FERMENTATION STUDIES ON *CLOSTRIDIUM ACETOBUTYLICUM*

by

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ABSTRACT

The initial aim of this work was to develop a laboratory system for the study of the ABE fermentation under laboratory conditions. The development of defined and simple laboratory inoculation and build-up procedures for the ABE process was investigated. A defined spore preparation in distilled water gave solvent yields comparable to the yields obtained in the commercial ABE process. A laboratory inoculation procedure was developed which avoided the lengthy culture build-up procedures presently utilised. An investigation into solvent production by *Clostridium acetobutylicum* in clostridial basal medium (CBM), reinforced clostridial medium (RCM), Leung and Robson media was undertaken with the aim of developing a partially defined laboratory medium which produced solvent yields comparable to the molasses fermentation medium (MFM). The solvent yields obtained in the partially defined laboratory media were substantially lower than those obtained in MFM. It became apparent that the initial aim of trying to identify and manipulate a few key factors to give better solvent yields would not be easily attained. Both the solvent levels and the overall pattern of cell development were markedly different in the various laboratory systems. In view of these differences, a more detailed investigation of the growth patterns, morphological and physiological changes were undertaken.

The second part of the study deals with the physiology of the ABE fermentation. In particular the relationship between

physiological, cell growth, cell morphology changes and solvent production was investigated. The different solvent levels produced by *C.acetobutylicum* P262 in MFM and CBM were associated with the different patterns of cell developments which were observed in the two systems. The appearance of solvents in the two systems coincided with the appearance of the first phase bright clostridial forms during late exponential growth. In the CBM system the phase bright cells were 50 to 70% of the total cell population compared with 70 to 90% in MFM. Studies on the morphological changes in the ABE fermentation system suggested that the clostridial forms in *C.acetobutylcium* were involved in the conversion of acetate and butyrate to acetone and butanol. This was confirmed by the isolation of sporulation mutants which either failed to form clostridial forms and produced no solvents or formed reduced numbers of clostridial forms and produced intermediate levels of solvent.

The third part of this study involved a comparison of *C.acetobutylicum* P262 and autolytic-deficient strains in the ABE fermentation. Autolytic-deficient mutants were isolated after mutagenesis and lacked substantial levels of autolytic enzyme(s) and grew in long chains. One of these mutants was highly pleiotropic. The pleiotropic characteristics include reduced cell free and cell bound autolysin levels, growth at increased glucose levels and an increased tolerance to butanol. The effect of butanol on the clostridial forms was determined and a relationship

between butanol toxicity and clostridial degeneration was shown. Clostridial forms from the autolytic-deficient mutant *lyt*1 were not induced to degenerate by concentrations of butanol which increase the rate of degeneration of *C.acetobutylicum* P262 clostridial forms. It is suggested that the *lyt*1 clostridial forms are more resistant to butanol-induced degeneration and one of the effects of butanol is the induction of autolysis and degeneration of *C.acetobutylicum* P262 clostridial forms. Initial fermentation studies have indicated that the mutant may be useful in producing higher solvent yields.

Growth of *Clostridium thermocellum* under laboratory conditions was also investigated. These studies were undertaken to develop the techniques necessary for future physiological and genetic investigations on *C.thermocellum*. These results have been included as an appendix.

CHAPTER I

1.1 GENERAL INTRODUCTION

1.1.1 FERMENTATION TECHNOLOGY AND ANAEROBES

There has been renewed interest in biotechnology which involves the use of living organisms or their components in industrial processes. The food processing, chemical and pharmaceutical industries are the three major users of fermentation biotechnology today, the food industry being the first to exploit microorganisms to produce alcoholic beverages and fermented foods. A number of factors have been identified in a recent review on fermentation technology (Impacts of Applied Genetics, 1981) which may influence the introduction of fermentation processes to replace present chemical synthetic processes. These include:

1. Abundance of a potentially useful raw material;
2. Scarcity of an established product;
3. Discovery of a new product;
4. Environmental concerns;
5. Scarcity of currently used materials.

Each of these factors has tended to accelerate the application of fermentation. Our present dependence on petroleum in particular has increased interest in microbial fermentations for chemical and fuel production. An important requirement for the production of petrochemicals and fuels by microbial fermentation is the availability of abundant fermentable substrates. Although abundant but finite sources of coal do exist they are not utilisable as substrates for fermentation unless first pyrolyzed. As a result, more interest has centred around renewable biomass fermentations.

The major advantages of using fermentation includes: the use of renewable resources; the need for less extreme conditions during conversion; the use of one step production processes; and a reduction in pollution. Fermentation of carbohydrates to chemicals provides an alternative route for the production of C_3 and C_4 chemicals currently derived from fossil fuels. Tong (1978) lists nine C_3 and C_4 chemicals that can be produced from carbohydrate raw materials by microorganisms. These include isopropanol, *n*- butanol, acetone, butadiene, 2,3 butylene glycol, methylethyl ketone, glycerol, fumaric acid and maleic anhydride. The major carbohydrate sources for fermentation are starch from corn, wheat, barley and sorgum, sucrose and molasses from beet and cane.

The bioconversion of carbohydrate raw material to chemicals can occur by three processes: aerobic fermentation, anaerobic fermentation and chemical modification of fermentation products of both aerobic and anaerobic fermentations. Aerobic fermentations which produce enzymes, vitamins, pesticides, growth regulators, amino acids, nucleic acids and other speciality chemicals are clearly well established (Impacts of Applied Genetics, 1981). Speciality chemicals such as interferons which are derived through recombinant DNA methods, may well represent the next great advance in clinical medicine and in the pharmaceutical industry (Aharonwitz and Cohen, 1981). Of the nine chemical products mentioned by Tong (1978), 2,3 butylene, glycerol and fumaric acid are aerobic fermentation chemicals. These processes involve relatively high exothermic reactions and are thus energy intensive requiring refrigerant cooling, aeration and agitation. Anaerobic fermentations produce organic acids, methane and solvents and are the industry's area of greatest current growth. Already 40% of the ethanol manufactured in the United States is produced in this way. (Impacts in Applied Genetics, 1981).

Chemical modification of the fermentation products of both aerobic and anaerobic fermentations has rarely been used to date on a commercial scale (eg. conversion of ethanol to butadiene).

In anaerobic fermentations the microorganisms recover less energy per unit of carbon substrate utilized than in aerobic fermentations. There is a tendency in these fermentations for carbon substrates to undergo only partial decomposition, so that various organic acids and amines accumulate in the growth medium; these products can present problems in pH maintenance of the fermentation. As a result of the incomplete utilization of substrate and low cellular-energy yields these fermentations often require more carbon substrate to be decomposed for the growth and maintenance of the microbial cell. This phenomenon can be beneficial to fermentation yields in that a greater passage of substrate carbon through the metabolic pathways results in a high substrate to product conversion.

There have been fewer industrial fermentations developed for anaerobic microorganisms than for aerobic microorganisms. In part this is because there was relatively little known about the spectrum of anaerobic bacteria that may be of importance in biofuel and biochemical production. In the past, lack of interest and technological problems associated with pure culture growth of anaerobes prevented more detailed investigation of species' diversity and metabolism. The effect of air on anaerobic bacteria that are not able to catabolize oxygen is quite varied (Morris and O'Brien, 1971). Oxygen is lethal to certain anaerobes (eg. *Methanosarcina* species), inhibits the metabolism of *Clostridium* species and has no effect on *Lactobacillus* species. Today cultural techniques (Hungate, 1966; Daniels and Zeikus, 1975; Edwards and McBride, 1975; Balch and Wolfe, 1976) and the use of the anaerobic glove box have resulted in the growth of the most oxygen-sensitive bacteria. Oxygen transfer is a process limiting step with aerobes which are therefore not ideal organisms for large scale fermentations.

The most interesting species for biotechnology include cellulose and starch fermenters, thermophiles and acid tolerant anaerobes. The genus *Clostridium* forms a major group of the anaerobes and a number of *Clostridium* strains are of particular interest. *Clostridium thermocellum* is of interest because it produces extracellular cellulases (Ng *et al.*, 1977; Gomez, 1980). The use of *C.thermocellum* in the production of ethanol from biomass is reviewed in

Appendix A. *Clostridium thermohydrosulfuricum* is of interest due to the high ethanol yields (1.95 mole ethanol/ mole glucose) (Zeikus, 1980; Zeikus *et al.*, 1980). *Clostridium acetobutylicum* ferments starches, hexoses and pentoses to butanol, acetone and ethanol (Spivey, 1978). *Clostridium butylicum* ferments hexoses or starch and produces largely butanol and smaller quantities of isopropanol.

The first step in the development of a novel fermentation system is finding a suitable organism. Until recently this selection was confined to organisms already producing the required product. However,through genetic manipulation strains can be engineered and microorganisms can be made to produce substances beyond their natural capacities. Although the majority of cloning experiments at the present time have utilised *Escherichia coli* as the host vector, this host is not particularly suited to the large scale production of enzymes, proteins or chemical feedstocks *C.acetobutylicum*, on the other hand, has potential for large scale fermentations and may be useful for the exploitation of genetic engineering (Allcock, 1981). Features which make it suitable for fermentation include:

1. The organism is non-toxic and non-pathogenic.
2. Being an anaerobe, it is suitable for large scale fermentations as no expensive aeration system is required.
3. The organism is non-exacting nutritionally and can utilise a number of different cheap substrates.
4. The organism shows rapid growth under industrial conditions.

5. The organism forms spores and is easily maintained as a spore suspension.
6. The organism has a long history as a fermentation organism in the acetone-butanol-ethanol (ABE) process.
7. As extracellular proteins are produced during growth, the appropriate genes that influence excretion of products are present.
8. Finally, a single strain could be used for a variety of fermentations provided that the development of a genetic system allowed the introduction of useful foreign genes into the strain.

1.1.2. THE ABE FERMENTATION

The ABE fermentation has a long history as an industrial fermentation process. Normal butanol was discovered as a regularly occurring constituent of fossil oil by Wurtz in 1852 (Prescott and Dunn, 1940). Bacterial production of butanol was first studied by Pasteur in 1861 (Ross, 1961). He was studying a butyric acid organism and identified butanol as one of the products formed. The early history of the ABE process has been reviewed by Prescott and Dunn (1940). From 1876 to 1884 Fitz investigated the fermentation process of *Bascillus butylicus*. The bacterium was a spore former producing butanol, butyric acid and small quantities of ethanol. Between 1887 and 1897 various butanol producing fermentations involving different bacterial strains were investigated. Among them Beijernick described a species named by him *Granulobacter butylicum*. (Granulobacter was the term applied to microorganisms that demonstrated a blue colour with iodine and showed a distinct swelling of the cell at sporulation). Winogradsky in 1902 reported on *Clostridium pasteurianum* and described it as a butyric acid producer. Acetone was first discovered as a fermentation product by Schardinger in 1905 when studying the fermentation of *Bacillus marcerans*.

The need for synthetic rubber supplied the impetus which resulted in the first successful commercial process (Artzberger *et al.*, 1920; Gabriel, 1928; Kelly, 1936). Synthetic rubber was obtained through polymerization of isoprene and butadiene and those compounds were best prepared from isoamyl alcohol and butanol respectively. Fernbach and Weizmann in 1911, (Prescott and Dunn, 1940), who were working on the above process discovered bacteria that fermented potato starch yielding acetone, ethanol and butanol. In 1912 Weizmann continued research on the fermentation and isolated an organism producing four times the amount of acetone than previous isolates. This organism was able to ferment starches other than potato starch and was named *Bascillus granulobacter pectinovorum*. During 1913 and 1914 factories were established for the production of solvents by fermentation. With the outbreak of World War I in 1914, the production of acetone was of interest to England for the manufacture of cordite (McCutchan and Hickey, 1954). However the demands for acetone could not be met using potatoes as the raw material. Consequently all the factories were replaced with the Weizmann strain which used maize as the raw material. At the end of the war there was no further demand for acetone production and as butanol had never been much in demand many factories closed down. Shortly after the end of the war, E I Du Pont de Nemours and Company developed nitrocellulose lacquers for the automobile industry, and it was found that butyl acetate was the solvent of choice for this coating system (Walton and Martin, 1979). In order to supply the butanol for conversion to butyl acetate the ABE fermentation was reestablished. After the Weizmann patent had expired in 1936, new ABE plants were built in Philadelphia, Pensylvania, Baltimore, Maryland, Puerto Rico and Japan. The industry was founded in Germiston, South Africa in 1944 and is still viable today despite the closure of many factories overseas.

Many early attempts were made to produce acetone and butanol by the fermentation of molasses which was a cheaper substrate than corn or maize. However, it was not until 1938 that organisms were isolated capable of utilizing diluted molasses as a feedstock, and a molasses based process was developed (Monick, 1968; Walton and Martin, 1979). From 1926-1930 McCoy *et al.* undertook a study of the acetone-butyl organism and suggested *Clostridium acetobutylicum* as a suitable name for the Weizmann strain.

Cummins and Johnson (1971) analysed cell wall sugar composition, DNA homology and nutritional requirements of the genus *Clostridium* to determine if they could be used as a more accurate means of classifying the butyric acid producing bacteria than the criteria used by McCoy *et al.* (1926 and 1930). They determined that *C.butyricum* strains could be divided into two main groups. Group I included strains containing glucose as the only cell wall sugar, and growth occurred in mineral salts glucose medium supplemented with biotin. The addition of amino acids improved growth. Group II included strains containing both glucose and galactose as cell wall sugars. In this group growth did not occur in a minimal salts glucose medium supplemented with amino acids and vitamins unless yeast extract was present. Several other clostridial strains could be classified in one of the two groups and it was suggested that group I be known as *Clostridium butyricum* and the second group as *Clostridium beijerinckii*. Some strains, e.g. *Clostridium fallax*, *C.acetobutylicum*, *Clostridium aurantibutyricum*, *C.pasteurianum* and *Clostridium tyrobutyricum* did not belong to either group and therefore the species *C.acetobutylicum* was retained.

Industrial production of acetone and butanol via fermentation has not been significant for the past 20 years due to the superior economic position of petrochemical based processes (Lonz and Moroira, 1980). However the demand for butanol and acetone from biomass fermentation has increased because of the high costs of petroleum based feedstocks. Butanol in particular is used in a number of industrial processes, ranging from the production of lacquers to extraction of antibiotics from media, and as an intermediate for the synthesis of butyl acetate, butyl acrylate (Rose, 1961; Compere and Griffith, 1979). There is substantial requirement for butanol as a co-agent in tertiary oil recovery systems (Compere and Griffith, 1979). Butanol may have a possible use in extending fuel supplies by mixing butanol with gasoline forming gasohol (Lonz and Moroira, 1980).

The production of acetone and butanol by *C.acetobutylicum* follows a rather complex mechanism which leads to a number of end products namely carbon dioxide, water, hydrogen, acetic acid and butyric acid in addition to acetone, ethanol and butanol (Doelle, 1975; Stanier *et al.*, 1976). Lonz and Moroira (1980) did an analysis comparing the energy of combustion of glucose with the energy of combustion of the fermentation products using available product yield data (Doelle, 1975). In spite of the large number of products formed during the ABE fermentation, it is interesting to note that 97% of the energy content of the glucose fermented is recovered in the chemicals produced during the fermentation. The fractional energy recovery in the ABE fermentation is shown in Table 1.2 (Lonz and Moroira, 1980). A similar analysis for yeast alcohol fermentation shows that 98% of the energy present in glucose is conserved in the ethanol produced during the fermentation. This concentration of chemical energy in a single compound is a distinct advantage for the ethanol fermentation relative to the ABE process. As seen in Table 2.1, butanol accounts for only 55.1% of the total energy recovered; this means that applications must be developed for the remaining fermentation products, especially hydrogen and acetone (Lonz and Moroira, 1980).

Lonz and Moroira (1980) reported in an economic evaluation of the ABE fermentation that the economics of producing these solvents by fermentation of high quality molasses was unattractive when current prices of petroleum based commodities were used in the calculation. The total production costs were found to be slightly higher than the

TABLE 2.1

Fractional energy recovery in the ABE fermentation (Lonz and Moroira, 1980).

Fermentation Products	% Total energy recovered in product
Butyric acid	3.2
Ethanol	3.5
Acetic acid	4.5
Acetoin	5.0
Hydrogen	14.2
Acetone	14.5
Butanol	55.1
Total	100.0

total annual income even when all the fermentation by-products were taken into account. This was mainly due to the high costs of molasses feedstocks.

Most of the acetone and butanol made by fermentation has been from one of the following carbohydrate sources: corn, black-strap molasses or high test molasses. Other common sources of carbohydrates such as wheat, rice, horse chestnuts, Jerusalem artichokes (Wendland *et al.*, 1941), and beet molasses can also be readily used (Walton and Martin, 1979). However when the fermentation process is considered as a possible replacement for those based on hydrocarbon feedstocks, carbohydrates derived from sources now considered to be waste products should be investigated. These include hydrolyzed wood (Leonard *et al.*, 1947; Sjolander *et al.*, 1938), hydrolyzed corn cobs (Dunning and Lathrop, 1945; Langlykke *et al.*, 1948), hydrolyzed cabbage (Prescott and Dunn, 1940), whey (Lonz and Moroira, 1980), sulphite liquor (Wiley *et al.*, 1941) and hydrol, a by-product from the manufacture of glucose (Walton and Martin, 1979).

Lonz and Moroira (1980) also evaluated the fermentation process utilizing liquid whey waste as a feedstock. The study showed that whey waste could be economically attractive and suggested that other low grade, or waste-type feedstocks should be further investigated. The utilization of waste-type materials is plagued by two fundamental problems; they are not usually available in concentrated forms and may have a seasonal character.

Although economically attractive when based on waste-type materials, the ABE fermentation has a number of drawbacks which must be addressed before any attempts of commercial production is made. The major problem is the very low level of solvents produced in the final fermentation broth. With current technology such levels are only about 1.9% (w/v) (Ryder, 1958; Hastings, 1978). This results in the need for large size vessels for fermentation and an energy-intensive distillation recovery process for solvents. Additional difficulties with the fermentation include the need for strict anaerobic conditions, delicate culture maintenance and propagation, and a tendency for infection by bacteriophages (Douglas, 1975) and lactobacilli (Thaysen, 1921; Fred *et al.*, 1926). If improved upon however, the acetone-butanol fermentation has the potential of becoming a major source of highly valuable chemicals and fuels in future years.

This study was undertaken with the view of further characterising the ABE fermentation under laboratory conditions and improving the fermentation.

CHAPTER II

THE DEVELOPMENT OF LABORATORY SYSTEMS FOR THE STUDY OF THE ABE FERMENTATION

The development of defined and simple laboratory inoculation and build-up procedures for the ABE process was investigated. A defined spore preparation in distilled water gave the same solvent yields as the factory spore/soil preparation. A laboratory inoculation procedure was developed which obviated the lengthy factory build-up procedure. The solvent yields obtained in the partially defined laboratory media were substantially lower than those obtained in MFM.

2.1 INTRODUCTION

In order to study solvent production by *C.acetobutylicum* it was necessary to obtain reliable laboratory systems involving small culture volumes which produced solvent levels comparable to those obtained under factory conditions.

The industrial fermentation process (Table 2.1A) is a lengthy process and consists of 4 inoculum build-up stages, A_1, A_2, B and C stages. An initial scaling down of the factory procedure was reported by Barber (1977), and van der Westhuizen (1978), Barber *et al.*, (1979) and Webster (1980), and fermentations were carried out in either 400 or 1 330 mℓ of molasses fermentation medium (MFM). Barber (1977)

TABLE 2.1

The factory inoculation procedure (A) and the scaled down laboratory procedure (B) for solvent production in MFM by *C.acetobutylicum*.

(A) Factory procedure

Culture Stage	Volume (ℓ)	Duration (h)	Transfer Volume (ℓ)	Medium
A_1	0.150	12	0.150 (to A_2)	Potato
A_2	0.500	6	0.650 (to B)	Molasses (3.5% TIS)*
B	3.5	6	1.38 (to 3xC)	Molasses (3.5% TIS)*
C	3x9	9	*c* 30 (to plant fermentor)	Molasses (3.5% TIS)*
Plant Fermentor	90 000	36		Molasses (6.5% TIS)*

(B) Scaled down factory procedure

Culture Stage	Volume (ℓ)	Duration (h)	Transfer Volume	Medium
A_1	0.015	12-18	0.015 (to A_2)	Potato
A_2	0.025	6	0.040 (to B)	Molasses (3.5% TIS)
B	0.350	6	0.040 (to C)	Molasses (3.5% TIS)
C	0.300	10	0.004 (to MFM)	Molasses (3.5% TIS)
MFM	0.400	36-48		Molasses (3.5% TIS)

* TIS : Total Invert Sugar.

found that the build-up stages could not be omitted from the fermentation without adversely affecting the solvent production. Her results were similar to those of Beech (1953) who, using *C.acetobutylicum* in a corn mash fermentation, determined the effect of varying the number of build-up stages (from 2 to 12 h) on solvent production. There was a decrease in the solvent production with decreasing number of transfers.

In the industrial production of acetone and butanol by *C.acetobutylicum*, the spores are maintained on sterile soil and germinated by heat shocking in liquid medium. Heat shock selection has been used to select cultures which give better fermentation yields (Ryden, 1955). Weizmann advocated heat treating a culture 100 to 150 times in order to improve its fermenting ability (Prescott and Dunn, 1940). In a study of six different strains of *C.acetobutylicum* (Weizmann, 1945), Weyer and Rettger (1927) showed that storage of spores for a period of over 6 months decreased their ability to produce solvents. The cultures could be "rejuvinated" by alternate pasteurization and subculturing. It was thought that pasteurization destroyed the vegetative forms and the weaker spores, and that the most prodigous producers of solvents are those cultures whose spores are in general the most heat resistant (Prescott and Dunn, 1940). Curran and Evans, (1945), were the first to recognise that some spore suspensions germinated more rapidly following sublethal heating (heat shocking). The term "germination" applied to a bacterial spore describes the rapid and irreversable process whereby the spore loses its typical dormant and resistant properties. The resulting cell is metabolically active, heat labile , non refractory and stainable but is nevertheless distinct from a typical vegetative cell (Campbell, 1957; Keynan and Holvorsen, 1965). Ungerminated spores appear phase bright whereas germinated spores are often swollen (Steinberg *et al.*, 1969) and phase dark (Pulvertoft and Hayes, 1951).

In clostridia the germination requirements are complex (Holland *et al.*, 1969; Rowley and Feeherry, 1970) and the spores are unable to germinate in distilled water or physiological saline solutions.

Germination and outgrowth are entirely distinct processes. This has been demonstrated by differences in metabolic and in nutritional requirements (Demain and Newkirk, 1960). Spores for example will germinate in the presence of compounds which inhibit protein synthesis and nucleic acid synthesis in outgrowth cells (Keyman and Halvorson, 1965). In general defined media for the growth of vegetative cells are satisfactory for outgrowth (Halvorson *et al.*, 1966; Gould, 1971). In the genus *Bacillus* (Steinberg *et al.*, 1969) the transition from spore to active growth occurs in three distinct phases: (1) activation, a period in which those mechanisms responsible for initiating germination are potentiated; (2) germination, a stage characterized by degradative reactions which break the dormant state; and (3) outgrowth, a stage which is dependent on the commencement of biosynthetic activity and which covers the period of development after germination until the first cell division.

Abou-Zeid *et al.*,(1980) compared different carbohydrates in the ABE fermentation. Molasses was the most suitable carbon source for the production of acetone, butanol and ethanol. The suitabilities of the different carbon sources were arranged in descending order according to their efficiency of production of acetone and butanol as follows: molasses > flour > fructose > glucose > pure flour > soluble starch > maltose > sucrose > mannose > lactose > stillage > glycerol > citric acid. Molasses is a complex substrate (Dekker, 1957; MacGillvray and Matic, 1970) and in order to study the factors affecting solvent production it is essential to have a chemically defined medium which produced high solvent yields.

In 1940, Oxford *et al.*, employed a synthetic medium for studies on the nutritional requirements of *C.acetobutylicum*. Their studies, along with those of Rubbo *et al.* (1941) and Porter (1946) indicated that biotin and p-amino benzoic acid (paba) were essential factors for growth of *C.acetobutylicum*. Oxford *et al*. (1940) observed that there was no strict correlation between growth and fermentation and postulated that the presence of other factors may be required for normal solvent production to take place following normal growth.

Rubbo *et al.* (1941) investigated the ability of *C.acetobutylicum* to ferment a synthetic glucose medium containing paba and found that normal levels of butanol were found but that acetone production was suppressed. A factor was isolated from yeast and was found to restore acetone yields. Davies and Stephenson (1941) demonstrated that L-asparagine was essential for acetone production by *C.acetobutylicum* in in glucose but not in starch containing media. Davies and Stephenson (1941) also reported that higher yields were obtained with maize meal than with 2% glucose and yeast extract medium. Frequently yields were very low (acid fermentations) and in order to convert an acid fermentation to a "normal" fermentation the addition of several growth supplements present in liver, maize and yeast extract were required. *C.acetobutylicum* has been studied during batch cultivation in Clostridial Basal Medium (CBM), containing glucose, casein hydrolosate, vitamins and salts (O'Brien and Morris, 1971). They found that acetate and butyrate, but not butanol and acetone, are produced during exponential growth of *C.acetobutylicum*, the products formed during stationary growth were not assayed. In a pH controlled batch fermentation of *C.acetobutylicum* ATCC strain 824 solvent yields of >20 $g\ell^{-1}$ have been obtained by Leung (1980) in a 10% glucose basal medium.

This chapter describes the development of inoculation and build up procedures for laboratory studies and the effect of spore activation on germination and solvent production. The solvent yields of different culture media for laboratory fermentation systems were compared with MFM.

2.2 MATERIALS AND METHODS

2.2.1 BACTERIAL STRAINS

The *C.acetobutylicum* P262 spores were supplied by National Chemical Products Limited (NCP), Germiston, South Africa. Spores were maintained on sterile soil at room temperature or in distilled water at 4°C.

2.2.2 MEDIA

All media used are listed in Appendix B. The A_2 molasses medium was used for the A_2, B and C stages of the fermentation. The bacterium was grown in the molasses fermentation media (MFM) of Barber *et al.* (1979), modified *Clostridium* basal medium (O'Brien and Morris, 1971),(CBM) reinforced clostridial media (Difco) (RCM).

Leung Medium (Leung, 1980) and Robson medium (Robson, 1981).

2.2.3 DISTILLED WATER SPORE PREPARATION

Distilled water spore suspensions were prepared from sporulating colonies on CBM plates. The spores were washed several times in distilled water, and finally resuspended in distilled water.

2.2.4 HEAT ACTIVATION OF SPORES (HEAT SHOCKING)

The spore suspension was diluted (1/600) and 0.5 mℓ was placed in a Bijou bottle and heated in a water bath at 70-75°C for 2 min before cooling in ice for 45 s. The heat activated spore suspension (10 µℓ) was added to 10 mℓ CBM in a Hungate tube under stringent anaerobic conditions in the anaerobic glove box (Forma Scientific, Ohio). The cultures were incubated at 37°C until they reached an absorbance of 0.4-0.5 at 600 nm (10^8-10^9 cells mℓ$^{-1}$). A 0.1% inoculum was used to inoculate the various experimental media.

2.2.5 FERMENTATION PROCEDURE

All media used were placed in the anaerobic glove box, after autoclaving at 121°C for 20 min, for 12-18h before inoculating. Cultures incubated under stringent anaerobic conditions were incubated at 34°C in the anaerobic glove box. Cultures incubated aerobically were first incubated under anaerobic conditions for 2-6h at 34°C before being incubated aerobically.

2.2.5.1. Fermenter

A Gallenkamp (London) modular fermenter with a working volume of 200 to 800 mℓ was used. The medium was agitated at *c* 100 rpm during the fermentations. The pH of the culture was controlled above 5.0 by means of a pH control module with 2 M NaOH. The temperature was maintained at 34°C by a temperature control module which regulated the flow of hot or cold water through an immersion coil located inside the fermenter. During the start up of the fermentation mixed gas consisting of 12% CO_2, 80% N and 5% H_2 (Air Products Limited, South Africa) was sparged into the fermenter to maintain anaerobiosis. The sparging was terminated once the culture was observed to be growing. After this time, sufficient gas (CO_2 + H_2) was produced to provide a reduced environment for the culture.

2.2.6 PHYSICAL MEASUREMENTS

2.2.6.1. pH

The pH of the samples were determined using the Orion Research Digital ionanalyzer/501.

2.2.6.2. Titratable acid

Titratable acids were determined by titration of 10 mℓ supernatant fractions plus 50 mℓ distilled water with 0.02 N NaOH to pH 7.0. The titratable acidity was expressed as acid equivalents (in g) ℓ^{-1}.

2.2.6.3. Analysis of Solvents

Aqueous samples (1 μℓ) of the fermentation medium were injected into a Hewlett-Packard 5830A Chromatograph equipped with a flame ionization detector. The quantitative evaluation of the peaks was performed automatically by the automatic integrator using n-propanol as an internal standard. A calibration mixture (Appendix B) comprising acetone, ethanol and *n*-butanol, *n*-propanol as internal standard.

