ROLE OF MOTILITY AND CHEMOTAXIS IN SOLVENT PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM*

NOEMI A. GUTIERREZ

A Thesis Presented In Partial
Fulfilment of the Requirements for the
Degree of Doctor of Philosophy
in Biotechnology at
Massey University

1989

To those who love me and whom I love,
especially my husband Ian and
my parents, Luding and Turing.

ABSTRACT

The motility of Clostridium acetobutylicum P262 and its relationship to solventogenesis were investigated. Motility was monitored in a typical batch fermentation process using sulphuric acid casein whey permeate as substrate. The motile behaviour of C. acetobutylicum was characterized by "runs" wherein the cells were observed to swim in a long, smooth line, then the cells "tumbled" by thrashing around for a few seconds before running again. The "runs" were particularly associated with the early phase of sugar utilization and acid production, while "tumbles" were associated with the onset of solventogenesis. During solvent production, the cells tumbled more frequently and the runs progressively became shorter and slower. The proportion of cells in the culture which exhibited motility increased to almost 100% up to 13h after inoculation, but decreased considerably after this time.

Assays for positive chemotaxis (chemoattraction) and negative chemotaxis (chemorepulsion) were performed to identify the chemoeffectors of C. acetobutylicum. Motile cells of C. acetobutylicum were observed to migrate towards glucose, galactose, and lactose. These sugars were identified as attractants. Acetate and butyrate elicited a dual response. Cells were repelled from the dissociated form and attracted towards the undissociated form above a minimum threshold concentration. Chemoattraction to butyric acid was observed at a threshold concentration of 9×10^{-2} M which is similar to the concentration of undissociated butyric acid inside the cell (1.3×10^{-2} M) at which solventogenesis is reported to be initiated, suggesting that the intracellular butyric acid concentration is the likely switch for

solventogenesis to commence. The solvents acetone, butanol and ethanol were identified as repellents.

The behavioural response of C. acetobutylicum towards the sugars, acids and solvents demonstrates that the motility observed during fermentation is a chemotactic response. Chemotaxis appears to provide survival advantage to C. acetobutylicum.

A non-motile mutant was isolated by mutagenesis using ethyl methane sulfonate. This mutant was morphologically indistinguishable from the motile parent strain, such that it possesses flagella in typical number and shape as those of the parent, and is capable of producing clostridial forms and endospores. This type of mutant is a paralyzed mutant and the mutation may be a defect in any of the genes that code for flagellar rotation.

The non-motile mutant was capable of solvent production suggesting that motility is not a regulatory mechanism for the switch to solvent production, but merely a behavioural chemotactic response. However, the maximum butanol concentration achieved, the initial rate of butanol production, the yield, and the sugar utilization observed with the mutant were poorer than those of the parent strain. These confirm the positive relationship between motile, chemotactic cultures and solvent production. The low butanol production by the non-motile mutant suggests that the mutant has a lower butanol tolerance than does the parent.

Inhibition studies have shown that both growth and solvent production of C. acetobutylicum are subject to end-product toxicity. Growth inhibition studies confirmed that the non-motile mutant was less tolerant to solvents than was the parent.

A deficiency in membrane-bound ATPase activity was observed with the non-motile mutant but not in the parent strain. This deficiency in ATPase activity, lack of motility, and lower butanol tolerance may explain the low butanol production by the mutant.

On a percentage basis, greater inhibition of solvent production was observed in the parent than in the mutant suggesting that butanol toxicity during the solvent production phase is more profound in the presence of another target site (i.e. ATPase) in addition to the cell membrane. It was further suggested that during growth, butanol inhibition due to membrane disruption was more important than inhibition of ATPase.

Thus, chemotaxis prevents C. acetobutylicum from being confined in a toxic situation. Motile cells are more solventogenic because they can chemotactically respond to changes in their environment, and are less susceptible to product inhibition.

ACKNOWLEDGEMENTS

I sincerely thank my supervisor, Dr. Ian S. Maddox, for his guidance, enthusiasm and encouragement throughout the course of this study.

I also appreciate the cooperation from Dr. Lindsay Pearce and Dr. Graham Manderson.

I gratefully acknowledge the New Zealand Ministry of Foreign Affairs for the financial support towards this degree and the University of the Philippines at Los Banos for allowing me the time to pursue my academic advancement.

I am thankful to the NZ Globus Group for the Award for Higher Studies in 1986.

Appreciation is also extended to the following:

- Mr Doug Hopcroft of Biotechnology Division, Department of Scientific and Industrial Research, for the electron microscope photographs.
- Dr. Brett Ennis, Miss Kerrie Hancock, Mr Sunthorn Kanchanatawee and Dr. Nasib Qureshi for sharing their enthusiasm on Clostridium acetobutylicum and their friendship.
- Mr M. Stevens, Mrs A.-M. Jackson, Mrs J. Collins, Mr J. Sykes and Mr J. Alger for their assistance in the laboratory.
- Miss V. Davis for typing my thesis and Mr L. Diamante for the illustrations.

- TM for 'recharging' me.
- My parents, brothers and sisters for their love, constant support and encouragement.
- My husband, Ian, and our Keir for their love, support and understanding.

TABLE OF CONTENTS

Page

Page

Page

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABE	Acetone-Butanol-Ethanol
ATP	Adenosine 5'-tri-phosphate
ATPase	ATP phosphohydrolase
CCCP	Carbonylcyanidene-m-chloro-phenylhydrazone
CFU	Colony forming unit
CM	Chemotaxis Medium
CMMG	Cooked Meat Medium, supplemented with glucose
CoA	Coenzyme A
CW, CCW	Clockwise, Counterclockwise rotation
DCCD	N, N'-Dicyclohexylcarbodiimide
EDTA	Ethylenediaminetetraacetate
EMS	Ethyl-methanesulfonate
FCCP	Carbonylcyanide-p-tri-fluoromethoxyphenylhydrazone
MCP	Methyl-accepting chemotaxis protein
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NAD^+, NADH	Nicotinamide adenine dinucleotide, and its reduced form
NYG	Nutrient Broth Yeast Extract Agar
OD	Optical density
PEP	Phosphoenolpyruvate
pHi	Internal or intracellular pH
Pi	Inorganic phosphate
PIPES	Piperazine N N'-bis-2-ethane-sulphonic acid
RCA	Reinfored Clostridial Agar
SSM	Semi-Synthetic Medium
TA	Tryptone Agar
WPA	Sulphuric acid-casein Whey Permeate Agar

CHAPTER 1

INTRODUCTION

Part I BACKGROUND AND OBJECTIVES OF THE THESIS

The finite supply of fossil fuels and the oil crisis of the mid-1970s sparked new interest in the microbial production of chemicals, such as butanol, that can be used as alternative fuel. However, the production of acetone-butanol-ethanol (ABE) by fermentation is limited not only by high substrate costs but also by severe product inhibition and inefficient butanol recovery systems. These limitations need to be relieved before the process can economically compete with cheaper chemical synthetic processes using petrochemical feedstocks.

The 'biotechnology' of the ABE fermentation typically employs biological and engineering approaches to overcome its limitations. In the engineering approach, technologies are being developed and intensified such as continuous reactor systems (using free cells, immobilized cells and cell recycle), and novel cost-efficient extraction of solvents from the fermentation broth. Product recovery processes can be integrated with optimized fermentation processes to achieve both efficient substrate utilization and increased solvent production. The biological approach entails fundamental studies on the organism Clostridium acetobutylicum to understand the factors involved in the onset and maintenance (i.e. regulation) of solvent production. It also involves understanding the physiological and molecular nature of acid and solvent toxicity and tolerance. Genetic manipulation using

mutation and/or recombinant DNA technologies are carried out not only to elucidate the regulatory mechanisms of solvent production, but also to develop strains with superior solvent producing ability, versatile substrate utilization or increased solvent tolerance.

Many reactor designs have been applied to the fermentation process, while novel ones are continually being devised. Likewise, many of the biological aspects of the fermentation, namely, acid production, solvent production, end-product inhibition and cell differentiation have been investigated. However, with regard to cell differentiation, one morphological stage that makes the ABE fermentation a fascinating phenomenon has been recognized but not investigated on its own merits - the motility of C. acetobutylicum.

Spivey (1978) observed a relationship between vigorous motility and strong solvent production in the industrial strain C. acetobutylicum P262. The absence of motility at any time during the inoculum development stage was a clear indication that poor solvent yields would result despite a normal cell population. Spivey, however, found the biochemical basis of this relationship obscure. Recently, the relationship has been confirmed after significantly higher butanol concentrations and production rates were obtained by ensuring the use of a highly motile culture during inoculum development (Gutierrez, 1985; Gutierrez and Maddox, 1987).

Thus, the present study was conceived to explain the 'obscure' relationship between motility and solvent production in C. acetobutylicum. Some fundamental studies on the motility of other

bacteria such as Escherichia coli and Salmonella typhimurium were applied to C. acetobutylicum in order to characterize its motile behaviour. Furthermore, this study hoped to contribute additional knowledge of the physiology of C. acetobutylicum which may aid in identifying the regulatory devices involved in solvent production, and in understanding the mechanism of butanol tolerance.

Part II THE ACETONE-BUTANOL-ETHANOL (ABE) FERMENTATION

The purpose of this section is to review some recent studies leading to an understanding of the physiology of C. acetobutylicum and to highlight those aspects which are relevant to the present study. A broader overview of the ABE fermentation can be obtained in recent reviews by Linden et al (1986); Ennis et al (1986); Jones and Woods (1986); McNeil and Kristiansen (1986); and Awang et al (1988).

1.II.1 History of the ABE Fermentation

The development of the ABE fermentation holds a real historical significance which is well documented (Gabriel, 1928; Gabriel and Crawford, 1930; Prescott and Dunn, 1959; Rose, 1961; Hastings, 1978; Compere and Griffith, 1979; Jones and Woods, 1986). Butanol was first discovered as a fermentation product by Pasteur in 1861. In 1905, Schardinger discovered acetone as an additional product (Jones and Woods, 1986). The ABE fermentation process was developed at the turn of the century due to the shortage of natural rubber. With this shortage, the English company Strange and Graham Ltd recruited the services of Weizmann, Perkins, Fernbach and Schoen to investigate the production of the synthetic rubber. They decided then that the best route for production of butadiene or isoprene was from butanol or isoamyl alcohol (Gabriel and Crawford, 1930). This led to the development of a microbial fermentation process for butanol production utilizing Fernbach's isolate which was able to ferment

potatoes, but not maize starch. Between 1912 and 1914, Weizmann isolated a culture which produced higher concentrations of butanol and acetone than that of Fernbach's culture. This organism is now known as Clostridium acetobutylicum.

During World War I, the emphasis on butanol production decreased while the demand for acetone sharply increased, since acetone was used to dissolve cordite in the manufacture of explosives. The fermentation process, initially established in England, was subsequently moved to the U.S.A. and Canada. The demand for acetone decreased after the war, but with the development of the automobile industry, butanol production gained importance again due to its use in the manufacture of solvent (butyl acetate) for nitrocellulose lacquers.

Apart from production problems such as contamination by phage and bacilli (Beesch, 1953; McCutchan and Hickey, 1954; Walton and Martin, 1979), the ABE fermentation industry was active until after World War II. It started to decline, however, due to the unprecedented growth of the economically attractive petrochemical processes, and the increased use of grains and molasses for human and animal consumption. In the 1960's, the fermentation-based processes in Europe and North America were terminated. The National Chemical Products in South Africa continued to operate until 1981 (Jones and Woods, 1986).

Today, acetone and butanol are produced by synthetic processes from petrochemicals. Acetone is produced by either the cumene hydroperoxide process or the catalytic degradation of isopropanol.

Butanol is produced by either the oxo process from propylene or the aldol process from acetaldehyde (Moreira, 1983).

1.II.2 Organisms

Almost all species of the genus Clostridium carry out the ABE fermentation and are commonly known as 'butyl' organisms. The classification of these organisms is still in a confused state because of the lack of sufficient data necessary for a thorough taxonomic study. However, two species have been developed for solvent production, namely, Clostridium acetobutylicum and Clostridium beijerinckii. C. acetobutylicum ferments starch, hexoses, or pentoses to butanol, acetone, and ethanol in the general ratio of 6:3:1 (Spivey, 1978), while C. beijerinckii produces solvents in approximately the same ratio as the former, but isopropanol is produced in place of acetone. George et al (1983) revealed that the strains labelled 'C. butylicum', such as those listed in the American Type Culture Collection, are actually C. beijerinckii and, therefore, the species is no longer recognized, nor is it considered a synonym for an existing species.

Other species isolated recently which could be potentially useful for solvent production include C. aurantibutyricum which produces both acetone and isopropanol in addition to butanol (Cummins and Johnson, 1971), and C. tetanomorphum which produces butanol and ethanol only (Nakamura et al, 1979).

C. acetobutylicum has been extensively used for ABE production. It is described as a Gram-positive, anaerobic straight rod (bacillus) measuring 0.6-0.9 μm by 2.4-4.7 μm (Smith and Hobbs, 1974). The vegetative cells are motile with peritrichous flagella. Division occurs by transverse fission resulting in chains of organisms which break apart into single cells in liquid medium during fermentation. Subterminal ovoid spores are formed which usually distend the bacilli.

Optimum growth of the organism occurs at a temperature of 30°C (Smith and Hobbs, 1974), at pH 6.5 and in the Eh range of -250 mV to -400 mV (Spivey, 1978; Gottschal and Morris, 1981b).

1.II.3 Fermentation Substrates

C. acetobutylicum can ferment a variety of sugars (Compere and Griffith, 1979; Mes-Hartree and Saddler, 1982). Conventional starch (such as corn, wheat, millet, rye) or molasses used to be utilized as raw material for the commercial ABE fermentation. Cheaper alternative raw materials are now being studied including whey (Maddox, 1980; Gapes et al, 1983; Schoutens, 1984; Welsh and Veliky, 1984; Ennis and Maddox, 1985); lignocellulosic materials (Saddler et al, 1983; Yu et al, 1984; Marchal et al, 1984; Yu et al, 1985); Jerusalem artichokes (Marchal et al, 1985); sulfite waste liquor (Wayman and Yu, 1985); and steam treated peat (Forsberg et al, 1986).

1.II.4 Course of Fermentation

The normal batch fermentation process using C. acetobutylicum is characterized by two distinct phases (Prescott and Dunn, 1959; Spivey, 1978). Initially, there is an acidogenic phase where the organism grows rapidly, producing acetic and butyric acids over the period of 7-18 h. The pH of the culture decreases from pH 6.8 to pH 5.1. The second phase, called the solventogenic phase, occurs from 18 h to 36-60 h. The growth rate decreases and acids are assimilated and metabolized to solvents, resulting in an increase in pH (pH "breakpoint"). It has been suggested that the uptake of acids functions as a detoxification process initiated in response to the accumulation of acid end-products resulting in conditions inhibiting to growth (Hartmanis et al, 1984). Hydrogen and carbon dioxide gases are released during the fermentation, and release becomes maximal during the solventogenic phase.

Microbial activity ceases eventually due to the accumulation of toxic concentrations of solvents (approx. 20 g/l). Thus, the fermentation can be considered to proceed in three phases (Awang et al, 1988).

Sugar is utilized throughout the fermentation. The maximum theoretical solvent yield for C. acetobutylicum has been calculated at 0.39 g/g sugar utilised (Jones and Woods, 1986). Typical solvent productivities of batch fermentation processes are in the range of 0.2 - 0.6 g/l.h depending on the operating conditions (Spivey, 1978; Ennis and Maddox, 1985; Yu et al, 1985).

1.II.5 Biochemical Pathways

The biochemical steps (Fig. 1.1) involved in the production of fatty acids, solvents, carbon dioxide, and hydrogen in C. acetobutylicum are now well established (Thauer et al, 1977; Lenz and Moreira, 1980; Doelle, 1975; Gottschalk and Bahl, 1981; Kim et al, 1984; Papoutsakis, 1984; Hartmanis and Gatenbeck, 1984; Petitdemange and Gay, 1986; Rogers, 1986; Jones and Woods, 1986).

Hexose sugars are metabolized, via the Embden-Meyerhof glycolytic pathway, to pyruvate. Two mol of ATP and 2 mol of NADH + H^+ are produced per mol of sugar fermented. Pentose sugars are metabolized via the pentose phosphate pathway (Zeikus, 1980; Volesky and Szcnesny, 1983) and converted through a series of steps, to fructose-6-phosphate and glyceraldehyde-3-phosphate, which enter the glycolytic pathway. A net yield of 5 mol of ATP and 5 mol NADH is obtained from 3 mol of pentose. Through a phosphoroclastic reaction, pyruvate is cleaved by pyruvate ferredoxin oxidoreductase in the presence of coenzyme A (CoA) to form acetyl-CoA, CO_2 and reduced ferredoxin. Acetyl-CoA serves as the precursor for all the fermentation products. Two acetyl-CoA molecules undergo a condensation reaction to form acetoacetyl-CoA initiating a cyclic mechanism that leads to the formation of butyric acid. As the pH drops due to acid formation, acetoacetyl-CoA is diverted from the normal cyclic mechanism and is utilized via a transferase system for the production of acetoacetate. In an irreversible step, acetoacetate is decarboxylated to produce acetone.

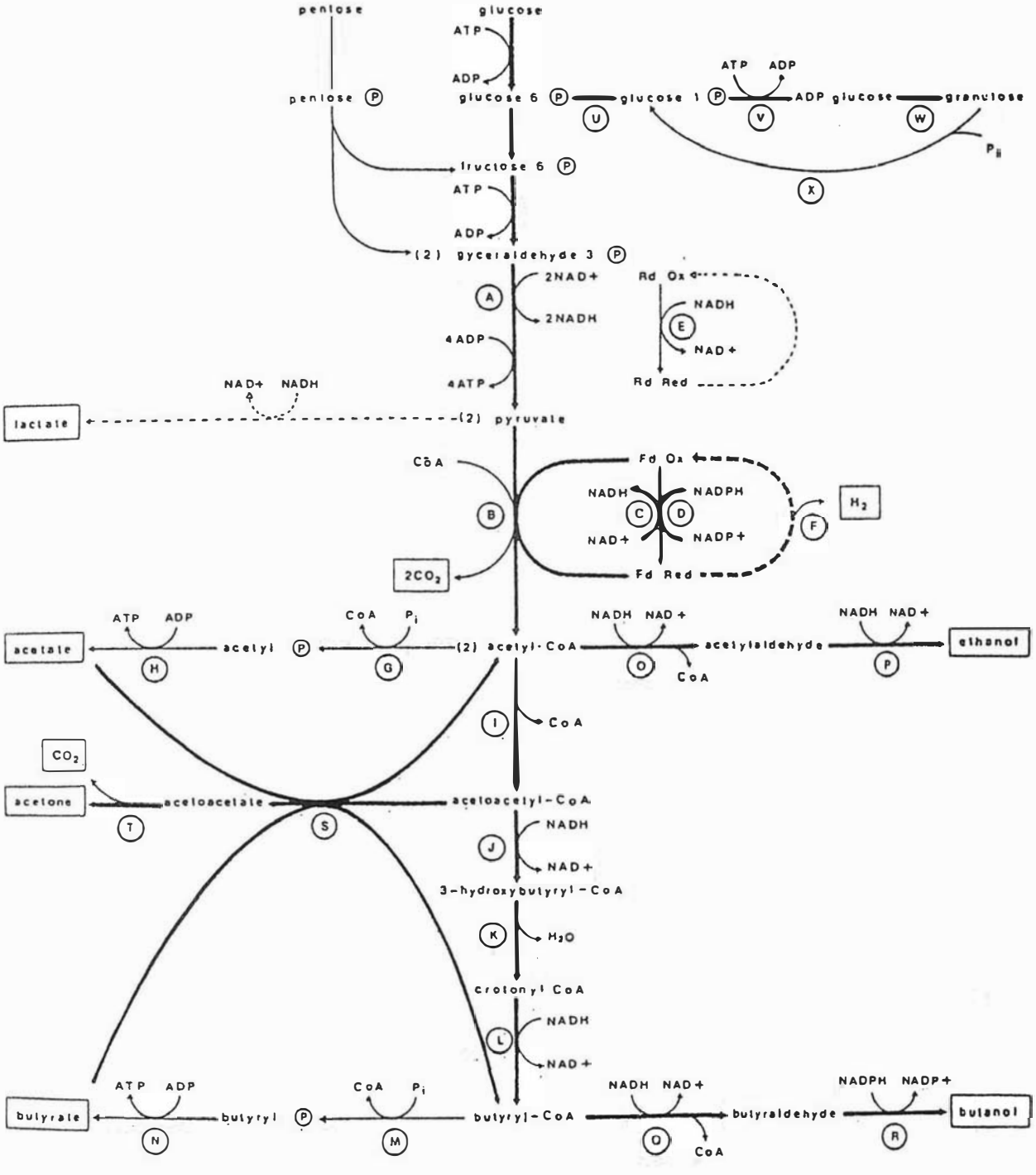


Fig 1.1 Biochemical pathways in C. acetobutylicum. (Jones and Woods, 1986). Reactions which predominate during the solventogenic phase of the fermentation are shown by thick arrows.

Fig. 1.1

Biochemical pathways in C. acetobutylicum (Jones and Woods, 1986).

Enzymes are indicated by letters as follows:

(A) glyceraldehyde-3-phosphate dehydrogenase;

(B) pyruvate-ferredoxin oxidoreductase

(C) NADH-ferredoxin oxidoreductase

(D) NADPH-ferredoxin oxidoreductase

(E) NADH-rubredoxin oxidoreductase

(G) phosphate acetyltransferase (phosphotransacetylase)

(H) acetate kinase

(I) thiolase (acetyl CoA acetyltransferase)

(J) 3-hydroxybutyryl-CoA dehydrogenase

(K) crotonase

(L) butyryl-CoA dehydrogenase

(M) phosphatebutyryl transferase (phosphotransbutyrylase)

(N) butyrate kinase

(O) acetaldehyde dehydrogenase

(P) ethanol dehydrogenase

(Q) butyraldehyde dehydrogenase

(R) butanol dehyrogenase

(S) acetoacetyl-CoA : acetate/butyrate CoA transferase

(T) acetoacetate decarboxylase

(U) phosphoglucomutase

(V) ADP-glucose pyrophosphorylase

(W) granulose (glycogen) synthase

(X) granulose phosphorylase

As the cyclic mechanism is diverted to form acetone, butyric acid formation ceases. Consequently the NAD^+ regenerating sequence from two electron-accepting reactions (the reduction of acetoacetyl-CoA to β-hydroxybutyryl-CoA and the reduction of crotonyl-CoA to butyryl-CoA) are also stopped. Thus, to regenerate NAD^+ the organism reconverts butyrate to butyryl-CoA, and then reduces the latter to butanol. Simultaneously, further sugar metabolism is directed to butanol rather than butyrate production, and ferredoxin is regenerated via NADH, rather than hydrogen production.

Ethanol is produced as the reaction branches off from acetyl-CoA, releasing CoA as acetyl-aldehyde is formed. Acetylaldehyde is further reduced to ethanol. This series of reaction involves the oxidation of 2 mol of $NADH^+ + H$ to NAD^+.

1.II.6 Regulation of Solvent Production.

The regulation of solvent production involves triggering the metabolic transition from acid production to solvent production and maintaining this solventogenic phase. Numerous investigations have been directed towards elucidating the factors controlling solventogenesis, but it is evident that the exact mechanisms are still not completely understood (Gottschal and Morris, 1981a; Gottschal and Morris, 1981b; Andersch et al, 1982; Bahl et al, 1982a; Monot et al, 1982; Monot et al, 1984; Fond et al, 1985; Long et al, 1984b). These factors include the external pH, concentration of acetic and butyric acids in the medium, internal pH, nutrient limitation, and retention of reducing power.

II.6.1 External pH

The effect of culture pH has been implicated in many reports as a key factor in the production of solvent, but some of these reports are conflicting (Bahl et al, 1982a; Andersch et al, 1983; Monot et al, 1984). It has been observed that cultures maintained at high pH values produce mainly acids, while cultures maintained at low pH values produce mainly solvents. However, the pH range over which solvent formation may occur appears to vary depending on the strain and culture conditions used (Jones and Woods, 1986). Solvent production may occur over a pH range of about pH 3.8 to 5.5 (Bahl et al, 1982a; Nishio et al, 1983; Monot et al, 1984). In laboratory conditions, the industrial strain C. acetobutylicum P262 produces solvents within a higher pH range of pH 5.0 to 6.5 (Jones et al, 1982; Jones and Woods, 1986).

Gottschal and Morris (1981b) reported that although a low pH may be prerequisite, low pH itself is not the trigger for the shift from acidogenesis to solventogenesis. The addition of a non-metabolizable acid 5,5-dimethyloxazolidine-2,4-dione which could mimic the effect of acetate and butyrate did not induce solventogenesis as much as did acetate and butyrate. The onset of solvent production has been shown to be accelerated when C. beijerinckii VPI 12436, maintained at pH 6.8, is supplemented with acetate and butyrate (George and Chen, 1983). Similar results have been observed by Holt et al (1984) when C. acetobutylicum ATCC 824 produced solvents at pH 7.0 when the 2.0% (w/v) glucose minimal medium was supplemented with 100 mM each of acetate and butyrate, or when the glucose concentration was

increased to 4.0% (w/v). Hence, the increasing concentrations of acetate and butyrate which accompany the decrease in pH can be implicated as another key factor affecting solvent production.

II.6.2 Acid End-Products

The acetic and butyric acids formed during the fermentation are weak organic acids which are able to diffuse passively across the cell membrane in their undissociated (protonated) form (Thauer et al, 1977; Herrero et al, 1985). At low concentrations, the accumulation of acid end-products and the associated decrease in pH lead to a decrease in growth rate until growth eventually ceases, although sugar utilization and cell metabolism continue (Herrero, 1983; Herrero et al, 1985). At higher acid concentrations, the pH gradient across the membrane collapses, resulting in complete inhibition of cellular metabolism. Hence, it has been suggested that solvent production is a detoxification mechanism of the cell to avoid the inhibitory effects of high concentrations of acids (Costa, 1981; Bahl et al, 1982a; George and Chen, 1983; Hartmanis et al, 1984; Long et al, 1984a).

Monot et al (1983) have suggested that the concentration of undissociated butyric acid plays a central role in the regulation of solvent production. Cell growth was inhibited by undissociated butyric acid at a concentration of 0.5 g/l to 0.8 g/l while solvent production was induced by a concentration of 1.6 g/l to 1.9 g/l (Monot et al, 1984).

However, since the undissociated acid is a biochemically inert compound, it seems likely that it is the elevated concentrations of butyric and acetic acids inside the cell, rather than outside the cell, which are more important (George and Chen, 1983; Gottwald and Gottschalk, 1985; Huang et al, 1985; Terrraciano and Kashket, 1986). The intracellular concentration of undissociated acids is affected by the external acid concentration and the intracellular pH value. Considering the metabolic steps from butyryl-CoA via butyryl phosphate to butyrate, an elevated butyrate concentration could result in elevated concentrations of butyryl-CoA and butyryl phosphate. Gottwald and Gottschalk (1985) have suggested that this situation would lead to a drastic decrease in the CoA and phosphate pools, and that this is the signal for solvent production.

II.6.3 Internal pH

The regulation of internal pH (pHi) in any organism is essential for the stability and functioning of metabolic enzymes. However, instead of maintaining a constant pHi, bacteria which produce weak acids, such as acetic and butyric acids, maintain a constant pH gradient (alkaline interior) (Riebeling et al, 1975; Baronofsky et al, 1984; Herrero et al, 1985). In fermentative bacteria, the pH gradient across the membrane is maintained by the extrusion of protons, at the expense of ATP, mediated by the proton-translocating ATPase system. Since the membrane is rather impermeable to ions, such as protons and hydroxyl ions, proton translocation leads to the generation of a proton motive force. The proton motive force is a metabolic energy intermediate

which is composed of an electrical component (the membrane potential) and a chemical component (the pH gradient). To compensate for the expense of ATP during proton translocation, the proton motive force may be translated into other forms of energy to drive energy-requiring cellular processes such as motility, phosphotransferase transport system for sugars, transmembrane transport of proteins, and others (Hellingwerf and Konings, 1985).

When acetic and butyric acids are produced by C. acetobutylicum, the external pH decreases and most of the acids are present in the undissociated form. These accumulate inside the cell (through passive diffusion) and lower the pHi. In studies using different strains of C. acetobutylicum, it has been observed that under conditions when the external pH decreases, the pHi also decreases appropriately, thus maintaining a more or less constant pH gradient (Gottwald and Gottschalk, 1985; Bowles and Ellefson, 1985; Huang et al, 1985; Terraciano and Kashket, 1986). Furthermore solvent production appears not to occur when the pHi decreases below a minimum value (Gottwald and Gottschalk, 1985). This suggests that maintenance of a pH gradient is important to maintain the pHi above a threshold value for solventogenesis to occur (Terraciano and Kashket, 1986).

The internal pH, the pH gradient across the membrane and the rate of acid production would affect the intracellular concentrations of acids (Fond et al, 1985). High concentrations of undissociated acids can act as protonophores resulting in the collapse of the pH gradient and metabolic functions (Baronofsky, 1984; Herrero et al, 1985). Hence, solvent production is considered an efficient detoxification mechanism

(Costa, 1981; Bahl et al, 1982a; George and Chen, 1983; Hartmanis et al, 1984; Long et al, 1984a). Monot et al (1984) modified the pHi by using the ATPase inhibitor, dicyclohexylcarbodiimide (DCCD) to effect changes in intracellular concentrations of undissociated acids. Lowering of the pHi by inhibiting the membrane-bound ATPase resulted in an increased production of acetone and butanol. Huesemann and Papoutsakis (1986) also observed that solvent production was enhanced by the addition of uncoupler carbonylcyanide-p-trifluoro-methoxy-phenylhydrazone (FCCP).

Although many studies have determined the pH gradient of C. acetobutylicum, the actual significance of these measurements in the regulation of the shift to solvent production still waits to be determined.

Clearly, if a pH gradient is not maintained the pHi will become too low for any metabolic functions to occur. Thus, it is possible that when the pHi approaches the "danger level", the cell responds by shifting from acidogenesis to solventogenesis. Kim and Zeikus (1985) and Lovitt et al (1988) have suggested that pHi may regulate hydrogenase and alcohol dehydrogenase activities such that at more neutral pHi values the organism regulates for hydrogen and butyrate consumption. However, it is not yet known whether the synthesis of solvent-producing enzymes is regulated by the concentration of butyric or acetic acids or protons or their specific production rates.

II.6.4 Retention of Reducing Power

During the acidogenic phase, excess reducing equivalents (NADH) are produced, which must be rapidly reoxidized to allow glycolysis to proceed. NADH ferredoxin oxidoreductase mediates electron transfer from NADH to generate reduced ferredoxin which is coupled with hydrogenase to release molecular hydrogen. This situation is unfavorable for solventogenesis to occur, since the formation of butanol and ethanol depends on the availability of reduced pyrimidine nucleotides. In the solvent-producing phase, less hydrogen is produced and it appears that the switch in carbon flow from acidogenesis to solventogenesis is linked to the decrease in hydrogen production.

Theoretically, inhibition of hydrogenase should result in increased solvent production to use up accumulating reducing power. In a reversible reaction, NADH will be formed from reduced ferredoxin, and this excess NADH will be oxidized by alcohol formation (Jones and Woods, 1986; Rao and Mutharasan, 1989).

Therefore, approaches to increase solvent production by controlling electron flow have been directed towards inhibiting hydrogenase. Approaches used, which successfully inhibited hydrogenase, include maintaining a positive head-space pressure (Maddox et al, 1981; Doremus et al, 1985); use of carbon monoxide (Kim et al, 1984; Datta and Zeikus, 1985; Meyer et al, 1985); and use of methyl and benzyl viologen dyes (Rao and Mutharasan, 1986, 1987, 1988; Kim and Kim, 1988).

Possibly, the role of the pHi in regulating hydrogenase and alcohol dehydrogenase activites, coupled to the role of the intracellular acid concentration in the value of the pHi, may provide a unifying theory to describe the shift from acidogenesis to solventogenesis.

II.6.5 Nutrient Limitation

Studies using batch and continuous cultures have demonstrated that the carbon source should be in excess to favor the shift from acid to solvent production (Gottschal and Morris, 1981a; Bahl et al, 1982b; Monot et al, 1983; Long et al, 1983; Fond et al, 1984; Long et al, 1984a; Ennis and Maddox, 1987). Glucose limitation probably results in deficiency of ATP which is required for solvent production.

There have been conflicting reports regarding the role of other nutrient limitations in the regulation of solvent production, and the situation is rather confusing.

Monot and Engasser (1983) reported that strong solvent production occurred after exhaustion of nitrogen from the medium. In contrast, Long et al (1984a) concluded that nitrogen limited cultures did not produce solvents. Their results showed that there must be a minimum nitrogen concentration remaining after the growth phase for solventogenesis to occur. In contrast again, results of experiments in which the ratio of nitrogen to glucose was varied suggested that an excess of nitrogen is detrimental to solvent production, and that as the ratio of nitrogen to glucose decreases, the rate of solvent production

may be enhanced (Roos et al, 1985). Hence, the possible role of nitrogen limitation in solvent production remains to be clarified.

Bahl et al (1982b) observed that solvents could be produced in batch and continuous cultures under conditions of phosphate limitation. They suggested that under these conditions, the level of CoA may be decreased within the cell, which could serve as an important factor for the induction and maintenance of solvent production. In fact, recent evidence suggests that although solventogenesis can occur under a variety of nutrient-limited conditions, such limitation is not essential (Clarke and Hansford, 1986). Thus, it is doubtful whether nutrient limitation plays any major role in the shift from acidogenesis to solventogenesis.

1.II.7 Solventogenesis and ATP Generation

A low net ATP production is generated during fermentation by anaerobes. The conversion of glucose to acetate and butyrate typically results in a net production of 3.25 mol ATP per mol of glucose (Thauer et al, 1977). The ATP produced is utilized in biosynthesis leading to cell growth and in membrane energization via the proton translocating ATPase to generate a pH and electrical gradient across the cell membrane (Herrero, 1983; Jones and Woods, 1986). During acid production, much of the ATP is diverted to membrane energization so as to maintain a critical internal pH of the cell. This diversion of ATP probably explains the decrease in growth rate prior to solventogenesis. The solvent- producing phase yields only 2 mol of

ATP per mol of glucose which could explain the accompanying reduction in growth rate.

1.II.8 Solventogenesis and Cell Differentiation

C. acetobutylicum exhibits distinct morphological variations during the course of the fermentation process (Spivey, 1978). The degree of variation differs in different strains. In the industrial strain P262, cell differentiation can be used as a convenient index to monitor the progress of the fermentation (Spivey, 1978).

The morphological changes in strain P262 have been studied and correlated with growth and solvent production (Jones et al, 1982; Long et al, 1983; Long et al, 1984b). Jones et al (1982) observed that the cells appeared as long chains with sluggish motility immediately after inoculation into the molasses fermentation medium. As the acidogenic phase starts, the growth rate increases and short phase-dark dividing rods, which are highly motile, appear. Cells remain motile for about 24h-30h. Motility starts to decrease before the pH breakpoint, and the cell starts to accumulate granulose, a glycogen-like storage product (Jones et al, 1982; Long et al, 1984a; Reysenbach et al, 1986). Spivey (1978) noted that there is a relationship between motility and good solvent production. The absence of motility at any time of the inoculum development procedures resulted in poor solvent yields.

The shift to solventogenic phase is characterized by the formation of swollen, phase-bright, cigar-shaped 'clostridial forms' which are

believed to be responsible for solvent production (Jones et al, 1982; Long et al, 1983). The clostridial forms continue to accumulate granulose and produce an extracellular capsule or slime layer (Long et al, 1983; Reysenbach et al, 1986).

Granulose accumulation has been observed to occur when growth is inhibited in the presence of excess carbon source, and is believed to be essential for sporulation. However, the isolation of sporulation granulose-deficient mutants has suggested the contrary (Reysenbach, 1987). Haggstrom and Forberg (1986) have suggested that the polysaccharide polymer which comprises the capsule acts as a sink for the storage of non-reduced compounds when excess reducing power is needed during the production of butyric acid and butanol, and as a reserve carbohydrate source.

The ability to form endospores has been associated with strong solvent production (Beesch, 1953; Prescott and Dunn, 1959). Gottschal and Morris (1981a) observed that the ability to produce solvents in continuous culture is lost when there is a loss in the ability to form spores. However, the isolation of a sporulation mutant, which was blocked after the clostridial stage, demonstrated that sporulation is not necessary for solvent production (Long et al, 1984b). This was confirmed when asporogenous mutants, selected during continuous culture, were not affected in their solvent production (Meinecke et al, 1984). Inhibition of DNA synthesis (Long et al, 1984b) blocked the initiation of sporulation but not solvent production, formation of the clostridial stage, granulose accumulation, or capsule formation, indicating that these events are independent of each other.

1.II.9 Product Inhibition

One of the problems associated with the ABE fermentation process is that it suffers from product inhibition (Herrero, 1983; Costa and Moreira, 1983). At the completion of batch fermentation, the solvent concentration in the broth rarely exceeds 20 g/l (Spivey, 1978; Moreira et al, 1981). Considering a typical solvents yield of 30%, the maximum initial carbohydrate concentration in the fermentation broth is approximately 60 g/l. This dilute solution leads to high energy demands during product recovery (by distillation).

Butanol, acetic acid, and butyric acid are the most toxic products of the fermentation (Lin and Blaschek, 1982; Moreira et al, 1981; Costa and Moreira, 1983; Kuhn and Linden, 1986). Cell growth was inhibited by 50% when butanol was added at 7 to 13 g/l. Concentrations of acetic acid and butyric acid causing a 50% inhibition of cell growth were determined as 8.0 g/l and 6.0 g/l, respectively (Costa and Moreira, 1983). Hence, butanol, acetic acid and butyric acid are inhibitory to cell growth at levels normally produced during fermentation. Leung and Wang (1981) reported concentrations of added acids resulting in 50% inhibition of growth which were about twice the levels observed by Costa and Moreira (1983). This difference was attributed to the different methods used to stabilize the pH of the fermentation media during the acid challenge.

Unlike butanol, ethanol and acetone are not inhibitory at concentrations typically produced during the fermentation (Moreira et al, 1981; Costa and Moreira, 1983).

It has been observed that there is a threshold concentration of products below which growth inhibition does not occur. Above the threshold concentration, there is a linear decrease in growth rate with an increase in product concentration (Costa and Moreira, 1983). Added solvents are considered to have a less toxic effect on growth than those produced naturally during the fermentation. This suggests that some synergism occurs among the fermentation products and it is probably the total concentration of products which is important in determining the toxic effect on growth rate. The difference in product concentrations within and outside the cell may also influence their toxic effects.

II.9.1 Mechanism of Solvent - and Acid - Toxicity

The exact mechanisms of toxicity of solvents and acids are not yet known. Their inhibitory "target sites" appear to be different (Herrero, 1983). Organic acids such as acetic and butyric acids are known to behave as uncouplers due to their proton - ionophoretic properties. Protons are allowed to enter the cell from the medium and thus counteract the proton pump mechanism and interfere with the establishment and maintenance of a functional pH gradient across the membrane (Herrero, 1983; Herrero et al, 1985). The proposed mechanism by which organic acids act as uncouplers includes the following steps (Herrero et al, 1985):

1. partition of the undissociated form of the acid in the membrane bilayer
2. passive diffusion of the undissociated acid into the cytoplasm

3. dissociation of the acid in the relatively higher cytoplasmic pH
4. effective anion exclusion, leaving behind a proton and reducing the prevailing pH gradient.

It has been suggested that when the internal pH of the cell is reduced the cell attempts to restore it by pumping out more protons at the expense of ATP that must be consumed via ATPase for membrane energization. As a consequence, less energy is available for biosynthesis. The altered internal pH also inhibits enzyme functions. It is in this regard that the shift from acidogenesis to solventogenesis has been suggested as a detoxifying mechanism of C. acetobutylicum (Bahl et al, 1982a; George and Chen, 1983; Hartmanis et al, 1984).

Alcohols have been known to disrupt the phospholipid component of the cell membrane (chaotropic effect) and thereby inhibit membrane-linked cellular functions (Herrero, 1983; Ingram and Buttke, 1984; Baer et al, 1987). Ingram and Buttke (1984) suggested that the short-chain alcohols (1 to 3 carbons) solidify or freeze the membrane by intercalating between and restricting movement of the fatty acid chains. Longer-chain alcohols (4 to 9 carbons) fluidize the membrane by anchoring at the phospholipid head groups and increasing the distance between the fatty acid chains.

Vollherbst-Schneck et al (1984) observed that in the presence of butanol there was an increase in the ratio of saturated to unsaturated fatty acids in the membranes of C. acetobutylicum ATCC 824. The alteration in the composition of the cell membrane seems to be the biological response of the microrganism to offset the fluidizing effect

of butanol. This response is similar to the response of cells when grown in elevated temperatures (Baer et al, 1987; Lepage et al, 1987). The butanol tolerance of SA-2 mutant derived from C. acetobutylicum ATCC 824 was attributed to its ability to synthesize an increased amount of saturated fatty acids, thus maintaining a more stable environment for cellular functions (Baer et al, 1987).

The sequence of inhibition and disruption of several interrelated cellular functions affected by butanol and the relationship of these events are not known (Bowles and Ellefson, 1985; Jones and Woods, 1986). Thus, butanol appears to have complex, multiple effects. Studies on the effects of butanol on C. acetobutylicum ATTC 4259 demonstrated that by disrupting membrane fluidity, butanol inhibited the ability of the cell to maintain the internal pH, partially inhibited the membrane ATPase, caused collapse of the intracellular ATP levels, and caused a decreased glucose uptake (Bowles and Ellefson, 1985). Gottwald and Gottschalk (1985), likewise, observed that the pH gradient is abolished in the presence of growth-limiting butanol concentrations. Kuhn and Linden (1986) found that at toxic levels of butanol (18 g/l) there was a two-order-of-magnitude increase in the initial rate of passive proton influx, attributed to membrane leakiness.

Another membrane-linked function affected by butanol is sugar uptake. Ounine et al (1985) reported that uptake of glucose and xylose decreased in the presence of inhibitory butanol concentrations and that the inhibitory effect of butanol was more pronounced in cells grown on xylose. Hutkins and Kashket (1986) demonstrated that glucose is transported in C. acetobutylicum by a phosphoenolpyruvate (PEP) -

dependent phosphotransferase system. Butanol did not inhibit this transport system but rather caused leakage of phosphoenolpyruvate and 2-deoxyglucose-6-phosphate. These results lend support to the view that the loss of membrane integrity (membrane leakiness) is the primary cause of butanol toxicity in C. acetobutylicum (Kuhn and Linden, 1986; Linden, 1987).

II.9.2 Butanol Tolerance

To minimize the problem of solvent toxicity, two approaches have been employed, namely, an engineering approach and a biological approach (Rogers, 1986). The engineering approach involves the elimination of the toxic products and their inhibitory effect by continuous extraction of the product from the fermentation broth. Appendix 1 provides a close examination of the integration of continuous in-situ or in-line solvents recovery processes with batch or continuous fermentation processes. The biological approach, which seems to lag behind the engineering efforts, involves an understanding of the physiological and molecular nature of solvent toxicity as a tool for further development of solvent or acid-tolerant organisms.

Three mechanisms have been used generally to amplify the tolerance of C. acetobutylicum to butanol, namely, classical mutation, continuous cultivation, and serial transfer in batch culture with increasing concentrations of butanol (Lin and Blaschek, 1982; Lemmel, 1985; Soucaille et al, 1987). There is some evidence that butanol tolerance can be enhanced by manipulation of growth

conditions during fermentation. Kuhn and Linden (1986) reported a two-fold increase in butanol tolerance after enrichment of the growth medium with a saturated fatty acid. However, the isolation of a butanol-tolerant mutant which can also produce higher concentrations of butanol has not been successful, probably, due to the complex genetic control of solvent tolerance (Rogers, 1986).

1.II.10 Genetics and Strain Improvement

This section is a brief attempt of an overview of the genetic studies on C. acetobutylicum. More comprehensive treatment of this subject can be obtained from reviews by Rogers (1986), Jones and Woods (1986) and Awang et al (1988). Genetic studies on obligate anaerobes, such as the clostridia, have lagged behind those of aerobic organisms (Jones and Woods, 1986) and, therefore, deserve attention. Knowledge of the genetic system of C. acetobutylicum may provide a tool to alleviate the limitations to the economical use of the ABE fermentation. Using genetic manipulation, the butanol tolerance, substrate range, and solvent yield may be improved.

