	0,426
0,15	6,013	4,900	0,249	0,710	0,771
0,20	4,971	3,494	0,133	0,566	0,896
0,25	4,382	3,581	0,175	0,687	1,043
0,30	3,925	2,790	0,218	0,697	1,019
0,35	2,732	1,697	0,196	1,305	1,433

Figure 4.12: Total solvents produced as a function of glucose utilised during continuous fermentation of C. acetobutylicum P262 over the range of dilution rates from 0,05 hr^{-1} to 0,35 hr^{-1}

glucose (gl^{-1})	total solvents (gl^{-1})
8,2	11,555
14,1	10,065
17,9	11,161
23,6	8,598
24,9	8,138
28,4	6,933
33,0	4,625

Figure 5.1: Growth and physiological changes in C. acetobutylicum P 262 during fermentation after the switch over from batch to continuous operation at a dilution rate of 0,1 hr^{-1}

time (hrs)	total cells (cells ml^{-1})	cell dry mass (gl^{-1})	glucose (gl^{-1})	total solvents (gl^{-1})	total acids (gl^{-1})
0,0	$7,68.10^8$	6,52	0,05	13,552	0,725
4,0	$1,08.10^9$	3,56	15,87	9,568	0,436
7,0	$1,16.10^9$	2,48	24,87	7,051	0,320
10,0	$6,25.10^8$	1,66	31,33	5,081	0,232
13,0	$2,62.10^8$	1,22	35,41	4,122	0,146
19,0		0,69	41,81	2,433	0,111
22,5	$1,29.10^8$	0,55	44,10	1,715	0,060
25,5	$1,07.10^8$	0,36	47,06	1,295	0,040
28,5	$4,13.10^7$	0,30	47,50	0,636	0,034
31,5	$3,00.10^7$	0,24	47,83	0,818	0,030
34,5		0,19	48,22	0,580	0,032
37,5	$4,50.10^7$	0,21	47,82	0,643	0,128
43,7	$9,10.10^8$	1,19	45,17	1,054	1,490
46,5	$1,99.10^9$	2,93	37,67	3,765	0,831
49,5	$2,84.10^9$	6,22	26,53	8,182	0,466
52,5	$2,07.10^9$	8,12	19,00	10,233	0,187
55,0	$1,83.10^9$	7,28	19,47	10,592	0,176
58,5	$9,70.10^8$	4,44	25,07	8,769	0,122
61,5	$5,53.10^8$	3,26	30,33	6,530	0,106
67,8	$5,20.10^8$	1,85	39,42	3,489	0,411
70,5	$6,93.10^8$	1,86	41,00	2,780	1,058
73,5	$1,24.10^9$	2,21	38,22	3,791	1,105
77,0	$1,74.10^9$	3,14	28,24	4,914	0,533

time (hrs)	butanol (gl^{-1})	acetone (gl^{-1})	ethanol (gl^{-1})	butyrate (gl^{-1})	acetate (gl^{-1})
0,0	9,226	3,907	0,419	0,425	0,300
4,0	6,372	2,691	0,505	0,366	0,070
7,0	4,705	2,003	0,343	0,263	0,057
10,0	3,476	1,466	0,139	0,232	0,000
13,0	2,675	1,145	0,302	0,146	0,000
19,0	1,530	0,653	0,250	0,111	0,000
22,5	1,096	0,495	0,124	0,060	0,000
25,5	0,847	0,416	0,032	0,044	0,000
28,5	0,523	0,051	0,062	0,034	0,000
31,5	0,463	0,237	0,118	0,030	0,000
34,5	0,339	0,209	0,032	0,032	0,000
37,5	0,244	0,178	0,221	0,072	0,056
43,7	0,667	0,305	0,082	0,566	0,924
46,5	2,221	1,407	0,137	0,269	0,562
49,5	4,875	2,842	0,465	0,189	0,277
52,5	6,335	3,378	0,520	0,088	0,099
55,0	6,753	3,362	0,477	0,117	0,059
58,5	5,523	2,670	0,576	0,058	0,064
61,5	4,183	1,958	0,389	0,057	0,049
67,8	2,280	1,044	0,165	0,258	0,153
70,5	1,755	0,812	0,213	0,648	0,410
73,5	2,398	1,256	0,137	0,277	0,828
77,0	3,020	1,790	0,104	0,153	0,380

