STUDIES ON AN AUTOLYSIN PRODUCED BY *CLOSTRIDIUM ACETOBUTYLICUM*

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ABSTRACT

An extracellular bacteriocin-like substance produced by *Clostridium acetobutylicum* was detected during studies on an industrial fermentation process. The bacteriocin-like substance was not inducible by either ultraviolet light or mitomycin C, and its production was not associated with the induction of a protease.

Studies on the mode of action of the bacteriocin-like substance indicated that it had no significant effect on DNA, RNA, or protein synthesis, and it did not cause the loss of intracellular ATP. However, the bacteriocin-like substance was able to lyse SDS-treated cells and cell walls of *C. acetobutylicum* and was identified as an autolysin.

Some of the characteristics of this extracellular autolysin were determined, and after purification it was shown to be a glycoprotein with a molecular weight of 28 000.

CHAPTER I

INTRODUCTION

1.1 BACTERIAL INHIBITORY SUBSTANCES

Bacteria produce a wide range of substances which are inhibitory to other bacteria and some of these also affect the producer strain itself. Bacterial inhibitory products include low molecular weight antibiotics, metabolic products, lytic agents, enzymes, bacteriocins and defective bacteriophages. Classification of a particular inhibitory substance is often difficult as it may possess characteristics that overlap from one division to another. In their reviews on bacteriocins, Nomura (1967), Reeves (1972), and Tagg *et al.* (1976) discuss the difficulties in distinguishing bacteriocins from other inhibitory substances. Standard tests used for bacteriocin detection are nonspecific and do not differentiate between the inhibitory effects of bacteriocins, and those of other bacterial products. Metabolic by-products such as ammonia, lactic acid, and hydrogen peroxide are also capable of exhibiting bacteriocin-like antagonism, and several investigators testing for bacteriocin production in β-haemolytic *Streptococci* did not exclude the effect of hydrogen peroxide released by the test strains (Tagg *et al.*, 1976).

Bacteriolytic enzymes are produced by a number of bacteria and have been confused with bacteriocins. *Staphylococcus aureus* species have been shown to produce a variety of bacteriolytic and autolytic enzymes, including lysostaphin (Schindler and Schuhardt, 1965), lysozyme (Hawiger, 1968), endo-β-N-acetylglucosaminidase (Wadström and Hisatsune, 1970),

and a phage-associated lysin (Stonstein *et al.*, 1971), as well as bacteriocins (Moore, 1970; Hale and Hinsdill, 1973; Dajani and Wannamaker, 1974). Tagg *et al.* (1976) pointed out that what one investigator might describe as a bacteriocin may well be described by another as a defective phage, a bacteriolytic enzyme, or a classical antibiotic. They suggest that until an incompletely defined antagonistic substance is fully characterised it should be described as "bacteriocin-like". The full extent of the confusion in nomeculture and definition is apparent in a report describing a specific lysin of *Clostridium perfringens* in which bacteriophage, bacteriocins and autolysins are discussed in relation to the lysin (Nakamura *et al.*, 1977).

1.2 AUTOLYSINS

Unless otherwise indicated, the information on autolysins or bacteriolytic enzymes has been obtained from the reviews of Stolp and Starr (1965), Strominger and Ghuysen (1967), Ghuysen (1968) and Ghuysen and Shockman (1973).

Autolysins are defined as enzymes that are capable of hydrolysing the cell walls of the producer strain and in particular the peptidoglycan. Bacterial cell walls may contain between 5% and 90% peptidoglycan, and the rest is made up of proteins, lipoproteins, polysaccharides, lipopolysaccharides and polyol-phosphate polymers such as teichoic acid. The peptidoglycan (mucopeptide, glycopeptide or murein are synonymous with peptidoglycan) layer forms the insoluble matrix of the cell wall. The peptidoglycan layer is composed of two acetamido sugars, N-acetylglucosamine and N-acetylmuramic acid, and four to eight different

amino acid residues such as L-alanine, D-alanine, D-glutamic acid, L-lysine and meso-diaminopimelic acid (DAP). The components are normally present in equimolar amounts. Although the peptidoglycan structure varies from one organism to another, the basic structure is provided by linear strands of alternating β-1-4-linked pyranoside-N-acetylglucosamine and N-acetylmuramic acid residues called the glycan strand. The N-acetymuramic acid residues of this structure are ether-linked to lactyl groups, and the carboxyl of the lactyl group provides the point to which peptide subunits are amide-linked to the glycan strand. Peptidoglycan is made up of several layers of glycan strands linked together, either by interpeptide bridges attached to the peptide subunits of each strand, or by direct peptide bonds between peptide subunits. Fig 1.1 shows the glycan strands interconnected through peptide bridges, and Fig 1.2 represents a small portion of one glycan strand.

The peptidoglycan layer of the cell wall provides the rigidity, shape and osmotic stability of the cell. When autolysins are permitted to act, cells lose their osmotic protection and autolyse. Although there are exceptions, autolysis generally occurs whenever the metabolism of the cell is disturbed drastically. Sudden removal of oxygen from aerated cultures in exponential phase causes lysis of *Bacillus subtilis* (Kaufmann and Bauer, 1958). Autolysis may also be induced by sudden cold storage ($0^{o}C$) of aerated growing bacteria, or by the exposure of psychrophilic bacteria to temperatures above the optimum. In some bacterial species, autolysis may be induced by the addition of univalent cations to the bacterial culture (Ogata and Hongo, 1973; Hebeler and Young, 1975) and autolysis can often be demonstrated by resuspending cells in buffers such as sodium acetate or phosphate buffer (Higgins *et al.*, 1970, Kawata and

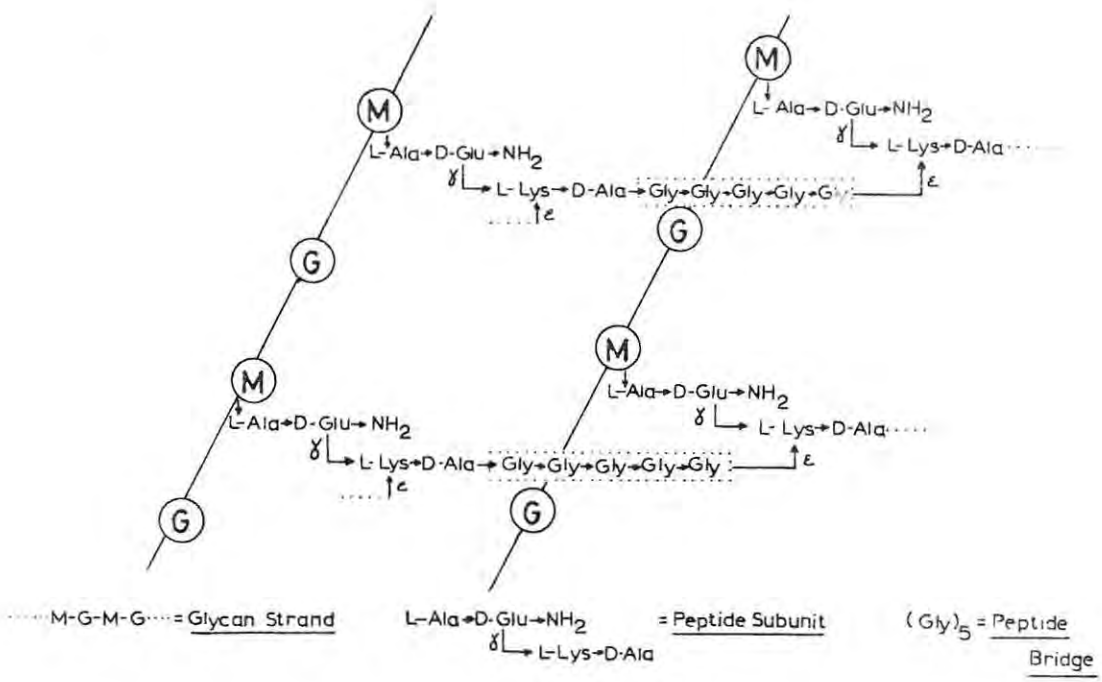


Fig 1.1 Primary structure of the wall peptidoglycan in *S. aureus*. The glycan strand consists of alternate residues of N-acetylglucosamine (G) and N-acetylmuramic acid (M). Usual α-peptide bonds are represented by horizontal arrows; other peptide bonds (eg α or ε) are also indicated. The pentaglycine bridges, which extend from the ε-amino group of L-lysine on one peptide subunit to the carboxyl group of D-alanine on another, are enclosed by a dotted rectangle (Ghuysen and Shockman, 1973).

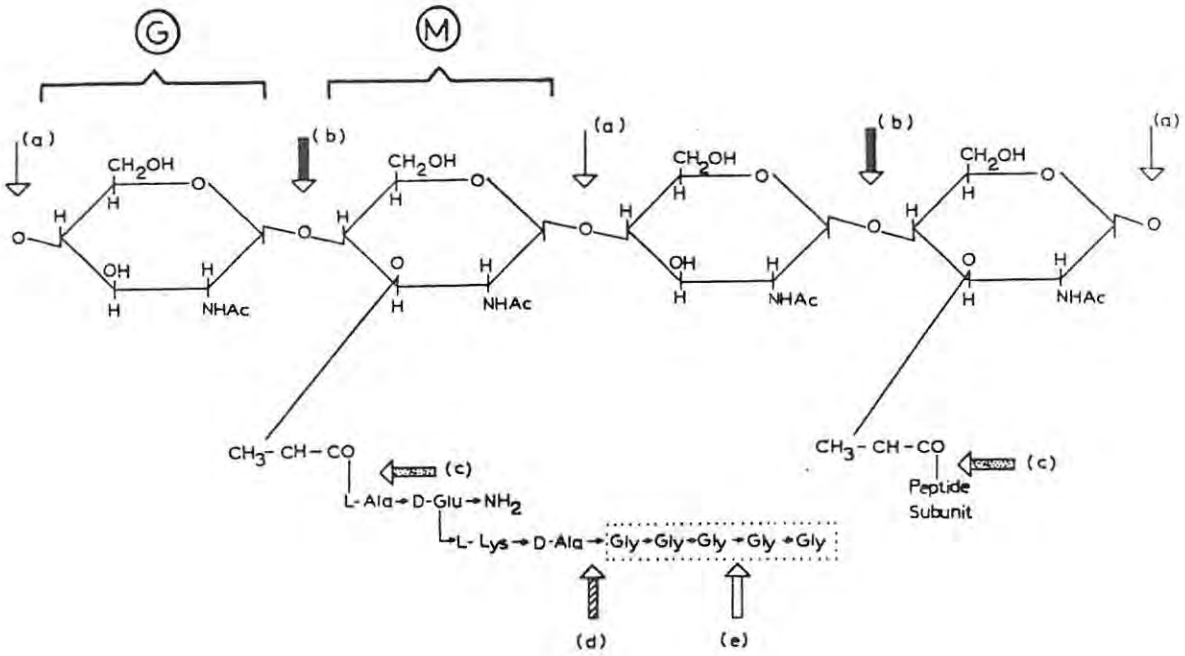


Fig 1.2 A small section of a glycan strand of *S. aureus* showing sites of action of endo-N-acetylmuramidases at arrows (a), endo-N-acetylglucosaminidases at arrows (b), N-acetylmuramyl-L-alanine amidases at arrows (c), and endopeptidases at arrows (d) and (e); adapted from Ghuysen and Shockman (1973).

Takumi, 1971; Coyette and Shockman, 1973). The inhibition of continued peptidoglycan synthesis by the addition of specific inhibitors such as penicillins or D-cycloserine may result in cellular autolysis. If cells are deprived of peptidoglycan precursors such as glucose, glucosamine, lysine, alanine, glutamic acid or DAP, the cells become sensitive to osmotic lysis. Certain *Escherichia coli* mutants were shown to lyse if deprived of DAP (Meadow *et al.*, 1957) and the absence of D-alanine, aspartic acid, glutamic acid, L-lysine or glucose, resulted in osmotic fragility of *Streptococcus faecalis* cells (Shockman *et al.*, 1961; Shockman *et al.*, 1968). Ghuysen and Shockman (1973) suggest that the inhibition of peptidoglycan synthesis does not in itself result in cellular lysis, but that the active participation of one or more peptidoglycan hydrolysase (autolysin) is required. Evidence for this is provided by the observation that bacteria with supressed or deficient autolytic systems are more resistant to lysis than normal cells when treated with antibiotics that prevent cell wall synthesis (Tomasz *et al.*, 1970; Rogers and Forsberg, 1971; Fein and Rogers, 1976).

1.3 CLASSES OF AUTOLYTIC ENZYMES AND THEIR SITES OF ACTION

Many bacterial species have been reported to possess more than one autolysin, each autolysin having a different specificity. Three major classes of autolysins have been discovered so far and these are glycosidases, N-acetylmuramyl-L-alanine amidases and endopeptidases. Their sites of action on the peptidoglycan of cell walls are shown in Fig 1.2.

1.3.1 Glycosidases

Glycosidases (or hexosaminidases as they are sometimes called) are either endo-N-acetylmuramidases or endo-N-acetylglucosaminidases. Endo-N-acetylmuramidases hydrolyse the glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan, releasing fragments with N-acetylmuramic acid residues at the reducing end. Endo-N-acetylglucosaminidases also hydrolyse glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine, but release fragments with N-acetylglucosamine at the reducing end. N-acetylglucosaminidase activity has been detected in *Bacillus cereus* (Kawagishi *et al.*, 1980), *Clostridium botulinum* (Takumi *et al.*, 1971), *C. perfringens* (Williamson and Ward, 1979), and *E. coli* (Ghuysen and Shockman, 1973) whilst *S. faecalis* (Shockman *et al.*, 1967a), *Lactobacillus acidophilus* (Coyette and Ghuysen, 1970), and *Arthrobacter crystallopoietes* (Krulwich and Ensign, 1968) were all shown to possess endo-N-acetylmuramidase activity.

1.3.2 N-acetylmuramyl-L-alanine amidases

This class of autolytic enzyme is able to hydrolyse the linkage between the carboxyl of the D-lactyl group of N-acetylmuramic acid and the amino group of the L-alanine residue at the amino terminus of the peptide unit. N-acetylmuramyl-L-alanine amidases have been detected in a wide variety of bacteria including *B. subtilis* (Lindsay and Glaser, 1976), *Bacillus licheniformis* (Forsberg and Ward, 1972), *E. coli* (van Heijenoort and van Heijenoort, 1971), *Diplococcus pneumoniae* (Mosser and Tomasz, 1970), *C. botulinum* (Takumi *et al.*, 1971) and *Neisseria gonorrhoeae* (Hebeler and Young, 1976).

1.3.3 Endopeptidases

There are two types of endopeptidases. Some endopeptidases are able to hydrolyse the peptide bonds in the interior of the peptide cross-linking bridges, and others hydrolyse the peptide bonds between the peptide subunits or the peptide bonds between peptide cross-linking bridges and peptide subunits (see Fig 1.2). Examples of the latter are the SA endopeptidase of *Streptomyces albus* (Petit *et al.*, 1966) and the *E. coli* endopeptidase (Weidel and Pelzer, 1964), whereas lysostaphin produced by *S. aureus* is known to split the glycyl-Gyl linkages in the interior of the peptide cross-linking bridges of the peptidoglycan of *Staphylococci* (Browder *et al.*, 1965).

The type of autolytic enzyme(s) produced by a specific organism may be determined by identifying the residues released after its action on the cell wall. This involves detailed analytical procedures which are fully described by Guhysen *et al.*, (1966).

1.4 AUTOPLAST FORMATION

Once a bacterial cell's autolytic system has been activated, lysis may be prevented if the external medium is osmotically buffered (Marquis and Corner, 1976). Under these conditions, although the bacterium may lose its cell wall, it quickly comes to an osmotic equilibrium with the medium and is stabilised. Complete loss of the cell wall results in protoplast formation whereas bacteria with some defect in their peptidoglycan are called sphaeroplasts, both forms appearing as spherical bodies. Autoplast formation (spontaneous formation of protoplasts or sphaeroplasts)

has been used by several investigators to study the autolytic systems of various bacteria (Dark and Strange, 1957; Kawata *et al.*, 1968; Joseph and Shockman, 1974), and in particular the initiation sites of autolysis (Kawata and Takumi, 1970) and autolysin location (Joseph and Shockman, 1976) have been determined by this method.

All bacterial strains that have been examined experience difficulty in restarting new wall synthesis once all the old wall has been lost. There is evidence to suggest that the further secretion of autolysin(s) prevents the accumulation of the threshold level of cross-linked peptidoglycan necessary to set sustained wall synthesis in motion (DeCastro-Costa and Landman, 1977).

1.5 ROLES OF AUTOLYSINS

Although autolysins are potentially dangerous to the cell, they are thought to participate in cellular functions related to growth and division. They have also been associated with other cellular functions such as sporulation, the ability of a cell to become competent for transformation, and the excretion of toxins and extracellular enzymes. The various roles proposed for autolysins in cell wall growth, cell division and other cellular functions are discussed individually.

1.5.1 Cell separation and division

One of the main approaches used to assess the roles of autolysins is the analysis of mutants with deficient autolytic systems. Fein and Rogers (1976) isolated mutants of *B. subtilis* which were 90 to 95% deficient in

the autolytic enzymes N-acetylmuramyl-L-alanine amidase and endo-β-N-acetylglucosaminidase. These mutants grew at normal rates but formed very long chains of unseparated cells. Isolated walls of the mutants had the same chemical composition as the wild type, which suggests that the failure of the cells to separate was not due to some change in the wall chemistry. Two autolytic deficient mutants (*lyt*$^-$)of *B. licheniformis* also grew as very long chains of unseparated bacilli, but the cell wall chemistry of these mutants differed from the wild type (Forsberg and Rogers, 1971; Forsberg and Rogers, 1974). The same effect was reported for a *S. faecalis* autolysin-defective mutant which was shown to have similar wall chemistry to that of the wild type, but grew in chains of 8 to 40 cells compared to the diplococci formed by the wild type (Pooley *et al.*, 1972). These results support the theory that there is a correlation between autolytic activity and cell separation. Further evidence is provided by the observation that when partially purified autolysin was added back to a *B. subtilis* mutant growing in long chains, the chains were unlinked and the long filaments were converted into short cells (Fan, 1970).

Studies on DNA replication and cell division in *E. coli* and *S. faecalis* (*S. faecium*) have revealed that there is an association between autolysins and cell division (Hinks *et al.*, 1978a; Hinks *et al.*, 1978b). The results obtained using synchronised populations of *S. faecalis* suggested that increased autolytic capacity at the beginning of the cell cycle was not related to the initiation of chromosome replication, whereas decreased autolytic capacity at the end of the cell cycle was related to the termination of chromosome replication, closure of the cross wall, and cell division.

1.5.2 Cell wall growth and cell enlargement

It has been proposed that growth of the bacterial cell surface requires some controlled degradation of the cell wall to open "gaps" where new wall material can be added. Rogers (1970) suggested that one role of autolysins may be to ensure that new wall is added at the right place, rather than being added randomly over the surface of the cell. Autolysins may accomplish this function by providing acceptor ends for disaccharide units resulting in growth, or by breaking bonds to allow realignment and rearrangement of existing peptidoglycan. Ghuysen and Shockman (1973) pointed out that the only autolysin that would provide suitable additional "ends" (non-reducing N-acetylglucosamine) would be an N-acetylmuramidase and that only a few species possess this enzyme (*S. faecalis*, *L. acidophilus*, *A. crystallopoietes* and *Bacillus thuringiensis*).

If autolysin(s) play a role in cell wall synthesis by cleaving the existing glycan backbone, a reduced rate of such a cleavage may result in a greater proportion of the synthesis going into wall thickening at the expense of cell wall area. A number of *lyt*$^-$ mutants have been isolated that show enhanced cell wall thickening in comparison to their wild types (Chatterjee *et al.*, 1969; Fan and Beckman, 1971; Fan *et al.*, 1972; Pooley *et al.*, 1972). Several properties of the autolysin produced by *S. faecalis* support the involvement of autolysin in cell wall growth. Activity of the *S. faecalis* autolysin was maximal during the exponential growth phase, and virtually absent in walls from stationary phase cultures. The autolysin was found almost exclusively in the wall fraction, and was localised at the site of cell wall synthesis (Shockman *et al.*, 1967a; Shockman *et al.*, 1967b).

Autolysins are not only associated with cell wall synthesis, but there is evidence to suggest that they are involved in cell wall turnover in some bacteria. Mauck *et al.* (1971) showed that in *B. subtilis* the products of wall turnover resulted from the action of an N-acetylmuramyl-L-alanine amidase. A correlation between autolytic activity and cell wall turnover has also been detected in *S. aureus* (Wong *et al.*, 1974; Chatterjee *et al.*, 1976; Wong *et al.*, 1978) and it was observed by Wong *et al.* (1978) that the presence of chloramphenicol, which inhibits whole cell autolysis, also inhibited turnover. However, the evidence for autolysin involvement in cell wall turnover is not consistent in all bacterial species studied. In a *L. acidophilus* strain, autolytic activity and turnover was not related, since a slowly autolysing mutant showed a turnover rate comparable to the controls (Ghuysen and Shockman, 1973). A similar result was obtained in *B. subtilis* mutants which were reported to have autolysin levels equal to, or greater than the wild type, but those mutants that were protease-deficient had a decreased rate of peptidoglycan turnover (Jolliffe *et al.*, 1980). In this case, it is suggested that the turnover of cell walls in *B. subtilis* may be regulated by extracellular proteases and not autolytic activity.

1.5.3 Development of a genetically competent state

Young *et al.* (1963) reported that those strains of *B. subtilis* which were highly susceptible to genetic transformation by deoxyribonucleic acid (DNA), contained higher activity of an autolytic enzyme than those strains with a low frequency of transformation. It was reasoned that autolytic involvement in transformation was required to provide a sufficient relaxation of the peptidoglycan structure, to permit the occurrence of

"gaps" in the cell wall through which the negatively charged segments of DNA could pass (Young *et al.*, 1964). Autolytic activity has also been associated with competence in group H *Streptococci* (Ranhand *et al.*, 1971; Ranhand, 1973). Seventeen strains of group H *Streptococci* were tested, and it was found that autolysis was only observed in competent cells, or cells that had passed their peak of competence. No strains were found that transformed but did not autolyse. In *Pneumococci* both protoplast formation and cellular leakage were induced by the same substance that induces competence (Seto and Tomasz, 1975). Further evidence is required to clarify the proposal that there is a correlation between autolysin activity and competence, as the information available at present is ambiguous. The ambiguity is demonstrated by two reports: Fein and Rogers (1976) describe *lyt*$^-$ mutants of *B. subtilis* which were able to transform, whilst Yoneda and Maruo (1975) describe mutants of *B. subtilis* with decreased autolytic activity which cannot transform.