A 1.84m stainless steel column with a 3.18 mm outside diameter, was packed under negative pressure with chromsorb W/AW

(80-100 mesh) coated with 15% carbowax 20 M. New columns were conditioned at 90°C for 24 h with a flow of 60 mℓ min^{-1} N_2 to remove volatile impurities. Analysis of solvents was achieved under the following conditions: column temperature, 250°C; detector temperature, 250°C; N_2 (carrier gas) flow rate 30 mℓ min^{-1}; H_2 flow rate, 40 mℓ min^{-1}; air flow rate 500 mℓ min^{-1}.

2.2.7. Microscopic Methods

A Zeiss photomicroscope fitted with phase contrast and interference contrast optics was used. Ungerminated spores appeared phase bright and germinated spores phase dark (Gould, 1971). Differential microscopic counts allowed the percentage germination in the spore suspension to be assayed.

The sequence of events following heat activation of *C.acetobutylicum* P262 spores was monitored. A drop of heat activated spore suspension on a coverslip was placed onto a pad of CBM agar (*c* 1mm thick) in a germination chamber slide. The edge of the coverslip was sealed with clear varnish. All manipulaitons were carried out under stringent anaerobic conditions. The sealed slide remained anaerobic and could be incubated at 37°C in the air.

2.3 RESULTS

2.3.1 THE DEVELOPMENT OF INOCULATION PROCEDURES FOR LABORATORY STUDIES.

2.3.1.1. Spore preparation

Spore production by *C.acetobutylicum* was compared on CBM agar plates containing different carbohydrates in the presence and in the absence of $CaCO_3$. The pH of the buffered plates was *c* pH 6.4 and the unbuffered plates (no $CaCO_3$) *c* pH 5.0. The addition of 2% glucose or 2% gelatin produced spore forming colonies with a maximum of 50% sporulation on the unbuffered plates. The addition of $CaCO_3$ to the plates increased sporulation to 60-65%. The addition of 2% or 5% sucrose to CBM agar in unbuffered plates produced 25-40% sporulation. Buffering of the CBM sucrose plates increased the sporulation to 60-70%.

The density of the colonies on the plates affected the degree of sporulation. The colonies were classified as either confluent; crowded (5 mm apart); spaced (10 mm apart); and isolated (20 mm apart). The % sporulation in the different colonies increased from 10% in the confluent colonies through 40% in crowded and 50% in spaced colonies to 60% in the isolated colonies. Plates with "spaced" colonies were used to prepare spore stocks.

The solvent yields produced by the distilled water spore preparation and the factory soil spores in 400 mℓ of MFM were compared and found to be similar (16.0 -18.0 $g\ell^{-1}$

after 48 h). The laboratory spore stocks maintained in distilled water at 4°C were viable for up to 36 months and reproducible solvent levels of 16-18 $g\ell^{-1}$ were obtained in MFM (48 h).

2.3.1.2. Spore activation and germination

Germination studies carried out in CBM indicated that *c* 95% of the spores in heat shocked cultures become constricted (Fig 2.1C) which was indicative of germination occurring. Approximately 55% of the constricted spores underwent outgrowth (Fig 2.1D) to produce motile vegetative cells (Fig 2.1E). In spore cultures which were not heated <10% of the spores germinated. It took *c* 30 min for heat activated spores to change from phase dark spores (Fig 2.1B) to phase grey spores. Within 1.5 h the majority of the spores had germinated and were phase dark, swollen and constricted (Fig 2.1C). The spores ruptured along the constriction and outgrowth occurred (Fig 2.1D) leading to the production of phase dark motile rods (Fig 2.1E). The appearance of the first vegetative cells occurred *c* 2 h after the heat activation step.

The effect of different cooling procedures following heat activation of spores on solvent production in MFM (400 $m\ell$) was compared. The heat activated spores were either cooled in an ice/ethanol bath or under running tap water for 45 s. The final solvent yields after 48 h were 16.812 $g\ell^{-1}$ and 16.774 $g\ell^{-1}$ for spores cooled in ice/ethanol and running tap water respectively.

Figure 2.1

Photomicrographs of *C.acetobutylicum* P262 sporulation events (A-D) and different cytological stages (E-F). (A) Sporulating rods with phase bright spores from CBM sucrose plates. (B) Distilled water, phase bright spore preparations. (C) Phase dark spores 1 h after heat activation; (⇧) spore constricted. (D) Spores 2 h after heat activation: (⇧) remains of spore; (⬆) newly emerged motile vegetative rod. (E) Actively growing motile vegetative phase dark rods. (F) Iodine stained clostridial forms showing granulose accumulation (⬆) and unstained forespore (⇧).

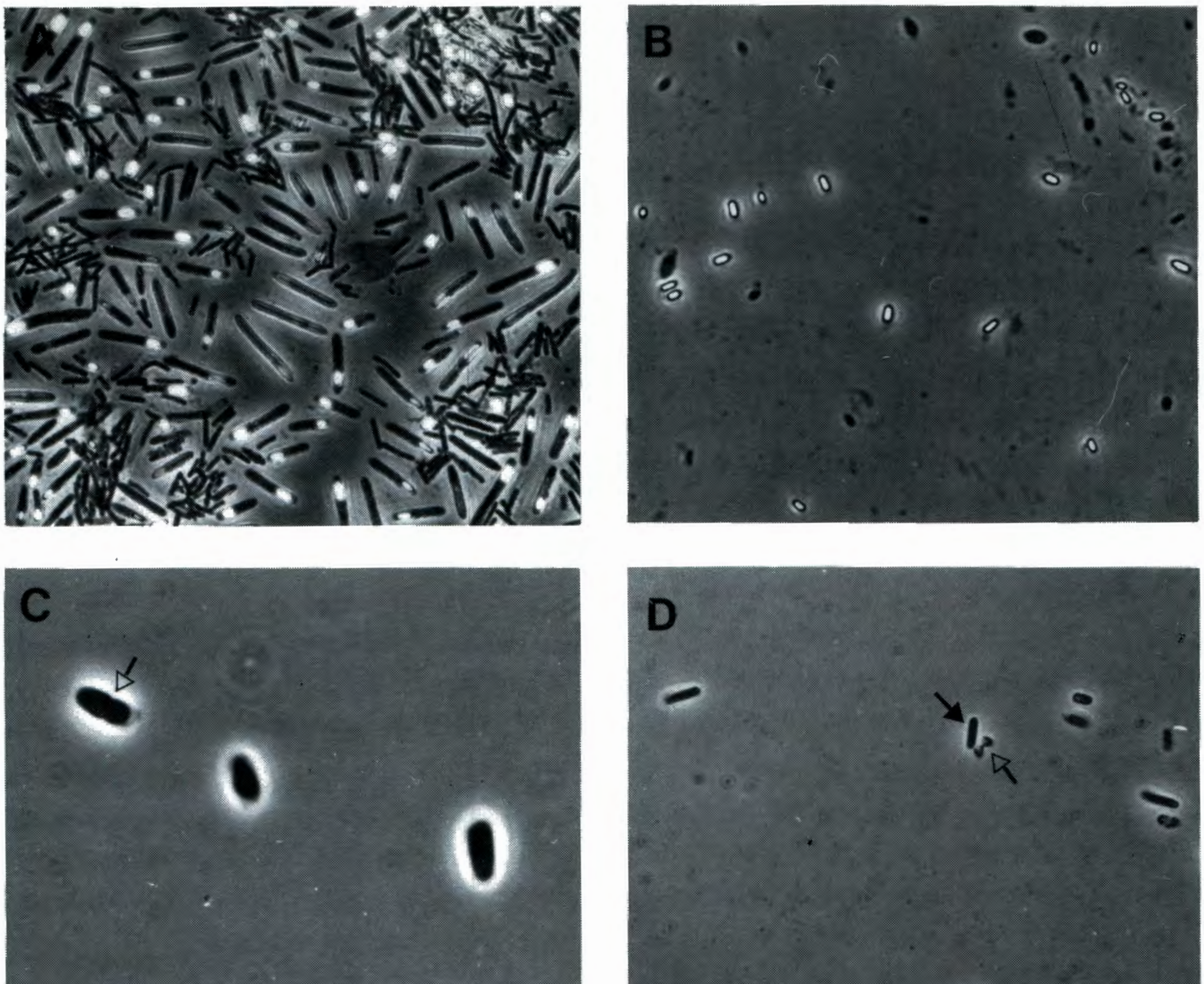
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TABLE 2.2

The effect of different inoculation procedures on solvent production by *C.acetobutylicum* P262 in MFM.

Inoculum	$g\ell^{-1}$ Acetone	$g\ell^{-1}$ Ethanol	$g\ell^{-1}$ Butanol	Total $g\ell^{-1}$
A_1	2.794	0.419	8.607	11.820
A_2	2.666	0.388	7.119	10.074
B	2.264	0.385	6.590	9.240
C	4.972	0.469	10.839	16.280
CBM (OD 0.5)	4.264	0.547	11.244	16.055

A_1, A_2, B, C - different stages in the factory build up procedures.

2.3.1.3. Simplification of inoculation procedures for laboratory studies

The factory build up stages A_1, A_2, or B were not suitable as inocula, as the solvent yields after 48 h in MFM were <12 $g\ell^{-1}$ (Table 2.2). The C stage inoculum produced <16.0 $g\ell^{-1}$ solvents in MFM after 48 h (Table 2.2).

Fermentation without the factory build up stages could be carried out successfully using exponential, actively motile cells grown in CBM (Table 2.2). Optimum transfer of the CBM grown inoculum to MFM was at an absorbance between 0.4-0.5 (*c* 10^8 cells $m\ell^{-1}$). At this stage the cells are actively motile phase dark (Fig 2.1E) and strongly Gram-positive.

2.3.2 DEVELOPMENT OF FERMENTATION SYSTEMS FOR LABORATORY STUDIES

2.3.2.1. Comparison of different culture media for laboratory fermentation systems

The pH, solvent yield and % sporulation were compared in the following media: MFM, RCM, CBM, Robson medium and Leung medium (Table 2.3). The pH and solvent yields were measured after 48 h and the % sporulation after 72 h. The results obtained (Table 2.3) show that MFM was the most suitable medium for the production of solvents. The suit=abilities of the different laboratory media were arranged in descending order according to their efficiency for the production of solvents as follows: MFM > Robson medium > RCM > CBM > Leung medium. The final pH value was related

TABLE 2.3

Comparison of different culture media for laboratory fermentation systems.

Media	pH (0h)	pH (48h)	Solvents $g\ell^{-1}$ (48h)	% Sporulation (72h)
MFM (6.5% TIS)	6.2	5.9	16-18	< 5
Robson Medium (6% glucose)	6.6	5.8	13-15	< 5
RCM (6% glucose)	5.9	5.2	8-10	20-30
CBM (6% glucose)	6.5	4.8	7-8	20-30
Leung Medium (6% glucose)	6.6	4.7	7-8	20-30

to solvent yields: the greater the solvent yields the higher the final pH. There was also a correlation between solvent yields and sporulation. High solvent yields were associated with low sporulation levels.

2.3.3 FACTORS EFFECTING FERMENTATION UNDER LABORATORY CONDITIONS

Solvent yields between 16-18 $g\ell^{-1}$ were obtained in batch fermentations of MFM in flask cultures. It was unnecessary to use a laboratory fermentor to control the pH levels in MFM.

The effect of different volumes of MFM on solvent production by *C.acetobutylicum* P262 was compared under aerobic and stringent anaerobic conditions (Table 2.4). *C.acetobutylicum* P262 did not grow in less than 100 $m\ell$ of MFM under aerobic conditions, while growth occurred in 10 $m\ell$ of MFM under anaerobic conditions. The volume of MFM affected the final solvent yields. The optimum volume of MFM was dependent on the culture vessel size and conditions of incubation. Under aerobic conditions 400 $m\ell$ of MFM were required in a 500 $m\ell$ flask in order to obtain *c* 17.0 $g\ell^{-1}$ solvents compared with 800 $m\ell$ of MFM in a 1ℓ flask to obtain 17.0 $g\ell^{-1}$ under the same conditions. Under stringent anaerobic conditions 300-400 $m\ell$ of MFM were required to produce optimum solvent levels in 500 $m\ell$ and 1 ℓ flasks. The solvent yields were slightly higher in the 500 $m\ell$ container (19.193 $g\ell^{-1}$) compared with fermentations carried out in 1 ℓ flasks (18.863 $g\ell^{-1}$) under stringent anaerobic conditions.

TABLE 2.4

The effect of culture volume on solvent production in MFM by *C. acetobutylicum* under aerobic and stringent anaerobic conditions.

Media Volume (mℓ)	Container Volume (mℓ)	Aerobic Total Solvent $g\ell^{-1}$	Anaerobic Total Solvent $g\ell^{-1}$
10	Hungate (15 mℓ)	NG [a]	7.026
25	500	NG	13.904
50	500	NG	14.119
100	500	12.501	17.421
200	500	14.703	18.762
300	500	14.979	19.224
400	500	17.301	19.193
400	1 000	16.488	18.863
800	1 000	17.225	18.865

a NG : No Growth

The final solvent yields produced in 400 mℓ of MFM were compared at 34°C and 37°C under stringent anaerobic conditions. The solvent yields were consistently higher at 34°C, 19.193 $g\ell^{-1}$ compared to 18.737 $g\ell^{-1}$ at 37°C.

When the fermentation of *C.acetobutylicum* in MFM was left undisturbed (i.e. no mixing) for 48 h the majority of the fermentations produced less than 16 $g\ell^{-1}$ solvents. The effect of intermittent and continuous agitation of the MFM cultures were compared under aerobic and stringent anaerobic conditions. Continuous mixing of the MFM culture during the fermentation had an adverse effect on the growth and solvent production. Continuous mixing under stringent anaerobic conditions produced a 10 h lag in solvent production, while continuous mixing under aerobic conditions inhibited growth. Intermittent mixing at intervals ranging from 1 to 12 h under aerobic and anaerobic conditions resulted in optimal solvent production (17-18 $g\ell^{-1}$). The solvent yields were consistently higher under anaerobic conditions, 18.762 $g\ell^{-1}$, compared with 17.189 $g\ell^{-1}$ under aerobic incubation.

2.3.3.1. pH Control

Solvent production and pH were compared in MFM and in Leung medium with and without pH control (Fig. 2.2). In MFM (Fig. 2.2a) the period between 7 to 18 h was associated with a gradual decrease in pH from pH 6.0 to *c* 5.1. Solvents were detected after 18 h which was associated with the break in the pH. The solvent levels increased between 18 to 36 h before plateauing at *c* 19 $g\ell^{-1}$. During the production of

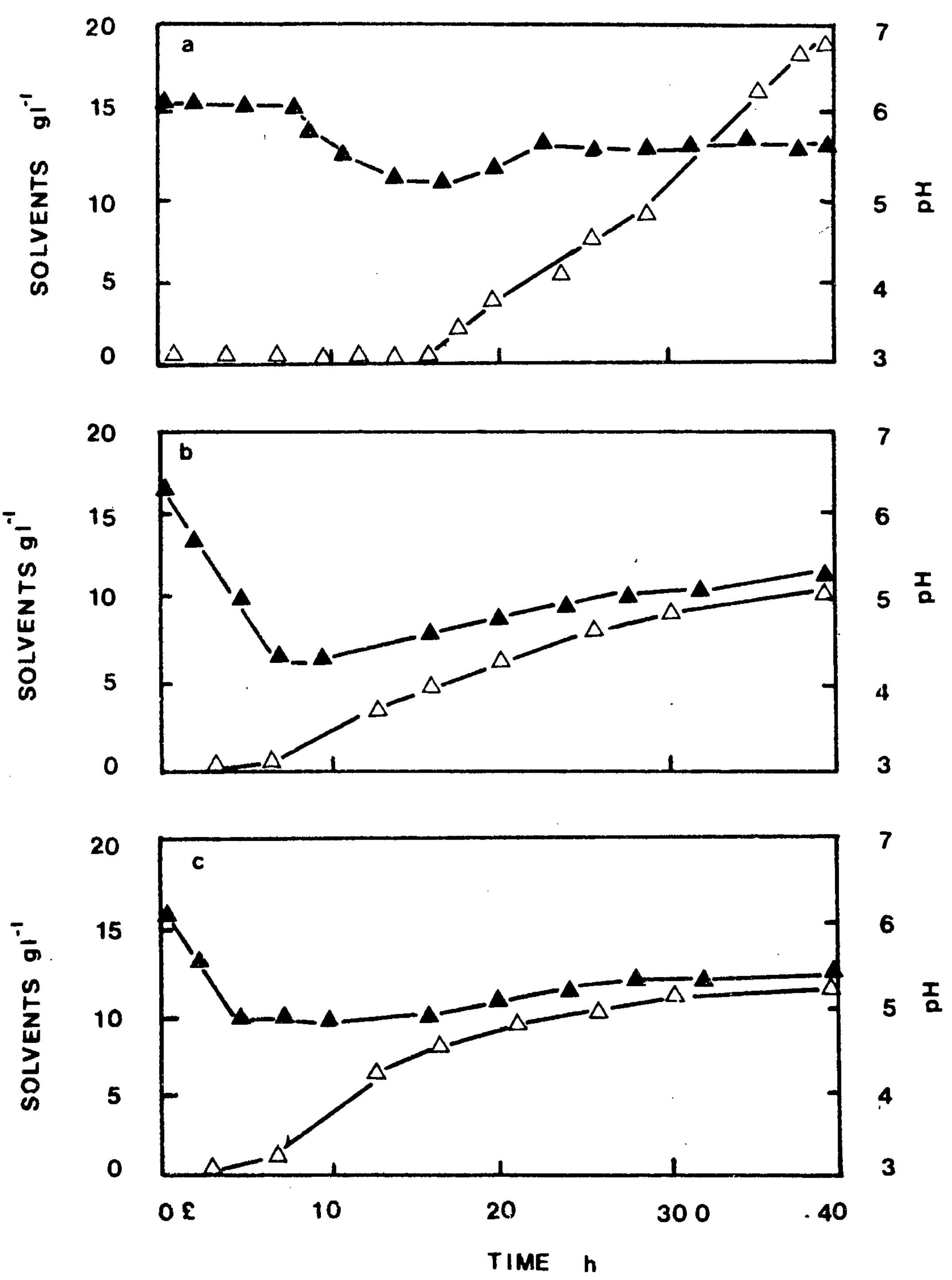

Figure 2.2

A comparison of solvent production, (△), and pH, (▲) in: (a) MFM; (b) Leung medium without pH control; (c) Leung medium with pH control maintained above pH 5.

solvents the pH increased to *c* pH 5.8. In Leung medium (Fig. 2.2b), without pH control, there was a sharp decrease in pH from *c* pH 6.2 to *c* pH 4.5. Solvents were detected after 7 h and correlated with the break in the pH. The final solvent yield and pH after 40 h were *c* 10 $g\ell^{-1}$ and pH 5.4 respectively. If the pH of the culture was controlled above pH 5.0 by the addition of 2 N, NaOH, solvents were detected after 7 h (Fig. 2.2c). The final solvent yields and pH were *c* 10.4 $g\ell^{-1}$ and pH 5.4 respectively.

2.4 DISCUSSION

The inoculation procedure for the ABE fermentation has been simplified for laboratory use. The *C.acetobutylicum* P262 spores for general laboratory use were prepared from sporulating colonies on CBM plates. The spores were resuspended in distilled water and maintained at 4°C. The factory soil/spore system and the clean spore system produced normal solvent yields in MFM. The clean spore system has the advantage in that it enables microscopic examination to check for contamination, facilitates germination and activation studies, and allows the spore suspension to be diluted accurately.

The clean spores were viable and produced normal solvent yields in MFM over a period of 36 months. This finding differs from Weyer and Rettger (1927). In a study of six different strains of *C.acetobutylicum*, Weyer and Rettger (1927) showed that storage of spores, in soil, for six months reduced their ability to produce solvents.

The heat activation of spores prior to inoculation was necessary to obtain good solvent yields. Ryden (1958) suggested that spores subjected to heat activation (shocking) kills the weaker spores and produces a better fermentation. The improved fermentation may also be due to the fact that the heat activation produces a synchronised culture. The sequence of events following heat activation of *C.acetobutylicum* spores are similar to the sequence found for the genus *Bacillus* (Steinberg *et al.*, 1969). The sequence of events occurring after heat activation of *C.acetobutylicum* spores are diagramatically illustrated in Fig. 2.3.

The factory build up stages were not necessary to obtain normal solvent yields in MFM. The laboratory fermentation procedure adapted for routine laboratory use, consists of a 12-18 h CBM *C.acetobutylicum* culture in which the cells are actively motile. The inoculation system developed was reliable and gave reproducible results.

The solvent yields obtained in the fermentation of molasses under laboratory conditions corresponds with the yields produced at the N.C.P. factory. Under factory fermentation conditions solvents are produced in the ratio 6:3:1; *n*-butanol; acetone; ethanol. The laboratory fermentation produced approximately the same ratios of butanol and acetone as in the factory, but the ethanol levels were lower. The fermentations were carried out in simple culture vessels and it was not necessary to use laboratory fermentors. The disadvantage of the system was that small volumes, less than 400 mℓ, can not be used to obtain optimum solvent yields in MFM. Anaerobic facilities and mixing once every 12 h was required for 400 mℓ cultures to obtain optimum solvent yields.

An investigation into the solvent production by *C.acetobutylicum* in CBM, RCM, Leung and Robson media was undertaken with the aim of developing a partially defined laboratory medium which produced solvent yields comparable to MFM. The final solvent yields obtained in the laboratory media were substantially lower than those obtained in MFM. The most successful of the laboratory media used was Robson medium which produced

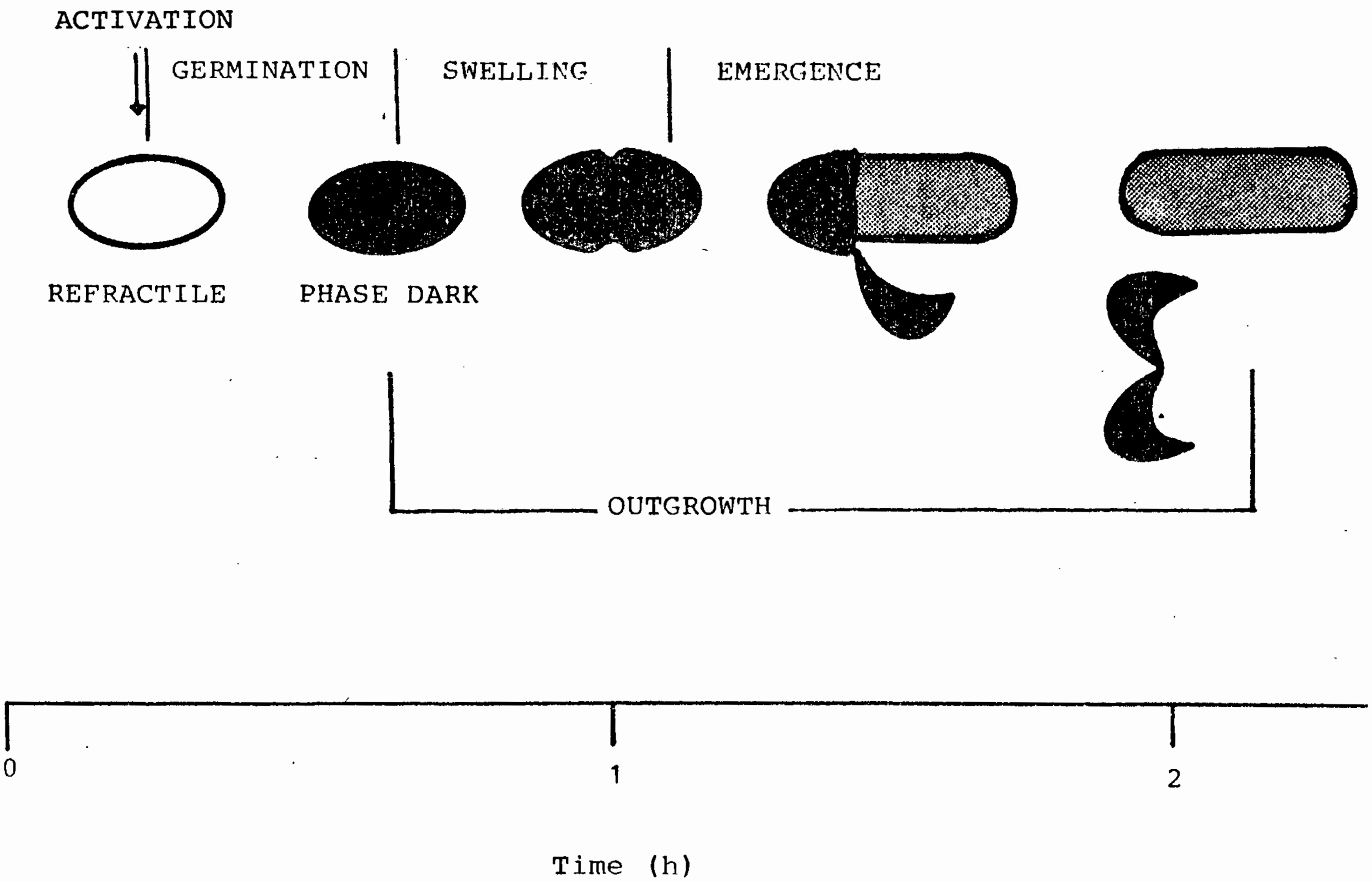

Figure 2.3

Diagramatic representation of the sequence of events following heat activation of *C.acetobutylicum* P262 spores in CBM.

c 14 $g\ell^{-1}$ compared with *c* 18 $g\ell^{-1}$ obtained in MFM. Davies and Stephenson (1941) found that when solvent yields were low the pH of the medium was high. In MFM the decrease in pH was gradual and did not decrease below pH 5.0. However in the other media tested the pH decreased rapidly to a pH below 5. Leung (1980) utilizing *C.acetobutylicum* ATCC 824 in Leung medium containing 10% (w/v) glucose (pH controlled above pH 5), obtained *c* 20 $g\ell^{-1}$ after 24 h fermentations. Under the same culture conditions *C.acetobutylicum* P262 produced less than 10 $g\ell^{-1}$ solvent after 48 h.

It became apparent that the initial aim of trying to identify and manipulate a few key factors to give better solvent yields would not be easily obtained. Not only did the solvent levels differ but the overall pattern of cell development appeared to be markedly different in the various laboratory systems. In view of these differences, a more detailed investigation of the growth patterns, morphological and physiological changes were necessary.

CHAPTER III

THE STUDY OF THE ABE FERMENTATION UNDER LABORATORY CONDITIONS

> Studies on the morphological changes in the ABE fermentation system suggested that the clostridial stages in *C.acetobutylcium* were involved in the conversion of acetate and butyrate to acetone and butanol. This was supported by the isolation of sporulation mutants. The different solvent levels produced by *C.acetobutylicum* in MFM and CBM appear to be associated with the different patterns of cell development which were observed in the two systems.