Figure 5.2: Morphological and cytological changes in C. acetobutylicum P 262 during fermentation after the switch over from batch to continuous operation at a dilution rate of 0,1 hr^{-1}

time (hrs)	granulose positive rods (cells ml^{-1})	granulose negative rods (cells ml^{-1})	forespore septa (cells ml^{-1})	mature forespores (cells ml^{-1})	free spores (spores ml^{-1})	forespore septa (%)	mature forespores (%)	free spores (%)
43,7	$8,45.10^8$	$6,46.10^7$	0,0	0,0	0,0	0,0	0,0	0,0
46,5	$1,68.10^9$	$1,05.10^8$	$2,09.10^8$	0,0	0,0	10,5	0,0	0,0
49,5	$2,20.10^9$	0,0	$6,46.10^8$	0,0	0,0	22,6	0,0	0,0
52,5	$1,47.10^9$	$1,86.10^7$	$5,88.10^8$	0,0	0,0	28,4	0,0	0,0
55,0	$1,23.10^9$	$2,01.10^7$	$5,82.10^8$	0,0	0,0	31,8	0,0	0,0
58,5	$4,40.10^8$	$3,40.10^7$	$3,83.10^8$	$1,13.10^8$	0,0	39,5	11,6	0,0
61,5	$2,31.10^8$	$1,88.10^7$	$2,10.10^8$	$9,51.10^7$	0,0	38,0	17,2	0,0
67,8	$2,54.10^8$	$1,04.10^8$	$1,15.10^8$	$4,73.10^7$	$1,35.10^7$	22,1	9,1	2,6
70,5	$1,98.10^8$	$4,55.10^8$	$3,40.10^7$	$6,99.10^6$	$6,93.10^6$	4,9	1,0	1,0
73,5	$5,27.10^8$	$6,86.10^8$	$2,73.10^7$	0,0	0,0	2,2	0,0	0,0
77,0	$1,41.10^9$	$3,29.10^8$	0,0	0,0	0,0	0,0	0,0	0,0

Figure 5.3: Growth and physiological changes in C. acetobutylicum P 262 during extended continuous fermentation at a dilution rate of 0,1 hr^{-1}

time (hrs)	total cells (cells ml^{-1})	cell dry mass (gl^{-1})	glucose (gl^{-1})	total solvents (gl^{-1})	total acids (gl^{-1})
0,0	$8,63.10^8$	2,52	30,25	5,921	0,286
3,0	$8,00.10^8$	3,23	24,50	8,160	0,115
6,0	$6,38.10^8$	3,37	21,00	9,432	0,004
9,0	$5,08.10^8$	3,16	18,92	10,026	0,025
12,0	$3,21.10^8$	2,80	20,50	9,378	0,023
15,0	$2,75.10^8$	2,39	23,56	8,427	0,078
18,0	$2,56.10^8$	2,10	26,75	6,750	0,163
21,0	$3,06.10^8$	1,96	30,17	5,684	0,376
24,0	$5,08.10^8$	1,92	30,95	5,182	0,595
27,0	$6,56.10^8$	2,30	29,67	5,712	0,264
30,0	$7,21.10^8$	2,81	25,42	7,293	0,199
33,0	$7,17.10^8$	3,29	20,33	9,149	0,133
36,0		3,54	15,50	10,621	0,107
39,0	$4,33.10^8$	3,58	13,92	11,184	0,134
42,0	$3,00.10^8$	3,25	15,17	11,319	0,039
45,7	$2,29.10^8$	2,86	19,42	9,604	0,085
48,3		2,49	22,81	8,385	0,095
51,0	$3,17.10^8$	2,41	25,69	7,414	0,182
54,0	$5,00.10^8$	2,37	27,92	5,645	0,350
57,0	$6,50.10^8$	2,88	25,83	6,652	0,489

time (hrs)	butanol (gl^{-1})	acetone (gl^{-1})	ethanol (gl^{-1})	butyrate (gl^{-1})	acetate (gl^{-1})
0,0	3,434	2,236	0,251	0,038	0,248
3,0	4,546	3,488	0,126	0,022	0,093
6,0	5,269	4,021	0,142	0,004	0,000
9,0	5,562	4,275	0,189	0,009	0,016
12,0	5,202	4,000	0,176	0,017	0,006
15,0	4,685	3,585	0,157	0,018	0,060
18,0	3,787	2,855	0,108	0,067	0,096
21,0	3,209	2,217	0,258	0,200	0,176
24,0	2,990	1,996	0,196	0,358	0,237
27,0	3,171	2,206	0,335	0,059	0,205
30,0	4,011	3,186	0,096	0,043	0,156
33,0	4,961	4,065	0,123	0,025	0,108
36,0	5,728	4,699	0,194	0,023	0,084
39,0	6,059	4,915	0,210	0,018	0,116
42,0	6,091	5,047	0,181	0,019	0,020
45,7	5,153	4,260	0,191	0,025	0,060
48,3	4,561	3,713	0,111	0,037	0,058
51,0	3,896	2,932	0,586	0,072	0,110
54,0	3,437	1,957	0,251	0,133	0,217
57,0	3,767	2,771	0,114	0,155	0,334