1.5.4 Excretion of toxins

Little information is available to support the theory that autolysis is related to toxin release. Boroff (1955) was the first investigator to describe the relationship between autolysis and toxin production in *C. botulinum*. Further studies by Bonventre and Kempe (1960) established that autolysis was an important mechanism for the liberation of toxin by *C. botulinum*. They found that the toxicity of the culture filtrates correlated with the degree of autolysis of the culture, and that the artifical disruption of cultures at the end of exponential phase, resulted in the release of large quantities of extracellular toxin.

1.5.5 Sporulation and germination

Studies on the cell wall lytic enzymes of *B. cereus* revealed the presence of two enzymes, enzyme V and enzyme S (Strange and Dark, 1957). Neither of the enzymes were detected in non-sporulating cells, and lysis of the sporulating cells resulting in spore release appeared to be due to one or both of these intracellular enzymes. The authors suggest that the V enzyme is mainly concerned with the release of free spores, and the S enzyme with the lytic process which accompanies spore germination. There have been several other reports of peptidoglycan hydrolysases involved in spore germination, and spore release in *Bacillus* species (Gould *et al.*, 1966; Kingan and Ensign, 1968; Brown and Young, 1970; Hsien and Vary, 1975). Recent studies on *C. perfringens* showed that the exudate of fully germinated spores contained a large amount of spore lytic enzyme which caused a decrease in optical density of alkali-treated spores (Ando, 1979). However, it is uncertain if the same enzymes that are involved with sporulation and germination are also involved with functions of the vegetative cell. Both mutants of *B. subtilis* which were 90% to 95% deficient in the autolytic enzymes N-acetylmuramyl-L-alanine amidase and endo-β-N-acetylglucosaminidase sporulated and germinated normally (Fein and Rogers, 1976). No definite conclusions can be drawn from these results because of the residual autolytic activity of the mutants (5% to 10%), but the authors suggest that there is a special "switching on" of autolytic enzymes during germination, rather than initiation of the formation of the usual vegetative cell enzymes.

1.5.6 Multiple roles of autolysins and the pleiotropic phenomena in *lyt*⁻ mutants

Mutants defective in the ability to autolyse, as well as those

showing mutations affecting wall chemistry, morphology, or function, are often pleiotropic. When choline is replaced by ethanolamine in walls of *D. pneumoniae* there is a considerable pleiotropic effect (Tomasz, 1968; Mosser and Tomasz, 1970; Tomasz *et al.*, 1970). Choline is required for growth and is a component of the wall teichoic acid in *D. pneumoniae*. The replacement of choline with ethanolamine resulted in resistance of isolated walls to the action of the autolytic enzyme, loss of the ability to autolyse, chain formation of the daughter cells, loss of the ability to transform, and resistance to cell wall antibiotic-induced lysis. Similar effects have been observed in a *B. subtilis* mutant which is thought to have a single gene mutation. These effects include a decreased content of autolytic enzymes, loss of flagellation, and the hyperproduction of extracellular enzymes (α amylase and protease), and it is suggested that the genetic control of all these proteins may be co-ordinately regulated (Yoneda and Mauro, 1975; Ayusawa *et al.*, 1975).

It is reasonably clear that autolysins are involved in cell separation and cell growth, and evidence is mounting for their involvement in other cellular functions. However, it is difficult to determine whether the same enzymes are implicated in numerous cellular functions, or whether different enzymes play various roles.

1.6 REGULATION OF AUTOLYTIC ACTIVITY

Autolytic activity must be strictly controlled by the cell so that the protective nature of the cell wall is maintained. This is particularly necessary for those species possessing extracellular autolysins, as there must be some mechanism that protects the wall, both during and after

excretion of the enzyme. The well-characterised autolytic system of *S. faecalis* allows some insight into possible mechanisms of control. In this organism, which possesses only one autolysin, the results obtained using ^{14}C labelled walls suggested that there was a latent and active form of the N-acetylmuramidase (Pooley and Shockman, 1969). The synthesised autolysin was found to be located in and near the cell wall as an inactive latent precursor (proenzyme). As illustrated in Fig 1.3, it is thought that the proenzyme is transported to sites on the cell wall associated with wall biosynthesis where it is activated (Shockman *et al.*, 1967b; Pooley and Shockman, 1969; Joseph and Shockman, 1974; Joseph and Shockman, 1976). It has been shown that the latent form is activated by proteases such as trypsin, and both the active and latent enzymes have a strong affinity for the cell wall (Shockman *et al.*, 1967a;Shockman and Cheney, 1969; Joseph and Shockman, 1976). The ability of *S. faecalis* cells to autolyse required protein synthesis, and this ability decreased sharply when cultures entered stationary phase (Pooley and Shockman, 1970; Higgins and Daneo-Moore, 1980). However, autolysin activity was detected in isolated walls long after the inhibition of protein synthesis, indicating that the correlation between protein synthesis, and cellular autolysis cannot just be due to the inhibition of autolysin synthesis, or its activation. This suggests that the enzyme has a relatively long half-life and that autolysin activity is inhibited by some other mechanism. Sayare *et al.* (1972) investigated the effect of DNA and RNA inhibition, and could find no correlation between RNA or DNA synthesis and cellular autolysis. Ghuysen and Shockman (1973) mention that there is an unidentified low molecular weight substance that inhibits autolytic activity in cells of *S. faecalis*. Larger amounts of this inhibitor could be extracted from chloramphenicol-inhibited cells than from normal exponential phase cells, indicating that the availability of the inhibitor

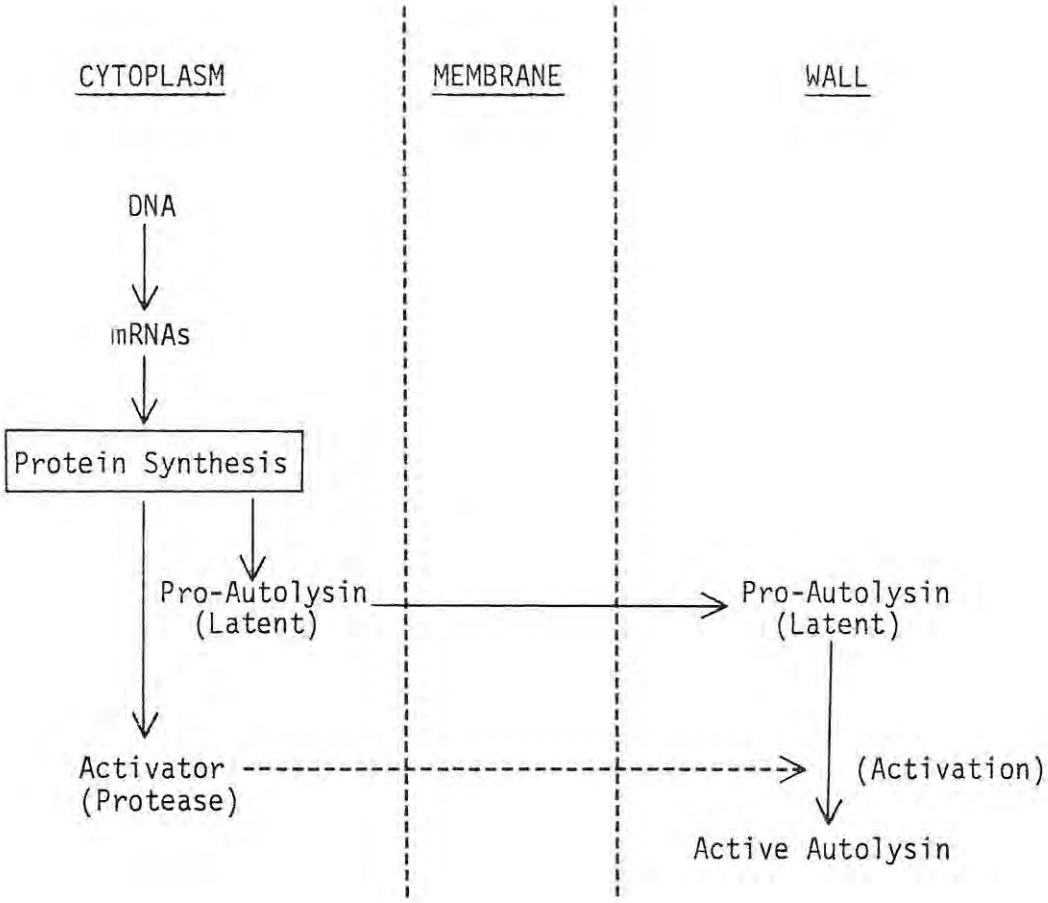


Fig 1.3 Possible relationship between the latent and active forms of the *S. facealis* autolytic enzyme. The latent form is shown as a precursor (proenzyme) of the active form. Activation of the latent enzyme, by means of a protease, takes place after the enzyme has passed through the membrane and become bound to the cell wall. Reprinted from Pooley and Shockman (1969).

could be inversely related to protein synthesis.

Few details are available on the regulation of autolysins in other bacterial species. Seto and Tomasz (1975) reported that the addition of an activator protein to *Pneumococcus* cells resulted in an induction of protoplast formation, and they suggest that the activator protein accelerates the normal transport process of autolysin into the periplasmic space. However, autolysin activation cannot always be demonstrated, and *L. acidophilus* which possesses the same autolysin as *S. faecalis* (an N-acetylmuramidase) lacks a latent form of the enzyme. Although these cells rapidly lose their ability to autolyse when the culture reaches stationary phase, walls isolated from stationary phase *L. acidophilus* cells contain substantial amounts of autolysin (Coyette and Ghuysen, 1970). The regulation of this autolysin and most others has still to be determined.

1.7 HETEROLYTIC AND BACTERIOLYTIC ENZYMES

Some bacteria produce heterolytic enzymes which are only active against bacteria of other species or strains. Both the ML endopeptidase and the KM endopeptidase produced by *S. albus* G attack bonds that do not occur in the peptidoglycan of *Streptomyces*. The endo-β-N-acetylglucosaminidase of *S. aureus* strain M18 is active against strains of *Micrococcus lysodeikticus*, *B. subtilis*, *Bacillus megaterium*, *Sarcina lutea*, *Staphylococcus epidermidis* and shows some activity against *S. aureus* strain 3528, but has virtually no activity against its own cell walls (Wadström and Hisatsune, 1970b). Lysozyme, which may be obtained from a variety of sources ranging from hens' eggs to bacteriophages (Chipman and Sharon, 1969), is a glycosidase and attacks cell walls of many Gram-positive and Gram-negative bacteria.

1.8 USE OF BACTERIOLYTIC ENZYMES IN DETERMINATION OF BACTERIAL WALL STRUCTURES

Elucidation of cell wall structures has involved detailed analysis of the various peptidoglycan types present in different bacterial species. The use of a number of specific hydrolytic enzymes has been necessary, and the numerous bacteriolytic and autolytic enzymes isolated over the years have successfully fulfilled this role. Using specific endopeptidases (each acting on different linkages), glycosidases, and N-acetylmuramyl-L-alanine amidases, the peptidoglycan structure in cell walls of *S. aureus*, *E. coli*, *B. megaterium*, *S. faecalis*, *C. perfringens*, *Micrococcus roseus*, *S. albus* and other bacterial species have been determined (Ghuysen, 1968; Ghuysen and Shockman, 1973). The enzymes were allowed to act in a specific sequence, and after the addition of each enzyme to the peptidoglycan being studied, the degradation products were analysed. For example, the peptidoglycan of *S. aureus* was quantitatively dismantled using the following enzymes in consecutive order: SA endopeptidase, aminopeptidase, endo-N-acetylmuramidase, and finally a N-acetylmuramyl-L-alanine amidase (Ghuysen and Shockman, 1973). As all peptidoglycans have the same basic structure, the use of specific bacteriolytic enzymes has permitted an exhaustive survey of the peptidoglycan structures in a wide variety of bacterial species.

CHAPTER II

PRODUCTION OF A BACTERIOCIN-LIKE SUBSTANCE BY *CLOSTRIDIUM ACETOBUTYLICUM* IN AN INDUSTRIAL FERMENTATION PROCESS

High titres of a noninducible bacteriocin-like substance were produced by *Clostridium acetobutylicum* in a molasses fermentation medium used for the industrial production of solvents. Release of the bacteriocin-like substance towards the end of the exponential growth phase was accompanied by lysis of the culture and the inhibition of further solvent production. The majority of the bacteriocin-like substance was extracellular and lysis of the culture was not associated with protease production.

2.1 INTRODUCTION

The production of solvents by a fermentation process was first described by Pasteur in 1861. He was studying butyric acid-producing organisms and identified butanol as one of the products formed. Acetone production in potato starch medium was first reported in 1905 by Schardinger, but at the beginning of this century there was little commercial interest in either butanol or acetone (Ross, 1961). The first World War provided a great impetus for developments in the conversion of carbohydrates to butanol and acetone on a commercial scale, as acetone was in demand for the manufacture of the explosive cordite (McGutchan and Hickey, 1954).

The production of butanol and acetone by a fermentation process was one of the first large-scale microbiological processes to be developed, and was pioneered by Chaim Weizmann during the period 1910-1920 (Rose, 1961). Since then, it has frequently been referred to as the Weizmann fermentation process, utilizing the anaerobic spore-forming bacillus, *Clostridium acetobutylicum* (Prescott and Dunn, 1940; Beesch, 1953; Ross, 1961).

Many saccharolytic members of the genus *Clostridium*, including *Clostridium acetobutylicum*, *Clostridium felsineum* and *Clostridium butylicum*, are known to convert carbohydrates to acetone, butanol and ethanol via the same biochemical pathway (Fig. 2.1). However, *C. acetobutylicum* is the species principally used for the commercial production of solvents. These bacteria are Gram-positive rods, motile with peritrichous flagella and are found in the soil and intestinal tract of man and animals. In the vegetative stage the organism is approximately 4.0μm x 1.0μm. The spores, formed at or near the ends of the cells when conditions are no longer suitable for growth, measure 1.5μm x 1.0μm. Strains of this bacterium vary considerably in their ability to produce solvents and are specially selected for each fermentation process, depending on the substrate (Rose, 1961). Much of the research determining suitable substrates, growth requirements and conditions necessary for solvent production by *C. acetobutylicum* took place during the period 1920-1950 and has been extensively reviewed by Prescott and Dunn (1940), Beesch (1953), and McGutchan and Hickey (1954).

Since the 1940's interest in the acetone-butanol fermentation has fluctuated according to the availability and cost of the substrates

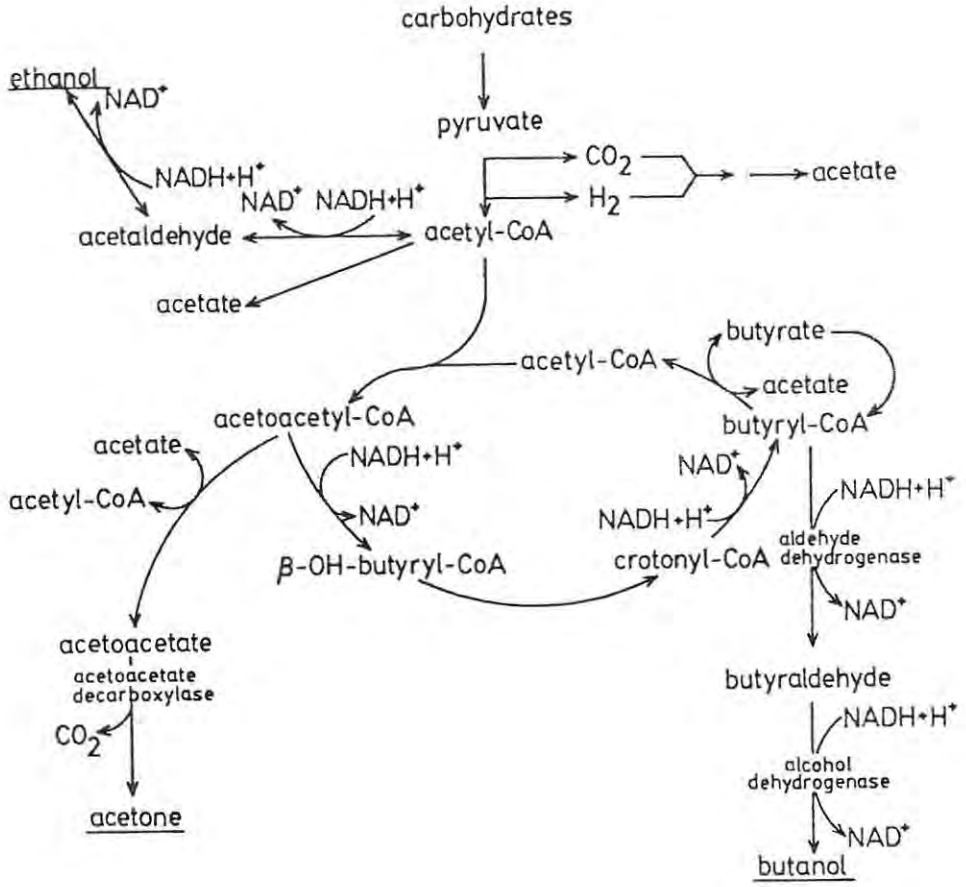


Fig 2.1 The formation of acetone, butanol and ethanol by species of the genus *Clostridium*. (Doelle, 1975).

(chiefly maize and molasses). Before the second World War there was a world surplus of molasses and the fermentation industry was able to obtain this substrate cheaply (Hastings, 1978). After the war, competition from the petrochemical industry seriously affected the fermentation industry and resulted in a rapid decline of the Weizmann process in America, Great Britain and Europe. By 1970 very few countries continued to operate the process. Now that the era of cheap petroleum has been abruptly terminated,there has been a dramatic revival of interest in the process (Abou-Zeid *et al.*, 1978; Hastings, 1978; Spivey, 1978).

A factory fermentation plant producing acetone and butanol and using molasses as the substrate is still functional in South Africa. National Chemical Products of South Africa have produced solvents by fermentation since 1936 utilizing strains of *C. acetobutylicum*. One of the major disadvantages associated with the acetone-butanol fermentation is the low yield of solvents obtained. This is partially due to the inability of the bacterium to tolerate high concentrations of solvents (Rose, 1961; Abou-Zeid *et al.*, 1978).

The fermentation process currently used by National Chemical Products (NCP), described by Spivey (1978),has been adapted to laboratory conditions for monitoring the production of solvents in attempts to increase the normal yield. Studies on the fermentation process using *C. acetobutylicum* in molasses medium revealed the production of a bacteriocin-like substance, which affected the producer strain, and resulted in an inhibition of further solvent production.

This chapter describes the production of solvents and a bacteriocin-like

substance by *C. acetobutylicum* in a laboratory scale molasses fermentation.

2.2 METHODS

2.2.1 Media

As given in Appendix B listed under Factory fermentation media.

2.2.2 Bacterial Strains

The *C. acetobutylicum* strains were supplied by National Chemical Products Ltd., Germiston, South Africa.

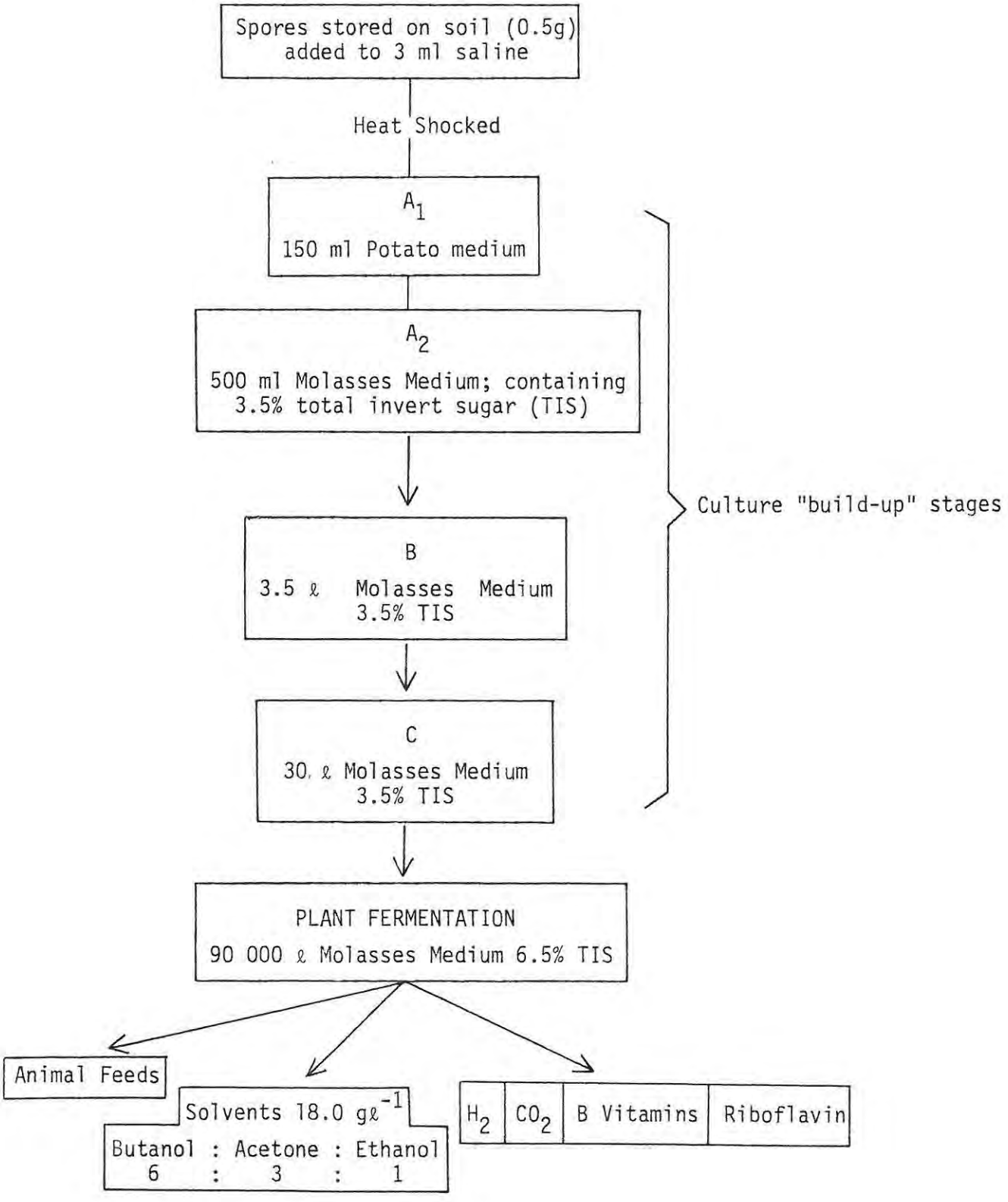
2.2.3 Fermentation Experiments

Factory fermentation conditions, described in detail by Spivey (1978) and summarized in Fig. 2.2 and Table 2.1(a), were adapted to a scale suitable for laboratory experimentation. The factory process involved several stages to build up the inoculum volume and to condition the organism to molasses medium. The initial potato medium was inoculated with heat-shocked spores of *C. acetobutylicum*. Various volumes of medium and inoculum were tested for each stage of the fermentation process, until it was possible to obtain a normal solvent yield in molasses medium under laboratory conditions.