Mutation and selection have been used to obtain mutants of C. acetobutylicum for genetic studies and improved industrial performance. Recent studies indicate that direct mutagens such as the alkylating agents N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and ethyl methane-sulfonate (EMS) are the most effective mutagens for C. acetobutylicum (Bowring and Morris, 1985; Lemmel, 1985). Mutagens such as ultraviolet irradiation and mitomycin have been

found to be relatively ineffective. This suggests that Clostridium species may be deficient in error-prone DNA repair such as the SOS repair in E. coli (Jones and Woods, 1986; Awang et al, 1988). Most mutants of C. acetobutylicum which have been utilized in physiological studies have been obtained using EMS. These mutants include auxotrophic mutants (Bowring and Morris, 1985; Jones et al, 1985); antibiotic resistant mutants (Bowring and Morris, 1985; Long et al, 1984b); granulose mutants, capsule mutants, sporulation mutants (Long et al, 1984b) and phage resistant mutants (Ogata and Hongo, 1979).

The relative effectiveness of the mutagen may be affected by strain differences or culture procedures. Therefore, optimal conditions should be evaluated and developed before carrying out any strain improvement program utilizing mutagens.

Chemostat selection can also be used to obtain mutants but it has not been applied to C. acetobutylicum (Awang et al, 1988). Strains which utilize substrates more efficiently may be selected by applying conditions of nutrient limitation in a chemostat.

Mutation and selection strategies in C. acetobutylicum still wait to be refined before they can be applied to improve the ABE fermentation.

Recent studies on genetic engineering in C. acetobutylicum have mostly been concerned with the development of gene transfer and cloning methods. The genetic transfer techniques that can be applied include conjugation (Oultram and Young, 1985; Reysett and Sebald, 1985); protoplast transformation (Lin and Blaschek, 1984);

transfection (Reid et al, 1983); and protoplast fusion (Jones et al, 1985). Transduction has not been reported but several phages which can infect solvent-producing clostridia may be developed as cloning vectors for C. acetobutylicum (Jones and Woods, 1986).

Conjugation between bacteria requires either large, self-transmissible plasmids or mobilization plasmids which promote transfer of smaller expression vectors. C. acetobutylicum has been reported to possess large and small plasmids (Truffaut and Sebald, 1983) but self-transfer to, or maintenance of plasmids in, this species has not been reported. In addition, these plasmids are still cryptic as no known function can be ascribed to their presence. Conjugative transfer of broad host-range plasmids from various streptococcal species has been accomplished (Oultram and Young, 1985; Yu and Pearce, 1986).

Successful transformation in C. acetobutylicum has been hampered by a number of problems. Firstly, there is a lack of DNA uptake by any method except protoplast transformation (Jones and Woods, 1986). Secondly, there is a strong deoxyribonuclease (DNAse) activity in Clostridium species which limits the isolation of both plasmids and chromosomal DNA, and interferes with DNA uptake (Urano et al, 1983; Luczak et al, 1985). Heating of protoplasts at 55°C for 15 min was utilized by Lin and Blaschek (1984) to inhibit DNAse activity and they successfully transformed the butanol-tolerant strain SA-1 to kanamycin resistance using the Bacillus subtilis plasmid pUB 110.

Transformation of C. acetobutylicum P262J protoplasts with bacteriophage DNA (transfection) has been demonstrated by Reid et al

(1983). However, the frequency of transfection could not be determined due to lack of expression of transfection as plaques on regeneration medium. Transfection still waits to be optimized as a system for genetic transfer, and development of a cosmid process may be necessary (Awang et al, 1988).

Jones et al (1985) demonstrated protoplast fustion of C. acetobutylicum P262 auxotrophs, producing stable recombinants and segregating biparentals. The isolation of stable recombinants indicates that the organism is capable of undergoing homologous recombination at a frequency similar to that obtained with aerobic species such as B. subtilis. Jones and Woods (1986) have suggested that plasmid transfer with Clostridium species can be facilitated by protoplast fusion, and it could be utilized to protect plasmids from the high DNAse levels produced by C. acetobutylicum strains.

It is essential to resolve the technical problems involved in gene transfer and expression before gene cloning can be successfully applied to C. acetobutylicum . Cloning foreign genes in this organism may impart new desirable industrial characteristics while cloning genes out will aid in studies of its genome. A number of genes from saccharolytic solvent-producing clostridia have been cloned and expressed in the Gram-negative Escherichia coli. These include the genes for β-isopropylmalate dehydrogenase and hydrogenase from C. butyricum (Ishii et al, 1983; Karube et al, 1983); a ferredoxin gene from C. pasteurianum (Graves et al, 1985); and cellulases from C. thermocellum (Millet et al, 1985). A number of chromosomal genes from strain P262 have been cloned in E. coli . These include genes

involved in cellulose-, hemicellulose- and starch degradation, genes involved in complementation of E. coli *arg* and *his* mutations, and the glutamine synthetase gene (Usdin et al, 1986; Zappe et al, 1986). The glutamine synthetase gene plays a role in nitrogen metabolism regulation which is important for solvent production and sporulation in C. acetobutylicum (Long et al, 1984b).

Genetic manipulation in C. acetobutylicum will be useful in expanding its substrate range, improving its efficiency of substrate utilization, and possibly increasing end-product tolerance. Solvent tolerance appears to be under complex genetic control (Rogers, 1986). However, assuming that there are sensitive targets within this complex system, genetic manipulation may be able to change or replace these targets in order to increase butanol tolerance.

Part III MOTILITY AND CHEMOTAXIS IN BACTERIA

Chemotaxis, defined as the movement towards or away from chemicals, is a universal biological sensory response. Man is attracted to perfume and repelled by rotting food; the slime mold Dictyostelium, which at one stage in development is comprised of single amoeboid cells, moves toward cyclic adenosine monophosphate (AMP) which influences cell aggregation and differentiation; leukocytes move toward invaders and destroy them by phagocytosis (Koshland, 1980, 1982).

Motile bacteria migrate through concentration gradients of attractant (positive chemotaxis) and repellents (negative chemotaxis) (Adler, 1975). Bacterial chemotaxis was discovered by Pfeffer and Engelman in the 1880's (Koshland, 1980). Pfeffer demonstrated chemotaxis by placing a capillary containing an attractant in a beaker with uniformly distributed bacteria. After an appropriate interval of time, he examined the capillary under the microscope and observed that the bacteria swam into the capillary in numbers far in excess of what would be expected by simple random motion. Owing to its simplicity, chemotaxis has been the most intensively studied behavioural system (Weibull, 1960; Adler, 1975; Berg, 1975; Chet and Mitchell, 1976; Koshland, 1980, Parkinson, 1981; Boyd and Simon, 1982, Koshland, 1982; Macnab and Aizawa, 1984; Ordal, 1985). A wide variety of bacterial species have been examined for their chemotactic ability but the most widely investigated are the Gram-negative bacteria Escherichia coli and Salmonella typhimurium (Adler, 1966; Enomoto, 1966; Adler and Templeton, 1967; Adler et al , 1973; Silverman

and Simon, 1976; Melton et al, 1978; Kihara and Macnab, 1979; Clancy et al, 1981; Chelsky and Dahlquist, 1980; Stock et al, 1984; Brass and Manson, 1984; Kehry et al, 1985). Other species, such as Bacillus subtilis, which is Gram-positive, have been shown to have chemotactic mechanisms very similar to those of the Gram-negative species (de Jong et al, 1976; Ordal et al, 1977; Shioi et al, 1978).

1.III.1 Bacterial Motility in a Chemical Gradient

There are several distinct types of bacterial motility, including flagellar, spirochetal and gliding (Macnab and Aizawa, 1984). To date, the most extensively studied is motility generated by external organelles called flagella. Flagellated bacteria are capable of translational movement through a homogenous liquid medium without requiring an interface in order to exert thrust. Speeds of swimming bacteria range from 20 to as much as 60 μm/sec (Macnab and Aizawa, 1984). Demonstration of this movement can be done microscopically by eye, recorded by microcinematography, or followed as motility tracks that form on photographic film after time exposure (Berg and Brown, 1972; Vaituzis and Doetsch, 1969). Quantitative and faster observations of motility have been facilitated by the invention of the tracking microscope (Macnab and Koshland, 1972; Lovely and Dahlquist, 1974). Schneider and Doetsch (1974) developed a method for velocity measurements using a videotape recording technique which avoids the problems of the other methods.

In the absence of a stimulus, bacteria such as E. coli and S. typhimurium exhibit two types of motion known as "runs" and "tumbles". When the organism "runs", it swims in a smooth, straight line for a number of seconds, then it "tumbles" by thrashing around for a fraction of a second. It again swims in a straight line, but in a new randomly chosen direction (Berg and Brown, 1972). An individual cell, therefore, exhibits a three-dimensional random movement (Armstrong et al, 1967; Dahlquist et al, 1972).

In the presence of gradients of chemical stimuli, the probability per unit time (rate) of undergoing a tumble depends on the direction in which the cell moves in the gradient. For a cell moving towards an increasing concentration of attractant, the tumbling frequency is less than in the absence of the gradient. A cell encountering increasing concentration of repellent tumbles more frequently (Berg and Brown, 1972; Macnab and Koshland, 1972; Tsang et al, 1973). By varying the tumbling frequency in this manner, there is a net flux of the cells in the favorable direction. Since the bacteria are not able to turn directly toward the favorable direction, they increase the time spent going in favorable direction by suppressing tumbling.

Bacteria sense gradients of chemicals in time (temporal) rather than in space (spatial) (Macnab and Koshland, 1972). Temporal sensing has been determined by mixing bacteria quickly with increasing or decreasing concentration of attractant (Macnab and Koshland, 1972) or repellent (Tsang et al, 1973) and then immediately observing the alteration of tumbling frequency. After a short time of response (increased tumbling with repellents, decreased tumbling with

attractant), the tumbling frequency was observed to return to the unstimulated state. It was concluded that the cells maintain a record of their chemical environment over the recent past (similar to "memory"). If the current environment is detected to be more favorable than the previously recorded one, tumbles are suppressed; if it is more unfavorable, tumbling is enhanced. This "memory" is continually updated, after which a comparison of the current environment with the record is made. If there is no difference, the behaviour returns to a random swimming pattern.

Thus the chemotactic response to temporal stimuli consists of two phases - an excitatory phase that leads to modification of tumbling frequency, followed by an adaptation phase where the cell updates its record of the chemical environment during the recent past.

1.III.2 The Bacterial Flagella

Flagella are long, thin external appendages embedded in the cell wall and the cytoplasmic membrane of the bacteria (Berg, 1975). They extend 15-20 μm from the cell surface (Silverman and Simon, 1977). Flagella are arranged in the bacteria in different manners, depending on the species. The simplest arrangement is a single flagellum at one end of the cell, or both ends, as in Spirillum and Pseudomonas (polar flagellation). Flagella may originate from random points around the cell surface as in E. coli and S. typhimurium (peritrichous flagellation). The vegetative cells of C. acetobutylicum, likewise, are motile with peritrichous flagella (Prevot, 1966).

III.2.1 Morphology and Function of Flagellar Components

Electron microscopy has revealed that each flagellar organelle is composed of three morphologically distinct substructures - the filament, the hook and the basal structure (Dimmitt and Simon, 1971; DePamphilis and Adler, 1971; Silverman and Simon, 1977). Fig 1.2 shows the general morphology of the filament - hook - basal body complex and the orientation of these components with regard to the cell membrane.

The flagellar filament is a long, thin, relatively rigid helical structure which extends out of the cell. The maximal length depends upon the culture conditions but it generally ranges from 16-22 μm. The typical filament measures 20 nm in diameter and has a wavelength of 2.3 μm (DePamphilis and Adler, 1971). In E. coli and S. typhimurium, and numerous other bacteria, the filament is comprised of identical subunits of a single protein called flagellin (Abram and Koffler, 1964). These subunits assemble in a polar manner into the characteristic cylindrical structure of the filament. The filament acts as a semi-rigid propeller.

The flagellar hook is a short (about 90 nm) curved structure which connects the base of the filament to the basal body (DePamphilis and Adler, 1971). The hook has slightly greater diameter than the filament and is made up of a different type of protein than the flagellin. Very little is known about the function of the hook. It is thought to act as a "universal joint" at the base of the filament, that is, it allows the efficient transmission of rotational motion of the motor (located in the

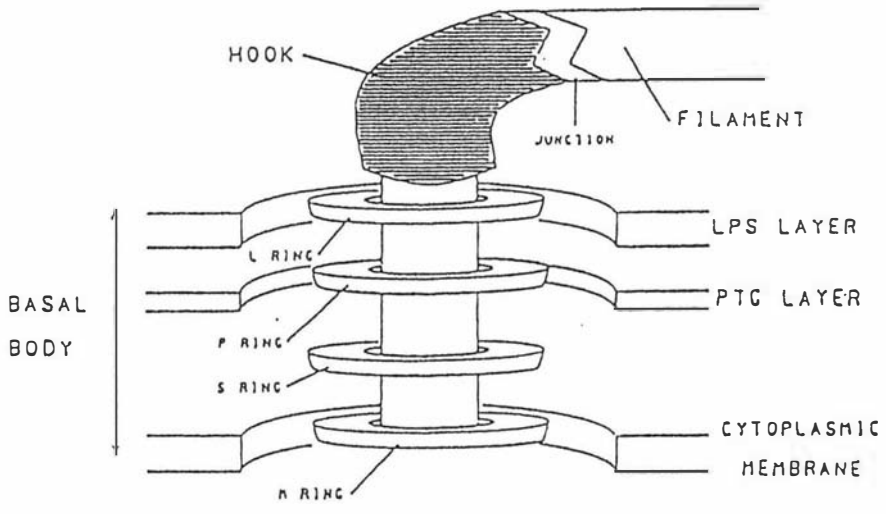


Fig 1.2 Schematic representation of the flagellar-basal body complex of S. typhimurium and E. coli. The major components of the flagellar-basal body complex and their orientations with respect to the inner and outer membranes are shown. (Stewart and Dahlquist, 1987).

Abbreviations:

LPS lipopolysaccharide layer of the outer membrane;

PTG peptidoglycan layer of the outer membrane.

membrane plane) to the filament so that the filament rotates about an axis appropriate for propelling the cell (Berg and Anderson, 1973; Silverman and Simon, 1977).

The basal body is a complex structure involved both in the attachment of the flagellar organelle to the cell membrane and in the generation of motion. As shown in Fig 1.2, it consists of a small central rod which passes through a system of rings. In Gram-negative organisms, there are four rings designated as L,P,S and M which appear to have specific orientations within the cell wall and membrane. In Berg's model of the flagellar rotary motor, the M ring serves as the rotor - it rotates freely in the cytoplasmic membrane. Attached to the M ring is the rod that serves as the driveshaft - it ends at the "universal joint" (hook). The S ring is mounted rigidly in the cell wall proximal to the M ring, and can therefore function as the stator (stationary part of the motor). The motor is driven by generating torque between the M and S rings.

In the Gram-positive B. subtilis, which lacks the outer lipopolysaccharide layer, there are only two rings (Dimmit and Simon, 1971).

III.2.2 Molecular Biology of Flagellar Components

There are over 30 genes necessary for the assembly and operation of the bacterial flagellum (Macnab and Aizawa, 1984). Fig 1.3 shows the organization of these genes in E. coli and S. typhimurium. There is almost total homology between the two species (Adler et al, 1973;

DeFranco et al, 1979). The genes belong to three classes - *fla, mot* and *che.* Macnab and Aizawa (1984) have listed all of the genes in E. coli and S. typhimurium with their corresponding products and functions. Mutations or deletions at most of the loci result in nonflagellated cells (*fla-* phenotype, genes are designated as *fla, flb*, or *hag*) (Armstrong and Adler, 1969). In most cases, such mutants lack any detectable flagellar precursors. Mutants exhibiting a *mot*$^-$ phenotype (genes designated *mot*) have morphologically normal flagella, but are 'paralyzed' (incapable of rotating them). *Che*$^-$ mutants (genes designated *che*) are motile but have abnormal bias in the direction of flagellar rotation and are incapable of chemotactic response to any stimuli. A mutation in a structural gene for one of the signal components (*tsr, tar, trg, tap*) generally affects response only to those stimuli mediated by a specific signal component (Parkinson, 1981; Stewart and Dahlquist, 1987).

III.2.3 Mechanism of Flagellar Movement

The flagellar filament is a rigid structure which does not flex at all in a wavelike manner. Therefore, movement is accomplished with the filament propelling the cell by rotating. Evidence for this mechanism has been provided by tethering experiments which involved the use of antibody to flagella to 'fix' the flagella to a glass slide. Such technique allowed measurement of the rotation resulting from the torque generated by the flagellar organelle (Silverman and Simon, 1974; Larsen et al, 1974b; Berg, 1974; Berg, 1975). Mutant cells of E. coli, which either had straight filaments or unusually long hooks, were used.

When the hook or the filament structure was tethered to a microscope slide, the bacterial body rotated rapidly. The rotation could be clockwise or counterclockwise. The regulation of the direction of rotation has been suggested to be the basis for the mechanism of chemotaxis (Silverman and Simon, 1974). Smooth swimming, in response to an increase in attractant concentration, results from counterclockwise (CCW) rotation of flagella; tumbling in response to repellents results from clockwise (CW) rotation (Larsen et al, 1974b).

Berg (1975) has proposed that the rotary motion of the flagellum is imparted from the basal body, which must act in some way like a motor. It is likely that the two inner rings located at the membrane (S and M) of E. coli and S. typhimurium, rotate in relation to each other so that the M ring acts as the rotor, and the S ring acts as the stator.

When there are two or more flagella, the flagella associate or bundle together to propel the bacterium from behind. Apparently the bundle of flagella survives counterclockwise rotation of the individual flagella but comes apart as a result of clockwise rotation (Macnab and Aizawa, 1984).

III.2.4 Energy Source for Motility and Chemotaxis

In contrast to eucaryotes in which the chemomechanical energy involves ATP, the source of energy for bacterial motility (rotation) is the proton motive force; either of its components (a pH gradient or a

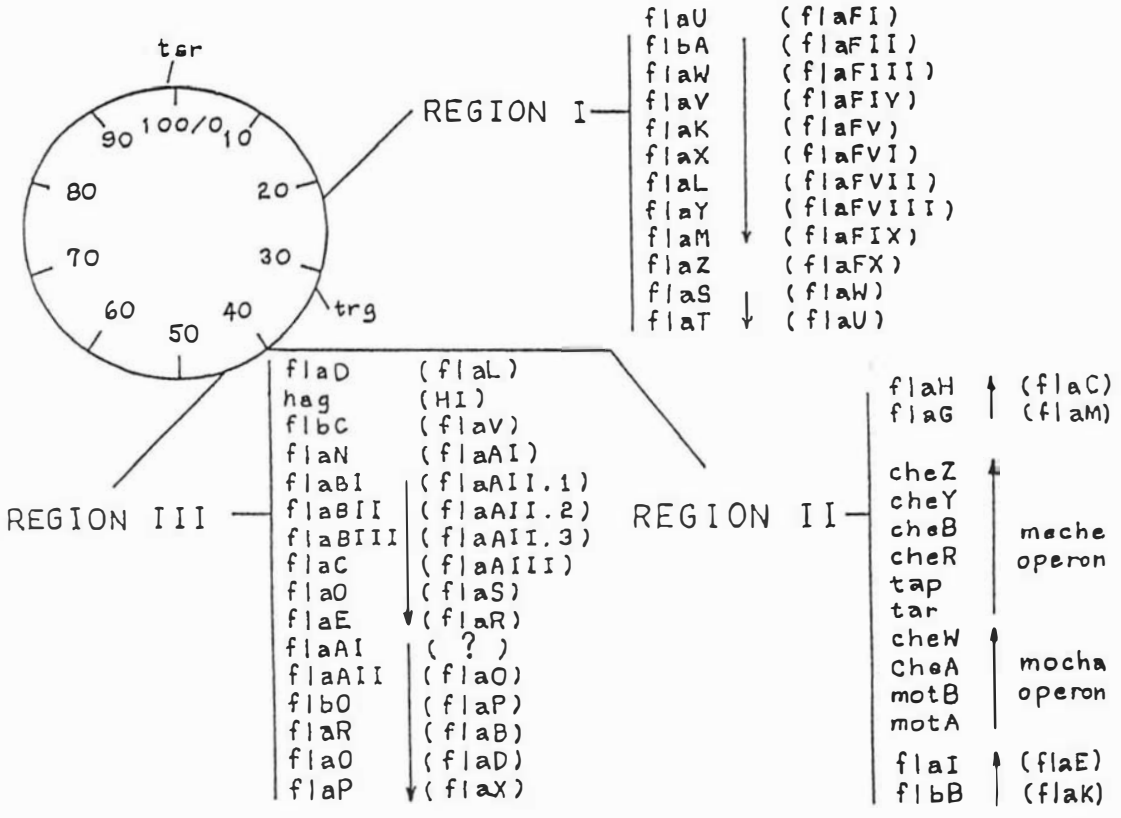


Fig 1.3 Distribution of flagellar and chemotaxis genes on the genetic maps (100 min) of E. coli and S. typhimurium. E. coli genes are in the left column and the corresponding genes in S. typhimurium are in the right column. Vertical arrows represent operons (Stewart and Dahlquist, 1987).

membrane potential) is effective (Larsen et al, 1974a; Manson et al, 1977; Shioi et al, 1980).

Mutants of E. coli and S. typhimurium defective in oxidative phosphorylation quickly became non-motile under anaerobic conditions even when given substrates that can generate ATP anaerobically, such as D-glucose or D-galactose (Larsen et al, 1974a; Thipayathasana and Valentine, 1974). In the presence of carbonylcyanide-m-chloro-phenylhydrazone (CCCP), which uncouples oxidative phosphorylation, there was complete inhibition of motility even though ATP remained present. Exposure to arsenate, which is known to reduce ATP levels in bacteria, resulted in cessation of motility; however, motility was regained after the addition of electron donors such as lactate, succinate and phenazine metosulphate containing ascorbate. These conclusions are supported by recent findings of Ravid and Eisenbach (1984b) using cell envelopes devoid of cytoplasmic contents, but which have functional flagella. These flagella can be made to rotate when a pH gradient is imposed across the cell membrane, indicating that proton flux itself is sufficient to drive the motor.

Chemotaxis, on the other hand, requires ATP (Larsen et al, 1974a; Aswad and Koshland, 1975). Complete inhibition of chemotaxis has been observed under conditions where cells treated with arsenate remained motile. This suggests a requirement for ATP in the formation of S-adenosyl-methionine which is required for the generation of tumbles (Larsen et al, 1974b; Adler, 1975).

The work output of the flagellar motor (i.e. the angular velocity in tethered cell) is approximately proportional to the applied proton motive force (Khan and Macnab, 1980; Shioi et al, 1980). The torque generated by the motor is independent of the load (viscous drag) experienced by the flagellum under high load conditions in tethered cells (Manson et al, 1980). There seems, therefore to be a fixed stoichiometry of proton flux per revolution of the motor. Values from 300 to 1000 protons per revolution have been proposed (Berg, 1974).

How proton motive force is coupled to flagellar rotation is not at all clear. A number of models have been proposed to explain how the components of the flagellar motor interact to achieve rotation of the filament and how proton motive force is utilized by the motor (Lauger, 1977; Glagolev and Skulachev, 1978; Macnab, 1978; Khan and Berg, 1983).

The model of Khan and Berg (1983) presents the most explicitly defined mechanism. In this model, there are two channels in a membrane-embedded "particle" that serves as the force generating unit; these channels mediate the proton flux. One channel enables protons from the outside to associate with or dissociate from proton binding sites on the M ring; the other channel makes these proton binding sites accessible to the cytoplasm. The S ring serves as an elastic connection between the channel complex and the cell wall such that when the channel complex is displaced from its equilibrium position, the S ring generates a force to restore the position. Movement of the channel occurs only when a proton sits in one of the

two binding sites exposed by the channels. The channel complex moves to the right when there is a negative pH gradient (protons flowing into the cell); the displacement exerts a force on the outside of the M ring. A positive pH gradient (protons flowing out of the cell) is expected to push the complex to the left of its equilibrium position and turn the motor in the "backward" direction. In other words, the flagella turn CCW when the pH gradient drives protons into the cell, whereas when the gradient drives protons out of the cell the flagella turn CW. These results suggest a chemotactic response of the cells to a decrease in pH.

1.III.3 Components of the Chemotaxis System

Extensive genetic and biochemical analyses of the chemotaxis machinery in E. coli and S. typhimurium have led to an intriguing picture of how bacteria detect and process sensory information. The genes involved in chemotaxis are shown in Fig 1.3. The chemotactic response of bacteria involves 3 phases, namely:

1. stimulus detection (chemoreception)
2. excitation
3. adaptation

The pathway of information flow through the components of the chemotaxis machinery is illustrated in Fig 1.4 (Taylor, 1983; Eisenbach et al, 1985; Stewart and Dahlquist, 1987).

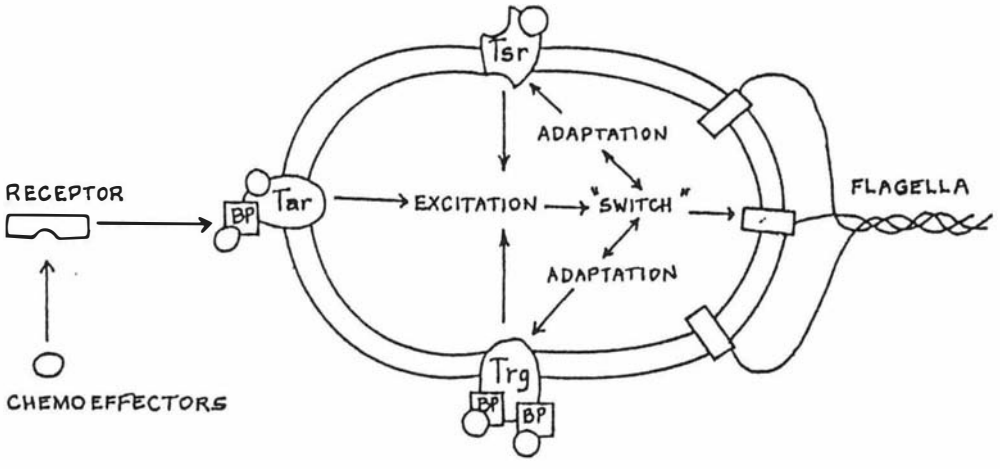


Fig 1.4 Sensory information pathway through the components of chemotaxis in E. coli and S. typhimurium (modified from Taylor, 1983; Eisenbach et al, 1985; Stewart and Dahlquist, 1987). *Tsr*, *Tar* and *Trg* are the known transducer proteins. BP designates soluble binding proteins to which chemoeffectors such as ribose, galactose and maltose bind before transduction.

III.3.1 Chemoreceptors

Motile bacteria are able to detect temporal changes in chemoeffector (attractant or repellent) concentrations. Concentration measurements are made by means of specific sensory devices called 'chemoreceptors' (Adler, 1969, 1975). They are protein molecules located either in the periplasmic space or in the inner membrane. The types of receptor vary with bacterial species. This diversity provides a sampling of conditions that will direct a bacterium to swim toward favorable conditions and away from unfavorable ones. It is assumed that the different receptors in different species reflect differences in their metabolism which demand different environmental conditions. A total of approximately 20 attractant receptors and 10 repellent receptors have been identified in E. coli, and the same diversity appears to be present in S. typhimurium, Table 1 (Adler, 1975; Koshland, 1980). Most of the receptors are highly specific for one or two chemicals but can react with a limited range of molecules at lower affinity.

The chemoreceptors so far identified biochemically fall into three distinct categories (Parkinson, 1981, Stewart and Dahlquist, 1987). The first includes some of the soluble periplasmic binding proteins such as the galactose, ribose, and maltose binding proteins. These binding proteins do not only function as specific sensory devices but also play a role in active transport. Not all periplasmic binding proteins are chemoreceptors and neither is sugar uptake required for chemotaxis. However, these two processes share a common component (Adler, 1969).

TABLE 1.1 List of receptors for chemotaxis of E. coli and S. typhimurium (Adler, 1975; Koshland, 1980).

Potential receptor for chemoeffector tested	Other compounds which interact strongly with receptor	Shown to be present in: E. coli	Shown to be present in: S. typhimurium	Attractant (A) or repellent (R)
Glucitol		√		A
N-Acetylglucosamine		√		A
D-Fructose		√		A
D-Galactose	D-glucose, D-fucose	√	√	A
D-Glucose		√		A
D-Mannose	D-glucose	√		A
Maltose		√		A
Mannitol		√		A
Ribose	Allose	√	√	A
D-Sorbitol		√		A
Trehalose		√		A
Aspartate	Glutamate	√	√	A
Serine	Homoserine	√	√	A
Acetate	Valerate, butyrate, propionate	√	√	R
Isopropanol	Ethanol, isobutanol	√		R
Leucine	Isoleucine, valine	√	√	R
Indole	Skatole	√	√	R
Phenol			√	A,R
Benzoate		√		R
H^+, OH^-		√	√	A,R
Mg^{2+}	Ca^{2+}, Sr^{2+}	√		A
Mg-Citrate	Ca-citrate		√	A
Citrate			√	A
Ni^{2+}, Co^{2+}		√		R
Nitrate			√	A
O_2		√	√	A
Arabinose	Fucose, xylose	√		
S^{2-}		√		R
Fumarate			√	A

The second class of chemoreceptors span the inner membrane. These receptor proteins provide the communication link between the periplasmic binding proteins and the cytoplasm. They can also function as primary receptors. For instance, the *Tar* proteins of E. coli mediate the signal from the periplasmic maltose binding protein, and it directly binds aspartate, one of the strong chemoattractants. They also serve as sites of reversible methylation reactions, which are involved in behavioural adaptation to some stimuli.

The third type of chemoreceptors are involved in responses to sugars such as glucose that are transported into the cell by a phosphotransferase system. The proteins are those involved in binding and phosphorylation of the sugars.

The existence of some repellent receptors such as those for fatty acids, alcohols, hydrophobic amino acids, and several divalent cations, has been identified only indirectly using competition studies (Tso and Adler, 1974). Competition experiments to classify repellents have been performed using the chemical-in-plug method wherein plugs of agar containing repellents create a clear area within a semi-solid plate of bacterial suspension (Tso and Adler, 1974). A plug of one repellent, A, at 50- to 200- fold above its threshold concentration for detection is placed in the suspension while a second repellent, B, is added at as close to saturating concentration as possible into the suspension and a plug. If the two repellents use the same chemoreceptor, the response to A should be inhibited; if they do not, the response should not be affected.

III.3.2 Transducers

Transducers are the components of the chemotaxis machinery which are responsible for transmitting any sensed information to the flagella for action via a possibly common sensory transduction system (Eisenbach et al, 1985). The excitation and adaptation phases actually constitute this transduction system. However, these steps are so far only defined phenomenologically. Changes in stimuli concentration are sent as a signal to the flagella and this information affects the direction their rotation. This is known as the excitation phase. It occurs within 0.2 sec in response to a large step change in the concentration of an attractant or a repellent (Eisenbach et al, 1985).

The transducer proteins are thought to act as the comparator in stimuli concentration. The *Tsr* and *Tar* transducers bind the amino acid attractants serine and aspartate and thus act as primary chemoreceptors for these compounds (Parkinson, 1981; Stewart and Dahlquist, 1987). *Tsr* also directly mediates the response to repellent stimuli including leucine. *Tar* also functions are receptor-transducer for the repellents Ni^{2+} and Co^{2+}. The *Trg* transducer binds with ribose- and galactose-receptor complexes to act as secondary chemoreceptors. A new gene, *tap*, has been found recently and may encode a transducer for another as yet unidentified chemoeffector (Boyd and Simon, 1982).

III.3.3 Methylation of Transducers

The transducer proteins of E. coli and S. typhimurium are subject to post-translational methylation and the degree of methylation reflects the cell's past environment (Springer et al, 1977). Hence, the transducers are often referred to as methyl-accepting chemotaxis proteins (MCP). The transducers are methylated at glutamyl residues to form carboxymethylesters. Methyl groups donated by S-adenosyl methionine are added by a methyltransferase which is encoded by the *che* R gene. The methyl groups from the transducers are removed by the *che* B gene product, a methylesterase. *che* R Mutants are unable to methylate and have a smooth-swimming phenotype (Springer and Koshland, 1977). *che* B Mutants are not able to de-methylate, therefore, have unusually high levels of methylation and exhibit a tumbly phenotype (Stock and Koshland, 1978).

During adaptation, which usually requires several seconds to a few minutes for completion, the unstimulated behaviour of the bacterial cell is restored i.e. smooth swimming with occasional brief periods of tumbling (Koshland, 1980). In E. coli and S. typhimurium, binding of an attractant to a transducer causes it to generate a CCW signal to the flagella; methylation of the MCP counteracts or turns off this signal so that the original signal level is reestablished and the bias of the flagellar motors returns to the prestimulus behaviour. Repellents cause CW rotation of the flagella, and are associated with decreased methylation (Boyd and Simon, 1982).

In B. subtilis, attractants cause decreased MCP methylation levels while repellents cause increased levels of methylation (Goldman et al, 1982).

Adaptations to oxygen and sugars transported by the phosphotransferase system in E. coli and S. typhimurium are not mediated by the MCP (Taylor, 1983). However the methylation-mediated transduction pathway and the methylation-independent pathway may converge before reaching the motor "switch" (a site which is closely associated with the flagellar motor and determines its direction of rotation).

III.3.4 Excitatory Signaling

Due to the absence of information about the steps that enable communication between the transducers and the "switch", a signal mechanism has been proposed to be generated or modulated by the transducers and which interacts with the "switch". The exact nature of this signal is still unclear. Eisenbach et al (1985) examined different excitatory signals and eliminated irrelevant mechanisms in an attempt to define what affects the motor directly.

III.3.4.1 Direct Interaction Between the MCP and the Switch

Direct communication necessitates a close physical contact between the MCP and the switch. In such a case there should be high levels of MCP molecules in the membrane area around the flagellar motor. However, Engstrom and Hazelbauer (1982) found that the MCPs were

distributed uniformly in all points of the membrane. This mechanism was further ruled out when Ravid and Eisenbach (1984a) observed that flagellar rotation in membrane envelopes devoid of cytoplasm was not affected by addition of chemoeffectors.

III.3.4.2 Electrical Signaling

Membrane potential is the transmembrane electrical component of the proton motive force (the pH gradient being the chemical component). Communication utilizing changes in membrane potential ($\Delta\psi$) have been considered because some eucaryotic cells (such as Paramecium) transmit these signals from one part of the cell to another (Kung and Saimi, 1982). This also occurs in some large, non-pertirichous bacteria. Chemotaxis in Spirochaeta aurantia and phototaxis (response to light) in Rhodospirillum rubrum are inhibited when the $\Delta\psi$ values are clamped close to zero (Eisenbach et al, 1985).

Early evidence indicated a correlation between $\Delta\psi$ and chemotactic behaviour in E. coli and S. typhimurium (Szmelcman and Adler, 1976; Snyder et al, 1981) and B. subtilis (Miller and Koshland, 1977). Addition of chemical agents to these organisms which change the membrane potential also altered their swimming behaviour. For instance, in B. subtilis, addition of valinomycin (to a potassium-free medium) resulted in an increase in $\Delta\psi$ (hyperpolarization) and led to a smooth swimming response followed by an adaptive return to random behaviour. Addition of an attractant to B. subtilis resulted in a similar swimming pattern as above but no change in the membrane potential was observed (Miller and Koshland, 1977). Conflicting results as to

the effect of chemoeffector on membrane potential have been observed in E. coli. Szmelcman and Adler (1976) detected changes in $\Delta\psi$ in response to attractants, but Snyder et al (1981) did not find such changes. However, Eisenbach (1982) has shown that the discrepancies were the consequence of differing experimental conditions.

If $\Delta\psi$ is the signal, it may be expected that all receptor signals go through the $\Delta\psi$. However, such is not the case since addition of a number of chemoeffectors has been shown to cause changes in behavioural response without any change in $\Delta\psi$ in B. subtilis (Miller and Koshland, 1977). To test if modulation of $\Delta\psi$ is the signal, Eisenbach et al (1985) clamped it in order to determine if chemotaxis would be inhibited. Valinomycin, a strong ionophore in the presence of K^+, was added to change the $\Delta\psi$ in E. coli and B. subtilis. Under conditions where the K^+ concentration was either high ($\Delta\psi$ was close to zero) or low ($\Delta\psi$ was clamped but not dissipated), the bacteria were still excitable and adaptable and chemotaxis was still normal. Therefore, $\Delta\psi$ was ruled out as the excitatory signal in bacterial species of small size.

III.3.4.3 Signaling by Intracellular pH

Changes in intracellular or extracellular pH have been observed to result in transducer-mediated behavioural response (Repaske and Adler, 1981; Kihara and Macnab, 1981). A decreased external pH after the addition of weak acid (acetate, benzoate, salicylate) evoked a repellent (tumbling) response in wild-type cells of E. coli. This

tumbling response also correlated with changes in intracellular pH (pHi). Kihara and Macnab (1981) have proposed a "proton receptor" theory wherein the receptor protein is protonated and altered in such a way as to initiate a signal to the motor. Sensory information from the receptor is transduced through the *Tsr* protein (Repaske and Adler, 1981; Sclonzewski et al, 1982). *Tsr* has two pH-sensitive regions that mediate responses to intracellular pH. The *Tsr* protein undergoes a change in conformation induced by pH which may be similar to that observed when (other) repellents are added (or attractants removed), such that there is a corresponding signal for the excitation and adaptation phases. *Tar* also has internal and external pH-sensitive regions which elicit responses that are opposite to those associated with *Tsr*. Decreasing pH causes an attractant response by the *Tar* protein. Such inverted response is observable only in *tsr*$^-$ mutants.

To verify if intracellular pH was the signal, Eisenbach et al (1985) used 0.1 M benzoate to produce acid accumulation and equilibrium between the intracellular and extracellular pH in E. coli. This condition promoted a highly buffered intracellular condition. However CCW rotation of the flagella in E. coli was still evoked in the presence of serine. Hence, these workers have concluded that intracellular pH appears to be involved in excitatory signaling only in the case of weak acid/base taxis. Repaske and Adler (1981) recognized the interrelationship of membrane potential, pH gradient and intracellular pH. They suggested that the effects ascribed to sensing of a $\Delta\psi$ could result from an altered pHi.

III.3.4.4 Signaling by Cytoplasmic Proteins

The most likely mechanism for excitatory signaling is through cytoplasmic proteins that are products of the chemotaxis genes. *Che* R, *Che* B, *Che* Y, *Che* Z, *Che* A and *Che* W are all cytoplasmic proteins that could, in principle, participate in signal generation and inactivation, or that could serve as the signal itself. This mechanism further involves diffusion of the signal through the cytoplasm to the flagellar switch (Eisenbach et al, 1985; Stewart and Dahlquist, 1987). However, *Che* R and *Che* B are unlikely candidates because *che* R, *che* B and *che* RB deletion mutants still exhibit normal excitatory response to attractants. (*che* R mutants lack methyl-transferase activity and show predominantly CCW rotation while *che* B mutants lack methylesterase activity and exhibit predominantly CW rotation).

Non-chemotactic mutants (*che*) are capable of flagellar rotation but exhibit aberrant swimming patterns characterized by too few or too frequent tumbling episodes.

Point mutation and deletions in *che* Y cause extreme CCW (smooth) bias in the swimming behaviour of E. coli and S. typhimurium (Parkinson, 1981). *Che* Y (protein) has been proposed as a "tumble factor". Eisenbach and Adler (1981) have prepared cell envelopes of S. typhimurium that have flagella which rotate exclusively CCW. Addition of purified *Che* Y from E. coli to these envelopes switched the rotation to CW (Ravid and Eisenbach, 1984a). Clegg and Koshland (1984) also observed that overproduction of *Che* Y in E. coli resulted in extreme CW bias to flagellar rotation. Similar results were obtained

when these *Che* Y genes were overexpressed in host cells that lack transducer proteins. These results, therefore, suggest that *Che* Y protein interacts directly with the flagellar switch and does not require additional components to mediate this interaction.

Point mutations and deletions in *che* Z cause extreme CW bias of flagellar rotation, which produces a tumbly phenotype (Parkinson, 1981). *Che* Z is thought to facilitate rapid inactivation of the "tumble factor" (*Che* Y) which leads to an increase in the CCW bias of the motor. However, Eisenbach et al (1985) suggested that *Che* Z is probably not a CCW signal but rather is involved in releasing *Che* Y from the switch since flagella in cell envelopes devoid of cytoplasm rotated CCW even in the absence of *Che* Z.

che A and *che* W Mutants are smooth swimming mutants which seldom tumble due to extreme CCW bias in flagella rotation (Parkinson, 1981). Only strong repellent stimuli such as benzoate can effect CW transient response in such mutants; other less potent stimuli cannot do so. Little is known about *che* A and *che* W gene products. *Che* A and *Che* W proteins do not seem to be required for CW flagellar rotation *per se* but they may be necessary for translating MCP - mediated signals to the flagellar switch. Stewart and Dahlquist (1987) observed that *che* A mutants were defective in regulation of methylesterase activity in response to repellent, which suggests a signaling role for *Che* A. Some *che* W mutants have overmethylated MCP, suggesting that the *Che* W protein is involved in regulation of methylesterase as well.

III.3.5 The flagellar Switch

The switch is conceived as the component which receives signals generated by the chemoreceptors during excitation. The *che* C and *che* V gene products may be the flagellar site for the receptor signals (Parkinson, 1981). Mutations at the *che* C and *che* V loci result in either excessively high or low tumbling rates which could be due to inability to respond correctly to the incoming receptor signals.

Using transductional crosses, *che* C and *che* V mutations were found to map in *fla* A and *fla* B loci, respectively (Armstrong and Adler, 1969; Silverman and Simon, 1973). These loci are altered in subtle ways by mutations in *che* C or *che* V, thereby permitting synthesis of flagella while interfering with normal rotational response and resulting in a chemotaxis defect. Parkinson (1976) and Rubik and Koshland (1978) found that the *che* C and *che* V switch mutants can still respond to chemical stimuli but exhibit higher than normal thresholds. A *che* C mutant of S. typhimurium exhibits inverted chemotactic response (Khan et al, 1978). CW rotation of their flagella results in smooth swimming and addition of attractant, which normally results in CCW rotation, leads to tumbling (Boyd and Simon, 1982).

III.3.6 Methylation - Independent Adaptation

In various organisms, behavioural response to oxygen (aerotaxis), light (phototaxis), proline taxis and electron acceptor taxis are mediated by proton motive force, instead of MCP (Taylor, 1983). In *che* R and *che*

B mutants of E. coli and S. typhimurium, adaptation to oxygen was shown to be normal but all methylation - dependent adaptation was impaired. Chemotaxis towards glucose and other sugars transported by a phosphotransferase system also involves a methylation-independent adaptation (Nirvano and Taylor, 1982). "Protometer" is the (putative) sensor that detects changes in the proton motive force (Glagolev, 1980). Phosphorylation or adenylation of the signaling protein in phosphotransferase chemotaxis, or of the protometer, are possible mechanisms for methylation - independent adaptation (Taylor, 1983).

The methylation-dependent and methylation-independent sensory transduction pathways probably converge at a point before reaching the flagellar motor switch. Inclusion of the MCP - independent pathways, namely proton motive force-dependent pathways and the phosphotransferase pathway, adds diversity to the sensory transduction pathway.

It has been suggested that behavioural responses mediated by the proton motive force occur primarily to guide cells away from environments which cannot support optimal energy production. In an environment where proton motive force can be generated, motile bacteria are guided by responses mediated by methylation-dependent pathways.

Taylor (1983) concluded that specialized responses of bacteria lead them to select one energy source over others and enable them to modify chemotactic responses to ensure that chemotaxis does not trap

bacteria in an environment which cannot supply an optimal rate of ATP production.

1.III.4 Chemotaxis in *Clostridium* and Other Bacteria

A review by Weibull (1960) listed the different genera of bacteria used in earlier investigations of chemotactic response. These include: Aerobacter, Bacillus, Beggiatoa, Bordetella, Chromatium, Clostridium, Corynebacterium, Escherichia, Klebsiella, Leptospira, Micrococcus, Pasteurella, Pseudomonas, Proteus, Rhizobium, Rhodospirillum, Salmonella, Serratia, Shigella, Spirillum and Vibrio.

Studies on Clostridium date back to 1893 when Beijerinck used microscopic techniques to study bacterial aerotaxis. Various bacteria were introduced into a test tube partially filled with distilled water or replaced by 0.1% agar. After about 24 h, motile aerobic organisms formed turbidity between the bottom of the tube and the surface of the liquid. Removal of oxygen resulted in a band toward the surface. Clostridia formed a band of motile organisms at the bottom of the test tube, leaving the rest of the liquid clear. Aside from this aerotactic response, no other studies on behavioural response, such as chemotaxis, of the motile, obligate anaerobic clostridia have appeared. Motility in C. acetobutylicum has been observed during the ABE fermentation (Spivey, 1978; Jones et al, 1982; Long et al, 1984a) but has not been perceived as a chemotactic response.