Figure 5.4: Product yields during extended continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}

time (hrs)	total solvent yield	total acid yield
0,0	0,281	0,014
3,0	0,304	0,004
6,0	0,311	0,000
9,0	0,309	0,001
12,0	0,304	0,001
15,0	0,303	0,003
18,0	0,275	0,007
21,0	0,269	0,018
24,0	0,254	0,029
27,0	0,264	0,012
30,0	0,281	0,008
33,0	0,295	0,004
36,0	0,296	0,003
39,0	0,299	0,004
42,0	0,313	0,001
45,7	0,301	0,003
48,3	0,294	0,003
51,0	0,289	0,007
54,0	0,241	0,015
57,0	0,261	0,019

Figure 5.5: Morphological and cytological changes in C. acetobutylicum P 262 during extended continuous fermentation at a dilution rate of 0,1 hr^{-1}

time (hrs)	granulose positive rods (cells ml^{-1})	granulose negative rods (cells ml^{-1})	short[a] rods (cells ml^{-1})	medium[b] rods (cells ml^{-1})	long[c] rods (cells ml^{-1})
0,0	$6,39.10^8$	$2,24.10^8$	$6,47.10^8$	$1,81.10^8$	$1,73.10^7$
3,0	$6,16.10^8$	$1,84.10^8$	$6,08.10^8$	$1,52.10^8$	$2,40.10^7$
6,0	$4,98.10^8$	$1,40.10^8$	$4,59.10^8$	$1,53.10^8$	$1,91.10^7$
9,0	$3,66.10^8$	$1,42.10^8$	$3,35.10^8$	$1,32.10^8$	$3,05,10^7$
12,0	$2,31.10^8$	$0,90.10^8$	$1,77.10^8$	$9,30.10^7$	$4,82.10^7$
15,0	$1,76.10^8$	$0,99.10^8$	$1,51.10^8$	$9,60.10^7$	$3,03.10^7$
18,0	$1,43.10^8$	$1,13.10^8$	$1,51.10^8$	$9,00.10^7$	$1,28.10^7$
21,0	$1,22.10^8$	$1,84.10^8$	$1,93.10^8$	$1,04.10^8$	$6,12.10^6$
24,0	$1,83.10^8$	$3,25.10^8$	$3,96.10^8$	$9,70.10^7$	$5,05.10^6$
27,0	$2,03.10^8$	$4,53.10^8$	$5,31.10^8$	$1,25.10^8$	0,0
30,0	$2,88.10^8$	$4,33.10^8$	$5,55.10^8$	$1,51.10^8$	$7,21.10^6$
33,0	$3,87.10^8$	$3,30.10^8$	$5,02.10^8$	$1,79.10^8$	$3,59.10^7$
39,0	$2,60.10^8$	$1,73.10^8$	$2,60.10^8$	$1,26.10^8$	$5,20.10^7$
42,0	$1,50.10^8$	$1,50.10^8$	$1,53.10^8$	$9,30.10^7$	$5,40.10^7$
45,7	$1,63.10^8$	$6,60.10^7$	$1,08.10^8$	$9,20,10^7$	$2,98.10^7$
51,0	$1,84.10^8$	$1,33.10^8$	$1,59.10^8$	$1,17.10^8$	$3,80.10^7$
54,0	$2,30.10^8$	$2,70.10^8$	$2,90.10^8$	$1,55.10^8$	$4,56.10^7$
57,0	$4,81.10^8$	$1,69.10^8$	$4,36.10^8$	$1,82.10^8$	$1,95.10^7$

a: 1 to 2 units in length
b: 2 to 6 units in length
c: >6 units in length

time (hrs)	short[a] rods (%)	medium[b] rods (%)	long[c] rods (%)
0,0	75	21	2
3,0	76	19	3
6,0	72	24	3
9,0	66	26	6
12,0	55	29	15
15,0	55	35	11
18,0	59	35	5
21,0	63	34	2
24,0	78	19	1
27,0	81	19	0
30,0	77	21	1
33,0	70	25	5
39,0	60	29	12
42,0	51	31	18
45,7	47	40	13
51,0	50	37	12
54,0	58	31	9
57,0	67	28	3

a: 1 to 2 units in length
b: 2 to 6 units in length
c: >6 units in length

Figure 5.6: Specific rate of cell division during extended continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}

time (hrs)	Specific rate of division (hr^{-1})
1,5	0,075
4,5	0,025
7,5	0,024
10,5	-0,050
13,5	0,049
16,5	0,076
19,5	0,159
22,5	0,265
25,5	0,186
28,5	0,130
31,5	0,098
40,5	-0,021
43,8	0,027
52,5	0,249
55,5	0,187