2.2.4 Analysis of Solvents

Aqueous samples of the molasses fermentation medium were injected into a Hewlett-Packard 5830 A Chromatograph equipped with a flame ionization

Fig 2.2 Factory fermentation system for production of solvents and other end products



detector, and the quantitative evalution of the peaks was performed automatically by an intergrator, using n-propanol as an internal standard. The stainless steel column (1.84m x 2mm) was packed with Chromosorb W/AW (80-100mesh) coated with 15% carbowax 20 M. Analysis of solvents was achieved under the following conditions:

Column temperature,	$90^{o}C$ (isothermal)
injector temperature,	$250^{o}C$
detector temperature,	$250^{o}C$
N_2 (carrier gas) flow rate,	130 ml min^{-1}
H_2 flow rate,	40 ml min^{-1}
Air flow rate,	500 ml min^{-1}

The retention times for acetone, ethanol, n-propanol and butanol were 0.98, 1.40, 2.19 and 3.89 min respectively.

2.2.5 Production of the Bacteriocin-like substance (BLS)

At different time intervals, samples from a 48 h fermentation in molasses medium were removed and centrifuged at 10 000 rev min^{-1} for 10 min at $4^{o}C$, and the supernatants were assayed for BLS.

2.2.6 Assay for the BLS

The BLS was assayed by the well method (Mayr-Harting *et al.*, 1972) in 10 ml potato medium plates containing 1% (wt/vol) agar and seeded with *C. acetobutylicum* P262 cells. A series of two-fold dilutions of the test culture supernatants were made in 0.01 M sodium acetate buffer, pH 4.5

and 25µl of each dilution was added to separate wells. The indicator strain suspension in 0.85% (wt/vol) saline was prepared from an overnight plate of *C. acetobutylicum* P262. The plates were incubated at 37°C in Gas Pak (Becton, Dickinson & Co.) jars or perfused with CO_2 and H_2 in large perspex anaerobic boxes (Plate 2.1). The activity of the BLS in arbitrary units (AU) was defined as the reciprocal of the highest dilution which gave a detectable zone of inhibition (Plate 2.1).

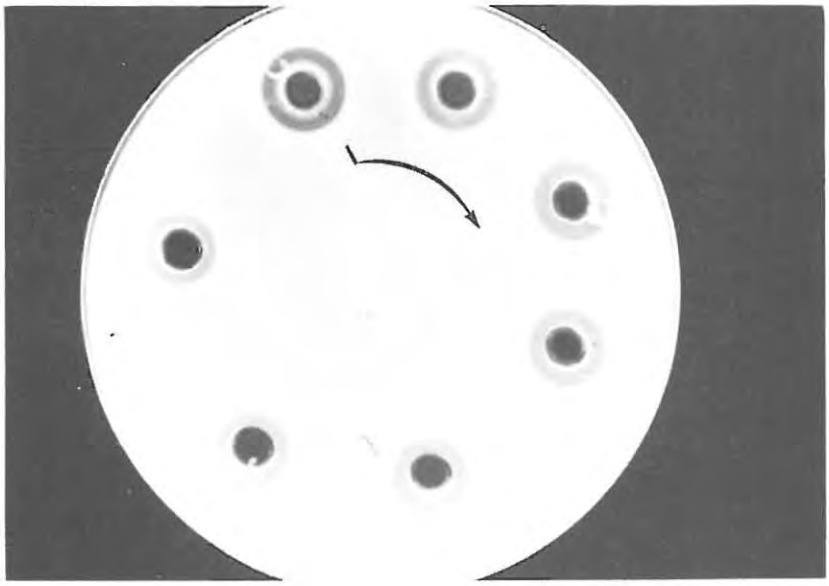
2.2.7 Induction of the BLS

Mitomycin C and ultraviolet irradiation were used to test for induction of the BLS. Exponential phase cells were added to potato media containing 0.1, 0.5, 0.75, 1.0, and 1.5 µg ml^{-1} of mitomycin C and incubated for 2 h. The cells were then transferred to the molasses or potato media (18 ml), and the supernatants were assayed after 48 h. Induction by ultraviolet irradiation was carried out on exponential phase cells in potato medium which were diluted 10^{-2} in 0.85% (wt/vol) saline. Samples (7 ml) were irradiated in glass petri dishes with a Hanovia UV lamp (2537 Å) at a distance of 25 cm and a dose rate of 1.15 Jm^{-2} sec^{-1} for 30, 60, 90 and 120 sec, before adding to the molasses and potato media (15 ml) and incubating for 48 h.

To determine if a component in molasses was responsible for inducing high titres of the BLS, molasses was clarified by centrifugation at 10 000 rev min^{-1} for 20 min, or dialysed for 3 d against distilled H_2O, then reconstituted with 2% (wt/vol) glucose and 4.5% (wt/vol)sucrose before addition to molasses medium.

Plate 2.1

Perspex anaerobic box



Zones of inhibition produced by *C. acetobutylicum* BLS.
The arrow indicates the direction of doubling dilutions.

Table 2.1(a) Different stages in the factory fermentation process for solvent production by *C. acetobutylicum* (Spivey, 1978)

Culture Stage	Volume	Duration	Transfer Volume	Medium
A_1	150 ml	12 h	150 ml (to A_2)	Potato
A_2	500 ml	6 h	650 ml (to B)	Molasses (3.5% TIS)
B	3.5 ℓ	6 h	1.38 ℓ (to 3 x C)	"
C	3 x 9 ℓ	9 h	*c.* 30 ℓ (to plant fermenter)	"
Plant Fermenter	90 000 ℓ	36 h	-	Molasses (6.5% TIS)

(TIS) = total invert sugar

Table 2.1(b) The modified laboratory fermentation process for solvent production by *C. acetobutylicum*

Culture Stage	Volume	Duration	Transfer Volume	Medium
A_1	15 ml	12-18 h	15 ml (to A_2)	Potato
A_2	25 ml	6 h	40 ml (to B)	Molasses (3.5% TIS)
B	350 ml	6 h	40 ml (to C)	"
C	300 ml	10 h	0.4 ml (to Fermenter medium)	"
Fermenter Medium (CFM)	200 ml	36-48 h	-	Molasses 6.5% TIS)

(TIS) = total invert sugar

2.2.8 Protease Determinations

Protease activity, at different time intervals throughout the 48 h fermentation in molasses medium was estimated using the synthetic nonspecific protease substrate Azocoll (Rawlings and Woods, 1978) and the haemoglobin method of Anson (1938). Positive controls using the protease trypsin were included with each assay.

The Azocoll assay was performed by weighing 15 mg of Azocoll into the assay tube, adding 2 ml of the fermentation supernatant and 3 ml of buffer (Tris-HCl, pH 6.5). After 18 h of gentle shaking at 30°C, the reaction mixture was centrifuged and the optical density of the supernatant was read at 560nm, against a blank containing 2 ml of sterile molasses medium.

The haemoglobin assay was carried out using 2% (wt/vol) haemoglobin in sodium acetate buffer, pH 5.5. The fermentation supernatant (0.1 ml) was added to 3.9 ml of the haemoglobin solution and incubated at 30°C for 18 h. The reaction was stopped by the addition of 4 ml 10% (wt/vol) trichloroacetic acid. The reaction mixture was filtered and the filtrate tested for absorbance at 280 nm.

2.2.9 Localisation studies

Cell-bound BLS was determined by washing the bacterial cells in 1M NaCl and assaying the supernatant. Intracellular BLS was determined in the supernatants of sonically disrupted cell samples, that had been clarified by centrifugation at 10 000 rev min^{-1} .

2.3 RESULTS

2.3.1 Fermentation Experiments

Solvent yields (16-18 $g\ell^{-1}$) normally obtained in the NCP factory process were achieved using the procedure described in Table 2.1(b). To obtain the required solvent concentration, it was necessary to increase the final inoculum ratio six-fold to $^{1}/500$ (0.4 ml to 200 ml), in comparison to $^{1}/3000$ (30ℓ to 90 000ℓ) used by the factory. When the removal of a large number of samples was required, the volume of the fermentation medium was increased from 200 ml to 400 ml with a corresponding increase in inoculum volume from 0.4 ml to 0.8 ml.

2.3.2 Production of the BLS

The production of the BLS by *C. acetobutylicum* P262 in molasses fermentation medium is shown in Fig 2.3. Low levels of BLS were detected after 24 h. The concentration of the BLS increased between 27 and 40 h, and high titres (4096 AU) were regularly obtained. The increase in titre of the BLS coincided with the end of the exponential growth phase and the decrease in turbidity of the bacterial culture. Mass lysis of the bacterial culture was not observed when titres of the BLS were low. The total production of solvents (acetone, butanol and ethanol) followed the bacterial growth curve, reaching a maximum of 16.9 $g\ell^{-1}$. Once cell lysis commenced, there was no further increase in the solvent concentration which plateaued at 34 h. The lysis of the cells was not caused by the solvents, since concentrations of 18.0$g\ell^{-1}$ did not affect the growth of *C. acetobutylicum* P262 cells or produce zones of inhibition when tested

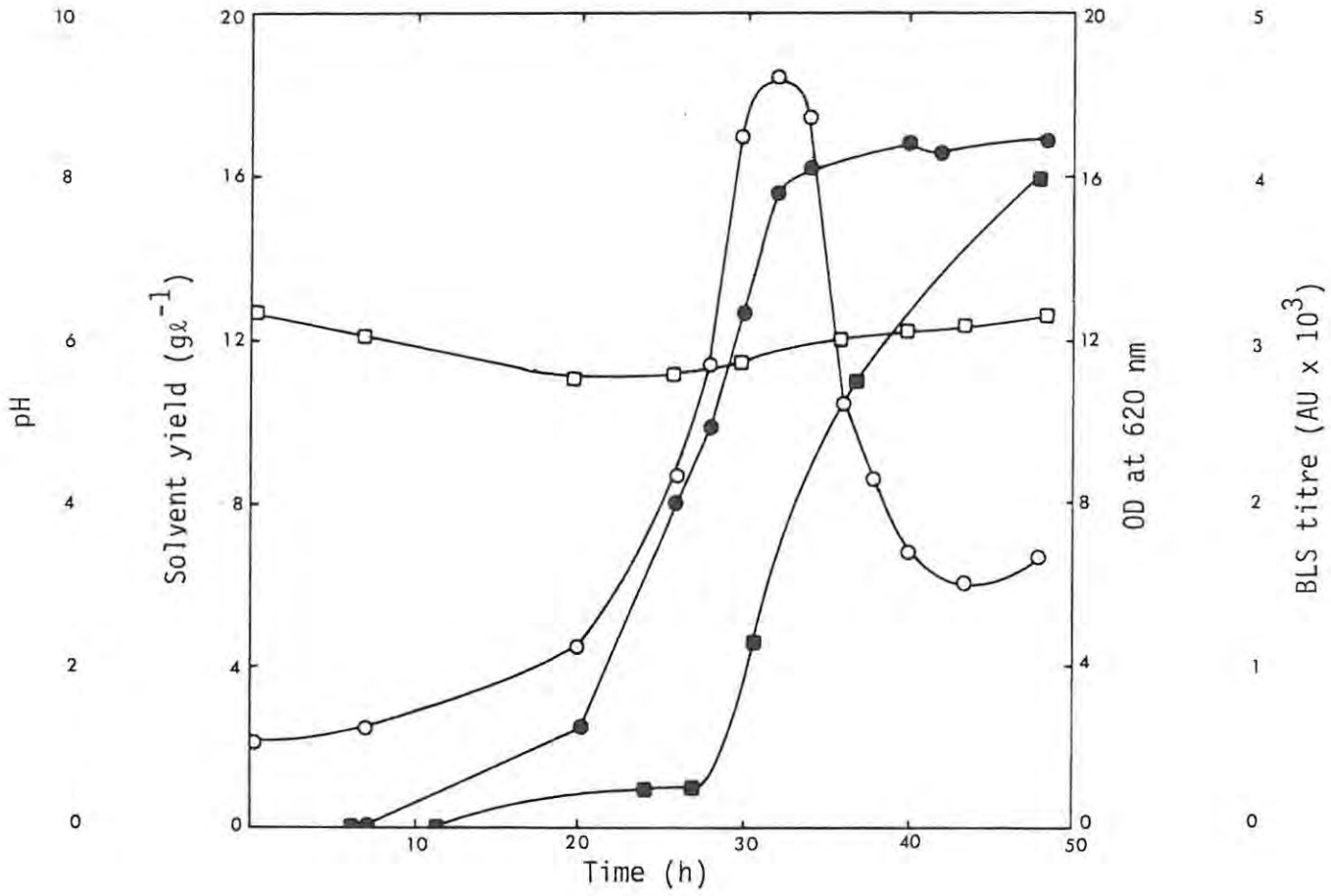


Fig 2.3 Detailed studies of pH changes (□), bacterial growth measured by turbidity (○), total solvent (●) and BLS (■) production during a *C. acetobutylicum* P262 molasses fermentation.

by the well assay. It should be noted that production of the BLS increased significantly shortly after the pH break point. This is the point at which solvent production commenced and the pH of the culture increased.

2.3.3 Induction of the BLS

Neither mitomycin C nor exposure to ultraviolet light induced the production of BLS (Table 2.2(a) and 2.2(b)). Exposure to ultraviolet light for 60 sec or more decreased the titre of BLS, probably due to inhibition of cell growth. High titres of BLS were obtained even when the solids in molasses were removed. However, if molasses was dialysed, and the carbohydrate content replaced by glucose and sucrose, extremely low levels of BLS were obtained (2 AU as compared to 1024 AU for clarified or normal molasses).

2.3.4 Protease Activity

No extracellular protease activity was detected at any stage during a molasses fermentation or in culture supernatants with high titres of BLS activity (2048 AU).

2.3.5 Localisation studies

Localisation studies carried out at 48 h in molasses fermentation medium indicated that the majority of the BLS was extracellular (2048 AU), compared to 512 AU intracellular and 32 AU cell bound.

Table 2.2(a) Induction of the BLS produced by *C. acetobutylicum* using mitomycin C

Mitomycin C (mg ml^{-1})	0	0.1	0.5	0.75	1.0	1.5
Activity (AU)*	128	256	256	256	128	128

Table 2.2(b) Induction of the BLS produced by *C. acetobutylicum* using ultraviolet light

Irradiation time (sec)	0	30	60	90	160
Activity (AU)*	512	512	128	128	64

* determined using the well plate assay described in this chapter.

2.4 DISCUSSION

It was possible to study the BLS produced by *C. acetobutylicum* in molasses medium using a "scaled down" factory fermentation system. The production of the BLS was concurrent with the lysis of the bacterial culture and the plateauing of solvent production at the end of the exponential growth phase. All strains of *C. acetobutylicum* tested, produced the BLS, but the highest titres were obtained using strain P262. The production of the BLS required a dialysable component of molasses, but was not induced by the inducing agents ultraviolet light and mitomycin C. Titres of bacteriocins and/or bacteriophages are normally inducible (Reeves, 1972). However, induction has also been shown to occur in the case of a specific lysin produced by a *C. perfringens* species (Nakamura *et al.*, 1977), whilst a bacteriocin produced by *Clostridium butyricum* was not inducible (Clarke *et al.*, 1975).

Barber *et al.*, (1979) described the production of the BLS by *C. acetobutylicum* P262 and noted that no lysis occurred when the BLS was added to actively growing cells of *C. acetobutylicum* P262. As cell lysis could not be demonstrated, the action of the BLS was described as "bactericidal", which is normally used to describe bacteriocin activity, rather than "bacteriolytic" which is used for autolysin activity. The lysis of the culture in a molasses fermentation was therefore assumed to be due to the production and release of the BLS as has been shown for other *Clostridium* species (Bonventre and Kempe, 1960; Ogata and Hongo, 1974; Clarke *et al.*, 1975).

However, the producer strain, when used as the indicator strain in the well plate assay, was sensitive to its own BLS. This sensitivity was thought

to be due to a breakdown in the immunity that a bacteriocinogenic organism usually has to its own bacteriocin. Immunity breakdown has been reported for several bacteriocins (Reeves, 1972; Tagg *et al.*, 1976) and is more prevalent among Gram-positive organisms than Gram-negative organisms (Hamon and Peron, 1963).

Lysis of the *C. acetobutylicum* P262 culture in molasses medium was not associated with protease production. This is in contrast to the finding of Manfeĭtelj (1941), in which autolysis of *C. acetobutylicum* occurred simultaneously to the production of a protease.

The release of an extracellular BLS by *C. acetobutylicum* during the critical stages of solvent production in a molasses fermentation could be inhibitory to the further production of acetone, butanol and ethanol. Barber *et al.* (1979) attempted to characterise the BLS, since prevention of the production of the BLS could result in a normal growth curve with the possibility of increased solvent yields. Characterisation in molasses medium indicated that the BLS was thermolabile ($t_{\frac{1}{2}}$ = 2 min at 60°C), had an optimum pH for stability between pH 4 and 5, and was proteinaceous. However, further characterisation was difficult in a molasses medium and it was necessary to establish alternative methods of BLS production for its complete identification and characterisation.

CHAPTER III

MODE OF ACTION OF THE BACTERIOCIN-LIKE SUBSTANCE PRODUCED BY *C. ACETOBUTYLICUM*

The bacteriocin-like substance produced by *C. acetobutylicum* had no effect on DNA, RNA or protein synthesis and no loss of intracellular ATP was detected. Both concentrated and partially purified BLS showed a broad spectrum of activity and the ability to lyse sodium dodecyl sulphate-treated cell walls. The production of BLS was associated with lysing or autoplasting cells and it was concluded that the BLS was an autolysin.

3.1 INTRODUCTION

Tagg *et al.*, (1976) wrote that

> "bacteria produce a wide variety of different bacteriolytic enzymes and low-molecular weight inhibitors, some of which have bacteriocin-like properties and may prove difficult to categorise".

The confusion in defining some inhibitory bacterial products has been discussed in Chapter I, and the division into any one category may be extremely difficult, as many of the properties of an inhibitory substance may overlap from one category to another. The BLS produced in molasses medium by *C. acetobutylicum* P262 was initially described as a bacteriocin

(Barber *et al.*, 1979), as the preliminary investigations on this substance indicated properties which were consistent with the classical definition of a bacteriocin. This definition is based on the characteristics of colicins (those bacteriocins produced by strains of *E. coli*) and may be summarised by the following criteria (Tagg *et al.*, 1976):

1. A narrow inhibitory spectrum of activity centred about homologous species.

2. The presence of an essential, biologically active protein moiety.

3. A bactericidal mode of action.

4. Attachment to specific cell receptors.

5. Plasmid-borne genetic determinants for bacteriocin production and host cell bacteriocin immunity.

6. Production by lethal biosynthesis (i.e commitment of a bacteriocinogenic strain to produce a bacteriocin will ultimately lead to cell death).

In our initial studies the first three criteria appeared to be satisfied, and only a detailed confirmation of these properties remained to be done. This involved determining the exact mode of action of the BLS, as extensive investigations on bacteriocins have defined their general biochemical effects. These fall into the following groups (Reeves, 1972):

a) Bacteriocins which affect energy flux. Normally macromolecular synthesis is also inhibited as a result of a rapid decrease in intracellular ATP levels. (eg colicins E1, K, A and I)

b) Bacteriocins which affect DNA metabolism. The effect is almost immediate and pre-existing DNA may also be degraded. (eg colicin E2, megacins C-C4 and KP-33, and pesticin P1)

c) Bacteriocins which affect protein synthesis (eg colicin E3, cloacin DF13 and pneumocins S6 and S8)

d) Bacteriocins which affect protein and RNA synthesis, eg pneumocin G196 and a *Bacteriodes fragilis* bacteriocin, isolated in our laboratory, which has an unusual mode of action as it specifically inhibits RNA synthesis, whilst protein synthesis is inhibited only as a result of RNA inhibition (Mossie *et al.*, 1979; Mossie *et al.*, 1980)

In order to study the mode of action of the BLS, it was necessary to find an alternative to molasses medium, as this contained insoluble material making it unsuitable for optical density estimations and for filter-disc experiments. Clostridium basal medium (CBM), described by O'Brien and Morris (1971), was routinely used in this study and was found to yield high

titres of the BLS after the addition of 5% (vol/vol) molasses medium.

3.2 METHODS

3.2.1 Media and materials as described in Appendix B.

3.2.2 Bacterial strains

The BLS producer and indicator strain was *C. acetobutylicum* P262. The bacterial strains used in the activity spectrum studies were stock cultures from the Microbiology Department, Rhodes University, Grahamstown, or strains supplied by the Microbiology Department, Medical school, University of Natal, Durban.