Recent studies on chemotaxis using non-peritrichous bacteria such as Spirillum (Krieg et al, 1967), Pseudomonas (Moench and Konetzka, 1978; Craven and Montie, 1983) and Spirochaeta (Goulborne and Greenberg, 1981) have dealt mainly with the basic mechanism of chemotaxis. The role and significance of chemotaxis have been studied in the denitrifying bacteria such as Pseudomonas aeruginosa, P. fluorescens, and P. stutzeri (Moulton and Montie, 1979; Kennedy and Lawless, 1985) and in the symbiotic nitrogen-fixing bacteria such as Rhizobium meliloti (Ames et al, 1980), R. leguminosarum (Bowra and Dilworth, 1981), R. trifolii and Bradyrhizobium japonicum (Parke et al, 1985). Results suggest that chemotaxis offers a mechanism by which denitrifiers successfully compete for available NO_3^- and NO_2^- and may facilitate the survival of naturally occuring populations of some denitrifiers. Similarly, attraction to sugars and amino acids and plant phenolics present in most exudates may provide competitive advantage in nodulation by nitrogen-fixing bacteria.

It appears that solventogenesis during the ABE fermentation and bacterial chemotaxis share some common features. As fields of study, they are both widely investigated.

As physiological phenomena, they both require signal(s) to occur and the nature of the signal(s) still waits to be identified. The proton motive force or either of its components (membrane potential and pH gradient) may be a common component of the two phenomena. Proton motive force is generated in strictly fermentative bacteria, such as C. acetobutylicum, through proton extrusion across the membrane to

maintain a functional pH gradient, and thereby drive energy-requiring metabolic processes. In motile bacteria, the proton motive force is converted into mechanical force that rotates the bacterial flagella.

The intracellular pH has been proposed as a possible signal, transmitted by the transducers to the flagella, which determines the direction of flagellar rotation. In the ABE fermentation, the intracellular pH has been proposed as a signal for the transition from the acidogenic phase to the solventogenic phase.

The cell membrane plays a vital function in both chemotactic and solvent-producing cells. The flagella, chemoreceptors and transducers are located in the cell membrane. Butanol seems to be most toxic to the cell membrane, thereby inhibiting membrane-linked cellular functions.

Although metabolism of attractant is not required in chemotaxis, chemotaxis has been suggested to provide survival advantage to bacteria by leading them towards sources of energy (such as sugars) and guiding them away from toxic substances (such as phenol and acids). Likewise, the shift to solvent production appears to provide survival advantage to C. acetobutylicum as it acts as a detoxification mechanism to avoid the inhibitory effects that would occur when acid and products reach toxic levels.

The purpose of this thesis, therefore, is to investigate this perceived link between motility and solventogenesis in C. acetobutylicum.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 MICROORGANISM

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

2.2 CULTURE MEDIA

A Semi-Synthetic Medium, SSM, (Ennis and Maddox, 1985) was used in various experiments. The composition is shown below:

	Concentration (g/l)
Glucose	50.0
Yeast extract	5.0
Ammonium acetate	2.0
L-Cysteine hydrochloride hydrate	0.5
NaCl	1.0
K_2HPO_4	0.75
KH_2PO_4	0.75
$MgSO_4$	0.2
$MnSO_4.7H_2O$	0.01
$FeSO_4.7H_2O$	0.01
Distilled water	

The yeast extract was obtained from GIBCO Diagnostics (Wisconsin, U.S.A.).

The slopes used for production of spores were prepared either from Reinforced Clostridial Agar, RCA (BBL Microbiology Systems, Cockeysville, Maryland, U.S.A) or Sulphuric acid-casein Whey Permeate Agar, WPA. The whey permeate was obtained from the New Zealand Dairy Research Institute (Palmerston North, New Zealand). For RCA, Agar (Davis Gelatine New Zealand Ltd, Christchurch, New Zealand) was added to give a final agar concentration of 17 g/l. This served to minimize shattering of agar, due to gas production, and swarming of colonies. The WPA was prepared by adding agar to whey permeate at 17 g/l and adjusting the pH to 6.5 using aqueous ammonia prior to autoclaving.

The Cooked Meat Medium, CMMG, (Difco Laboratories, Michigan, U.S.A.) used in inoculum development was supplemented with glucose at 10 g/l.

2.3 CHEMICALS

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

Sigma Chemical Company	Glucose
(St. Louis, Missouri, U.S.A.)	Galactose
	Lactose

BDH Chemicals Ltd	Acetic acid
(Palmerston North, New Zealand)	Butyric acid
	Butanol
	Ethanol
	Acetone
	Orthophosphoric acid
	L-cysteine hydrochloride
	$MgSO_4$
	$FeSO_4.7H_2O$
May & Baker Ltd	Ammonium acetate
(Victoria, Australia)	NaCl
Ajax Chemicals	KH_2PO_4
(Sydney, Australia)	K_2HPO_4
	$MnSO_4.7H_2O$

2.4 ANAEROBIC INCUBATION

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

Anaerobic condition was obtained by using a BBL GasPak 100 disposable envelope which generates hydrogen and carbon dioxide. Development of anaerobic atmosphere was checked with a BBL GasPak anaerobic indicator. The palladium catalyst in wire gauze capsule was

rejuvenated either by flaming until red hot or placing in an oven at 160-180°C for 2 h, prior to use.

2.5 HEAT SHOCKING

All spore stock cultures were revived by heat shocking an aliquot of 0.2 ml in 20 ml CMMG at 75°C for 2 min. This was followed immediately by cooling in iced water for 1 min.

2.6 PRODUCTION OF SPORES AND CULTURE MAINTENANCE

The stock culture was heat shocked as described in Section 2.5. The culture was then incubated anaerobically at 37°C until vigorous gassing was observed (24-48 h).

Slopes of RCA or WPA were streaked with the revived culture and incubated anaerobically at 37°C. Spore formation was monitored by Bartholomew and Mittwer's spore staining technique using malachite green (Harrigan and McCance, 1966). After 8 days incubation, the sporulated cultures were aseptically scraped off into sterile distilled water and dispensed at 2 ml into sterile screw-capped Kimax test tubes (16 x 100 mm). The haemocytometer spore counts from RCA and WPA were approximately 2.4×10^7 and 5×10^5 per ml, respectively.

The spore suspensions were maintained at 4°C.

2.7 CULTURE CONDITIONS

Cultures were grown in 3 stages as follows:

a) An aliquot of stock culture (0.2 ml) was inoculated into 20 ml of CMMG and heat shocked at 75°C for 2 min. This was followed by cooling in iced-water for 1 min. The culture was incubated anaerobically at 37°C for 16-19 h after which time highly motile cells were present.

b) One ml of the culture was transferred to 20 ml of SSM. This was incubated at 24°C for 16-19 h.

c) Ninety nine ml of freshly autoclaved SSM was then inoculated with 1 ml of the above culture and incubated at 34°C.

2.8 STERILIZATION OF MEDIA AND GLASSWARE

All culture media were sterilized in the autoclave at 121°C for 15 min.

Pipettes, test tubes and bottles were sterilized in the hot air oven at 160°C for 2 h.

2.9 ANALYTICAL METHODS

2.9.1 pH Measurement

All pH measurements were done using a Metrohm pH meter E520 (Metrohm A. G., Herisau, Switzerland).

2.9.2 Cell Counts

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

2.9.3 Analysis of Solvents and Acids

Solvent and acid fermentation products were determined by gas chromatography (GC) using a Shimadzu GC (Shimadzu Corporation, Kyoto, Japan, Model GC-8APF) equipped with a flame ionization detector. A stainless steel column (1 m x 0.15 cm internal diameter) packed with Porapak Q was used at a carrier gas (nitrogen) flow rate of 60 ml/min. The column was maintained at 200°C, and the injector and detector temperature at 220°C. Samples were injected at 2 µl aliquots.

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

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

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

where:

$\left(\frac{IS}{P}\right)_h$, is the average of the ratio of peak heights of internal standard: solvent or acid from duplicate injections.

$\left(\frac{IS}{P}\right)_c$, is the concentration ratio of the internal standard: solvent or acid in the standard solution.

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

$$c = R_f \cdot IS_c \cdot \left(\frac{c\,H}{I\,S\,H}\right)$$

where:

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

IS_c, is the concentration of the internal standard in the sample.

$\frac{c\,H}{I\,S\,H}$, is the ratio of peak heights of solvent or acid: internal standard.

2.9.4 Analysis of Sugars

Quantitative analysis of glucose or lactose was performed using a Waters Associates Model ALC/GPC 244 high performance liquid chromatograph (HPLC) with a Model 590 solvent delivery system and

Model U6K septumless injector (Waters Associates, Inc., Milford, Massachusetts, U.S.A.).

A Sugar-PAK 1 carbohydrate column (Water Associates) was used. The column was operated at 90°C using a solvent system of calcium acetate (20 mg/l) at a flow rate of 0.5 ml/min.

The detector was a Waters Associates differential refractometer Model R401.

Culture supernatants were suitably diluted to give a sugar concentration around 5 g/l. The samples were filtered through a 0.45 µm membrane using a Swinney Filter Kit (Millipore Corporation) before injecting at 25 µl volume.

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

A $\pm$ 5% error was estimated.

CHAPTER 3

CHEMOTAXIS IN *CLOSTRIDIUM ACETOBUTYLICUM* P262

3.1 INTRODUCTION

A series of distinct morphological and cytological changes occur in certain strains of C. acetobutylicum, including the industrial strain P262, during fermentation (Spivey, 1978; Jones et al, 1982; Jones and Woods, 1986) suggesting a possible relationship between solvent production and cell differentiation.

The initial acidogenic phase coincides with the exponential growth phase. It is characterized by the presence of highly motile, actively dividing phase-dark cells. A few hours before the pH-breakpoint active growth ceases, motility decreases and the cell begins to accumulate granulose (Jones et al, 1982; Long et al, 1984a; Reysenbach et al, 1986). The shift to the solventogenic phase is accompanied by the conversion of the vegetative rods to swollen phase-bright clostridial forms which continue to accumulate granulose and produce clearly defined extracellular capsules. The formation of clostridial forms is normally followed by spore formation which includes development of forespore septum and mature endospores. Formation of mature endospores is not usually observed in industrial fermentations, probably due to inhibition of further development by the accumulation of toxic concentrations of butanol (Jones and Woods, 1986).

Studies have been carried out to investigate the link between induction of solvent production and induction of cell differentiation in C. acetobutylicum (Jones et al, 1982; Long et al, 1984a, Long et al 1984b, Haggstrom and Forberg, 1986; Reysenbach et al, 1986), while the number of clostridial forms has been directly related to solvent concentration (Jones et al, 1982). Cells that failed to differentiate to clostridial forms underwent degradative changes. The development of the clostridial stage in C. acetobutylicum P262 has also been associated with the production of capsules (Jones et al, 1982; Long et al, 1984b). However, Haggstrom and Forberg (1986) observed that capsule production in strain ATCC 824 occurred not only during the solvent-production phase but also during the exponential and acid-production phase. The onset of endospore formation has been shown to be not essential for solvent production (Jones et al, 1982; Gottschal and Morris, 1982; Long et al, 1984b; Meinecke et al, 1984). Solvent production could still occur in mutants blocked in granulose accumulation, capsule production and sporulation.

Studies concerning motility of C. acetobutylicum lag behind those of other morphological stages despite a possible relationship suggested by Spivey (1978). Vigorous motility which developed in the first stage of inoculum development correlated with good solvent production. The absence of motility at the first stage (after heat shocking), or indeed at any time in the subsequent stages, was a clear indication of poor solvent yields despite a normal cell population. This relationship was confirmed when significantly higher concentrations of butanol and higher production rates were observed when transfers between stages of inoculum development were made while the cells were highly motile (Gutierrez, 1985).

Spivey (1978) found the biochemical basis of this relationship obscure. However, such a relationship could be explained by the phenomenon of chemotaxis (Appendix 2). Chemotaxis is defined as movement towards or away from chemicals and is a ubiquitous biological phenomenon (Adler, 1975; Koshland, 1980).

Motile bacteria are known to detect temporal changes in concentrations of specific chemicals, behaviourally respond to these changes, and then adapt to the new concentration of the chemical stimulus. In positive chemotaxis, bacteria migrate towards attractants while in negative chemotaxis, bacteria swim away from repellents. Typical attractants for various bacteria include sugars and some amino acids (Mesibov and Adler, 1972; Adler et al, 1973; Moulton and Montie, 1979). Repellents include alcohols and weak acids (Tso and Adler, 1974). In an environment lacking a stimulus, the movement of a motile bacterium is characterized by a series of "runs" in smooth, rather straight lines for a few seconds, and "tumbles", when the cell thrashes around for a fraction of a second. Cells migrating towards increasing concentration of attractant swim in longer runs and tumble less frequently. In the presence of increasing repellent concentration, bacteria tumble more often.

Due to the simplicity of bacterial cells compared to higher organisms, bacterial chemotaxis as a behavioural response has been widely investigated in an effort to provide an explanation for more complex behavioural systems.

In this study, the motility of C. acetobutylicum has been investigated during a typical batch fermentation process. Chemotaxis experiments

were performed using various fermentable sugars and fermentation products to obtain data which could help explain the motile behaviour of the organism during the fermentation process.

3.2 MATERIALS AND METHODS

3.2.1 Chemotaxis Medium

The chemotaxis medium (CM) used for the assays consisted of the following:

	Concentration
Potassium phosphate buffer, pH 5.0	0.01 M
Sodium thioglycollate	0.5 g/l
Resazurin	0.005 g/l

The medium was maintained anaerobic by flushing with carbon dioxide.

The CM agar used for the plugs in certain assays was prepared by solidifying CM with agar at 30 g/l. The semi-solid CM was prepared by adding agar to the CM at 2.5 g/l.

3.2.2 Fermentation Medium

Sulphuric acid - casein whey permeate obtained from the New Zealand Dairy Research Institute (Palmerston North, New Zealand), and prepared according to Matthews et al (1978), was used as the

fermentation substrate. Before being autoclaved, the permeate was supplemented with yeast extract at 5 g/l and adjusted to pH 6.5 with aqueous ammonia.

3.2.3 Chemicals, Gases, and Other Materials

All chemicals used for chemotaxis assays were of analytical grade. Sources of glucose, lactose, galactose, acetic acid, butyric acid, butanol, ethanol, and acetone are described in Section 2.3. Resazurin, sodium acetate, sodium hydroxide, and potassium ethylenediaminetetraacetate were obtained from BDH Chemicals Ltd (Poole, U.K.); sodium thioglycollate was from Difco Laboratories (Michigan, U.S.A.).

Oxygen-free nitrogen gas (N_2) and carbon dioxide gas (CO_2) were supplied by New Zealand Industrial Gases Ltd (Palmerston North, New Zealand).

Micropipettes were obtained from Clay Adams, Becton, Dickinson and Company (Parsipanny, N.J., U.S.A.). Parafilm seal was obtained from American Can Company (Greenwich, Connecticut, U.S.A.).

3.2.4 Anaerobic Manipulations

An anaerobic cabinet (Forma Scientific Inc., Ohio, U.S.A.) was used for anaerobic manipulations. The oxygen-free atmosphere was achieved by using N_2 and CO_2 at a ratio of 80 : 20, respectively.

3.2.5 Inoculum Preparation for Batch Fermentation

C. acetobutylicum P262 was grown in 3 stages to develop the inoculum for fermentation:

a) The stock culture was heat shocked as described in Section 2.5. The culture was incubated anaerobically at 34°C until cells were highly motile (19 h).

b) Five ml of the culture were transferred to 95 ml of fermentation medium (Section 3.2.2). The culture was incubated at 34°C for 22 h to obtain highly motile cells.

c) The fermentation medium (5.2 liters) was inoculated with 100 ml of the motile culture.

3.2.6 Batch Fermentation

Fermentation was performed in a 7 - liter Microferm fermenter (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.) containing 5.2 liters of whey permeate prepared as described in Section 3.2.2. The vessel containing the medium was assembled onto the fermenter unit immediately after autoclaving. During cooling, sterile oxygen-free N_2 gas was passed across the surface of the medium, and this gassing was continued after inoculation until visible gassing due to bacterial growth was observed. Fermentation was carried out at 34°C without any agitation or pH control.

3.2.7 Preparation of Chemotactic Cells

For chemotaxis experiments, C. acetobutylicum was grown in 2 culture stages:

a. The stock culture was inoculated into 20 ml CMMG and heat shocked as described in Section 2.5. The culture was incubated at 37°C until highly motile cells were present (16-19 h).

b. A 5 ml sample of the culture was transferred to 95 ml of SSM and incubated at 30°C for 17 h to obtain highly motile cells.

Cells were harvested by centrifugation at 3,000 x g for 10 min at room temperature. The pellet was gently suspended in 5 ml of CM. Cells were washed in CM five times for positive chemotaxis experiments and two times for negative chemotaxis experiments. The final pellet was suspended in CM to give an OD_{650} equivalent to approximately 10^8 cells per ml. Between 70 and 90% of the harvested cells remained motile for at least 1 h.

3.2.8 Positive Chemotaxis

A modified 'chemical-in-capillary' method (Fig 3.1) was used for positive chemotaxis (Adler, 1973). The chamber was formed by placing a bent (U-shaped) melting-point capillary tube between a microscope slide and a cover slip. This was filled with approximately 0.2 ml of the bacterial suspension. The capillary tubes for containing the attractant solutions were 5-µl micropipettes with an internal diameter of 0.17 mm. After filling with attractant dissolved in CM, one end was sealed with parafilm, and the tube was inserted into the

chamber. The assay was performed at 20°C in the anaerobic cabinet. After the appropriate incubation period of 30 or 45 min, the contents of the capillaries were ejected into 10 ml of peptone water (5 g/l). The cells were counted using a haemocytometer. Control tubes filled with CM alone were used to assess the background motility. To assess reproducibility of the method, nine capillaries were used to measure the response to 10^{-2} M glucose (Appendix 3). The standard error was 30%. This is rather high but not unusually so for this method (Mesibov and Adler, 1972). The results reported are the average of those obtained on at least three different days.

3.2.9 Negative Chemotaxis

For negative chemotaxis, experiments were performed using a modified "chemical in plug" method (Tso and Adler, 1974). The test chemicals were added into molten CM agar just before the agar set (about 40°C). The agar was poured into plates and allowed to solidify before incubating overnight in anaerobic jars. The agar plugs were cut using a stainless steel borer with a diameter of 10 mm.

Washed (chemotactic) cells were plated in semi-solid CM. The plugs were placed in this suspension as illustrated in Fig 3.2. The set-up was incubated at 30°C for 30 min. Plugs containing CM alone were used as controls.

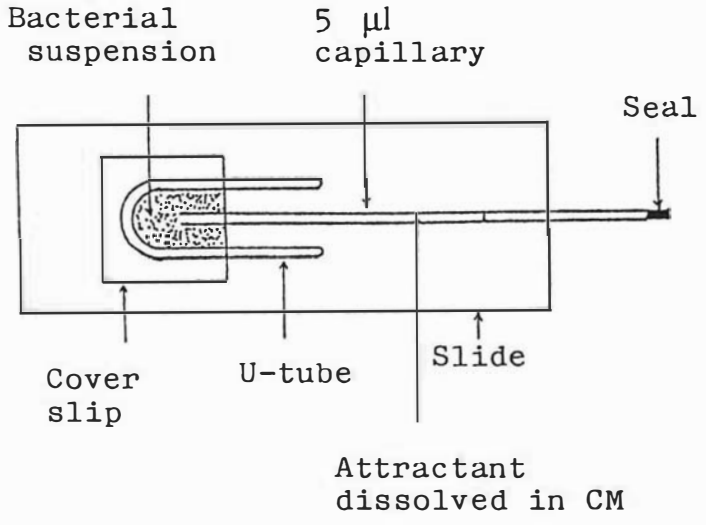


Fig 3.1 Positive chemotaxis assay: Modified "chemical in capillary" method (Adler, 1973).

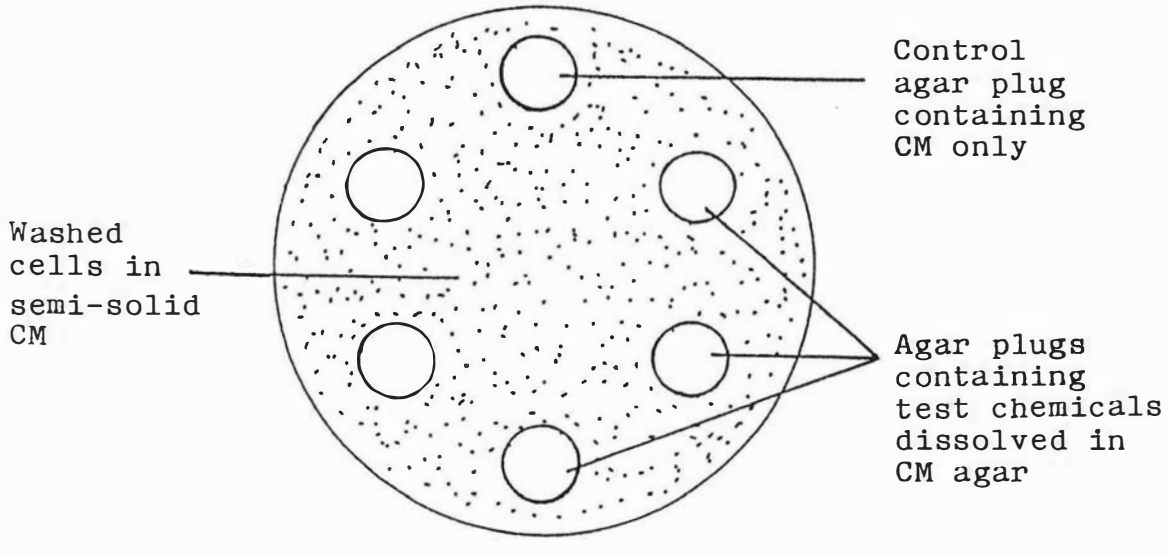


Fig 3.2 Negative chemotaxis assay: Modified "chemical in plug" method (Tso and Adler, 1974).

3.2.10 Chemical Analyses

Analyses for solvents and acids were performed as described in Section 2.9.3. Lactose was determined quantitatively as described in Section 2.9.4.

3.3 RESULTS

3.3.1 Batch Fermentation

The motility of C. acetobutylicum P262 was monitored closely during a typical batch fermentation process using a substrate of whey permeate. Figure 3.3 shows the initial 28 h of the process. The inoculum consisted of 100% motile cells. Upon inoculation, most cells became nonmotile, but after 2 h of fermentation, motility was restored, with cells moving in long, fast runs without changing direction. The cells again became nonmotile after 2 h but had fully recovered by 6 h. The motility was again characterized by long, fast runs, with very little tumbling being observed. The reason for the hiatus in motility is not clear, but it may be peculiar to the substrate used; i.e., a compound other than lactose may be the initial carbon source, followed by lactose utilization. Virtually 100% of the cells remained motile until after 13 h of fermentation, but the motility changed from running to tumbling during this time. Most cells exhibited both running and tumbling motions, so the fraction of tumbling cells can be taken as the tumbling frequency. Running cells were associated with the early period of the lactose utilization, acid production, and pH decrease. The frequency of tumbling increased as solvent production commenced and the runs

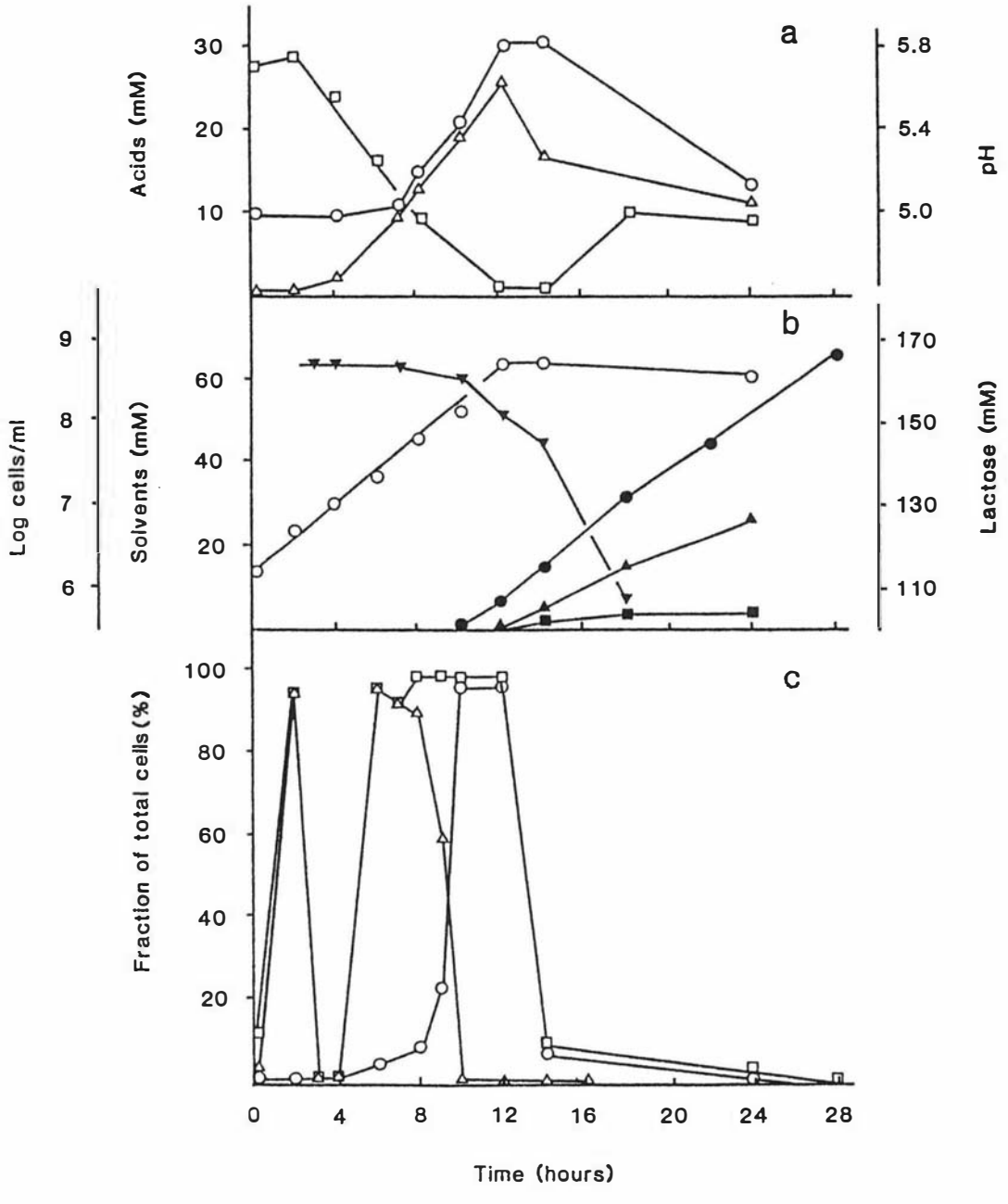


Fig 3.3 Time course of batch fermentation process.

Symbols: (a) □ pH; ○ acetate; △ butyrate

(b) ○ log cell number; ▼ lactose; ● butanol; ▲ acetone; ■ ethanol

(c) □ motile cells; △ running cells; ○ tumbling cells.

The equivalent concentrations in g/l of 1 mM of each chemical are: acetate, 0.060; butyrate, 0.088; lactose, 0.342; butanol, 0.074; acetone, 0.058; ethanol, 0.046.

became short and slow. After 10 h of fermentation, only tumbling was observed. The proportion of motile cells in the population decreased considerably by 14 h, but some individual cells remained motile for much longer. After 24 h, the vast majority of cells were in the clostridial form.

3.3.2 Optimum Conditions for Chemotaxis of *C. acetobutylicum*

In order to identify attractants and repellents, it was first necessary to determine the conditions and chemicals needed for optimum motility and chemotaxis of C. acetobutylicum. When grown under the conditions described in Section 3.2.7 cells from the exponential phase were the most motile.

A medium which supported motility and which contained only known chemicals, so as not to allow growth during the assay, was necessary to study bacterial motility. A wash medium designed for E. coli (Adler and Templeton, 1967; Adler, 1973) was used for C. acetobutylicum. This consisted of potassium phosphate buffer (pH 7.0) 10^{-2} M; MgSO4, 10^{-3} M; and potassium ethylenediaminetetraacetate (EDTA), 10^{-4} M. However, motility of cells harvested after centrifugation ceased immediately upon suspension in medium containing EDTA.

Assay conditions similar to those of growth conditions were provided by adjusting the pH of the wash medium to pH 5.0 (approximate pH when motile cells were harvested). However the motility of C. acetobutylicum cells was not maintained in wash medium at pH 5.0.

Under the conditions used, EDTA seemed to inhibit the motility of C. acetobutylicum.

After deleting EDTA, further modifications to the wash medium were made. $MgSO_4$ was deleted since, as for E. coli (Adler, 1973), $MgSO_4$ did not seem to be required by C. acetobutylicum for motility or chemotaxis. Additions of sodium thioglycollate at 0.5 g/l, resazurin at 0.05 g/l and flushing with CO_2 were made to ensure anaerobic conditions.

In this way, motility was maintained and this solution was subsequently used as chemotaxis medium. Assays were performed at 20°C to retard cell differentiation.

Cells prepared using the modified conditions remained motile for at least 1 h.

In the positive chemotaxis assay, the number of cells which migrated to the capillaries was counted using a haemocytometer instead of a plate count method, to avoid failure to obtain colony forming units. Each chemical concentration was sampled four times for counting.

3.3.3 Rates of Accumulation Towards Sugars

The rates of accumulation of C. acetobutylicum cells towards glucose, lactose and galactose were determined by incubating the capillaries containing different sugar concentrations for different time periods to optimize the chemotaxis assays. Results show that the number of cells

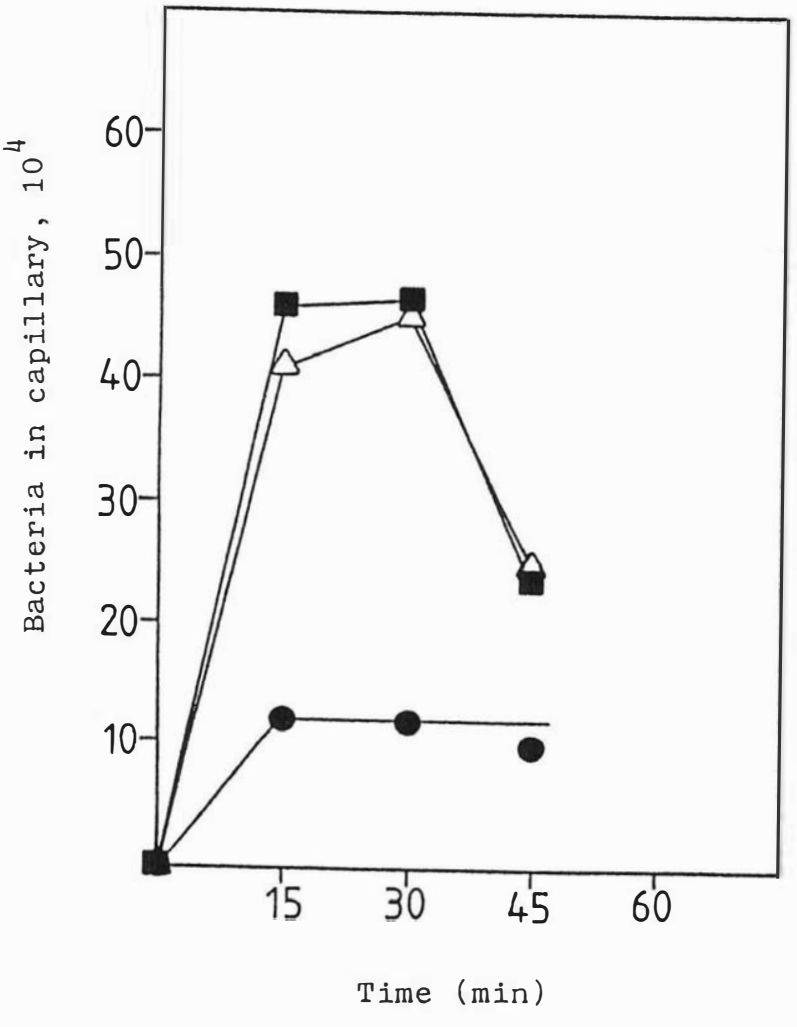


Fig 3.4 Rate of bacterial migration towards glucose: ●, 0 M (control); △, 10^{-2} M; ■, 10^{-1} M. (10^{-1} M glucose is equal to 18.0 g/l).

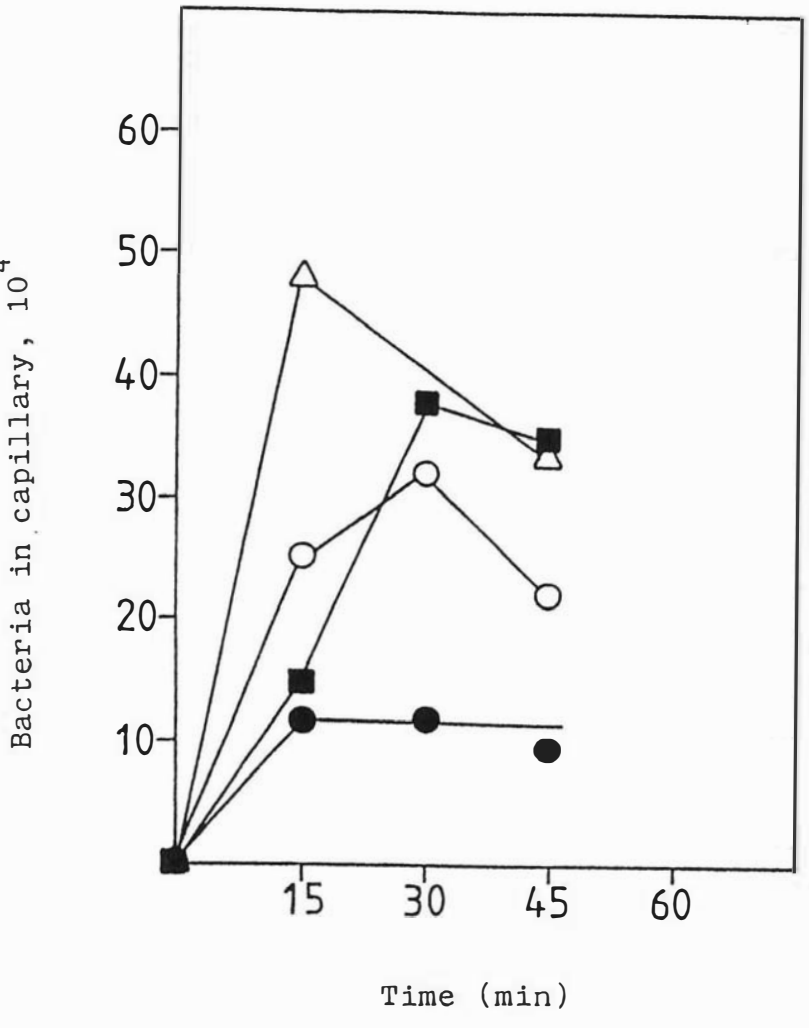


Fig 3.5 Rate of bacterial migration towards galactose: ●, 0M (control); ○, 10^{-3} M; △, 10^{-2} M; ■, 10^{-1} M. (10^{-1} M galactose is equal to 18.0 g/l).

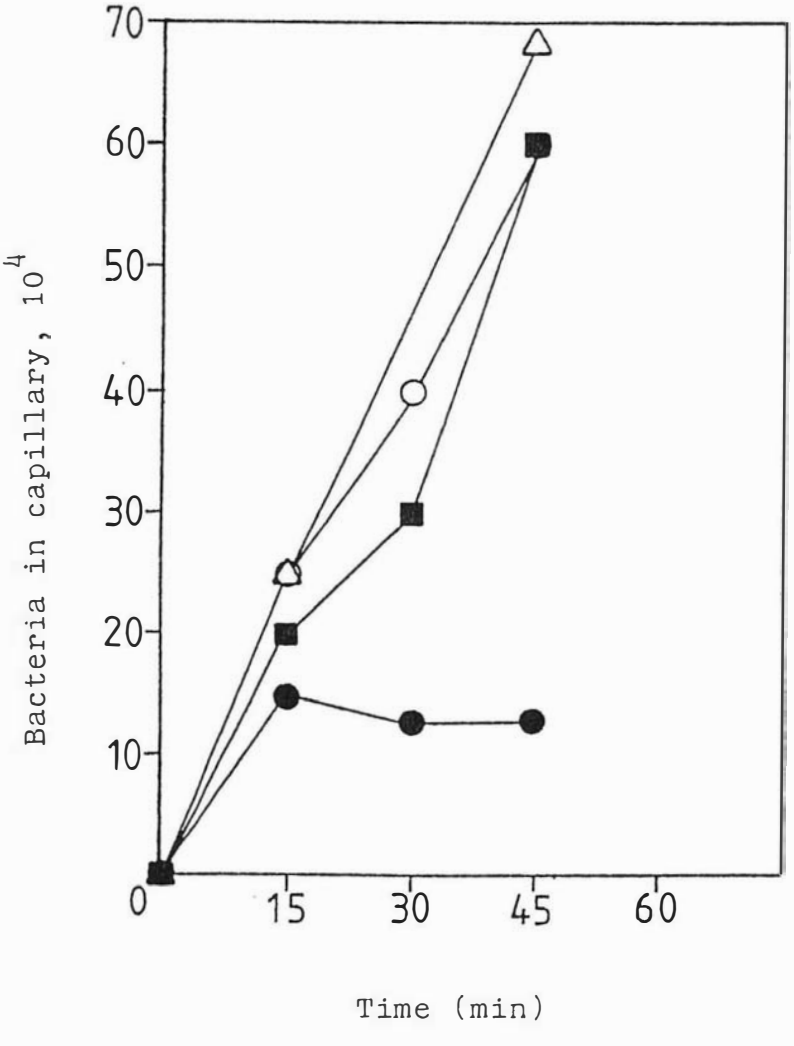


Fig 3.6 Rate of bacterial migration towards lactose:
●, 0 M (control); ○, 10^{-3} M; △, 10^{-2} M; ■, 10^{-1} M. (10^{-1} M lactose is equal to 34.2 g/l).

attracted to glucose increased linearly for up to 30 min of incubation after which a decline was observed (Fig 3.4). When the capillary contained no attractant, a relatively small number of bacteria entered the capillary. This 'background' accumulation occurred presumably by random swimming. An incubation period of 30 min was chosen for the chemotaxis assay using glucose.

Fig 3.5 shows the rate of accumulation of cells towards galactose. Migration of cells declined after about 30 min. The chemotaxis experiments for galactose were, therefore, performed for 30 min.

The rates of accumulation towards different concentrations of lactose are shown in Fig 3.6. The cells appeared to be attracted to lactose at a more or less constant rate until the number of cells migrating declined after 45 min of incubation. Chemotaxis assays for lactose were thus performed by incubating for 45 min.

3.3.4 Positive Chemotaxis

The chemotactic response of C. acetobutylicum towards different fermentable sugars was investigated. The concentration - response curves for glucose, galactose and lactose are shown in Fig 3.7. All three sugars were attractants. The threshold concentration for each sugar was determined as the lowest concentration which gave an accumulation of cells in the capillary significantly greater than that of the background (Adler, 1973), as judged by a t - test (Appendix 4). There was also a peak concentration of each sugar where the response was maximal. The results are shown in Table 3.1.

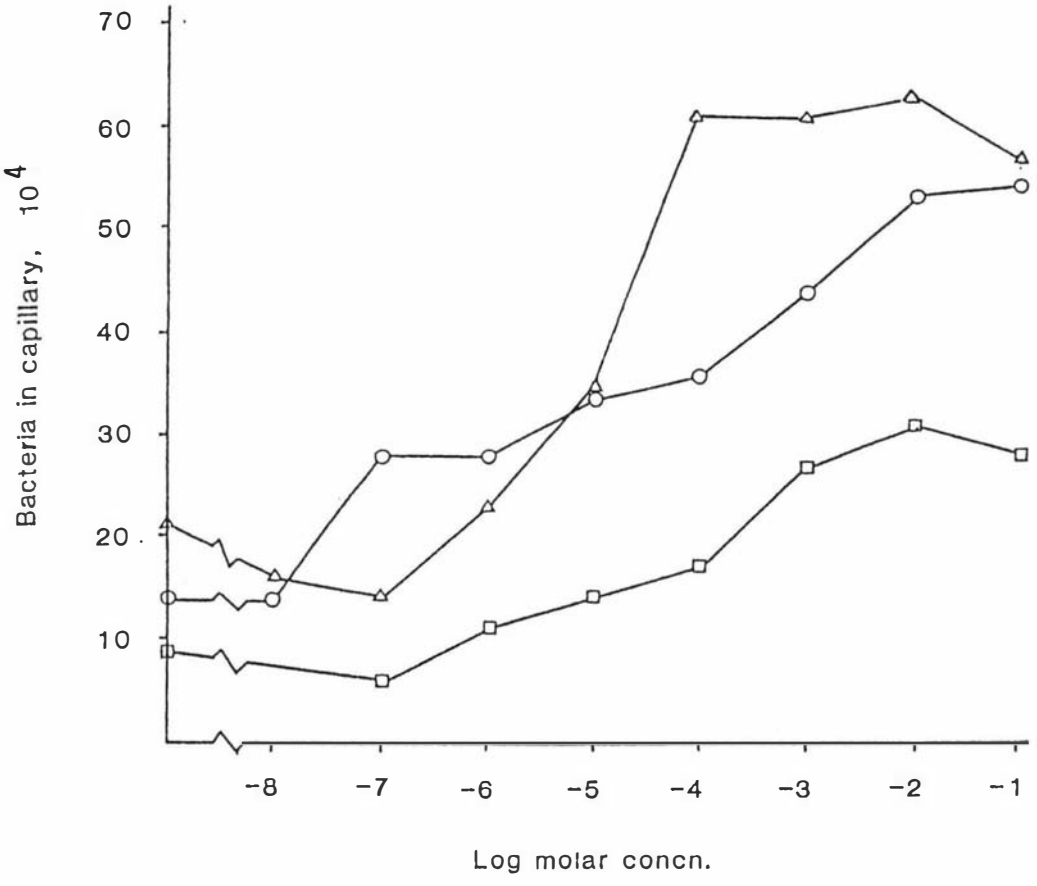


Fig 3.7 Concentration-response curves for glucose (○), galactose (□), and lactose (△).

Table 3.1 Response values for sugars.

Sugar	Threshold concentration (M)	Peak concentration (M)	Relative response
Glucose	1×10^{-7}	1×10^{-1}	2.0
Galactose	1×10^{-4}	1×10^{-2}	1.9
Lactose	1×10^{-5}	1×10^{-2}	1.7

The minimum concentrations where motile cells were observed to accumulate were 1 x 10^{-7} M for for glucose; 1 x 10^{-4} M for galactose; and 1 x 10^{-5} M for lactose. The t - tests suggest that the number of cells which migrated to these concentrations were significantly greater than those present in the capillary containing no attractant. Thus, the response was to a concentration gradient. Values for the relative response (Table 3.1) were also determined to normalize for day-to-day differences in motility and to allow comparison between attractants. The relative response was calculated as the ratio of cell accumulation at the threshold concentration to the background accumulation. Glucose and galactose seem to have greater chemoattracting ability for C. acetobutylicum than does lactose.

Similar experiments were performed using the metabolic products acetone, butanol and ethanol, but none of these solvents was an attractant.

For acetate and butyrate, initial experiments were performed using buffers of 0.1 M concentration at various pH values. The buffers were sodium acetate - acetic acid and sodium hydroxide - butyric acid for acetate and butyrate, respectively. The results for acetate and butyrate (Table 3.2 and Table 3.3, respectively) show that positive chemotaxis occurred towards those buffers of decreasing pH values. Statistical analysis of the data using ANOVA (analysis of variance) confirmed that the observed differences for acetate and butyrate were significant at $\alpha = 0.05$ (Appendix 5 and Appendix 6, respectively).

Table 3.2 Chemotactic response of C. acetobutylicum to acetate at various pH values[a].

pH	Concentration of undissociated acid, CH_3COOH (M)	Concentration of dissociated acid, CH_3COO^- (M)	Accumulation of bacteria in capillary, (10^4)
4.0	0.0849	0.0151	39
4.5	0.0640	0.0360	35
5.0	0.0360	0.0640	27
5.5	0.0151	0.0849	20
6.0	0.0053	0.0947	13
Control[b]			11

a All buffers were 0.1 M

b CM (Section 3.2.1) was used as control

Table 3.3 Chemotactic response of C. acetobutylicum to butyrate at various pH values[a].

pH	Concentration of undissociated acid, $CH_3(CH_2)_2$ COOH (M)	Concentration of dissociated acid, $CH_3(CH_2)_2COO^-$ (M)	Accumulation of bacteria in capillary, (10^4)
4.0	0.0868	0.0132	37
4.5	0.0676	0.0324	24
5.0	0.0398	0.0602	21
5.5	0.0173	0.0817	15
6.0	0.0062	0.0938	15
Control[b]			18

a All buffers were 0.1 M

b CM (Section 3.2.1)

The concentrations of dissociated and undissociated species of acetic and butyric acids present in the buffers, as calculated by the Henderson-Hasselbach equation (Conn and Stumpf, 1976) are shown in Tables 3.2 and 3.3, respectively. Calculations were based on pKa values of 4.75 for acetate and 4.82 for butyrate.