3.1 INTRODUCTION

Based on the information available in the literature (Doelle, 1975; Stanier *et al.*, 1976; Gottschalk, 1978), the most probable metabolic reactions occurring during glucose utilisation by *Clostridium* organisms are shown in Fig. 3.1. Initially glucose is metabolized via the Ebden-Meyerhof-Parnas (EMP) pathway with the formation of pyruvate which is then broken down to acetyl-CoA with the release of carbon dioxide and hydrogen. Acetyl-CoA itself plays a central roll in the metabolism of the *Clostridium* bacteria by serving as a precursor to ethanol, acetate and all the fermentation solvents. Saccharolytic clostridia ferment glucose to butyric acid in preference to acetate as the latter is a stronger acid. From an energetics point of view the production of acetate as a

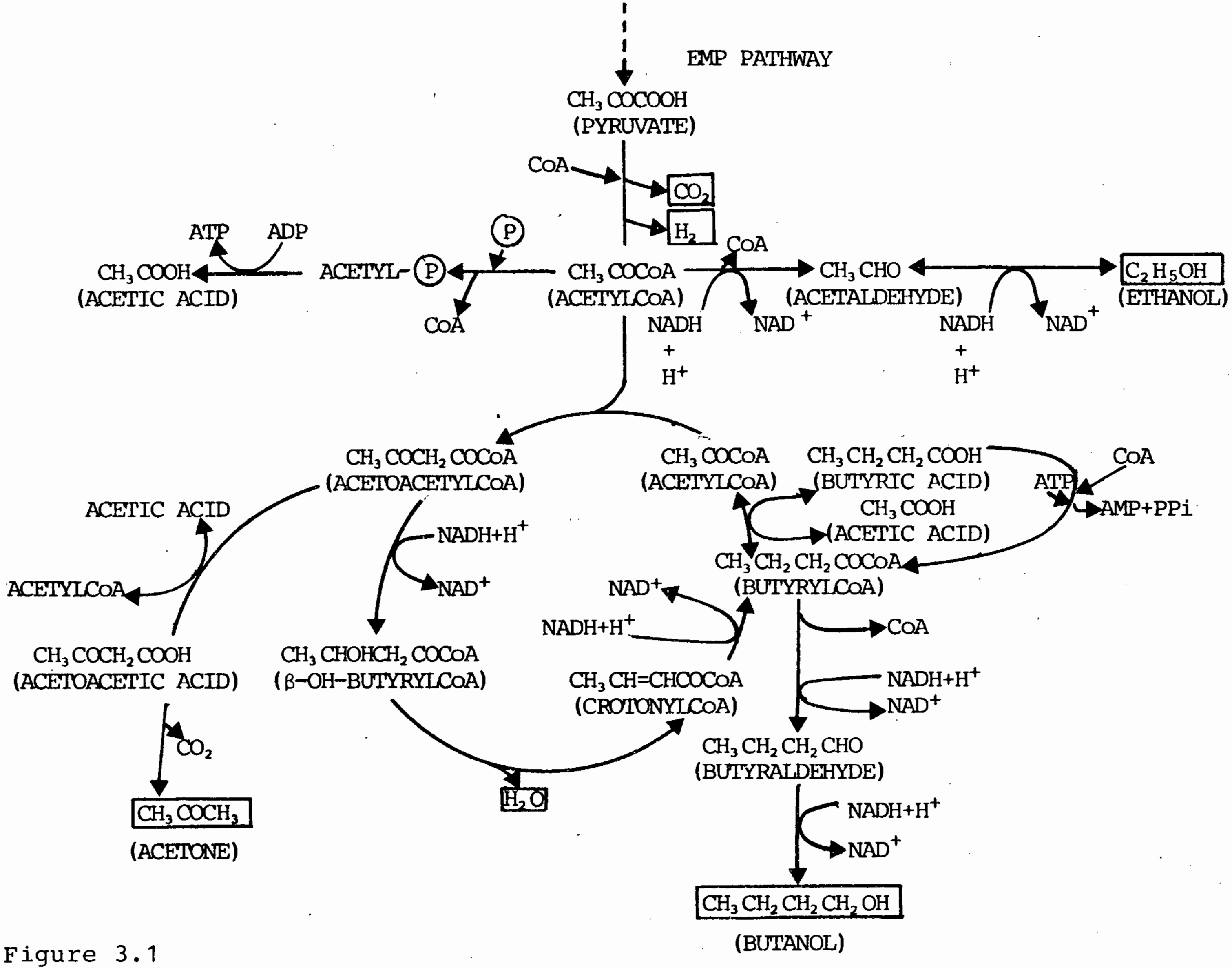

Figure 3.1
Metabolic pathway for glucose utilisation by *Clostridium* bacteria.

sole end product would not be satisfactory since it becomes more difficult to reoxidize the NADH as the pH drops towards the acid region. The clostridia have developed a similar cyclic mechanism to that found in the propionibacterium which brings about the formation of butyric acid. Two acetyl-CoA molecules undergo a combination to form acetoacetyl CoA initiating the cyclic mechanism that leads to the production of butyric acid. A number of saccharolytic clostridia, which normally ferment carbohydrates to butyric acid, are able to change their systems, favouring the production of acetone, and concurrently converting the butyrate already produced to butanol. This new system comes into operation as soon as the butyrate production has lowered the pH of the medium to *c* pH 4.0, at which time a new enzyme system is activated leading to the formation of acetone and butanol. In this system, acetoacetyl CoA is diverted from the normal cyclic mechanism and is utilised via a transferase system for the production of acetoacetate which is then decarboxylated to acetone. The diversion of the original cyclic system to form acetone stops further production of butyric acid. As a result of the interuption of the cycle two steps creating NAD^+ are eliminated and consequently the clostridia must find alternative reducing reactions for NAD^+ regeneration (i.e. the production of butanol). This involves three consecutive reactions where butyryl-CoA and butyraldehyde are formed as intermediate compounds. The last reaction in the cycle is reversed and the enzyme CoA-transferase transfers the co-enzyme (CoA) from the acetyl group to the butyl group. The acetate formed is used for the regeneration of acetyl-CoA in the production of acetone. Butyryl-CoA may be formed by an alternative pathway if there is a deficiency in the amount of acetyl-CoA available. ATP and enzyme CoA are required for the process. The reduction of butyryl-CoA to butyraldehyde is catalysed by the same aldehyde dehydrogenase that reduces acetyl-CoA to acetaldehyde, the final reduction to butanol is carried out by the NAD-linked alcohol dehydrogenase. This production in butanol occurs after the change to the production of acetone has taken place.

The pathway to ethanol production differs from that in yeast, where ethanol is produced from pyruvate which is converted by a decarboxylase to acetaldehyde and carbon dioxide, the acetaldehyde being reduced by a NAD-linked reaction to ethanol. *C.acetobutylicum* forms acetyl-CoA from pyruvate which is converted by the enzyme aldehyde dehydrogenase to acetaldehyde which in turn is converted by NAD-dependent alcohol dehydrogenase to form ethanol. It is known that the final solvent ratios obtained are a function of the *Clostridium* utilised (Beech, 1952). Most of the bacteria that have been used in the commercial ABE fermentation give a mixture composed of *c* 65% butanol, 30% acetone and 5% ethanol (Rose, 1961).

The biochemistry and physiology (Prescott and Dunn, 1940; Walton and Martin, 1979) associated with solvent production in the ABE process have been well documented. However, little has been reported about the relationship between the morphological changes and solvent production in the ABE process. This chapter describes the morphological changes which occur during the ABE fermentation and the isolation of *C.acetobutylicum* sporulation mutants which can be used to elucidate the relationship between morphology, growth and solvent production.

3.2 MATERIALS AND METHODS

3.2.1. BACTERIAL STRAINS

As described in 2.2.1.

3.2.2. MEDIA

All media used are listed in Appendix B.

3.2.3. GENERAL METHODS

The fermentation methods, heat shocking and inoculation procedures outlined in Chapter II were used unless stated otherwise.

3.2.4. GROWTH MEASUREMENTS

Cell growth in laboratory media was monitored turbidometrically at 600 nm in a Corning Colorimeter. Total bacterial counts were carried out using a Thoma counting chamber (Webb, England) and a Zeiss photomicroscope fitted with phase contrast and interference contrast optics. Viable counts were carried out by diluting the cells into fresh CBM and plating onto CBM plates under stringent anaerobic conditions.

3.2.5. MORPHOLOGY

Clostridial stage counts and spore counts were carried out microscopically using the Thoma counting chamber. Cells were investigated for the presence of capsules by negative staining with Indian ink, for granulose by staining with iodine and forespore formation by the methods of Smith and Ellner (1957) and Hoeniger and Headley (1968). The Gram reaction of the bacterium was determined by the method described by Cruikshank *et al.*, (1969). The % motility on a wet slide mount was calculated by a differential count of motile and non-motile cells in a given microscopic field.

3.2.6. CHEMICAL ANALYSIS

Titratable acids, pH and solvent analyses were carried out as described in 2.2.6.

3.2.7. CELL FREE AUTOLYSIN ASSAY

The autolysin was assayed by the well method (Mayr-Harting *et al.*, 1972) in 10 mℓ CBM plates containing 1% (w/v) agar and seeded with a 0.8 mℓ suspension of *C.acetobutylicum* indicator strain (Webster *et al.*, 1981). A series of two-fold dilutions of the test culture supernatants (centrifuged at 10 000 rpm for 10 min at 4°C) were made in 0.01 M sodium acetate buffer, pH 4.5 and 25 μℓ of each dilution was added to separate wells. The indicator strain suspension (OD_{600} 0.5-0.7) in sterile distilled water was prepared from an overnight plate of the *C.acetobutylicum* indicator strain. The plates were incubated at 37°C in Gas Pack (B Dickinson and Co.) jars. The activity of the cell free autolysin in arbitrary units (AU) was defined as the reciprical of the highest dilution which gave a detectable zone of inhibition.

3.2.8. ISOLATION OF SPORULATION MUTANTS

Exponential phase CBM cultures (5 x 10^7 cell $m\ell^{-1}$) of *C.acetobutylicum* P262 were treated with ethyl methane sulfonate (EMS) (2.5% v/v) for 20 min at 37°C to obtain 10% survival. The washed cells were resuspended in CBM and incubated for 18 h before diluting and plating onto CBM agar containing rifampin (100 μg $m\ell^{-1}$). Spontaneous rifampin resistant mutants were selected on gradient plates containing rifampin. Rifampin-resistant colonies were examined to determine whether they were also defective in sporulation. Granulose mutants were isolated by the method of Mackay and Morris (1971). The EMS treated cells were washed and diluted to give discrete colonies when plated onto CBM agar and incubated for 18 h. The colonies were then exposed to I_2 vapour for 1 min. The granulose positive colonies turned blue black while the granulose negative colonies remained a creamy white colour.

3.3. RESULTS

3.3.1. STUDY OF THE ABE FERMENTATION IN MOLASSES MEDIUM

3.3.1.1. GROWTH AND PHYSIOLOGICAL CHANGES

The total and viable cell counts, titratable acid concentration, pH and solvent production were monitored in MFM (Fig. 3.2a

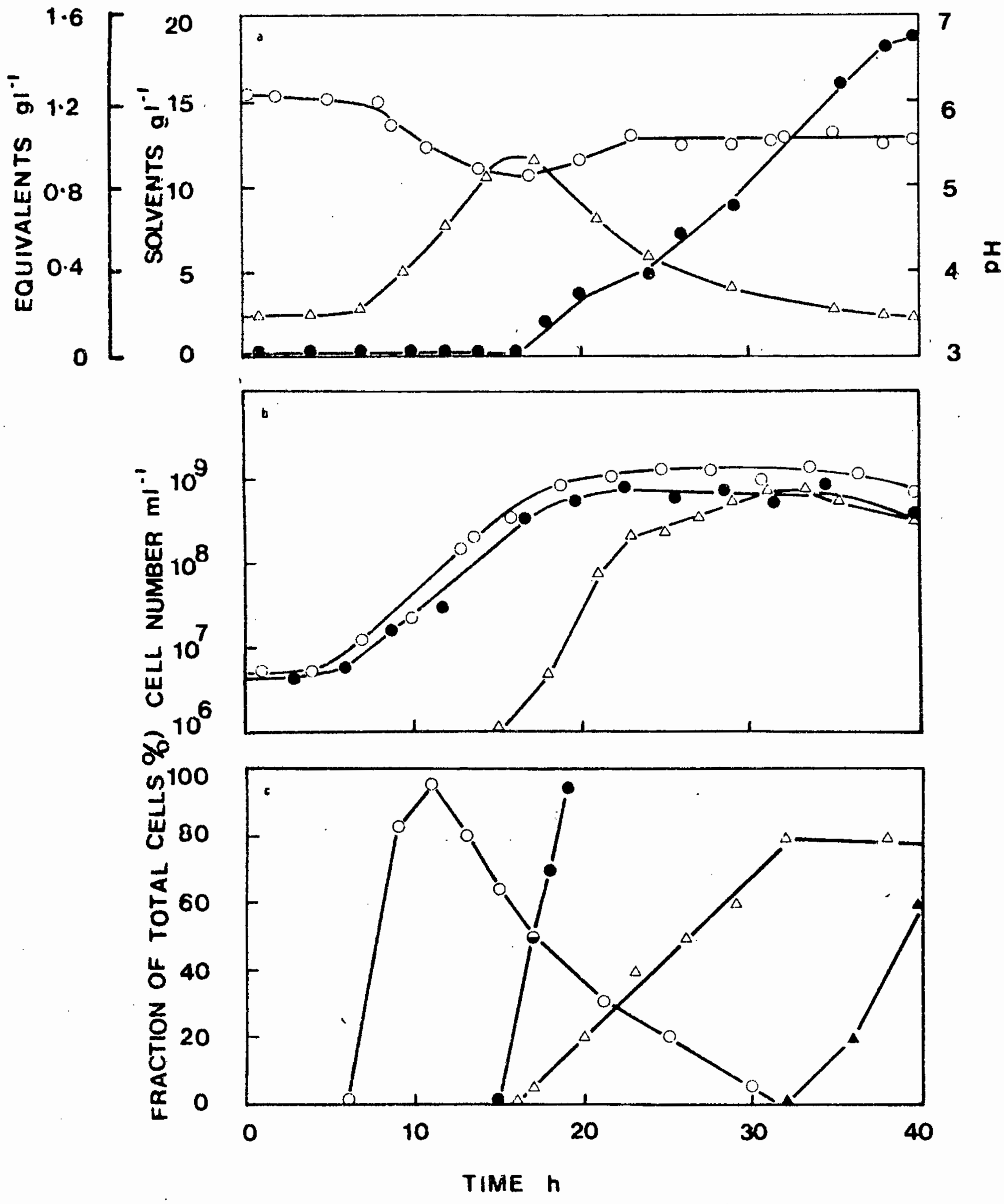

Figure 3.2

Physiological (a) growth (b) and morphological (c) changes in *C.acetobutylicum* during the ABE fermentation in MFM. (a) pH (○); titratable acids (△) and total solvents (●). (b) Total cell count (○); c.f.u. (●) and total clostridial form count (△). (c) Motility (○); onset of granulose accumulation (●); appearance of phase bright clostridial forms (△) and appearance of forespores (▲).

and 3.2b). Exponential phase cells in CBM were diluted 10^{-4} fold to give an initial cell concentration of between 10^5 to 10^6 cells $m\ell^{-1}$ in the MFM. Cultures inoculated in this way were characterised by a short lag period of *c* 5 h. During the exponential growth phase (between 4-8 h) the doubling time in the culture was *c* 90 min. From 8 to 12 h the doubling time began to increase and from 18 to 20 h no further increase in cell number occurred. During the exponential growth phase, the titrable acidity increased rapidly to a maximum after 13-18 h. This increase in titratable acid corresponded to a decrease in the pH from pH 6.8 to pH 5.1. After 18 h the quantity of titratable acid decreased. Coincidental with the drop in the level of titratable acids there was an increase in neutral solvents and the pH increased to *c* pH 5.7. Solvents were only detected after *c* 18h when the cultures were entering the stationary growth phase. Solvent production correlated with a change in cellular morphology and the appearance of clostridial forms (Fig. 3.2a and b). The final solvent yield varied between 16 and 18 $g\ell^{-1}$. The ratio of the solvents produced were *c* 6:3:1; *n*-Butanol (11.858 $g\ell^{-1}$): Acetone (5.519 $g\ell^{-1}$): Ethanol (0.514 $g\ell^{-1}$). The solvent levels increased between 18 to 36 h before plateauing. During this 12 h period the majority of the vegetative rods were converted to clostridial forms.

3.3.1.2. MORPHOLOGICAL CHANGES

Following inoculation into MFM the cells appeared as elongated rods which developed septa and resulted in the formation of long chains of phase dark, strongly Gram-positive cells with

Figure 3.3

Cytological changes in *C.acetobutylicum* P262 during the fermentation in MFM. (A) (6h) Actively growing phase dark rods (⬆) (400 x). (B) (18h) Phase grey rods (⬆) (400 x). (C) (18h) Interference contrast optics of phase grey rods (⬆) (400 x). (D) (33h) Phase bright clostridial (⇧) forms with phase dark (⬆) (600 x). (E) (38h) Phase bright clostridial forms (⇧) and degenerating cells (⬆) (600 x). (F) (38h) Interference optics of phase bright clostridial forms (⇧) and degenerating cells (⬆).

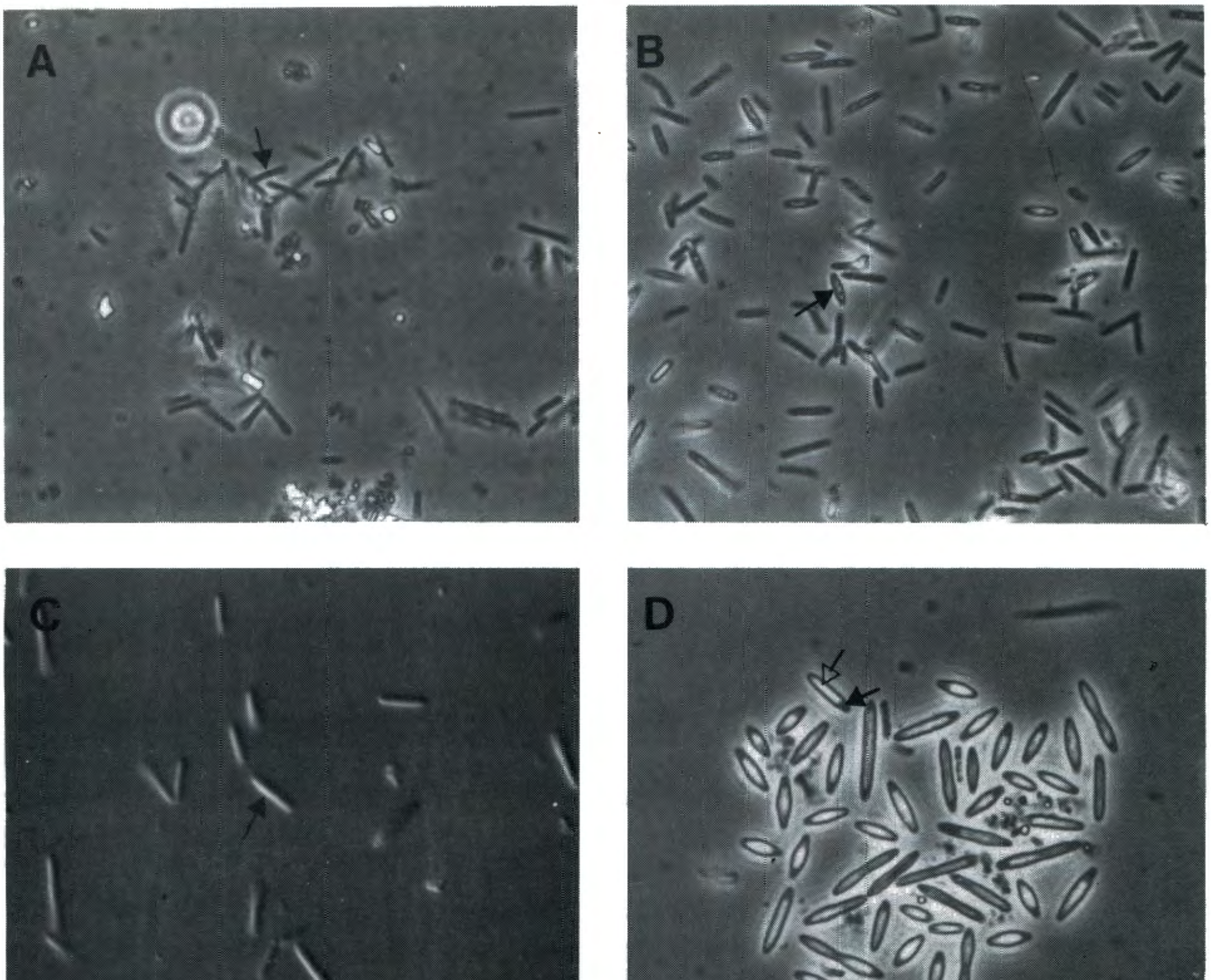
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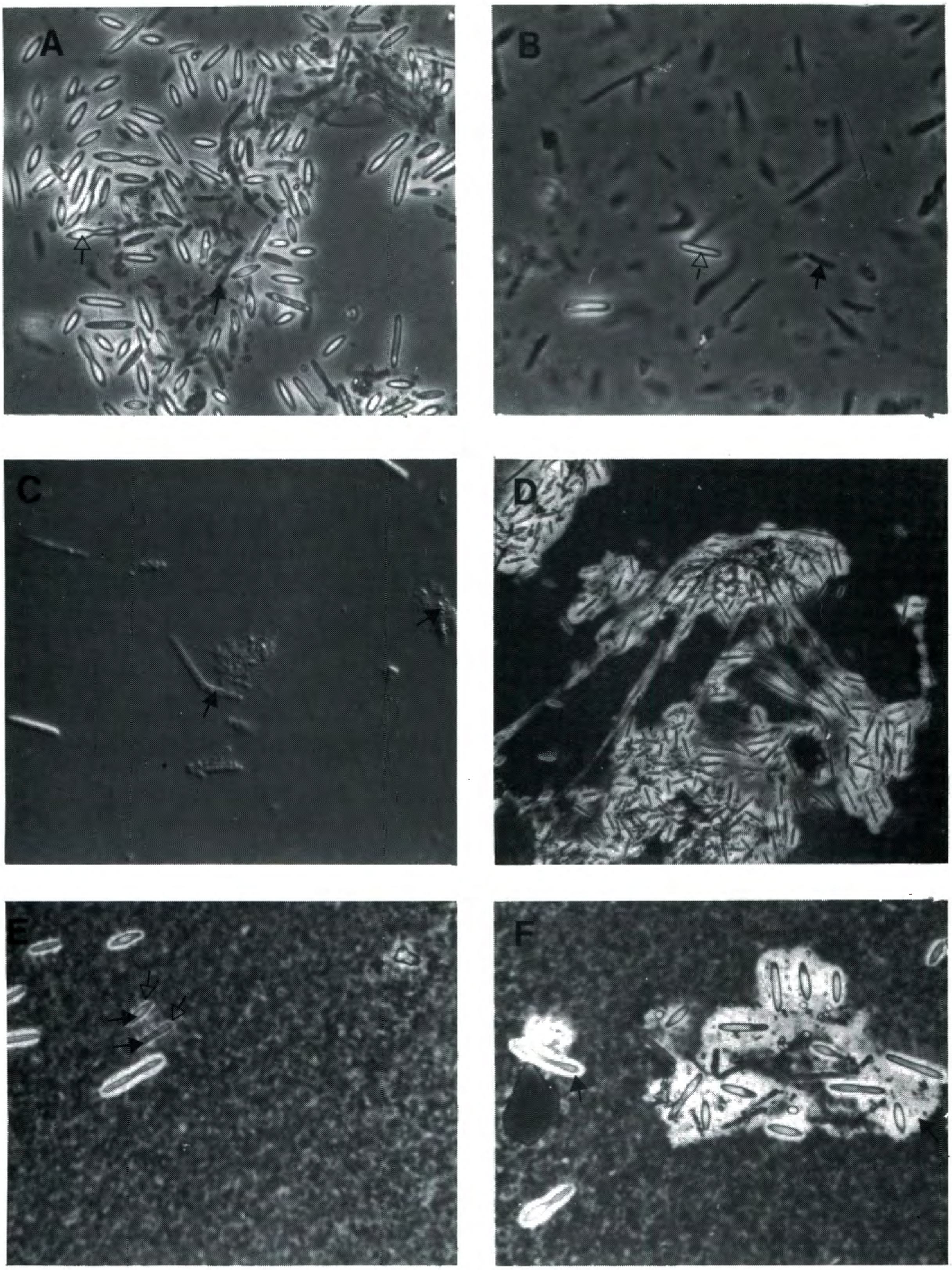
Figure 3.4

Cytological changes in *C.acetobutylicum* P262 during the ABE fermentation in MFM. (A) (48h) Phase bright clostridial forms (⇧) and phase dark degenerating clostridial forms (⬆) (400 x). (B) (60h) Majority of cells have degenerated (⬆) and were phase dark. Few phase bright clostridia (⇧) with no forespores (600 x). (C) (60h) Interference contrast optics of degenerating cells (⬆) (600 x). (D) (48 h) Negatively stained culture showing how the cells clump together. (E) (20h) Negatively stained culture showing thin capsule (⇧) associated vegetative cells (⬆). (F) (36h) Negative stained culture during peak of clostridial formation showing thick capsule (⬆).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sporadic and sluggish motility. About 6 h after inoculation the chains began to break up resulting in a mixture of individual rods and pairs which were highly motile (Fig. 3.3A). The peak in the number of motile cells occurred at 12 h (Fig. 3.2c). At this stage the growth rate was at its maximum and the rods were actively dividing. Cessation of active growth between 18 and 22 h correlated with the decrease in motility and at 22 h the majority of the cells were non-motile and the rest were sluggishly motile (Fig. 3.2c).

From 15 to 18 h the phase dark cells became phase grey with a granular appearance (Fig. 3.3B), and under interference contrast have a smooth appearance (Fig. 3.3C). Between 30 and 36 h the majority of the cells were phase bright and swollen and formed the characteristic "cigar-shaped" clostridial forms (Fig. 3.3D). A small minority of the cells (<10%) which did not form clostridial forms became Gram-negative and underwent degenerative changes. From 36 to 40 h the clostridial forms started degenerating (Fig 3.2b). The degenerating cells were phase dark (Fig.3.3E) and had a "rough" appearance under interference contrast optics (Fig. 3.3F). After 48 h the majority of the cells were phase bright clostridial forms (Fig. 3.4A), however after 60 h there were less than 10% phase bright clostridial forms present and the majority of the cells were phase dark (Fig. 3.4B). The phase dark cells have a "rough", ill-defined outline under interference contrast optics (Fig. 3.4C).

Granulose accumulation within the cells was first detected 1 to 2 h before the pH breakpoint (Fig. 3.2c) as small isolated phase bright granules (Fig. 3.3B) which stained a brownish-purple with iodine. Within 2 h granulose accumulation could be detected in over 90% of the cells (Fig 3.2c) which stained a uniform deep purple with iodine (Fig 2.1F). This accumulation of granulose was associated with the change from vegetative cells to the typical swollen cigar shaped clostridial forms. Another cytological change which was associated with the conversion of vegetative rods into clostridial forms was the production of a clearly defined extracellular capsule. From 16 to 18 h very faint capsules were associated with bacteria which were still in the vegetative form (Fig 3.4E), during this stage the capsule thickness varies. At the peak of clostridial formation the capsules were well-formed and were two to three times the thickness of the width of the clostridial forms (Fig 3.4F). The encapsulated clostridial forms stick together and form clumps which rise and fall in the media because of the gas associated with these clumps (Fig. 3.4D).

In the factory fermentations which are usually stopped after 36 h, spores were not normally observed. In laboratory fermentations which were allowed to continue for 60 h, less than 5% sporulation occurred. The sporulation was low even though the forespores developed in the clostridial forms between 30-35 h (Fig 3.2c and Fig. 3.3D). The forespores did not develop into mature phase bright spores but degenerated before sporulation occurred.

3.3.1.3. RELATIONSHIP BETWEEN SOLVENT PRODUCTION, AUTOLYSIN PRODUCTION AND CLOSTRIDIAL FORM DEGENERATION

The production of autolysin by *C.acetobutylicum* P262 in MFM is shown in Fig. 3.5. Low levels of autolysin were detected after 24 h. The concentration of autolysin increased between 24 and 50 h. The increase in the titre of autolysin coincided with the end of exponential growth phase. This increase in autolysin also coincided with a decrease in the total count and the clostridial form count, due to cell lysis. Once cell lysis commenced, there was no further increase in the solvent concentration which plateaued at 45 h.