Figure 5.7: Specific rate of growth during extended continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}

time (hrs)	specific rate of growth (hr^{-1})
1,5	0,182
4,5	0,114
7,5	0,079
10,5	0,060
13,5	0,047
16,5	0,057
19,5	0,077
22,5	0,093
25,5	0,160
28,5	0,167
31,5	0,152
34,5	0,124
37,5	0,104
40,5	0,068
43,8	0,065
47,0	0,047
49,6	0,088
52,5	0,094
55,5	0,165

Figure 5.8: Relationship of redox potential with specific division rate, product yields and cell numbers during extended continuous fermentation of C. acetobutylicum P 262 at a dilution rate of 0,1 hr^{-1}

time (hrs)	redox potential[a] (mV)	specific division rate (hr^{-1})	total solvent yield	total acid yield	total cells (cells ml^{-1})
0,0	-458		0,281	0,014	$8,63.10^8$
1,5		0,075			
3,0	-458		0,304	0,004	$8,00.10^8$
4,5		0,025			
6,0	-452		0,311	0,000	$6,38.10^8$
7,5		0,024			
9,0	-460		0,309	0,001	$5,08.10^8$
10,5		-0,050			
12,0	-460		0,304	0,001	$3,21.10^8$
13,5		0,049			
15,0	-450		0,303	0,003	$2,75.10^8$
16,5		0,076			
18,0	-448		0,275	0,007	$2,56.10^8$
19,5		0,159			
21,0	-429		0,269	0,018	$3,06.10^8$
22,5		0,265			
24,0	-430		0,254	0,029	$5,08.10^8$
25,5		0,186			
27,0	-452		0,264	0,012	$6,56.10^8$
28,5		0,130			
30,0	-458		0,281	0,008	$7,21.10^8$
31,5		0,098			
33,0	-458		0,295	0,004	$7,17.10^8$
36,0	-458		0.296	0,003	
39,0	-455		0,299	0,004	$4,33.10^8$
40,5		-0,021			
42,0	-449		0,313	0,001	$3,00.10^8$
43,8		0,027			
45,7	-433		0,301	0,003	$2,29.10^8$
48,3	-423		0,294	0,003	
51,0	-421		0,289	0,007	$3,17.10^8$
52,5		0,249			
54,0	-426		0,241	0,015	$5,00.10^8$
55,5		0,187			
57,0	-450		0,261	0,019	$6,50.10^8$

a: silver/silver chloride reference electrode

Figure 5.9: Growth and physiological changes in C. acetobutylicum P 262 during extended continuous fermentation at a dilution rate of 0,25 hr^{-1}

time (hrs)	total cells (cells ml^{-1})	cell dry mass (gl^{-1})	glucose (gl^{-1})	total solvents (gl^{-1})	total acids (gl^{-1})
0,0	$1,15.10^9$	4,03	23,60	7,676	0,881
2,0	$1,15.10^9$	3,93	22,93	8,522	0,502
4,0		3,64	23,80	8,082	0,562
6,0	$1,17.10^9$	3,46	24,20	7,056	0,342
8,0			24,80	7,050	0,476
12,0	$1,07.10^9$	3,22	27,13	6,556	0,516
15,0	$1,10.10^9$	3,22	27,47	6,374	0,588
22,0	$1,65.10^9$	3,64	24,40	7,272	0,738
24,5	$1,59.10^9$	3,82	23,55	7,302	0,882
26,5	$1,31.10^9$	3,71	24,20	7,574	0,562
28,5	$1,02.10^9$	3,60	24,00	7,046	0,528

time (hrs)	butanol (gl^{-1})	acetone (gl^{-1})	ethanol (gl^{-1})	butyrate (gl^{-1})	acetate (gl^{-1})
0,0	4,454	2,699	0,523	0,376	0,505
2,0	4,990	3,447	0,085	0,231	0,271
4,0	4,780	3,190	0,112	0,280	0,282
6,0	4,178	2,630	0,248	0,180	0,162
8,0	4,226	2,598	0,226	0,204	0,272
12,0	3,718	2,446	0,392	0,234	0,282
15,0	3,656	2,660	0,058	0,256	0,332
22,0	4,162	3,002	0,108	0,316	0,422
24,5	4,196	2,776	0,330	0,444	0,438
26,5	4,358	3,102	0,114	0,300	0,262
28,5	4,078	2,630	0,338	0,250	0,278