When similar experiments were performed using only inorganic buffers, such as phosphate, no differences were observed for different pH values (Table 3.4). No pH effect was detected even in the presence of 0.1 M glucose. Thus, C. acetobutylicum exhibits positive chemotaxis towards the undissociated forms of acetic and butyric acids, and the effect is not entirely an effect of pH value. The concentration - response curves, performed at pH 4.0 are shown in Fig 3.8, while the threshold concentrations and relative response values are given in Table 3.5. In contrast to the sugars (Fig 3.7), saturation points were not observed in the concentration range likely to be attained under physiological conditions.

3.3.5 Negative Chemotaxis

Negative chemotaxis was determined by using a modified "chemical in plug" method (Section 3.2.9). Typical results are shown in Fig 3.9 where movement of cells repelled by the chemical was indicated by clear zones around the plugs containing the repellent. Agar plugs containing acetate and butyrate at different pH values were used to determine the chemotactic response of C. acetobutylicum cells to these acids. For acetate (pH 4.0) and butyrate (pH 4.0), both at 0.1M, negative chemotaxis was not observed. Acetate (pH 6.0) and butyrate

Table 3.4 Effect of pH on chemotaxis.

Chemical in capillary	Bacteria in capillary, 10^4
0.01 M phosphate, pH 4.0	11
0.01 M phosphate, pH 5.0	14
0.01 M phosphate, pH 6.0	10
deionized water, pH 7.0	9

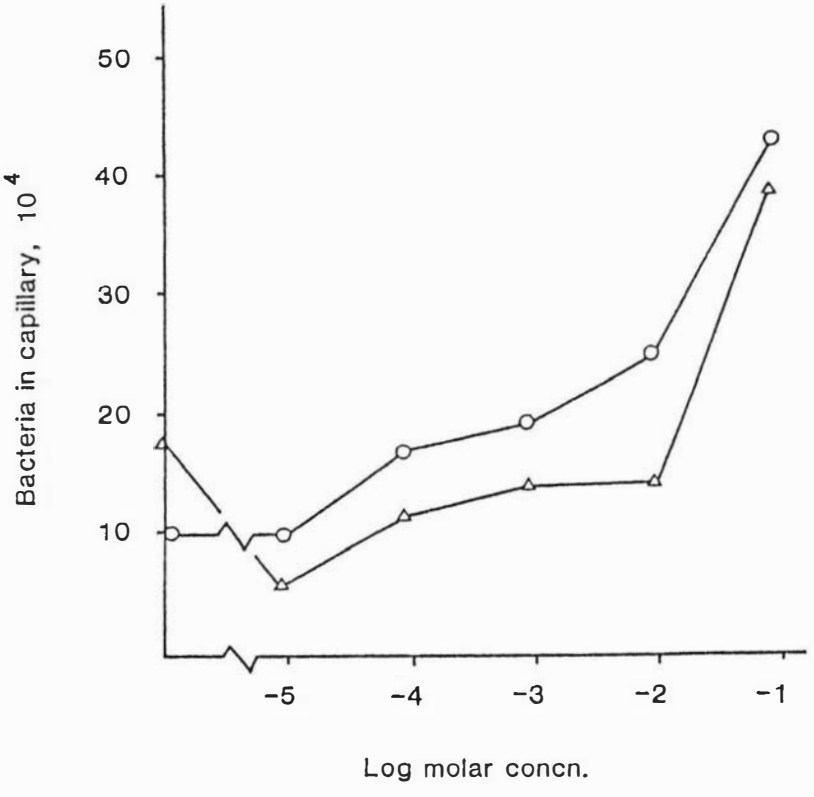


Fig 3.8 Concentration-response curves for acetic acid (○) and butyric acid (△) at pH 4.0.

Table 3.5 Response values for acetic acid and butyric acid

Chemical	Threshold concentration (M)	Relative response
Acetic acid, pH 4.0	9×10^{-4}	1.9
Butyric acid, pH 4.0	9×10^{-2}	2.0

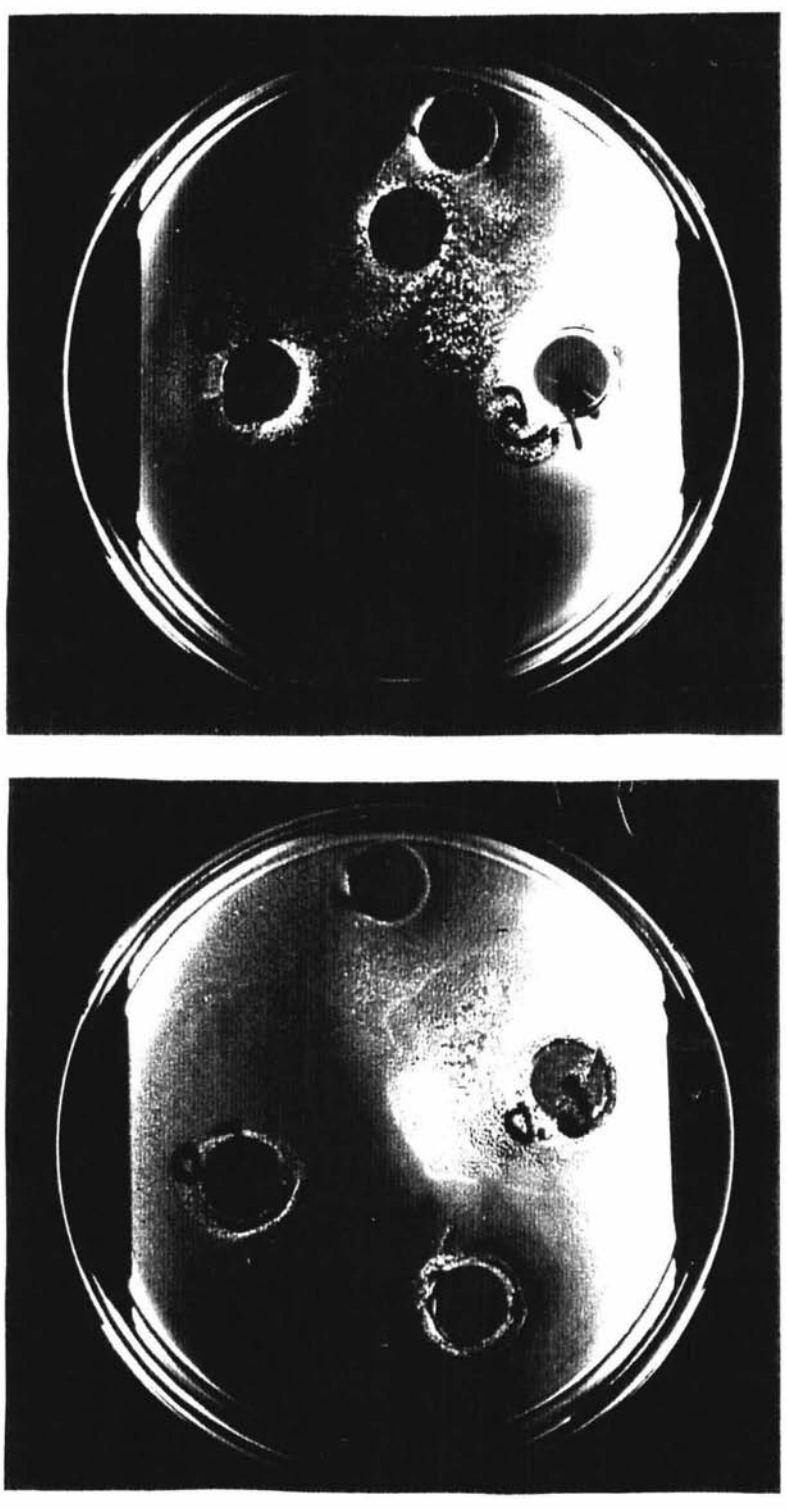


Fig 3.9 Negative chemotaxis assay using modified "chemical in plug" method (Tso and Adler, 1974) showing typical clear zones around plugs containing repellents.

a) From top clockwise: two plugs of CM as controls; pH 4.5; pH 4.0. No clear zones were present as acetic acid at pH 4.0 and pH 4.5 was found to be an attractant (Section 3.3.4).

b) From top clockwise: CM (control); pH 6.0; pH 5.5; pH 5.0. Clear and mottled zones were observed around plugs with acetate buffer as dissociated acids were found to be repellents (Section 3.3.5). Photos which could show better contrast between the suspension and clear zones were difficult to obtain.

Table 3.6 Threshold concentrations for some chemorepellents.

Chemical	Threshold concentration (M)[a]
Acetate (pH 6.0)	8.5×10^{-7}
Butyrate (pH 6.0)	8.7×10^{-8}
Butanol	1×10^{-11}
Ethanol	1×10^{-12}
Acetone	1×10^{-9}

a Concentrations are expressed as dissociated acids, calculated by using pK_a values of 4.75 and 4.82 for acetate and butyrate, respectively.

(pH 6.0), both at 0.1 M, were observed to be repellents. Clear zones ranging in size from 0.5 to 5.0 mm were observed for the dissociated acids.

The solvents acetone, butanol and ethanol were likewise observed to repel cells. Mottled, irregular clearing was formed around the solvents, rather than discrete clear zones.

Values of threshold concentrations varied considerably from day to day. For this reason, experiments were performed on up to eight different days, and the lowest values observed are shown in Table 3.6.

3.4 DISCUSSION

The need to optimize the assay conditions for motility and chemotaxis of C. acetobutylicum cells demonstrated different requirements from those of other bacteria. The addition of EDTA to the chemotaxis medium, as done with E. coli (Adler, 1973), was not essential with C. acetobutylicum. On the contrary, the cessation of motility in the presence of EDTA suggests an inhibitory effect of this agent in C. acetobutylicum. Similar effects have been observed using Rhizobium species (Parke et al, 1985). To summarize, the conditions required for optimum motility of C. acetobutylicum P262 are: pH of 5.0; temperature of 20^{o}C to retard cell differentiation to clostridial forms which are less motile and chemotactic; and stringent anaerobic conditions.

This study has demonstrated that motility of C. acetobutylicum cells during the fermentation process is a chemotactic response and that motility changes from being predominantly runs to predominantly tumbles. The runs are associated with the early period of lactose utilization and acid production, concurrent with a pH decrease. Such runs are characteristic of positive chemotaxis (Adler, 1973, 1975), and the sugars and undissociated acids have now been shown to be attractants. As the fermentation proceeds, the motility becomes characterized by tumbles. This is associated with solventogenesis, and the solvents have now been shown to be repellents. The results strongly suggest that the motility of C. acetobutylicum cells during fermentation is a chemotactic response and this may help to explain the relationship between motility and solventogensis.

All the changes in chemical composition of the environment are detected by specific receptors or sensors, and the sensed information is transmitted to the flagella for action through the sensory transduction system (Adler, 1969; Boyd and Simon, 1982; Eisenbach et al, 1985; Stewart and Dahlquist; 1987). In E. coli and S. typhimurium, receptors for the attractants glucose and galactose have been identified (Adler, 1969; Stewart and Dahlquist, 1987). The galactose receptor is a periplasmic binding protein which has also a role in transport of galactose. The chemoreceptors for glucose are the proteins involved in the phosphotransferase transport of sugars. Attraction to lactose in E. coli has been suggested to result from chemotaxis toward the galactose and probably glucose which are produced from hydrolysis of lactose. Receptors for ethanol and isobutanol in E. coli and S. typhimurium have also been identified although indirectly using competing chemicals

(Tso and Adler, 1974; Koshland, 1980). The receptor for the weak acids acetate, propionate, butyrate, benzoate and salicylate, which in E. coli are repellents, have been recently reassigned, not in terms of an external or membrane-bound receptor (as in the case of the attractants), but in terms of a 'proton receptor', located either within the cytoplasm or on the cytoplasmic side of the membrane, which when protonated is altered in such a way as to initiate a signal to the flagellar motor. Decreases in intracellular pH, therefore, act as a signal for the behavioural (repellent) response to weak acids in E. coli (Kihara and Macnab, 1981; Repaske and Adler, 1981).

All bacteria may share the same general types of receptors although even the very similar E. coli and S. typhimurium differ slightly, and it is not surprising that more distantly related organisms show greater differences in specific receptors. It is possible that C. acetobutylicum has the same receptors towards the sugar examined and the weak acids as has E. coli.

Chemotaxis is not a consequence of the metabolism of attractants or toxicity of repellents (Tsang et al, 1973; Koshland, 1980). There is no one-to-one correspondence of every compound that serves as an attractant or repellent with a rational explanation. However, positive chemotaxis becomes a futile event if the compound is not transported and utilized. In the case of C. acetobutylicum, chemoattraction seems to offer the organism a competitive advantage in natural environments. Sugar taxis must allow the cells to compete effectively, for source of energy, against other organisms found in their natural habitat, such as

soil where spatial or temporal chemical gradients occur. Such selective advantage has been suggested for several species of Rhizobium (Parke et al, 1985) and Pseudomonas (Kennedy and Lawless, 1985).

The dual response to the acids (attraction towards the undissociated form and repulsion from the dissociated form) is an interesting result, and differs from that observed in E. coli. In the latter organism, weak acids such as acetic acid and butyric acid are repellents, and this repellent effect is increased at lower pH values, that is, there is no attractant effect of the undissociated acid (Kihara and Macnab, 1981; Rapaske and Adler, 1981). This apparent contradiction between the two bacteria requires an explanation.

It is known that weak acids are transported into the cell in the undissociated form (Thauer et al, 1977; Gottwald and Gottschalk, 1985; Herrero et al, 1985). Due to the more alkaline pH within the cell, dissociation occurs in the cytoplasm, releasing a proton. This lowers the intracellular pH, and if the "proton receptor" hypothesis described above is correct, the cell would exhibit negative chemotaxis. On this basis, it would be easy to understand why the repellent effect of weak acids is increased at lower pH values, in E. coli. The problem, then, is to explain why acids are attractants in C. acetobutylicum. One possible explanation involves describing, and interpreting, the present data in terms of the concentration of the undissociated acid, and neglecting the anion, on the basis that weak acids are not transported into the cell in the charged form.

Thus, at low concentrations, weak (undissociated) acids are repellents in C. acetobutylicum, as they are in E. coli. At high concentrations, however (such as the threshold concentrations given in Table 3.5), these acids become attractants. Possibly, this is related to the "trigger" concentration of undissociated acetic and/or butyric acid, which is required for the initiation of solventogenesis (Monot et al, 1983, 1984; Fond et al, 1985; Terraciano and Kashket, 1986). At these concentrations, the acids become further metabolized within the cell, and may thus be compared with other attractants such as sugars. Terraciano and Kashket (1986) observed that the switch from acid to solvent production in C. acetobutylicum occurred when the concentration of undissociated butyric acid inside the cell, (which was equal to that in the extracellular medium), reached 1.3×10^{-2} M. This is in the same region as the threshold concentration for chemoattraction to butyric acid which is 9×10^{-2}M (Table 3.5).

Thus, although acetate and butyrate may not be used directly as nutrients in C. acetobutylicum, their uptake has been suggested as being a detoxification mechanism, by metabolizing them to solvents (Costa, 1981; Bahl et al, 1982a; George and Chen, 1983). On this basis, then, an increased intracellular concentration of undissociated acid, may be the signal for initiation of solventogenesis. Whichever it is, a relationship between solventogenesis and motility/chemotaxis is apparent.

A further relationship may involve the mechanism by which the cell prevents the intracellular pH value from decreasing too far. Extrusion

of protons is via the membrane-bound ATPase complex (Hellingwerf and Konings, 1980; Herrero et al, 1985; Jones and Woods, 1986), and this allows generation of a proton motive force, which is the driving force for many energy - requiring metabolic processes such as flagellar synthesis, flagellar rotation and chemotaxis (Adler, 1975; Hellingwerf and Konings, 1980).

Chemorepulsion from toxic solvent products during the onset of solventogenesis may present a survival mechanism since butanol, acetone and ethanol have been shown to inhibit growth of C. acetobutylicum (Moreira et al, 1981; Lin and Blaschek, 1982; Costa and Moreira, 1983; Ounine et al, 1985).

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF N0N-MOTILE MUTANT OF *CLOSTRIDIUM ACETOBUTYLICUM* P262

4.1 INTRODUCTION

One approach that has been used towards understanding the possible relationship between solvent production and morphological changes in C. acetobutylicum is to obtain mutants and study their specific characteristics. Isolation of sporulation mutants (*spo*$^-$) of C. acetobutylicum P262 has shown that the ability to sporulate is not required for the development of a normal solventogenic phase (Jones et al, 1982; Long et al, 1984b). A relationship between production of clostridial forms and solventogenesis has been identified through the use of *cls*$^-$ mutants which were unable to produce solvents, spores, clostridial forms, granulose or capsules.

Reysenbach et al (1986) utilized sporulating and non-sporulating granulose-deficient mutants (*grn*$^-$) of C. acetobutylicum P262 to distinguish between the regulation of granulose accumulation, solvent production and sporulation. Results indicated that granulose accumulation was not essential for sporulation, and that the absence of granulose production and sporulation ability did not affect the production of normal levels of solvents.

This chapter describes the isolation of a non-motile mutant of C. acetobutylicum P262, in an attempt to establish the relationship between vigorous fermentation and motile cultures of C. acetobutylicum.

4.2 MATERIALS AND METHODS

4.2.1 Culture Media

The composition of Cooked Meat Medium (CMMG), Semi-Synthetic Medium (SSM) and Reinforced Clostridial Agar (RCA) used in these experiments are described in Section 2.2. Extra agar was added to RCA to increase the concentration to 15 g/l.

The composition of other media used are given below:
Semi-Solid Tryptone Agar (TA) used for swarm plates

	Concentration (g/l)
Tryptone	10.0
NaCl	5.0
Glucose	5.0
Sodium thioglycollate	3.0
Agar	0.5

Nutrient Broth Yeast Extract Glucose Agar (NYG) used for characterization of colonies

	Concentration (g/l)
Difco nutrient broth	8.0

Yeast extract	1.0
Glucose	4.0
Agar	3.0 or 15.0

Tryptone Broth (TB) used as diluent

	Concentration (g/l)
Tryptone	5.0
Sodium thioglycollate	0.5

4.2.2 Chemicals

All chemicals used in these experiments were of analytical grade. Ethyl methane sulfonate was obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.).

4.2.3 Anaerobic Manipulation

All anaerobic manipulations were carried out in the anaerobic chamber as described in Section 3.2.4.

4.2.4 Mutagenesis

The stock spore suspension was heat shocked as described in Section 2.5, and the culture was incubated in anaerobic jars until highly motile

cells were present. This culture (0.05 ml) was transferred to 95 ml of SSM and then incubated at 34°C for 24 h. To obtain clostridial cells for mutagenesis, the culture was further incubated at 25°C for 24 h. The clostridial cells were harvested by centrifugation at 4000 x g for 5 min using bottles previously flushed with sterile nitrogen gas. The cells were resuspended in deoxygenated chemotaxis medium consisting of phosphate buffer and thioglycollate, pH 5.0 (Section 3.2.1).

Mutagenesis was carried out by treating the cells with a final concentration of 5.0% (v/v) ethyl methane sulfonate (EMS) and incubating at 37°C inside the anaerobic chamber for 45 min. The cells were then diluted in TB and spread plated on RCA. The cultures were incubated at 34°C for 4 days.

4.2.5 Isolation of Non-Motile Mutants

Pinpoint colonies from cultures mutagenized with EMS were transferred to CMMG and incubated at 37°C for 48 h. Isolates were then purified by streaking onto RCA plates. Well-isolated colonies were picked using sterile toothpicks and transferred to CMMG. The cultures were incubated at 37°C for 48 h.

In a modification of the procedure used by Armstrong et al (1967), the isolates were spotted onto swarm plates consisting of soft Tryptone Agar, and incubated anaerobically at 34°C for 24-48 h. Isolates were screened for the absence of minimal swarming by repeated spotting from the center of the colonies to fresh swarm plates. Respotting was

performed up to four times, until the isolates formed colonies of an approximate diameter of 1 mm.

All mutants isolated by the procedure were grown in CMMG to ensure that their growth was comparable to that of the parent strain.

4.2.6 Maintenance of Mutant

Spores of the non-motile mutant were produced from slopes of RCA, and kept in distilled water at 4°C as described in Section 2.6.

4.2.7 Characterization of Mutant

All experiments performed to characterize the non-motile mutant were carried out in parallel with the parent P262 strain.

4.2.7.1 Culture Conditions

The parent and mutant strains were grown using the following procedure. For the parent strain, all transfers were done while the cells were highly motile as observed microscopically.

a) Stock spore suspension was heat shocked in CMMG as described in Section 2.5. The culture was incubated anaerobically at 37°C for 16 - 19 h.

b) A range of 0.01 ml to 0.05 ml of the culture was transferred to 20 ml SSM and incubated at 34°C until smooth swimming single or paired cells were obtained (17 - 19 h, OD_{650} approximately 1.1).

c) One ml of the culture was then transferred to 99 ml of SSM. The culture was incubated at 34°C.

4.2.7.2 Presence of Flagella

The presence of flagella was demonstrated using transmission electron microscopy. Cultures were centrifuged at 3000 x g for 5 min, the supernatant was discarded, and the cells were washed with deionized water. The cells were strained using 1% (v/v) potassium phosphotungstic acid. Bovine serum albumin (0.5%) was added to the specimen suspension as a spreading agent. A drop of the specimen was placed on a copper grid (200 mesh) with a Formvar support film. The drop was withdrawn using filter paper to leave a thin film which dried at room temperature in no longer than 30 seconds. The specimens were examined in a Philips 201 C electron microscope with double condenser illumination at an accelerating voltage of 60 kv.

4.2.7.3 Morphological Aspects

The production of clostridial forms was observed using a phase-bright microscope. Counts were obtained as described for total cell counts in Section 2.9.2.

The presence of forespores was determined using the method of Smith and Ellner (1956).

The formation of spores was determined either by phase-bright microscopy or by staining a 1 cm^2 smear with Bartholomew and Mittwer's malachite green for a Breed count (Harrigan and McCance, 1966).

4.2.7.4 Colony Characteristics

Distinction between the parent and mutant strains by swarm and colony morphologies was determined by cultivating the organisms in both hard and semi-soft culture media. The following media were used:

a) Reinforced Clostridial Agar, RCA

b) Nutrient Broth Yeast Extract Agar, NYG

c) Tryptone Agar, TA

The composition of the media are described in Section 4.2.1. Agar at a concentration of 15.0 g/l or 3.0 g/l was used for hard or semi-soft agar, respectively.

4.2.7.5 Heat Sensitivity of Spores

Spores of parent and mutant strains from 72 h cultures in SSM were harvested by centrifugation at 16,000 x g for 30 min. The spores were resuspended in sterile distiled water.

Heat treatments were carried out by transferring 3 ml of spore suspension to a Kimax glass tube (16 x 100 mm). A set of 10 tubes, closed by screw caps, was heated in a stirred water bath at 75°C or 90°C. After the prescribed temperature / time treatment, the tubes were cooled in iced-water for 1 min.

Dilutions were made in 0.01 M phosphate buffer, pH 7.0, before plating the survivors in RCA. The cultures were incubated anaerobically at 34°C.

4.2.7.6 Solvent-producing Ability

Fermentations were performed in 100 ml cultures in SSM. Samples were taken at appropriate time intervals, and analyzed for acids, solvents and sugars as described in Section 2.9.3 and Section 2.9.4, respectively.

4.3 RESULTS

4.3.1 Mutagenesis and Isolation of Non-Motile Mutant

Several experiments were performed to develop a procedure by which mutation in C. acetobutylicum P262 grown in conditions described in Section 4.2.7.1 could be induced using EMS. At a final concentration of 2.5% (v/v) EMS, and using a mid-exponential phase culture, no reduction in numbers of viable cells was observed.

During the attempts to modify the procedure for mutagenesis, it was observed that no colonies could be isolated on different media tested if cells were harvested from a culture consisting of highly motile, early-exponential phase cells. This result suggested that a mutation program with C. acetobutylicum P262 should involve the clostridial stage and not the vegetative stage. Hence, cultures were grown for 48 h to obtain mainly clostridial forms.

In a previous study, Lemmel (1985) performed the mutagenesis by adding EMS directly to culture media. In the present work, to further increase the mutagenic effect of EMS, the clostridial forms were washed and resuspended in phosphate buffer, pH 5.0 (Section 3 2.1), and EMS was added to a final concentration of 5.0% (v/v). Although very little kill (less than 10%) was obtained, despite exposure to EMS for 1 h, an exposure time of 45 min was used (Bowring and Morris, 1985) in subsequent experiments to achieve some induction of mutation.

To obtain non-motile mutants, pinpoint colonies from mutagenized cultures were isolated. The isolates were screened by the swarm plate technique (Section 4.2.5) modified from the procedure of Armstrong et al (1967). If motile chemotactic bacteria are deposited on a semi-solid medium containing an energy source, the bacteria will migrate from the site of inoculation in one or more rings. Wild-type E. coli has been observed to produce three or more rings on semi-solid tryptone agar (TA). The rings are interpreted as due to consumption of glucose, followed by amino acids. Non-chemotactic mutants were observed as not capable of forming rings or developing large swarms, while non-

motile mutants did not swarm, hence, formed compact colonies, usually with defined boundaries.

The diameter of the colonies as the isolates were repeatedly spotted onto the soft TA were measured and compared with that of the parent strain. The size of the swarm of the parent strain was typically about 75 mm. There were 57 isolates which consistently failed to swarm, and colony size did not increase to greater than 1.0 mm diameter. However, after microscopic examination only 1 isolate did not show any motility when grown in CMMG. This non-motile mutant was designated as isolate NG 12.

Mutant strain NG 12 was characterized for the following properties: presence of flagella, morphological changes during growth, colony characteristics, heat sensitivity of spores, and solvent-producing ability.

4.3.2 Presence of Flagella

Flagella were negatively stained, using 1.0% potassium phosphotungstate, for electron microscopy. Electron micrographs showed that the parent strain P262 has peritrichous flagellation (Fig 4.1). Although the exact number and length of the flagella were difficult to ascertain, it is apparent that there are more than 16 flagella arranged on the sides and ends of each cell. The length seems to be not much longer than the entire length of the cell.

The mutant strain NG 12 also possessed flagella (Fig 4.2). Although no chemical analysis was performed on the flagella, microscopic

Fig 4.1 Electronmicrographs of the parent strain P262 showing the flagella. (a), x 7,800; (b), x 9,000.

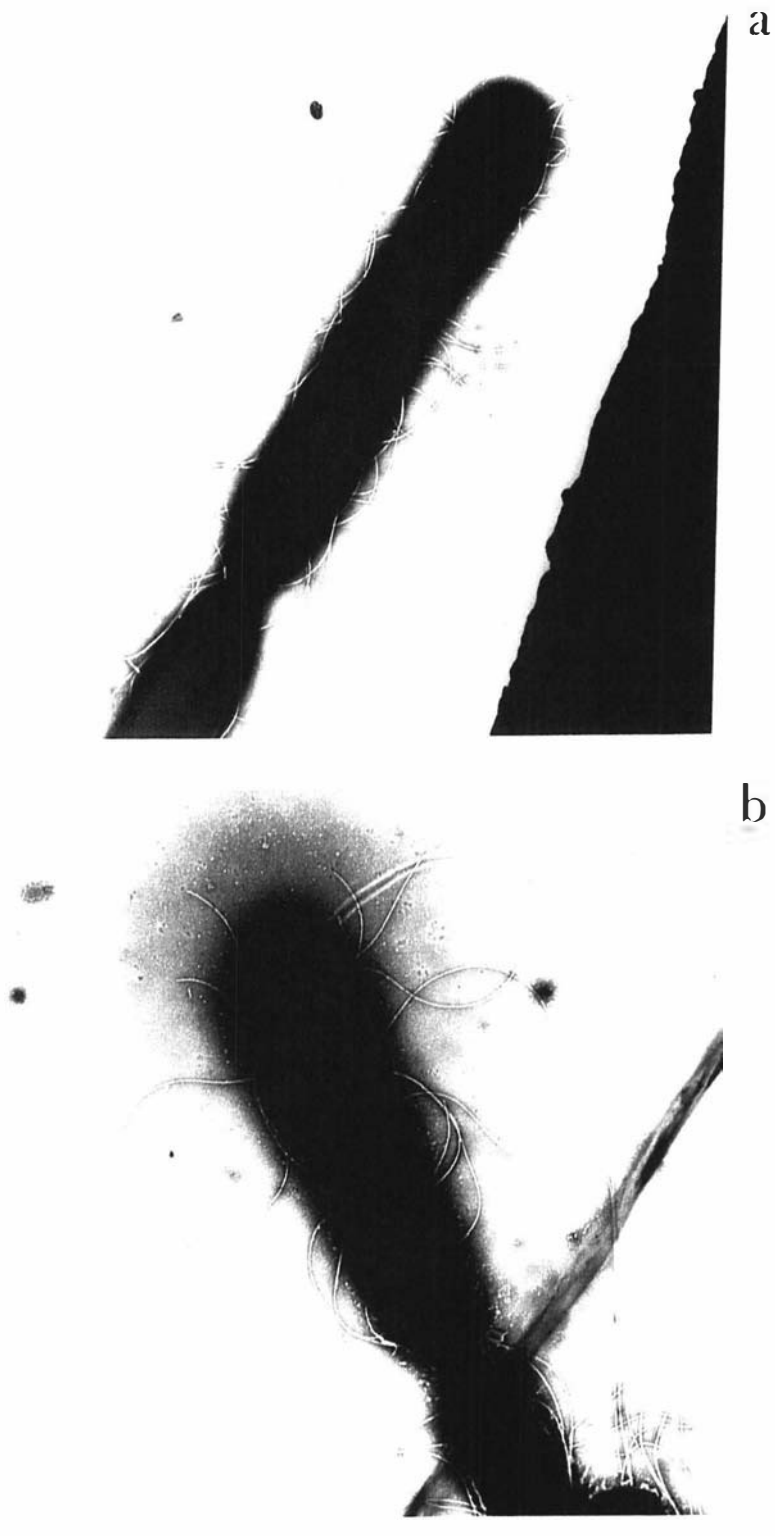


Fig 4.2 Electronmicrographs of the mutant strain NG 12 showing the flagella. (a), x 7,800; (b), x 9,000.

examination revealed that those of the mutant complement, i.e. resemble, those of the parent strain in all respects.

This type of mutant, therefore, can be classified as a paralyzed mutant (*mot*$^-$ mutant). They possessed flagella that look normal, but are not capable of motility (Armstrong and Adler, 1967).

4.3.3 Cellular Morphology

The morphological changes associated with growth of NG 12 (Fig 4.4) in SSM were determined and compared with those of the parent strain (Fig. 4.3). NG 12 showed no detectable motility while the parent strain demonstrated running and tumbling responses similar to those observed in whey permeate (Section 3.3.1). Within 3 h of inoculation, P262 exhibited swimming in straight long lines (runs). Some of the cells started to tumble after 5 h, and some cells remained motile for up to 19 h after inoculation. The decrease in motility coincided with the cessation of active growth (between 8-13 h).

In all other aspects of morphology, NG 12 and P262 were indistinguishable. During exponential growth, the cells appeared as phase-dark rods which were actively dividing, thus forming long chains of cells. Cells started to break off the chain after 7-9 h, and started to develop a swollen configuration. This marked the formation of early clostridial stage. After 10 h, the majority of the cells (> 80%) had assumed the swollen, cigar-shaped clostridial form.

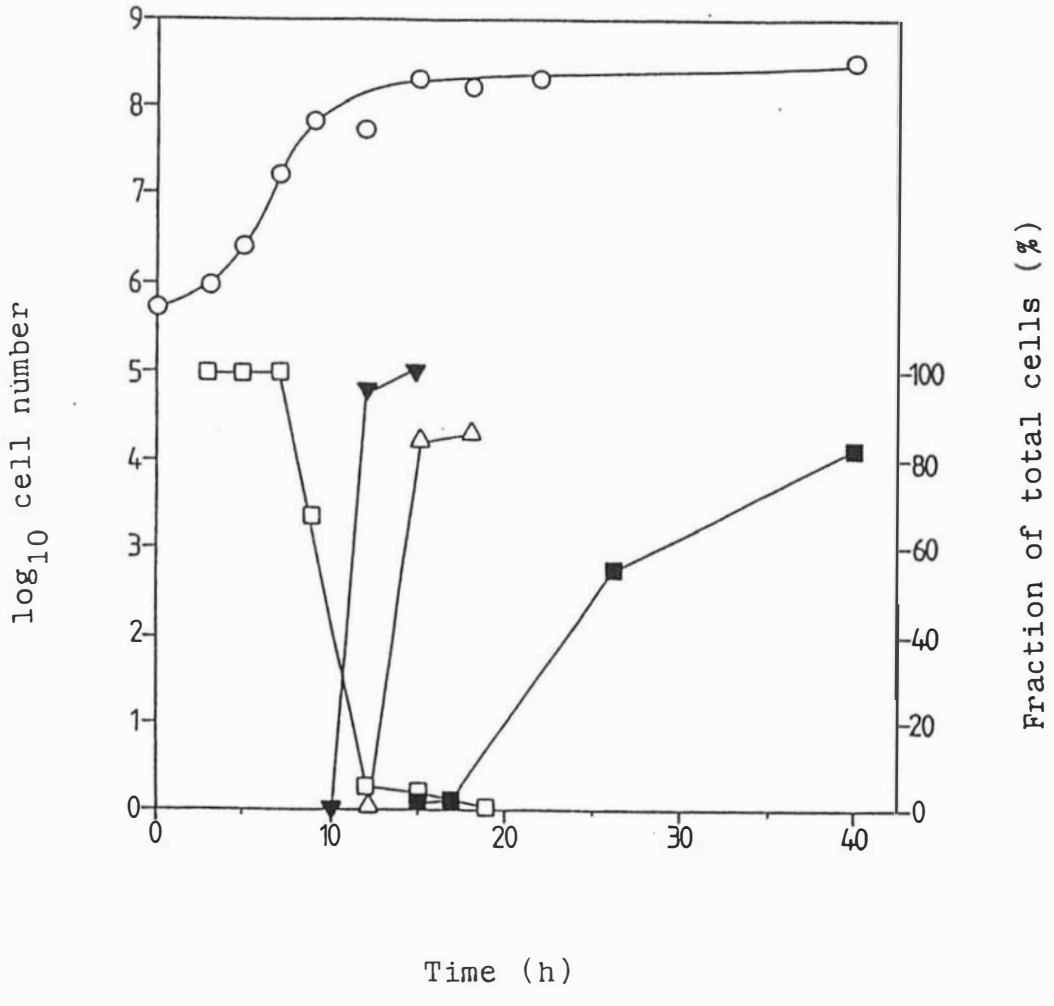


Fig 4.3 Morphological changes in C. acetobutylicum P262 grown in SSM. ○, log total cell number; △, % clostridial forms; □, % motile cells ▼, % forespores; ■, % spores.

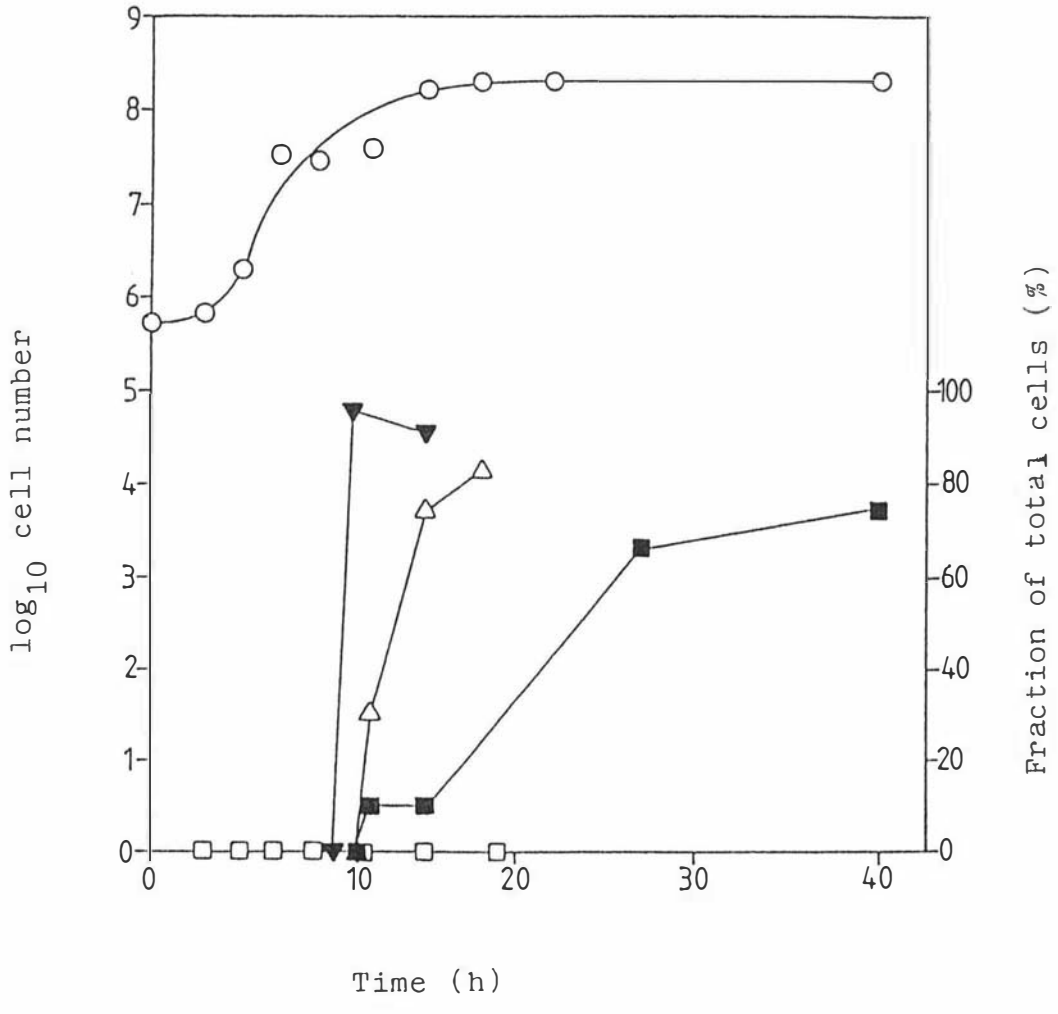


Fig 4.4 Morphological changes in mutant NG 12 grown in SSM. ○, log total cell number; △, % clostridial forms; □, % motile cells; ▼, % forespores; ■, % spores.

NG 12 formed forespores to the same extent as did P262. The presence of forespore septa was detected shortly before clostridial formation. By 10 h, almost all cells had formed forespores, and these eventually developed into mature spores. After 40 h, the extent of sporulation in the mutant strain did not differ markedly from that of the parent strain (74% and 82%, respectively).

4.3.4 Cultural Characteristics

The distinguishing properties of the parent P262 and mutant NG 12 were examined in agar and broth cultures.

4.3.4.1 Colony Morphology

Comparison of colony growth of the parent and mutant strains was performed after 5 days of incubation in three different media - RCA, NYG and TA. In addition to solid media containing a typical agar concentration (15.0 g/l), semi-solid (soft) media, containing agar at 3.0 g/l, were prepared to demonstrate any swarm morphology.

The parent P262 swarmed in both hard and soft media. Fig 4.5 a, b, c show the colony growth on soft RCA, NYG, and TA, respectively. The bacteria migrated from the point of inoculation toward the sides of the plates and did not leave a dense origin. The size of the swarm in hard media ranged from 20 mm to 50 mm in diameter (Table 4.1). In soft media, the parent formed larger swarms (approximately 80 mm) which almost covered the entire surface of the plates. Colonies in RCA

and NYG had a band of outgrowth which comprised their undulating margins. The surface of the colony was smooth in RCA and granular in NYG.

The chemotactic behaviour of the parent was well demonstrated in soft TA, where two morphologically distinct rings of colony were formed (Fig 4.5 c). The bacteria moved from the site of inoculation to about one-third of the plate forming an inner, granular ring. The outer ring was radially striated with edges which appeared powdery. The size of the swarm measured from 40-80 mm. For comparison, in E. coli, one or more rings were reported to be formed on semi-solid TA. When glucose served as the energy source, one ring formed where the cells consumed all the glucose as they traveled. When tryptone served as the energy source, three or more rings were formed according to the amino acids consumed (Armstrong et al, 1967). Swarming, particularly the formation of the outer ring, in the parent strain may not only be an indication of chemotaxis toward nutrients (although clostridia are known to be proteolytic, Smith and Hobbs, 1974) but also of chemotaxis away from metabolic end-products (Section 3.3.5). In Proteus, swarming has been suggested as a negatively chemotactic response induced by toxins formed during growth of the culture (Hoeniger, 1964).

The mutant strain was easily distinguishable from the parental strain as it expectedly failed to swarm even in soft media (Fig 4.6 a, b, c). The size of the colonies on the semi-solid agar did not differ much from those on the hard agar (Table 4.1). The mutant formed the same colony margin and texture in RCA and NYG as did the parent. On soft TA (Fig 4.6 c), NG 12 formed a small compact colony with an entire

(smooth) edge, in contrast to the concentric growth of P262 on the same medium (Fig 4.5.c).

Microscopic examination revealed that cells from colonies of the mutant were indistinguishable from those of the parent strain. Cells from the center of the colonies on RCA and NYG showed high number of mature spores. Cells obtained from the outgrowth contained a mixture of mostly late clostridial cells (containing forespores) and some mature spores. No spores were observed from cells grown on TA medium.

Spontaneous reversion to motile cells was not observed with NG 12.

A summary of the colonial characteristics is given in Table 4.1.

4.3.4.2 Growth in Broth

Another distinguishing cultural characteristic of the parent and mutant strains was the growth in broth (liquid medium). After 8 to 10 h of growth in SSM, the parent P262 produced uniform turbidity in the medium. In contrast, the mutant NG 12 formed a slight to moderate amount of granular sediment. The bulk of the broth was otherwise clear (Fig 4.7). However, upon further growth, vigorous gassing occurred (about 14 h) and NG12 cultures became as turbid as those of P262.

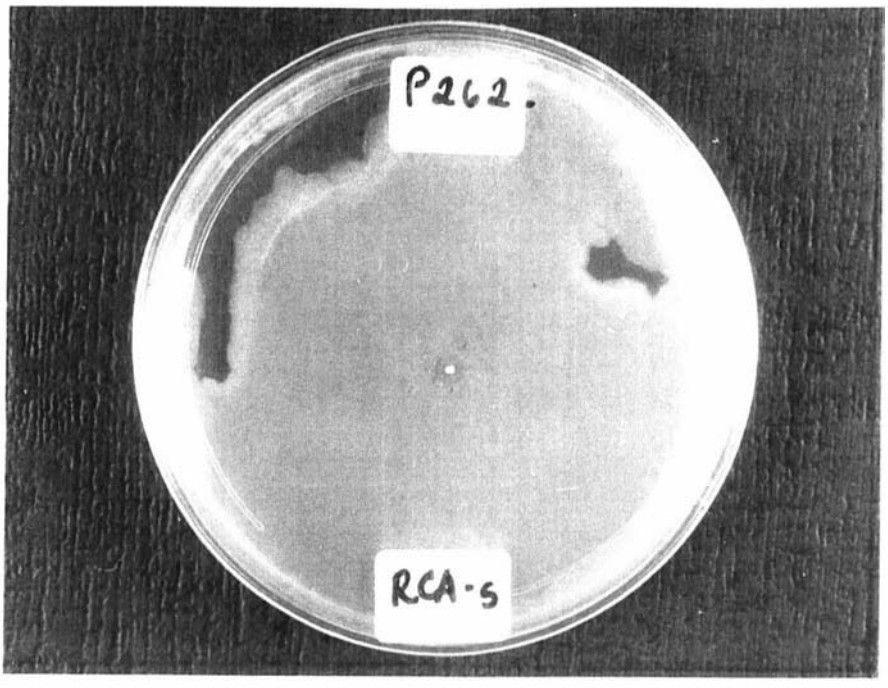


Fig 4.5 a Colony morphology of C. acetobutylicum P262 on soft RCA.