3.3.2. ABE FERMENTATION IN THE LABORATORY MEDIUM CBM

3.3.2.1. GROWTH AND PHYSIOLOGICAL CHANGES

The same growth and physiological changes monitored in MFM were observed in CBM (6% glucose) (Fig. 3.6 a and b). Growth was measured turbidometrically as well as by viable and total counts. CBM fermentations were characterised by having no lag period. The rate of cell growth and the initial decrease in pH was more rapid in the CBM cultures than in MFM cultures. During exponential growth the doubling time was *c* 40 min compared with 90 min in MFM cultures. From 10-12 h there was a peak in the total number of bacteria (Fig 3.6b) compared with a peak in absorbance readings (600 nm) at *c* 14 h (Fig 3.6a). The viable count, colony forming units (c.f.u.) coincided with the total bacterial count for the

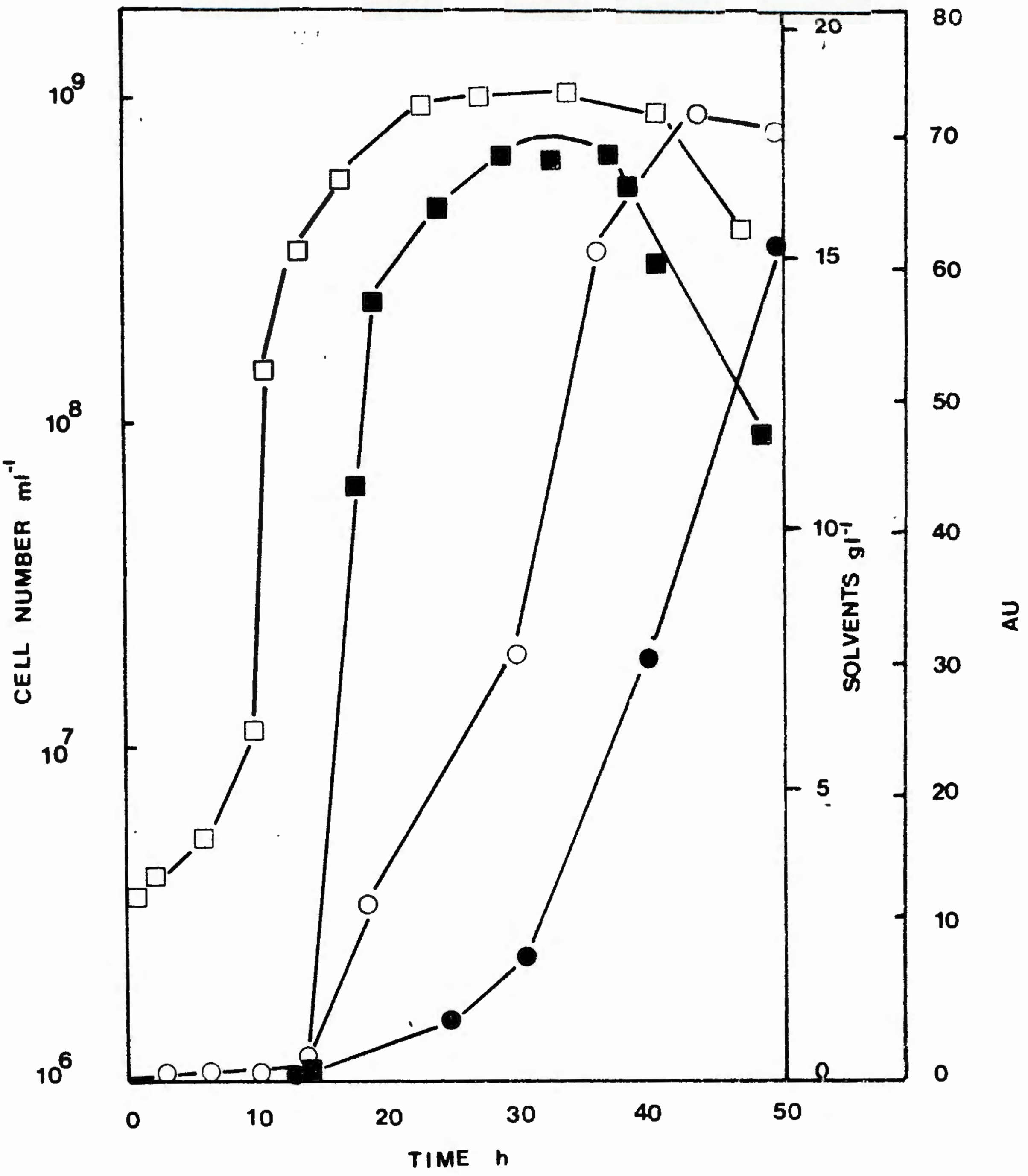

Figure 3.5

C.acetobutylicum P262 growth, solvent production and autolysin production in MFM. Total count (□); clostridial form count (■); total solvents (○); cell free autolysin concentration (●).

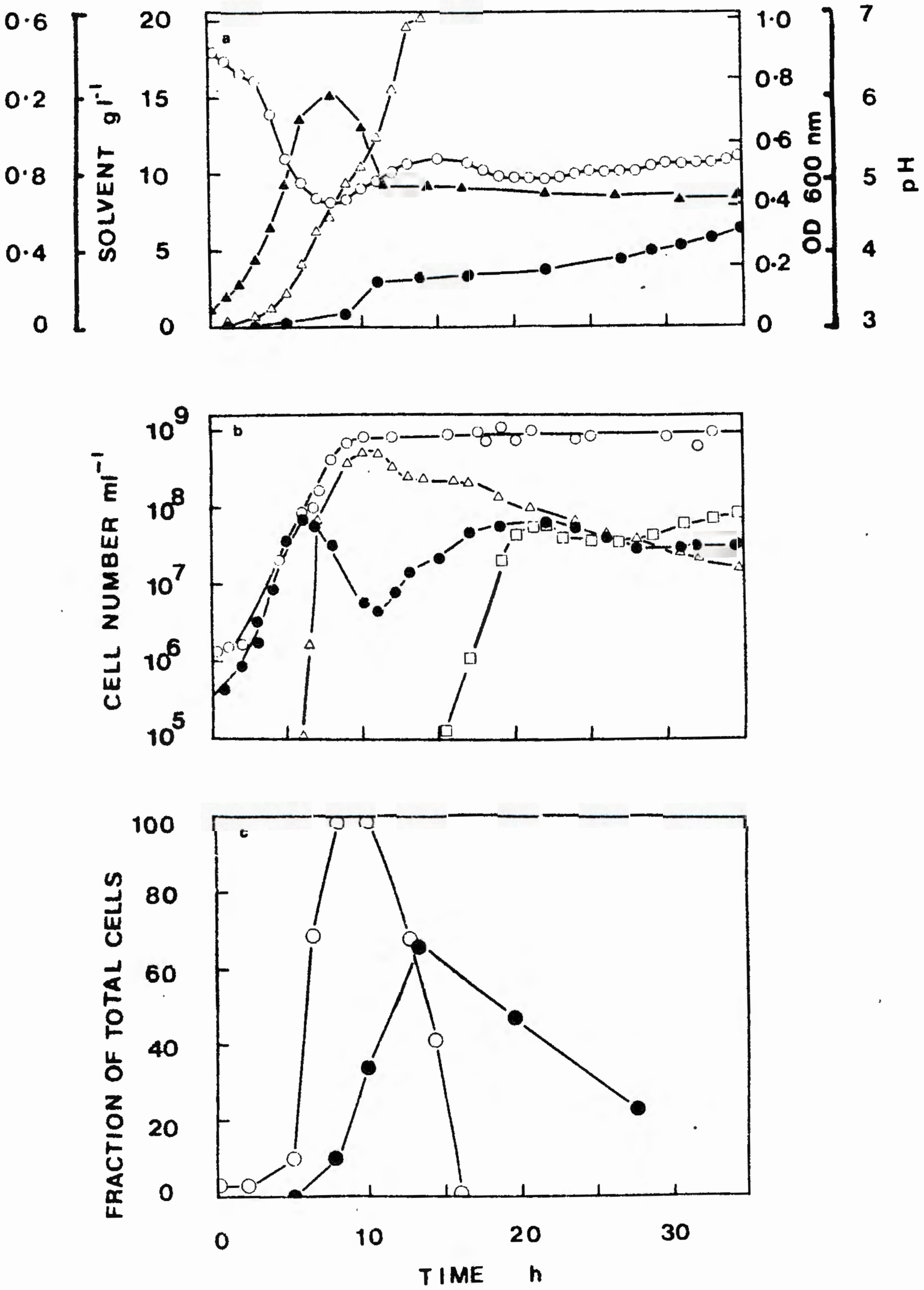

Figure 3.6

Physiological (a) growth (b) and morphological (c) changes in *C.acetobutylicum* during the ABE fermentation in CBM. (a) pH (○); Titratable acids in equivalents ℓ^{-1} (▲); Total solvents ( ); OD (△). (b) Total cell count (○); clostridial form count (△); c.f.u. (●); phase bright spores (□). (c) Motility (○); granulose accumulation ( ).

first 6-8 h after which there was a decrease in the number of c.f.u. up to 11-12 h when the c.f.u. increased and plateaued at *c* 20 h. This decrease in viability was associated with the peak in titratable acids (Fig 3.6a and b).

The period between 0-8 h was associated with the production of titratable acids and a decrease in the pH from *c* pH 6.5 to *c* pH 4.5. Low levels of solvent were detected from 8-10 h when the cultures were entering the stationary growth phase. Solvent production in CBM, as in MFM correlated with a change in cellular morphology and the appearance of phase bright clostridial forms (Fig 3.6b). The solvent yield obtained varied between 5-8 $g\ell^{-1}$. The ratio of the solvents obtained were *c* 5:1:<1; *n*-Butanol (5.054 $g\ell^{-1}$): Acetone (1.374 $g\ell^{-1}$): Ethanol (0.217 $g\ell^{-1}$). During the formation of solvents the titratable acids decreased slightly and the pH increased to pH 4.5. The break in the pH was not as marked as in the MFM system.

3.3.2.2. MORPHOLOGICAL CHANGES

After inoculation in CBM the short dark rods elongated (Fig. 3.7A) and resulted in the formation of long chains, which were strongly Gram-positive with sporadic motility. About 2 h (compared with 6 h in MFM). After inoculation the chains broke up and the cells were actively motile. At this stage the growth rate was at its maximum. As in MFM a reduction in the growth rate after 8-10 h (*c* 20 in MFM) correlated with a decrease in motility. After 15 h none of the cells were motile (Fig 3.6c).

Figure 3.7

Cytological changes in *C.acetobutylicum* during the ABE fermentation in CBM containing 5% glucose. (A) (2h) Elongated, actively growing phase dark vegetative rods (⬆) (160 x). (B) (5h) Phase grey vegetative rods (⬆) (400 x). (C) (10h) Phase grey rods (⬆) and degenerative, phase dark rods (⇧) (400 x). (D) (12h) Phase bright cells (⇧) with phase dark forespore (⬆) (400 x). (E) (15h) Phase bright cells (⇧) and degenerating cells (⬆) (400 x). (F) (24h) Sporulating cells showing phase bright spore (⬆) and phase dark spore mother cell (⇧) (400 x).


A
B
C
D
E
F

Between 6-10 h after inoculation the cells changed from phase dark to phase grey (Fig 3.7B). A small proportion of the vegetative cells degenerated and became Gram-negative and had a phase dark granular appearance (Fig 3.7C). The phase bright clostridial forms in CBM were long and thin and did not have the characteristic "cigar-shaped" appearance (Fig 3.7D). Phase dark forespores (Fig. 3.7D) appeared after 12 h.

Granulose accumulation within the cell occurred at the pH breakpoint (Fig 3.6c) and was correlated with the appearance of phase bright cells. Capsule formation was not as well-defined in the CBM system as in the MFM system.

In the CBM system developmental sequences leading to the production of mature free spores could be followed and the stages of spore development could be identified. The stages were similar to those reported for other bacilli and clostridial strains (Murrel, 1967; Kay and Warren, 1968; Fitz-James and Young, 1969; Walker, 1970; Mackey and Morris, 1971). The stages include stage II cells which showed septum formation and forespore engulfment. Stage III cells contained phase dark forespores (Fig 3.7D). Stage V cells were characterised by the appearance of phase bright forespores and at the same time the spore mother cell appeared phase dark (Fig. 3.7F). A proportion of the stage II/III cells (*c* 20%) underwent degeneration and did not form mature spores (Fig. 3.7E).

3.3.3. THE USE OF DEVELOPMENTAL STAGE MUTANTS TO STUDY THE RELATIONSHIP BETWEEN GROWTH, MORPHOLOGICAL STAGES

AND SOLVENT PRODUCTION

3.3.3.1. ISOLATION AND CHARACTERISATION OF MUTANTS

Two groups of mutants were obtained after treatment of the *C.acetobutylicum* P262 strain with EMS (Table 3.1). These mutants were characterised in CBM. The first group of mutants were blocked before stage II and were unable to produce phase bright swollen clostridial forms. These mutants were designated as *clos* mutants and remained as vegetative rods which were unable to synthesize granulose and did not produce capsules or forespore septa. The *clos* mutants which were obtained from rifampin plates (*clos*1 and *clos*2) were resistant to rifampin and were presumably RNA polymerase mutants. The *clos* mutants which were isolated as granulose negative mutants (*clos*3 and *clos*4) were rifampin sensitive and were presumably mutants in the granulose biosynthetic pathway.

The second group of mutants which were blocked after stage II, were able to accumulate granulose and produced typical clostridial forms surrounded by a capsule but were totally or partially blocked in the production of mature spores (Table 3.1). These mutants were designated as *spo* mutants and were either asporogenous (*spo*1 and *spo*2) or oligosporogenous (*spo*3 and *spo*4) mutants depending on whether their ability to produce mature spores was completely or partially blocked.

TABLE 3.1

Characteristics of the *C.acetobutylicum* sporulation mutants in CBM.

Strain	Clostridial forms	Granulose	Capsule	Septum	Forespore	Mature spore	Rifampin
P262	++[a]	++	++	++	++	++	s[d]
*Clos*1	-[c]	-	-	-	-	-	r[e]
*Clos*2	-	-	-	-	-	-	r
*Clos*3	-	-	-	-	-	-	s
*Clos*4	-	-	-	-	-	-	s
*Spo*1	++	++	++	+	-	-	r
*Spo*2	++	++	++	+	-	-	r
*Spo*3	+[b]	+	+	+	+	+	r
*Spo*4	+	+	+	+	+	+	r

[a] normal wild type levels of sporulation features

[b] reduced levels of sporulation features

[c] zero levels of sporulation features

[d] sensitive to 20 ng $m\ell^{-1}$ rifampin

[e] resistant to 100 μg $m\ell^{-1}$ rifampin

[f] solvent determined after 48 h

In all the *clos* and *spo* mutants, except for the *clos*2 mutant, the growth rate vegetative cell development and final cell concentrations were similar to that of *C.acetobutylicum* P262. The *clos*2 mutant grew more slowly and produced a lower final cell concentration. The characteristics of the *C.acetobutylicum* P262 sporulation mutants were similar in MFM and CBM except that the *spo*3 and *spo*4 mutants produced no phase bright mature spores in MFM.

3.3.3.2. SOLVENT PRODUCTION BY SPORULATION MUTANTS

The solvent production of the 8 sporulation mutants and *C.acetobutylicum* P262 were compared in MFM. In all experiments the MFM was inoculated with exponential cells growing in CBM. *C.acetobutylicum* P262 produced the same levels of solvent in MFM when inoculated with exponential CBM cultures which were initially inoculated with heat activated spores or Beef Liver Medium stock cultures of *C.acetobutylicum* P262 (Fig 3.8). The four *clos* mutants which were unable to form clostridial stages did not produce solvents. The two asporogenous *spo* mutants which produced clostridial forms at the same frequency as the *C.acetobutylicum* P262 but were unable to sporulate, produced similar amounts of solvent to *C.acetobutylicum* P262. The two oligosporogenous *spo* mutants which formed low numbers of clostridial stages and spores produced intermediate levels of solvents.

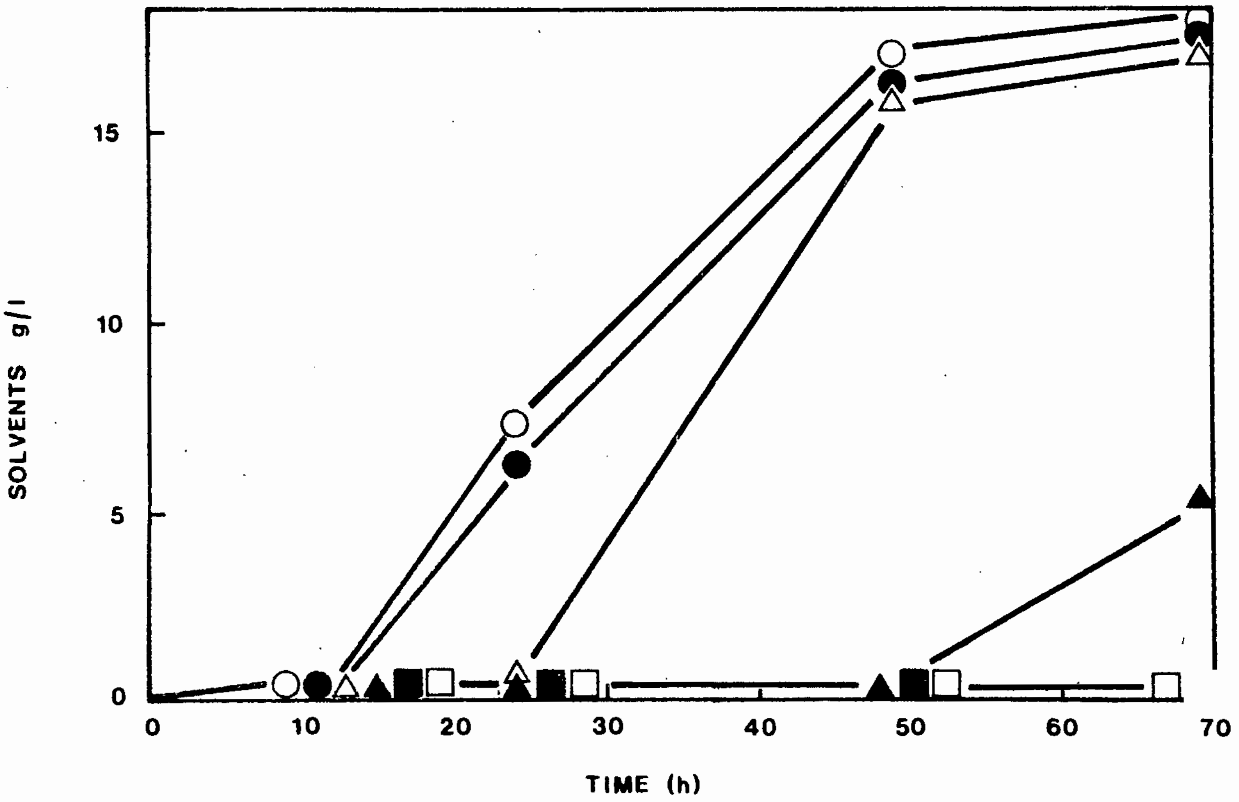

Figure 3.8

Solvent production by sporulation mutants of *C.acetobutylicum* P262 strain propagated from a spore suspension (●) and beef liver culture (○). *Spo*1 mutant (△); *spo*3 mutant (▲); *clos*1 mutant (□) and *clos*3 mutant (■).

3.4 DISCUSSION

The results obtained from monitoring the various growth and physiological changes in the laboratory scale fermentation of molasses correlated well with the results obtained from the factory fermentation (Spivey, 1978). Initial studies on the physiology of the ABE fermentation were carried out by Speakman (1920) and Peterson and Fred (1932). Three physiological phases similar to those proposed by Speakman (1920) could be distinguished in the laboratory fermentations.

The first phase was associated with exponential growth and the accumulation of titratable acids and a decrease in the pH. The bacteria reproduce rapidly during this period. Gas production begins almost immediately, but is not visible until after several hours have elapsed (Speakman, 1920).

During the second phase the fermentation enters the pH "break-point" after which the pH begins to increase again and the titratable acidity drops as the acids are converted to the corresponding solvents. Different hypotheses have been proposed for the process of solvent formation from the acids. The addition of acetic acid increased acetone yields but did not effect butanol production (Prescott and Dunn, 1940). Similarly the addition of butyric acid only increased butanol production (Prescott and Dunn, 1940). Bernhaeur and Kushner in 1953 (Nakmanovich and Shcheblykina, 1960) showed that the efficiency of conversion was dependent on the activity of the *C.acetobutylicum* used. Bernhaeur and Kurshner (1953) do not define what is meant by an active culture, presumably it is an actively growing culture. They showed that an active culture would convert 97% of the acetic acid while weak cultures would only convert 30% to acetone. Speakman (1920) and Peterson and Fred (1932) showed that the ratio of acetic acid and butyric acid varies during this period. Butyric acid disappears from the fermentation much more rapidly than acetic acid and accounts for the production of greater proportions of butanol than acetone (Reiley *et al.*, 1920).

The break in the pH could also be utilised to mark the change over between the early (active growth) and late (zero growth) stages in the fermentation process.

The final phase of the ABE fermentation is less distinct. According to Speakman (1920) the third phase was associated with a decrease in solvent production and an increase in titratable acid. In the factory fermentation the total titratable acidsreach a peak at *c* 18 h then decreases slightly (Spivey, 1978). However, in the laboratory fermentation of molasses, the titratable acid decreases to *c* zero in the medium. These differences may be due to the buffers produced by the hydrolysis of corn or other proteins (Speakman, 1920) in corn fermentations and the pH regulation with ammonia under factory conditions.

In the industrial fermentation of molasses it was necessary to maintain a pH of 5.0 to 6.2, and preferably of 5.5 to

5.85 for optimum solvent production (McCutchan and Hickey, 1954). In the factory liquid ammonia is fed into the vessels as a nitrogen source and a means of pH control (Spivey, 1978). In addition to ammonia, calcium carbonate is also utilised. Under laboratory conditions pH regulation with ammonia was unnecessary as solvent levels similar to those produced in the factory were obtained.

The rate of cell growth and the initial decrease in the pH of the medium was found to occur faster in CBM than in MFM. This rapid increase in titratable acids may be a critical factor in the low solvent yields obtained in CBM.

The different solvent levels produced by *C.acetobutylicum* P262 in MFM and CBM appear to be associated with the different patterns of cell development which are observed in the two systems. The appearance of solvents in the two systems coincided with the appearance of the first phase bright clostridial forms during the late exponential growth. In the CBM system the phase bright cells make up 50 to 70% of the total cell population compared with 70 to 90% in MFM. The clostridial forms in CBM do not form the characteristic "cigar shaped" clostridial forms associated with good solvent production in MFM (Spivey, 1978).

One of the most obvious differences between the two systems was the lack of mature spore formation in the MFM system in spite of the high proportion of clostridial forms produced, which are sporulation intermediates (Gunsalus and Stanier, 1960). In the CBM system the decrease in the number of phase bright clostridial forms was associated with the formation of mature spores. In the MFM system the decrease in the number of clostridial forms was associated with the production of autolysin and the inhibition of solvent production. It has been shown previously that the release of autolysin towards the end of exponential growth was accompanied by the lysis of the culture (Barber *et al.*, 1979; Webster *et al.*, 1980). The relationship between sporulation and solvent production and the production of autolysin and solvent is complex and needs to be studied in more detail.

Studies on the morphological changes in the ABE fermentation system suggested that the clostridial forms in *C.acetobutylicum* P262 were involved in the conversion of acetate and butyrate to acetone and butanol. This was confirmed by the isolation of sporulation mutants which either failed to form clostridial forms and produced no solvents or formed reduced numbers of clostridial forms and produced intermediate levels of solvent. The results confirm those of Davies and Stephenson (1941) and O'Brien and Morris (1971) who reported that acetate and butyrate but not butanol and acetone are produced by *C.acetobutylicum* during exponential growth. *Clostridium thermosaccharolyticum* is similar in that butyric acid is produced as a major fermentation product during exponential growth (Hsu and Ordal, 1970). Induction of sporulation in *C.thermosaccharolyticum* was associated with the formation of glucose-6-phosphate dehydrogenase and ethanol dehydroganase and the production of ethanol instead of butanol as the major fermentation product.

Sporulation and solvent production are stationary phase events. Associated with solvent production is the formation of phase bright clostridial forms. In common with other saccharolytic *Clostridium* species *C.acetobutylicum* synthesises large quantities of a starch-like polymer (granulose) which accumulates extracellularly (Hobson and Nsar, 1951; Darvill, *et al.*, 1977). The swelling and conversion to phase bright clostridial forms appears to be directly related to the onset of the accumulation of granulose. It is likely that the swelling and the change in refractile index which characterise the clostridial forms are due to the accumulation of granulose in the cell.

The production of extracellular capsular material associated with the appearance of clostridial forms appears to coincide with the change from extreme oxygen sensitivity to aerotolerance (O'Brien and Morris, 1971).

The acquisition of both granulose and capsules appears to be directly linked to the conversion of the vegetative rod to the clostridial form and the commitment to sporulation. In sporulation minus mutants (Jones, *et al.*, 1981) which are unable to produce clostridial forms no storage material or capsules are produced.

The identification of distinct morphological stages associated with solvent production and the isolation of *spo* mutants which could be maintained as clostridial forms suggests the possibility of the development of a novel ABE fermentation process involving dual continuous fermentations. In the first continuous culture a *clos* mutant could be utilised to produce acetate and butyrate which would be converted in the second continuous culture by an immobilised *spo* mutant maintained in the clostridial stage.

The identification of the morphological and cytological stages of *C.acetobutylicum* P262 can be used as a simple and practical means of monitoring the progress of the ABE fermentation and assisting in the identification of the causes of failed fermentations (Spivey, 1978).

A limiting factor in the ABE fermentation is butanol toxicity. An approach to overcome this problem is to search for butanol-tolerant strains or mutants. The results indicate that the screening programmes must involve the clostridial stage and not the vegetative cell.

CHAPTER IV

COMPARISON OF *C.ACETOBUTYLICUM* P.262 AND AUTOLYTIC DEFICIENT STRAINS IN THE ABE FERMENTATION

Autolytic deficient mutants which produced less autolysin than the parent strain were isolated. Pleiotropic effects of the *lyt*1 mutant include increased resistance to lysis by butanol and a higher glucose tolerance. The *lyt*1 mutant produced higher levels of solvents than the parent strain.

4.1 INTRODUCTION

A major problem associated with the ABE fermentation is the relatively low yield of solvents obtained. A contributing factor to the low yields is the inability of the bacterium to tolerate concentrations of butanol exceeding 13 $g\ell^{-1}$ (McCutchan and Hickey, 1954; Ryden, 1958; Abou-Zeid *et al.*, 1980). This butanol concentration is the highest final concentration obtained with commercially useful strains (Ryden, 1958), and determines the initial sugar concentration that can be used (6% w/v). In batch fermentation studies it has been shown that the butanol and butyrate concentrations are critical to the growth of *C.acetobutylicum* (Leung, 1980). The concentration of each of the ABE products which caused a 50% inhibition of growth were: ethanol, 60 $g\ell^{-1}$; acetone, 40 $g\ell^{-1}$; acetate, 15 $g\ell^{-1}$; butyrate, 14 $g\ell^{-1}$ and butanol, 13 $g\ell^{-1}$ (Leung, 1980).

Alcohols and other lipophylic agents interact directly with the membrane lipid bilayer (Sheetz and Singer, 1974). Lypophilic agents either fluidise or increase the membrane's internal viscosity depending on the site at which these molecules partition and interact (Sheez and Singer, 1974). This change in the degree of fluidity of the membrane is induced by the direct insertion of lypophilic agents into cellular membranes. Benzyl alcohol (Hubbel *et al.*, 1970; Paterson *et al.*, 1972), as well as ethanol and other alcohols (Grisham and Barnett, 1973; Hui and Barton, 1973) have been shown to directly affect membrane fluidity. The presence of alcohols in rich growth media results in considerable changes in fatty acid composition of *Escherichia coli* K-12 (Ingram, 1976). The degree of this variation in fatty acid composition is strongly dependent upon alcohol concentration. The alcohol concentration required to cause a given change in fatty acid composition is inversely related to chain length, as is toxicity (Ingram, 1976).

These changes in fatty acid composition in *E.coli* K-12 in the presence of alcohol represents an adaptive membrane alteration compensating for the direct physiochemical interaction of alcohols with the membrane. Similar adaptive responses of membrane lipids are proposed as a possible biochemical basis for tolerance to alcohol and related lypophilic agents (Ingram, 1976). Attempts have been made, notably by Ryden (1958), to find a way of overcoming the toxic effect of butanol in batch culture fermentations of *C.acetobutylicum*. Selection of mutants (Ryden, 1958) after ultraviolet irradiation and adaptation of cultures have proved unsuccessful. Higher levels of butanol 14.4 $g\ell^{-1}$ (as compared with 13.0 $g\ell^{-1}$) were obtained when large numbers of bacteria in their most active state were used as inocula (Ryden, 1958). Jerusalemskii (1958) grew *C. acetobutylicum* in continuous cultivation in the presence of increasing amounts of butanol. At first the culture withstood only 8 $g\ell^{-1}$ butanol; after 21 days in the presence of 6 $g\ell^{-1}$ butanol it withstood up to 10 $g\ell^{-1}$ butanol. After 200 days the bacteria were able to withstand 25 $g\ell^{-1}$ of butanol. Jerusalemskii (1958) found that this resistance was transferred through the spores to further generations. It is likely that during continuous cultivation a certain selection took place and that mutant strain(s) tolerating a high butanol concentration outgrew the wild type strain.

Continuous cultivation of *C. acetobutylicum* is a possible means whereby the butanol toxicity can be overcome by employing high dilution rates. A continuous ABE fermentation was patented as long ago as 1932 (Wheeler and Goodale, 1932). It was known that high solvent yielding strains of *C. acetobutylicum* were unstable and lost their ability to produce large quantities of solvent when repeatedly transferred at 24 h intervals. Consequently continuous cultivation of these strains did not seem possible. This was achieved in 1958 and Finn and Nowrey (1959) demonstrated that a strain of *Clostridium saccharoacetobutylicum* showed less tendency to degenerate when held in exponential growth in continuous propagation than when serially transferred at 24 h intervals. Dyr *et al.* (1958), working in Prague, have been able to show that strains of *C.acetobutylicum* can be induced to retain their characteristics throughout repeated vegetative transfer. The Czech workers have successfully carried out continuous ABE fermentations on a laboratory scale, using both one-stage and multi-stage systems. The toxicity of butanol to *C.acetobutylicum* has been overcome in this continuous process by employing high dilution rates, so that the toxic end products in the culture medium are kept as low as possible. If the increased productivity from a given fermentation volume and the increased ratio of butanol to acetone claimed in the literature (Dyr *et al.* 1958) using continuous cultivation of *C.acetobutylicum* can be verified, it would show a significant improvement in the economics of the fermentation process.

The *in situ* removal of butanol from the medium using activated charcoal and/or corn oil can increase the final butanol concentration to 25.8 $g\ell^{-1}$ (Hongo and Nagata, 1958) and 19.0 $g\ell^{-1}$ (Leung, 1980).