3.2.3 Production of the BLS

The BLS was prepared by the addition of exponential phase cells (4 ml) to CBM (400 ml) containing 5% (vol/vol) molasses medium. After growth for 48 h at 37^{o}C, the supernatant was collected by centrifugation, then concentrated by 60% acetone (vol/vol) precipitation. The acetone precipitate was pelleted, freeze dried and stored at 4^{o}C. The crude BLS was reconstituted in 0.01M sodium acetate buffer (pH 4.5) before use, and partially purified BLS was prepared as described in Chapter V.

3.2.4 Assay of the BLS

The BLS was assayed by the well plate technique described in Chapter II. CBM containing 1% (wt/vol) agar was used instead of potato medium for the

preparation of the well plates.

3.2.5 Determination of protein and nucleic acid synthesis

Exponential phase cells of *C. acetobutylicum* P262 in CBM or molasses medium (containing 3.5% TIS) were assayed for protein synthesis by the incorporation of ^{14}C-leucine (0.25 μCi ml^{-1}) into trichloracetic acid (TCA) precipitable material. Samples (0.2 ml) were removed at 10 min intervals, added to 0.2 ml cold 10% (wt/vol) TCA containing 1 mg ml^{-1} unlabelled leucine, and kept on ice for 30 min. The samples were then filtered on Whatman glass fibre filters and the precipitates washed twice with 10 ml cold 5% (wt/vol) TCA, and once with 10 ml 1% (vol/vol) acetic acid. The filters were dried, added to 10 ml scintillation cocktail and counted using a Beckman model LS 3150T liquid scintillation counter.

RNA and DNA synthesis was determined after incorporation of ^{3}H-adenine (2 μCi ml^{-1}) into TCA precipitable material. The procedure used for total nucleic acid synthesis was the same as that used for protein synthesis, except that the samples were added to 0.2 ml cold 10% (wt/vol) TCA containing 1 mg ml^{-1} unlabelled adenine. To determine DNA synthesis, 0.2 ml samples were added to 0.2 ml 1N NaOH, and incubated at 37^{o}C overnight to hydrolyse both RNA and protein. The samples were then neutralised with 13 μl of 1% (vol/vol) acetic acid, added to 0.2 ml 10% (wt/vol) TCA containing 1 mg ml^{-1} unlabelled adenine, and kept on ice for 30 min before filtering. RNA synthesis was estimated by subtracting the DNA counts from the total nucleic acid counts.

The effect of BLS on macromolecular synthesis was routinely determined by the addition of 1638 AU (final concentration) 11 min after the

radioactively-labelled precursor was added. Heat-inactivated BLS was used as a control.

3.2.6 Estimation of intracellular levels of ATP

Intracellular levels of adenosine triphosphate (ATP) were determined before and 30 min after the addition of BLS (final concentration 1638 AU), or heat-inactivated BLS, to exponential phase cells in CBM. ATP was extracted from 0.5 ml culture samples by boiling with 4.5 ml of distilled H_2O as described by Fields and Luria (1969). ATP was measured by the Luciferin-luciferase assay (Foulds, 1971), counting the flashes of light emitted from mixtures of cell extracts and firefly lantern extracts using a Beckman scintillation counter. The assays were performed by mixing 1 ml of the sample in a scintillation vial with 2.5 ml of buffer (0.05 M potassium arsenate, 0.02 M $MgSO_4$ and 0.001 M EDTA, pH 7.4). Firefly lantern extract (0.5 ml), reconstituted in 0.05 M potassium arsenate (pH 7.4) and 0.02 M $MgSO_4$ was added, the contents of the vial mixed and counted immediately. A linear curve was obtained between 0.0125 nM and 0.1 nM ATP with 0.05 nM of ATP producing 100 000 counts per min (CPM). Extreme care was taken to maintain the anaerobic conditions of the cultures, as aeration of *C. acetobutylicum* is known to result in a marked decrease in intracellular ATP (O'Brien and Morris, 1971).

3.2.7 Effect of BLS on exponential phase *C. acetobutylicum* cells

The optical density (OD) at 600 nm, of exponential phase cells in CBM, was monitored after the addition of crude, partially purified and

heat-inactivated BLS. The cultures were incubated at 37°C for 90 min.

3.2.8 Autolysis of exponential phase *C. acetobutylicum* cells

The ability of a bacterial culture to autolyse has often been determined by resuspending the cells in autolysing buffers such as phosphate buffer (Kawata *et al.*, 1968; Higgins *et al.*, 1970; Ogata and Hongo, 1974). Exponential phase cells of *C. acetobutylicum* P262, harvested by centrifugation, were resuspended in 0.02 M phosphate buffer (pH 6.4), and the decrease in OD was monitored as described in 3.2.7. After incubation at 37°C for 60 min, the supernatant was collected by centrifugation at 12 500 rev min^{-1}, and assayed for activity by the well plate technique.

3.2.9 Effect of BLS on sodium dodecyl sulphate-treated cell walls

Preparation of cell walls

Cell walls were prepared from exponential phase *C. acetobutylicum* P262 cells using the method described by Kawata *et al.* (1968). The cells were collected from 9 h CBM cultures (400 ml), washed at least three times in cold 0.05 M phosphate buffer (pH 7.0), and then resuspended in the same buffer. The washed cells were sonically disrupted using a MSE sonicator at *c* 6Kc for 10 min. To remove the unbroken cells, the suspension was centrifuged at 4 000 rev min^{-1}. The resulting supernatant, containing cell walls, was pelleted by centrifugation at 12 500 rev min^{-1} for 30 min. The crude cell wall pellet was then washed twice in 1 M NaCl, twice in distilled H_2O, and resuspended in 10 ml 0.05 M phosphate buffer. This was followed by treatment with 10 ml 2% (wt/vol) sodium dodecyl sulphate (SDS) for 30 min at 20°C. To remove the SDS, the walls were washed

three times in 0.05 M phosphate buffer (pH 6.0), resuspended in the same buffer and dialysed overnight at 20°C. Crude, partially purified and heat-inactivated BLS were added to SDS-treated cell walls at 37°C and the OD at 660 nm was monitored.

3.2.10 Induction of BLS with molasses medium or sucrose

Exponential phase cells (0.1 ml) were added to two tubes containing 10 ml of CBM. After 6 h incubation at 37°C, molasses medium was added to one of the tubes to give a final concentration of 5% (vol/vol). The remaining tube was used as a control. The cultures were assayed for BLS activity and examined microscopically at 6 h intervals.

Tubes of CBM (10 ml) containing sucrose at final concentrations of 4, 6, 8, 10 and 15% (wt/vol) were inoculated with exponential phase cells and incubated at 37°C for 24 h. Samples were removed from each tube, assayed for BLS, and examined microscopically.

CBM (400 ml) containing 15% (wt/vol) sucrose was inoculated with 4 ml of exponential phase cells and incubated at 37°C for 48 h. At various time intervals the culture was monitored for BLS activity, OD, pH and also examined microscopically.

3.2.11 Activity spectrum of BLS

Clostridium and *Bacillus* species were tested by the well plate assay for sensitivity to the BLS. Both crude and partially purified preparations were tested,and heat-inactivated BLS was used as the control. The

Clostridium species were grown in brain heart infusion medium (BHI) containing 1% (wt/vol) agar; and the *Bacillus* species in potato medium containing 1% (wt/vol) agar. Incubation was at 37°C under anaerobic conditions.

3.3 RESULTS

3.3.1 Effect of BLS on protein and nucleic acid synthesis

Addition of the BLS to exponential phase cells of *C. acetobutylicum* P262 did not significantly inhibit the synthesis of DNA, RNA or protein, but there was a slight transient effect (Fig 3.1, 3.2 and 3.3). Similar results were obtained if the BLS was added to cells in molasses medium or CBM.

3.3.2 Effect of BLS on intracellular ATP

The BLS had no effect on intracellular levels of ATP. Thirty minutes after the addition of BLS to the culture, 90% of the initial ATP level was still detectable.

3.3.3 Effect of BLS on exponential phase cells

No lysis was observed when crude or partially purified BLS were added to exponential phase *C. acetobutylicum* P262 cells, but the growth rate was inhibited (Fig 3.4). Heat-activated BLS did not affect the growth rate of exponential phase cells. Microscopic examination of the BLS-treated culture showed healthy motile cells, and no protoplasts were visible.

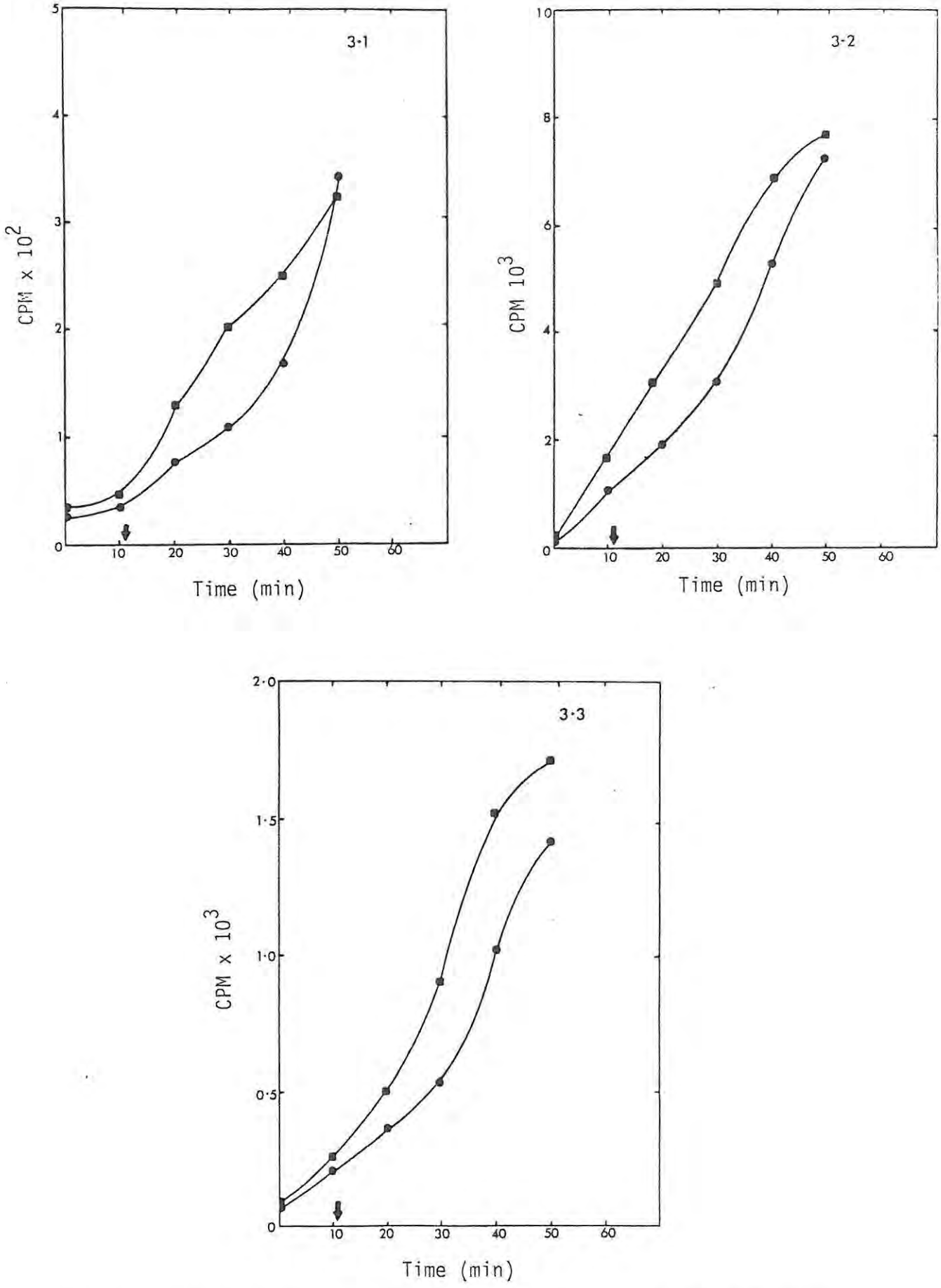


Fig 3. Effect of the *C. acetobutylicum* BLS on DNA (3.1), RNA (3.2) and protein (3.3) synthesis of *C. acetobutylicum* (●), compared to control cells to which heat-inactivated BLS was added (■).

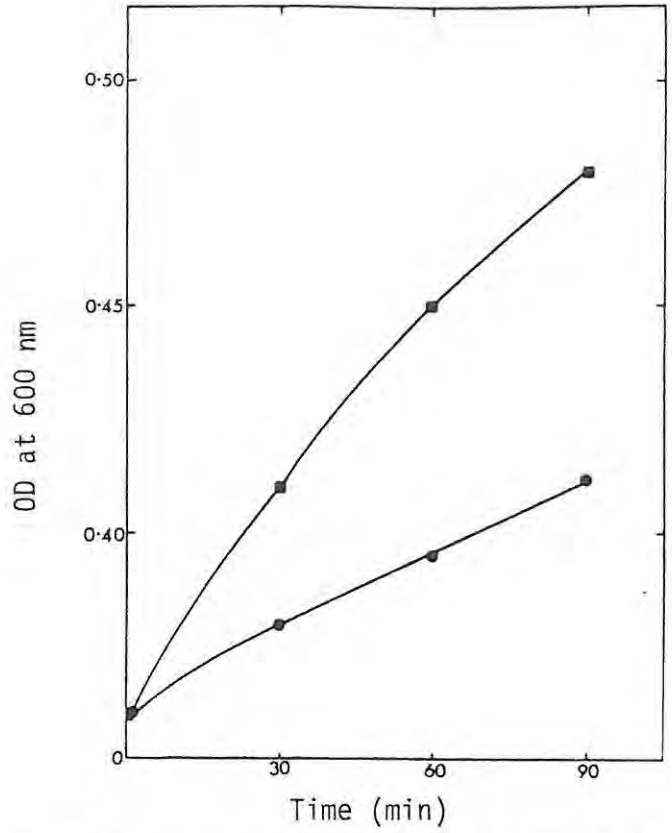


Fig 3.4 Effect of partially purified BLS on the growth rate of exponential phase *C. acetobutylicum* cells. Heat-inactivated BLS (■) and BLS (●) were added to the cells at 0 min.

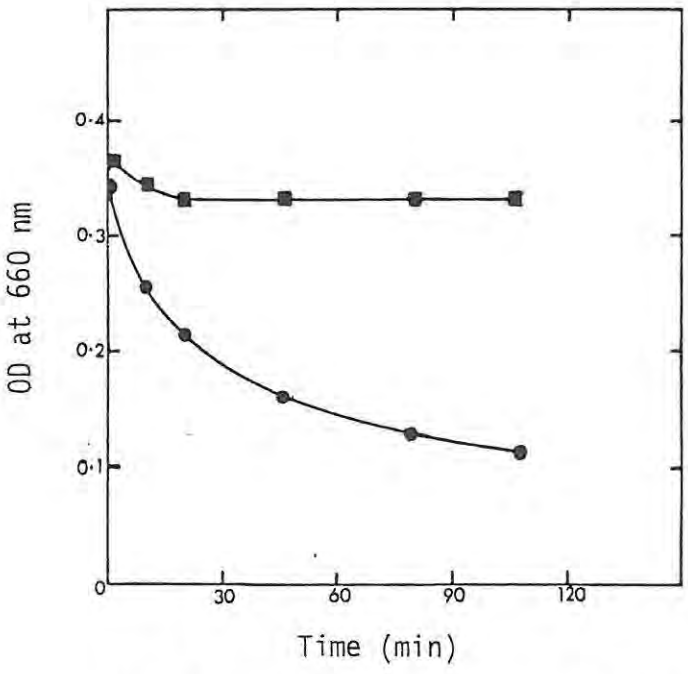


Fig 3.5 Lysis of SDS-treated walls of *C. acetobutylicum* by the partially purified BLS. Heat-inactivated BLS (■) and BLS (●) were added at 0 min.

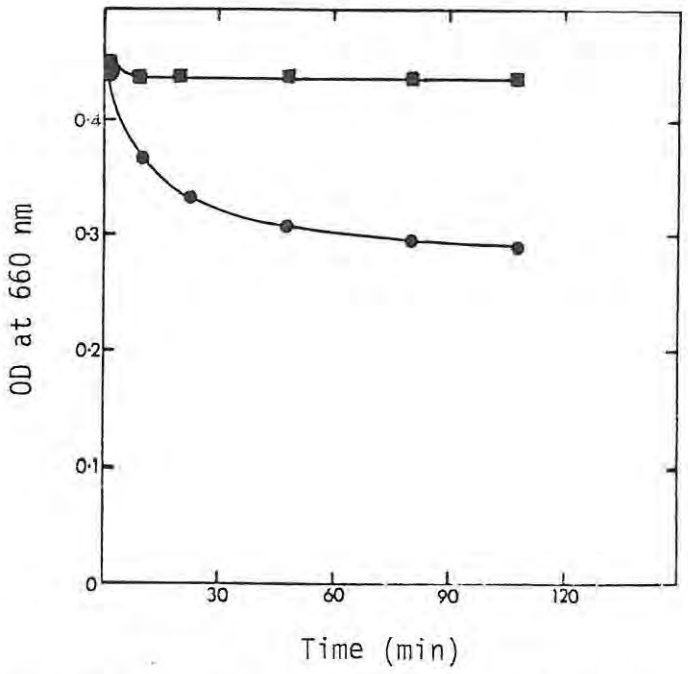


Fig 3.6 Lysis of SDS-treated cell walls of *C. acetobutylicum* by the crude BLS. Heat-inactivated BLS (■) and BLS (●) were added at 0 min.

3.3.4 Effect of BLS on SDS-treated cell walls

The crude and partially purified BLS lysed SDS-treated cell walls of *C. acetobutylicum* P262. The partially purified BLS was more active than the crude preparation (Figs 3.5 and 3.6).

3.3.5 Autolysis of exponential phase *C. acetobutylicum* P262 cells

A marked decrease in optical density was detected when exponential phase cells were resuspended in phosphate buffer (Fig 3.7).

The supernatant (lysate) obtained after 60 min in phosphate buffer was assayed by the well plate technique and had a titre of 512-1024 AU. The lysate was also able to lyse SDS-treated cell walls.

3.3.6 Induction of BLS by molasses medium or sucrose

High titres of BLS were obtained when CBM contained 5% (vol/vol) molasses medium (Fig 3.8). Microscopic examination at 6 h revealed healthy motile cells (Plate 3.1a), but 12 h after the addition of molasses medium, when the BLS titre had reached its peak, mass lysis of the culture was apparent (Plate 3.1b). The BLS titre in CBM with no molasses medium added remained low (8 AU) , and healthy sporulating cells were observed after 18 h (Plate 3.1c).

The addition of sucrose to CBM also increased the titre of BLS, and there was a concomitant increase of both BLS and autoplast formation as the concentration of the sucrose was increased (Table 3.1). There were no

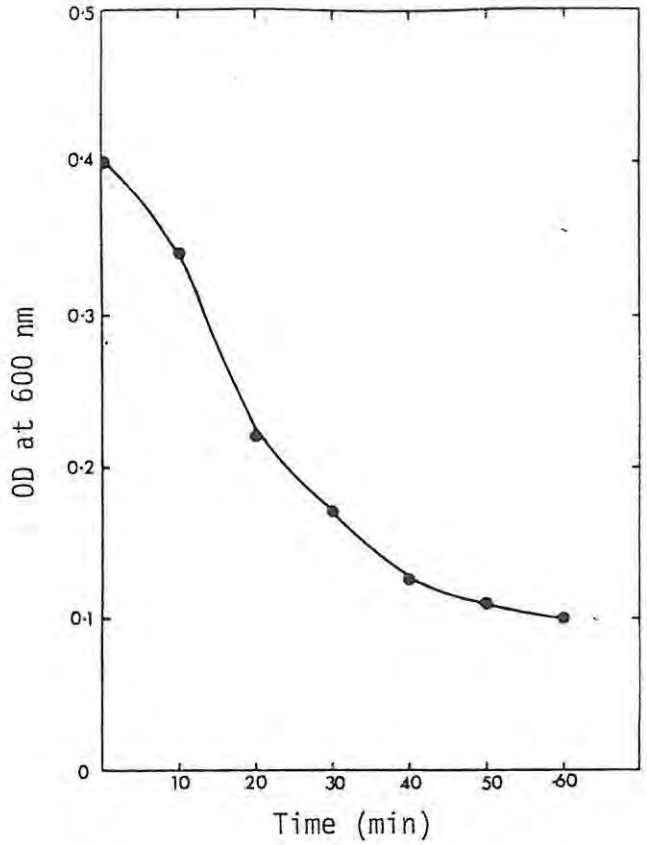


Fig 3.7 Autolysis of exponential phase *C. acetobutylicum* cells resuspended in phosphate buffer.

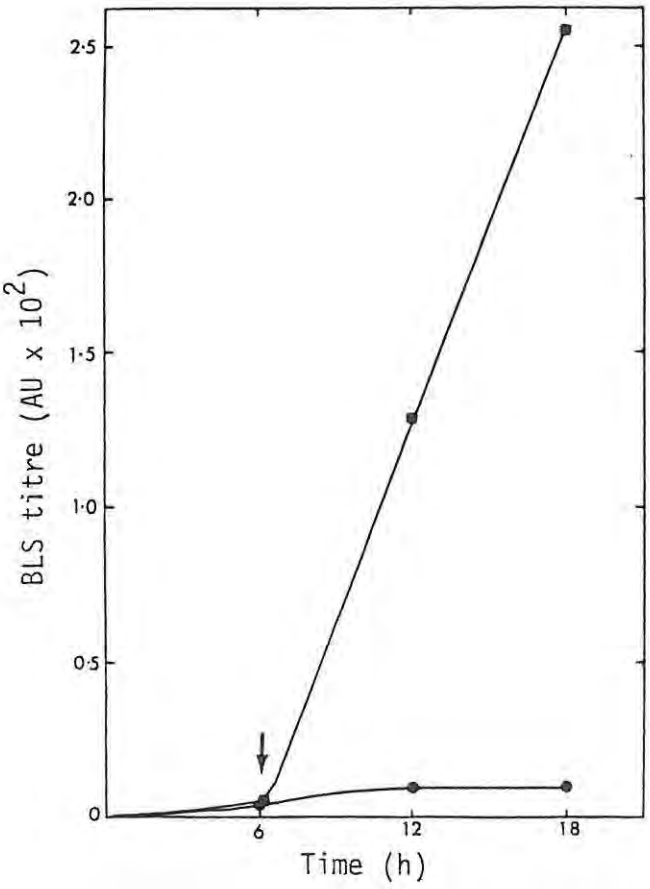


Fig 3.8 Induction of *C. acetobutylicum* BLS production by the addition of 5% (wt/vol) molasses medium to *C. acetobutylicum* cells in CBM. The arrow indicates the time of addition of the molasses medium to the test culture (●), the control culture (■) was kept molasses-free.