Figure 5.10: Morphological and cytological changes in C. acetobutylicum P 262 during extended continuous fermentation at a dilution rate of 0,25 hr^{-1}

time (hrs)	granulose positive rods (cells ml^{-1})	granulose negative rods (cells ml^{-1})	short[a] rods (cells ml^{-1})	medium[b] rods (cells ml^{-1})	long[c] rods (cells ml^{-1})
0,0	$1,10.10^9$	$4,60.10^7$	$5,29.10^8$	$4,26.10^8$	$2,45.10^7$
2,0	$1,10.10^9$	$4,60.10^7$	$5,41.10^8$	$5,18.10^8$	0,0
6,0	$9,80.10^8$	$1,90.10^8$	$4,68.10^8$	$4,80.10^8$	$5,85.10^7$
12,0	$7,70.10^8$	$3,00.10^8$	$6,10.10^8$	$2,14.10^8$	$4,28.10^7$
15,0	$8,14.10^8$	$2,86.10^8$	$6,16.10^8$	$1,54.10^8$	$4,40.10^7$
22,5	$1,44.10^9$	$2,15.10^8$	$1,06.10^9$	$2,97.10^8$	$4,95.10^7$
24,5	$1,45.10^9$	$1,43.10^8$	$9,70.10^8$	$3,02.10^8$	$7,95.10^7$
26,5	$1,27.10^9$	$3,93.10^7$	$7,86.10^8$	$3,67.10^8$	$5,24.10^7$
28.5	$9,89.10^8$	$3,06.10^7$	$5,41.10^8$	$3,16.10^8$	$3,06.10^7$

time (hrs)	short[a] rods (%)	medium[b] rods (%)	long[c] rods (%)
0,0	46	37	3
2,0	47	45	0
6,0	40	41	5
12,0	57	20	4
15,0	56	14	4
22,5	64	18	3
24,5	61	19	5
26,5	60	28	4
28,5	63	31	3

a: 1 to 2 units in length
b: 2 to 3 units in length
c: >3 units in length

Figure 5.11: Morphological changes in C. acetobutylicum P 262 during continuous fermentation at a dilution rate of 0,10 hr^{-1} as the pH is increased by 0,2 units

time (hrs)	total cells with forespore septa (cells ml^{-1})	short[a] cells with forespore septa (cells ml^{-1})	medium[b] cells with forespore septa (cells ml^{-1})	long[c] cells with forespore septa (cells ml^{-1})
21,0	$2,00.10^6$	$2,00.10^6$	0,0	0,0
24,0	$2,00.10^6$	$2,00.10^6$	0,0	0,0
27,0	$2,00.10^6$	$2,00.10^6$	0,0	0,0
28,0	0,0	0,0	0,0	0,0
30,0	0,0	0,0	0,0	0,0
32,0	0,0	0,0	0,0	0,0
34,0	$4,20.10^7$	$3,40.10^7$	$8,00.10^6$	0,0
36,0	$2,08.10^8$	$2,08.10^8$	0,0	0,0
38,0	$7,47.10^8$	$6,71.10^8$	$3,80.10^7$	$3,80.10^7$
40,0	$7,14.10^8$	$6,32.10^8$	$6,60.10^7$	$1,60.10^7$
43,0	$4,96.10^8$	$4,83.10^8$	$1,30.10^7$	0,0
46,5	$3,99.10^8$	$3,39.10^8$	$5,40.10^7$	$6,00.10^6$
49,0	$2,95.10^8$	$2,42.10^8$	$5,30.10^7$	0,0
52,0	$1,74.10^8$	$1,68.10^8$	$6,00.10^6$	0,0
55,0	$1,20.10^8$	$1,03.10^8$	$1,70.10^7$	0,0
58,0	$1,06.10^8$	$5,90.10^7$	$4,20.10^7$	$5,00.10^6$
61,0	$1,38.10^8$	$8,90.10^7$	$4,90.10^7$	0,0

a: 1 to 2 units in length
b: 2 to 6 units in length
c: >6 units in length

time (hrs)	total cells with forespore septa (%)	short[a] cells with forespore septa (%)	medium[b] cells with forespore septa (%)	long[c] cells with forespore septa (%)
21,0	0,8	0,8	0,0	0,0
24,0	0,8	0,8	0,0	0,0
27,0	1,1	1,1	0,0	0,0
28,0	0,0	0,0	0,0	0,0
30,0	0,0	0,0	0,0	0,0
32,0	0,0	0,0	0,0	0,0
34,0	4,0	3,2	0,8	0,0
36,0	11,0	11,0	0,0	0,0
38,0	39,6	35,6	2,0	2,0
40,0	48,9	43,2	4,5	1,1
43,0	52,9	51,4	1,4	0,0
46,5	60,9	51,8	8,2	0,9
49,0	57,4	47,1	10,3	0,0
52,0	43,2	41,9	1,4	0,0
55,0	34,3	29,5	4,8	0,0
58,0	22,9	12,8	9,1	1,0
61,0	18,4	11,9	6,5	0,0