C.acetobutylicum P262 produces an autolysin(s) in the industrial fermentation (Barber, *et al.*, 1979). The autolysin was similar to a bacteriocin (Webster, *et al.*, 1981), and the similarity between bacteriocins and lytic agents has been described by Tagg *et al.* (1976). Autolysins are defined as enzymes that are capable of hydrolysing the cell walls of the producer strain and in particular the peptidoglycan layer. Bacterial cell walls may contain between 5 and 90% peptidoglycan, and the rest is made up of proteins, lipoproteins, polysaccharides, lipopolysaccharides and polyolphosphate polymers such as teichoic acid (Stolp and Starr, 1965; Ghuysen, 1968; Rogers, 1979). The peotidoglycan layer of the cell wall provides the rigidity, shape and osmotic stability for the cell. When autolysins are permitted to act, cells lose their osmotic protection and autolyse. Although there are exceptions, autolysins are generally over-produced whenever the metabolism of the cell is disturbed drastically. Sudden removal of oxygen from aerated exponential phase cultures of *Bacillus subtilis* causes lysis (Kaufman and Bower, 1958). Autolysins may also be induced by a sharp decrease in temperature of aerated growing bacteria, or by exposure of psychrophilic bacteria to temperatures above optimum. In some bacterial species, autolysins may be induced by the addition of univalent cations to bacterial cultures (Ogata and Hongo, 1973 and 1974; Hebeler and Young, 1976) and autolysis can be demonstrated by resuspending cells in buffers such as sodium acetate and phosphate buffer (Higgins *et al.*, 1970; Kawata and Takuni, 1971; Coyette and Shockman, 1973).

C.acetobutylicum P262 has been shown to produce cell bound autolytic activity (Allcock, 1981) and cell free autolytic activity (Webster *et al.*, 1981). The autolytic activity of *C.acetobutylicum* P262 has been studied mainly in CBM and the factory fermentation medium, MFM. The pattern of autolytic activity differs in these two systems. It was shown by Webster *et al.*, (1981) that high titres of cell free autolysin (4156 arbitrary units (AU)) were produced by *C.acetobutylicum* in MFM. In contrast low titres (<8AU) of autolysin were obtained in CBM (Webster *et al.*, 1981). Cell free autolytic activity (256AU) could be induced in CBM by 5% (v/v) molasses medium or 15% sucrose (Webster, 1980).

The autolysis of *C.acetobutylicum* P262 (grown in CBM) in sodium phosphate buffer is similar to that in *Streptococcus faecalis* (Pooley and Shockman, 1970) and *Lactobacillus acidophilus* (Coyette and Ghuysen, 1970) in that the ability of the cells to autolyse decreases sharply when the cultures enter the stationary growth phase (Allcock, 1981). The autolysis of *C.acetobutylicum* P262 in sodium phosphate buffer was the method used to assay cell bound autolytic activity (Allcock, 1981). Cell free autolysin activity could be detected in the sodium phosphate autolysate of *C.acetobutylicum* P262. In MFM, the release of cell free autolysin was associated with the onset of the stationary growth phase (Webster, 1980).

In common with a number of bacteria (Ghuysen and Shockman, 1973; Rogers, 1979; Rogers *et al.*, 1980) the *C.acetobutylicum* strain produces latent and active forms of autolysin. Proteases are able to activate the enzyme(s). Iso-electric focussing and PAGE of cell free and cell bound autolysates of *C.acetobutylicum* P262 cultures have indicated the presence of two autolytic enzymes of different iso-electric points (Allcock, 1981). These proteins band closely together on gel electrophoresis at a position corresponding to a molecular weight of 28 000.

The production and release of an extracellular autolysin by *C.acetobutylicum* in molasses medium has important implications for the industrial production of solvents. High titres of autolysin were always obtained at the end of exponential growth when solvent levels were maximal (Barber *et al.*, 1979). It has been suggested that the inhibition of autolytic activity at the stage when solvent production is maximal may result in increased solvent yields. The isolation and characterisation of autolytic deficient mutants (*lyt*) is important in order to establish the function(s) of the autolysin, and to determine the correlation between autolysin activity, solvent production and sporulation.

4.2 MATERIALS AND METHODS

4.2.1. Bacterial strains.

As described in 2.2.1.

4.2.3. General methods.

The fermentation methods, heat shocking and inoculation procedures outlined in Chapter II were used unless stated otherwise.

4.2.4. Growth measurements.

Turbidometric readings and total bacterial counts described in 3.2.4. and the clostridial form count described in 3.2.5. were utilised.

4.2.5. Physical measurements.

Titratable acid, pH and solvent analysis were carried out as described in 2.2.6.

4.2.6. Cell free autolysin assay.

The cell free autolysin was assayed by the well method described in 3.2.8.

4.2.7. Cellular autolysis activity.

Mid-exponential phase cells (5 to 10 mℓ) were cooled on ice, harvested by membrane filtration, washed twice with 10 mℓ cold double distilled deionized water and resuspended in

4 to 6 mℓ of 0.64 M sodium phosphate buffer, pH 6.3. Cellular autolysis at 37°C was monitored turbidometrically. One unit of cellular autolysis is defined as the loss of 0.001 OD units h^{-1}. In order to compare the autolysis of different samples, results are expressed in units of autolysin per 0.1 optical density of cell suspension. One unit is then equivalent to a loss of 1% of the initial turbidity h^{-1}.

4.2.8. Amylase activity

The reagents used are listed in Appendix B. Samples were centrifuged at 10 000 rpm at 4°C and 1 mℓ samples of the supernatants were added to 10 mℓ of 1% soluble starch solution at 55°C. The samples were shaken and the enzyme reaction stopped by placing 1 mℓ of the reaction mixture into 10 mℓ N/10-HCl. One mℓ of this solution was mixed with 10 mℓ of iodine solution and allowed to stand for 2 min before the OD was determined at 660 nm. The units of amylase activity was determined from the following equation:

$$\alpha \text{ - amylase activity} = \frac{D - D_1}{D} \times \frac{100}{10} \times n$$

Where D = optical density obtained without enzyme

D^1 = optical density obtained for the sample.

n = dilution.

One α - amylase unit was defined as that activity which causes a 1% drop in OD_{660} of a 1% soluble starch solution held at 55°C for 1 min.

4.2.9. Isolation of Autolytic-deficient mutants

Mutants deficient in autolytic activity were isolated from exponential-phase CBM cultures (5 x 10^7 cells $m\ell^{-1}$) of *C.acetobutylicum* P262 treated with ethyl methane sulfonate (EMS) (2.5% v/v) for 20 min at 37°C to obtain 10% survival. The washed cells were resuspended in the minimal salt medium and incubated for 18 h before diluting and plating onto CBM plates overlayed with 5 mℓ CBM containing autoclaved exponential phase *C.acetobutylicum* P262 cells. The plates were incubated at 37°C for 18 h. Colonies which produced small or no zones of lysis in the overlay were selected and tested for cellular autolytic activity.

4.2.10. Effect of Sugar concentration

The sugar tolerance of *C.acetobutylicum* P262 and the *lyt*1 mutant was determined by monitoring bacterial growth (OD) in CBM containing different concentrations of glucose and sucrose.

The effect of adding sucrose, glucose or starch to MFM (TIS 6.5%) on solvent production by *C.acetobutylicum* P262 and the *lyt*1 mutant strain were compared. The effect of increasing the initial TIS on solvent production by *C.acetobutylicum* P262 and the *lyt*1 mutant was also investigated. The percentage TIS was determined by N.C.P. using the modified Lane and Eynon method (Mann and Saunders, 1967). The percentage conversion of TIS to solvents was used as a measure of the efficiency of the fermentation. Percentage conversion was calculated as follows:

$$\% \text{ Conversion} = \frac{\text{Solvent yield } \% \text{ (w/v)}}{\text{TIS } \% \text{ (w/v)}} \times \frac{100}{1}$$

4.2.11. Effect of solvents on growth, cellular autolysis and clostridial degeneration

4.2.11.1. Effect on growth

Oxygen free solutions of butanol and acetone were prepared by placing the prewarmed solvents (60°C) under stringent anaerobic conditions in the anaerobic glove box for 48 h.

The effect of acetone and butanol on growth of *C.acetobutylicum* P262 and the *lyt*1 strain were compared. Hungate tubes containing 8 mℓ of CBM broth at 1.25 times its normal concentration and pre-equilibrated at 34°C was inoculated with 1 mℓ of an exponential phase CBM culture. The cultures were incubated for 0.5 to 1.0 h before being challenged with oxygen free acetone or butanol. The volume was made up to 10 mℓ with oxygen free distilled water. Oxygen free distilled water (1 mℓ) was used with control cultures. The OD_{600} was measured as a function of time.

4.2.11.2. Effect on cellular autolysis

Samples were prepared as in 4.2.7. The cells were resuspended in 0.04 M sodium phosphate buffer, pH 6.3 containing varying concentrations of acetone and butanol. Cellular autolysis was monitored turbidimetrically as in 4.2.7.

4.2.11.3. Effect on clostridial degeneration.

Different volumes of oxygen free butanol were added to 400 mℓ MFM cultures of *C.acetobutylicum* P262 and the *lyt*1 mutant at 28-36 h. Samples were assayed for clostridial forms (3.2.5) and for total solvents before and after the addition of butanol.

4.3 RESULTS

4.3.1. The isolation and characterisation of autolytic-deficient mutants

Autolytic-deficient mutants, which did not produce or produced decreased halos of lysis on *C.acetobutylicum* P262 indicator plates, were isolated after EMS treatment of *C.acetobutylicum* P262. Cellular autolysis of the autolytic mutants (*lyt*) was reduced and the mutants *lyt*1 and *lyt*8 showed the least autolytic activity (Table 4.1). The growth rates of all the autolytic mutants were similar to that of *C.acetobutylicum* P262 in CBM. The morphology of the mutants was altered and in CBM the vegetative cells formed long chains. This effect was most pronounced in the *lyt*1 and *lyt*8 mutants. All the *lyt* mutants were motile. The *lyt*1 and *lyt*2 mutants produced clostridial forms in MFM similar in shape and size to the clostridial forms produced by *C.acetobutylicum* P262. Clostridial forms produced by the *lyt*8 mutant in MFM were 2 to 3 times the length of the *C.acetobutylicum* P262 clostridial forms. The *lyt*9 and *lyt*10 mutants produced a mixture of long and short clostridial forms. The clostridial forms produced by the *lyt*4 and *lyt*5 mutants were not well formed and were phase grey.

The amount of solvent produced in MFM by the different autolytic mutants was compared (Table 4.2). The mutants *lyt*1 and *lyt*10 produced the highest solvent levels. After 48 h fermentation the *lyt*1 mutant produced 18.351 gℓ^{-1} and the *lyt*10 mutant produced 19.752 gℓ^{-1}. When the

TABLE 4.1

Cellular autolytic activity of *C.acetobutylicum* P262 and the *lyt* mutant strains.

Strain	Autolytic activity (%) [a]
P262	100
*Lyt*1	27.2
*Lyt*2	83.9
*Lyt*4	74.8
*Lyt*5	73.8
*Lyt*8	18.8
*Lyt*9	43.6
*Lyt*10	57.4

[a] Autolytic activity expressed as a % of *C.acetobutylicum* P262 activity.

TABLE 4.2

Solvent levels produced by *C.acetobutylicum* P262 and the *lyt* mutants in MFM.

Strain	Total solvents $g\ell^{-1}$ 48 h	Total solvents $g\ell^{-1}$ 72 h
C.acetobutylicum P262	18.791	18.750
*Lyt*1	18.351	20.096
*Lyt*2	0.287	12.815
*Lyt*4	5.807	6.294
*Lyt*5	5.840	8.262
*Lyt*8	13.674	15.823
*Lyt*9	12.597	17.551
*Lyt*10	19.752	20.608

fermentations were continued for 72 h both the *lyt*1 and *lyt*10 mutants produced *c* 20 gℓ^{-1} of solvents. The *lyt*2, *lyt*4, *lyt*5 and *lyt*8 mutants produced solvent levels lower than that produced by *C.acetobutylicum* P262. The *lyt*1 mutant was chosen for further analysis.

4.3.2. Amylase production by *C.acetobutylicum* P262 and the *lyt*1 strain

The levels of amylase produced by *C.acetobutylicum* P262 and the *lyt*1 mutant were compared in MFM supplemented with 2% (w/v) starch. *C.acetobutylicum* P262 produced 48.6 units of amylase activity compared with 73.7 units of amylase activity produced by the *lyt*1 mutant over 48 h.

4.3.3. Autolytic activity of *C.acetobutylicum* P262 and the *lyt*1 strain

The cell free autolysin produced in MFM by *C.acetobutylicum* P262 under stringent anaerobic conditions varied between 64 and 2048 AU in 48 h fermentations. The *lyt*1 mutant produced cell free autolysin levels of < 8 AU over 48 h. The effect of sucrose induction on the production of cell free autolysin by *C.acetobutylicum* and the *lyt*1 mutant in CBM was compared (Table 4.3). *C.acetobutylicum* P262 showed an increase in the cell free autolytic activity as the concentration of sucrose was increased. No cell free autolysin was detected for the *lyt*1 mutant. The addition of sucrose, up to 3% (w/v), did not increase the autolysin

TABLE 4.3

Effect of sugar concentration on cell free autolytic activity (arbitrary units - AU) of *C.acetobutylicum* P262 and the *lyt*1 mutant.

Sucrose concentration (% w/v)	AU P262	AU *lyt*1
4	8	0
6	32	0
8	64	0
10	128	0
15	256	0

titres produced by *C.acetobutylicum* P262 or the *lyt*1 mutant in MFM.

Cellular autolysis of the *lyt*1 mutant strain was markedly reduced when compared with *C.acetobutylicum* P262. Exponential phase *lyt*1 cells showed between 46 and 80 units of autolytic activity compared with 118 to 256 units of autolytic activity shown by *C.acetobutylicum* P262.

4.3.4. Cell degeneration of *C.acetobutylicum* P262 and the *lyt*1 strain.

The autolysis of exponential phase and stationary phase cells (clostridial forms) of *C.acetobutylicum* P262 and the *lyt*1 strain in sodium phosphate buffer were compared (Fig. 4.1). A marked decrease in optical density was detected when exponential phase cells of *C.acetobutylicum* P262 were resuspended in the sodium phosphate buffer. The autolysates of mid-exponential cells of the *C.acetobutylicum* P262 strain and the *lyt*1 mutant strain obtained after 60 min in the sodium phosphate buffer were assayed for cell free autolysin by the well plate technique. The autolysate from *C.acetobutylicum* P262 had a titre of 512 AU. No zones of inhibition were produced by the *lyt*1 mutant autolysate. Stationary phase cells showed a small decrease in optical density, 0.2 OD units in 1 h for the *C.acetobutylicum* P262 strain and 0.1 OD units in 1 h for the *lyt*1 strain.

The total number of bacteria and the proportion of phase bright clostridial forms produced by *C.acetobutylicum* P226 and

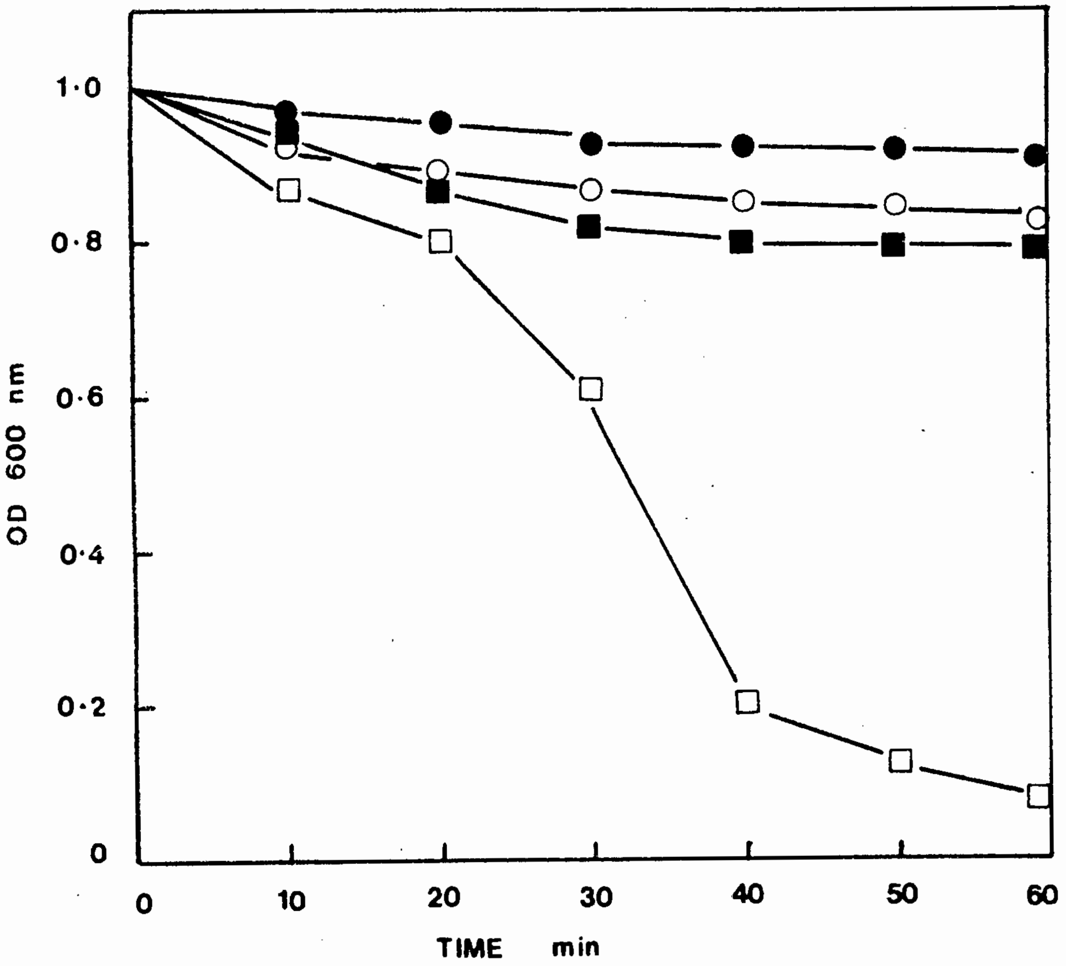

Figure 4.1

Lysis of vegetative cells and clostridial forms of *C.acetobutylicum* P262 and the *lyt*1 mutant in sodium phosphate buffer (0.04 M, pH 6.3). P262 vegetative cells (□); P262 clostridial forms (○); *lyt*1 vegetative cells ( ); and *lyt*1 clostridial forms (●).

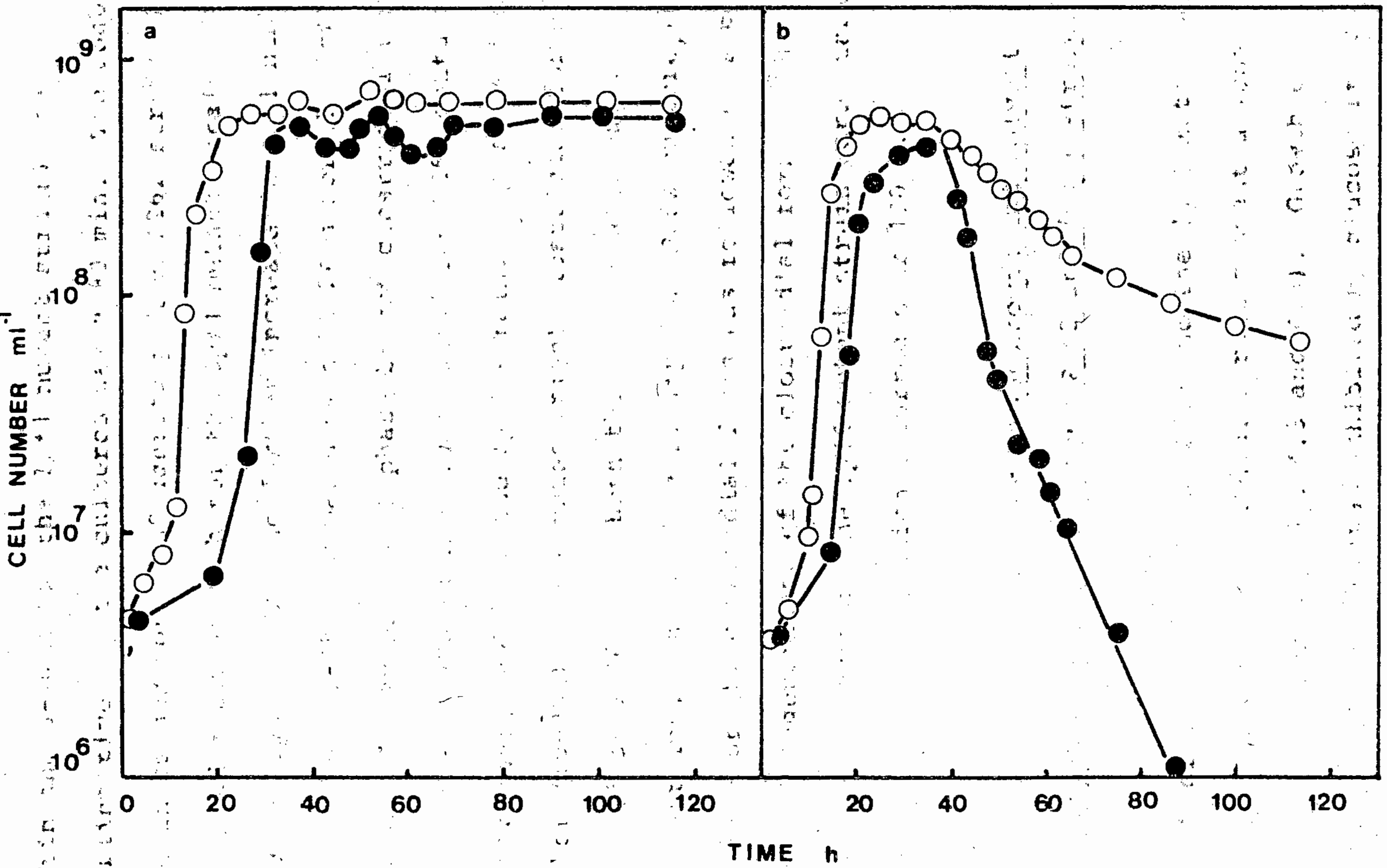

Figure 4.2

Growth of the *lyt*1 mutant (a) and *C.acetobutylicum* P262 (b) in MFM with a T.I.S. of 6.5% (w/v). Total count (○) and clostridial form count (●).

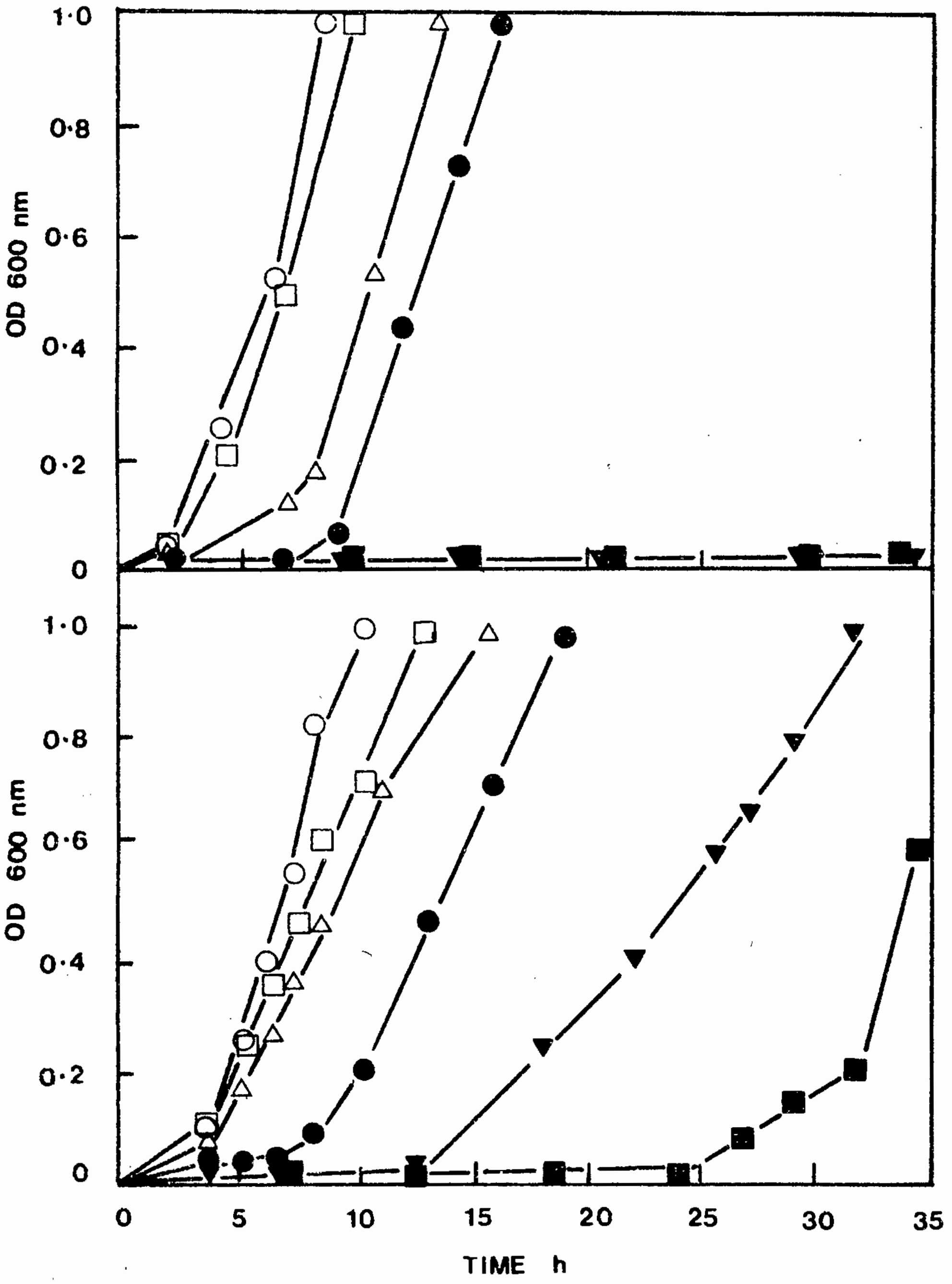

Figure 4.3

Effect of glucose levels on the growth of *C.acetobutylicum* P262 (a) and the *lyt*1 (b) mutant in CBM. Glucose concentration (% w/v); 1-4% (○); 6% (□); 10% (△); 12% (●); 14% (▼) and 16% (■).

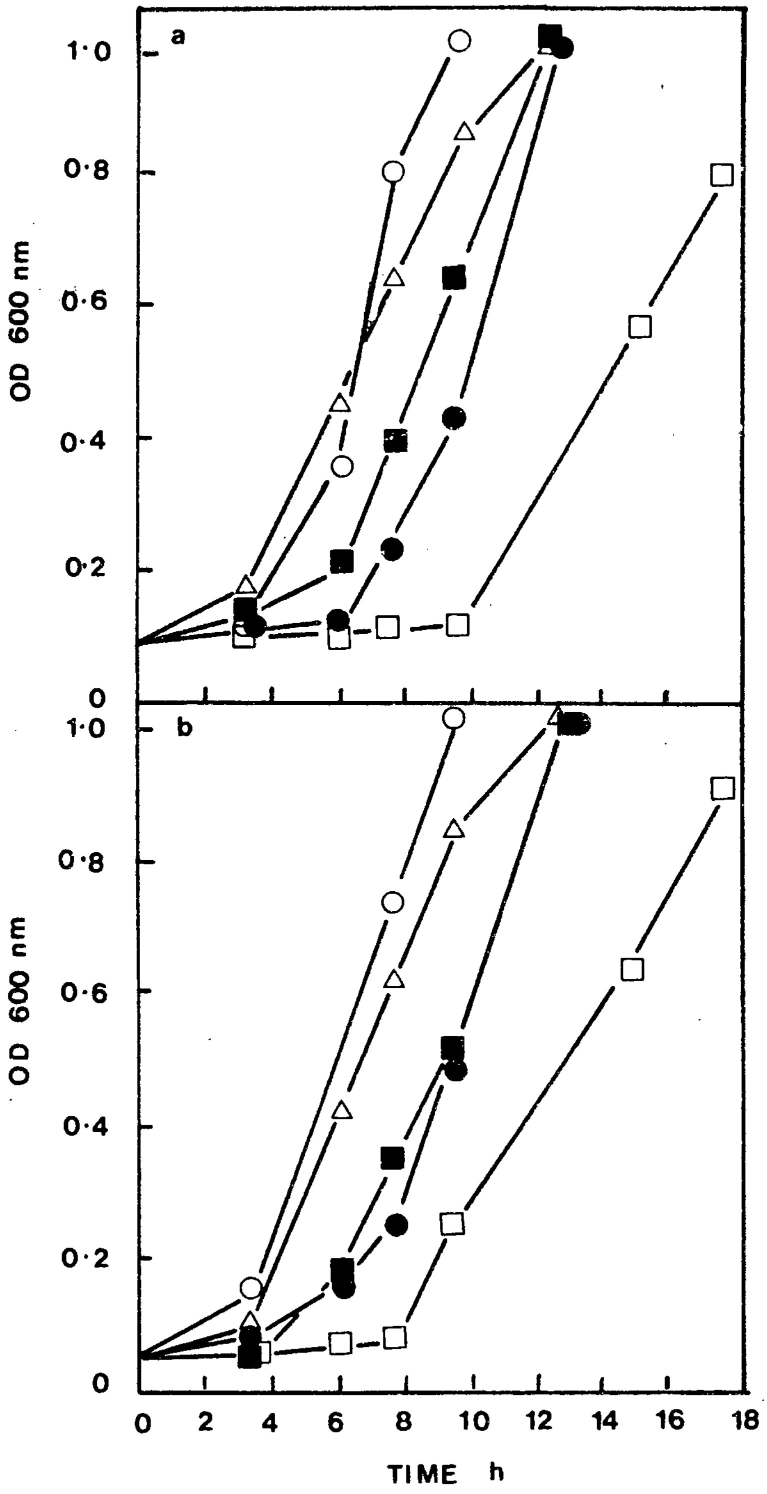

Figure 4.4

Effect of sucrose concentration in CBM on the growth of *C.acetobutylicum* P262 (a) and the *lyt*1 strain (b). Sucrose concentrations (% w/v); 1-8% (○); 10% (△); 12% (■); 14% (●) and 18% (□).