Plate 3.1

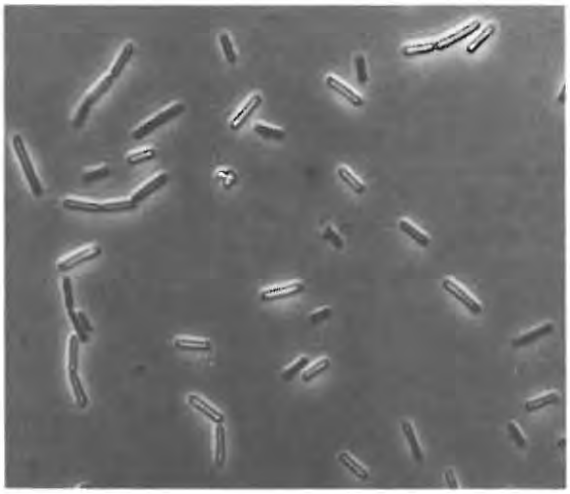


Plate 3.1a Healthy exponential-phase *C. acetobutylicum* cells in CBM. Photographed at a magnification of 400x

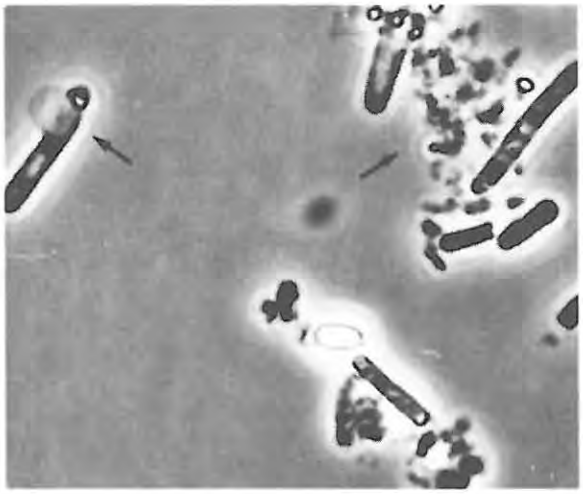


Plate 3.1b Lysing *C. acetobutylicum* cells in CBM containing 5% (vol/vol) molasses medium. The arrows indicate a cell undergoing lysis and cell debris. Photographed at a magnification of 2000x

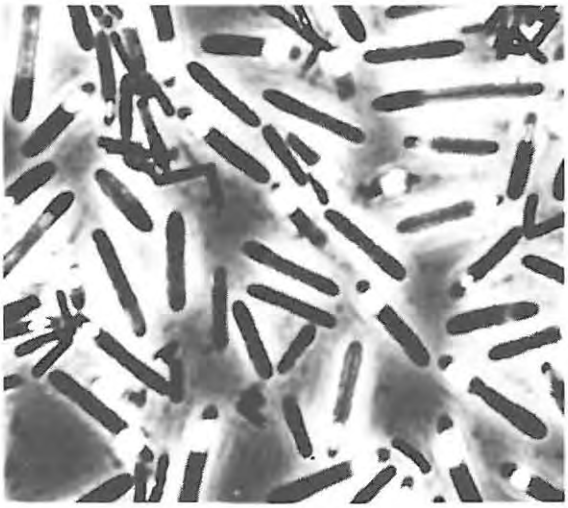


Plate 3.1c Healthy sporulating *C. acetobutylicum* cells in CBM. Photographed at a magnification of 1000x

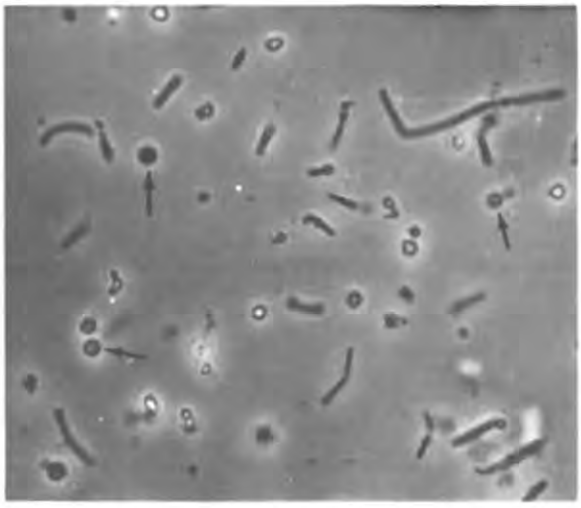


Plate 3.1d *C. acetobutylicum* autoplasts (arrows) in CBM containing 15% (wt/vol) sucrose. Photographed at a magnification of 400x

Table 3.1 Autoplast formation and BLS titres with increasing concentrations of sucrose in CBM

Sucrose concentration (% wt/vol)	BLS (AU)*	Autoplast formation (%)+
4	8	0
6	32	<1
8	64	5
10	128	20
15	256	50

* measured using the well plate method described in Chapter II

+ expressed as a percentage of the total cells counted in a representative microscopic field.

autoplasts present in medium containing 4% (wt/vol) sucrose, and the BLS titre was only 8 AU. When the medium contained 15% (wt/vol) sucrose, the BLS titre increased to 256 AU and the culture contained 50% autoplasts.

In CBM containing 15% (wt/vol) sucrose, the BLS titre and the autoplast population both reached a maximum after 24 h (Fig 3.9). The pH of the medium decreased significantly over the first 12 h, but remained between pH 4.6 to 5.0 thereafter. Although cell lysis was observed at 24 h, the OD increased over the 48 h fermentation period, possibly because many of the cells were able to autoplast and remain intact in the isotonic sucrose-rich medium. Plate 3.1d shows the characteristic autoplast formation in a 15% (wt/vol) sucrose culture containing a high titre of BLS.

3.3.7 Activity spectrum of BLS

Crude BLS, concentrated by acetone precipitation to an activity of 8192 AU, produced zones of inhibition in the well plate test against 4 other *Clostridium* strains and 7 *Bacillus* species (Table 3.2). However, a *C. perfringens* strain and a *Clostridium sporogenes* strain were resistant to the BLS. Using partially purified BLS (256 AU), all but one of the *Clostridium* strains were resistant, whereas all the *Bacillus* strains were sensitive. A strain of *Clostridium oedematiens* was sensitive to partially purified, but not crude, BLS. Heat-inactivated BLS used as a control had no effect on any of the strains tested.

In addition, *S. aureus, Samonella typhi, Proteus vulgaris, Serratia marcescens* and *Pseudomonas fluorescens* were resistant to the BLS.

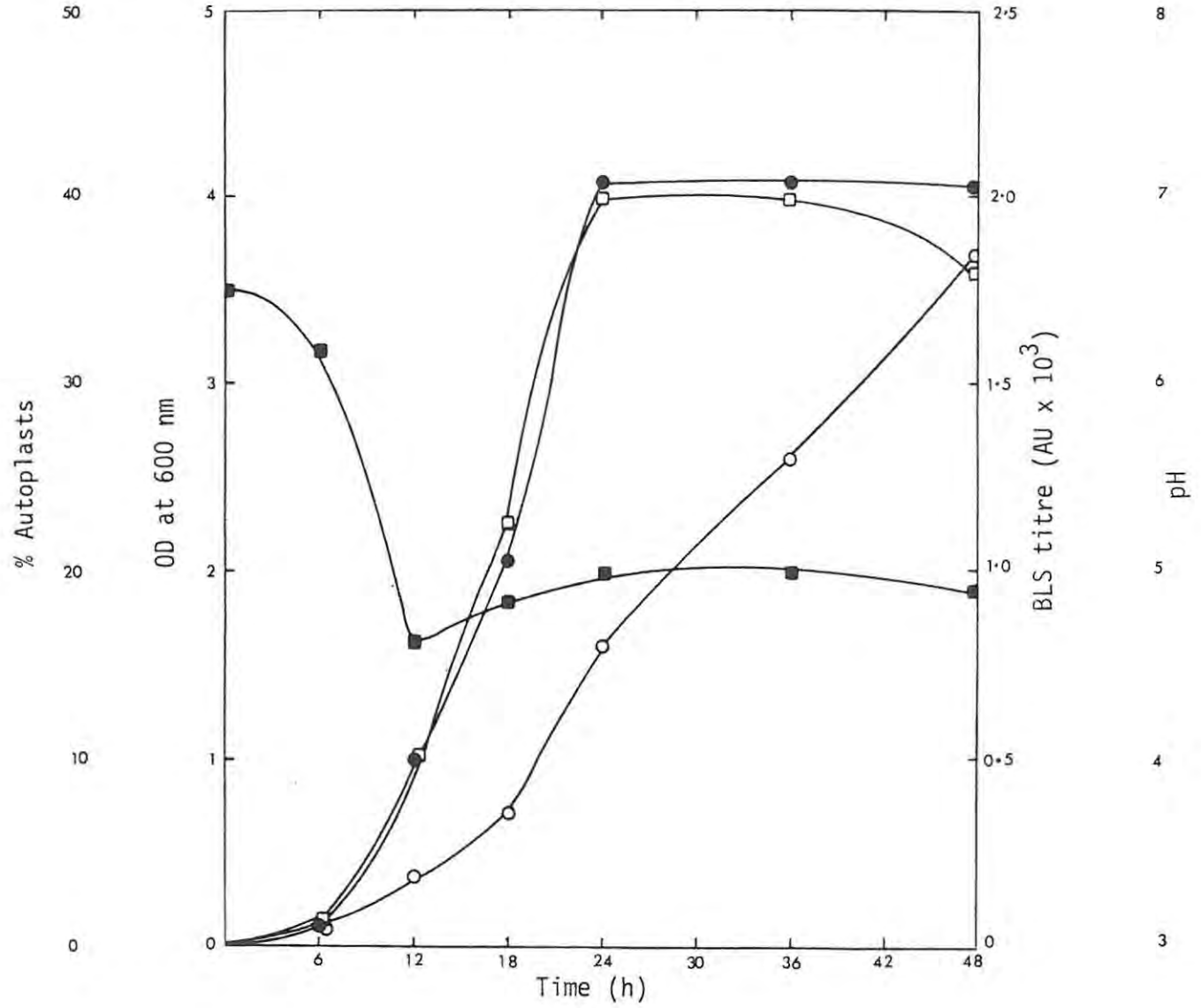


Fig 3.9 Detailed studies of pH changes (■), bacterial growth measured by OD at 600 nm (○), BLS production (●), and autoplast formation (□) by *C. acetobutylicum* in CBM containing 15% (wt/vol) sucrose.

Table 3.2 Activity spectrum of concentrated crude and partially purified BLS from *C. acetobutylicum*

	Heat Inactivated Crude BLS	Crude BLS (8192 AU)	Partially Purified BLS (256 AU)
Clostridium butyricum	-	+	-
Clostridium perfringens Strain 1	-	+	-
Clostridium perfringens Strain 2	-	-	-
Clostridium sporogenes Strain 1	-	+	-
Clostridium sporogenes Strain 2	-	-	-
Clostridium histolyticum	-	+	-
Clostridium oedematiens	-	-	+
Bacillus subtilis	-	+	+
Bacillus polymyxa	-	+	+
Bacillus laterosporus	-	+	+
Bacillus coagulans	-	+	+
Bacillus cereus	-	+	+
Bacillus lichenformis	-	+	+
Bacillus megaterium	-	+	+

(+) Sensitive

(-) Resistant

3.4 DISCUSSION

The mode of action of the BLS produced by *C. acetobutylicum* P262 was not consistent with that of a bacteriocin. The BLS was unable to inhibit DNA, RNA or protein synthesis and no decrease in intracellular ATP was detected when the BLS was added to actively growing cells of *C. acetobutylicum* P262. The activity spectrum obtained, using concentrated and partially purified BLS, was much broader than originally reported (Barber *et al.*, 1979). Initially, culture supernatants only showed activity against *C. acetobutylicum* strains and one other *Clostridium* species *(C. felsineum)*. This wider spectrum of activity, including strains from another Gram-positive genus, is also inconsistent with the normally accepted definition of a bacteriocin. The production of BLS was not controlled by a plasmid gene, as no plasmid DNA could be detected in *C. acetobutylicum* P262 cells, either by dye-bouyant density gradient centrifugation or by gel electrophoresis of labelled DNA (Webster *et al.*, 1980). No immunity substance was produced by the host cell, as the producer strain was sensitive to its own BLS when tested by the well plate technique (Barber *et al.*, 1979).

Although the BLS was not able to lyse exponential phase cells of *C. acetobutylicum* P262, it was able to lyse SDS-treated cell walls. This activity is associated with the action of an autolysin, and SDS-treated cell walls are commonly used for studying autolysins (Brown and Young 1970; Kawata and Takumi, 1971; Brown, 1972; Williamson and Ward, 1979). Other indications that *C. acetobutylicum* P262 possessed an autolytic system were demonstrated by autolysis of exponential phase cells in phosphate buffer. The resulting lysate was active against the same strain when

tested by the well plate assay or SDS-treated cell walls. The spontaneous formation of protoplasts (autoplasts) in an osmotically-stabilised medium suggested that the organism was able to hydrolyse the insoluble peptidoglycan layer in its own cell wall. This is an enzymatic action caused by active autolysin(s) produced by the organism (Dark and Strange, 1957; Joseph and Shockman, 1976). High titres of BLS were always associated with lysing cells or autoplast formation. These results suggest that the BLS is an autolysin and not a bacteriocin as previously reported (Barber *et al.*, 1979).

Many *Clostridium* species have been shown to possess autolysins and these include *C. perfringens* (Williamson and Ward, 1979), *C. botulinum* (Kawata and Takumi, 1971), *Clostridium saccharoperbutylacetonicum* (Ogata and Hongo, 1974), *C. sporogenes* (Galli and Hughes, 1965) and *Clostridium sordellii* (Novotný and Čáslavská, 1967). However, in contrast to other genera, very few autolysins produced by members of the genus *Clostridium* have been characterised. This is probably due to the difficulties involved in working with anaerobes.

The extracellular autolysin produced in CBM supplemented with molasses medium was identical to that produced in CBM containing 15% (wt/vol) sucrose (Chapter V). Therefore, further characterisation was carried out using autolysin produced in the latter medium, as it was more defined and more amenable to experimental manipulation.

CHAPTER IV

CHARACTERISATION OF THE AUTOLYSIN PRODUCED BY *C. ACETOBUTYLICUM*

The extracellular autolysin produced by *C. acetobutylicum* was most active in 0.05 M sodium acetate buffer (pH 4 to 5) at 37°C. The action of the autolysin was partially inhibited by the diavalent ions Cu^{2+} and Fe^{2+} and the chelating agent EDTA. SDS completely inhibited the autolytic action, whereas the proteolytic enzymes pronase and proteinase K caused partial inhibition, and the sulphydryl reagent mercaptoethanol had no effect. The autolysin had a different mode of action from lysozyme in that it affected an interior site on the cell wall.

4.1 INTRODUCTION

The properties of various autolysins have been defined using either crude or partially purified preparations (Shockman *et al.*, 1967; Kingan and Ensign, 1968; Coyette and Shockman, 1973). The autolytic system of *C. botulinum*, which is the most extensively characterised system of the genus *Clostridium* was also studied using a partially purified preparation (Kawata and Takumi, 1971; Takumi *et al.*, 1971). In this chapter, the characteristics of the *C. acetobutylicum* extracellular autolysin were determined using partially purified preparations and some of these properties were compared to the lytic enzyme, hen's egg white lysozyme (3.2.1.17).

4.2 METHODS

4.2.1 Materials and media as described in Appendix B

4.2.2 Bacterial strain

The autolysin producer and indicator strain was *C. acetobutylicum* P262.

4.2.3 Production of autolysin

The crude autolysin was produced by 60% (vol/vol) acetone precipitation of a 48 h culture supernatant of *C. acetobutylicum* P262 in CBM containing 15% (wt/vol) sucrose. The precipitate was collected by centrifugation, freeze dried and stored at $4^{o}C$. Partially purified autolysin was prepared by DEAE-cellulose column chromatography as described in Chapter V. The partially purified autolysin was used at a titre of 256 to 512 AU, which contained 1.5 to 4.0 $\mu g\ ml^{-1}$ protein.

4.2.4 Autolysin assay

The autolysin was assayed by either the well plate method described in Chapter II, using CBM containing 1% (wt/vol) agar as the background growth medium, or by the decrease in OD of SDS-treated cell walls in 0.05 M sodium acetate buffer (pH 5.0). The initial turbidity of the cell wall suspension was adjusted to an OD of 0.4 to 0.6 at 660 nm using an MSE Spectro Plus spectrophotometer. Turbidity was measured at various time intervals, and one unit of lytic activity was defined as a decrease in OD of 0.001 h^{-1} at $37^{o}C$. The assays were performed in matched

quartz cuvettes with a 10 mm light path, and 200 µl of the autolysin was added to 800 µl of cell walls. A microtechnique using 50 µl of autolysin and 200 µl of cell walls in 1.0 mm quartz cuvettes was also utilised. The OD was measured on an expanded scale, and multiplied by ten to compensate for the shorter light path. A control with heat-inactivated autolysin was included with each assay, and the number of units was calculated from the difference between the change of OD (Δ) of the test and the ΔOD of the control.

4.2.5 Effect of physical and chemical agents on autolysin activity

The effect of pH on the lytic activity of the autolysin was studied using SDS-treated cell walls suspended in the following buffers (0.05 M): sodium phosphate pH 6, 7 and 8, sodium acetate pH 5 and 4, and sodium citrate pH 3. The optimum temperature for activity was determined in 0.05 M sodium acetate buffer (pH 5.0) at 20, 30, 37 and 42°C. The effect of ionic strength was determined using SDS-treated cell walls in 0.01, 0.025, 0.05, 0.075 and 0.1 M sodium acetate buffer (pH 5.0).

The inhibitory or stimulatory effect of divalent cations on autolysin activity was tested using the following salts: $MgSO_4$, $CaCl_2$, $MnCl_2$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$ (10^{-3}M). The effects of mercaptoethanol (0.1% vol/vol), EDTA (10^{-2}M), SDS (0.1% wt/vol), and the proteases; trypsin, pronase, pepsin (1 mg ml^{-1}) and proteinase K (0.5 mg ml^{-1}) were also determined.

4.2.6 Stability of the autolysin

The stability of the autolysin was determined at -20°C and 4°C.

The stabilising effect of glycerol (2% vol/vol), bovine serum albumin, ovalbumin (0.5 mg ml^{-1}) and SDS-treated cell walls was tested by adding them to the partially purified autolysin.

4.2.7 Effect of the autolysin on SDS-treated, formalin-treated and heat-inactivated whole cells

SDS-treated whole cells were prepared from exponential phase cells grown in CBM. The cells were harvested by centrifugation, and after three washes in distilled water were resuspended and treated with an equal volume of 2% (wt/vol) SDS. The treated cells were incubated at 20°C for 30 min and then washed three times with distilled water, resuspended in 0.05 M sodium acetate buffer (pH 5.0), and dialysed against the same buffer to remove the SDS.

Washed whole cells were treated with formalin (10% vol/vol) for 30 min at 20°C. The cells were washed three times before resuspending and dialysing against the sodium acetate buffer.

Heat-inactivated whole cells were prepared by resuspending washed exponential phase cells in the sodium acetate buffer, and heating in a boiling water bath at 100°C for 10 min. In order to increase the susceptibility of heat-inactivated cells they were pretreated with lysozyme (200 μg ml^{-1}) before the addition of autolysin.

4.2.8 Comparison of the action of the autolysin and lysozyme on exponential phase cells and SDS-treated cell walls

Actively growing cells of *C. acetobutylicum* were assayed for cell wall

damage after treatment with autolysin and/or lysozyme using the method of Shockman *et al.* (1968). Exponential phase cells (4 ml) in CBM were treated with 1 ml 0.01 M sodium acetate buffer (pH 4.5), 1 ml of partially purified autolysin (in buffer), or 1 ml of lysozyme at 1 mg ml^{-1} in buffer (final concentration 200µg ml^{-1}). The autolysin and lysozyme preparations (1 ml of each) were added simultaneously to 3 ml of cells in CBM. Autolysin and lysozyme preparations were perfused with H_2 and CO_2 to achieve anaerobic conditions. The OD was monitored at 37°C for 1 h and the cells were then pelleted by centrifugation and resuspended in the same volume of distilled water. The pH was increased to *c* pH 12 by the addition of 0.1 ml 2N NaOH which resulted in lysis of the cells that had sustained cell wall damage. This damage was measured by the change in OD after 1 min exposure to alkali.

Lysozyme (200 µg ml^{-1}) was also added to SDS-treated cell walls with and without the addition of autolysin, and the lytic activity was monitored over 1 h at 37°C.

4.2.9 Protease activity of the autolysin

The partially purified autolysin was tested for protease activity using the non-specific protease substrate Azocoll. The assays were performed as described in Chapter II, except that the buffer used was 0.01 M sodium acetate (pH 4.5). Trypsin (1 mg ml^{-1}) was used as a positive control.

4.2.10 Determination of a mutant screening technique

Isolated colonies of *C. acetobutylicum* were grown on CBM agar, or CBM agar

containing 5% (vol/vol) molasses medium. After various time intervals the colonies were overlayed with CBM containing 0.75% (wt/vol) agar seeded with *C. acetobutylicum* P262 cells. The plates were incubated at 37°C for a further 18 h, and examined for zones of inhibition around the original colonies.