a: 1 to 2 units in length
b: 2 to 6 units in length
c: >6 units in length

Figure 5.12: Growth and physiological changes in C. acetobutylicum P 262 during continuous fermentation at a dilution rate of 0,1 hr^{-1} as the pH is increased by 0,2 units

time (hrs)	total cells (cells ml^{-1})	cell dry mass (gl^{-1})	glucose (gl^{-1})	total solvents (gl^{-1})	total acids (gl^{-1})
0,0	$7,18.10^8$	2,37	32,47	4,372	0,662
2,0	$7,73.10^8$	2,76	29,73	5,365	0,553
4,0	$1,01.10^9$	3,49	25,83	7,334	0,239
6,0	$1,20.10^9$	4,45	18,63	9,739	0,066
9,0	$1,18.10^9$	4,86	12,48	12,029	0,007
12,0	$9,68.10^8$	4,58	11,13	12,479	0,004
15,0	$6,20.10^8$	3,92	12,65	12,714	0,016
18,0	$3,93.10^8$	3,51	15,90	12,201	
21,0	$3,03.10^8$	2,93	20,47	10,131	0,187
24,0	$2,23.10^8$	2,51	25,92	8,299	0,350
27,0	$2,20.10^8$	2,27	29,69	7,153	0,601
28,0	$2,90.10^8$	2,18	30,83	6,651	0,825
30,0	$3,85.10^8$	2,27	31,20	6,129	1,693
32,0	$7,13.10^8$	2,62	29,85	6,058	2,504
34,0	$1,05.10^9$	3,49	25,83	7,581	2,022
36,0	$1,90.10^9$	4,89	18,74	10,317	1,080
38,0	$1,89.10^9$	6,33	11,40	12,579	0,508
40,0	$1,46.10^9$	7,06	6,82	13,966	0,257
43,0	$9,39.10^8$	6,93	6,67	14,442	0,241
46,5	$6,55.10^8$	5,94	10,38	13,372	0,271
49,0	$5,14.10^8$	5,21	12,98	12,669	0,658
52,0	$4,02.10^8$	4,55	16,65	11,289	0,704
55,0	$3,49.10^8$	4,19	18,75	9,380	0,891
58,0	$4,58.10^8$	4,06	19,99		1,019
61,0	$7,48.10^8$	4,21	17,73	10,527	0,923

time (hrs)	butanol (gl^{-1})	acetone (gl^{-1})	ethanol (gl^{-1})	butyrate (gl^{-1})	acetate (gl^{-1})
0,0	2,615	1,567	0,190	0,413	0,249
2,0	3,146	1,933	0,286	0,230	0,323
4,0	4,141	3,090	0,103	0,088	0,151
6,0	5,360	3,934	0,445	0,037	0,029
9,0	6,766	5,151	0,112	0,007	0,000
12,0	7,035	5,283	0,161	0,004	0,000
15,0	7,141	5,118	0,455	0,016	0,000
18,0	6,826	4,825	0,550	0,128	
21,0	5,641	3,996	0,494	0,107	0,080
24,0	4,632	3,369	0,298	0,174	0,176
27,0	3,917	2,855	0,381	0,300	0,301
28,0	3,603	2,625	0,423	0,451	0,374
30,0	3,157	2,249	0,723	0,944	0,749
32,0	3,285	2,166	0,607	1,136	1,368
34,0	4,407	2,803	0,371	0,474	1,548
36,0	5,900	4,020	0,397	0,307	0,773
38,0	7,258	4,898	0,423	0,184	0,324
40,0	8,229	5,318	0,419	0,092	0,165
43,0	8,691	5,219	0,532	0,128	0,113
46,5	8,220	4,745	0,407	0,097	0,174
49,0	7,727	4,516	0,426	0,375	0,283
52,0	6,775	4,067	0,447	0,328	0,376
55,0	6,065	2,901	0,414	0,429	0,462
58,0				0,408	0,611
61,0	6,256	3,520	0,751	0,335	0,588