12% respectively. The *lyt*1 mutant grew in CMB containing 16% glucose. The rate of growth of the *lyt*1 mutant decreased at glucose concentrations greater than 10%. Associated with the decrease in growth rate was a lag in growth varying from 7.5 h at 12% glucose to 27 h at 16% glucose. The *C.acetobutylicum* P262 strain and the *lyt*1 mutant strain grew in sucrose concentrations up to 18% (w/v) (Fig. 4.4).

The production of solvents, titratable acids and the pH were compared in MFM (6.5% TIS) (Fig. 4.5). The production of titratable acids and the pH profile of the *lyt*1 mutant were similar to the *C.acetobutylicum* P262 strain. The peak in titratable acid and the pH break point occurred between 15-18 h. The break in pH coincided with the onset of solvent production in *C.acetobutylicum* P262 and the *lyt*1 mutant. The solvent levels in the *C.acetobutylicum* P262 culture increased from *c* 15 to 36 h before plateauing at a solvent level of 18 $g\ell^{-1}$. In the *lyt*1 mutant strain the solvent level increased rapidly from 15-40 h after which the rate of solvent production decreased and plateaued. The solvent concentration at 60 h was *c* 20 $g\ell^{-1}$.

The effect of increasing the TIS concentration in the MFM on solvent production by *C.acetobutylicum* P262 and the *lyt*1 mutant was compared after 48 h and 72 h (Fig. 4.6). There was an increase in the solvent yields at 7.5% TIS from 17.105 to 17.784 $g\ell^{-1}$ and 18.289 to 19.044 $g\ell^{-1}$ for *C.acetobutylicum* P262 and the *lyt*1 mutant respectively. Increasing the TIS to 8.5 and 9.5% decreased the amount of

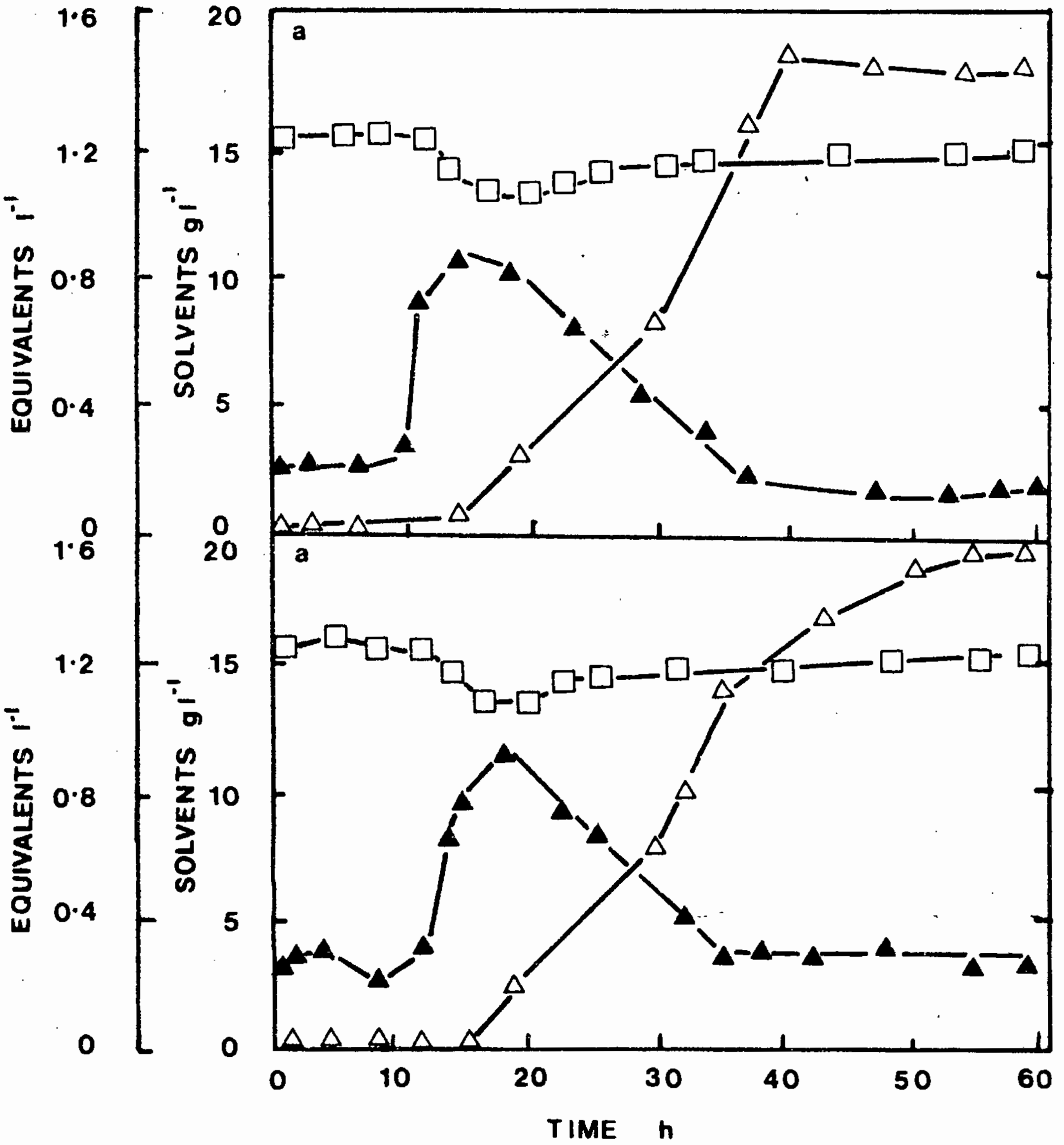

Figure 4.5

Growth of *C.acetobutylicum* P262 (a) and the *lyt*1 (b) mutant strains in MFM (6.5% (w/v) T.I.S.) pH (□); titratable acid (▲) and total solvent gℓ^{-1} (△).

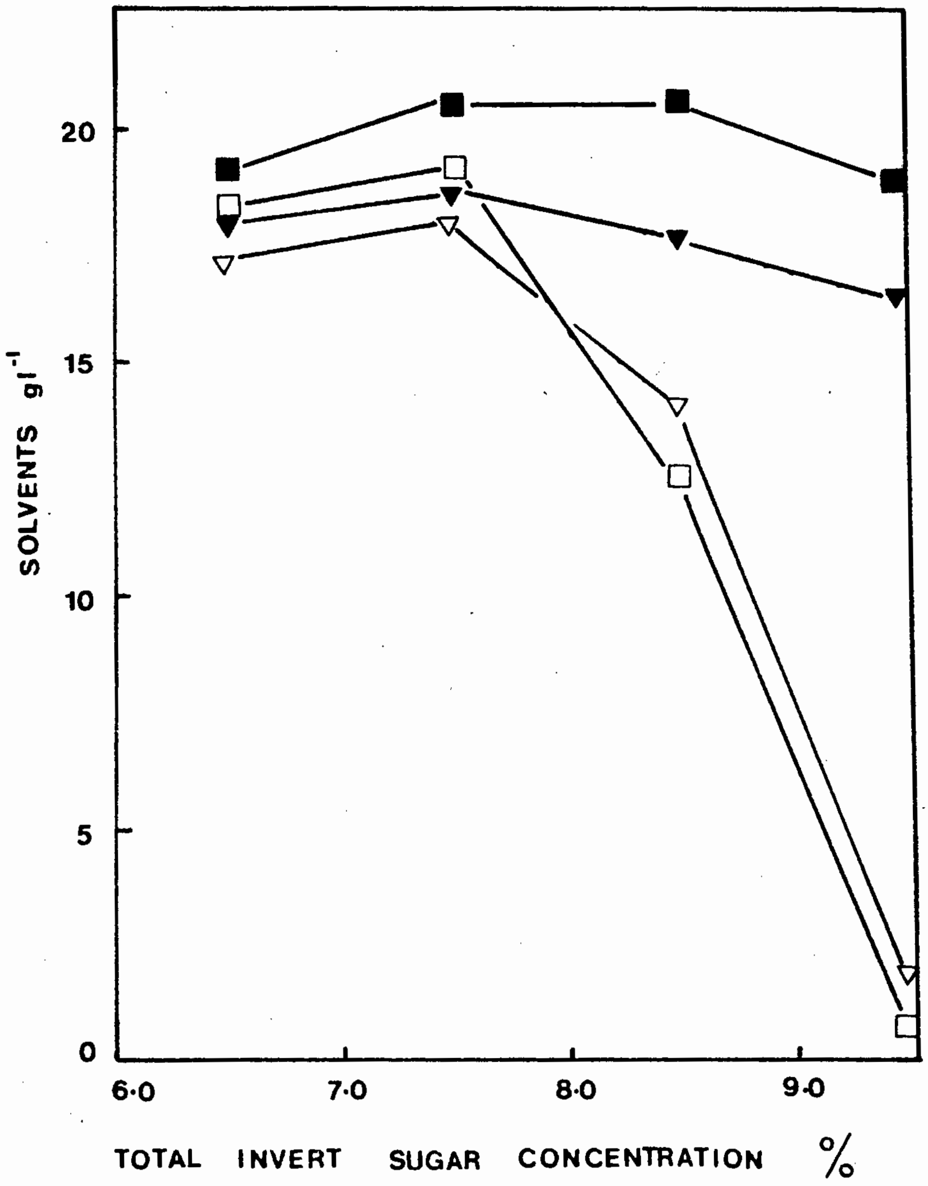

Figure 4.6
Effect of increased T.I.S. on solvent production by *C.acetobutylicum* P262 and the *lyt*1 mutant strains. *C.acetobutylicum* P262 48h (▽) and 72h (▼); *lyt*1 mutant 48h (□) and 72h (■).

solvents produced by *C.acetobutylicum* P262 and the *lyt*1 mutant; both strains produced *c* 1-2 $g\ell^{-1}$ after 48 h fermentation.

When the fermentations were allowed to continue for 72 h there was a large increase in the solvents produced at the higher TIS concentrations. At TIS concentrations of 9.5% the solvent level at 72 h increased from *c* 1 $g\ell^{-1}$(48 h) to 16.68 $g\ell^{-1}$ in *C.acetobutylicum* P262 cultures and 19.44 $g\ell^{-1}$ in the *lyt*1 mutant culture. The *C.acetobutylicum* P262 strain produced 18.80 $g\ell^{-1}$ and 17.99 $g\ell^{-1}$ of solvents at TIS concentrations of 7.5 and 8.5% respectively compared with 20.51 $g\ell^{-1}$ and 20.46 $g\ell^{-1}$ produced by the *lyt*1 strain. The percentage conversion of TIS to solvents was used as a measure of the efficiency of the fermentations. The percentage conversion after 72 h at TIS concentrations of 6.5 , 7.5, 8.5 and 9.5% were 27.7 , 24.7 , 20.5 and 17.26% for *C.acetobutylicum* P262 and 29.6 , 27.3 , 24.1 and 18.42% for the *lyt*1 mutant respectively.

The effect of supplementing MFM (6.5% TIS) with sucrose on the final solvent yields were compared (Table 4.4). The highest solvent yields obtained after 48 h by *C.acetobutylicum* P262 was 18.44 $g\ell^{-1}$ in MFM supplemented with 2% sucrose compared with 18.73 $g\ell^{-1}$ solvent produced by the *lyt*1 mutant in MFM supplemented with 2% sucrose. When the fermentations were continued for 72 h the highest solvent yield of 18.840 $g\ell^{-1}$ was obtained with the *C.acetobutylicum* P262 strain in MFM supplemented with 1% sucrose (w/v). In the *lyt*1 cultures, after 72 h the solvent yields increased from 17.983 $g\ell^{-1}$ in

TABLE 4.4

Effect of supplementing MFM (TIS 6.5%) with sucrose on solvent production by *C.acetobutylicum* P262 and the *lyt*1 mutant.

Concentration of Sucrose (% w/v)	Total solvents $g\ell^{-1}$			
	P262		*Lyt*1	
	48h	72h	48h	72h
0	17.381	17.654	17.163	17.983
1	17.841	18.840	18.328	19.446
2	18.444	18.148	18.729	19.504
3	17.840	18.381	18.070	20.091
4	16.179	18.492	16.910	21.647

the unsupplemented MFM control through to 21.647 gℓ^{-1} in MFM cultures supplemented with 4% sucrose (w/v) (Table 4.4). The efficiency of conversion decreased from 27.5% conversion in the unsupplemented control through to 20.5% conversion in the MFM supplemented with 4% sucrose.

The addition of 2% starch to MFM (TIS of 6.5%) increased the solvent yields in the *lyt*1 mutant by 2.8 gℓ^{-1} giving a total solvent yield after 48 h of 21.035 gℓ^{-1} (with an efficiency of *c* 24.7%) compared with an increase of 1.9 gℓ^{-1} by *C.acetobutylicum* P262 giving a final solvent yield of 19.714 gℓ^{-1} (with an efficiency of *c* 23.2%) after 48 h (Table 4.5).

The highest butanol level recorded in MFM for the *lyt*1 mutant was 14.196 gℓ^{-1} compared with 13.261 gℓ^{-1} for *C.acetobutylicum* P262.

4.3.6. The effect of air on autolysin levels and solvent production

Autolysin levels were compared in MFM cultures incubated under aerobic and stringent anaerobic conditions for 48 h. The cell free autolysin levels of *C.acetobutylicum* P262 were higher (2048 AU) under aerobic conditions compared with 512 AU under stringent anaerobic conditions. The cell free autolysin levels produced by the *lyt*1 mutant were not affected by air. The cell free autolysin titres of the *lyt*1 mutant were < 8 AU under aerobic or stringent anaerobic conditions.

TABLE 4.5

Effect of supplementing MFM with 2% starch on solvent yields by *C.acetobutylicum* P262 and the *lyt*1 mutant.

Strain	Media MFM (6.5% TIS)	Fermentation Time(h)	Acetone ($g\ell^{-1}$)	Ethanol ($g\ell^{-1}$)	Butanol ($g\ell^{-1}$)	Total ($g\ell^{-1}$)
*Lyt*1	+2% starch	48	6.662	0.722	13.650	21.035
*Lyt*1	Control	48	5.712	0.622	11.955	18.289
P262	+2% starch	48	6.727	0.725	13.261	19.714
P262	Control	48	5.119	0.591	12.086	17.796

TABLE 4.6

Effect of air on solvent production by *C.acetobutylicum* P262 and the *lyt*1 mutant in MFM enriched with 2% (w/v) starch.

Strain	Fermentation Time (h)	Acetone ($g\ell^{-1}$)	Ethanol ($g\ell^{-1}$)	Butanol ($g\ell^{-1}$)	Total ($g\ell^{-1}$)	
*Lyt*1	48	7.042	0.691	13.579	21.312	Aerobic
*Lyt*1	72	7.232	0.722	13.814	21.769	
P262	48	4.573	0.503	10.921	15.997	
P262	72	4.722	0.571	11.296	16.589	
*Lyt*1	48	6.662	0.722	13.650	21.035	Anaerobic
*Lyt*1	72	6.892	0.716	14.028	21.636	
P262	48	6.727	0.725	13.261	19.714	
P262	72	6.519	0.734	13.155	20.208	

The effect of air on solvent production by *C.acetobutylcium* P262 and the *lyt*1 mutant in MFM supplemented with 2% starch with intermittent mixing (*c* 4 hourly) were compared (Table 4.6). The total solvents produced by the *lyt*1 mutant strain under anaerobic and aerobic conditions were similar, *c* 21.0 gℓ^{-1}. The acetone levels were slightly higher (0.4 to 0.6 gℓ^{-1}) under aerobic conditions. Air had a marked effect on the solvent yields of *C.acetobutylicum* P262. Under aerobic conditions the solvent yields produced by *C.acetobutylicum* P262 were less than 17 gℓ^{-1} after 48 and 72 h. When cultures of *C.acetobutylicum* P262 or the *lyt*1 mutant were stirred continuously under aerobic conditions growth was inhibited.

4.3.7. Effect of solvents on growth, cell degeneration and cellular autolysis.

A comparison of the effects of acetone and butanol on the growth of *C.acetobutylicum* P262 and the *lyt*1 mutant was made in CBM (Fig. 4.7 and 4.8). The growth rates of *C.acetobutylicum* P262 and the *lyt*1 mutant were not effected by 20 gℓ^{-1} acetone (Fig. 4.7). Increasing the concentration of acetone to 30 gℓ^{-1} reduced the growth rate of *C.acetobutylicum* P262 and the cultures plateaued at an OD of 0.9. The growth of the *lyt*1 mutant at 30 gℓ^{-1} acetone was inhibited slightly and the cultures reached a final OD of 1.0 after 7 h incubation compared with 5.5 h in the control without the addition of acetone.

Butanol was more toxic than acetone against mid log cells of

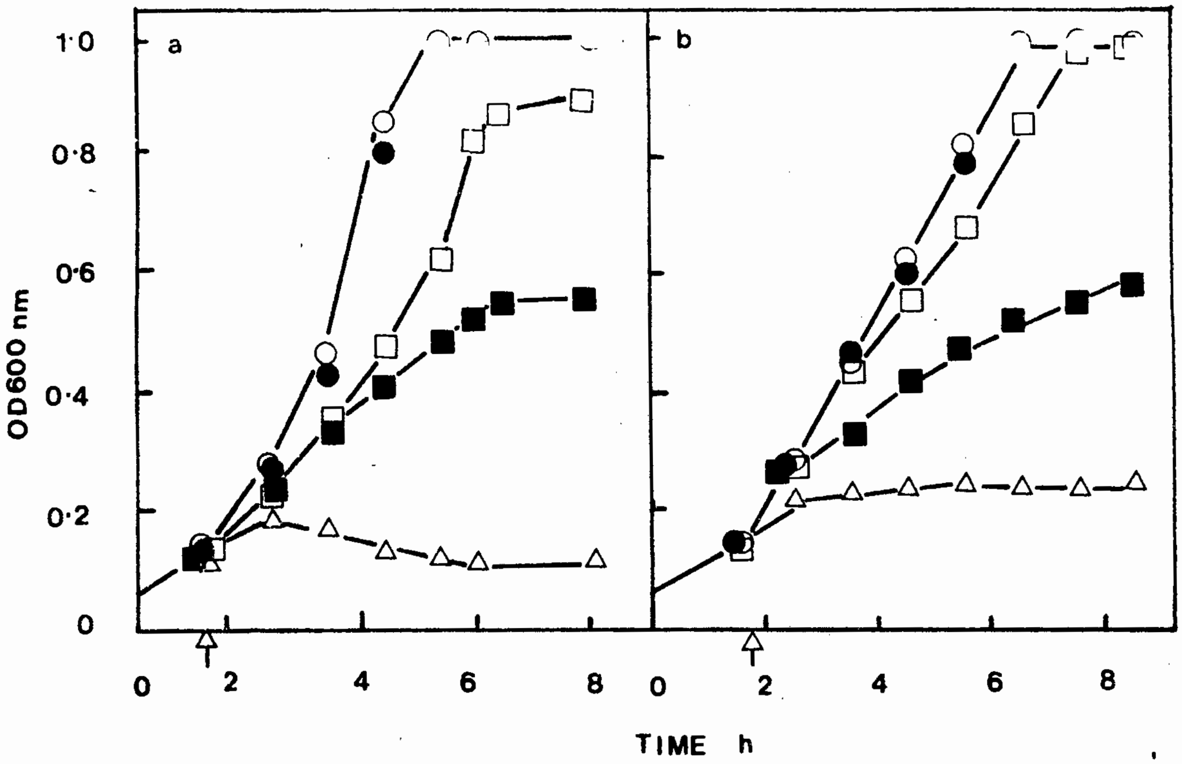

Figure 4.7
Effect of acetone on the growth of *C.acetobutylicum* P262 (a) and the *lyt*1 mutant (b). Acetone concentration: Control (○); 20 $g\ell^{-1}$ (●); 30 $g\ell^{-1}$ (□); 40 $g\ell^{-1}$ (■); 50 $g\ell^{-1}$ (△). Time of acetone addition (↑).

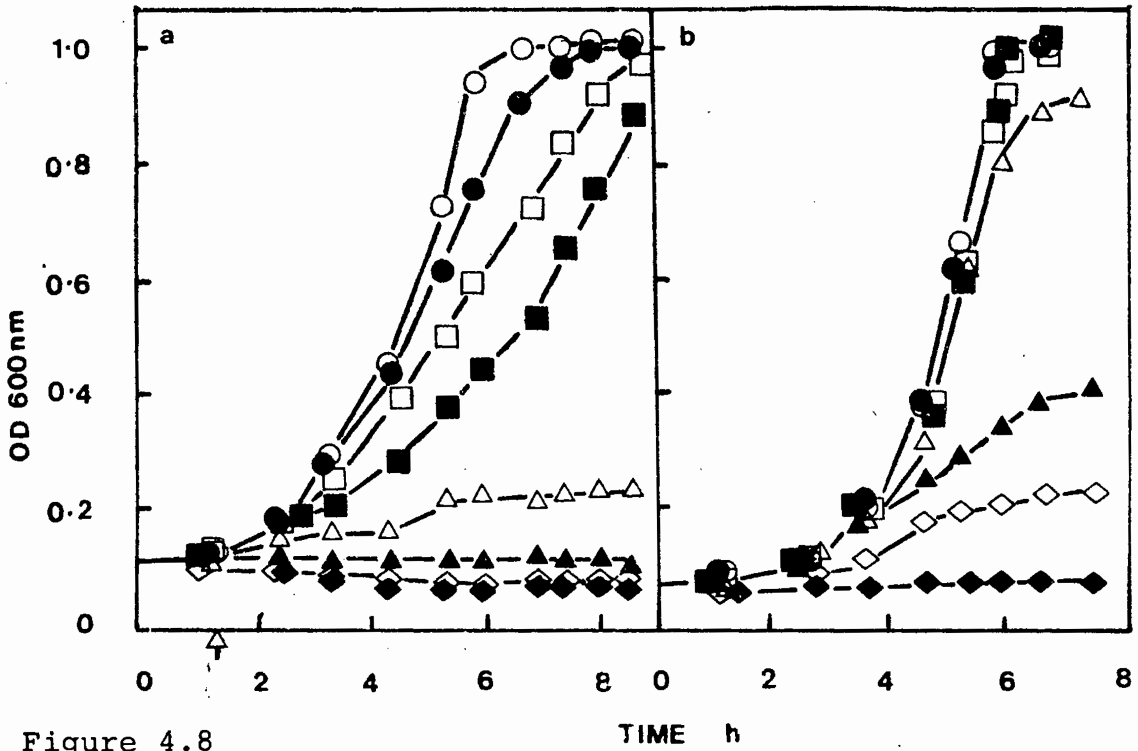

Figure 4.8
Effect of butanol on the growth of *C.acetobutylicum* P262 (a) and the *lyt*1 mutant (b) in CBM. Butanol concentration: Control (○); 4 $g\ell^{-1}$ ( ); 6 $g\ell^{-1}$ (□); 8 $g\ell^{-1}$ ( ); 10 $g\ell^{-1}$ (△); 12 $g\ell^{-1}$ (▲); 15 $g\ell^{-1}$ (◇) and 18 $g\ell^{-1}$ ( ).

C.acetobutylicum P262 and *lyt*1 mutant cultures (Fig 4.8). Butanol at a concentration of 8 $g\ell^{-1}$ reduced the growth of *C.acetobutylicum* P262 and growth was inhibited by 10 $g\ell^{-1}$ butanol. However, 10 $g\ell^{-1}$ butanol had little effect on the growth of the *lyt*1 mutant. Butanol at a concentration of 15 $g\ell^{-1}$ almost totally inhibited the growth of the *lyt*1 mutant.

The effect of butanol on the autolysis of mid-exponential phase cells, in sodium phosphate buffer, was investigated (Fig. 4.9). The addition of butanol to the sodium phosphate buffer reduced the rate of autolysis of *C.acetobutylicum* P262 and the *lyt*1 mutant. The reduction in the rate of autolysis depended on the concentration of butanol added. Cellular autolysis of *C.acetobutylicum* P262 and the *lyt*1 mutant cells in sodium phosphate buffer was completely inhibited by 40 $g\ell^{-1}$ butanol.

The effect of acetone on cellular autolytic activity of *C.acetobutylicum* P262 in sodium phosphate buffer was investigated. Cellular autolytic activity in sodium phosphate buffer was less sensitive to acetone. Acetone at 40 $g\ell^{-1}$ reduced the cellular autolytic activity from 155 units to 120 units.

The effect of butanol on the clostridial forms of *C.acetobutylicum* P262 and the *lyt*1 mutant in MFM was compared (Fig. 4.10). The butanol was added to *c* 36 h cultures which corresponded to the peak in the number of clostridial forms

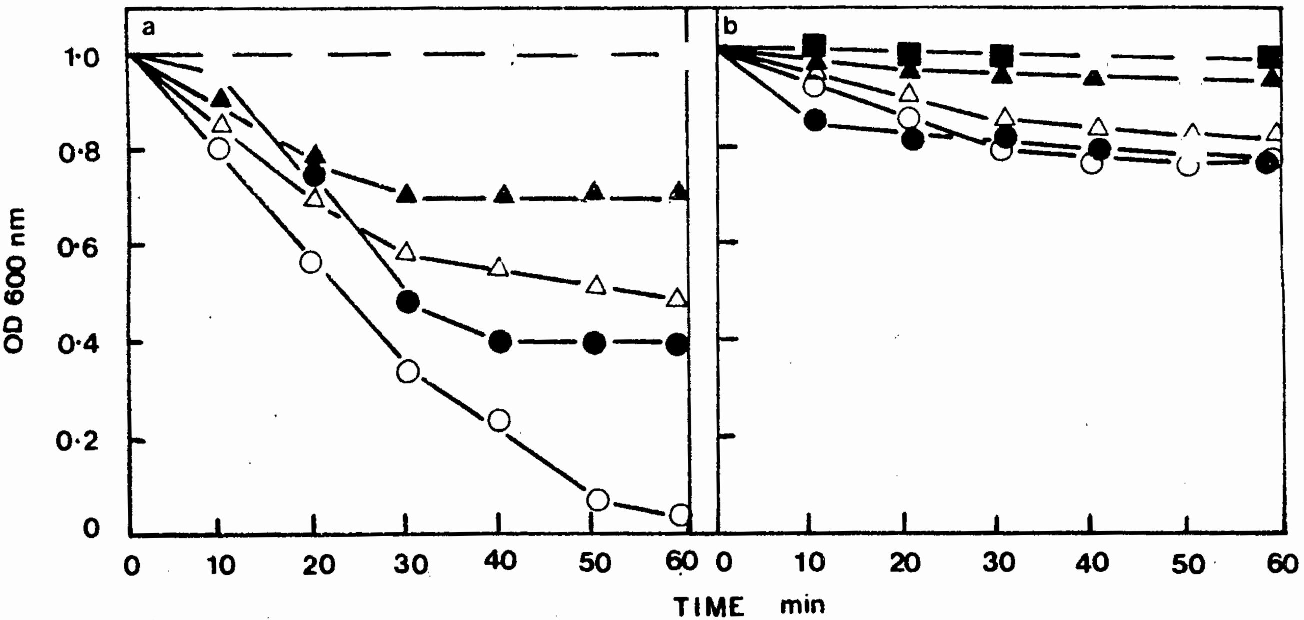

Figure 4.9
Effect of Butanol on the autolysis of exponential phase cells of *C.acetobutylicum* P262 (a) and the *lyt*1 mutant strains in sodium phosphate buffer (0.04 M, pH 6.3). Cells were harvested and resuspended in sodium phosphate buffer. Control, no butanol added (○); 10 $g\ell^{-1}$ (●); 20 $g\ell^{-1}$(△); 30 $g\ell^{-1}$ (▲) and 40 $g\ell^{-1}$ ( ) butanol.