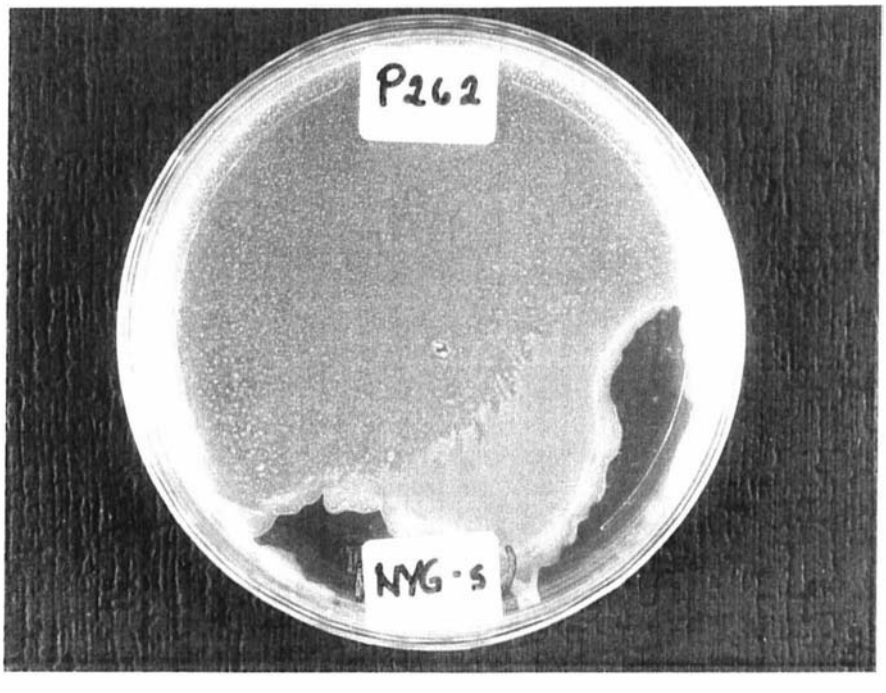


Fig 4.5 b Colony morphology of C. acetobutylicum P262 on soft NYG.

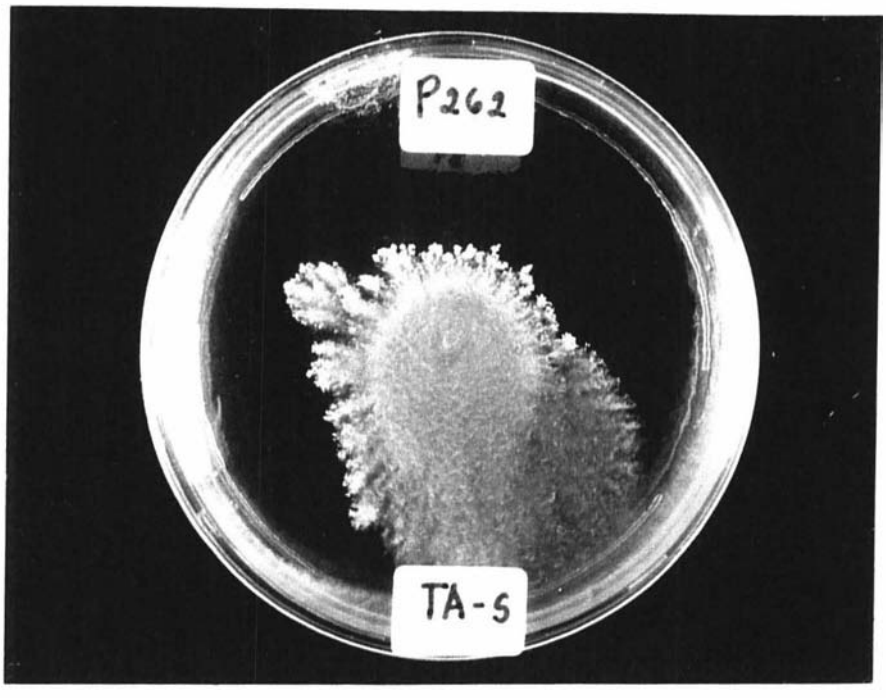


Fig 4.5 c Colony morphology of C. acetobutylicum P262 on soft TA.

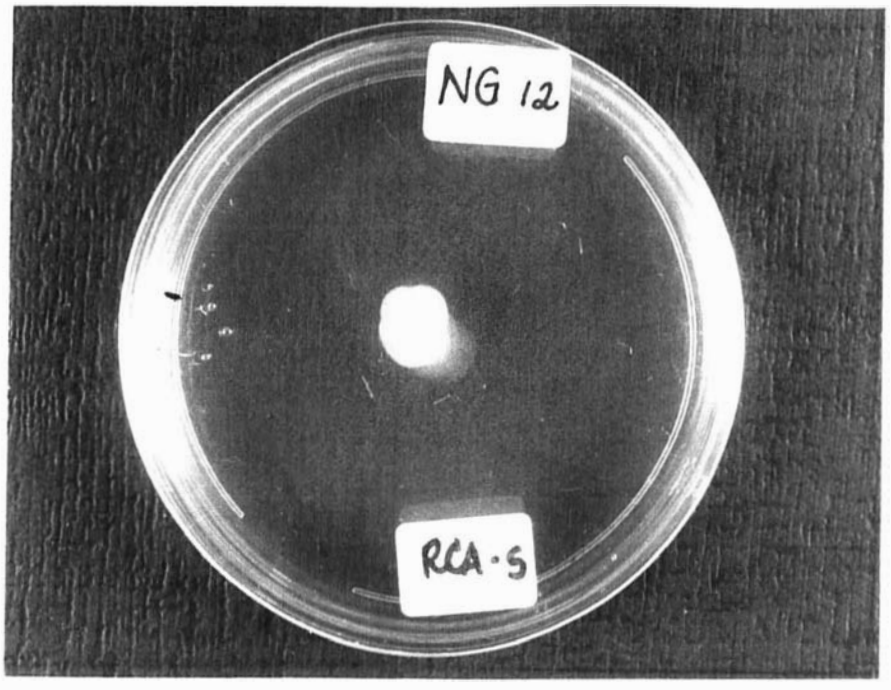


Fig 4.6 a Colony morphology of mutant NG 12 on soft RCA.

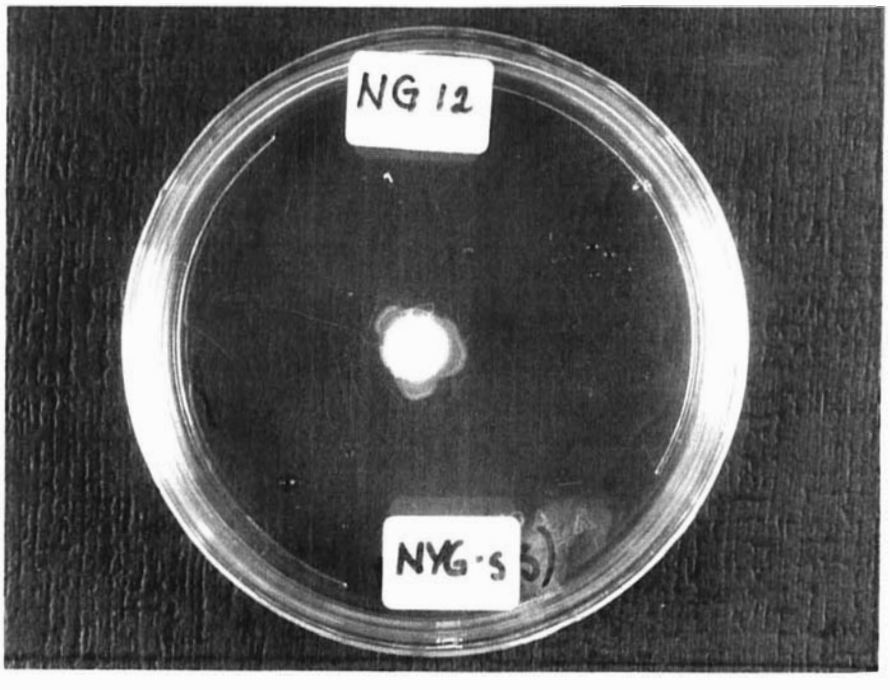


Fig 4.6 b Colony morphology of mutant NG 12 on soft NYG.

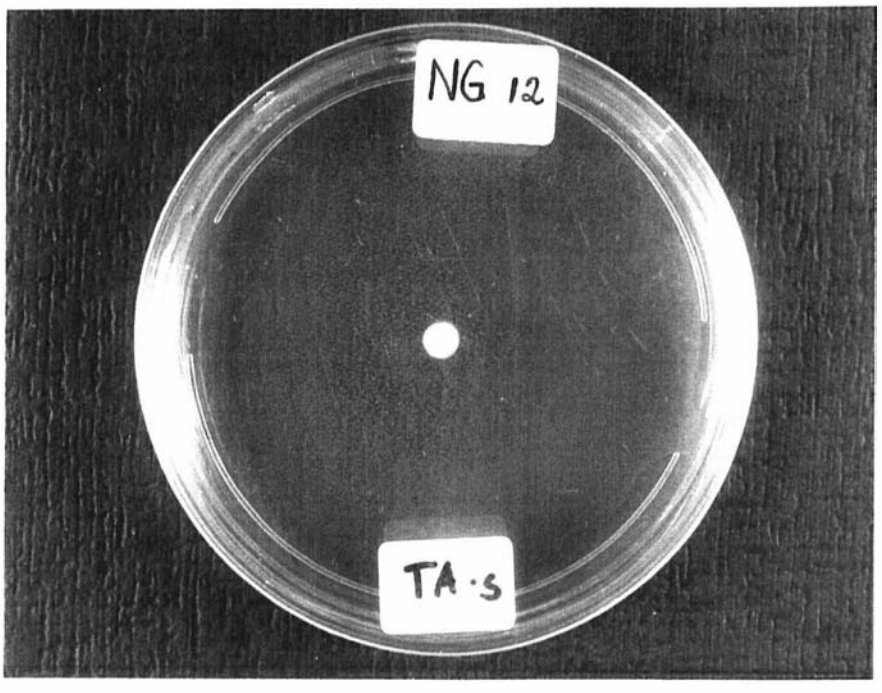


Fig 4.6 c Colony morphology of mutant NG 12 on soft TA.

Table 4.1 Characteristics of colonies of the parent strain P262 and mutant strain NG 12.

Medium	Colony Morphology	
	Parent P262	Mutant NG 12
RCA (hard)	Diameter, 40-50 mm; smooth surface, undulate edge	Diameter, 11-12 mm; smooth surface, undulate edge
NYG (hard)	Diameter, 20-30 mm; granular surface, undulate edge	Diameter, 12-14 mm; granular surface, undulate edge
TA (hard)	Diameter, 20-30 mm; rhizoid inner ring and radially striated outer ring	Diameter, 10-11 mm; smooth surface, entire edge
RCA (soft)	Diameter, 75-80 mm; smooth surface, undulate edge	Diameter, 18-20 mm; smooth surface; undulate edge
NYG (soft)	Diameter, 75-80 mm; granular surface, undulate edge	Diameter, 11-14 mm; granular surface, undulate edge
TA (soft)	Diameter, 40-80 mm; rhizoid inner ring and radially striated outer ring	Diameter, 10-14 mm; smooth surface, entire edge

Fig 4.7 Sediment formation of NG 12 (left) and turbidity of P262 (right) after 8h growth in SSM.

4.3.5 Heat Sensitivity of Spores

Heat treatment of spores can lead either to destruction or activation of germination, i.e. sublethal heat can induce dormant spores to germinate. Usually temperatures above 100°C are required to kill spores. However, spores of Clostridium species have been observed to be sensitive at temperatures below 100°C (Russel, 1982).

The spores of the parent strain P262 and mutant NG12 were subjected to heat treatments at 75°C and 90°C. D-values (decimal reduction time) were computed from the survivor curves (90°C) using linear regression analysis.

Results show that heating at 75°C activated the spores of both organisms to a similar extent. Destruction of spores was not observed even after heating for 30 min (Table 4.2).

The time-survival curves for P262 and NG 12 at 90°C are shown in Fig 4.8. In both strains, there was no initial increase in colony forming units as an effect of heat activation. Rather, the spores appeared to be destroyed relatively quickly. The D-values were calculated as 1.4 min and 1.3 min for spores of P262 and of NG 12, respectively. It was concluded that the spores of P262 and NG 12 displayed similar heat resistance.

Table 4.2 The effect of heating at 75°C on the spores of the parent P262 and mutant NG 12.

Time of heating (min)	Number of survivors (cfu/ml)	
	P262	NG 12
0	3.0×10^5	5.0×10^5
3	5.2×10^6	4.0×10^6
5	$> 1 \times 10^7$	$> 1 \times 10^7$
7	$> 1 \times 10^7$	$> 1 \times 10^7$
10	$> 1 \times 10^7$	$> 1 \times 10^7$
15	$> 1 \times 10^7$	$> 1 \times 10^7$
20	$> 1 \times 10^7$	$> 1 \times 10^7$
25	$> 1 \times 10^7$	$> 1 \times 10^7$
30	$> 1 \times 10^7$	$> 1 \times 10^7$

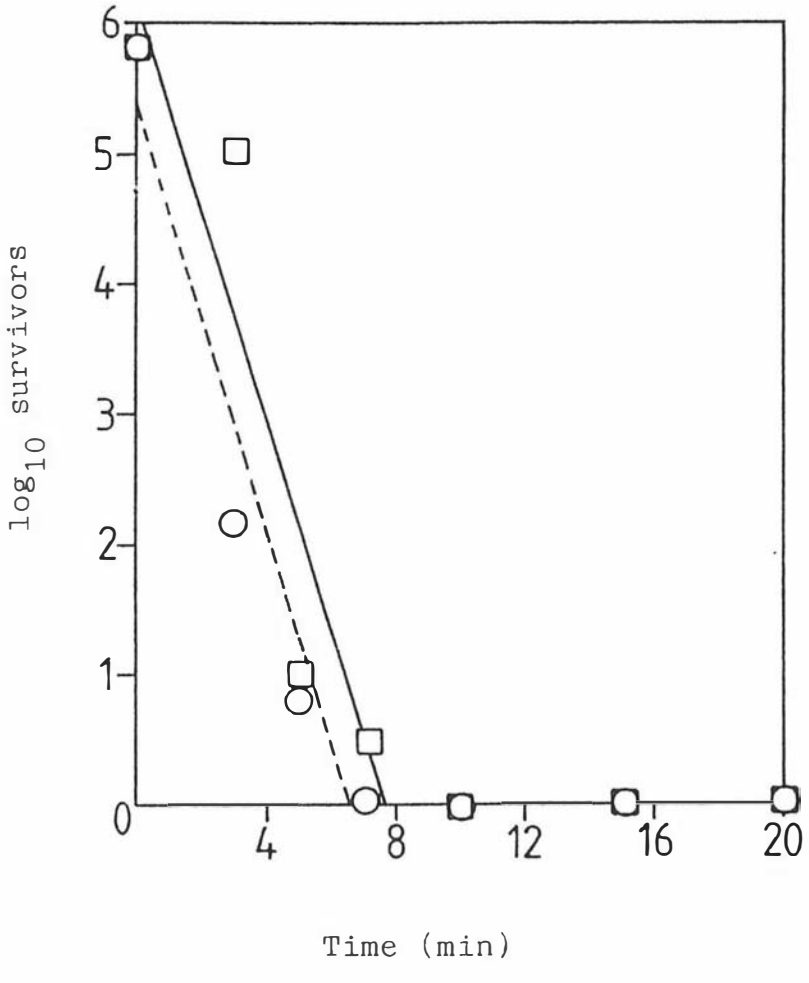


Fig 4.8 Heat resistance of spores of P262 (□) and NG12 (○) at 90°C.

1.3.6 Solvent Production

The ability of the non-motile mutant NG12 to produce solvents from the semi-synthetic medium containing glucose was investigated and compared with that of the parent strain P262. The fermentations were performed on a 100-ml scale at 34°C. Inoculation was done at a time when P262 showed mostly running cells, and NG 12 cells were morphologically similar to P262 cells. The fermentation profiles for P262 and NG 12 are shown in Fig. 4.9 and Fig 4.10, respectively, while Table 4.3 provides a summary of the data.

The growth rate of the mutant was lower than that of the parent. However, towards the completion of growth, the mutant produced the same number of cells as did the parent (approx. 10^8 cells/ml). The solvent production of the mutant was also observed to be lower than that of the parent. As the process proceeded, the solvent production rate of the mutant decreased more rapidly than that of the parent, and production ceased after 72 h. In contrast, solvent production by the parent continued until after 100 h of fermentation.

The parent strain produced 18.2 g/l of total solvents after 120 h of fermentation, while the non-motile strain was able to produce solvents at a lower total concentration of 7.1 g/l. The maximum butanol concentration observed for the mutant was 5.6 g/l which was less than 50% of that observed with its parent strain. Sugar utilization and butanol yield were poorer for NG 12 (56.6% and 0.20, respectively) than for P262 (86.4% and 0.28, respectively). The overall fermenter productivity obtained for the mutant was 50% lower than that for the

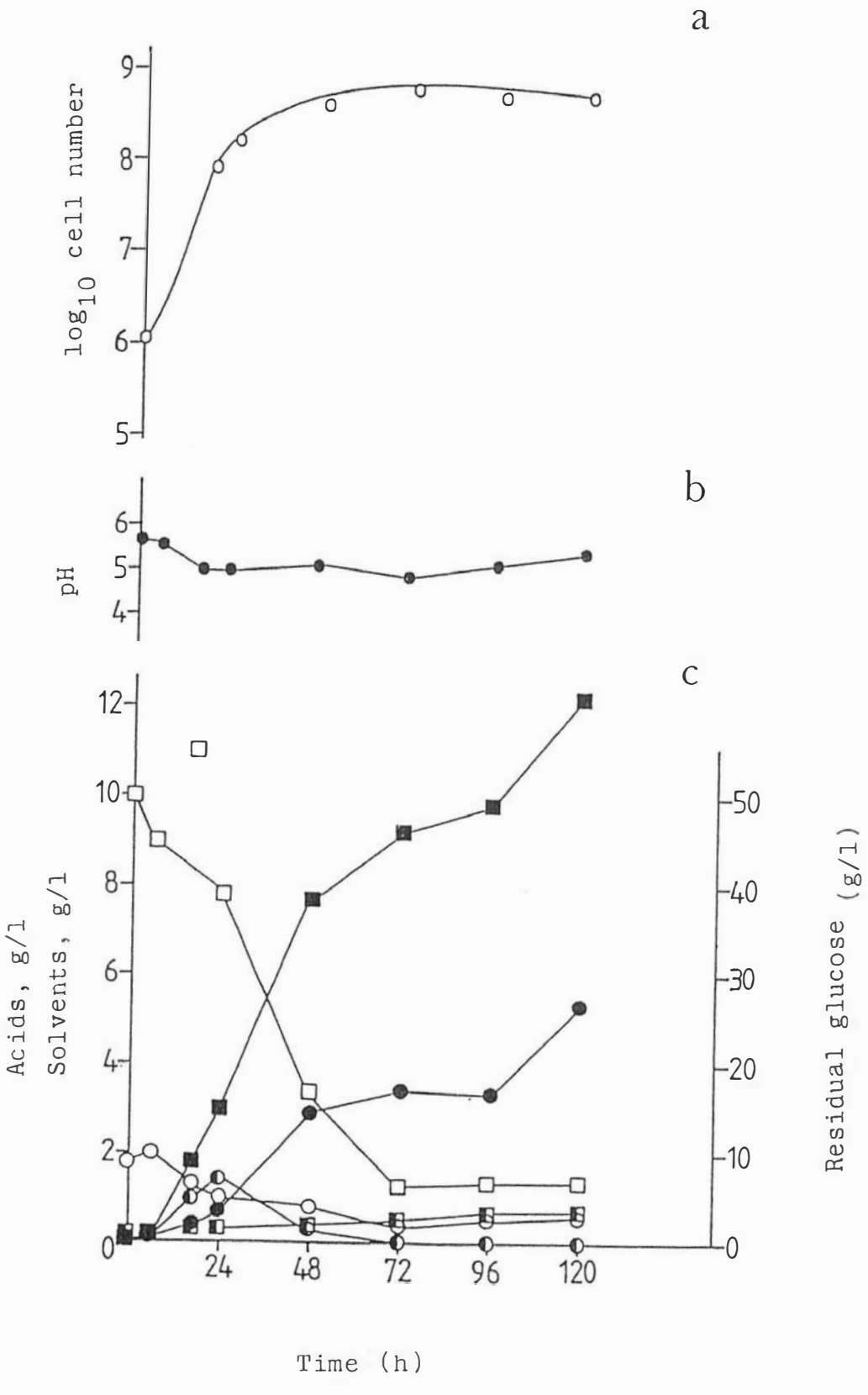


Fig 4.9 Fermentation profile of the parent strain C. acetobutylicum P262 in Semi-Synthetic Medium. (a) ○, log cell number; (b) ●, pH; (c) ■, butanol; ◧, ethanol; ●, acetone; □, glucose; ○, acetic acid; ◐, butyric acid.

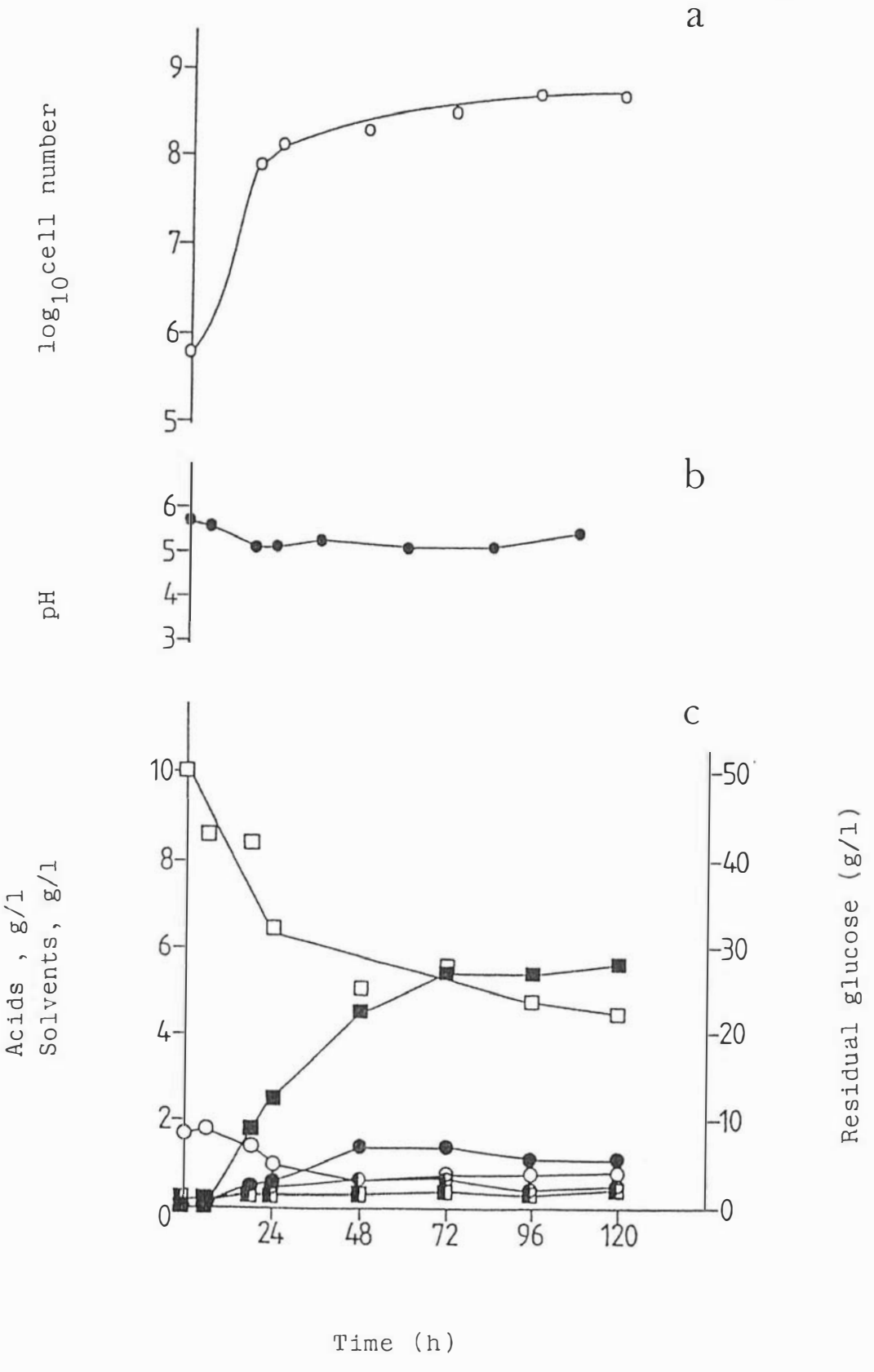


Fig 4.10 Fermentation profile of the non-motile mutant NG 12 in Semi-Synthetic Medium. (a)○ , log cell number; (b) ● , pH; (c)□ , butanol; ◧ , ethanol; ● , acetone; □ , glucose; ○ , acetic acid; ◐ , butyric acid.

Table 4.3 Fermentation parameters for parent strain P262 and mutant strain NG 12, after 120 h fermentation.

	P262	NG 12
μ	0.279	0.240
initial pH	5.67	5.69
final pH	5.42	5.45
ethanol, g/l	0.7	0.4
acetone, g/l	5.3	1.1
butanol, g/l	12.2	5.6
acetic acid, g/l	0.6	0.8
butyric acid, g/l	0	0.5
glucose utilized, g/l	43.2	28.3
yield (butanol), g butanol / g glucose utilized	0.28	0.20
initial rate of butanol production, g/l.h	0.188	0.139
overall butanol productivity (120 h) g/l.h	0.1	0.05

parent although comparable amounts of biomass were produced by each organism.

4.4 DISCUSSION

A non-motile mutant of C. acetobutylicum P262 has been isolated. This mutant is morphologically indistinguishable from the motile parent strain. It possesses flagella in typical number and shape as the parent, and is capable of forming clostridial stage and spores. The isolation of a non-motile mutant could provide a tool in establishing a relationship between motility and solventogenesis.

The non-motile strain was obtained by mutagenesis using EMS. Ethyl methane sulfonate has been used as the mutagen of choice for the induction and isolation of physiological mutants of C. acetobutylicum (Jones and Woods, 1986). EMS is a directly acting alkylating agent, which reacts with a nucleotide so as to generate a directly mispairing analogue. EMS induces predominantly base transition (the replacement of one pyrimidine by the other or of one purine by the other). It acts by ethylating G (guanine) on the O-6 position which then mispairs with T (thymine). This results in transition from G-C to A-T (Springer and Kusmierek, 1982).

The difficulties encountered while optimizing conditions for mutagenesis of C. acetobutylicum confirm previous observations that mutants of obligate anaerobes are difficult to obtain (Awang et al, 1988). The mutation protocol in this study proved successful when it was developed in line with the recommendation of Jones et al (1982)

that mutation screening programs with strain P262 should involve the clostridial stage and not the vegetative cells. No specific mention of this observation was made with other strains of C. acetobutylicum, such as ATCC 824 and ATCC 39.236, in which mutagenesis was carried out using mid-exponential cells (Lemmel, 1985; Bowring and Morris, 1985). Such a recommendation should not be overlooked for a strain such as P262 due to its highly differentiated morphological stages (Jones et al, 1982; Long et al, 1984a).

The non-motile mutant is classified as a paralyzed mutant, that is, a mutant which although flagellated, is non-motile (Enomoto, 1966; Iino, 1977). The mutation may be a defect on the *mot* gene, which controls the locomotive function of flagella without affecting their overall structure. Studies on E. coli and S. typhimurium have identified that the defect in paralyzed mutants lies on any of the *mot* A, *mot* B, or *fla* A cistrons (Armstrong and Adler, 1967; Iino, 1977; Parkinson, 1981; Yamaguchi et al, 1986; Enomoto, 1966; Kuo and Koshland, 1986). The *mot* A and *mot* B cistrons give rise to a single phenotype (paralyzed), in contrast to the *fla* A cistron which is multifunctional and, therefore, gives rise to several types of mutants, namely flagellated, paralyzed or non-chemotactic (Yamaguchi et al 1986; Kuo and Koshland, 1986). The *mot* A and *mot* B proteins are found in the cytoplasmic membrane; the locations of *fla* A products are still unknown.

The genes mentioned are required for flagellar rotation. Studies are still in progress to define the exact roles of the *mot* genes. Dean et al (1984) have suggested that they control part of the intact motor complex which lies at the basal region of each flagellum. Ishihara et al

(1981) observed that the flagella of both a *mot*⁻ mutant and wild-type strain of S. typhimurium (in the presence of respiratory inhibition in the latter) could be rotated passively by applying an external rotary driving force generated by a flow of viscous fluid. However, *mot*⁻ mutants of B. subtilis remained non-motile after applying an artificial proton motive force (Matsuura et al, 1977) while in the wild type, motility which is inhibited by starvation of endogenous energy source could be reactivated by such means. These results suggest that the defect in *mot*⁻ mutants is not simply a loss of proton motive force. This is confirmed in the present work by the occurrence of relatively normal growth and acidogenic phase in the *mot*⁻ mutant, similar to those of the parent strain, demonstrating that a proton motive force can be typically generated in the mutant during the efflux of end products of metabolism and protons (Sections 3.4).

The flagella of *mot*- mutants seem to maintain their rotary freedom. Hence, non-motility of flagella on *mot*⁻ cells could be caused by a defect in the flagellar motor itself, namely, by a failure in the process that converts proton motive force into rotary driving force. (Ishihara et al, 1981).

The isolation of the *mot*⁻ mutant of C. acetobutylicum which produces a lower concentration of solvents than the motile parent strain has confirmed the relationship between motile, chemotactic cultures and solvent production. The *cls*⁻ mutants of strain P262 studied by other workers were unable to form the clostridial stage and did not produce granulose, capsules or spores. These mutants were unable to produce solvents, suggesting that these events are linked by some common regulatory mechanism (Jones et al, 1982; Long et al, 1984b). The

isolation of sporulation mutants blocked in granulose and capsule production, and that are capable of solvent production, indicates that solventogenesis, granulose accumulation and capsule formation are not sporulation - specific events. These pathways probably function independently of each other (Jones and Woods, 1986). In the present study, the *mot*$^-$ mutant was morphologically indistinguishable from the motile parent. It was able to produce the clostridial stage and sporulate to a similar extent as the parent, and the spores exhibited similar heat resistance. However, there is no evidence that motility is directly involved in the regulation of solvent production, that is, motility does not seem to be a trigger for solventogenesis, but is simply a behavioural response. This behaviour could provide a basis for a method for predicting solvent production during the ABE fermentation.

The lower level of solvent production in the *mot*$^-$ mutant could not be attributed to the difference in the heat resistance of spores used to start up a culture, as the spores of the *mot*$^-$ mutant showed the same sensitivity to heat as did the spores of the parent. This confirms the results obtained by Adler and Crow (1987) that the spores produced by solventogenic colonies were indistinguishable from those produced by less efficient solvent producing colonies on the basis of heat sensitivity. The heat shocking treatment, used in the inoculum development protocol for the mutant and parent strains, therefore, did not provide an advantage to one organism over the other.

Lower growth rate and initial rate of butanol production in the *mot*$^-$ mutant seem to account for its lower level of solvent production when compared to that of the parent. Such low rates may be due to the absence of motility and chemotactic response in the mutant such that the

mutant could not migrate towards source of energy, such as sugars, or towards the acids which could be detoxified by conversion to neutral solvents. In the previous chapter, the parent has been shown to respond positively in the presence of sugars and increasing concentration of undissociated acids. Furthermore, it appears that in the absence of motility and chemotaxis the mutant could not escape from the toxic effects of solvents. Hence, the *mot*$^-$ mutant seems to have a lower butanol tolerance than does the parent. The isolation of a non-motile mutant could provide a tool in further understanding the fundamental problem of end-product inhibition in the ABE fermentation.

CHAPTER 5

EFFECTS OF BUTANOL ON THE NON-MOTILE MUTANT OF *CLOSTRIDIUM ACETOBUTYLICUM:* A COMPARISON WITH THE PARENT STRAIN

5.1 INTRODUCTION

End-product inhibition has long been recognized as a limiting factor in attaining high productivity in many industrial fermentations (Herrero, 1983). Among the end-products of the ABE fermentation, butanol is the most toxic (Moreira et al, 1981; Lin and Blaschek, 1982; Costa and Moreira, 1983; Kuhn and Linden, 1986). Solvent production ceases when the concentration of butanol reaches about 13 g/l (Walton and Martin, 1979; Jones and Woods, 1986).

Butanol was found to inhibit cell growth by 50% when added at 7 to 13 g/l (Moreira et al, 1981; Lin and Blaschek, 1982; Costa and Moreira, 1983; Ou nine et al, 1985). There appears to be a threshold concentration (4.0 to 4.8 g/l) below which no growth inhibition occurs, and above which a linear decrease in growth rate is observed with increasing butanol concentration. Complete inhibition of growth results from the presence of butanol at 12 to 16 g/l. Butanol toxicity is not completely understood but, like other alcohols, it probably affects the cell membrane of C. acetobutylicum by disrupting its structure (chaotropic effect), and thereby inhibiting membrane-linked cellular functions. These functions include the maintenance of physical integrity of the cell, nutrient uptake and generation of proton motive

force. The mechanism by which the deleterious effects on these functions are brought about is still an enigma. Ingram and Buttke (1984) suggested that the short-chain alcohols (1 to 3 carbons), solidify or freeze the membrane while longer chain alcohols (4 to 9 carbons) e.g. butanol, fluidize the membrane.

Butanol has been shown to cause an increase in the fluidity of the cell membrane of C. acetobutylicum (Baer et al, 1987; Vollherbst-Schneck et al 1984). The response of the organism to this disruptive effect of butanol is an increase in the synthesis of saturated fatty acids at the expense of unsaturated fatty acids.

A decrease in glucose uptake in the presence of butanol has been reported (Moreira et al, 1981; Bowles and Ellefson, 1985; Hutkins and Kashket, 1986). In a recent study, Hutkins and Kashket (1986) demonstrated that glucose uptake in C. acetobutylicum is mediated by a phosphoenol-pyruvate (PEP) - dependent phosphotransferase system and that butanol does not inhibit this transport protein, but rather causes cellular PEP and 2-deoxyglucose-6-phosphate to leak out of the cell, resulting in growth inhibition.

An interior alkaline pH gradient has been shown to be essential for growth and metabolism of C. acetobutylicum (Bowles and Ellefson, 1985; Huang et al, 1985; Gottwald and Gottschalk, 1985). The ability to maintain this gradient is destroyed in the presence of growth-limiting butanol concentrations. The pH gradient can be abolished if the proton extrusion mechanism is deficient and/or there is an increased influx of protons. The ATPase, a membrane-bound proton-

translocating enzyme, which is essential to maintain a functional pH gradient, has been reported to be inhibited by butanol (Moreira et al, 1981; Bowles and Ellefson, 1985; Huang et al, 1985). Kuhn and Linden (1986) found that butanol enhances the passive influx of protons into C. acetobutylicum cells. The intracellular ATP levels also decreased markedly in the presence of butanol (Bowles and Ellefson, 1985).

Butanol has also been linked to the activity of autolysin which causes the degradation of solvent-producing cells (Van der Westhuizen et al, 1982; Barber et al, 1979; Soucaille and Goma, 1986; Soucaille et al, 1987). However, the nature of this link is unclear.

The lower level of butanol production by the *mot*$^-$ mutant compared to the parent strain P262 (Chapter 4) could be due to the differing butanol tolerance in the two organisms. The objective of this study was to investigate the effects of solvent, particularly butanol, on the *mot*$^-$ mutant. The study of this mutant, which seems to be less tolerant to butanol, will not only provide new insights into the mechanism of butanol toxicity, but will also be instrumental in further understanding of the physiological factors involved in the tolerance to higher concentrations of butanol.

5.2 MATERIALS AND METHODS

5.2.1 Microorganisms

The wild-type strain of C. acetobutylicum P262 and the non-motile mutant NG 12 (Chapter 4) were used in these studies.

5.2.2 Culture Media

CMMG was used to cultivate the organisms and SSM was used as fermentation medium (Section 2.2).

5.2.3 Chemicals

All chemicals used in these experiments were of analytical grade. The sources are listed below.

Sigma Chemical Company (St. Louis, Missouri, U.S.A.)	Mutanolysin
	Adenosine-5'-triphosphate (ATP)
	N N'-Dicyclohexylcarbodiimide (DCCD)
	Carbonylcyanide-m-chloro-phenylhydrazone (CCCP)
	Carbonylcyanide-p-trifluoro-methoxylphenylhydrazone (FCCP)

	Nigericin
	Valinomycin
	Lithium acetoacetate
BDH Chemicals Ltd	Tris (hydroxymethylamine)
(Palmerston North,	Hydrochloric acid
New Zealand)	Ethylene diaminetetraacetate disodium salt (EDTA)
	Piperazine N N'-bis-2-ethane-sulphonic acid (PIPES)
	Magnesium chloride
	Sodium molybdate
	Hydrazine sulphate
	Sulphuric acid
Ajax Chemicals	Trichloroacetic acid
(N.S.W., Australia)	

5.2.4 Culture Conditions

Both the parent and mutant strains were cultivated using an identical procedure to that described in Section 4.2.7.1.

5.2.5 Inhibition of Growth by Solvents

Inhibition of growth by solvents was determined by adding butanol, ethanol and acetone at different concentrations to 100 ml cultures in SSM 10-20 min after inoculation with the parent and mutant strain. Growth was measured as optical density at 650 nm (OD_{650}) using a Shimadzu spectrophotometer UV-120 (Shimadzu Corporation, Kyoto, Japan).

Appropriate dilution of the cultures using sterile culture medium was done immediately prior to OD measurements to ensure that OD_{650} readings were in the range 0-0.7 OD units.

5.2.6 End-product Inhibition of Solvent Formation

Cultures were allowed to grow for 18 h in 100 ml SSM before they were challenged with various concentrations of butanol, ethanol and acetone.

Fermentation samples were analyzed for solvents as described in Section 2. 9.3. Analysis for glucose was performed as described in Section 2.9.4.

5.2.7 Determination of Membrane-bound ATPase Activity

ATPase activity was determined using a modification of the method of Bowles and Ellefson (1985).

5.2.7.1 Preparation of Crude Bacterial Membranes

The parent and mutant strains were grown in SSM supplemented with glycine (0.4% w/v) to facilitate subsequent lysis (Allcock et al, 1982). For the parent, smooth running cells, grown to approximately 10^5 cells per ml, were harvested by centrifugation at 12,000 x g for 10 min at 4°C. For the *mot*$^-$ mutant, cells at the same stage of growth were used. The cells were suspended in 10 ml of 50 mM Tris hydrochloride buffer (pH 7.0) containing 20 mM EDTA (TE buffer) The cells were lysed by incubating with mutanolysin (100 µg/ml for 20-30 min at 37°C. To obtain the membrane fraction, the lysed suspension was centrifuged at 12,000 x g for 30 min at 4°C.

5.2.7.2 Assay of ATPase Activity

The crude membrane fraction (Section 5.2.7.1) was suspended in 1.0 ml of 100 mM PIPES buffer, pH 5.95. The assay for ATP hydrolysis was performed by mixing the following in a total reaction volume of 0.5 ml:

100 mM PIPES buffer, pH 5.95
10 mM MgCl
5 mM ATP
50 µl crude bacterial membrane

The mixture was incubated at 37°C for 20 min. The reaction was stopped by adding 1.0 ml of ice-cold trichloroacetic acid (15% w/v).

The mixture was centrifuged for 3 min in a mini-centrifuge (Kempthorne KMS Medical Supplies, U.S.A.). The inorganic phosphate in the supernatant was determined using the method of Vogel (1961) as described in Section 5.2.7.3.

5.2.7.3 Determination of Inorganic Phosphate

The amount of inorganic phosphate released from hydrolysis of ATP was determined by measuring the blue color intensity formed by the reduction of molybdenum blue (of uncertain composition) in the presence of hydrazine sulphate (Vogel, 1961). The intensity of the blue color is proportional to the phosphate concentration in the sample. Sample and standard solutions containing up to 0.1 mg of phosphorus as the orthophosphate were prepared containing the following in a total reaction volume of 50 ml:

25 ml sample or standard solution
5 ml molybdate solution (12.5 g Na molybdate in 5.0 M sulphuric acid)
2 ml hydrazine sulphate solution (1.5 g hydrazine sulphate in 1.0 liter deionized water)
deionized water to dilute

The mixtures were incubated in a boiling water bath for 10 min, then cooled rapidly in iced-water. The absorbance of the samples was measured at 830 nm against the standard solution without any

phosphorus. The standard curve was linear in the range 0 to 4.0 mg/l phosphorus.

5.2.8 Effect of ATPase Inhibitor and Protonophores on Solvent Production

Cultures were grown in SSM for 18 h before adding the inhibitor and protonophores. The chemicals were added at the following final concentrations: 300 μM, DCCD; 75 μM, CCCP; 40 μM, FCCP; 20 μM, valinomycin; 20 μM nigericin; 10 g/l, acetoacetate. DCCD, CCCP, FCCP, and valinomycin were dissolved in absolute ethanol while nigericin was dissolved in acetone.

Samples were collected for analyses of solvents and sugar utilization as described in Section 2.9.3 and Section 2. 9.4, respectively.

5.3 RESULTS

5.3.1 Growth Inhibition by Solvents

The growth responses of C. acetobutylicum P262 and its non-motile mutant in the presence of different concentrations of solvents were compared (Fig 5.1 to Fig 5.6). The maximum specific growth rates (μ) were calculated from the slopes of the growth curves of OD_{650} plotted against time (Table 5.1). Inhibition by added solvents was determined by calculating the growth rate ratio, $\frac{\mu_i}{\mu_o}$, where μ_i is the

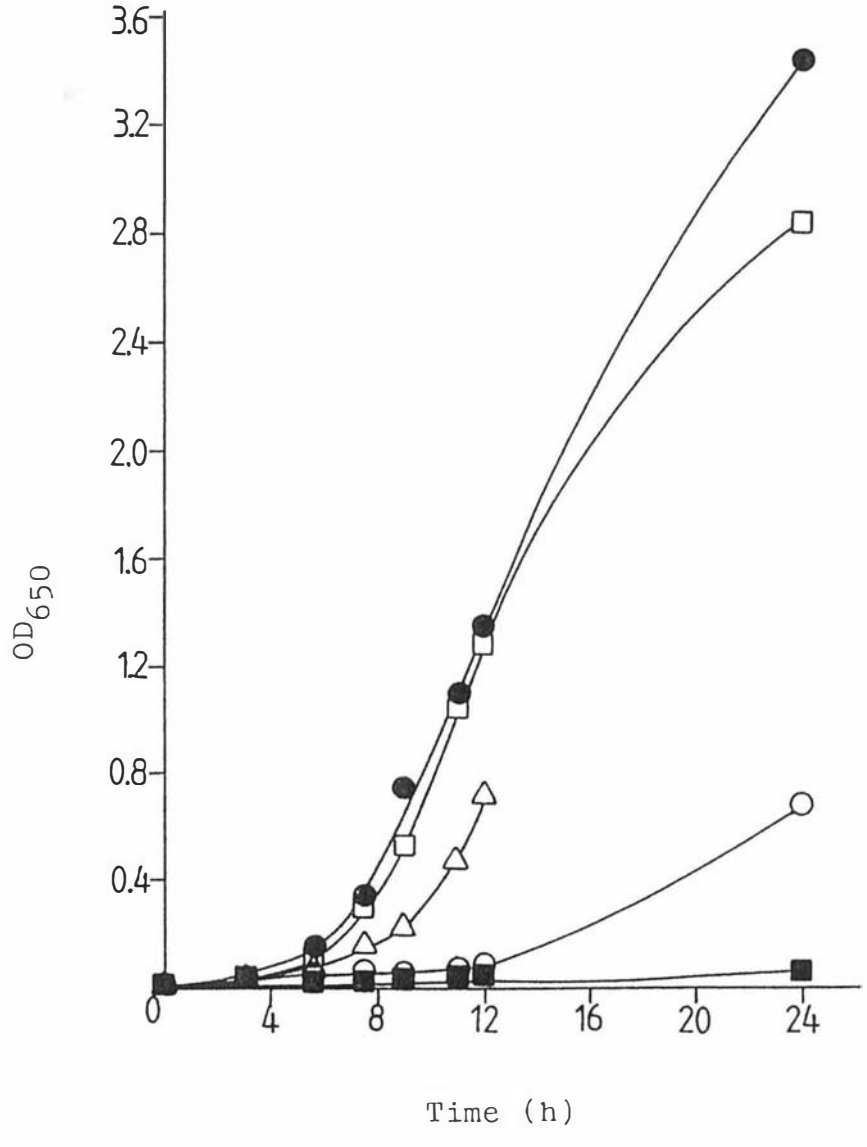


Fig 5.1 Growth of C. acetobutylicum P262 challenged with various concentrations of butanol: ●, 0 g/l; □, 5 g/l; △, 8 g/l; ○, 10 g/l; ■, 12 g/l.