Figure 5.13: Morphological and cytological changes in C. acetobutylicum P 262 during continuous fermentation at a dilution rate of 0,1 hr^{-1} as the pH is increased by 0,2 units

time (hrs)	granulose positive rods (cells ml^{-1})	granulose negative rods (cells ml^{-1})	short[a] rods (cells ml^{-1})	medium[b] rods (cells ml^{-1})	long[c] rods (cells ml^{-1})
0,0	$3,83.10^8$	$3,35.10^8$	$3,35.10^8$	$2,74.10^8$	$1,10.10^8$
2,0	$4,07.10^8$	$3,66.10^8$	$5,64.10^8$	$1,75.10^8$	$2,30.10^7$
4,0	$7,48.10^8$	$2,62.10^8$	$8,76.10^8$	$1,13.10^8$	$1,40.10^7$
6,0	$9,56.10^8$	$2,39.10^8$	$9,95.10^8$	$1,43.10^8$	$3,90.10^7$
9,0	$9,92.10^8$	$1,83.10^8$	$7,99.10^8$	$3,28.10^8$	$4,80.10^7$
12,0			$5,23.10^8$	$3,55.10^8$	$9,10.10^7$
15,0	$5,51.10^8$	$6,90.10^7$	$2,51.10^8$	$2,89.10^8$	$5,30.10^7$
18,0	$3,40.10^8$	$5,30.10^7$	$1,00.10^8$	$2,27.10^8$	$6,60.10^7$
21,0	$2,59.10^8$	$4,40.10^7$	$2,50.10^7$	$2,25.10^8$	$5,10.10^7$
24,0	$1,89.10^8$	$3,40.10^7$	$3,40.10^7$	$1,45.10^8$	$4,30.10^7$
27,0	$1,40.10^8$	$8,00.10^7$			
28,0	$1,59.10^8$	$1,31.10^8$	$8,00.10^7$	$1,75.10^8$	$3,50.10^7$
30,0	$1,82.10^8$	$2,03.10^8$	$1,88.10^8$	$1,54.10^8$	$4,40.10^7$
32,0	$4,91.10^8$	$2,23.10^8$	$5,37.10^8$	$1,67.10^8$	$9,00.10^6$
34,0	$9,32.10^8$	$1,18.10^8$	$8,15.10^8$	$1,68.10^8$	$2,50.10^7$
36,0	$1,83.10^9$	$6,10.10^7$	$1,35.10^9$	$2,46.10^8$	$9,50.10^7$
38,0	$1,82.10^9$	$7,00.10^7$	$8,58.10^8$	$1,68.10^8$	$1,11.10^8$
40,0	$1,45.10^9$	$1,60.10^7$	$4,49.10^8$	$2,17.10^8$	$8,30.10^7$
43,0	$9,01.10^8$	$3,80.10^7$	$1,88.10^8$	$1,75.10^8$	$8,10.10^7$
46,5	$6,31.10^8$	$2,40.10^7$	$7,70.10^7$	$1,37.10^8$	$4,20.10^7$
49,0	$5,06.10^8$	$8,00.10^6$	$1,50.10^7$	$1,29.10^8$	$7,60.10^7$
52,0	$3,86.10^8$	$1,60.10^7$	$6,00.10^6$	$1,30.10^8$	$9,20.10^7$
55,0	$3,24.10^8$	$2,50.10^7$	$5,40.10^7$	$8,50.10^7$	$9,10.10^7$
58,0	$3,90.10^8$	$6,80.10^7$	$1,04.10^8$	$1,23.10^8$	$1,26.10^8$
61,0	$6,72.10^8$	$7,60.10^7$	$3,01.10^8$	$1,97.10^8$	$1,13.10^8$

a: 1 to 2 units in length
b: 2 to 6 units in length
c: >6 units in length

time (hrs)	short[a] rods (%)	medium[b] rods (%)	long[c] rods (%)
0,0	47	38	15
2,0	73	23	3
4,0	87	11	1
6,0	83	12	3
9,0	68	28	4
12,0	54	37	9
15,0	41	47	9
18,0	26	58	17
21,0	8	74	17
24,0	15	65	19
28,0	28	60	12
30,0	49	40	11
32,0	75	23	1
34,0	78	16	2
36,0	71	13	5
38,0	46	9	6
40,0	31	15	6
43,0	20	19	9
46,5	12	21	6
49,0	3	25	15
52,0	1	32	23
55,0	15	24	26
58,0	23	27	28
61,0	40	26	15

a: 1 to 2 units in length
b: 2 to 6 units in length
c: >6 units in length

Figure 6.1: Growth and physiological changes in C. acetobutylicum cls⁻ during batch fermentation at pH 5,0

time (hrs)	ln (cell dry mass)	ln (total cells.10^{-8})	glucose (gl^{-1})	butanol plus acetone (gl^{-1})	total acids (gl^{-1})
1,5	-1,17	-0,39	45,44	0,042	0,679
3,5	-0,04	1,55	43,13	0,137	2,457
5,5	0,68	2,19	39,42	0,095	4,294
7,5	1,02	2,60	34,75	0,308	6,012
9,5	1,13	3,00	32,13	0,698	7,460
11,5	1,14	3,17	28,42	1,146	8,146
13,5	1,13	3,18	25,42	1,556	8,671
15,5	1,01	3,25	24,20	1,923	9,272
17,5	0,85	2,91	23,20	1,973	9,687
19,5	0,77	2,77	23,00	1,999	9,490