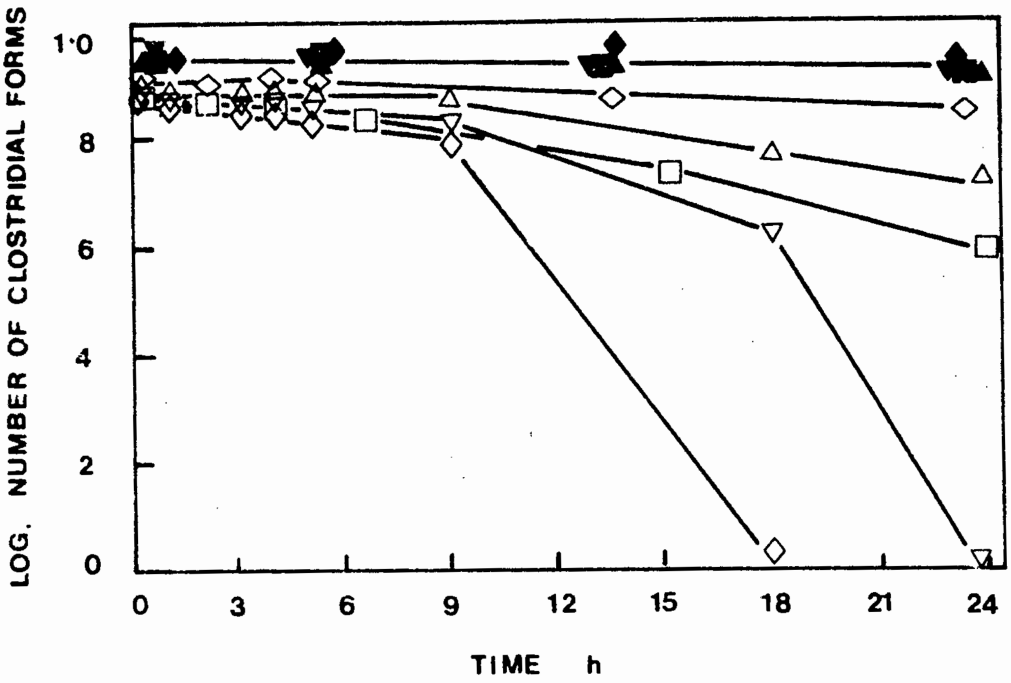

Figure 4.10
Effect of butanol on clostridial forms of *C.acetobutylicum* P262 and the *lyt*1 mutant. Butanol was added to clostridial forms to give the following final concentrations ($g\ell^{-1}$): P262 control, no extra addition of butanol 7.02 (△); P262, 11.68 (▽); 16.72 (◇); 28.06 (□); 36.59 (◇); *lyt*1 mutant control, no extra addition of butanol, 7.12 (▲); *lyt*1 14.27 ( ); *lyt*1 25.64 (▼); *lyt*1 38.21 ( ).

produced by *C.acetobutylicum* P262 and the *lyt*1 mutant. The normal level of butanol produced by *C.acetobutylicum* P262 and the *lyt*1 mutant in MFM at 36 h was *c* 7 $g\ell^{-1}$. In *C.acetobutylicum* P262 without the addition of extraneous butanol the peak in phase bright clostridial forms was followed by a period of degeneration (Fig. 4.2). The phase bright clostridial forms became phase dark and granulated and had an irregular outline. The addition of butanol to the *C.acetobutylicum* P262 cultures to give a final butanol concentration of 11.68 $g\ell^{-1}$ and 16.72 $g\ell^{-1}$ increased the rate of clostridial degeneration (Fig. 4.10). The addition of 18.52 $g\ell^{-1}$ of butanol (final butanol concentration of 25.64 $g\ell^{-1}$) to the *lyt*1 mutant had no effect on the clostridial forms. However the addition of butanol to *C.acetobutylicum* P262 cultures, giving a final concentration of butanol of 36.59 $g\ell^{-1}$ inhibited clostridial form degeneration.

DISCUSSION

The *lyt*1 mutant is similar to the majority of autolytic-deficient mutants (Ghuysen and Shockman, 1973; Rogers, 1979; Rogers *et al.*, 1980) which have been described in that they all possess reduced but detectable levels of autolytic activity and from long chains of cells (Tomasz, 1968; Pooley *et al.*, 1972). The autolytic-deficient mutant, *lyt*1 is pleitropic in that it produces less autolysin than the parent strain and it has an altered cell wall (Allcock, 1981). The autolytic activity of *C.acetobutylicum* P262 was induced by air when grown in MFM. Air had no effect on the low levels of autolysin produced by the *lyt*1 strain.

The *lyt*1 mutant differs from mutants of *S.faecuim* with reduced autolytic activity which show reduced growth and yields in complete media (Shungu *et al.*, 1979). The growth rates of the *lyt*1 mutant are similar to *C.acetobutylicum* P262 in minimal medium, (Allcock, 1981) and in CMB.

Autolysis of *C.acetobutylicum* P262 in sodium phosphate buffer is similar to the autolysis of *S. faecalis* (Pooley and Shockman, 1970) and *Lactobacillus acidophillus* (Coyette and Ghuysen, 1970) in that the ability of the cells to autolyze decreases when cultures enter the stationary growth phase. Little or no autolysis occurred when *C.acetobutylicum* P262 or *lyt*1 mutant clostridial forms were resuspended in phosphate buffer.

In previous studies the clostridial forms of *C.acetobutylicum* were responsible for the conversion of acids to neutral solvents and sporulation mutants which were unable to form clostridial stages did not produce solvents (Chapter III; Jones *et al.*, 1981). In the ABE process butanol toxicity will be most important and apparent during the stationary growth phase when clostridial forms predominate. Therefore the effect of butanol on the clostridial forms was determined and it could be concluded that there is a relationship between butanol toxicity and clostridial degeneration. Clostridial forms from the *lyt*1 mutant were not induced to degenerate by concentrations of butanol which increased the rate of degeneration of *C.acetobutylicum* P262 clostridial forms. It is suggested that the *lyt*1 clostridial forms are more resistant to butanol induced degeneration and that one of the effects of butanol concentrations between 7 - 16 $g\ell^{-1}$ is the induction of autolysis and degeneration of *C.acetobutylicum* P262. The concentrations of butanol which induce degeneration of clostridial forms were within the concentration ranges obtained in the factory fermentations. High concentrations of butanol had the reverse effect and prevented degeneration. However these high concentrations would not be produced by *C.acetobutylicum* P262 as it would have degenerated before these levels were obtained.

The complete inhibition of solvent production after *c* 39 h observed with *C.acetobutylicum* P262 in MFM suggests that a threshold level is reached which results in the inhibition of further solvent production. The gradual decrease in solvent production obtained with the *lyt*1 mutant suggests that solvent production in this strain is not subject to inhibition by the same mechanism.

Although not important as regards solvent production and butanol toxicity, it is significant that vegetative cells of the *lyt*1 mutant were also more resistant to butanol than *C.acetobutylicum* P262 cells. The mechanism of increased resistance of the *lyt*1 vegetative cells is unclear since butanol did not induce or increase autolysis of the vegetative cells. Nevertheless the increased butanol tolerance shown by the *lyt*1 vegetative cells supports our conclusion that there is a relationship between butanol tolerance and autolytic activity.

Although the *lyt*1 clostridial forms do not degenerate and remain phase bright and look healthy in the presence of concentrations of butanol between 0 and 35 $g\ell^{-1}$ the physiological state of the clostridial forms at the higher butanol concentrations has not been determined. Nevertheless this pleiotropic *lyt*1 mutant is butanol tolerant and its potential to produce higher solvent levels should be investigated.

CHAPTER V

CONCLUSION AND GENERAL DISCUSSION

The molasses laboratory scale fermentation systems which were developed were found to produce approximately the same levels of solvents but slightly less ethanol than the yields obtained at the N.C.P. factory. Three main types of culture systems are currently employed in the genetic, molecular biology and fermentation studies being carried out on *Clostridium acetobutylicum*. There are: factory type fermentation media, eg. MFM; complete complex laboratory media, eg. CBM (O'Brien and Morris, 1971); chemically defined media, eg. minimal medium (Long *et al.*, 1981). Although there are many advantages in carrying out such studies in a "clean" laboratory medium such as CBM, a major drawback is that the final solvent yields obtained are substantially lower than those obtained in a factory type fermentation. Initial attempts to increase solvent levels in laboratory media by manipulation of physical, chemical and nutritional factors has met with limited success. In the initial studies the level of solvent produced was the main criterion used to monitor the effect of the manipulations. Further studies showed that the relationship of the various factors is complex and it became apparent that the initial aim of trying to identify and manipulate a few key factors to give better solvent yields would not easily be obtained. Later studies showed that not only do the solvent levels differ but the overall patterns of growth and cell development are markedly different in the various laboratory systems which have been compared. In view of the observed differences,

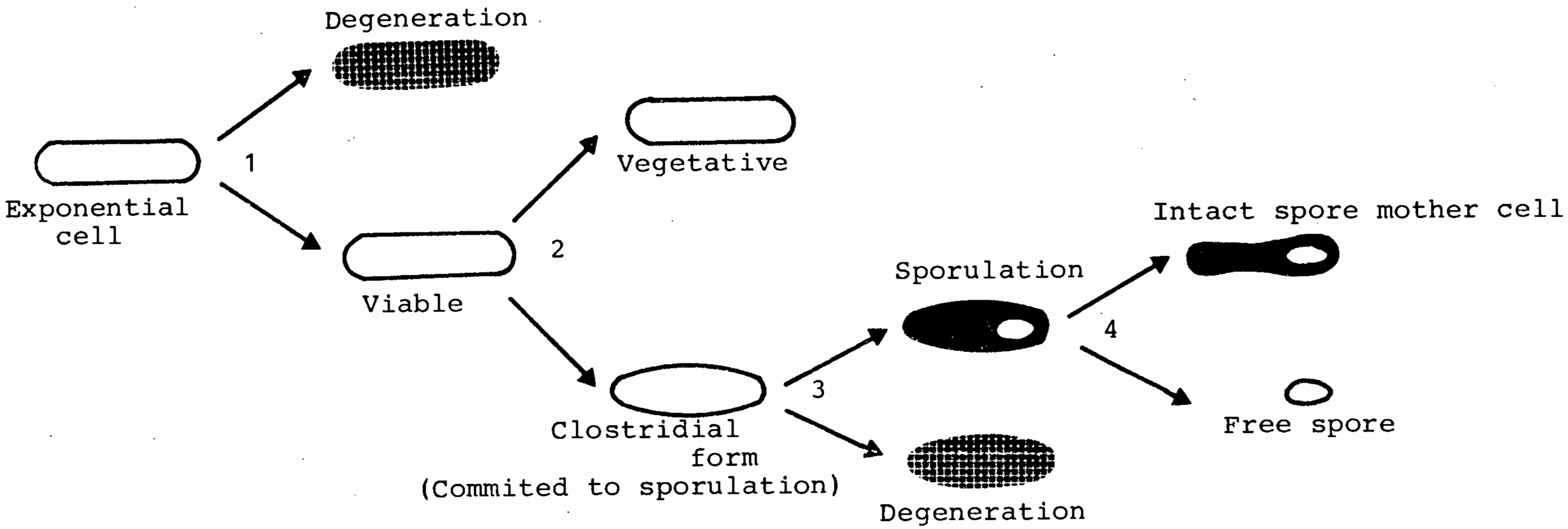

Figure 5.1

Diagram indicating four control points in the developmental cycle of *C.acetobutylicum* P262.

more detailed investigations of the growth pattern and the morphological and physiological changes were undertaken in the MFM and CBM systems.

Studies on the morphological changes in the ABE fermentation system suggested that the clostridial forms in *C.acetobutylicum* P262 were involved in the conversion of acetate and butyrate to acetone and butanol. This was confirmed by the isolation of sporulation mutants which either failed to form clostridial stages and produced no solvents or formed reduced numbers of clostridial forms and intermediate levels of solvent.

These studies have lead to the identification of four probable control points in the developmental stages of the culture during the fermentation process (Fig. 5.1). The first control point determines whether the exponential cells undergo degenerative changes or remain viable. The second control point determines whether the cells remain as vegetative cells or become committed to sporulation, i.e. form clostridial forms which are sporulation intermediates (Gunsalus and Stanier, 1960). The third control point determines whether the clostridial forms complete sporulation or undergo degenerative changes. Long *et al.* (1981) have shown the induction of sporulation of *C.acetobutylicum* P262 following exposure to air. The induction of cell free autolysin by air in MFM has been demonstrated for *C.acetobutylicum* P262. Whether or not this autolytic activity is associated with the sporulation process needs to be determined. The fourth control point is the release of the mature spore from the spore mother cell.

Degenerative changes in the cell population of *C.acetobutylicum* may occur at two distinct stages in the fermentation process. The relationship between these two stages is not known. Two autolytic systems have been identified (Allcock *et al.*,1981b; Allcock,1981). The relationship between the autolytic systems and the degenerative changes has not been determined. Autolytic activity may also be induced experimentally in actively growing vegetative rods which results in the production of protoplasts (Allcock *et al.*, 1981a).

Autolytic deficient mutants were isolated after mutagenesis and lacked substantial levels of autolytic enzymes and grew in long chains. One of these mutants was highly pleiotropic and four phenotypic characteristics were affected. These include reduced cell free and cell bound autolysin levels, an altered cell wall which was more resistant to its own and *C.acetobutylicum* P262 autolysin (Allcock *et al.*, 1981b), growth at increased glucose levels and an increased tolerance to butanol. Initial fermentation studies have indicated that the mutant may be useful in producing higher solvent yields.

The future of the ABE fermentation is likely to depend on the ability of the organism to produce higher solvent concentrations (Ryden, 1958; Spivey, 1979). The recovery of 2% solvents by distillation requires considerable expenditure of energy. The economic production of solvents would obviously be more easily obtained if some means of increasing solvent yields could be developed. The *lyt*1 mutant or other butanol resistant strains may be potentially important in increasing the solvent yields obtained because of the inability of the bacterium to tolerate concentrations of butanol exceeding 13 $g\ell^{-1}$. The potential of continuous cultures for the production of solvent has yet to be exploited and coupled with the continuous removal of solvent by, for example reverse osmosis or absorption on active carbon, continuous cultures may enable much higher sugar concentrations to be used with a consequent increase in productivity.

Finally it should be noted that the development of a genetic system in *C.acetobutylicum* (Allcock, 1981) enables this industrial microorganism to be manipulated utilising recombinant DNA techniques. This development and the demonstration that *C.acetobutylicum* P262 produces cellobiase and carboxymethyl cellulase enzyme (Allcock and Woods, 1981) enhances its potential in the emerging field of biotechnology.

APPENDIX A

GROWTH OF *CLOSTRIDIUM THERMOCELLUM* UNDER LABORATORY CONDITIONS

A.1. INTRODUCTION

The energy crisis in the world today is, to a large extent, a consequence of the need for vast quantities of liquid fuels. These liquid fuels are derived mostly from petroleum. In order to lessen our dependence on petroleum it is necessary to both increase conservation of energy and to develop alternatives and, preferably, renewable energy resources.

Ethanol can be mixed with gasoline to obtain "gasohol" a motor fuel as good as gasoline. Ethanol can be produced from ethylene derived from hydrocarbons or fermented by fermentation of sugars derived from plant matter. Sugars derived from two classes of plant material, starch and cellulose, can be fermented and distilled into ethanol. The most commonly proposed approach for the conversion of plant materials to ethanol is a two step process, where in the first step the polymeric material is hydrolyzed either by chemical or by enzymatic treatment and the second step, the product of hydrolysis (reducing sugars) are then subjected to fermentation to ethanol by the yeast *Saccharomyces cerevisiae* (Gomez, 1980).

Cellulose is the most abundant biopolymer on earth and exists as a highly ordered crystalline structure composed of glucose moities linked by β1-4 bonds (Cowling, 1975). Renewed interests in both basic and applied studies of ethanol production from cellulose bioconversion has arisen because of the abundance of cellulosic substrates in biomass derived residues (Wilke, 1975; Zeikus, 1980).

Most microbial cellulases are comprised of three components which when purified have readily distinguishable activities (Reese, 1975). These include the following: 1. Endo-β-1,4-glucanases that act randomly on crystaline cellulose and are assayed using carboxymethyl-cellulose as substrate; 2. exo-β-1,4 glucanases that are either glucohydrolases or cellohydrolases that act on the non reducing end of the cellulose chain that are assayed using a variety of crystalline celluloses as substrates (e.g. filter paper); 3. Cellobiase or β-glucosidase, which cleaves cellobiose into glucose. The components act synergistically (Wood, 1975) to effectively solubilize crystalline cellulose.

Anaerobic micro-organisms capable of degrading cellulose to useful products have not received the same attention in recent years as their aerobic counterparts. Considerable research efforts have been devoted to systems using cellulase enzymes produced predominantly by aerobic, mesophillis fungi (Mandells and Weber, 1969; Mandells *et al.*, 1974). Nevertheless effective utilization of cellulosic biomass, via these processes, has yet to become a reality.

Evidence suggests that cellulolytic activity is common among thermophiles (Stutzenberger *et al.*, 1970; Cooney and Ackerman, 1975; Cooney and Wise, 1975; Romanelli *et al.*, 1975).

Thermophillic anaerobic, cellulolytic bacteria have been isolated by Mc Bee (1948, 1950), and Lee and Blackburn (1975). At present *Clostridium thermocellum* is the only taxonomically recognised thermophillic anaerobic species (Buchanan and Gibbons, 1974), although other clearly distinct bacteria probably exist (Lee and Blackburn, 1975).

An approach to achieve conversion of cellulose to fermentable sugars and the fermentation of these sugars to ethanol in a single step by using *C.thermocellum* has been proposed by Wang *et al.*, (1976-1980). Since *C.thermocellum* is an anaerobic micro-organism, the catabolic products from cellulose are alcohol and organic acids (Weimer and Zeikus, 1977). Thus the conversion of cellulose to alcohol could be achieved in a single step.

The advantages and disadvantages of *C.thermocellum* as an organism for the utilization of cellulose are listed in Table 1. Preliminary purification studies have suggested that *C.thermocellum* cellulase is a complex, but the mechanism of action of individual components have not been described (Ait *et al.*, 1979). The supernatant cellulase of *C.thermocellum* differs significantly from that of the mould *Trichoderma viridae* in both the types of activities present and their specific activities. *C.thermocellum* supernatant cellulase, unlike that of *T.viridae* lacks cellobiase activity (Ng *et al.*, 1977). *C.thermocellum* contains both endo- and exo-glucanase activities but the ratio of endoglucanase to exoglucanase is greater in *C.thermocellum* than in

TABLE 1

Characteristics of *C.thermocellum* cellulase production.

	Advantages	Disadvantages
1.	Cellulase constitutive.	Cellulase activity is low compared with *T.viridae*.
2.	Cellulase not product inhibited.	Pentoses not utilized.
3.	Cellulase-hydrolyses a) Cellulose b) Hemi-cellulose	Ethanol sensitive.
4.	Ethanol is a fermentation end product.	Other fermentation products other than ethanol.
5.	Oxygen transfer not required.	
6.	Heat removal not necessary.	
7.	High metabolic rates, physically (i.e. thermally stable) and chemically stable enzymes.	
8.	Facilitated end product recovery.	

T.viridae (Ng. *et al.* 1979 ; Shinmyo and Garcia-Martinez, 1979). Filter paper activity is significantly higher in *T.viridae* hypercellulase mutants than in strains of *C.thermocellum* (Ng and Zeikus, 1979). However, the criteria of cellulase activity, moles of reducing sugar equivalents per unit time produced from a crystaline cellulose, may not be adequate to apply to *C.thermocellum*. Results have indicated that the cellulase of *C.thermocellum* produces oligosaccharides in addition to glucose and cellobiose from cellulose (Gomez, 1980). This characteristic will result in an underestimate of the ability of *C.thermocellum* cellulase to solubilize cellulose. This accumulation of soluble reducing sugar appears to have no effect on the activity of the *C.thermocellum* cellulases (Gordon *et al.*, 1979).

The ability of *C.thermocellum* to utilise glucose as the sole carbon source has been the subject of debate in the literature (McBee, 1948, 1950; Patni and Alexander, 1971; Ng *et al.*, 1977; Zeikus *et al.*, 1980). Gomez and Hernadez (1980) have shown that *C.thermocellum* is able to utilise glucose as the sole carbon source after an extended lag phase (12 to 30 h). After cultures were repeatedly grown on glucose this lag disappeared. Cultures which were not adapted to grow on glucose were unable to transport glucose, while glucose adapted cells transported glucose (Gomez and Hernadez, 1980).

A disadvantage that *C.thermocellum* presents is its inability to ferment 5-carbon sugars. Several strains of *C.thermocellum* have been described that actively ferment cellulose and cellobiose but not pentoses to ethanol (Ng *et al.*, 1977; Garcia-Martinez *et al.*, 1980; Lamed and Zeikus, 1980). Cellulose substrates are composed of approximately 50% cellulose, 25% hemicellulose and 25% lignins. Although *C.thermocellum* will degrade hemicelluloses, the resulting 5-carbon sugars accumulate in the fermentation broth (Gordon *et al.*, 1979). Clearly this is a problem that has to be overcome, since this would result in 25% of the substrate not being converted to ethanol. A means of overcoming this problem, other than via genetic manipulation of *C.thermocellum*, is to utilise a second microorganism which can utilise these sugars. The natural decomposition of cellulose by thermophillic bacteria is a symbiotic process (Enebo, 1949, 1951), indicating that co-culture studies are of importance (Wang *et al.*, 1979; Ng *et al.*, 1981). Co-culturing *C.thermocellum* and *Clostridium thermohydrosulfuricum*, a non-cellulolytic thermophile, (Ng *et al.*, 1981) have increased the ethanol production rate three-fold.

The bioconversion of cellulose to ethanol by *C.thermocellum* is limited by the strong inhibition that the fermentation end-product exerts on the growth of the bacteria (Herrero and Gomez, 1980a). Ethanol at concentrations of 5g per litre decreases the growth rate by 50%. An ethanol resistant *C.thermocellum* strain *C9* has been derived from the wild type strain which is capable of growing at ethanol concentrations of 25gℓ^{-1} (Herrero and Gomez, 1980b). Wang *et al.*, (1979) using ethanol ressitant strains of *C.thermocellum* and *C.thermosaccharolyticum* have obtained 40gℓ^{-1} of ethanol from cellulose fermentations.

Johnson *et al.*,(1981) have developed a chemically defined medium for *C.thermocellum*. The growth factors required are biotin, pyridoximine, vitamin B12 and p-amino-benzoic acid.

Chemical and fuel production from thermophillic anaerobic fermentations of biomass provides a promising approach for the use of renewable resources.

Allcock and Woods (1981) have shown that *C.acetobutylicum* possesses an inducible carboxymethyl cellulase and cellobiase but no activity on crystalline cellulose could be demonstrated. The cellulase required for crystalline cellulose degradation could be inserted by genetic manipulation (Eveleigh and Montecourt, 1979). *C.thermocellum* has the genes necessary for crystalline cellulase activity and could be the source of these genes. These studies were undertaken in order to be able to work with *C.thermocellum* prior to genetic manipulation studies.

A.2 MATERIALS AND METHODS

A.2.1. Organism *C.thermocellum* ATCC 27405, a thermophillic obligatory anaerobic Gram negative bacterium c0.6-0.7 µm by 4.0-5.0 µm.

A.2.2. Anaerobic methods. The anaerobic culture technique of Hungate (1969) as modified by Bryant (1972) were used throughout the investigation. Hungate anaerobic culture tubes were used for small culture volumes (10 mℓ) and 500 mℓ Erlenmeyer flasks converted for anaerobic use (Daniels and Zeikus, 1975) were used for 250 to 300 mℓ cultures.

A.2.3. Growth of organism. *C.thermocellum* was grown in biotin enriched Clostridium Medium 3 (CM3) (Weimer and Zeikus, 1977), which contained $g\ell^{-1}$ cellulose 9.72; Yeast extract, 2.0 (Difco); $(NH_4)_2SO_4$, 1.3; KH_2PO_4, 1.5; $K_2HPO_4 \cdot 3H_2O$ 2.9; $MgC\ell_2 \cdot 6H_2O$, 2.9; $MgC\ell_2 \cdot 6H_2O$ 1.0; $CaC\ell_2$, 0.15; resazurin 0.002; $FeSO_4$, 0.0125; Biotin, 2×10^{-6}; the pH was adjusted to 7.5 with 1N NaOH prior to dispensing 9.6 mℓ of the medium into Hungate tubes under constant vigorous gassing with N_2. Then 0.4 mℓ of a solution containing cysteine hydrochloride (1.25%) and $Na_2S \cdot 9H_2O$ (1.25%) was added to each tube. The tubes were sealed and autoclaved for 15 min at 105 kPa. Larger volumes of CM3 were autoclaved before reduction. After sterilisation the flasks were gassed under N_2, stoppered and reduced. Cellobiose was prepared by replacing cellulose with filter sterilised cellobiose. The following cellulosic substrates were utilised; avicel, microcrystalline cellulose (FMC Corporation, Food and Pharmaceutical Products, Philadelphia) α-cellulose (Sigma Chemical Co.); Whatman CC41 cellulose.

Growth was measured as optical density (OD) at 600 nm in a Corning Colorimeter 525. Cultures that contained cellulose were blended in a vortex mixer and allowed to stand upright for 2 h prior to taking OD readings. Cultures that contained cellobiose were measured immediately after blending.

Stock cultures of *C.thermocellum* for daily use were maintained by subculturing biweekly into fresh CM3 medium. The cultures were grown for 48 h at 60°C and stored at 4°C. Since the strain did not utilise glucose, contamination checks were performed in CM3 that contained glucose rather than cellulose. Turbidity and gas production after 72 h were taken as evidence for contamination.

Stock cultures were also maintained in chopped meat carbohydrate medium (appendix B), grown for 48 h at 60°C and stored in the dark at room temperature.

A.2.4. Sugar analyses.

D-glucose in the medium was determined by the colourimetric assay for D-glucose with glucose oxidase (Merkotest Diagnostic Kit Catalogue No. 3393). Total reducing sugars were calculated according to the dinitrosalycilic (DNS) method (Miller, 1959).

A.2.5. Measurement of cellulolytic activity.

The carboxymethyl cellulase activity (CMCase) assay described by Gordon *et al.*, (1979) and the filter paper assay described by Mandels *et al.*, (1976) were used. One unit of activity was defined as the quantity of enzyme required to catalyse the formation of 1 μmole of reducing sugar $m\ell^{-1}$ min^{-1}.

A.3 RESULTS

A.3.1. Characteristics of growth on cellulose.

Initial evidence of fermentation was a fluffing of the cellulose and the release of descrete gas bubbles. A yellow pigment which bound tightly to the cellulose was produced. As the fermentation became more vigorous, the cellulose was gradually solubilized and the liquid phase became turbid due to cell growth. No yellow pigment was formed when *C.thermocellum* was propagated on cellobiose media. Shaking the cultures decreased growth on cellulose but had no effect on cellobiose grown cultures. Microscopic examination indicated that the organism associated tightly with the cellulose fibres.

A.3.1.1. Transfer of growth factors from meat broth.

Inocula from meat broth cultures of *C.thermocellum* grew better in CM3 cellobiose and CM3 avicel than CM3 inocula (Fig. A1). The growth rate was faster and the final absorbance was greater in CM3 cellobiose medium. Serial transfer of *C.thermocellum* in CM3 decreased its growth. After 4 transfers into fresh media no growh occurred (Fig. A2). The addition of 0.5 mℓ of fresh uninoculated meat broth supernatant replaced the growth factors lost through serial transfer (Fig. A2).