4.3 RESULTS

4.3.1 Effect of physical and chemical agents on autolysin activity

The optimum conditions required for cell wall lysis were established by studying the effects of pH, temperature and ionic strength (Figs 4.1, 4.2 and 4.3). The optimum pH was between pH 4 and 5, and the maximum decrease in turbidity of the SDS-treated cell walls was obtained using sodium acetate buffer at an ionic strength of 0.05 M. Although there was no marked difference in autolytic activity using a temperature range of 20 to 42°C, the optimum temperature was 37°C.

Cu^{2+} and Fe^{2+} ions markedly inhibited the autolysin, and Mg^{2+} had a slight inhibitory effect (Table 4.1). No significant activation or inhibition was obtained with the other cations tested.

The sulphydryl reagent mercaptoethanol did not affect the autolysin, whereas the addition of the chelating agent EDTA resulted in a loss of over half the lytic activity, and the protein inhibitor SDS completely inactivated the autolysin (Table 4.2a).

The autolysin was partially inactivated by the non-specific proteases,

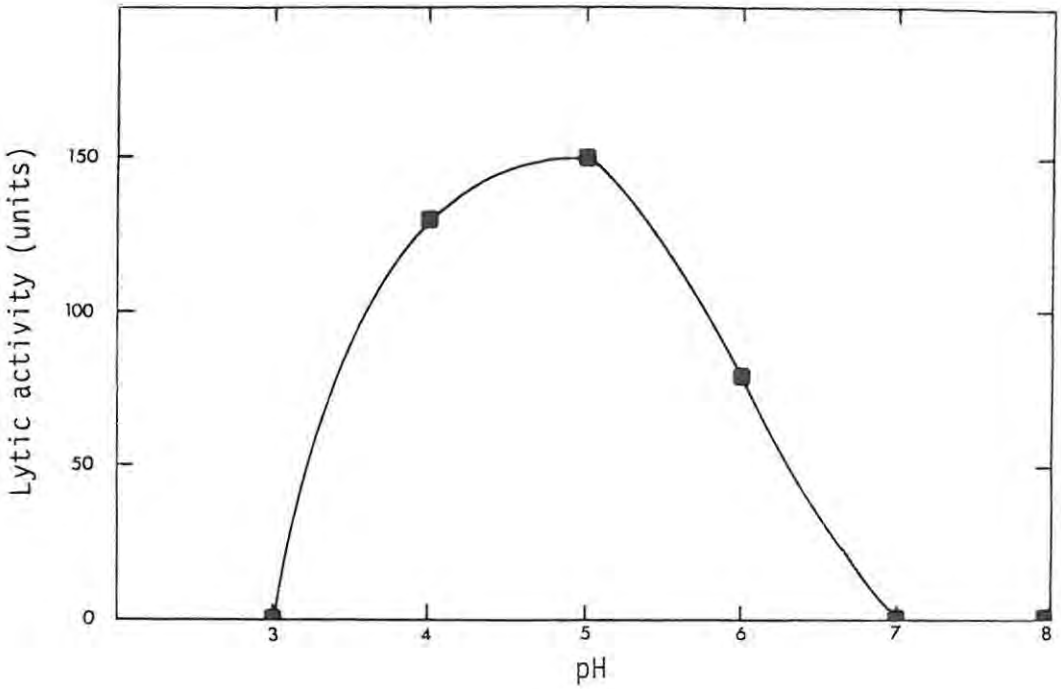


Fig 4.1 Effect of pH on the lytic activity of the *C. acetobutylicum* autolysin, determined using SDS-treated cell walls of *C. acetobutylicum*.

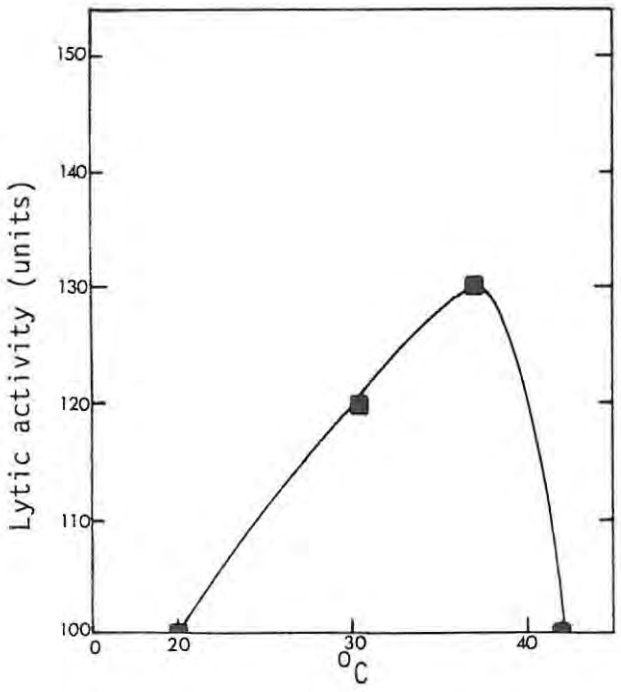


Fig 4.2 Effect of temperature on the lytic activity of the *C. acetobutylicum* autolysin, determined as for Fig 4.1.

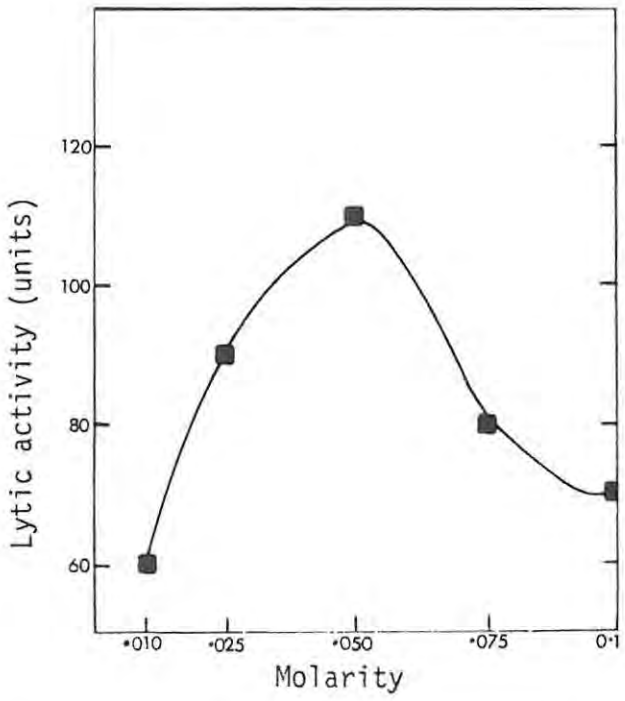


Fig 4.3 Effect of buffer concentration on the lytic activity of the *C. acetobutylicum* autolysin, determined as for Fig 4.1.

Table 4.1 Effect of divalent cations on autolysin activity

Compound (10^{-3}M)	Units of activity*
Control	230
Mg^{2+} ($MgSO_4$)	200
Ca^{2+} ($CaCl_2$)	220
Mn^{2+} ($MnCl_2$)	230
Cu^{2+} ($CuSO_4$)	130
Zn^{2+} ($ZnSO_4$)	240
Fe^{2+} ($FeSO_4$)	180

* activity was measured using the SDS-treated cell wall assay described in this chapter.

Table 4.2a Effect of inhibitors on autolysin activity

Compound	Units of activity*
Control	230
EDTA (10^{-2}M)	110
SDS (0.1%)	0
Mercaptoethanol (0.1%)	220

* measured as for Table 4.1

Table 4.2b Effect of proteases on autolysin activity

Protease	Units of activity*
Control	290
Pepsin	290
Trypsin	290
Pronase	200
Proteinase K	240

* measured as for Table 4.1

pronase and proteinase K, but not by the specific proteases, trypsin and pepsin (Table 4.2b).

4.3.2 Comparison of autolysin activity using the cell wall or well plate assay

The activity of the partially purified autolysin could just be detected at a dilution of $^{1}/50$ using the cell wall assay, whereas a zone of inhibition was still visible at $^{1}/256$ using the well plate assay. The well plate assay was a five times more sensitive than the cell wall assay.

4.3.3 Stability of the autolysin

Crude (culture supernatants) and concentrated autolysin preparations were stable and 100% activity remained after 24 h at -20 and 4°C. Partially purified autolysin was unstable and was inactivated after 24 h at -20 and 4°C. The addition of glycerol, bovine serum albumin, ovalbumin or SDS-treated cell walls did not increase the stability of the partially purified autolysin.

4.3.4 Effect of autolysin on SDS-treated, formalin-treated and heat-inactivated whole cells

The autolysin was able to lyse SDS-treated whole cells (Fig 4.4), but had no effect on formalin or heat-treated whole cells (Figs 4.6 and 4.7). Pretreating heat-inactivated cells with lysozyme did not make them sensitive to the autolysin. The most rapid decrease in OD was obtained with SDS-treated cell walls (Fig 4.5).

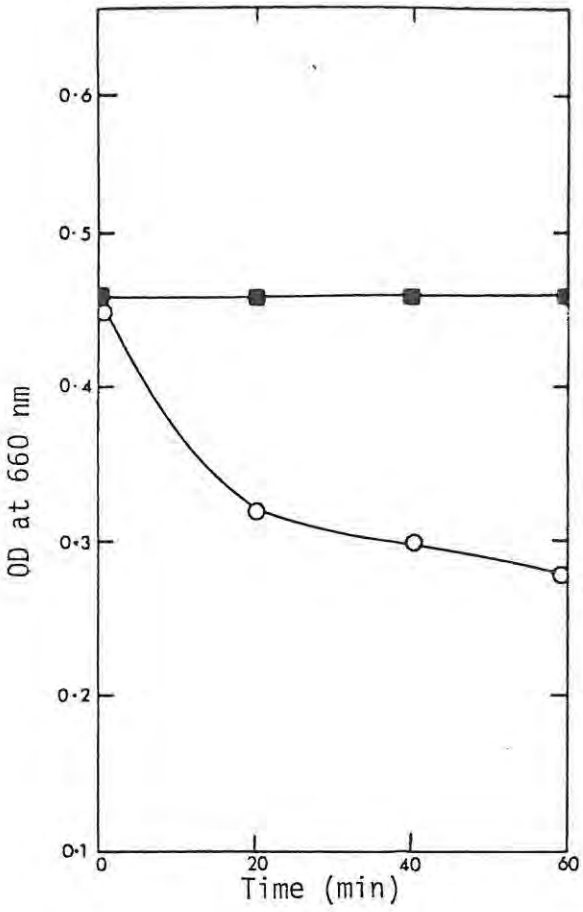


Fig 4.4 Effect of the autolysin (○) and heat-inactivated autolysin (■) on SDS-treated whole cells of *C. acetobutylicum*.

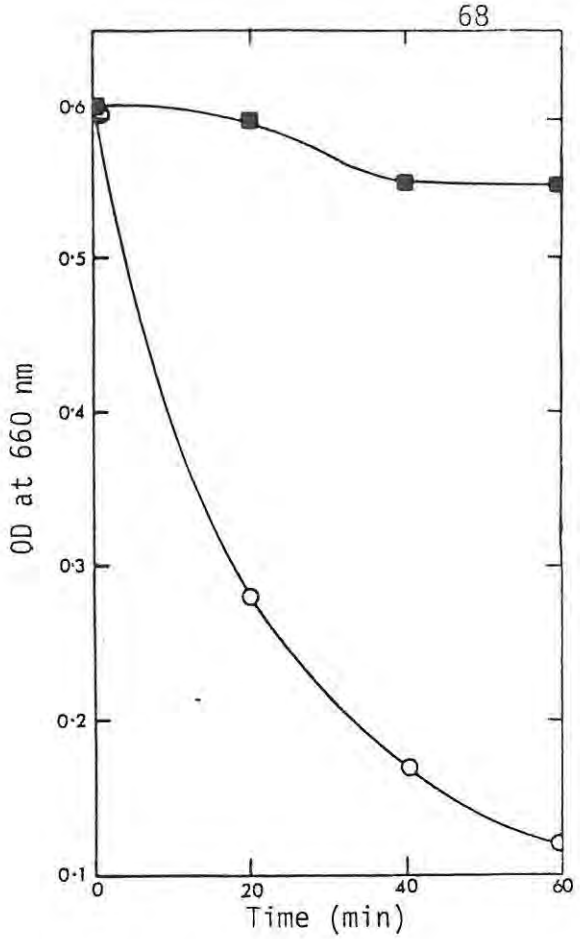


Fig 4.5 Effect of the autolysin (○) and heat-inactivated autolysin (■) on SDS-treated cell walls of *C.acetobutylicum*.

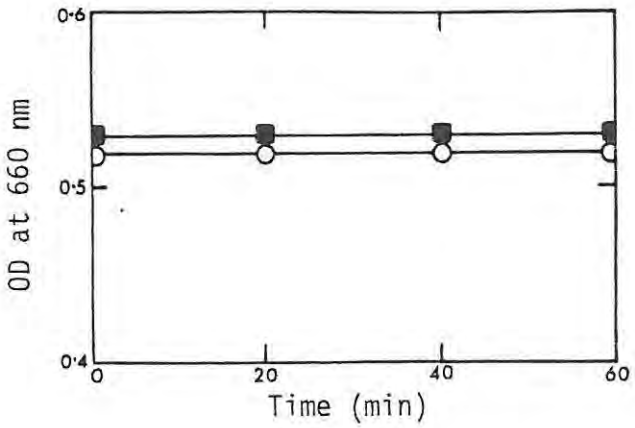


Fig 4.6 Effect of the autolysin (○) and heat-inactivated autolysin (■) on formalin-treated whole cells of *C. acetobutylicum*.

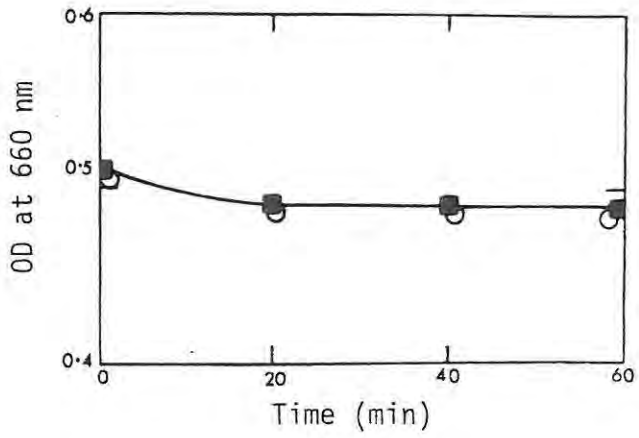


Fig 4.7 Effect of the autolysin (○) and heat-inactivaled autolysin (■) on heat-treated whole cells of *C.acetobutylicum*.

4.3.5 Comparison of the action of the autolysin and lysozyme on exponential phase cells and SDS-treated cell walls

No lysis occurred when either autolysin or lysozyme was added to actively growing cells (Fig 4.8 and Chapter III). However, this technique gives no indication of the degree of cell wall damage which can be detected by the exposure of the treated cells to a high pH. The decrease in OD after the addition of alkali indicates the degree of cell wall damage. There was no difference in the degree of lysis of cells at high pH after pretreatment with autolysin or buffer (Fig 4.8). However, cells pretreated with lysozyme showed a greater degree of lysis when compared with the buffer control. When cells were pretreated with a mixture of autolysin and lysozyme, the amount of lysis was markedly reduced, when compared with cells pretreated with lysozyme on its own. In contrast to the autolysin (see Fig 4.5) lysozyme had no effect on SDS-treated cell walls of *C. acetobutylicum*.

4.3.6 Protease activity of the autolysin

The partially purified autolysin was unable to degrade the synthetic protease substrate Azocoll, even after 18 h incubation.

4.3.7 Mutant screening technique

Zones of inhibition were detected around 24 h old colonies of *C. acetobutylicum* P262 overlayed with the same strain (Plate 4.1). Microscopic examination of the producer colony showed that the majority of the cells in the colony were lysed or lysing (Plate 4.1).

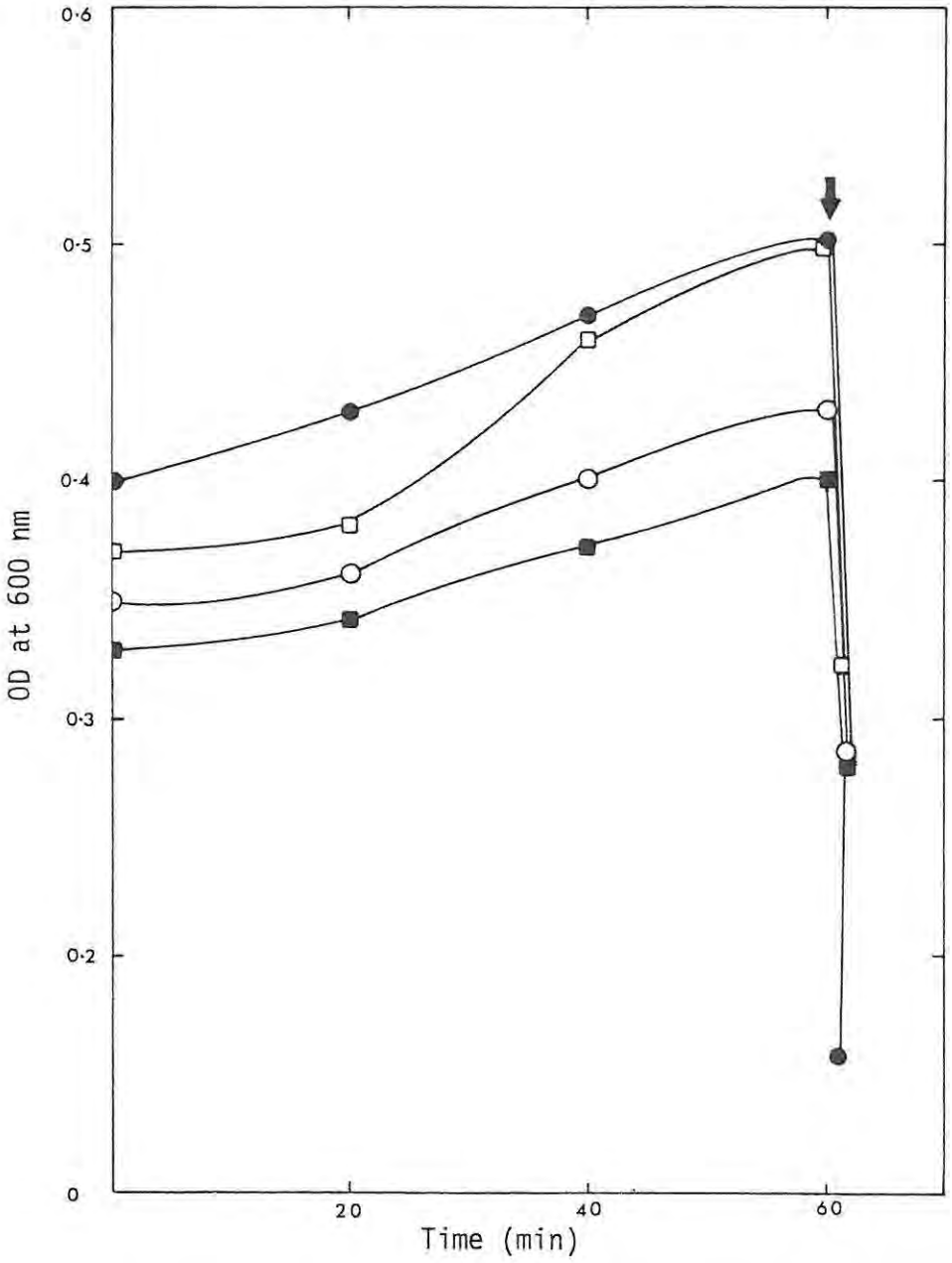
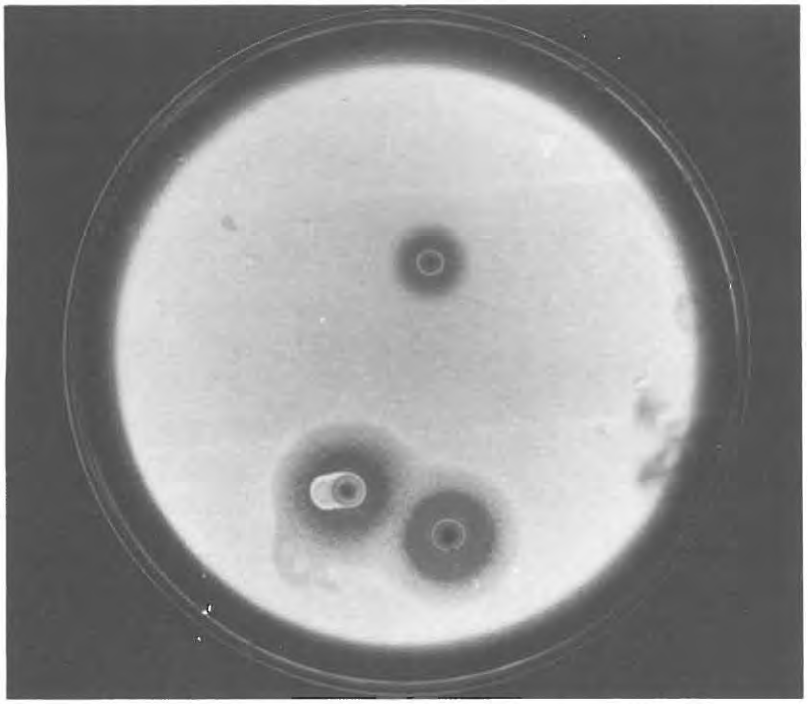
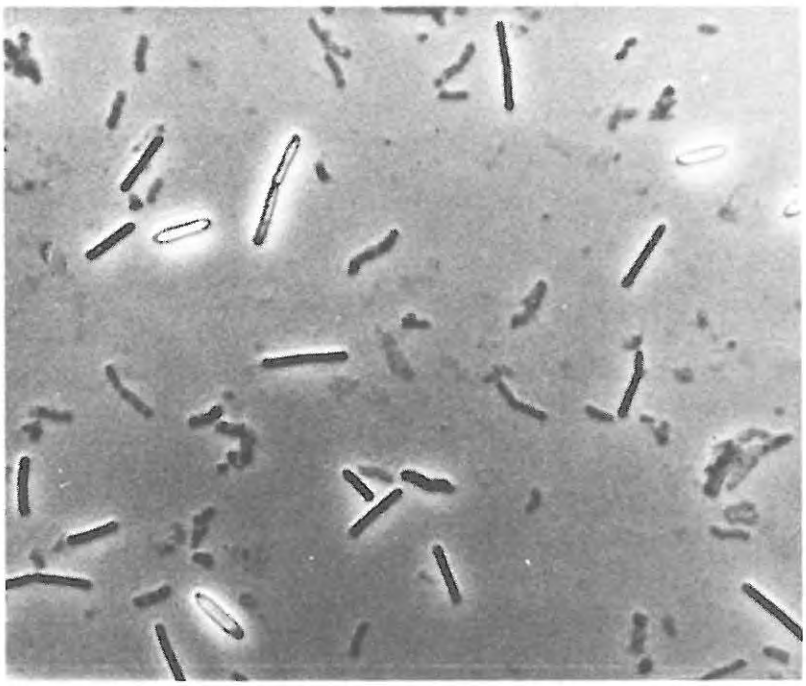


Fig 4.8 The effect on exponential phase cells of *C. acetobutylicum* of autolysin (○), lysozyme (●), and autolysin plus lysozyme (□), compared with control cells to which only buffer was added (■). The arrow indicates the time of addition of alkali, and the decrease in OD represents the extent of cell wall damage that had occurred.