Figure 6.2: Growth and physiological changes in C. acetobutylicum cls⁻ during batch fermentation at pH 6,0

time (hrs)	ln(cell dry mass)	ln(total cells.10^{-8})	glucose (gl^{-1})	butanol plus acetone (gl^{-1})	total acids (gl^{-1})
1,0	-2,04	-2,85	43,96	0,065	1,001
3,0	-0,89	-0,21	43,33	0,080	1,630
5,0	0,37	1,67	36,00	0,040	4,097
7,0	1,04	3,04	25,83	0,037	8,621
9,0	1,28	3,27	13,92	0,079	13,593
11,0	1,42	3,50	2,39	0,254	18,512
13,0	0,28	3,13	0,04	0,409	20,320
15,0	-0,05	2,94	0,00	0,444	19,947
17,0	-0,09	2,82	0,04	0,347	20,307

Figure 6.3: Growth and physiological changes in C. acetobutylicum P 262 during batch fermentation at pH 5,0

time (hrs)	ln (cell dry mass)	ln (total cells.10^{-8})	glucose (gl^{-1})	butanol plus acetone (gl^{-1})	total acids (gl^{-1})
1,8	-1,14	0,31	45,78	0,090	0,776
3,3	-0,31	1,24	43,58	0,073	1,476
4,8	0,39	1,71	40,92	0,264	2,349
6,8	1,24	2,56	34,17	2,725	1,640
8,8	1,59	2,81	26,67	5,677	0,316
10,8	1,87	3,02	21,33	7,506	0,244
12,8	1,86	3,23	17,13	8,982	0,189
15,8	1,80	3,08	13,88	10,210	0,284
18,8	1,66	2,73	12,92	10,430	0,100
21,8	1,51	2,38	12,92	10,350	0,151

Figure 6.4: Growth and physiological changes in C. acetobutylicum P 262 during batch fermentation at pH 6,0

time (hrs)	ln (cell dry mass)	ln (total cells.10^{-8})	glucose (gl^{-1})	butanol plus acetone (gl^{-1})	total acids (gl^{-1})
0,0	-2,53		47,3	0,070	0,413
2,0	-1,39	-0,67	44,6	0,062	0,557
4,0	-0,25	0,74	41,1	0,031	2,004
6,0	0,65	1,93	34,4	0,039	5,076
8,0	1,12	2,79	25,8	0,556	8,365
10,0	1,38	3,13	14,1	3,397	9,926
13,0	1,70	3,66	0,0	9,353	8,359
17,0	0,66		0,0	9 839	8,448

Figure 6.5: Comparison of the growth patterns of C. acetobutylicum P 262 during batch solventogenic fermentations

pH control	time (hrs)	ln (cell dry mass)	butanol plus acetone (gl^{-1})	total acids (gl^{-1})
none	1,0	-1,37	0,074	0,574
	2,5	-0,56	0,063	1,163
	4,0	-0,11	0,440	1,100
	6,0	0,53	2,012	0,579
	8,0	1,10	3,815	0,667
	10,0	1,58	5,837	0,413
	12,0	1,80	8,300	0,305
	15,0	1,99	11,588	0,206
	18,0	1,99	13,253	0,192
	21,0	1,97	14,015	0,152
5	1,8	-1,14	0,090	0,776
	3,3	-0,31	0,073	1,476
	4,8	0,39	0,264	2,349
	6,8	1,24	2,725	1,640
	8,8	1,59	5,677	0,316
	10,8	1,87	7,506	0,244
	12,8	1,86	8,982	0,189
	15,8	1,80	10,210	0,284
	18,8	1,66	10,430	0,100
	21,8	1,51	10,350	0,151
6	0,0	-2,53	0,070	0,413
	2,0	-1,39	0,062	0,557
	4,0	-0,25	0,031	2,004
	6,0	0,65	0,039	5,076
	8,0	1,12	0,556	8,365
	10,0	1,38	3,397	9,926
	13,0	1,70	9,353	8,359
	17,0	0,66	9,839	8,448