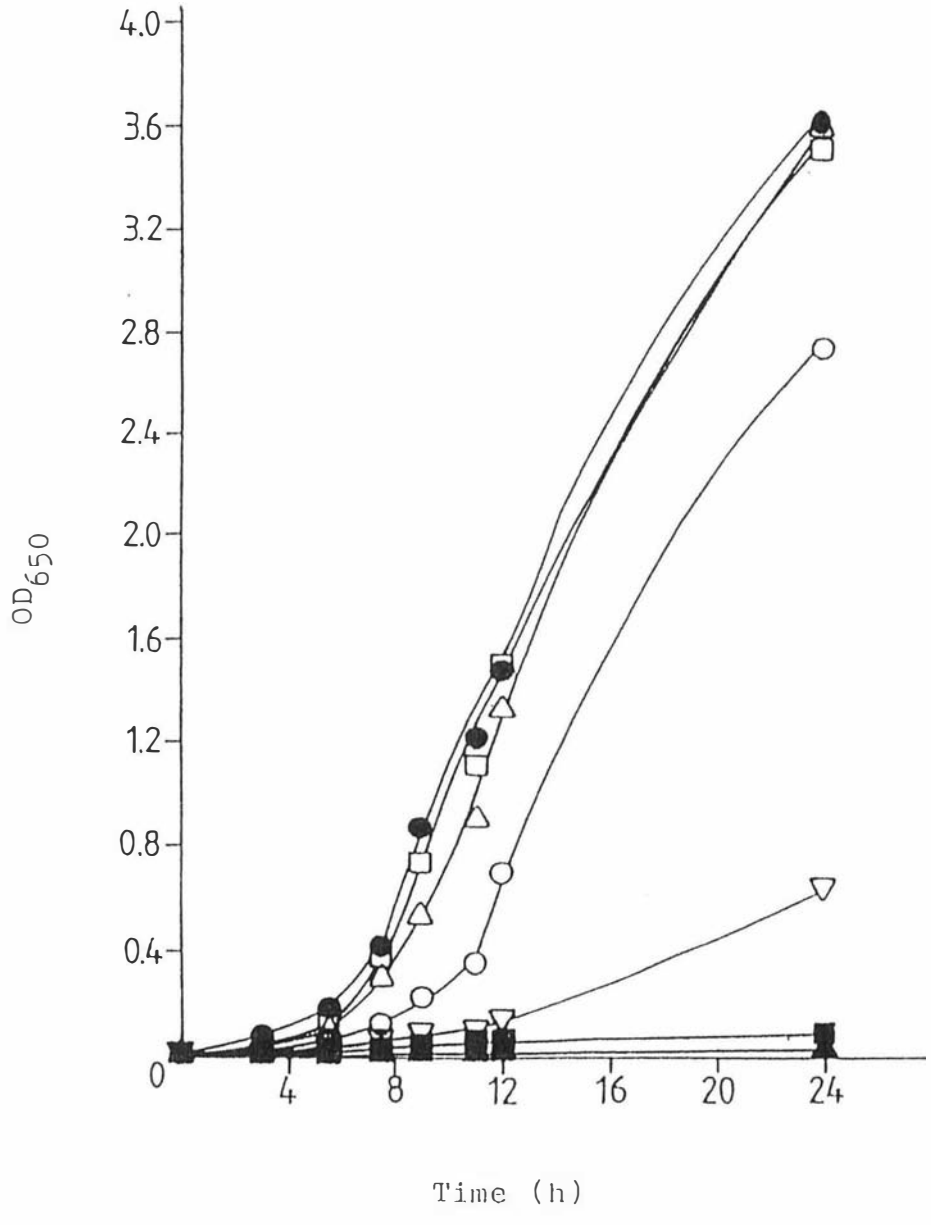


Fig 5.2 Growth of C. acetobutylicum P262 challenged with various concentrations of ethanol: ●, 0 g/l; □, 10 g/l; △, 20 g/l; ○, 30 g/l; ▽, 40 g/l; ■, 50 g/l; ▲, 60 g/l.

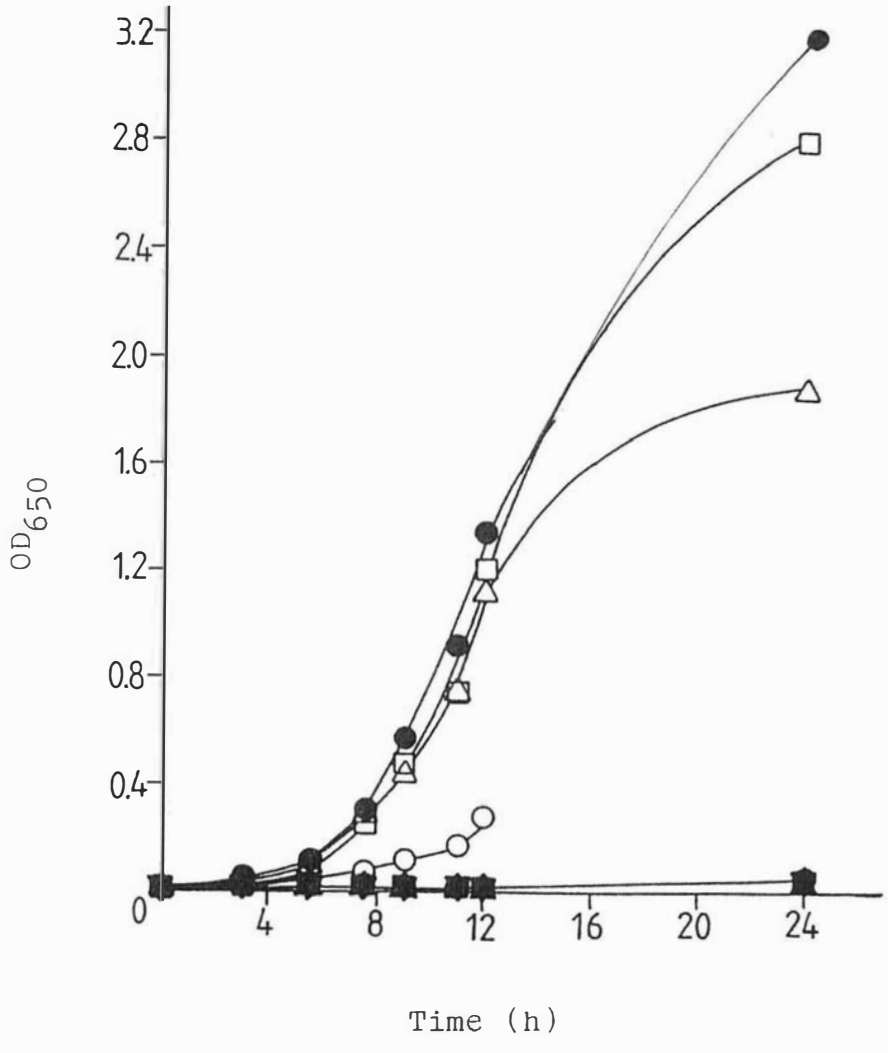


Fig 5.3 Growth of C. acetobutylicum P262 challenged with various concentrations of acetone: ●, 0 g/l; □, 10g/l; △, 20 g/l; ○, 30 g/l; ▽, 40 g/l; ■, 50 g/l; ▲, 60 g/l.

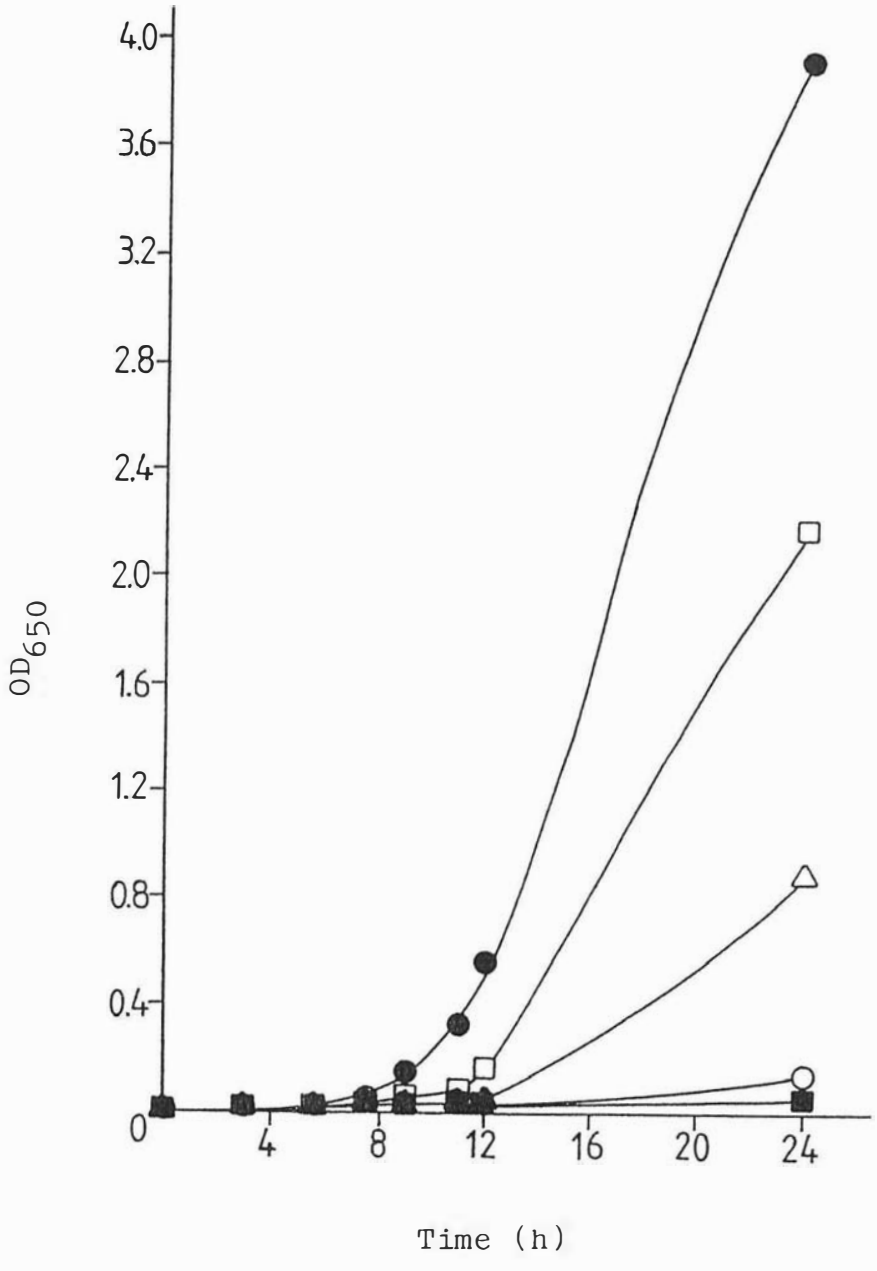


Fig 5.4 Growth of mutant NG 12 challenged with various concentrations of butanol: ●, 0 g/l; □, 5 g/l; △, 8 g/l; ○, 10 g/l; ■, 12 g/l.

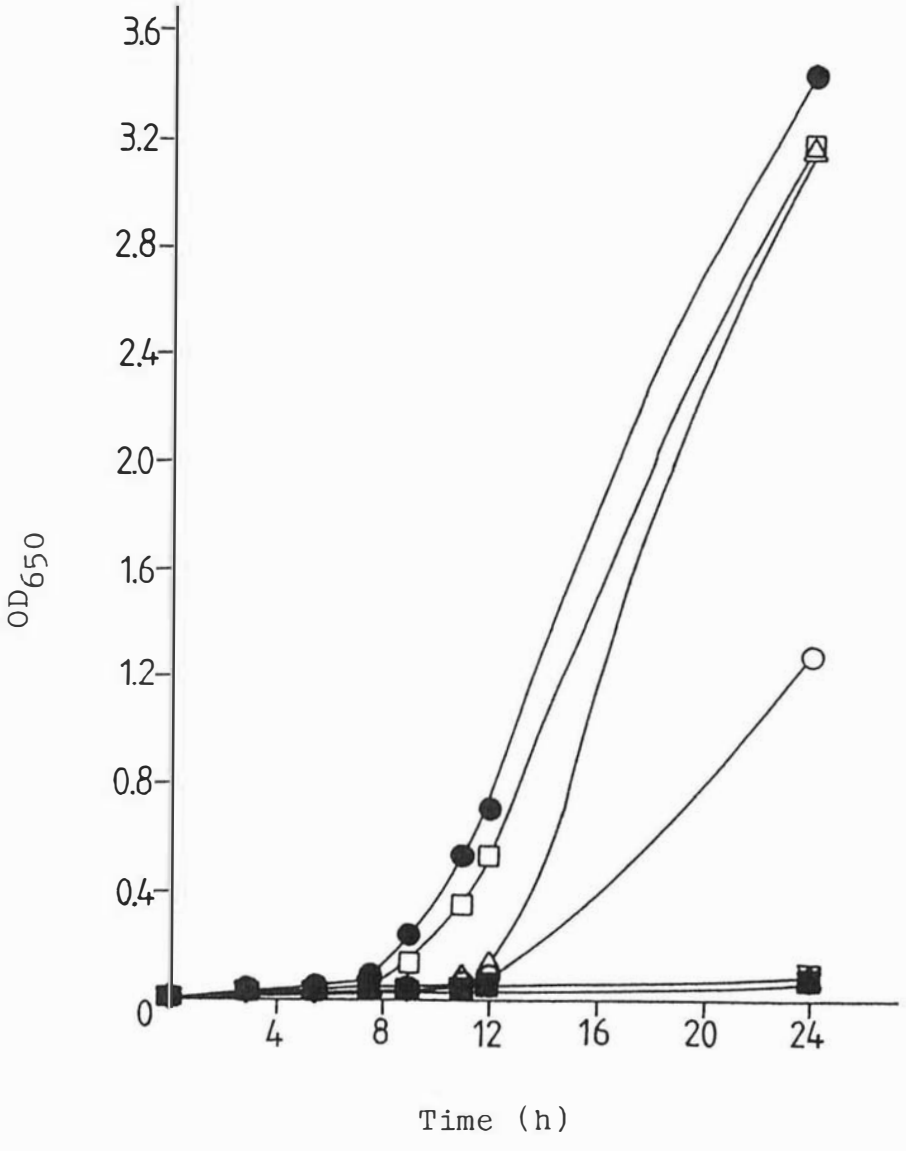


Fig 5.5 Growth of mutant NG 12 challenged with various concentrations of ethanol: ●, 0 g/l; □, 10g/l; △, 20 g/l; ○, 30 g/l; ▽, 40 g/l; ■, 50 g/l; ▲, 60 g/l.

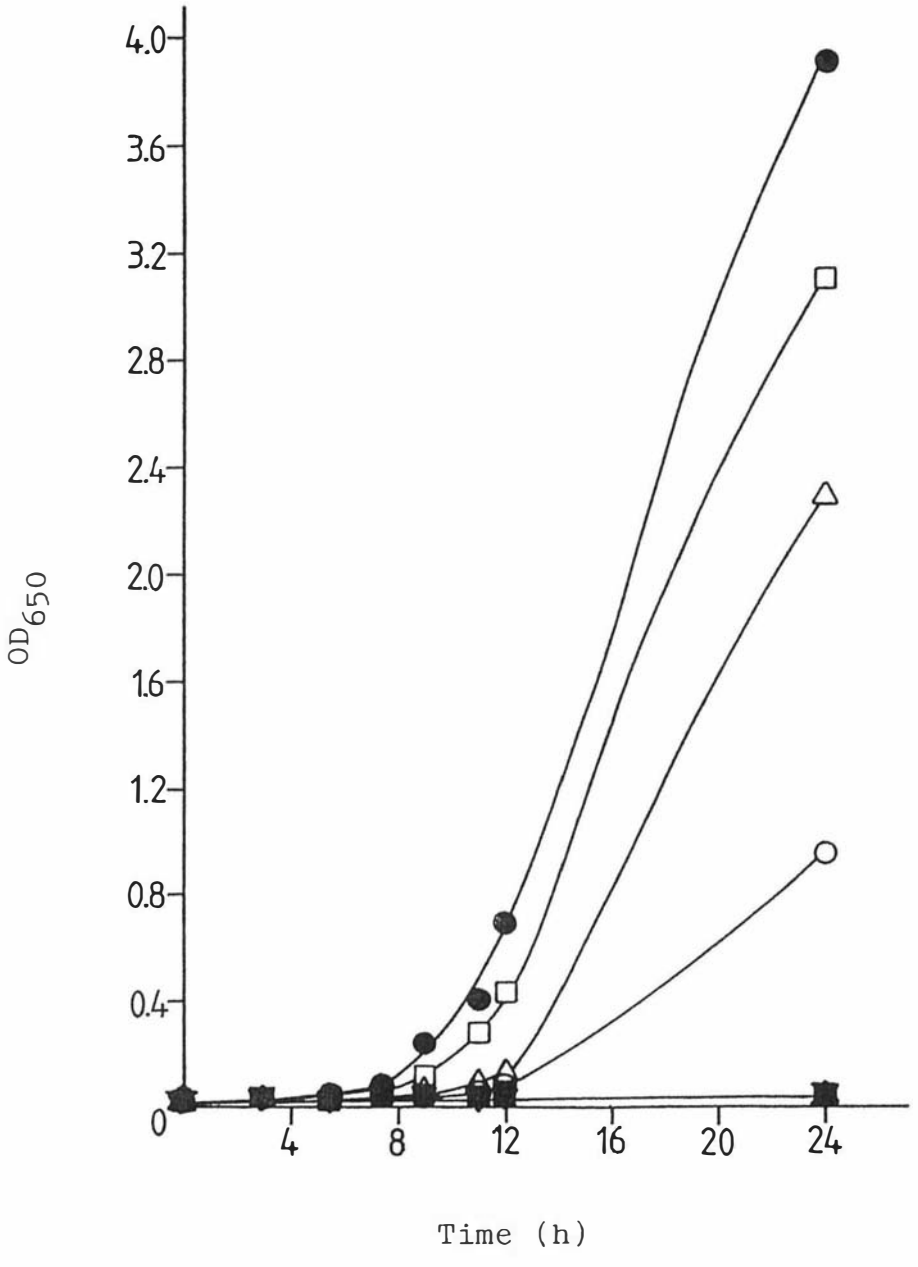


Fig 5.6 Growth of mutant NG 12 challenged with various concentrations of acetone: ●, 0 g/l; □, 10g/l; △, 20 g/l; ○, 30 g/l; ▽, 40 g/l; ■, 50 g/l; ▲, 60 g/l.

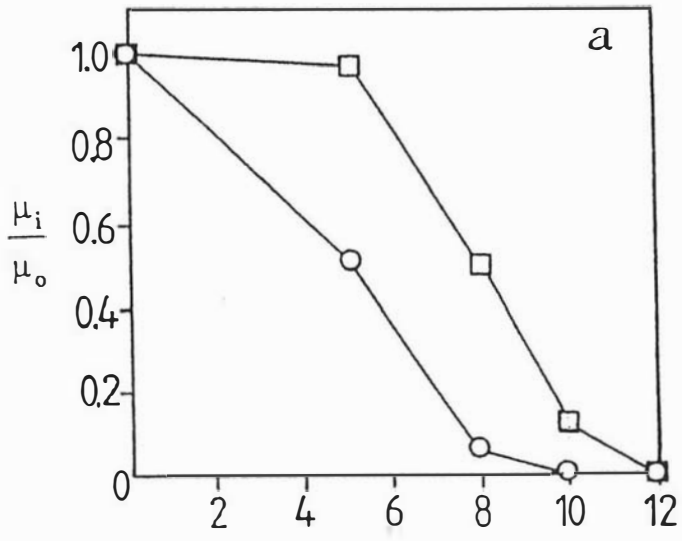
growth rate in the presence of inhibitor and μ_o is the growth rate in the uninhibited control.

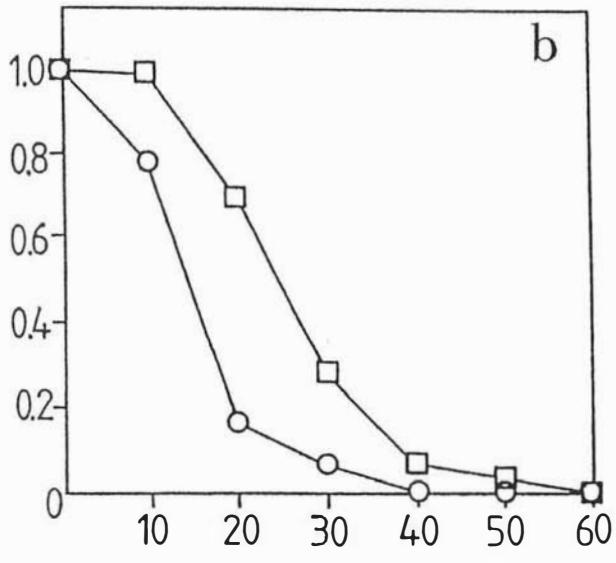
Fig 5.7a shows the inhibition of growth rates of the parent and mutant strains as a function of butanol concentration. The growth response of P262 indicates higher tolerance to butanol than does that of the non-motile NG 12. The specific growth rate of the mutant was inhibited by 50% at an added butanol concentration of 5 g/l, whereas at this concentration, the growth rate of the parent was hardly inhibited. A 50% inhibition of growth rate of P262 was observed at an added butanol concentration of 7.8 g/l. Growth of NG 12 ceased when the concentration of butanol added was 10.0 g/l while growth of P262 was completely inhibited at added butanol concentration of 12.0 g/l.

The inhibitory effects of ethanol and acetone were observed at concentrations far greater than those attained during a typical fermentation process. Threshold concentrations, below which very little or no growth inhibition occurred, were also observed. As with butanol, the mutant was similarly found to be more inhibited by added ethanol and acetone than was the parent. P262 was only slightly inhibited by ethanol and acetone at 10.0 g/l (Fig 5.7b and Fig 5.7c, respectively), whereas, NG 12 was already inhibited markedly at this concentration. Concentrations of added ethanol and acetone resulting in a 50% inhibition of growth rate of NG 12 were determined as 14.5 g/l and 13.0 g/l, respectively. P262 was inhibited by 50% at concentration of added ethanol and acetone of 24.5 g/l and 23 g/l, respectively.

Table 5.1 Growth rate values in the presence of added solvents.

Solvent added	Concentration (g/l)	μ (h^{-1}) P262	NG 12
Butanol	0	0.272	0.200
	5.0	0.263	0.100
	8.0	0.130	0.009
	10.0	0.003	0
	12.0	0	0
Ethanol	0	0.271	0.200
	10.0	0.267	0.154
	20.0	0.188	0.033
	30.0	0.076	0.013
	40.0	0.018	0
	50.0	0.009	0
	60.0	0	0
Acetone	0	0.253	0.167
	10.0	0.235	0.110
	20.0	0.180	0.027
	30.0	0.022	0.007
	40.0	0	0
	50.0	0	0
	60.0	0	0





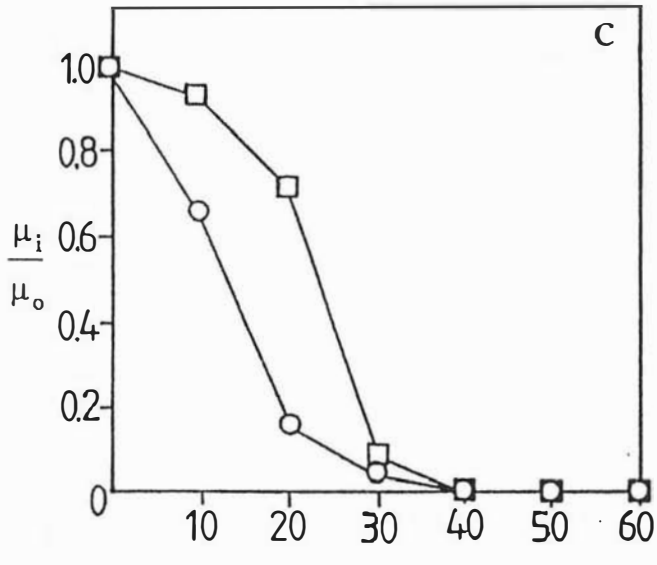


Fig 5.7 Effect of added solvents (a) butanol; (b) ethanol; (c) acetone on growth rates of the parent strain (□) and non-motile mutant (○).

Growth yield was also determined and was defined as the growth achieved after 24 h (OD_{650}) minus the growth (OD_{650}) at the time of inoculation. The growth yield values for P262 and NG 12 in the presence of solvents are shown in Table 5.2.

The % inhibition of growth yields of P262 and NG 12 in the presence of added solvents are shown in Fig 5.8. The growth yield of NG 12 was reduced to a greater extent than was P262, at a given butanol concentration (Fig 5.8a).

Ethanol and acetone also inhibited the growth yield of NG12 more than that of P262 (Fig 5.8b and Fig 5.8c, respectively). Similar to inhibition of growth rate by ethanol and acetone, inhibition of growth yield occurred at concentrations above those typically achieved during fermentation.

.3.2 End-Product Inhibition of Butanol Production

The effects of butanol, ethanol and acetone on the production of butanol by C. acetobutylicum P262 and its non-motile mutant were investigated and compared in an experiment separate from that of the effect on growth rate, wherein each solvent was added to the fermentation 18 h after inoculation. At this stage, cell growth was almost completed and butanol production was only about 1 g/l. Hence, any inhibitory effect on solvent production could be attributed solely to the effect of added solvent *per se*, and not to any differences in

Table 5.2 Growth yield values in the presence of added solvents.

Solvent added	Concentration (g/l)	OD_{650} P262	OD_{650} NG 12
Butanol	0	3.438	3.893
	5.0	2.831	2.179
	8.0	nd	0.885
	10.0	0.663	0.133
	12.0	0.047	0.038
Ethanol	0	3.503	3.441
	10.0	3.524	3.185
	20.0	3.595	3.173
	30.0	2.733	1.281
	40.0	0.641	0.071
	50.0	0.086	0.037
	60.0	0.047	0.048
Acetone	0	3.158	3.890
	10.0	2.826	3.075
	20.0	1.868	2.298
	30.0	nd	0.922
	40.0	0.056	0.030
	50.0	0.054	0.034
	60.0	0.043	0.014

nd not determined

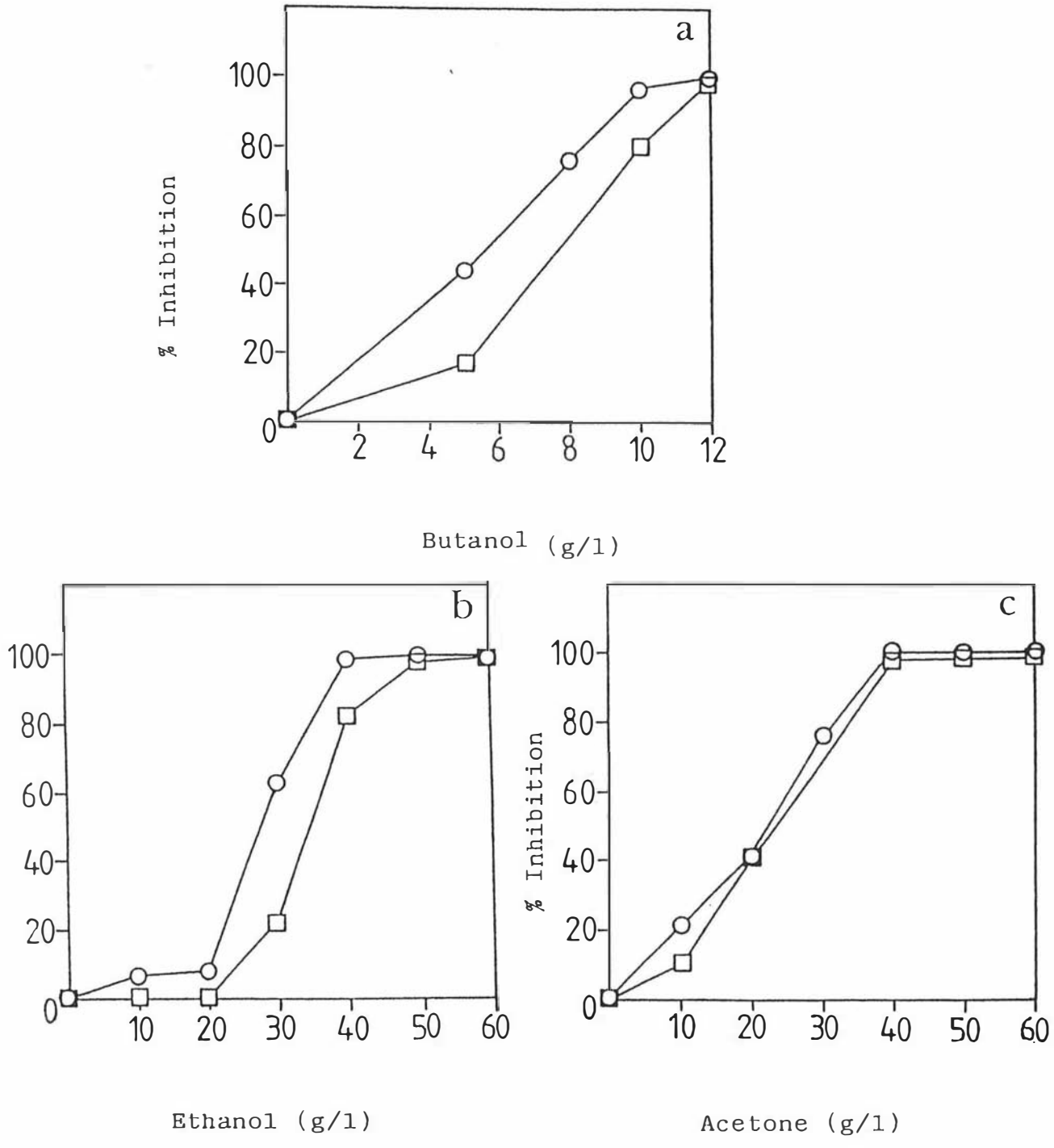


Fig 5.8 Inhibition (%) of growth yield by added solvents: (a) butanol; (b) ethanol; (c) acetone; □, parent strain; ○, non-motile mutant.

biomass concentration. At time = 18 h, the butanol concentration was taken as 0 g/l.

Fig 5.9, 5.10 and 5.11 show the concentration of butanol as a function of time when the parent strain was challenged with butanol, ethanol, and acetone, respectively. A marked decrease in butanol production was observed after the addition of each end-product. Similar results were observed with the mutant (Fig 5.12, 5.13 and 5.14). These results confirm that the production of butanol by C. acetobutylicum is subject to product inhibition.

The butanol production rate was calculated as the initial rate and the values are shown in Table 5.3. The amount of inhibition was determined by calculating the ratio $\frac{q_i}{q_o}$, where q_i is the rate in the presence of inhibitor and q_o is the rate in its absence. Fig 5.15a shows that the butanol production rate of NG 12 was less inhibited by the addition of butanol than was that of P262. The presence of added butanol resulted in 50% inhibition of the butanol production rate at 8.7 g/l for NG 12 and 5 g/l for P262.

Similar to their effects on growth, ethanol and acetone were found to be less toxic to solvent production than was butanol. Inhibitory concentrations were considerably greater than was observed with butanol. NG 12, when compared to P262, showed the same response (as with butanol) to ethanol and acetone in terms of butanol production rate (Fig 5.15b and Fig 5.15c), respectively. Ethanol, at an added concentration of 40.0 g/l, caused a 50% inhibition of production rate in NG 12, while for P262 a 50% inhibition was calculated to occur at

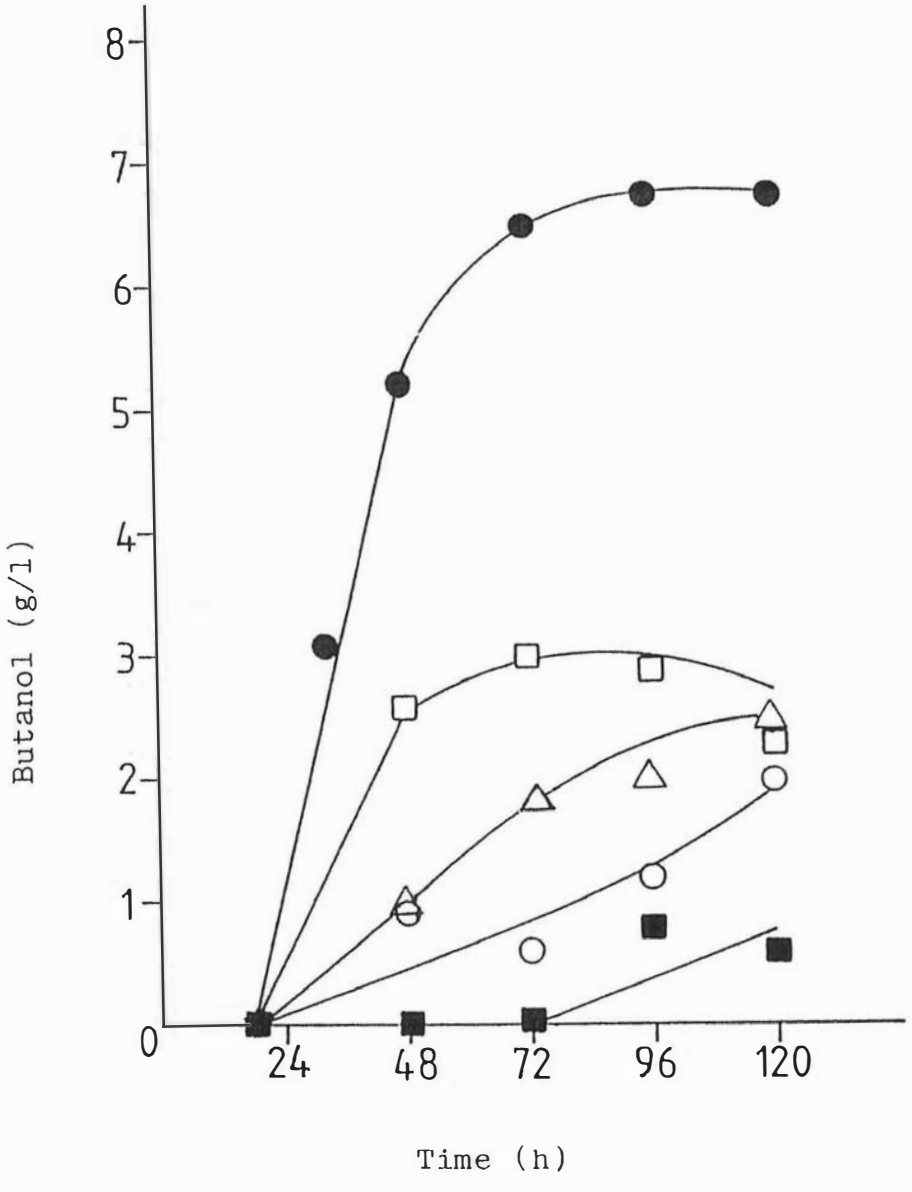


Fig 5.9 Butanol production by parent strain P262 in the presence of added butanol: ● , 0 g/l; □, 5 g/l; △, 8 g/l; ○, 10 g/l; ■, 12 g/l.

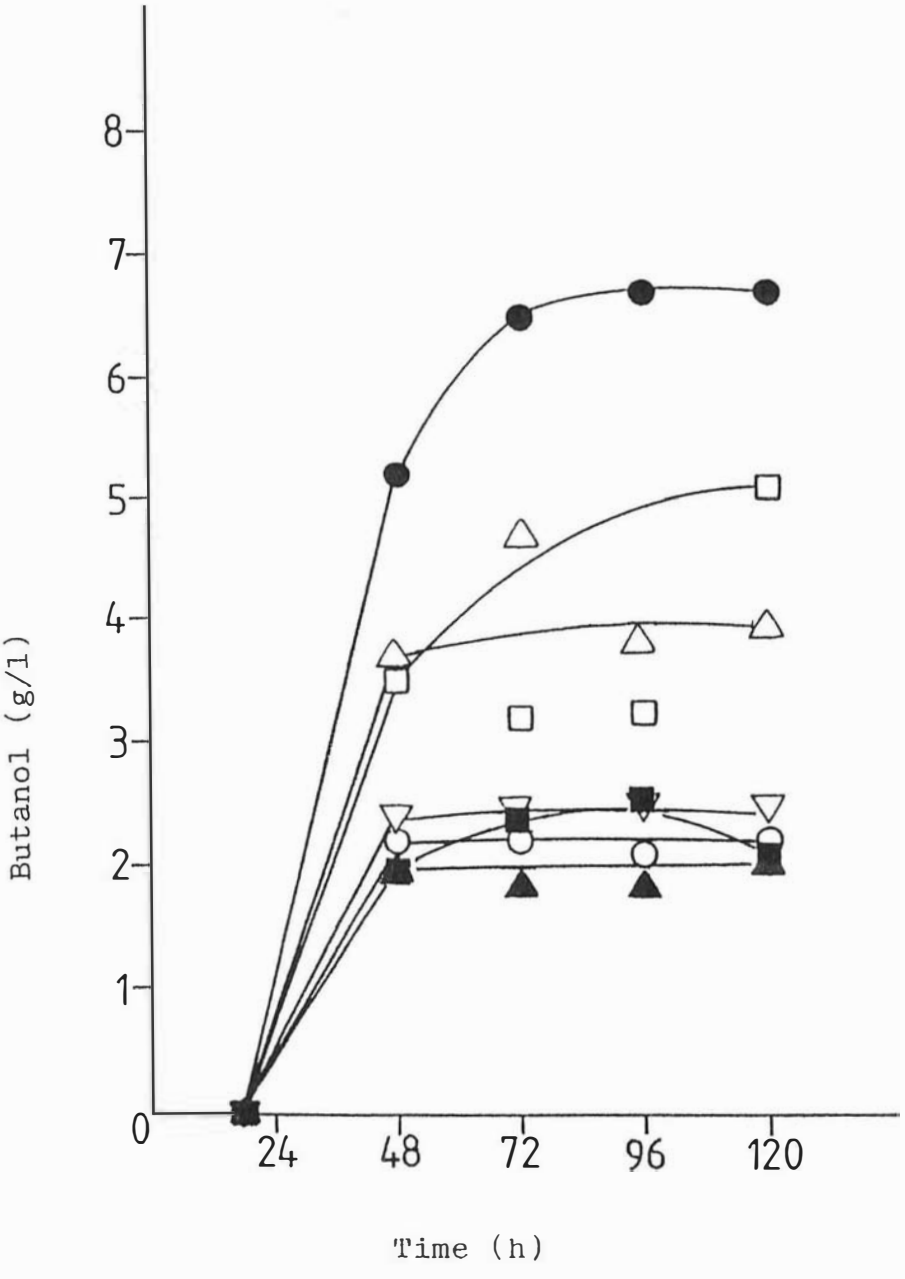


Fig 5.10 Butanol production by parent strain P262 in the presence of added ethanol: ●, 0 g/l; □, 10 g/l; △, 20 g/l; ○, 30 g/l; ▽, 40 g/l; ■, 50 g/l; ▲, 60 g/l.

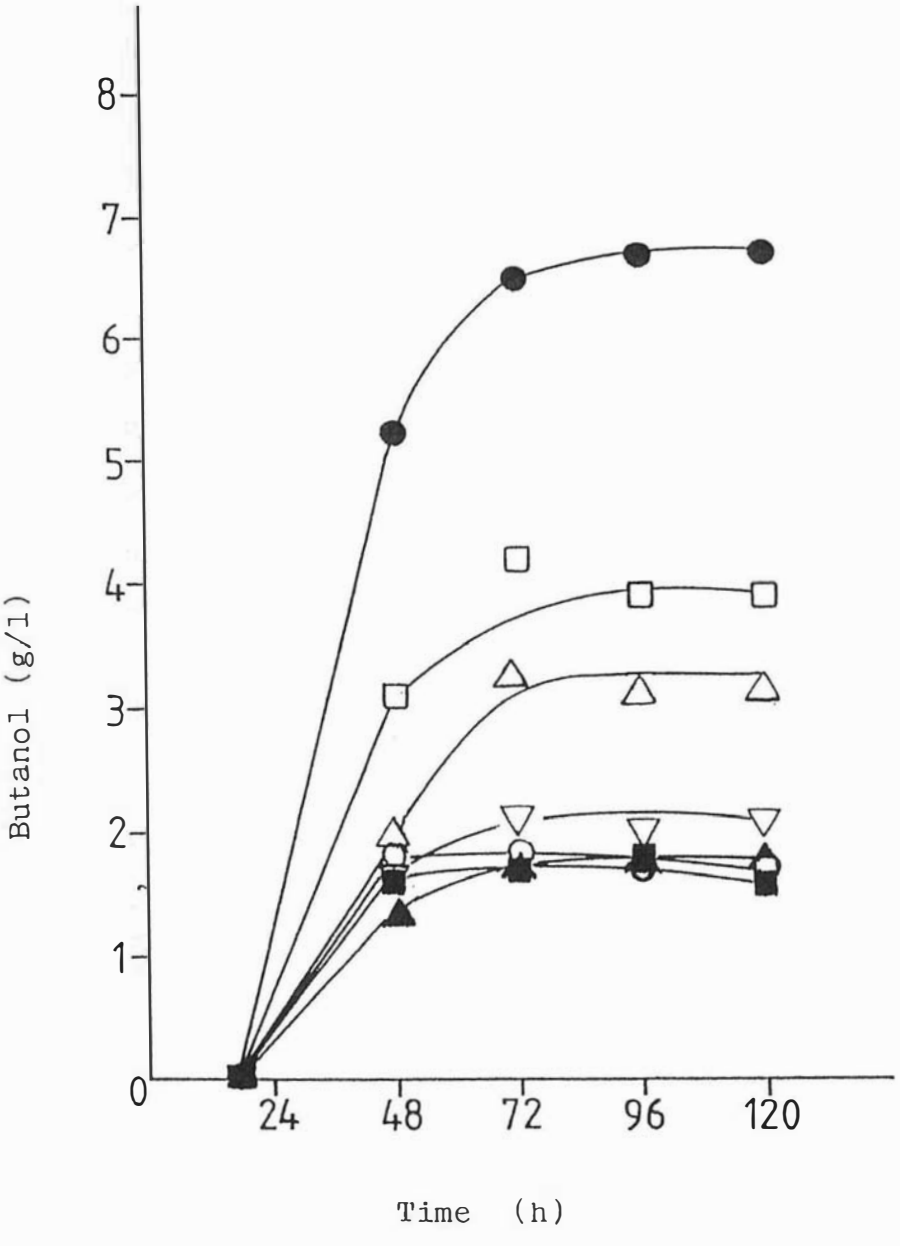


Fig 5.11 Butanol production by parent strain P262 in the presence of added acetone: ●, 0 g/l; □, 10 g/l; △, 20 g/l; ○, 30 g/l; ▽, 40 g/l; ■, 50 g/l; ▲, 60 g/l.

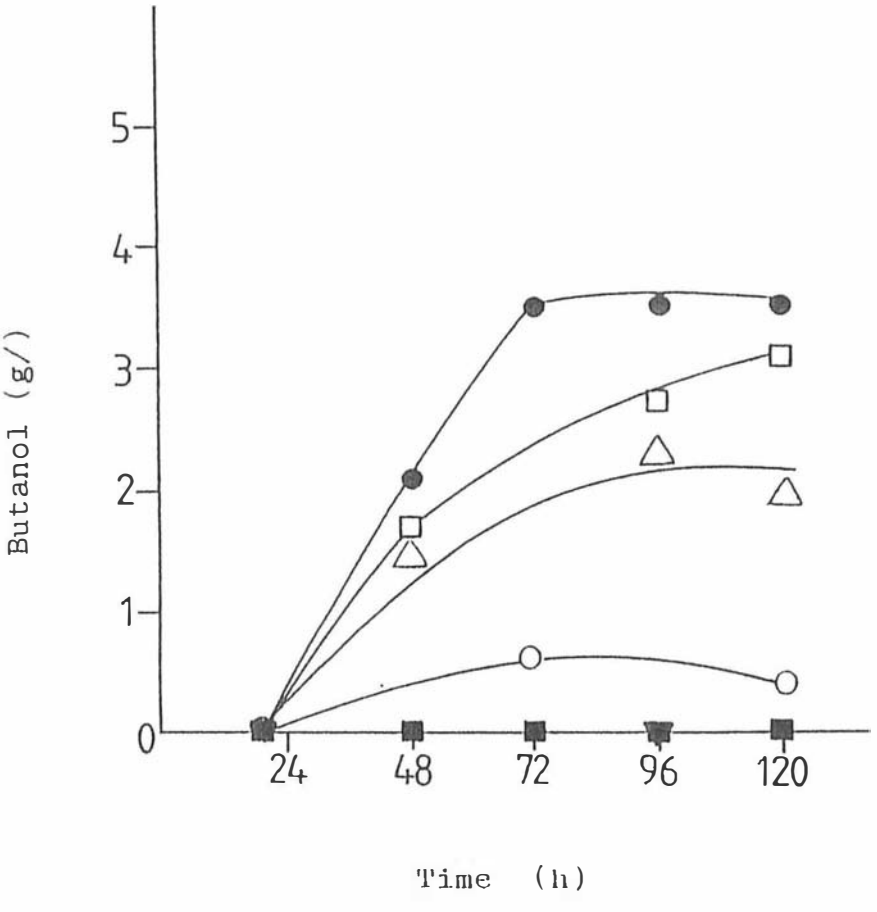


Fig 5.12 Butanol production by mutant NG 12 in the presence of added butanol: ●, 0 g/l; □, 5 g/l; △, 8 g/l; ○, 10 g/l; ■, 12 g/l.