Plate 4.1



Zones of inhibition produced by autolysin from colonies of *C. acetobutylicum*



Lysing cells and cell debris in autolysin producing colonies of *C. acetobutylicum*

4.4 DISCUSSION

In common with many autolysins the activity of the *C. acetobutylicum* autolysin was affected by divalent cations. Cu^{2+} and Fe^{2+} ions which were also able to inhibit protoplast formation (unpublished data) inhibited the activity of the autolysin. None of the cations tested were able to stimulate the autolysin, whereas the lytic activity of the autolysin(s) produced by *C. botulinum* was stimulated by Co^{2+} and Mg^{2+} at 10^{-4} M, but inhibited by Zn^{2+}, Ca^{2+} and Ni^{2+} ions (Kawata and Takumi, 1971). An endo-glucosaminidase produced by *B. cereus* was also stimulated by Co^{2+} and Mg^{2+} as well as Ca^{2+} and Ba^{2+} and inhibited by Ni^{2+} ions (Kawagishi *et al.*, 1980). Coyette and Shockman (1973) reported that the N-acetylmuramidase of *L. acidophilus* was inhibited by Cu^{2+} at 10^{-3} M, but Ca^{2+} and Mg^{2+} at the same concentration had no effect. Lysozyme, which is irreversibly inhibited by some heavy metal ions, is stimulated by Ca^{2+} and Mg^{2+} (Wadström, 1970).

The chelating agent EDTA has been reported to decrease the activity of a number of autolysins (Hawiger, 1968; Singer *et al.*, 1972; Coyette and Shockman, 1973) and the *C. acetobutylicum* autolysin was no exception. This suggests that one or more divalent cations are required for autolytic activity. Since the sulphydryl reagent mercaptoethanol did not affect the autolysin, no sulphydryl groups are required for its activity. This also appears to be the case for some other autolysins (Kawata and Takumi, 1971; Singer *et al.*, 1971), although the N-acetylmuramidase produced by *L. acidophilus* is a sulphydryl-containing enzyme (Coyette and Shockman, 1973).

Optimum conditions for autolysins vary considerably and some organisms

may produce several autolysins each with different pH optima. Brown and Young (1970) described three autolysins produced by a sporulation mutant of *B. subtilis* with pH optima at 5.5, 8.0 and 9.5. Two autolysins produced by *B. thuringiensis* are most active at pH 8.5, whilst one other has a pH optimum of 4.0 (Kingan and Ensign, 1968). A lytic enzyme of *S. aureus* has an optimum pH range of 6.0 to 9.4 which is almost identical to lysozyme (Hawiger, 1968). The optimum pH for the *C. acetobutylicum* autolysin was between pH 4 and 5.

Lysozyme is denatured by the surface-active agent SDS and the proteolytic enzyme pepsin, but not by trypsin, chymotrypsin and papain (Jollès, 1960). The protein nature of the *C. acetobutylicum* autolysin was demonstrated by its partial inactivation by pronase and proteinase K, and also by its complete inactivation by SDS.

The partially purified autolysin was unstable even at -20^{o}C. Instability is a common property of autolysins, although crude preparations are often very stable (Shockman and Cheney, 1969; Singer *et al.*, 1972; Yem and Henry, 1976). The autolysin produced by *L. acidophilus* is unstable at 37^{o}C, but the addition of bovine serum albumin increased its stability (Coyette and Shockman, 1973). The partially purified *C. acetobutylicum* autolysin could not be stabilized.

Treatment of whole cells with SDS, formalin or heat inactivates the autolysin(s) present in the walls of the indicator cells. Furthermore, SDS treatment is thought to extract associated autolytic enzymes (Williamson and Ward, 1979). The *C. acetobutylicum* autolysin was only able to lyse whole cells that had been treated with SDS. This may mean

that the sites required for autolysin action are occupied by autolysin(s) resident in exponential cell walls, and it is only after their removal that the effect of added autolysin can be demonstrated. Williamson and Ward (1979) characterised the autolytic enzymes produced by *C. perfringens* and the predominant autolysin was an endo-β-N-acetylglucosaminidase, although some endo-β-N-acetylmuramidase activity was detected. They found that these enzymes were unable to lyse SDS-treated cell walls of *C. perfringens*, but were active against cell walls treated with hot formamide at 160°C which extracts secondary wall polymers. They suggest that the complex secondary polymers of *C. perfringens* mask the peptidoglycan layer after wall isolation, and prevent the association of the enzyme with their binding site on the peptidoglycan. Hawiger (1968) also described an enzyme produced by *S. aureus* which would only lyse cell walls of *S. aureus* after treatment with hot 5% TCA, which exposed the peptidoglycan layer. Huff and Silverman (1968) characterised another cell wall lytic enzyme produced by *S. aureus*, which was not active against intact cells of *S. aureus*. They suggested that this enzyme acts on the cell wall inner surface. The *C. acetobutylicum* autolysin was unable to lyse intact whole cells, and no cell wall damage could be detected after its addition to actively growing cells. The action of the autolysin could only be demonstrated after SDS-treatment of whole cells or cell walls, suggesting that its site of action was situated in the cell wall interior and was exposed by SDS.

Westmacott and Perkins (1979) reported an enhancement of autolysin activity if heat-inactivated cells of *B. cereus* 569 were pretreated with lysozyme, even though lysozyme was unable to lyse *B. cereus* cells. However, pretreatment of *C. acetobutylicum* heat-inactivated cells with lysozyme

failed to increase the susceptibility of the cells to autolysin. Lysozyme could not lyse exponential phase cells of *C. acetobutylicum*, but, in contrast to the autolysin, was able to cause cell wall damage. Resistance to lysozyme-induced lysis has been reported for bacteria belonging to Gram-negative and Gram-positive genera, but lysozyme-induced cell wall damage has been described for many Gram-positive species (Shockman *et al.*, 1968). Although lysozyme caused cell wall damage in growing cells of *C. acetobutylicum*, it was unable to lyse SDS-treated cell walls, demonstrating a different site of action to the autolysin.

Determination of the exact site of action of the autolysin requires extensive studies involving the analysis of the cell wall degradation products (Ghuysen *et al.*, 1966). As discussed in Chapter I, cell wall lytic enzymes fall into three classes; glycosidases, N-acetylmuramyl-L-alanine amidases, and endopeptidases. The glycosidases are either endo-N-acetylmuramidases such as lysozyme, or endo-N-acetylglucosaminidases. Although it may depend on the structure of the cell wall of *C. acetobutylicum*, it would seem unlikely that the autolysin is an endo-N-acetylmuramidase since it attacks a different site to that of lysozyme. Ensign and Wolfe (1966) characterised a low molecular weight enzyme produced by a *Myxobacter* species, which was capable of both cell wall lytic activity and proteolytic activity. The proteolytic activity of this enzyme, which has recently been described as an endopeptidase hydrolysing D-alanyl-L-alanine linkages or D-alanyl-glycyl-LL-diaminopimelyl sequences (Ghuysen and Shockman, 1973), could be assayed using the synthetic substrate Azocoll. The *C. acetobutylicum* autolysin was not active against the non-specific proteolytic substrate Azocoll. This suggests that the

autolysin is not an endopeptidase, although proteolytic activity has not been reported for peptidases of *S. aureus*, *E. coli* and *Streptomyces* (Ensign and Wolfe, 1966).

Further characterisation, including the elucidation of the biological role of the autolysin, is required. The analysis of mutants defective in autolytic activity (*lyt*$^-$) has helped to indicate the biological roles of various autolysins (Ghuysen and Shockman, 1973). An overlay system for screening autolysin production by individual colonies has been described in this chapter and may prove to be useful for obtaining autolysin mutants of *C. acetobutylicum*.

CHAPTER V

PURIFICATION OF THE AUTOLYSIN PRODUCED BY *C. ACETOBUTYLICUM*

The extracellular autolysin of *C. acetobutylicum* was purified by acetone precipitation, followed by DEAE-cellulose column chromatography and polyacrylamide gel electrophoresis (PAGE). The molecular weight determined by SDS-PAGE was 28 000, and carbohydrate staining of the purified autolysin showed that it was a glycoprotein.

5.1 INTRODUCTION

In spite of the universal occurrence of autolysins, only a few have been purified. The classical methods for enzyme purification such as ammonium sulphate precipitation, gel filtration and ion exchange chromatography have not always been successful for autolysin purification. Huff and Silverman (1968) were unable to purify the autolysin produced by *S. aureus* using the methods just described, nor by sucrose gradient centrifugation or disc gel electrophoresis. Autolysin purification is often made more difficult by properties such as instability and strong association of the autolysin with cell wall components. Brown *et al.* (1970) showed that a highly purified autolytic enzyme (N-acetylmuramyl-L-alanine amidase) of *B. subtilis* remained tightly bound to teichoic acid which is a major polymer in *B. subtilis* cell walls. The autolysin(s) produced by *C. botulinum* was not purified by ammonium sulphate

precipitation, DEAE-cellulose chromatography or gel filtration, and the resulting partially purified preparation contained two major and two minor protein bands after PAGE (Kawata and Takumi, 1971). Mode of action studies performed with this partially purified preparation revealed the presence of a N-acetylmuramyl-L-alanine amidase and an hexosaminidase (Takumi *et al.*, 1971).

Using disc gel electrophoresis, Ensign and Wolfe (1966) showed that the wall lytic enzyme AL-1 of a *Myxobacter* species resolved into one major and two very faint protein bands. An unstained gel was cut into segments, eluted and each segment tested for cell wall lytic activity. Proteolytic and cell wall lytic activities were found only in the position which corresponded with the major protein band. No lytic activity was detected in the segments containing the faint bands. Although other lengthy and elaborate purification methods have been used for autolysin purification (Ghuysen *et al.*, 1970; Brown, 1972; Fan and Beckman, 1972), the extracellular autolysin produced by *C. acetobutylicum* was purified using the methods described above.

5.2 METHODS

5.2.1 Media and materials as described in Appendix B.

5.2.2 Bacterial strain and autolysin assay as described in Chapter IV.

5.2.3 Preparation of the concentrated crude autolysin

The concentrated crude autolysin was prepared from batches of culture

supernatants (400 ml) produced by *C. acetobutylicum* P262 grown anaerobically in CBM containing 15% (wt/vol) sucrose. The supernatant was collected by centrifugation at 10 000 rev min^{-1} for 15 min at $4^{o}C$, and cold acetone was added slowly to give a 60% (vol/vol) solution. After 2 h at $4^{o}C$ the precipitate was collected by centrifugation at 10 000 rev min^{-1} for 20 min. The precipitate was freeze dried and the normal yield of protein from 400 ml culture supernatants was between 0.2 and 0.3 gm. The concentrated crude autolysin was stored at $4^{o}C$ until required, and was stable for several weeks. For purification studies, the concentrated autolysin was resuspended in the appropriate buffer at 20 mg ml^{-1} which gave a titre of c 32 000 AU.

5.2.4 Ion exchange column chromatography

Concentrated autolysin (0.1 ml) was applied to small ion exchange columns (0.8 x 3.0 cm). DEAE-cellulose chromatography (anion exchange) was performed using three different columns conditioned with the following buffers (0.01 M): phosphate buffer pH 7 or 6.5, and sodium acetate buffer pH 4.5. Two cation exchange columns were tested, a CMC column conditioned with 0.01 M sodium acetate buffer pH 5.0, and a cellulose phosphate column conditioned with 0.01 M sodium acetate, pH 4.0. Each column was eluted with 2 ml of the appropriate buffer containing 0.1, 0.2, 0.3, 0.4, and 1.0 M NaCl. The fractions collected were assayed for protein at 280 nm, and for autolysin by the well plate method. All the column chromatography experiments were carried out at $4^{o}C$ and fractions were stored on ice until assayed.

5.2.5 Preparation of partially purified autolysin

Partially purified autolysin was routinely prepared by anion exchange

column chromatography using a column packed with DE-52 resin (see Appendix B) and conditioned with 0.01 M sodium acetate buffer, pH 4.5. The column (1.5 x 15.0 cm) was loaded with 1 to 2 ml concentrated autolysin and eluted with sodium acetate buffer. Fractions (2 ml) were collected using an ISCO Golden Retriever fraction collector, and protein-containing fractions (estimated by absorption at 280 nm) were assayed for autolysin activity.

5.2.6 Agarose gel electrophoresis

Agarose gels (1.0% wt/vol) were prepared in 0.05 M phosphate buffer, pH 6.0 on microscope slides (26 x 76 mm) using a Shandon gel spreader. Small wells were cut in the centre of the gels and 15 µl of concentrated, partially purified, or heat-inactivated autolysin was added to duplicate gels. The gels were electrophoresed in a Shandon electrophoresis tank containing 0.05 M phosphate buffer, pH 6.0 at 30 volts per slide for 2 h. After electrophoresis, the duplicate gels were either stained with amido black for 30 min and destained with 45% (vol/vol) methanol containing 10% (vol/vol) acetic acid, or overlayed with CBM containing 1% (wt/vol) agar seeded with *C. acetobutylicum* cells. The overlayed slides were incubated anaerobically at 37°C for 18 h and examined for zones of inhibition.

5.2.7 Sephadex column chromatography

Using the gel filtration methods described by Andrews (1965), a Sephadex G-100 column (1.5 x 21 cm) equilibrated with 0.01 M sodium acetate buffer, pH 4.5 was loaded with 0.5 ml partially purified autolysin and eluted

using the same buffer. This was repeated using a Sephadex G-200 column (1.6 x 28 cm). The void volumes were determined using blue dextran 2000 (MW 2 x 10^6) at a flow rate of 10 ml h^{-1}. The columns were calibrated with bovine serum albumin (MW 67 000) and cytochrome C (MW 14 500) before use.

5.2.8 Polyacrylamide disc gel electrophoresis

The partially purified autolysin concentrated by dialysis against polyethylene glycol was applied to 5% (wt/vol) polyacrylamide disc gels (0.8 x 8.0 cm) in 0.05 M phosphate buffer, pH 6.0 ($\sim$ 250 µg protein per gel). The gels were electrophoresed at 4°C towards the cathode in 0.01 M phosphate buffer, pH 6.0 at 27 volts per gel for 5 h. Cytochrome C ($\sim$ 150 µg) was electrophoresed simultaneously on a separate gel, and used as a visual marker to monitor electrophoretic mobility. The gels were then cut in half lengthwise, and one half was stained with coomassie blue for 2 h, and destained in 45% (vol/vol) methanol containing 7% (vol/vol) acetic acid. The remaining half of the gel was either stained for lipid or carbohydrate (see 5.2.10), or stored at -20°C until the complementary gel (stained for protein) was destained. The two halves were then matched and the corresponding sections opposite stained protein bands were cut out. The same unstained sections from three gels were macerated and eluted with 2 ml 0.01 M sodium acetate buffer, pH 4.5 for 6 h at 4°C. The gel was removed by centrifugation, and the supernatant was assayed for autolysin activity by the well plate technique. As a control, three sections of unstained gel not containing protein were eluted and tested for activity.

5.2.9 SDS-polyacrylamide slab gel electrophoresis

The purity and molecular weight of the protein eluted from the disc gels was determined by SDS polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli (1970). The eluted protein was concentrated by the addition of two volumes of acetone. After standing at -20°C for 18 h the precipitate was removed, resuspended in the dissociation buffer (see Appendix B) and electrophoresed on a 4% (wt/vol) acrylamide stacking gel with a 12% (wt/vol) acrylamide resolving gel. The following proteins were used as molecular weight markers and were electrophoresed simultaneously in adjacent wells: phosphorylase b (MW 94 000), bovine serum albumin (MW 67 000), ovalbumin (MW 43 000), aldolase (MW 40 000), carbonic anhydrase (MW 30 000), chymotrypsinogen (MW 25 700), soybean trypsin inhibitor (MW 20 100), and myoglobin (MW 17 200).

5.2.10 Analytical and staining methods

Protein was determined by the method of Lowry *et al.* (1951) and carbohydrate by the phenol-sulphuric acid procedure described by Hodge and Hofreiter (1969). Carbohydrate staining using the method of Upreti and Hinsdill (1973) was accomplished by adapting the colorimetric phenol-sulphuric acid procedure. The gels were heated for 5 min at 90°C in a mixture of 5% (wt/vol) phenol and conc. sulphuric acid at a ratio of 1:5. Carbohydrate, when present in the gel, stained dark brown. The glycoprotein lysozyme (Young, 1963) was also electrophoresed on disc gels and used as a positive control. Lipid was detected by staining with sudan black as described by Crowle (1961). The gels were stained for 2 h in a 60% (vol/vol) ethanol solution

saturated with sudan black, and destained in 50% (vol/vol) ethanol.

5.3 RESULTS

5.3.1 Ion exchange column chromatography

The highest yields of partially purified autolysin were obtained using an anion exchange column (DE-52) eluted with 0.01 M sodium acetate (pH 4.5). This procedure removed most of the contaminating proteins (Plate 5.1), and a single peak of autolysin activity was detected in the void volume which coincided with the major protein peak (Fig 5.1). Carbohydrate was detected in the fractions containing autolysin. A six-fold purification was obtained after acetone precipitation and DEAE chromatography (Table 5.1), but the recovery was very low (4%). This figure may not be a true reflection, as the autolysin was very unstable after ion exchange column chromatography (see Chapter IV).

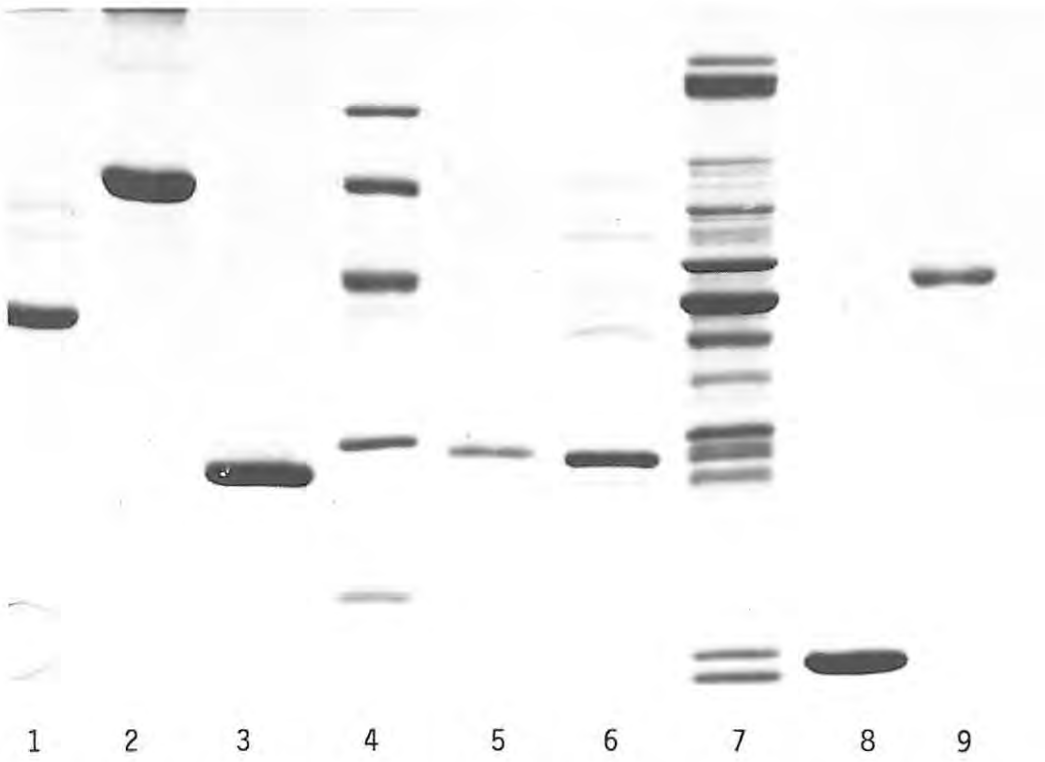
5.3.2 Sephadex column chromatography

The autolysin was not retained or purified by Sephadex G-100 or G-200, and the majority of the protein and autolytic activity was detected in the void volume.

5.3.3 Agarose gel electrophoresis

This technique established the charge of the autolysin at pH 6.0 in phosphate buffer and determined the conditions required for purification on polyacrylamide disc gels. The autolysin behaved

Plate 5.1 SDS-polyacrylamide electrophoresis of concentrated, partially purified and purified autolysin and proteins of known molecular weights.