The effect of adding biotin, thiamine and p-aminobenzoic acid to CM3 on the growh of *C.thermocellum* was determined. The addition of thiamine and p-aminobenzoic acid on their own or in combination had no effect on the growth of *C.thermocellum*. The addition of biotin at 2 μg/ℓ^{-1} was

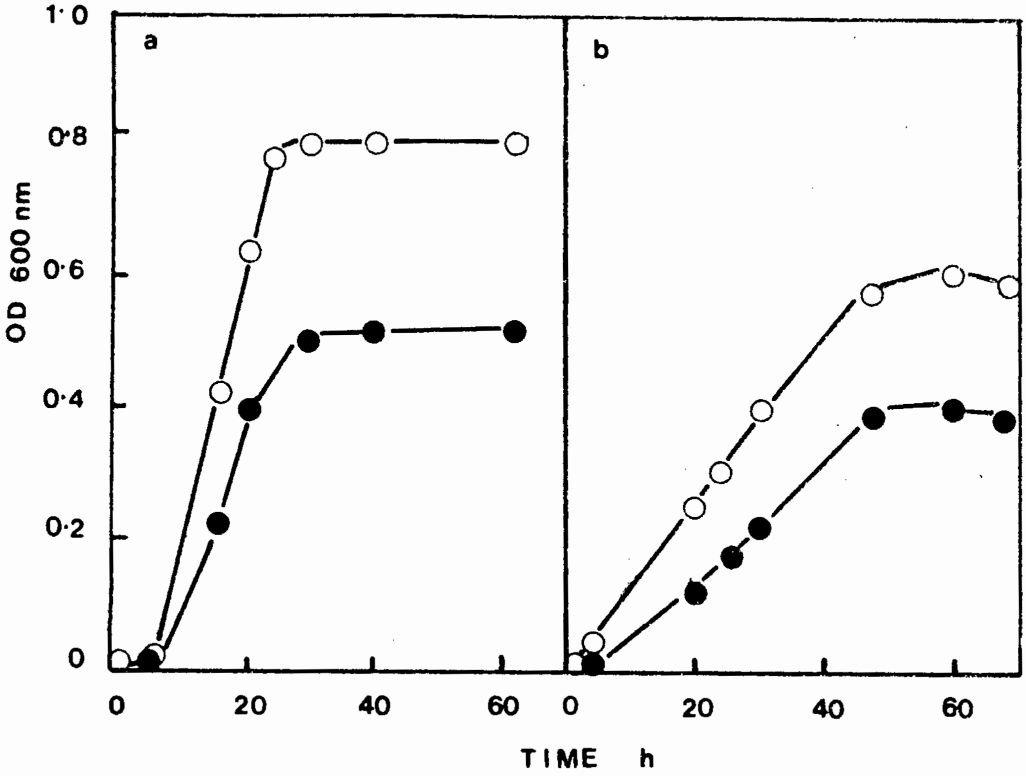

Figure A.1

Growth of *C.thermocellum* in CM3 1% cellobiose medium (a) and CM3 1% avicel medium (b). Both media were inoculated with a meat broth inoculum (○) and with a CM3 stock inoculum (●).

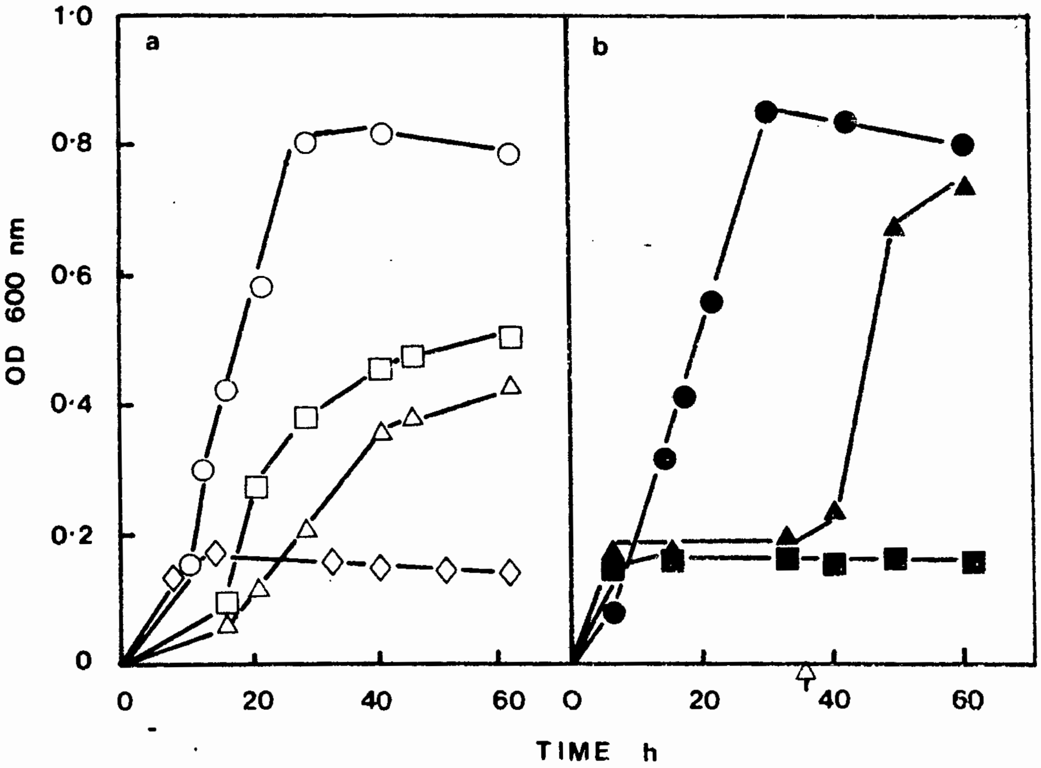

Figure A.2
(a) Effect of serial transfer on the growth of *C.thermocellum* in 1% cellobiose CM3 media in Hungate tubes. 1st transfer ( ) from meat broth stock culture into CM3. 2nd ( ), 3rd ( ) and 4th ( ) serial transfers into fresh CM3 media. (b) The addition ( ) of 0.5 mℓ uninoculated meat broth supernatant after 4th serial transfer ( ), no meat broth supernatant added ( ). Control ( ) meat broth stock inoculum.

necessary for optimum growth of *C.thermocellum*.

The optimum pH for growth of *C.thermocellum* was at pH 7.0 and pH values greater than 7.4 inhibited growth. The optimum growth temperature was 60°C for *C.thermocellum* grown in CM3-cellobiose and CM3-avicel media. *C.thermocellum* growth was inhibited by cellobiose concentrations greater than 2% (Fig. A3).

A.3.2. The effect of physical and chemical factors on cellulase production and activity in *C.thermocellum*.

The optimum temperature for cell-free CMCase activity of *C.thermocellum* was between 65 and 70°C (Fig. A4). The enzyme was thermally stable at 65°C for 50 min, but was completely denatured at 90°C after 10 min. At 60°C the pH optimum for CMCase activity was pH 5.7 in citrate buffer (Table A1).

In order to determine the most suitable cellulosic substrates for the production of cellulase, *C.thermocellum* was grown in CM3 containing 1% avicel, α-cellulose or Whatman CC41 cellulose (Table A2). The supernatant was assayed for CMCase activity and filter paper activity after 72 h. In comparison with the other substrates avicel gave the highest enzyme levels for CMCase activity and filter paper activity. *C.thermocellum* grown in CM3-cellobiose produced lower levels of CMCase (370 mU $m\ell^{-1}$ compared to 437 mU $m\ell^{-1}$ in avicel) and higher levels of filter paper activity (30 mU $m\ell^{-1}$ compared with 24 mU $m\ell^{-1}$ in avicel).

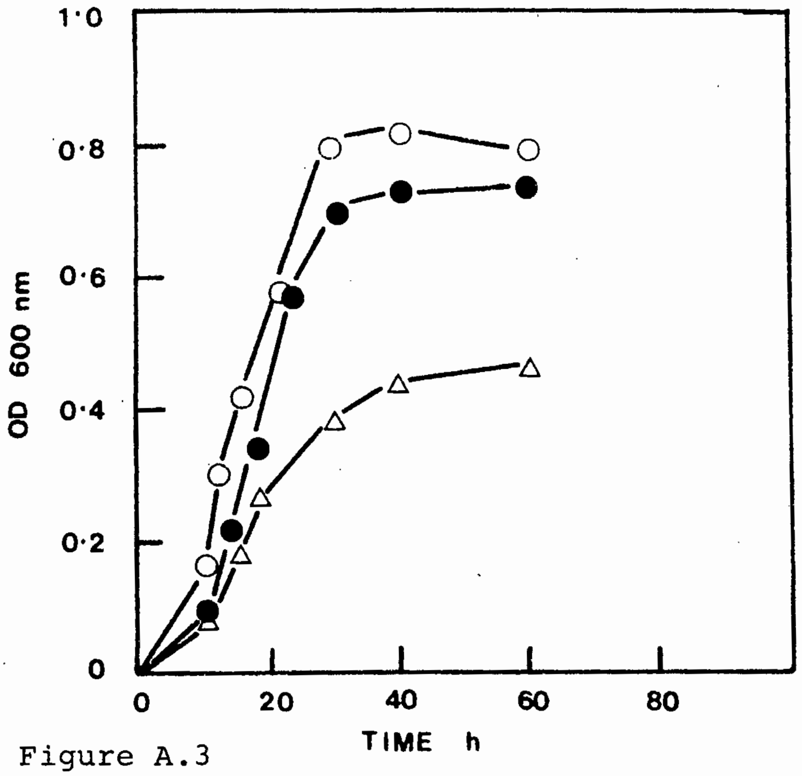

Figure A.3

The effect of increased cellobiose concentrations (% concentrations w/v) on the growth of *C.thermocellum*. 1% (○), 2% (●) and 3% (△).

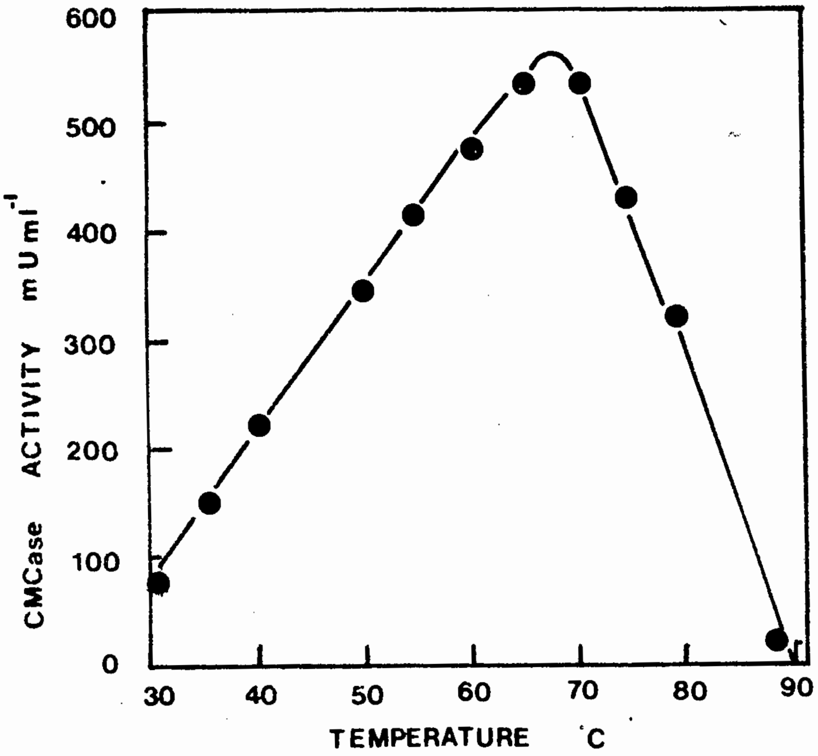

Figure A.4

The effect of temperature on cell free CMCase activity of *C.thermocellum* (●).

TABLE A1

Effect of pH on the cell-free CMCase activity of *C.thermocellum*.

Buffer	pH	CMCase mU $m\ell^{-1}$
Acetate 0.2M	2.4	0
Acetate 0.2M	3.8	130
Citrate 0.1M	4.3	194
Citrate 0.1M	5.1	380
Acetate 0.2M	5.1	361
Acetate 0.2M	5.6	398
Citrate 0.1M	5.7	462
Sodium phosphate 0.2M	5.9	407
Sodium phosphate 0.2M	7.0	370
Sodium phosphate 0.2M	8.1	305

TABLE A2.

Cellulase levels produced by *C. thermocellum* on different cellulose substrates and cellobiose.

Substrate	Final absorbance (600 nm)	CMCase mU $m\ell^{-1}$	Filter paper activity mU $m\ell^{-1}$
α-cellulose	0.68	372.2	22.6
Avicel	0.57	437.0	24.1
Whatman CC41	0.52	361.1	19.0
Cellobiose	0.79	370.1	30.0

Enzyme levels assayed after 72 h.

A.3.3. Reducing sugar accumulation in the fermentation media.

The levels of reducing sugar and glucose which accumulated after growth of *C.thermocellum* in CM3 containing 1% of avicel and cellobiose were compared. After 72 h growth of *C.thermocellum* on avicel there was a 10-fold increase in the level of reducing sugar (0.195 mg $m\ell^{-1}$ to 2.00 mg $m\ell^{-1}$ after 72 h). Cellobiose grown cultures showed a net loss of 1.25 mg $m\ell^{-1}$. There was however an accumulation of glucose in the cellobiose medium (2.4 mg $m\ell^{-1}$). No growth or cellulase activities occurred when *C.thermocellum* was grown in 1% glucose cultures incubated at 60°C for 1 week.

Since growth of *C.thermocellum* on cellobiose was more rapid than on insoluble cellulose substrates, the growth, cellulase activities and sugar levels were monitored in CM3-cellobiose cultures (Fig. A5). The CMCase activity was *c* 10-fold higher than the filter paper activity. The levels of reducing sugar decreased for the first 32 h after which it gradually plateaued. This plateau in the level of reducing sugar in the supernatant was associated with the appearance of glucose in the medium.

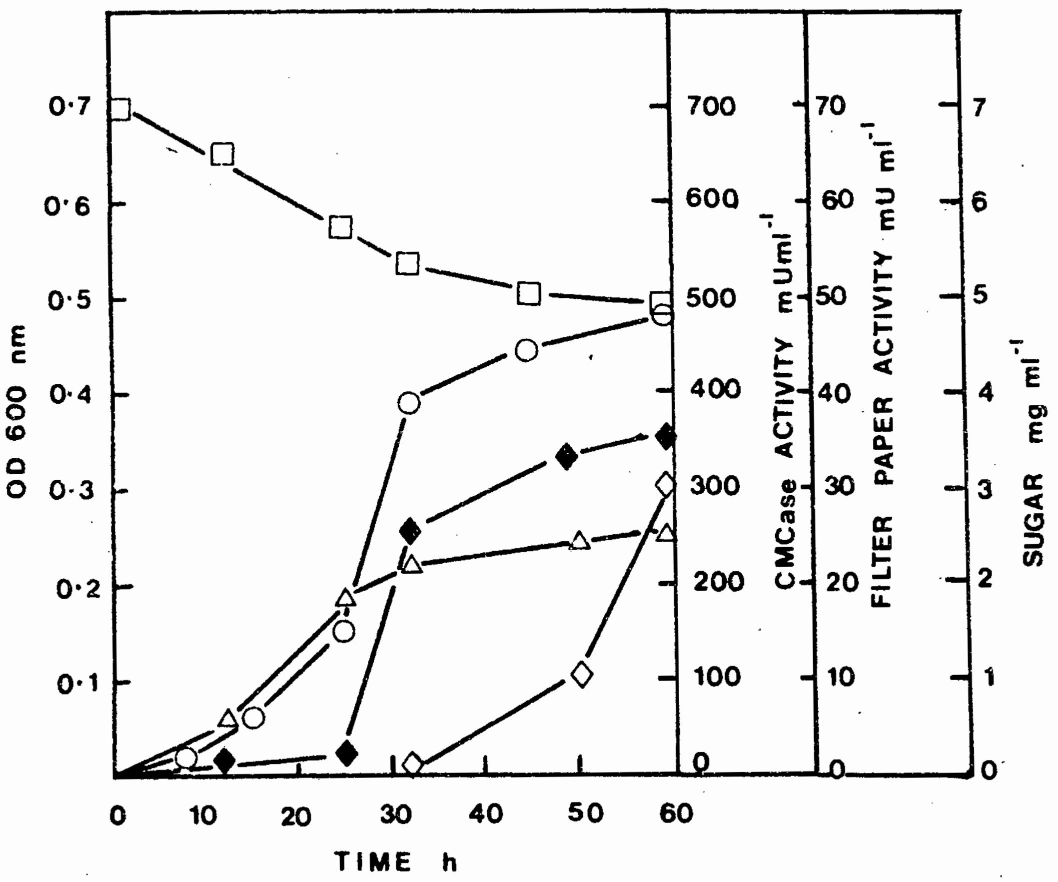

Figure A.5

Growth of *C.thermocellum* on 1% cellobiose; absorbance 600 nm (○); CMCase activity (◆); Filter paper activity (△); Reducing sugar concentration (□); Glucose concentration (◇).

A.3.4. Discussion.

C.thermocellum was successfully grown at 60°C and methods for the maintenance of the strain using meat broth medium have been developed.

Biotin had to be added to the CM3 medium developed by Weimer and Zeikus (1977) before successful serial transfer could be accomplished. The biotin requirement was necessary for growth in cellobiose and cellulose media. Growth beyond a single transfer could not be obtained if biotin was omitted from the medium. Similar results by Johnson *et al.*, (1981) found that biotin and vitamin B6 were required for growth of *C.thermocellum* in their chemically defined minimal medium.

The cellulolytic and physiological properties of *C.thermocellum* tested were similar to those found by other workers (Ng *et al.*, 1977; Gordon *et al.*, 1979; Johnson *et al.*, 1981; Ng *et al.*, 1981). Growth of *C.thermocellum* on cellulose and cellobiose resulted in the production of extracellular cellulase, which was thermally stable at 70°C. The highest cellulase yields were obtained on avicel. The temperature and pH optima for CMCase activity were 70°C and 5.7 respectively.

The accumulation of reducing sugar during the fermentation of cellulose was detected. This was not a new observation as in 1913 Pringheim working with enriched cultures demonstrated the accumulation of reducing sugar in a fermentation which consisted of glucose and cellobiose. This accumulation of reducing sugar was also noted by Woodman and Stewart (1928), Peterson *et al.*, (1930) and Tetrault (1930). The accumulation of reducing sugar, mainly glucose and cellobiose, in pure cultures of *C.thermocellum* was also reported by Gordon *et al.* (1979). This accumulation of glucose and cellobiose also occurred in the fermentation of cellulose by a cellulolytic member of the *Bacteroidaceae* family, isolated from sewage sludge (Saddler and Khan, 1979).

C.thermocellum accumulated glucose when grown on both cellulose and cellobiose. The accumulation of glucose in cellobiose cultures has not been reported previously. There have been conflicting reports on the ability of *C.thermocellum* to utilise glucose as the sole carbon source (see introduction). Gomez and Hernadez (1980) have shown that *C.thermocellum* is able to utilise glucose as the sole carbon source after an extended lag phase (12 to 30 h). We have been unable to repeat these findings. No extracellular cellobiase activity has been found in *C.thermocellum* fermentations (Reese, 1956). This suggests that cellodextrins are readily transported into cells before being degraded by the cellulases. This proposal is supported by the demonstration of intracellular cellodextrin phosphorylase in *C.thermocellum* by Alexander (1960, 1972a, 1972b). The cellobiase activity has been purified from the "periplasmic space" of *C.thermocellum* (Ait *et al.*, 1979). Thus, the accumulation of glucose during growth was not likely to be due to extracellular conversion of cellobiose to glucose. This was not tested and the possibility of membrane bound activity should be taken into consideration. The accumulation of glucose in the medium could be a means of accommodating accompanying symbiotic bacteria (Imsenecki, 1940; Enebo, 1951).

There is a possibility that if this glucose is due to efflux from *C.thermocellum* it may be advantageous energetically to *C.thermocellum*, as in the efflux of β-galactosidase products from *Escherichia coli* (Huber *et al.*, 1976). They suggest that the efflux of glucose which is excreted by proton linked permeases (Henderson *et al.*, 1977) drives the uptake of lactose which is energised by other transport systems acting in the opposite direction (Flagg and Wilson, 1978) In *E.coli* the glucose could re-enter the cell and become phosphorylated by the phosphoenolpyruvate-phosphotransferase system (Fraenkel *et al.*, 1964). This system could apply to *C.thermocellum* although there is no direct evidence, except the accumulation of glucose.

APPENDIX B

GENERAL METHODS

Preparation of Anaerobic Media and Solutions

Pre-reduced and anaerobically sterilised media were prepared according to the methods of Moore (1966) and Hungate (1969). Most of the oxygen was driven off by heating. The media were further reduced by the addition of cysteine hydrochloride. They were then flushed with a CO_2 + H_2 mixture and maintained in an anaerobic state in Hungate tubes, or by storing in anaerobic jars or boxes with silica gel in muslin bags.

All sterilisation was done by autoclaving at 121°C for 20 min at 105 kPa unless otherwise stated.

Factory Fermentation Media

Molasses medium for fermentation stages A_2, B and C

Molasses	73.0 g (or amount required to give 3.5% total invert sugar)
$(NH_4)_2SO_4$	7.0 g
$CaCO_3$	3.0 g
$(NH_4)_2PO_4$	0.2 g
H_2O (distilled)	1.0 ℓ

Molasses Fermentor Medium (MFM)

Molasses	134.0 g (or amount required to give 6.5% total invert sugar)
$(NH_4)_2\ SO_4$	2.0 g
$CaCO_3$	1.0 g
Steepwater concentrate	1.5 mℓ
H_2O (distilled)	1.0 ℓ

The pH of the medium was adjusted to pH 7.0 - 7.3 with IN NaOH before autoclaving.

Anaerobic Media

Clostridium basal medium (CBM) (O'Brien & Morris, 1971)

Glucose	10.0 g
Casein Hydrolosate	4.0 g
Yeast extract	4.0 g
Na HCO_3	1.0 g
Cystein HCl	0.5 g
H_2O (distilled)	1.0 ℓ
$MgSO_4.7H_2O$ Stock Solution	1.0 mℓ
$MnSO_4.4H_2O$ " "	1.0 mℓ
$FeSO_4.7H_2O$ " "	1.0 mℓ
p-aminobenzoic acid "	1.0 mℓ
Thiamine HCl Stock Solution	1.0 mℓ
Biotin " "	1.0 mℓ
Risazurin " "	10.0 mℓ

Sterilised by autoclaving and stored anaerobically.

Clostridium Basal Medium agar

Glucose	10.0 g
Casein hydrolosate	4.0 g
Yeast extract	4.0 g
Agar	15.0 g
H_2O (distilled)	1.0 ℓ
$MgSO\ .7H_2O$ Stock solution	1.0 mℓ
$MnSO\ .4H_2O$ " "	1.0 mℓ
$FeSO\ .7H_2O$ " "	1.0 mℓ
p-aminobenzoic acid stock solution	1.0 mℓ
Thiamine HCl Stock solution	1.0 mℓ
Biotin " "	1.0 mℓ

The medium was autoclaved, cooled and the following sterile stock solutions were added prior to pouring:

Cysteine HCl	10.0 mℓ
$NaHCO_3$	10.0 mℓ

Autolysin assay medium

Glucose	10.0 g
Casein hydrolosate	4.0 g
Agar	4.0 g
H_2O (distilled)	1.0 ℓ
$MgSO_4.7H_2O$ Stock Solution	1.0 mℓ
$MnSO_4.4H_2O$ " "	1.0 mℓ
$FeSO_4.7H_2O$ " "	1.0 mℓ
Thiamine HCl " "	1.0 mℓ
p-aminobenzoic acid Stock solution	1.0 mℓ
Biotin Stock solution	1.0 mℓ

The medium was autoclaved, cooled and the required volumes of sterile K_2HPO_4 (*c* 2.5 mℓ) and KH_2PO_4 (*c* 1.0 mℓ) stock solutions were added to give a pH of 6.8 - 7.0.

Robson medium (Robson 1981)

Sucrose	65.0 g
$CaCO_3$ (10% w/v soln)	20.0 mℓ
Calcium lactate	12.0 g
Yeast extract	2.0 g
Di-ammonium hydrogen phosphate	1.7 g
$MnSO_4$ (Stock solution)	1.0 mℓ
$FeSO_4$ " "	1.0 mℓ
$ZnSO_4$ (5% w/v ")	10.0 mℓ
p-aminobenzoic acid (stock solution)	1.0 mℓ
Biotin (Stock solution)	1.0 mℓ
Thiamine HCl (stock solution)	1.0 mℓ
K_2HPO_4 (5% w/v soln)	10.0 mℓ
KH_2PO_4 (5% w/v soln)	10.0 mℓ
Resazurin (Stock solution)	10.0 mℓ
H_2O (distilled) made up to	1.0 ℓ

The pH was adjusted to 6.5 with 25% NH_3 solution and the medium was autoclaved for 20 min.

Leung medium (Leung, 1980)

Glucose	50.0 g
KH_2PO_4	0.75 g

K_2HPO	0.75 g
MgSO	0.20 g
NaCl	1.00 g
Asparagine . H_2O	0.50 g
Cysteine hydrochloride	0.50 g
$FeSO_4.7H_2O$	0.01 g
$MnSO_4.3H_2O$	0.01 g
Yeast extract	15.00 g
Resazurin (stock solution)	1.0 mℓ
H_2O (distilled)	1.0 ℓ

The pH was adjusted to 6.5 with 1N NaOH prior to autoclaving.

<u>Chopped beef meat carbohydrate medium</u>

(*C. thermocellum* stock cultures)

Ground beef (fat free)	500.0 g
H_2O (distilled)	1.0 ℓ
1N NaOH	25.0 mℓ

Lean beef or horse meat was used. Fat and connective tissue were removed before grinding. Meat was mixed with water and NaOH and brought to the boil, stirring. It was cooled to room temperature, the fat skimmed off the surface and filtered, both meat particles and filtrate were retained. Sufficient distilled water was added to filtrate to restore original 1 ℓ volume. To this filtrate was added:

Peptone	30.0 g
Yeast extract	5.0 g
$K_2H\ PO_4$	5.0 g
Glucose	4.0 g

Cellobiose	1.0 g
Maltose	1.0 g
Starch	1.0 g
Resazurim solution (0.025% w/v)	4.0 mℓ

It was boiled, cooled, and 0.5 g cystein hydrochloride was added and the pH adjusted to 7.5 with 1N NaOH. 7 mℓ amounts were dispensed into Astell roll tubes containing meat particles under O_2 free 97% N_2 and 3% H_2 (1 part meat to 4-5 parts fluid). The tubes were stoppered under N_2 and H_2 and autoclaved in a press for 15 min.

Beef liver medium for anaerobes

(from the ATCC catalogue No. 38) (Used for *C.acetobutylicum* stock cultures)

Beef liver (cut up into small pieces)	500.0 g
Tap water	1.0 ℓ

The beef liver was soaked overnight at 4°C, the fat skimmed off the top, autoclaved for 10 min at 121°C and filtered through cheese cloth. Both meat particles and filtrate were retained. To the filtrate was added:

Peptone	10.0 g
K_2HPO_4	1.0 g

The pH was adjusted to 8.0 with 1 NaOH and the volume made up to 1 ℓ with tap water. A small amount (*c* 0.05 g) of $CaCO_3$ was placed in each tube, *c* 1.5 cm of meat was added and covered with the filtrate to a total depth of 5 cm. The tubes were capped with rubber stoppers and autoclaved in a press for 15 min.

Stock solutions

All stock solutions were stored at 4°C.

p-Aminobenzoic acid (paba) stock solution

p-Aminobenzoic acid	0.1 g
H_2O (distilled)	100.0 mℓ

Biotin stock solution

Biotin	200.0 μg
H_2O (distilled)	100.0 mℓ

Cysteine HCl stock solution

Cysteine HCl	5.0 g
H_2O (distilled)	100.0 mℓ

Autoclaved.

$FeSO_4.7H_2O$ stock solution

$FeSO_4.7H_2O$	1.0 g
H_2O (distilled)	100.0 mℓ

Stored at -20°C.

K_2HPO_4 stock solution

K_2HPO_4	25.0 g
H_2O (distilled)	100.0 mℓ

Autoclaved.

KH_2PO_4 stock solution

KH_2PO_4	12.5 g
H_2O (distilled)	100.0 mℓ

Autoclaved.

Resazurin stock solution

Resazurin	20.0 mg
H_2O (distilled)	100.0 mℓ

Sodium bicarbonate stock solution

$NaHCO_3$	10.0 g
H_2O (distilled)	100.0 mℓ

Autoclaved.

Thiamine hydrochloride stock solution

Thiamine HCl	0.1 g
H_2O (distilled)	100.0 mℓ

REAGENTS FOR AMYLASE ASSAY

1% Starch solution

1 g of soluble starch (Merck) was weighed into a 100 mℓ Erlenmeyer flask, and 20 mℓ of distilled H_2O was added with shaking. Then 5 mℓ of 2N NaOH was added to obtain

a completely dispersed solution. The mixture was heated for 3 min in boiling water, 25 mℓ of distilled H_2O added and the solution was cooled under running tap water. 5 mℓ of 2N CH_3 COOH (acetic acid) was added to neutralise the mixture, and the pH was adjusted to 5.5 by addition of 0.1N CH_3 COOH or 1N CH_3COOH. It was made up to 100 mℓ with distilled H_2O. This starch solution contained 0.1N CH_3 COOH - CH_3 COONa buffer.

Iodine solution

I_2	0.5 g
KI	5.0 g
H_2O (distilled)	100.0 mℓ

The concentrated iodine solution was stored in a dark bottle at 4°C. A 1/100 dilution was made up of the concentrated stock when required.

Calibration mixture for GC analysis

The solvents were added separately into a 100 mℓ volumetric flask containing 20 mℓ of distilled H_2O and weighed to 4 decimal places. The stock solution contained the following solvents:

Acetone	7.8202 $g\ell^{-1}$
Ethanol	0.7360 $g\ell^{-1}$
Butanol	7.9443 $g\ell^{-1}$
Propanol	7.8274 $g\ell^{-1}$

The calibration mixture was stored in sealed containers at 4°C.

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