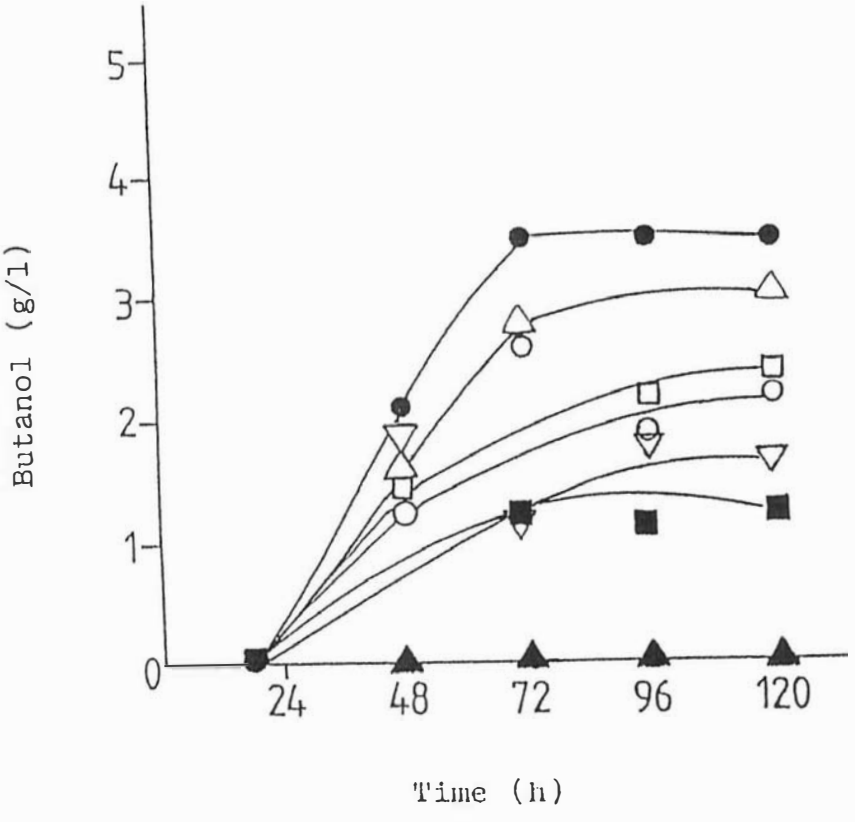


Fig 5.13 Butanol production by mutant NG 12 in the presence of added ethanol: ●, 0 g/l; □, 10 g/l; △, 20 g/l; ○, 30 g/l; ▽, 40 g/l; ■, 50 g/l; ▲, 60 g/l.

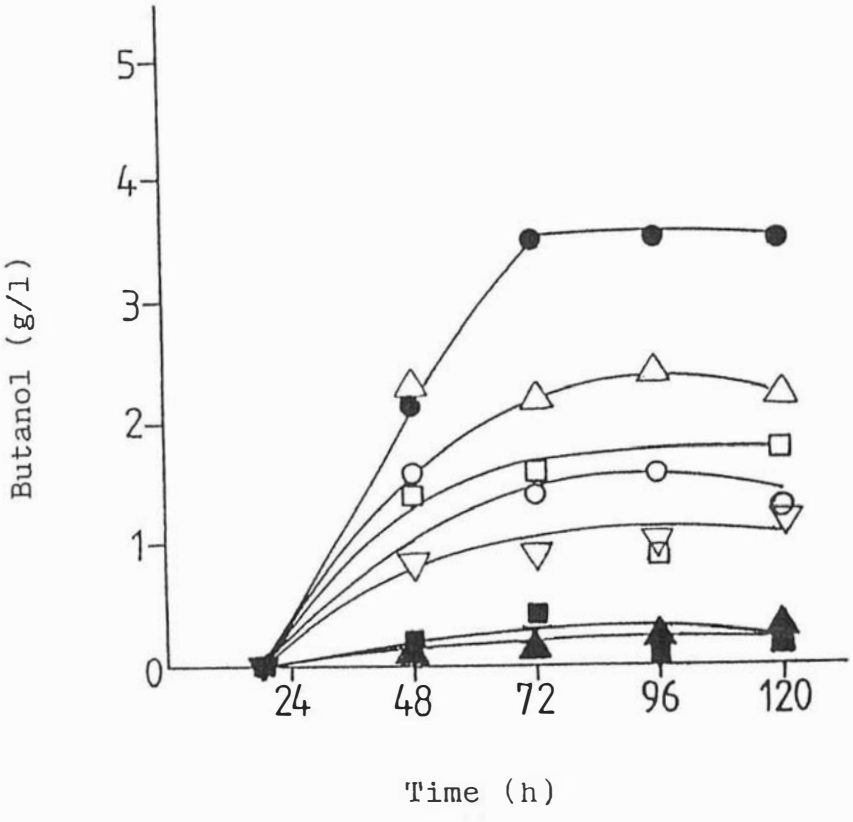


Fig 5.14 Butanol production by mutant NG 12 in the presence of added acetone: ●, 0 g/l; □, 10 g/l; △, 20 g/l; ○, 30 g/l; ▽, 40 g/l; ■, 50 g/l; ▲, 60 g/l.

Table 5.3 Butanol production rate values in the presence of added solvents.

Solvent added	Concentration (g/l)	q (g/l.h) P262	NG 12
Butanol	0	0.173	0.080
	5.0	0.087	0.062
	8.0	0.033	0.046
	10.0	0.017	0.020
	12.0	0.001	0
Ethanol	0	0.173	0.080
	10.0	0.117	0.050
	20.0	0.123	0.050
	30.0	0.073	0.043
	40.0	0.080	0.040
	50.0	0.067	0.043
	60.0	0.067	0
Acetone	0	0.173	0.080
	10.0	0.103	0.047
	20.0	0.067	0.053
	30.0	0.060	0.035
	40.0	0.057	0.030
	50.0	0.057	0.007
	60.0	0.047	0.007

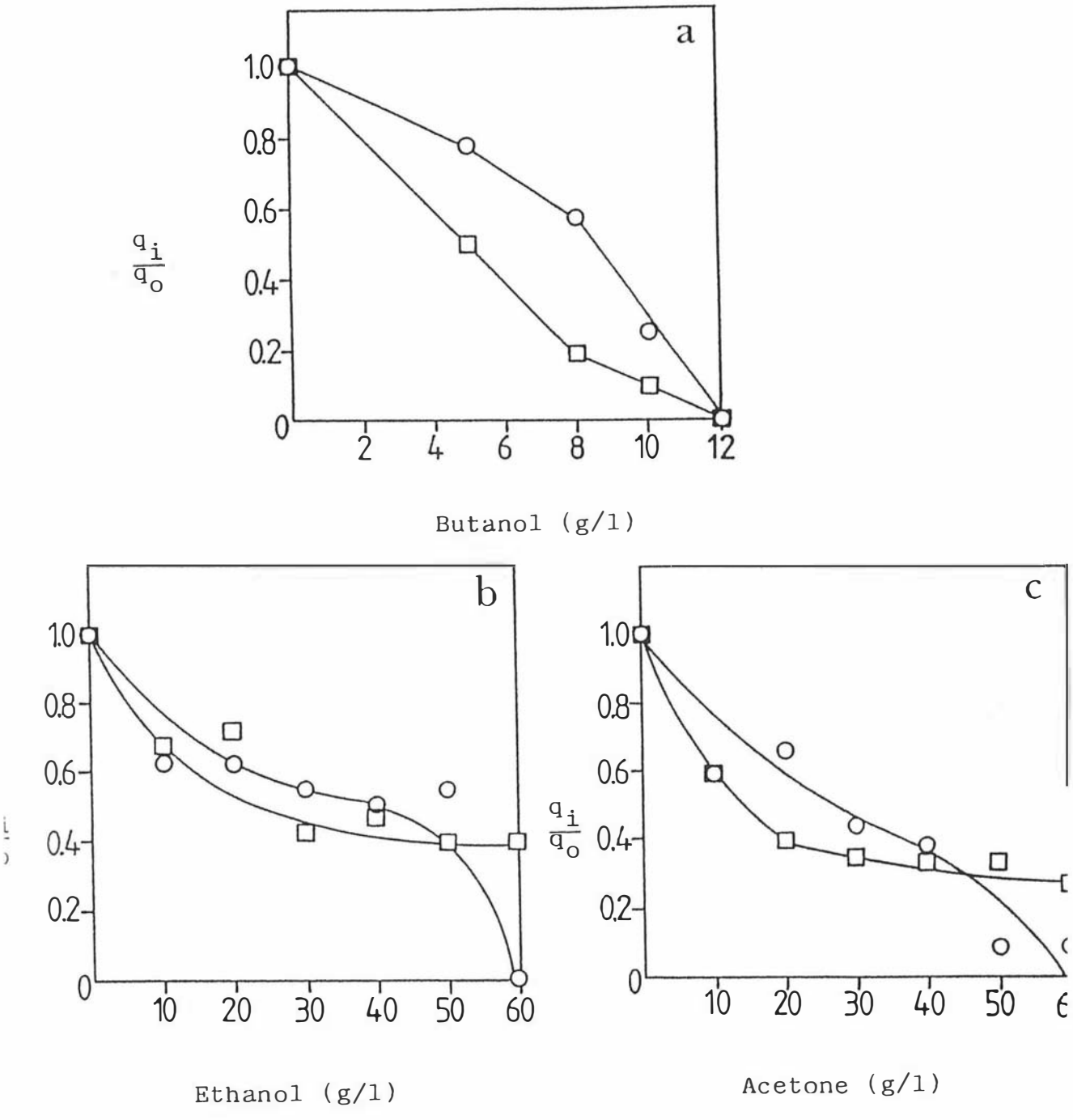


Fig 5.15 Effects of added solvents (a) butanol; (b) ethanol; (c) acetone on butanol production rates of the parent strain (□) and non-motile mutant (○).

an added ethanol concentration of only 23 g/l. However, at an added ethanol concentration of 60.0 g/l, butanol production by NG 12 was completely inhibited, while in P262 it was observed to persist. In the presence of added acetone, 50% inhibition of the rate of butanol production was observed in NG 12 at a concentration of 26 g/l, whereas in P262, it was at 14 g/l.

The inhibitory effects of added solvents were also apparent in the final concentrations of butanol obtained (referred to as butanol production in this study). NG 12 was less inhibited on a percentage basis than was P262 in its ability to produce butanol in the presence of added concentrations of butanol less than 9.4 g/l. At this concentration, a cross-over effect was observed where the ability of the mutant to produce butanol was more adversely affected than that of the parent (Fig 5.16a).

Added ethanol and acetone were also observed to have a less adverse effect on the ability of NG 12 to produce butanol than on P262. Similar cross-over effects were noted, although they occurred at very high concentrations of ethanol, 53 g/l (Fig 5.16b) and acetone, 40 g/l (Fig 5.16c).

5.3.3 Membrane-bound ATPase Activity

Previous studies have shown that butanol inhibits membrane-bound ATPase activity (Moreira et al, 1981; Bowles and Ellefson, 1985). ATPase couples the hydrolysis of ATP to the extrusion of protons

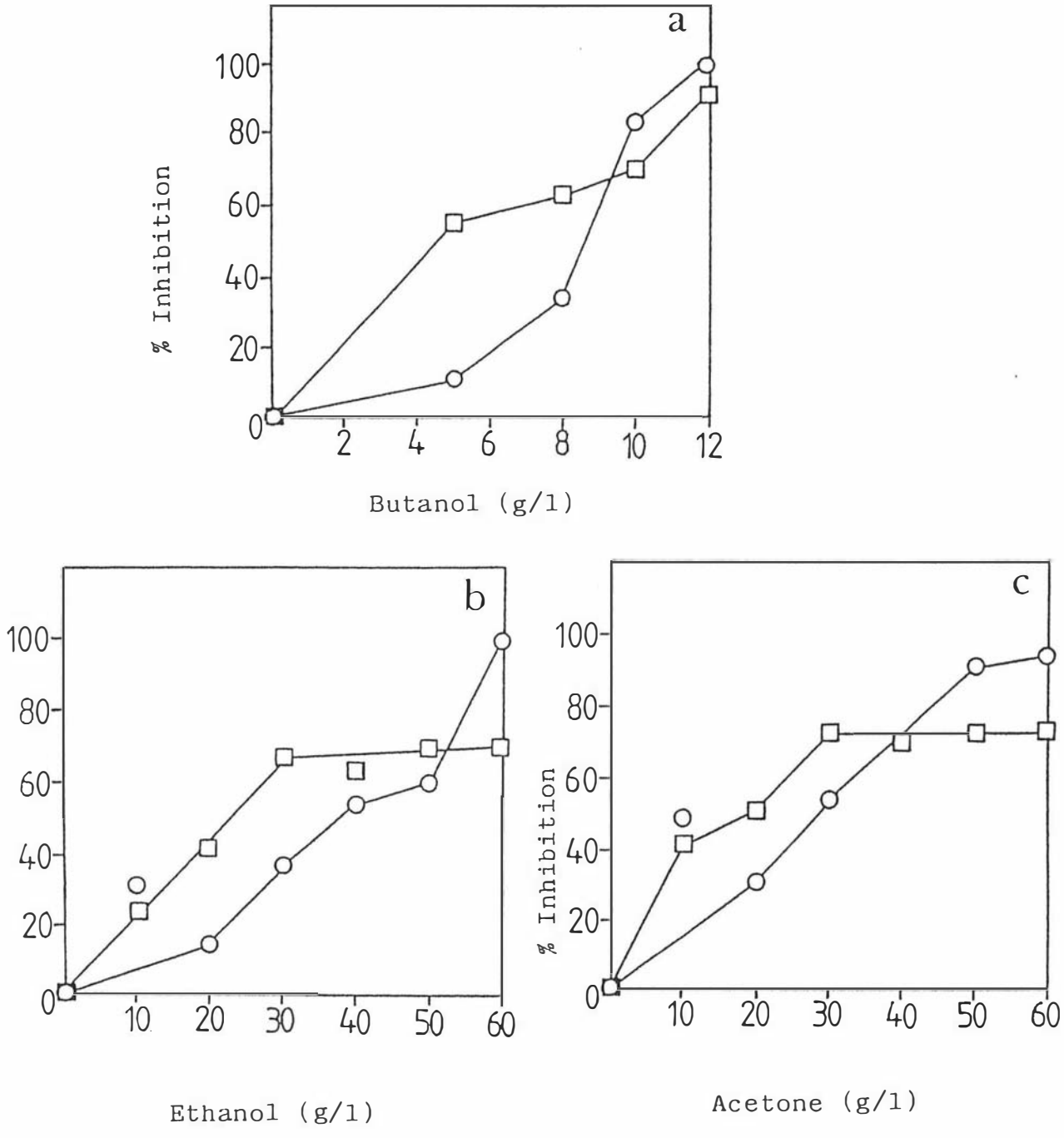


Fig 5.16 Inhibition (%) of butanol production in the presence of added solvent: (a) butanol; (b) ethanol; (c) acetone. □, parent strain; ○, non-motile mutant.

from the cell (Riebeling and Jungermann, 1976). This action generates a proton motive force which is the driving force for many energy-requiring processes such as motility (Hellingwerf and Konings, 1985). Since proton extrusion and motility are both mediated by proton motive force which is then mediated by ATPase, the membrane-bound ATPase activities in the parent and non-motile mutant were determined and compared.

The membrane-bound ATPase was solubilized by treating the crude bacterial membrane with PIPES buffer (pH 5.95). ATPase activity was then determined by measuring the release of inorganic phosphate (P_i) from ATP and the results are summarized in Table 5.4. A higher membrane-bound ATPase activity was observed with the parent strain. The level of ATPase activity in the non-motile strain was virtually not detected by the method employed in this study. The different values obtained for the different experiments are probably due to cells of slightly different stages of growth being used.

5.3.4 Effect of an ATPase Inhibitor and Protonophores on Butanol Production

The effects of the ATPase inhibitor, DCCD, and proton-ionophoretic compounds (protonophores) on the butanol production of the parent and mutant strain were investigated. The mutant and the parent strains were allowed to grow for 18 h after inoculation (i.e. until growth was complete), before DCCD and the protonophores were added at concentrations which were sub-inhibitory to growth. These

Table 5.4 ATPase activity in the parent and non-motile strains of C. acetobutylicum

Organism	Enzyme activity (x 10^{-6} mg Pi / h / cell)		
	I	II	III
P262	1.8	3.3	0.032
NG 12	nd	0.006	nd

Data were obtained from three experiments.
Pi was measured as PO_4^{3-}
nd signifies not detected.

concentrations were based on previous studies on C. acetobutylicum and E. coli: DCCD and CCCP (Reysenbach, 1987); FCCP, nigericin and acetoacetate (Huesemann and Papoutsakis, 1986); valinomycin (Miller and Koshland, 1977). To measure the effects of the compounds on butanol production, two ratios were calculated namely,

$\frac{B_i}{B_o}$, where B_i is the maximum butanol production in the presence of inhibitor or protonophore and B_o is the maximum butanol production in the absence of inhibitor or protonophore (control)

$\frac{q_i}{q_o}$, where q_i is the initial rate of butanol production in the presence of inhibitor or protonophore and q_o is the initial butanol production rate in the control.

The results are summarized in Table 5.5.

In the presence of DCCD, the ability of NG 12 to produce butanol was not affected whereas that of P262 was greatly reduced. Likewise, DCCD exerted a more adverse effect on the initial rate of butanol production of P262 than of NG 12.

CCCP and FCCP appeared to increase the butanol production of the mutant and reduce that of the parent. However, the initial rate of butanol production of the mutant was more severely inhibited than that of the parent. The higher culture pH values observed with the mutant after the addition of CCCP and FCCP imply that proton conduction into the mutant was greater than that into the parent (Fig 5.17).

Table 5.5 Effect of an ATPase inhibitor and protonophores on butanol production and initial rates of butanol production of strains P262 and NG 12.

Compound added	$\frac{B_i}{B_o}$ P262	$\frac{B_i}{B_o}$ NG 12	$\frac{q_i}{q_o}$ P262	$\frac{q_i}{q_o}$ NG 12
None	1.00	1.00	1.00	1.00
DCCD (300 μM)	0.46	1.00	0.57	0.75
CCCP (75 μM)	0.87	1.31	0.38	0.20
FCCP (40 μM)	0.70	1.10	0.46	0.28
Valinomycin (20 μM)	1.00	1.00	1.00	1.00
Nigericin (20μM)	0.22	0.05	0.09	0.02
Acetoacetate (10 g/l)	1.00	1.31	1.00	1.00

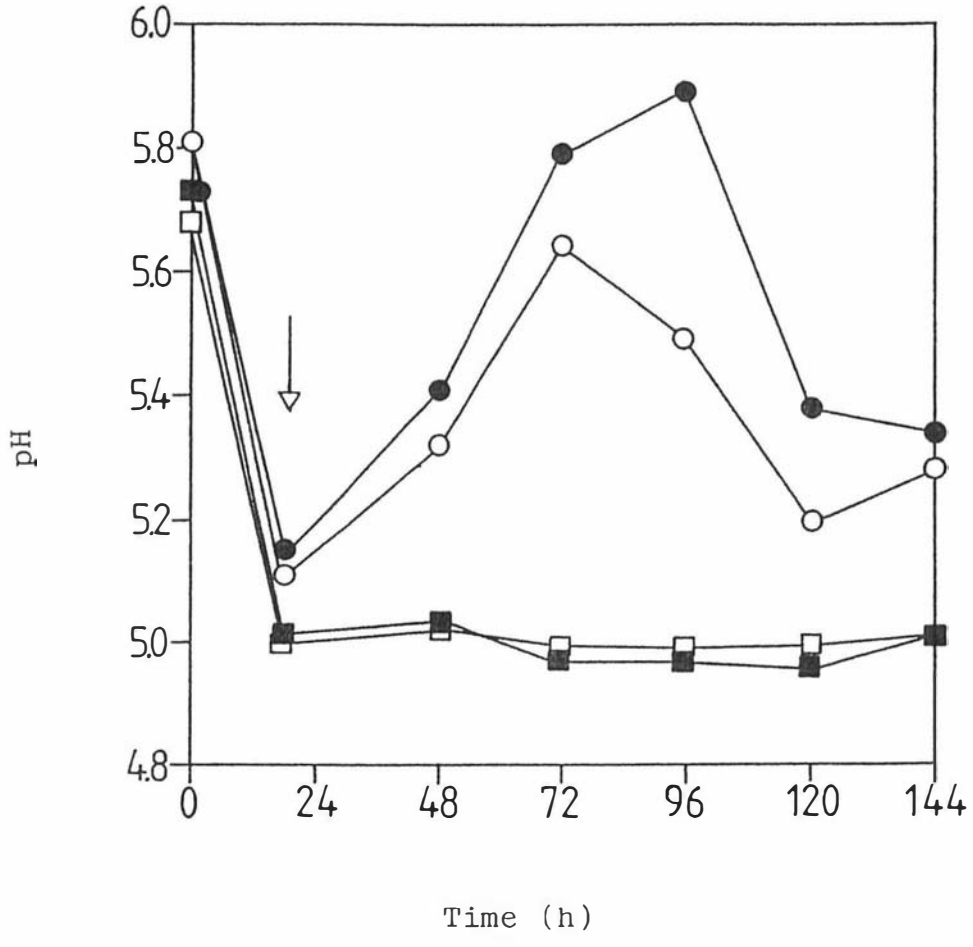


Fig 5.17 pH of the culture after addition of CCCP and FCCP. Arrow indicates time of addition of protonophores. ○, CCCP in NG 12 culture; ●, FCCP in NG 12 culture; □, CCCP in P262 culture; ■, FCCP in P262 culture.

In the presence of acetoacetate at 10 g/l, solvent production in the mutant increased but not in the parent. Valinomycin, at the concentration used in this experiment, had no effect on butanol production of either the mutant or the parent strain. On the other hand, the concentration of nigericin (and butyric acid) used virtually poisoned both organisms.

5.4 DISCUSSION

The results obtained from studies on solvent inhibition of growth rate (Section 5.3.1) suggest that the *mot*$^-$ mutant NG 12 has a lower tolerance to solvent toxicity than does the parent strain P262. This could explain why the *mot*$^-$ mutant produced a lower solvent concentration during fermentation than did the parent strain (Section 4.3.6).

Among the end-products of fermentation, butanol was the most potent inhibitor. Hence, this discussion will focus more on the effects of butanol than of the other products. At levels normally attained during fermentation (up to 18 g/l) both growth and solvent production were observed to be inhibited. This is in agreement with previous studies (Moreira et al, 1981; Costa and Moreira, 1983; Lin and Blaschek, 1983; Ounine et al, 1985; Kuhn and Linden, 1986). Results obtained from the present study on end-product inhibition of solvent production provide more legitimate evidence that the ABE fermentation is subject to end-product inhibition, since the cultures were allowed to establish before the toxic end-products were added. Inhibition of solvent

production, therefore, could not be attributed to inhibition of growth rate or growth yield, but only to the inhibitory effect of solvents on the solvent-producing machinery of the organisms.

Alcohols are known to exert antimicrobial activity through the disruption of the cell membrane structure of the microorganism (Harold, 1970; Ingram and Buttke, 1984). However, the exact mechanism of the chaotropic effect on the membrane bilayer is not known. The cell membrane acts as a permeability barrier and as an agent for selective transport or uptake of materials. The bacterial cell membrane also plays a key role in the generation of metabolic energy intermediates, one of which is the proton motive force, which can drive energy-requiring metabolic processes.

It has been shown that butanol addition inhibits growth by inhibiting glucose uptake of C. acetobutylicum cells (Bowles and Ellefson, 1985; Moreira et al, 1981; Ounine et al, 1985). The decrease in glucose uptake would result in a decreased growth rate, resulting in cell death and a decrease in fermentation activity. Little is known about the exact mechanism of glucose uptake in C. acetobutylicum. Moreira et al (1981) proposed that glucose transport is coupled to the hydrolysis of ATP by a membrane-bound ATPase, which brings about proton extrusion from the cell. This action of proton extrusion creates a proton motive force that drives the energy-dependent glucose transport. However, Bowles and Ellefson (1985) argued that glucose uptake in C. acetobutylicum is not mediated by an ATP-dependent system since the presence of 1 mM arsenate, which lowered the intracellular ATP levels by 80%, had no effect on the rate of glucose

uptake. Recently, Hutkins and Kashket (1986) demonstrated that C. acetobutylicum transports glucose by the phosphoenolpyruvate-dependent phosphotransferase system. Butanol concentration in the medium up to 2 % v/v did not inhibit the phosphotransferase activity, but caused cellular phosphoenolpyruvate to leak out of the cell due to the disruption of the cell membrane.

If butanol does not inhibit the phosphotransferase activity in C. acetobutylicum, it is quite unlikely that a defect in the phosphotransferase system in the mutant NG 12 can be postulated as an explanation for the reduced glucose uptake and solvent production observed in the previous chapter (Section 4.3.6). However, it may be postulated that the mutant is more sensitive to the disruptive effect of butanol because it could not efficiently produce a change in the fatty acid composition of its membrane in the presence of butanol. Microorganisms have been known to adjust their lipid composition to survive adverse environmental conditions. This response, known as homeoviscous adaptation, is believed to affect the physical changes caused by the environment and to permit the cell to maintain its membrane at the proper viscosity and surface ionic milieu for optimal cellular function. C. acetobutylicum has been observed to increase the ratio of saturated fatty acids to unsaturated fatty acid to counteract the fluidizing effect of butanol on the membrane (Baer et al, 1987; Kimelberg, 1977; Kuhn and Linden, 1986; Vollherbst-Schneck et al, 1984).

One result of this homeoviscous adaptation induced by the presence of alcohols is that the cell must expend energy to alter the composition of

this lipid membrane. This energy must be diverted from other processes, such as synthesis of new cell material. Cell yield, therefore, will be reduced in the presence of alcohol. This is in agreement with results observed in butanol-challenged experiments where NG 12 produced a lower growth yield than did P262, suggesting perhaps a greater diversion of energy.

Typically, bacteria also carry out homeostasis by which the cytoplasmic (intracellular) pH is maintained closer to neutrality than to the external medium. However, bacteria which produce weak acids such as acetic and butyric acids are unable to do this (Reibeling et al, 1975; Baronofsky et al, 1984). Instead, these bacteria maintain a relatively constant pH gradient across the membrane. In strictly fermentative bacteria, there are no electron transfer systems that function as proton pumps. In these organisms proton extrusion is carried out by the membrane-bound ATPase complex (Riebeling and Jungermann, 1976; Hellingwerf and Konings, 1985; Brink and Konings, 1986).

The chemiosmotic hypothesis of Mitchell (1961, 1966) postulates that proton translocation generates an electrochemical proton gradient, or proton motive force, across the cell membrane. This proton motive force consists of the membrane potential ($\Delta\psi$) and the pH gradient (ΔpH) with the inside of the cytoplasm electrically negative and alkaline, and the outside of the membrane electrically positive and acidic. The proton motive force can be used to drive endergonic cellular processes such as biosynthesis, motility, phosphotransferase-

mediated transport of sugars and transmembrane transport of protein (Hellingwerf and Konings, 1985).

The acetic acid and butyric acid produced during fermentation are weak organic acids which are able to diffuse passively across the cell membrane (Thauer et al, 1977; Baronofsky, et al, 1984; Herrero et al, 1985; Gottwald and Gottschalk, 1985). They are assumed to equilibrate on both sides of an energized membrane and to partition in the membrane bilayer. The undissociated acids diffuse passively into the cytoplasm. Since the intracellular pH is higher than that of the outside, the acids dissociate inside the cytoplasm. The anion moiety is effectively excluded to the outside of the cell, leaving behind a proton and thus reducing the prevailing ΔpH (Herrero et al 1985; Bahl and Gottschalk, 1985).

In the presence of a functioning membrane-localized ATPase, a functional ΔpH will be maintained. In C. acetobutylicum the physiological levels of acetate and butyrate do not affect the ΔpH. However, in the presence of growth-limiting butanol concentrations, the ability of C. acetobutylicum to maintain the ΔpH (interior alkaline) is destroyed (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985). Terraciano and Kashket (1986) found that in batch culture, the ΔpH of C. acetobutylicum did not decrease until accumulation of butanol occurred in the medium. Butanol was observed to inhibit the proton-translocating ATPase (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985) thereby inhibiting proton efflux.

Since the membrane-bound ATPase seems to play a key role in the growth and metabolism of C. acetobutylicum, the levels of ATPase activity in the parent and mutant strains were determined. The undetectable ATPase activity in the mutant may explain the differing butanol tolerance of the parent and mutant strains. Butanol itself acts as an uncoupler, that is, it enhances proton influx via the disruption of membrane integrity (Kuhn and Linden, 1986; Linden, 1987). Therefore, in the presence of butanol and the lack of a functional ATPase activity,the rate of proton efflux could not match the rate of proton influx leading to the collapse of the ΔpH in the mutant.

Thus far, butanol seems to exert its deleterious effect on two target sites, namely, the cell membrane as an osmotic barrier and the membrane-bound ATPase as a proton-translocating enzyme.

The complexity of the effects of butanol is emphasized in the results obtained from the study on end-product inhibition of solvent production. In the presence of added solvents, the mutant was less inhibited (on a percentage basis) than was the parent. The differing level of membrane-bound ATPase in the two organisms may account for this observed effect. Since ATPase is in an undetectable amount in the mutant, there is one target site less for butanol to act on. In the parent, the two target sites (cell membrane and ATPase) are present, hence, the effect of butanol seems more profound. Such an explanation may appear to be contradictory to the results obtained for the inhibition of growth. However, it may be possible that during active cell growth, the inhibitory effect of butanol due to membrane disruption is much more important than inhibition of the ATPase

activity (Linden, 1987). In contrast, after cell growth is completed, the effect on the activity of the ATPase becomes of increasing importance. Further, it is possible that during cell growth, some means of proton extrusion other than ATPase is actively involved.

It can also be speculated that in the mutant, one immediate effect of butanol addition (18 h after inoculation) was for it to act as an uncoupler, and thus, in the absence of a functioning ATPase lower the intracellular pH sufficiently so that solventogenesis commenced. A lowered intracellular pH is believed to be one of the triggers for solvent production (Gottwald and Gottschalk, 1985; Bowles and Ellefson, 1985; Huang et al, 1985; Terraciano and Kashket, 1986). In the parent, in contrast, the ATPase functions to remove protons from the cell, and thus the intracellular pH is lowered less quickly than in the mutant. Kuhn and Linden (1986) have shown that butanol enhanced the passive influx of protons into the cell. At added butanol concentrations of 0.05 M to 0.08 M (about 4 g/l to 6 g/l), proton influx into C. acetobutylicum cells was induced without any marked inhibition of growth rate. Through this proton influx, a low internal pH, a critical intracellular acid concentration and a pH gradient, which are essential to initiate solvent production, could be attained.

Another speculation can be made involving autolysin production in order to explain the lesser response of the mutant in this experiment. The release of cell-free autolysin was possibly not as great in the mutant as it was in the parent. Thus, inhibition of solvent production was less. Butanol toxicity has been linked to the autolytic degradation of solvent-producing cells in C. acetobutylicum (Van der Westhuizen et

al 1982; Souicalle et al, 1987). Barber et al (1979) suggested that inhibitory concentrations of butanol triggered the release of cell-free autolysin during solventogenesis. A synergistic effect between butanol and autolysin was proposed by Souicalle and Goma (1986), wherein butanol was assumed to cause greater permeability of the cell membrane to the bacteriocin. Either way, the lower amount of butanol produced by the mutant probably did not allow the production and effect of autolysin on solvent-producing cells in NG 12 as much as it did in the parent. A concentration of added butanol of approximately 10 g/l was probably sufficient to trigger production of autolysin in the mutant such that a cross-over effect was observed where the production by the mutant was more inhibited than was that of the parent (Fig. 5.16).

Furthermore, the low ATPase activity in NG 12 was confirmed in experiments using DCCD and protonophores. The effect of the addition of DCCD and protonophores to C. acetobutylicum cultures demonstrates the importance of the role of the membrane-bound ATPase to pump protons out of the cell and maintain a pH gradient essential for cellular functions. DCCD is a potent and highly selective inhibitor of the membrane-bound ATPase (Abrams and Smith, 1974; Riebeling and Jungermann, 1976). The uninhibited butanol production in the mutant confirms the lack or absence of a functional membrane-bound ATPase in this strain of organism. It is assumed that inhibition of ATPase in the parent strain resulted in the dissipation of the pH gradient. However, there appears to be only a partial dissipation since the decrease in butanol production in the presence of DCCD was not drastic. This is in agreement with the observation of Huang et al

(1985) that some other proton extrusion mechanism exists in C. acetobutylicum which may contribute to proton translocation at low pH values. It has been suggested that rubredoxin and NADH-rubredoxin oxidoreductase may participate in proton extrusion since their activity is highest only at low pH values (Huang et al, 1985).

Protonophores are compounds, usually lipid-soluble, which facilitate passive proton diffusion through the membranes. The exact mechanism by which proton conduction occurs in the presence of protonophores is unclear but they probably act as lipid-soluble proton carriers (Harold, 1970). It is expected that the presence of protonophores could create a proton pulse, increase the rate of proton entry into the cell and possibly dissipate the pH gradient. CCCP and FCCP have been known to completely abolish the pH gradient in C. acetobutylicum cells (Gottwald and Gottschalk, 1985; Huang et al, 1985). The presence of sub-inhibitory concentrations of CCCP and FCCP seemed to result only in partial dissipation of the pH gradient. The greater decrease in the initial rate of butanol production in the mutant than in the parent was not unexpected due to a greater dissipation of pH gradient in the mutant as a result of lower levels of ATPase activity. However, these protonophores did not seem to affect the final concentration of butanol produced by the mutant. It can be speculated that later in the solventogenic phase the sub-inhibitory concentrations of CCCP and FCCP were affecting the mutant towards enhancement of butanol production by creating a functional internal pH and pH gradient values. Huesemann and Papoutsakis (1986) obtained similar stimulation of butanol production using much lower concentrations of CCCP and FCCP on the parent strain or wild-type C.

acetobutylicum. The lower butanol production in the parent strain, in the presence of these protonophores, can be attributed simply to ATP drain. Since a functional ATPase was present in the parent strain, this enzyme was continuously active in the presence of a proton pulse to maintain or restore the membrane pH gradient. In other words, the presence of protonophores led to an increasing rate of proton motive force generation which, in turn, produced an increased rate of proton motive force consumption at the expense of ATP (Hellingwerf and Konings, 1985; Herrero et al, 1985).

Valinomycin is an antibiotic which increases the permeability of the membrane to alkali-metal ions, with greater perference for K^+. This antibiotic can also act as a highly selective carrier for H^+ if the pH values are low (Harold, 1970). The absence of drastic effect of valinomycin in either the parent or mutant strain probably indicates that it favored diffusion of K^+ across the membrane which did not affect the membrane pH gradient. Acetoacetate seemed to enhance butanol production in the mutant, in a similar way to CCCP and FCCP. Acetoacetate is taken up as a metabolic intermediate through the same mechanism as used for other organic acids, in which dissociation in the cytoplasm and anion exclusion leaves a proton inside the cell which is then excluded, creating a pH gradient favorable for butanol production. By virtue of a functional ATPase, addition of acetoacetate possibly created an ATP drain in the parent strain but not in the mutant.

At the concentration used, nigericin seemed to be a potent protonophore causing complete dissipation of the pH gradient and cessation of solvent production.

CHAPTER 6

FINAL DISCUSSION AND CONCLUSION

This thesis has examined the motile behaviour of C. acetobutylicum during the ABE fermentation. It has been demonstrated that motility during the fermentation is a chemotactic response of the cells towards nutrients and end-products (Chapter 3). C. acetobutylicum exhibits positive chemotaxis (attraction) toward sugars (glucose, galactose, and lactose) and the undissociated forms of acetic and butyric acids. This organism also exhibits negative chemotaxis (repulsion) from the solvents acetone, butanol and ethanol.

The chemotactic response to undissociated acids is contrary to that observed in E. coli (Kihara and Macnab, 1981; Repaske and Adler, 1981) and this can be explained as a survival mechanism of C. acetobutylicum to further metabolize these acids to neutral end-products before they reach inhibitory levels. Attraction to the undissociated acids occurs at a threshold concentration which is in the same region of intracellular concentration of acids, particularly that of butyric acid, where solvent production is initiated. Since both physiological (conversion of acids to solvents) and behavioural responses (chemotaxis) are elicited at this concentration of intracellular butyric acid, the most likely 'trigger' for the shift to solvent production seems to be the intracellular butyric acid concentration.

The relationship between motility and solvent production observed previously (Spivey, 1978; Gutierrez, 1985; Gutierrez and Maddox, 1987) has been confirmed and explained by the isolation of a non-motile mutant of C. acetobutylicum. The non-motile mutant was otherwise identical to the parent strain in all cell morphology aspects but which, although still solventogenic, exhibited a lower growth rate and produced lower butanol concentrations.

It can be concluded, therefore, that motile cells of C. acetobutylicum are more solventogenic because they can chemotactically respond to changes in their environment. Although nutrient transport is not required for chemotaxis to occur, chemotaxis to sugars leads the cells towards source of optimal energy. If cells are motile and chemotactic, they will migrate towards acetic and butyric acid, under conditions of low pH, to metabolize these acids to solvents in order to prevent the acids from reaching toxic intracellular levels.

The toxicity of the solvents, particularly butanol, has been demonstrated in Chapter 5. When toxic end-products start to accumulate, chemotaxis guides cells to migrate away from these end-products. Hence, the absence of motility or chemotaxis clearly puts the cells at a disadvantage since they become confined in an adverse environment. As a consequence non-motile or non-chemotactic cells appear to have lower butanol tolerance. Butanol is known to disrupt the cell membrane structure of microorganisms. Therefore in the presence of butanol, non-motile cells which cannot escape from the toxic effects of butanol are more subject to the chaotropic effect on the membrane. The non-motile cells then are expected to expend more

energy for homeoviscous adaptation to preserve cell membrane integrity which should otherwise be utilized for other energy-requiring cellular processes such as biosynthesis.

Chemotaxis, therefore, provides survival advantage to C. acetobutylicum.

The solvent inhibition studies have shown that the ABE fermentation is indeed subject to end-product toxicity and that the low butanol production observed with the non-motile mutant can be attributed to its lower butanol tolerance. The non-motile mutant was also observed to be deficient in the membrane-bound ATPase activity, and this may further explain its low solvent production. The membrane-bound ATPase mediates proton extrusion across the membrane and generates proton motive force. When ATPase activity is lacking, the rate of proton efflux would not match the rate of proton influx which is enhanced when the cell membrane is disrupted in the presence of butanol. This leads to the collapse of the pH gradient necessary for cellular functions.

The deficiency of membrane-bound ATPase in the mutant has been useful in highlighting the multiple effects of butanol. The apparent greater tolerance of the mutant to butanol inhibition of solvent production suggests that butanol toxicity during solventogenesis is more profound in the presence of two target sites, namely, the cell membrane and the membrane-bound ATPase. However, disruption of the membrane seems to be more important than toxicity to ATPase during active growth.

Although quantitative measurement of the intracellular pH, intracellular acids concentration and pH gradient in the non-motile mutant will be more conclusive, enhancement of butanol production in the ATPase-deficient mutant in the presence of protonophores has emphasized the importance of these interrelated factors in initiating solvent production.

However, the deficiency of ATPase in the non-motile mutant complicates the exact nature of the mutant. Loss of motility in *mot*$^-$ (paralyzed) mutants is not due to loss of the proton motive force that drives flagellar rotation, but to a defect in the conversion of proton motive force to rotary driving energy (Ishihara et aI, 1981). However, it is known that lack of detectable ATPase activity blocks anaerobic motility in *unc*$^-$ mutants of E. coli (Thipayathasana and Valentine, 1974). Whether the mutation in mutant NG 12 is in any of the genes which codes for flagellar rotation or in the gene which codes for ATPase, or in both genes (double mutation) is not known. Nor is it known whether proton motive force in NG 12 is lacking, but quantitative determination of its level will probably help identify the type of mutation. In any case, the usefulness of the non-motile mutant in identifying the relationship between motility and solvent production cannot be understated.

Chemoattraction to the acids and chemorepulsion from the solvents are behavioural responses which seem to be very crucial to solvent production, with the former linked to initiation of solventogenesis and the latter linked to solvent tolerance. Any alteration in the sensory information system will not offer any advantage to C. acetobutylicum.

Mutants blocked in sensing or adaptation to acids and solvents cannot respond appropriately or escape from their toxic effects. Generally the non-chemotactic (*che*) mutants (Chapter 1) are unable to carry out effective chemotactic responses to any compounds. All *che* mutants exhibit aberrant motility patterns and cannot regulate direction of flagellar rotation. A *che* mutant, therefore, that has either a running bias or a tumbling bias cannot regulate direction towards a more favorable environment. It would be useful, therefore, to identify the receptor genes for those crucial chemoeffectors, as this may help to improve the ABE fermentation process. Amplification of the chemoreceptor genes for the acids and solvents in C. acetobutylicum will probably improve both its solvent production and solvent tolerance.

LITERATURE CITED

Abram, D. and H. Koffler. 1964. In vitro formation of flagella-like filaments and other structure from flagellin. J. Mol. Biol. 9:168-185.

Abrams, A. and J. B. Smith. 1974. Bacterial membrane ATPase. In: The enzymes. vol. 10. P.D. Boyer (ed.) Academic Press. New York. San Francisco. London.

Adler, J. 1966. Effect of amino acids and oxygen on chemotaxis in Escherichia coli. J. Bacteriol. 92: 121-129.

Adler, J. 1969. Chemoreceptors in bacteria. Science 166: 1588-1597.

Adler, J. 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by Escherichia coli. J. Gen. Microbiol. 74: 77-91.

Adler, J. 1975. Chemotaxis in bacteria. Ann. Rev. Biochem. 44: 341-356.

Adler, H. I. and W. Crow. 1987. A technique for predicting the solvent-producing ability of Clostridium acetobutylicum. Appl. Environ. Microbiol. 53: 2496-2499.

Adler, J. and M. M. Dahl. 1967. A method for measuring the motility of bacteria and for comparing random and non-random motility. J. Gen. Microbiol. 46: 161-173.

Adler, J., G. L. Hazelbauer, and M. M. Dahl. 1973. Chemotaxis towards sugars in Escherichia coli. J. Bacteriol. 115: 824-847.

Adler, J. and B. Templeton. 1967. The effect of environmental conditions on the motility of Escherichia. coli. J. Gen. Microbiol. 46: 175-184.

Allcock, E. R., S. J. Reid, D. T. Jones, and D. R. Woods. 1982. Clostridium acetobutylicum protoplast formation and regeneration. Appl. Environ. Microbiol. 43: 719-721.

Andersch, W., H. Bahl, and G. Gottschalk. 1982. Acetone-butanol production by Clostridium acetobutylicum in an ammonium-limited chemostat at low pH values. Biotechnol. Lett. 4: 29-32.

Andersch, W., H. Bahl, and G. Gottschalk. 1983. Level of enzymes involved in acetate, butyrate, acetone and butanol formation by Clostridium acetobutylicum. Eur. J. Appl. Microbiol. Bitotechnol. 18: 327-332.

Ames, P., S. A. Schluederberg and K. Bergman. 1980. Behavioral mutants of Rhizobium meliloti. J. Bacteriol. 141: 722-727.

Armstrong, J. B. and J. Adler. 1967. Genetics of motility in Escherichia coli: complementation of paralyzed mutants. Genetics. 56: 363-373.

Armstrong, J. B. and J. Adler. 1969. Location of genes for motility and chemotaxis on the Escherichia coli genetic map. J. Bacteriol. 97: 156-161.

Armstrong, J. B., J. Adler, and M. M. Dahl. 1967. Nonchemotactic mutants of Escherichia coli. J. Bact. 93: 390-398.

Aswad, D. and D. E. Koshland, Jr. 1975. Evidence for an S-adenosylmethionine requirement in the chemotactic behaviour of Salmonella typhimurium. J. Mol. Biol. 97: 207-223.

Awang, G. M., G. A. Jones and W. M. Ingledew. 1988. The acetone-butanol-ethanol fermentation. Crit. Rev. Microbiol. 15: S33-S67.

Baer, S. J., H. P. Blaschek, and T. L. Smith. 1987. Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant Clostridium acetobutylicum. Appl. Environ. Microbiol. 53: 2854-2861.

Bahl, H., W. Andersch, K. Braun, and G. Gottschalk. 1982a. Effect of pH and butyrate concentration on the production of acetone and butanol by Clostridium acetobutylicum grown in continuous culture. Eur. J. Appl. Microbiol. Biotechnol. 14: 17-20.

Bahl, H., W. Andersch, and G. Gottschalk. 1982b. Continuous production of acetone and butanol by Clostridium acetobutylicum in a two-stage phosphate limited chemostat. Eur. J. Appl. Microbiol. Biotechnol. 15: 201-205.

Bahl, J. and G. Gottschalk. 1985. Energy partitioning effects during inhibition of anaerobic bacteria by their fermentation products. Biotech. Bioeng. Symp. 14: 215-223.

Barber, J. M., F. T. Robb, J. R. Webster, and D. R. Woods. 1979. Bacteriocin production by Clostridium acetobutylicum in an industrial fermentation process. Appl. Environ. Microbiol. 37: 433-437.

Baronofsky, J. J., W. J. A. Schreurs, and E. R. Kashket. 1984. Uncoupling by acetic acid limits growth and acetogenesis by Clostridium thermoaceticum. Appl. Environ. Microbiol. 48: 1134-1139.

Beesch, S. C. 1953. Acetone-butanol fermentation of starches. Appl. Microbiol. 1:85-96.

Berg, H. C. 1974. Dynamic properties of bacterial flagellar motors. Nature. 249: 77-79.

Berg, H. C. 1975. Bacterial behavior. Nature. 254: 389-392.

Berg, H. C. 1975. Chemotaxis in bacteria. Ann. Biophys. Biochem. 4: 119-136.

Berg, H. C. and R. A. Anderson. 1973. Bacteria swim by rotating their flagellar filaments. Nature. 245: 380-382.

Berg, H. C. and D. A. Brown. 1972. Chemotaxis in Escherichia coli analyzed by three-dimensional tracking. Nature. 239: 500-504.

Boyd, A. and M. Simon. 1982. Bacterial chemotaxis. Ann. Rev. Physiol. 44: 501-517.

Bowles, L. K., and W. L. Effefson. 1985. Effects of butanol on Clostridium acetobutylicum. Appl. Environ. Microbiol. 50: 1165-1170.

Bowra, B. J. and M. J. Dilworth. 1981. Motility and chemotaxis towards sugars in Rhizobium leguminosarum. J. Gen. Microbiol. 126: 231-235.

Bowring, S. N., and J. G. Morris. 1985. Mutagenesis of Clostridium acetobutylicum. J. Appl. Bacteriol. 58: 577-584.

Brass, J. M. and M. D. Mason. 1984. Reconstitution of maltose chemotaxis in Escherichia coli by addition of maltose-binding protein to calcium-treated cells of maltose regulation mutants. J. Bacteriol. 157: 881-890.

Brink, B. T. and W. N. Konings. 1986. Generation of a protonmotive force in anaerobic bacteria by end-product efflux. In: Methods in enzymology. vol. 125. S. Fleischer and B. Gleischer (ed.) Academic Press. New York.

Chelsky, D. and F. W. Dahlquist. 1980. Structural studies of methyl-accepting chemotaxis proteins of Escherichia coli: Evidence of multiple methylation sites. Proc. Nat. Acad. Sci. U.S.A. 77: 2434-2438.

Chet, I. and R. Mitchell. 1976. Ecological aspects of microbial chemotactic behaviour. Ann. Rev. Microbiol. 30: 221-239.

Clancy, M., K. A. Madill and J. M. Wood. 1981. Genetic and biochemical requirements for chemotaxis to L-proline in Escherichia coli. J. Bacteriol. 146: 902-906.

Clegg, D. O. and D. E. Koshland Jr. 1984. The role of a signaling protein in bacterial sensing: Behavioral effects of increased gene expression. Proc. Nat. Acad. Sci. U.S.A. 81: 5056-5060.

Conn, E. E. and P. K. Stumpf. 1976. Outlines of biochemistry. John Wiley and Sons. New York.

Compere, A. L., and W. L. Griffith. 1979. Evaluation of substrates for butanol production. Dev. Ind. Microbiol. 20: 509-517.

Costa, J. M. 1981. Solvent toxicity in the butanol-acetone fermentation. Proc. Annu. Biochem. Eng. 11: 83-90.

Costa, J. M., and A. R. Moreira. 1983. Growth inhibition kinetics of the acetone-butanol fermentation, p. 501-512. In: Foundations of biochemical engineering, kinetics and thermodynamics in biological systems. H. W. Blanch, E.T. Papoutsakis, and G. Stephanopoulos (ed.). ACS Symp. Ser. no. 207. American Chemical Society, Washington, D.C.

Craven, R. C. and T. C. Montie. 1983. Chemotaxis of Pseudomonas aeruginosa: involvement of methylation. J. Bacteriol. 154: 780-786.

Cummins, C. S. and J. L. Johnson. 1971. Taxonomy of the Clostridia: cell wall composition and DNA homologies in Clostridium butyricum and other butyric acid-producing Clostridia. J. Gen. Microbiol. 67: 33-46.

Dahlquist, F. W., P. Lovely, and D. E. Koshland, Jr. 1972. Quantitative analysis of bacterial migration in chemotaxis. Nature New Biol. 236: 120-123.

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

Datta, R., and J. G. Zeikus. 1985. Modulation of acetone-butanol-ethanol fermentation by carbon monoxide and organic acids. Appl. Environ. Microbiol. 49: 522-529.

Dean, G. E., R. Macnab, J. Stader, P. Matsumura, and C. Burks. 1984. Gene sequence and predicted amino acid sequence of the *mot* A protein, a membrane-associated protein required for flagellar rotation in Escherichia coli. J. Bacteriol. 159: 991-999.

DeFranco, A. T., J. S. Parkinson, and D. E. Koshland Jr. 1979. Functional homology of chemotaxis genes in Escherichia coli and Salmonella typhimurium. J. Bacteriol. 139: 107-114.

DeJong, M. H., C. van der Drift, and G. D. Vogels. 1976. Proton-motive force and the motile behaviour of Bacillus subtilis. Arch. Microbiol. 1: 7-11.

DePamphilis, M. L. and J. Adler. 1971. Purification of intact flagella from Escherichia coli and Bacillus subtilis. J. Bacteriol. 105: 376-383.

Dimmitt, K. and M. Simon. 1971. Purification and thermal stability of intact Bacillus subtilis flagella. J. Bacteriol. 105: 369-375.

Doelle, H. W. 1975. Bacterial metabolism, 2nd ed. Academic Press, Inc., New York.

Doremus, M. G., J. C. Linden and A. R. Moreira. 1985. Agitation and pressure effects on acetone-butanol fermentation. Biotechnol. Bioeng. 27: 852-860.

Eisenbach, M. 1982. Changes in membrane potential of Escherichia coli in response to temporal gradients of chemicals. Biochemistry. 21: 6818-6825.

Eisenbach, M. and J. Adler. 1981. Bacterial cell envelopes with functional flagella. J. Biol. Chem. 256: 8807-8814.

Eisenbach, M., Y. Margolin, and S. Ravid. 1985. Excitatory signaling in bacterial chemotaxis. In: Sensing and Response in Microorganisms. M. Eisenbach and M. Balaban (ed.) Elsevier Science Publishers. The Netherlands.

Engstrom, P. and G. L. Hazelbauer. 1982. Methyl-accepting chemotaxis proteins are distributed in the membrane independently from basal ends of bacterial flagella. Biochem. Biophys. Acta. 686: 19-26.

Ennis, B. M., N. A. Gutierrez, and I. S. Maddox. 1986. The acetone-butanol-ethanol fermentation: A current assessment. Process Biochem. 21: 131-147.

Ennis, B. M. and I. S. Maddox. 1985. Use of Clostridium acetobutylicum P262 for production of solvents from whey permeate. Biotechnol. Lett. 7:601-606.

Ennis, B. M. and I. S. Maddox. 1987. The effect of pH and lactose concentration on solvent production from whey permeate using Clostridium acetobutylicum. Biotech. Bioeng. 29:329-334.

Enomoto, M. 1966. Genetic studies of paralyzed mutants of Salmonella. I. Genetic fine structure of the *mot* loci in Salmonella typhimurium . Genetics. 54: 715-726.

Fond, O., G. Matta-Ammouri, H. Petitdemange, and J. M. Engasser. 1985. The role of acids on the production of acetone and butanol by Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 22: 195-200.

Fond, O., E. Petitdemange, H. Petitdemange, and R. Gay. 1984. Effect of glucose flow on the acetone butanol fermentation in fed batch culture. Biotechnol. Lett. 6: 13-18.

Forsberg, C. W., H. E. Schellhorn, L. N. Gibbins, F. Maine, and E. Mason. 1986. The release of fermentable carbohydrate from peat by steam explosion and its use in the microbial production of solvents. Biotech. Bioeng. 28: 176-184.

Gabriel, C. L. 1928. Butanol fermentation process. Ind. Eng. Chem. 20: 1063-1067.

Gabriel, C. L. and F. M. Crawford. 1930. Development of the butyl-acetonic fermentation industry. Ind. Eng. Chem. 22: 1163-1165.

Gapes, J. R., V. F. Larsen, and I. S. Maddox. 1983. A note on procedures for inoculum development for the production of solvents by a strain of Clostridium acetobutylicum. J. Appl. Bacteriol. 55: 363-365.

George, H. A., and J. S. Chen. 1983. Acidic conditions are not obligatory for onset of butanol formation by Clostridium beijerinckii (synonym, C. butylicum). Appl. Environ. Microbiol. 46: 321-327.

George, H. A., J. L. Johnson, W. E. C. Moore, L. V. Holdeman, and J. S. Chen. 1983. Acetone, isopropanol, and butanol production by Clostridium beijerinckii (syn, Clostridium butylicum) and Clostridium aurantibutyricum. Appl. Environ. Microbiol. 45: 1160-1163.

Glagolev, A. N. 1980. Cited in Taylor, B. L. 1983. Role of proton motive force in sensory transduction in bacteria. Ann. Rev. Microbiol. 37: 551-573.

Glagolev, A. N. and V. P. Skulachev, 1978. The proton pump is a molecular engine of motile bacteria. Nature. 272: 280-282.

Goldman, D. J., S. W. Worobec, R. B. Siegel, R. V. Hecker and G. W. Ordal. 1982. Chemotaxis in Bacillus subtilis: effects of attractants on the level of methylation and methyl-accepting chemotaxis proteins and the role of demethylation in the adaptation process. Biochemistry. 21: 915-920.

Gottschal, J. C. and J. G. Morris. 1981a. Nonproduction of acetone and butanol by Clostridium acetobutylicum during glucose-and ammonium-limitation in continuous culture. Biotechnol. Lett. 3: 525-530.

Gottschal, J. C. and J. G. Morris. 1981b. The induction of acetone and butanol production in cultures of Clostridium acetobutylicum by elevated concentrations of acetate and butyrate. FEMS Microbiol. Lett. 12: 385-389.

Gottschal, J. C. and J. G. Morris. 1982. Continuous production of acetone and butanol by Clostridium acetobutylicum growing in turbidostat culture. Biotechnol. Lett. 4: 477-482.

Gottschalk, G. and H. Bahl. 1981. Feasible improvements of the butanol production by Clostridium acetobutylicum. In: Trends in the biology of fermentation for fuels and chemicals. A. Hollaender, R. Rabson, P Rogers, A. San Pietro. R Valentine, and R. Wolfe (ed.). Plenum Publishing Corp., New York.

Gottwald, M. and G. Gottschalk. 1985. The internal pH of Clostridium acetobutylicum and its effects on the shift from acid to solvent formation. Arch. Microbiol. 143: 42-46.

Gottwald, M., H. Hippe, and G. Gottschalk. 1984. Formation of n-butanol from D-glucose by strains of "Clostridium tetanomorphum" group. Appl. Environ. Microbiol. 48: 573-576.

Goulbourne, E. A. Jr and E. P. Greenberg. 1981. Chemotaxis of Spirochaeta aurantia: involvement of membrane potential in chemosensory signal trasduction. J. Bacteriol. 148: 837-844.

Graves, M. C., G. T. Mullenbach, and J. C. Rabinowitz. 1985. Cloning and nucleotide sequence determination of the Clostridium pasteurianum ferredoxin gene. Proc. Natl. Acad. Sci. U.S.A. 82: 1653-1657.

Gutierrez, N. A. 1985. Methodology of culture maintenance and inoculum development for production of solvents by Clostridium acetobutylicum. M.Tech. Thesis. Massey University, Palmerston North, New Zealand.

Gutierrez, N. A. and I. S. Maddox. 1987. The effect of some culture maintenance and inoculum development techniques on solvent production by Clostridium acetobutylicum. Can. J. Microbiol. 33: 82-84.

Haggstrom, L. and C. Forberg. 1986. Significance of an extracellular polymer for the energy metabolism in Clostridium acetobutylicum: a hypothesis. Appl. Microbiol. Biotechnol. 23: 234-239.

Harold, F. M. 1970. Antimicrobial agents and membrane function. Adv. Microb. Physiol. 4: 45-104.

Harrigan, W.F. and M. E. McCance. 1966. Laboratory methods in food and dairy microbiology. Academic Press. London. New York. San Francisco.

Hartmanis, M. G. N. and S. Gatenbeck. 1984. Intermediary metabolism in Clostridium acetobutylicum: levels of enzymes involved in the formation of acetate and butyrate. Appl. Environ. Microbiol. 47: 1277-1283.

Hartmanis, M. G. N., T. Klason, and S. Gatenback. 1984. Uptake and activation of acetate and butyrate in Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 20: 66-71.

Hastings, J. H. J. 1978. Acetone-butyl alcohol fermentation. In: Economic microbiology. vol. 2. Primary products of metabolism. A. H. Rose (ed.). Academic Press, Inc., New York.

Hellingwerf, K. J. and W. N. Konings. 1985. The energy flow in bacteria: the main free energy intermediates and their regulatory role. Adv. Microb. Physiol. 26: 125-154.

Herrero, A. A. 1983. End-product inhibition in anaerobic fermentations. Trends Biotechnol. 1:49-53.

Herrero, A. A., R. F. Gomez, B. Snedecor, C. J. Tolman, and M. F. Roberts. 1985. Growth inhibition of Clostridium thermocellum by carboxylic acids: a mechanism based on uncoupling by weak acids. Appl. Microbiol. Biotechnol. 22: 53-62.

Holt, R. A., G. M. Stephens, and J. G. Morris. 1984. Production of solvents by Clostridium acetobutylicum cultures maintained at neutral pH. Appl. Environ. Microbiol. 48: 1166-1170.

Hoeniger, J. F. M. 1964. Cellular changes accompanying the swarming of Proteus mirabilis. Can. J. Microbiol. 10: 1-9.

Huang, L., L. N. Gibbins, and C. W. Forsberg. 1985. Transmembrane pH gradient and membrane potential in Clostridium acetobutylicum during growth under acetogenic and solventogenic conditions. Appl. Environ. Microbiol. 50: 1043-1047.

Huesemann, M., and E. T. Papoutsakis. 1986. Effect of acetoacetate, butyrate and uncoupling ionophores on growth and product formation of Clostridium acetobutylicum. Biotechnol. Lett. 8: 37-42.

Hutkins, R. W. and E. R. Kashket. 1986. Phosphotransferase activity in Clostridium acetobutylicum from acidogenic and solventogenic phases of growth. Appl. Environ. Microbiol. 51: 1121-1123.

Ingram, L. O. and T. M. Buttke. 1984. Effects of alcohols on microorganisms. Adv. Microb. Physiol. 25:253-300.

Iino. T. 1977. Genetics of structure and function of bacterial flagella. Ann. Rev. Genet. 11: 161-182.

Ishihara, A. , S. Yamaguchi, and H. Hotani. 1981. Passive rotation of flagella on paralyzed Salmonella typhimurium *(mot)* mutants by external rotatory driving force. J. Bacteriol. 145: 1082-1084.

Ishii, K., T. Kundo, H. Honda, and K. Horikoshi. 1983. Molecular cloning of b-isopropylmalate dehydrogenase gene from Clostridium butyricum M588. Agric. Biol. Chem. 47: 2313-2317.

Jones, D. T., W. A. Jones, and D. R. Woods. 1985. Production of recombinants after protoplast fusion in Clostridium acetobutylicum P262. J. Gen. Microbiol. 131: 1213-1216.

Jones, D. T., A. van der Westhuizen, S. Long, E. R. Allcock, S. J. Reid, and D. R. Woods. 1982. Solvent production and morphological changes in Clostridium acetobutylicum. Appl. Environ. Microbiol. 43: 1434-1439.

Jones, D. T. and D. R. Woods. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50: 484-524.

Karube, I., N. Urano.,T. Yamada, H. Hirochika, and K. Sakaguchi. 1983. Cloning and expression of the hydrogenase gene from Clostridium butyricum in Escherichia coli. FEBS Lett. 158: 119-122.

Kehry, M. R., T. G. Doak, and F. W. Dahlquist. 1985. Aberrant regulation of methylesterase activity in *che* D chemotaxis mutants of Escherichia coli. J. Bacteriol. 161: 105-112.

Kennedy, M. J. and J. G. Lawless. 1985. Role of chemotaxis in the ecology of denitrifiers. Appl. Environ. Microbiol. 49: 109-114.

Khan, S. and H. C. Berg. 1983. Isotope and thermal effects in chemiosmotic coupling to the flagellar motor of Streptococcus. Cell. 32: 913-919.

Khan, S. and R. M. Macnab, 1980. Proton chemical potential, proton electrical potential and bacterial motility. J. Mol. Biol. 138: 599-614.

Khan, S., R. M Macnab, A. L. DeFranco, and D. E. Koshland Jr. 1978. Inversion of a behavioral response in bacterial chemotaxis: explanation at a molecular level. Proc. Nat. Acad. Sci. U.S.A. 75: 4150-4154.

Kihara, M. and R. M. Macnab, 1979. Chemotaxis of Salmonella typhimurium towards citrate. J. Bacteriol. 140: 297-300.

Kihara, M. and R. M. Macnab. 1981. Cytoplasmic pH mediates pH taxis and weak-acid repellent taxis of bacteria. J. Bacteriol. 145: 1209-1221.

Kim, B. H., P. Bellows, R. Datta, and J. G. Zeikus. 1984. Control of carbon and electron flow in Clostridium acetobutylicum fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl. Environ. Microbiol. 48: 764-770.

Kimelberg, H. K. 1977. The influence of membrane fluidity on the activity of membrane-bound enzymes. Cell Surface Rev. 3: 205-293.

Kim, T. S. and B. Y. Kim. 1988. Electron flow shift in Clostridium acetobutylicum fermentation by electrochemically introduced reducing equivalent. Biotechnol. Lett. 10: 123-128.

Kim, B. H., and J. G. Zeikus. 1985. Importance of hydrogen metabolism in regulation of solventogenesis by Clostridium acetobutylicum. Dev. Ind. Microbiol. 26: 1-14.

Koshland, D. E. Jr. 1980. Bacterial chemotaxis as a model behavior system. Raven Press. New York.

Koshland, D. E., Jr. 1982. Special topic: chemotaxis and motility. Ann. Rev. Physiol. 44: 499-500.

Krieg, N. R., J. P. Tomelty and J. S. Wells Jr. 1967. Inhibition of flagellar coordination in Spirillum volutans. J. Bacteriol. 94: 1431-1436.

Kuhn, R. H. and J. C. Linden, 1986. Effects of temperature and membrane fatty acid composition on butanol tolerance of Clostridium acetobutylicum. Biotech. Bioeng. Symp. No. 17: 197-207.

Kung, C. and Y. Saimi, 1982. The physiological basis of taxes in Paramecium. Ann. Rev. Physiol. 44: 519-534.

Kuo, S. C. and D. E. Koshland Jr. 1986. Sequence of the *fla* A (*che* C) locus of Escherichia coli and discovery of a new gene. J. Bacteriol. 166: 1007-1012.

Larsen, S. H., J. Adler, J. J. Gargus, and R. W. Hogg. 1974a. Chemomechanical coupling without ATP: The source energy for motility and chemotaxis in bacteria. Proc. Nat. Acad. Sci. U. S. A. 71: 1239-1243.

Larsen, S. H., R. W. Reader, E. N. Kort, W.-W. Tso, and J. Adler. 1974b. Change in direction of flagella rotation is the basis of the chemotactic response in Escherichia coli. Nature. 249: 74-77.

Lauger, P. 1977. Ion transport and rotation of bacterial flagella. Nature. 268: 360-362.

Lemmel, S. A. 1985. Mutagenesis in Clostridium acetobutylicum. Biotechnol. Lett. 7: 711-716.

Lenz, T. G. and A. R. Moreira. 1980. Economic evaluation of the acetone-butanol fermentation. Ind. Eng. Chem. Prod. Res. Dev. 19: 478-483.

Leung, J. C. Y., and D. I. C. Wang. 1981. Production of acetone and butanol by Clostridium acetobutylicum in continuous culture using free cells and immobilized cells. Proc. 2nd World Congr. Chem. Eng. 1: 348-352.

Lapage, C., F. Fayoulle, M. Hermann, and J.-P. Vandecasteele. 1987. Changes in membrane lipid compositon of Clostridium acetobutylicum during acetone-butanol fermentation: effects of solvents, growth temperature and pH. J. Gen. Microbiol. 133: 103-110.

Lin, Y., and H. P. Blaschek. 1982. Butanol production by a butanol-tolerant strain of Clostridium acetobutylicum in extruded corn broth. Appl. Environ. Microbiol. 45: 966-973.

Lin, Y. L., and H. P. Blaschek. 1984. Transformation of heat-treated Clostridium acetobutylicum protoplasts with pUB110 plasmid DNA. Appl. Environ. Microbiol. 48: 737-742.

Linden, J. C. 1987. Biochemistry of alcohol effects of Clostridium acetobutylicum. Proc. 4th European Congr. Biotechnol. 3: 247-250.

Linden, J. C., A. R. Moreira, and T. G. Lenz. 1986. Acetone and butanol. In: Comprehensive biotechnology. The principles of biotechnology: engineering consideration. C. L. Cooney and A. E. Humphrey (ed.) p. 915-931. Pergamon Press, Oxford.

Long, S., D. T. Jones and D. R. Woods. 1983. Sporulation of Clostridium acetobutylicum P262 in a defined medium. Appl. Environ. Microbiol. 45: 1389-1393.

Long, S., D. T. Jones and D. R. Woods. 1984a. Initiation of solvent production, clostridial stage and endospore formation in Clostridium acetobutylicum P262. Appl. Microbiol. Biotechnol. 20: 256-261.

Long, S., D. T. Jones and D. R. Woods. 1984b. The relationship between sporulation and solvent production in Clostridium acetobutylicum P262. Biotechnol. Lett. 6: 529-534.

Lovely, P. and F. W. Dahlquist. 1974. Instrument for recording the motions of microrganisms in chemical gradients. Rev. Sci. Instrum. 45: 683-686.

Lovitt, R. W., B. H. Kim, G.-J. Shen and J. G. Zeikus. 1988. Solvent production by microorganisms. CRC Crit. Rev. Biotech. 7: 107-170.

Luczak, H., H. Schwarzmoser, and W. L. Staudenbauer. 1985. Construction of Clostridium butyricum hybrid plasmids and transfer to Bacillus subtilis. Appl. Microbiol. Biotechnol. 23: 114-122.

Macnab, R. M. 1978. Bacterial motility and chemotaxis: the molecular biology of a behavioral system. CRC Crit. Rev. Biochem. 5: 291-341.

Macnab, R. M. and S.-I. Aizawa. 1984. Bacterial motility and the bacterial flagellar motor. Ann. Rev. Biophys. Bioeng. 13: 51-83.

Macnab, R. M. and D. E. Koshland, Jr. 1972. The gradient-sensing mechanism in bacterial chemotaxis. Proc. Nat. Acad. Sci. U.S.A. 69: 2509-2512.

Maddox, I. S., J. R. Gapes and V. F. Larsen. 1981. Production of n-butanol from whey ultrafiltrate. In: Proceding of the 9th Australasian Conference on Chemical Engineering. Christchurch, New Zealand. p. 535-542.

Maddox, I. S. 1980. Production of n-butanol from whey filtrate using Clostridium acetobutylicum NCIB 2951. Biotechnol. Lett. 2: 493-498.

Manson, M. D., P. M. Tedesco, H. C. Berg, F. M. Harold, and C. van der Drift. 1977. A proton motive force drives bacterial flagella. Proc. Nat. Sci. U.S.A. 74: 3060-3064.

Manson, M. D., P. M. Tedesco, and H. C. Berg. 1980. Energetics of flagellar rotation in bacteria. J. Mol. Biol. 138: 541-561.

Marchal, R., D. Blanchet and J. P. Vandecasteele. 1985. Industrial optimization of acetone-butanol fermentation: a study of the utilization of Jerusalem artichokes. Appl. Microbiol. Biotechnol. 23: 92-98.

Marchal, R., M. Rebeller, and J. P. Vandecasteele. 1984. Direct bioconversion of alkali-pretreated straw using simultaneous enzymatic hydrolysis and acetone-butanol fermentation. Biotechnol. Lett. 6: 523-528.

Matthews, M. E., R. K. Doughty, and I. R. Hughes. 1978. Performance characteristics of a plate and frame system for ultrafiltration of sulphuric acid casein whey. N.Z. J. Dairy Sci. Technol. 13: 37-42.

Matsuura, S., J. Shioi, and Y. Imae. 1977. Motility in Bacillus subtilis driven by an artificial protonmotive force. FEBS Lett. 82: 187-190.

McCutchan, W. N. and R. J. Hickey. 1954. The butanol-acetone fermentations. Ind. Ferment. 1: 347-388.

McNeil, B. and B. Kristiansen. 1986. The acetone butanol fermentation. Adv. Appl. Microbiol. 31: 61-93.

Meinecke, B., H. Bahl, and G. Gottschalk. 1984. Selection of an asporogenous strain of Clostridium acetobutylicum in continuous culture under phosphate limitation. Appl. Environ. Microbiol. 48: 1064-1065.

Melton, T., P. E. Hartman, J. P. Stratis, T. I. Lee and A. T. Davis. 1978. Chemotaxis of Salmonella typhimurium to amino acids and some sugars. J. Bacteriol. 138: 708-716.

Mes-Hartree, M., and J. N. Saddler. 1982. Butanol production of Clostridium acetobutylicum grown on sugars found in hemicellulose hydrolysates. Biotechnol. Lett. 4: 247-252.

Mesibov, R., and J. Adler. 1972. Chemotaxis toward amino acids in Escherichia coli. J. Bacteriol. 112: 315-326.

Meyer, C. L., J. K. McLaughlin, and E. T. Papoutsakis. 1985. The effect of CO on growth and product formation in batch cultures of Clostridium acetobutylicum. Biotechnol. Lett. 7: 37-42.

Miller, J. B. and D. E. Koshland, Jr. 1977. Bacterial electrophysiology: Relationship of the membrane potential to motility and chemotaxis in Bacillus subtilis. Proc. Nat. Sci. U.S.A. 74: 4752-4756.

Millet, J., D. Petre, P. Beguin, O. Raynaud, and J. Aubert. 1985. Cloning of ten distinct DNA fragment of Clostridium thermocellum coding for cellulases. FEMS Microbiol. Lett. 29: 145-149.

Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature. 191: 144-148.

Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. 41: 445-502.

Moench, T. T. and W. A. Konetzka. 1978. Chemotaxis in Pseudomonas aeruginosa. J. Bacteriol. 133: 427-429.

Monot, F. and J. M. Engasser. 1983. Production of acetone and butanol by batch and continuous culture of Clostridium acetobutylicum under nitrogen limitation. Biotechnol. Lett. 5: 213-218.

Monot F., J. M. Engasser, and H. Petitdemange. 1983. Regulation of acetone butanol production in batch and continuous cultures of Clostridium acetobutylicum. Biotechnol. Bioeng. Symp. 13: 207-216.

Monot, F., J. M. Engasser, and H. Petitdemange. 1984. Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of Clostridium acetobutylicum. Appl. Microbiol. Biotechnol. 19: 422-426.

Monot, F., J. R. Martin, H. Petitdemange, and R. Gay. 1982. Acetone and butanol production by Clostridium acetobutylicum in a synthetic medium. Appl. Environ. Microbiol. 44: 1318-1324.

Moreira, A. R. 1983. Acetone-butanol fermentation, p. 385-406. In: Organic chemicals from biomasss. D. L. Wise (ed.) Benjamin/Cummings Publishing Co. Inc. Menlo Park. Calif.

Moreira, A. R., D. C. Ulmer, and J. C. Linden. 1981. Butanol toxicity in the butylic fermentation. Biotechnol. Bioeng. Symp. 11: 567-579.

Moulton, R. C. and T. C. Montie. 1979. Chemotaxis by Pseudomonas aeruginosa. J. Bacteriol. 137: 274-280.

Nakamura, S., I. Okado, T. Abe, and S. Nishida. 1979. Taxonomy of Clostridium tetani and related species. J. Gen. Microbiol. 113: 29-35.

Nishio, N., H. Biebl, and M. Meiners. 1983. Effect of pH on the production of acetone and butanol by Clostridium acetobutylicum in a minimum medium. J. Ferment. Technol. 61: 101-104.

Niwano, M. and B. L. Taylor. 1982. Novel sensory adaptation mechanism in bacterial chemotaxis to oxygen and phosphotransferase substrates. Proc. Nat. Acad. Sci. U.S.A. 79: 11-15.

Ogata, S., and M. Hongo. 1979. Bacteriophages of the genus Clostridium. Adv. Appl. Microbiol. 25: 241-273.

Ordal, G. W., D. P. Villani, and K. J. Gibson. 1977. Amino acid chemoreceptors of Bacillus subtilis. J. Bacteriol. 129: 156-165.

Ordal, G. W. 1985. Bacterial chemotaxis: biochemistry of behaviour in a single cell. CRC Crit. Rev. Microbiol. 12: 95-108.

Oultram, J. D., and M. Young. 1985. Conjugal transfer of plasmid pAMB1 from Streptococcus lactis and Bacillus subtilis to Clostridium acetobutylicum. FEMS Microbiol. Lett. 27: 129-134.

Ounine, K., H. Petitdemange, G. Raval, and R. Gay. 1985. Regulation and butanol inhibition of D-xylose and D-glucose uptake in Clostridium acetobutylicum. Appl. Environ. Microbiol. 49: 874-878.

Papoutsakis, E. T. 1984. Equations and calculations for fermentations of butyric acid bacteria. Biotechnol. Bioeng. 26: 174-187.

Parke, D., M. Rivelli and L. N. Ornston. 1985. Chemotaxis to aromatic and hydroaromatic acids: comparison of Bradyrhizobium japonicum and Rhizobium trifolii. J. Bacteriol. 163: 417-422.

Parkinson, J. S. 1976. *che* A, *che* B and *che* C genes of Escherichia coli and their role in chemotaxis. J. Bacteriol. 126: 758-770.

Parkinson, J. S. 1981. Genetics of bacterial chemotaxis. Soc. Gen. Microbiol. Sym. 31: 265-290.

Petitdemange, H. and R. Gay. 1986. Biochemical mechanism of the acetone-butanol fermentation and future prospects. In: Proceedings 3rd International Conference, Biotechnology in the Pulp and Paper Industry. Stockholm.

Prescott, S. G., and C. G. Dunn. 1959. Industrial microbiology, 3rd ed. McGraw-Hill Book Co., New York.

Prevot, A. R. 1966. Manual for the classification and determination of the anaerobic bacteria. Lea and Febiger. Philadelphia.

Rao, G. and R. Mutharasan. 1986. Alcohol production by Clostridium acetobutylicum induced by methyl viologen. Biotechnol. Lett. 8: 893-896.

Rao, G. and R. Mutharasan. 1987. Altered electron flow in continuous cultures of Clostridium acetobutylicum induced by viologen dyes. Appl. Environ. Microbiol. 53: 1232-1235.

Rao, G. and R. Mutharasan. 1989. NADH levels and solventogenesis in Clostridium acetobutylicum: new insights through culture fluorescence. Appl. Microbiol. Biotechnol. 30: 59-66.

Ravid, S. and M. Eisenbach. 1984a. Direction of flagellar rotation in bacterial cell envelopes. J. Bacteriol. 158: 222-230.

Ravid, S. and M. Eisenbach. 1984b. Minimal requirements for rotation of bacterial flagella. J. Bacteriol. 158: 1208-1210.

Reid, S. J., E. R. Allcock, D. T. Jones, and D. R. Woods. 1983. Transformation of Clostridium acetobutylicum protoplasts with bacteriophage DNA. Appl. Environ. Microbiol. 45: 305-307.

Repaske, D. R. and J. Adler. 1981. Change in intracellular pH of Escherichia coli mediates the chemotactic response to certain attractant and repellents. J. Bacteriol. 145: 1196-1208.

Reysenbach, A. L. 1987. The intracellular reserve polysaccharide of Clostridium acetobutylicum P262. Ph.D Thesis. University of Cape Town. Cape Town, South Africa.

Reysenbach, A. L., N. Ravenscroft, S. Long, D. T. Jones and D. R. Woods. 1986. Characterization, biosynthesis and regulation of granulose in Clostridium acetobutylicum. Appl. Environ. Microbiol. 52: 275-281.

Reysset, G. and M. Sebald. 1985. Conjugal transfer of plasmid-mediated antibiotic resistance from streptococci to Clostridium acetobutylicum. Ann. Inst. Microbiol. 136B.

Riebeling, V. and K. Jungermann. 1976. Properties and function of clostridial membrane ATPase. Biochim. Biophys. Acta. 430: 434-444.

Riebeling, V., R. K. Thauer, and K. Jungermann. 1975. The internal-alkaline pH gradient, sensitive to uncoupler and ATPase inhibitor, in growing Clostridium acetobutylicum. Eur. J. Biochem. 55: 445-453.

Rogers, P. 1986. Genetics and biochemistry of Clostridium relevant to development of fermentation processes. Adv. Appl. Microbiol. 21: 1-60.

Roos, J. W., J. McLaughlin, and E. T. Papoutsakis. 1985. The effect of pH on nitrogen supply cell lysis, and solvent production in fermentations of Clostridium acetobutylicum. Biotechnol. Bioeng. 27: 681-694.

Rose, A. H. 1961. Industrial microbiology. Butterworths, London.

Rubik, B. A. and D. E. Koshland Jr. 1978. Potentiation, desensitization and inversion of response in bacterial sensing of chemical stimuli. Proc. Nat. Acad. Sci. U.S. A. 75: 2820-2824.

Russel, A. D. 1982. The destruction of bacterial spores. Academic Press. London.

Saddler, J. N., E. K. C. Yu, M. Mes-Hartree, N. Levitin, and H. H. Brownell. 1983. Utilization of enzymaticaly hydrolyzed wood hemicelulloses by microorganisms for production of liquid fuels. Appl. Environ. Microbiol. 45: 153-160.

S z melcman, S. and J. Adler. 1976. Change in membrane protential during bacterial chemotaxis. Proc. Nat. Acad. Sci. U.S.A. 73: 4387-4391.

Schneider, W. R. and R. N. Doetsch. 1974. Velocity measurements of motile bacteria by use of a videotape recording technique. Appl. Microbiol. 27: 283-284.

Schoutens, G. H., M. C. H. Nieuwenhuizen, and N. W. F. Kossen. 1984. Butanol from whey ultrafiltrate: batch experiments with Clostridium beyerinckii LMD 27.6 Appl. Microbiol. Biotoechnol. 19: 203-206.

Sclonzewski, J. L., R. M. Macnab, J. R. Alger, and A. N. Castle. 1982. Effects of pH and repellent tactic stimuli on protein methylation levels in Escherichia coli. J. Bacteriol. 152: 384-399.

Shioi, J., Y. Imae, and F. Oosawa. 1978. Proton motive force and motility of Bacillus subtilis. J. Bacteriol. 133: 1083-1088.

Shioi, J., S. Matsuura, and Y. Imae. 1980. Quantitative measurements of proton motive force and motility in Bacillus subtilis. J. Bacteriol. 144: 891-897.

Silverman, M. and M. Simon. 1974. Flagellar rotation and the mechanism of bacterial motility. Nature. 249: 73-74.

Silverman, M. and Simon. 1973. Genetic analysis of flagellar mutants in Escherichia coli. J. Bacteriol. 116: 114-122.

Silverman, M. and M. Simon. 1976. Operon controlling motility and chemotaxis in Escherichia coli. J. Bacteriol. 130: 1317-1325.

Silverman, M and M. Simon. 1977. Bacterial flagella. Ann. Rev. Microbiol. 31: 397-419.

Smith, A. G. and P. D. Ellner. 1956. Cytological observations on the sporulation process of Clostridium perfringens. J. Bacteriol. 73: 1-7.

Smith, L. D. S. and G. Hobbs. 1974. In: Bergey's Manual of Determinative Bacteriology. R. E. Buchanan and N. E. Gibbons (ed.). The Williams and Wilkins Co. Baltimore.

Snyder, M. A., J. B. Stock and D. E. Koshland, Jr. 1981. Role of membrane potential and calcium in chemotactic sensing by bacteria. J. Mol. Biol. 149: 241-257.

Soucaille, P. and G. Goma. 1986. Acetonobutylic fermentation by Clostridium acetobutylicum ATCC 824: autobacteriocin production, properties and effects. Curr. Microbiol. 13: 163-169.

Soucaille, P., J. Gwenael, A. Izard, and G. Goma. 1987. Butanol tolerance and autobacteriocin production by Clostridium acetobutylicum. Curr. Microbiol. 14: 295-299.

Spivey, M. J. 1978. The acetone/butanol/ethanol fermentation. Process Biochem. 13: 2-5.

Springer, M. S., M. F. Goy, and J. Adler. 1977. Sensory transduction in Escherichia coli: A requirement for methionine in sensory adaptation. Proc. Nat. Acad. Sci. U.S.A. 74: 183-187.

Springer, W. R. and D. E. Koshland, Jr. 1977. Identification of a protein methyltransferase as the *che* R gene product in the bacterial sensing system. Proc. Nat. Acad. Sci. U.S.A. 74: 533-537.

Springer, B. and J. T. Kusmierek. 1982. Chemical mutagenesis. Ann. Rev. Biochem. 51: 655-693.

Stewart, R. C. and F. W. Dahlquist. 1987. Molecular components of bacterial chemotaxis. 87: 997-1025.

Stock, J. B., S. Clarke, and D. E. Koshland, Jr. 1984. The protein carboxylmethyltransferase involved in Escherichia coli and Salmonella typhimurium chemotaxis. Methods Enzymol. 106: 310-321.

Stock, J. B. and D. E. Koshland, Jr. 1978. A protein methylesterase involved in bacterial sensing. Proc. Nat. Acad. Sci. U.S.A. 75: 3659-3663.

Taylor, B. L. 1983. Role of proton motive force in sensory transduction in bacteria. Ann. Rev. Microbiol. 37: 551-573.

Terraciano, J. S., and E. R. Kashket, 1986. Intracellular conditions required for initiation of solvent production by Clostridium acetobutylicum. Appl. Environ. Microbiol. 52: 86-91.

Thauer, R. K., K. Jungermann, and K. Dekker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41: 100-180.

Thipayathasana, P. and R. C. Valentine. 1974. The requirement for energy transducing ATPase for anaerobic motility in Escherichia coli. Biochem. Biophys. Acta. 347: 464-468.

Truffaut, N., and M Sebald. 1983. Plasmid detection and isolation in strains of Clostridium acetobutylicum and related species. Mol. Gen. Genet. 189: 178-180.

Tsang, N., R. Macnab, and D. E. Koshland, Jr. 1973. Common mechanism for repellents and attractants in bacterial chemotaxis. Science. 181: 60-63.

Tso, W.-W. and J. Adler. 1974. Negative chemotaxis in Escherichia coli. J. Bacteriol. 118: 560-576.

Urano, N., I. Karube, S. Suzuki, T. Yamada, H. Hirochika. and K. Sakaguchi. 1983. Isolation and partial characterization of large plasmids in hydrogen-evolving bacterium Clostridium butyricum. Eur. J. Appl. Microbiol. Biotechnol. 17: 349-354.

Usdin, K. P., H. Zappe, D. T. Jones, and D. R. Woods. 1986. Cloning expression and purification of glutamine synthetase from Clostridium acetobutylicum. Appl. Environ. Microbiol. 52: 413-419.

Vaitazus, Z. and R. N. Doetsch. 1969. Motility tracks: technique for quantitative study of bacterial movement. Appl. Microbiol. 17: 584-588.

Van der Westhuizen, A., D. T. Jones, and D. R. Woods. 1982. Autolytic activity and butanol tolerance of Clostridium acetobutylicum. Appl. Environ. Microbiol. 44: 1277-1282.

Vogel, I. A. 1961. A textbook of quantitative inorganic analysis. Longman. London.

Volesky, B., and T. Szczesny. 1983. Bacterial conversion of pentose sugars to acetone and butanol. Adv. Biochem. Eng. Biotechnol. 27: 101-117.

Vollherbst-Schneck, K, J. A. Sands, and B. S. Montenecourt. 1984. Effect of butanol on lipid composition and fluidity of Clostridium acetobutylicum ATCC 824. Appl. Environ. Microbiol. 47: 193-194.

Walton, M. T., and J. L. Martin 1979. Production of butanol-acetone by fermentation. Microbial technology. 2nd ed., vol. 1. H. J. Peppler and D. Perlman (ed.). Academic Press Inc., New York.

Wayman, M., and S. Yu. 1985. Acetone-butanol fermentation of xylose and sugar mixtures. Biotechnol. Lett. 7: 255-260.

Weilbull, C. 1960. Movement. In: The Bacteria. vol. 1. Gunsalus, I. and R. Y. Stanier (ed.). Academic Press. New York.

Welsh, F. W., and I. A. Veliky. 1984. Production of acetone-butanol from acid whey. Bitoechnol. Lett. 6: 61-64.

Yamaguchi, S., H. Fujita, A. Ishihara, S.-I. Aizawa. and R. Macnab. 1986. Subdivision of flagellar genes of Salmonella typhimurium into regions responsible for assembly, rotation and switching. J. Bacteriol. 166: 187-193.

Yu, E. K. C., M. K. H. Chan and J. N. Saddler, 1985. Butanol production from cellulosic substrates by sequential co-culture of Clostridium thermocellum and C. acetobutylicum. Biotechnol. Lett. 7: 509-514.

Yu, E. K. C., N. Levitin, and J. N. Saddler. 1984 Utilization of wood hemicellulose hydrolyzates by microorganisms for the production of liquid fuels and chemicals. Dev. Ind. Microbiol. 25: 613-620.

Yu, P.-L. and L. E. Pearce. 1986. Conjugal transfer of streptococcal antibiotic resistance plasmids into Clostridium acetobutylicum. Biotechnol. Lett. 8: 469-474.

Zappe, H., D. T. Jones, and D. R. Woods. 1986. Cloning and expression of Clostridium acetobutylicum endoglucanase, cellobiase and amino acid biosynthesis genes in Escherichia coli. J. Gen. Microbiol. 132: 1367-1372.

Zeikus, J. G. 1980. Chemical and fuel production by anaerobic bacteria. Ann. Rev. Microbiol. 34: 423-464.

APPENDIX 3

PRECISION OF THE "CHEMICAL IN CAPILLARY" METHOD FOR POSITIVE CHEMOTAXIS.

Replicate	Bacteria in capillary, 10^4 Trials				Average
1	50	30	50	30	40
2	60	30	30	40	40
3	50	30	50	30	40
4	40	50	30	50	42
5	30	20	20	10	20
6	30	40	10	40	30
7	80	70	60	30	60
8	60	40	30	20	38
9	20	30	20	20	22

The experiment was performed using glucose at 10^{-2}M.

Mean, μ	37
Standard deviation	11
Variance, V	3
% Standard error	30

APPENDIX 4

T-TEST FOR THE THRESHOLD RESPONSE TO SUGARS

Concentration of sugar (M)	Bacteria in capillary, 10^4		
	Glucose	Galactose	Lactose
0	14	9	21
10^{-8}	14	-	16
10^{-7}	28*	6	14
10^{-6}	28	11	23
10^{-5}	34	14	35*
10^{-4}	35	17*	61
10^{-3}	44	27	61
10^{-2}	53	31	64
10^{-1}	54	28	57

$t\,(0.05,\ 4) > 2.132$

* Significant at $p = 0.05$

APPENDIX 5

ANOVA TEST FOR RESPONSE TO ACETATE

Table 1 Accumulation of bacteria towards different pH values of sodium acetate-acetic acid buffer.

Trial	Bacteria in capillary, 10^4					
		pH				
	Control[a]	4.0	4.5	5.0	5.5	6.0
1	12	40	25	25	20	18
2	10	48	60	38	28	10
3	11	39	35	27	20	13

a CM (Section 3.2.1) was used as control

Table 2 Summary of ANOVA results.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio Value	F Critical	Test Decision
Factor	5	25.943	5.189	7.12	3.11	Significant difference between different pH values
Error	12	8.740	0.728			
Total	17	34.683				

APPENDIX 6

ANOVA TEST FOR RESPONSE TO BUTYRATE

Table 1 Accumulation of bacteria towards different pH values of sodium butyrate-butryic acid buffer.

Trial	Bacteria in capillary, 10^4					
		pH				
	Control[a]	4.0	4.5	5.0	5.5	6.0
1	12	25	15	12	10	10
2	12	42	28	15	18	20
3	38	52	30	30	18	18
4	8	28	22	15	12	12

[a]CM (Section 3.2.1) was used as control

Table 2 Summary of ANOVA results.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio Value	F Critical	Test Decision
Factor	5	13.828	2.766	3.29	2.77	Significant difference between different pH values
Error	18	15.145	0.841			
Total	23	28.973				