1 Aldolase (MW 40 000)
2 Bovine serum albumin (MW 67 000)
3 Chymotrypsinogen (MW 25 700)
4 Phosphorylase b (MW 94 000)
Bovine serum albumin (MW 67 000)
Ovalbumin (MW 43 000)
Carbonic anhydrase (MW 30 000)
Soybean trypsin inhibitor (MW 20 100)
5 Purified autolysin
6 Partially purified autolysin
7 Concentrated crude autolysin
8 Myoglobin (MW 17 200)
9 Ovalbumin (MW 43 000)

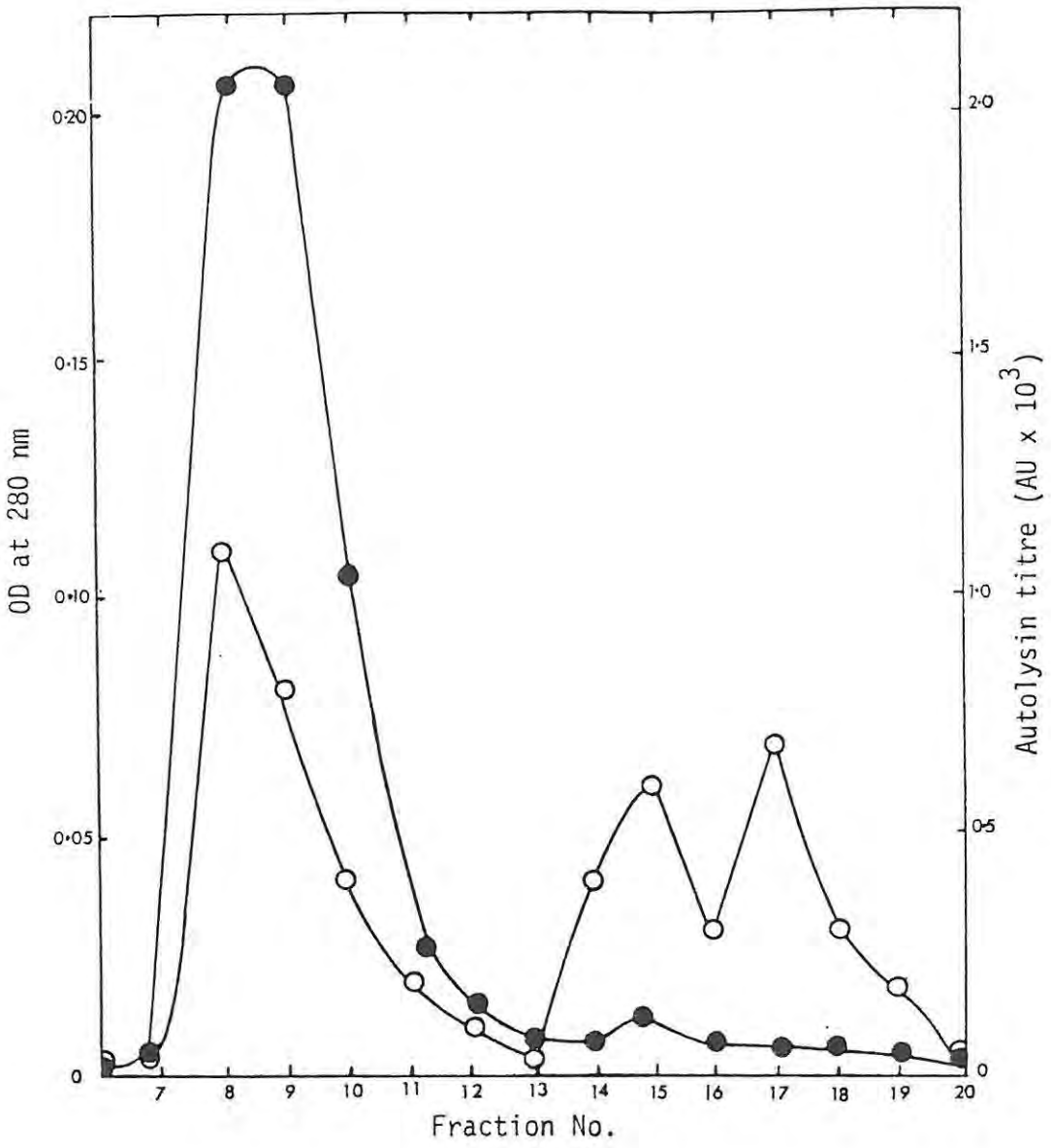


Fig 5.1 Autolysin titre (●) and protein concentration (O) after DEAE-column chromatography.

Table 5.1 Partial purification and specific activities of the *C. acetobutylicum* autolysin

	Total* activity (AU)	Total+ protein (mg)	% Recovery	Specific activity (AU mg^{-1})	Purification (x)
400 ml culture supernatant (CBM containing 15% (wt/vol) sucrose)	1.926×10^7	9.600×10^2	100	2.007×10^4	-
13 ml acetone-precipitate resuspended in sodium acetate buffer	1.664×10^7	2.600×10^2	86	6.400×10^4	3
19.5 ml DE-52 eluate in sodium acetate buffer	7.987×10^5	6.630	4	1.205×10^5	6

* measured using the well plate assay described in Chapter II

+ determined as described by Lowry *et al.* (1951)

as a cation at pH 6.0, and a zone of inhibition was detected between the origin and the cathode when electrophoresed agarose gels were overlayed with *C. acetobutylicum* in CBM (Fig 5.2).

5.3.4 Comparison of autolysin produced in CBM containing 5% (vol/vol) molasses medium or 15% (wt/vol) sucrose

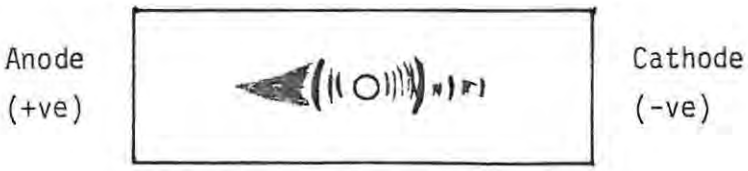
Partially purified autolysin was prepared from supernatants of CBM containing 5% (vol/vol) molasses medium or 15% (wt/vol) sucrose. After polyacrylamide disc gel electrophoresis, protein bands obtained from both preparations were compared and it was found that the same protein was present in each preparation. The proteins, when eluted from the gels, possessed autolysin activity and produced zones of inhibition when tested by the well plate assay.

5.3.5 Purification and molecular weight determination

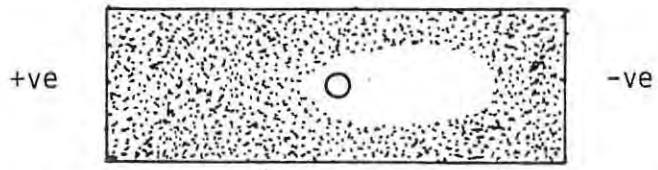
Preparative purification of the autolysin on polyacrylamide disc gels yielded a single protein band. This band, after elution from the unstained half of the gel, had autolysin activity and produced zones of inhibition in the well test. Carbohydrate staining of the complementary gels indicated that the autolysin was a glycoprotein. No lipid component was detected in the autolysin after staining with sudan black. The full purification procedure is illustrated in Fig 5.3. SDS-polyacrylamide gel electrophoresis of the concentrated autolysin after elution from preparative gels showed a single band, indicating that the autolysin had been purified to homogeneity (Plate 5.1). SDS-PAGE studies with proteins of known molecular weights established that the autolysin had a molecular weight of 28 000 (Fig 5.4).

Fig 5.2 Agarose gel electrophoresis of the autolysin

a) Concentrated crude autolysin added to the well and electrophoresed.

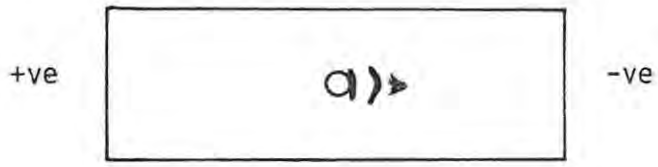


Stained for protein

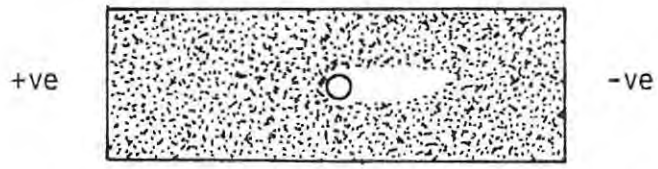


Overlayed with *C. acetobutylicum* in CBM

b) Partially purified autolysin added to the well and electrophoresed.

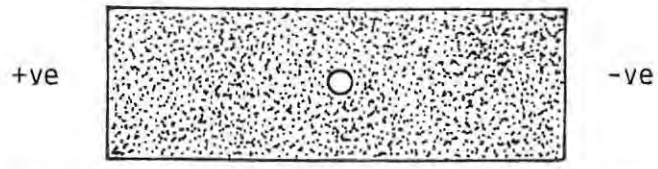


Stained for protein



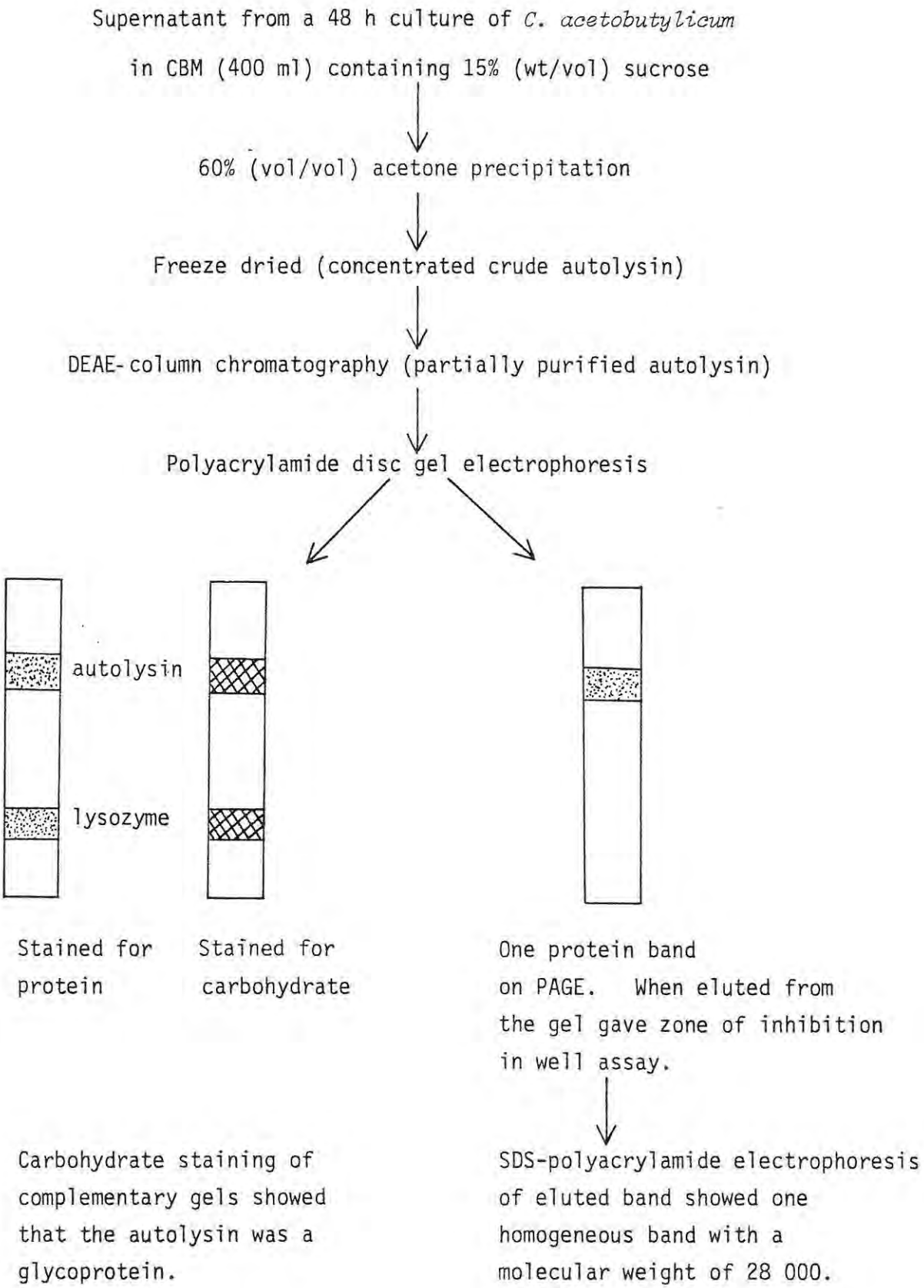
Overlayed with *C. acetobutylicum* in CBM

c) Heat-inactivated autolysin added to the well and electrophoresed.



Overlayed with *C. acetobutylicum* in CBM

Fig 5.3 Purification of the *C. acetobutylicum* autolysin



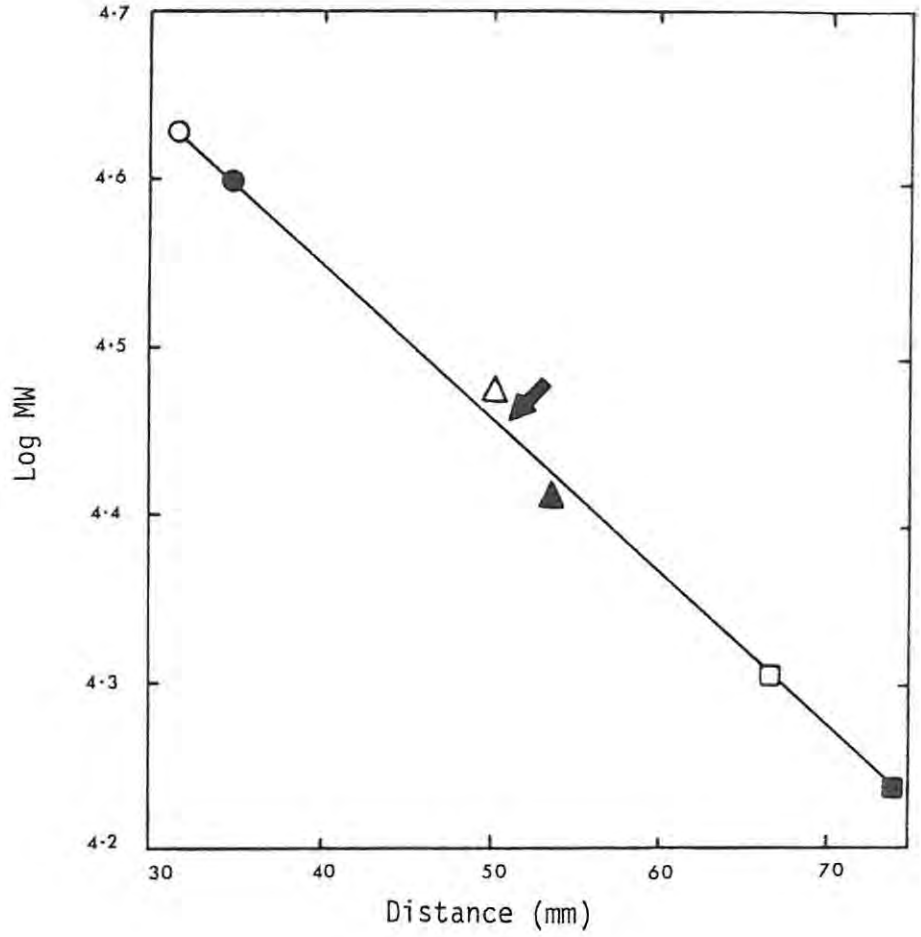


Fig 5.4 Estimation of the molecular weight (MW) of the *C. acetobutylicum* autolysin by SDS-PAGE. The protein markers used were ovalbumin, MW 43 000 (○); aldolase, MW 40 000 (●); carbonic anhydrase, MW 30 000 (△); chymotrypsinogen, MW 25 700 (▲); soybean trypsin inhibitor, MW 20 100 (□); myoglobin, MW 17 200 (■). The arrow indicates the position of the autolysin (MW 28 000).

5.4 DISCUSSION

Preparative purification of the extracellular autolysin on PAGE resolved only one band which had autolytic activity. This contrasts with the autolytic system of *C. botulinum*, as partially purified cell wall lysates have been shown to possess at least two autolysins (Kawata and Takumi, 1971). The autolytic cystem of *C. perfringens* also possesses two autolysins, an endo-β-N-acetylglucosaminidase and an endo-β-N-acetylmuramidase (Williamson and Ward, 1979). It is possible that *C. acetobutylicum* possesses more than one autolysin, but only one extracellular autolysin was detected under the conditions described. Some of the rod-shaped bacteria such as *E. coli* and *B. subtilis* have complex autolytic systems containing all three classes of autolysins (Coyette and Ghuysen, 1970; Kingan and Ensign, 1968). However, three bacterial species, *S. faecalis*, *L. acidophilus* and *A. crystallopoietes*, have been reported to contain only one autolysin, an N-acetylmuramidase (Coyette and Shockman, 1973). When the cell wall lytic activity in culture supernatants of a *Myxobacter* species was purified, only one extracellular lytic enzyme, an endopeptidase, was detected (Ensign and Wolfe, 1966).

Although the mode of action of the autolysin produced by *C. acetobutylicum* was different to that of lysozyme, these two lytic enzymes have some properties in common. They both possess characteristics of alkaline proteins, are more stable at acid pHs, and are glycoproteins, (Jollès, 1960; Young, 1963). During studies involving molecular weight determinations of various proteins by gel filtration, Andrews (1965) observed that some glycoproteins do not fit a V_e (elution volume) versus log MW curve

as shown by carbohydrate-free globular proteins. It was suggested that some glycoproteins have more expanded structures than those of typical globular proteins, which explained why some glycoproteins were excluded from gels with the widest pore size available. The *C. acetobutylicum* autolysin was excluded by Sephadex G-100 and G-200, although its molecular weight was only 28 000 daltons on SDS-PAGE. The discrepancy in molecular weight of 28 000 and >100 000 is too large to be explained by Andrews' findings alone. Brown *et al.* (1970) could not purify the *B. subtilis* N-acetylmuramyl-L-alanine amidase by Sephadex G-100 or G-200, and the bulk of the enzyme activity was restricted to the void volume in both cases. They suggest that the inability of the enzyme to penetrate gels of wide pore sizes was related to the teichoic acid which remained bound to the enzyme. Perhaps the partially purified autolysin of *C. acetobutylicum* was bound to some cell wall component which was only removed by PAGE.

The molecular weights of autolysins appear to vary considerably, although several autolysins have molecular weights of 30 000 or less (Singer *et al.*,1972). An N-acetylmuramic acid -L-alanine amidase from *B. subtilis* W-23 was found to have a molecular weight of 51 000 (Lindsay and Glaser, 1976), and an endo-β-N-acetyl-glucosaminidase produced by *S. aureus* has a molecular weight of 70 000 (Wadström and Hisatsune, 1970a). The extracellular endopeptidase of a *Myxobacter* species was a small molecular weight protein of 8 700 (Ensign and Wolfe, 1966), in contrast to an N-acetylmuramyl-L-alanine amidase from *S. aureus* H which had a molecular weight of 800 000 to 1 000 000, when estimated by sucrose gradient centrifugation, or Sephadex and Sepharose column chromatography (Singer *et al.*, 1972). Such a high molecular weight seems most unlikely, and the authors suggest that this large intracellular amidase may be a precursor of a smaller functional wall enzyme.

CHAPTER VI

CONCLUSIONS AND GENERAL DISCUSSION

The aim of this study was to identify, isolate and characterise the bacteriocin-like substance produced by *C. acetobutylicum* in molasses medium. The bacteriocin-like substance was identified as an autolysin with a molecular weight of 28 000. Although this is not the first report of autolytic activity in *Clostridium* species, it appears to be the first clostridial autolysin to have been purified.

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 phase when the solvent levels were maximal. It is suggested that the inhibition of autolysin activity at the stage when solvent production is maximal may result in increased solvent yields. A dialysable component of molasses (not sucrose or glucose) was required for autolysin production. However, as molasses is composed of such a wide variety of substances, it would be a major task to isolate and identify the component that induces autolysin production in molasses medium.

An alternative approach would be to gain some insight into the control of autolysin production and release. Autolytic enzyme systems may be regulated at several levels including autolysin synthesis, activation, transport of enzyme or activator (or both) and substrate effect (Sayare *et al.*, 1972). Any one or a combination of these mechanisms may be involved in the regulation of the *C. acetobutylicum* autolysin. Recent

studies on the inhibitory effects of bacterial lipids and lipoteichoic acid on the extracellular N-acetylmuramyl-L-alanine amidase of *S. aureus* indicated that the bacterial cardiolipin, which is a main component of cellular lipid in *S. aureus* cells, may participate in the regulation of this autolysin (Suginaka *et al.*, 1979). Similar effects have been demonstrated for the N-acetylmuramidase of *S. faecalis* using intact cells of *S. faecalis* (Cleveland *et al.*, 1976a; Cleveland *et al.*, 1976b; Carson and Daneo-Moore, 1980). Shungu *et al.* (1979) suggest that the increased levels of both cellular lipoteichoic acid and lipids were responsible for the decrease in autolytic activity by mutants of *S. faecalis* ATCC 9790 (*S. faecium*). It would be useful to determine if this inhibitory effect can be demonstrated in *C. acetobutylicum*.

The isolation and analysis of *lyt*$^-$ mutants is of prime importance to establish the function(s) of the autolysin, and in particular to determine if there is any correlation between autolysin activity, solvent production and sporulation. Solvent production in molasses medium is associated with the development of presporing cells called "clostridial forms" (Barber, 1977). Although this study has not revealed any direct evidence for the involvement of the extracellular autolysin in sporulation, other sporulating species require the activity of lytic enzymes for spore release and spore germination (see Introduction). It is interesting to note that "fruiting bodies" of *C. acetobutylicum* which mainly consist of sporulating cells and free spores (see Appendix A) produced large amounts of autolysin, which could be demonstrated by the overlay method described in Chapter IV. Whether this is the same autolysin detected at the end of exponential phase in liquid medium has still to be established.

It is also important to determine whether or not the autolysin is

associated with the ability of the cells to become competent for transformation, as this may be critical for the success of present genetic studies on *C. acetobutylicum*.

An unusual feature of the *C. acetobutylicum* autolysin was its activity spectrum. Although it had a broad activity spectrum against some species, it was also shown to be strain specific within a particular species (eg *C. perfringens* and *C. sporogenes*; Table 3.2). This specificity may be explained by variations in cell wall chemistry between one strain and another. It has been found that when choline was replaced by ethanolamine in cell walls of *D. pneumoniae*, the walls became resistant to the action of its autolytic enzyme (Mosser and Tomasz, 1970). The lytic activity of the autolysin(s) of *C. botulinum* type A on SDS-treated walls of *C. botulinum* and *C. perfringens* indicated a relatively high specificity (Kawata and Takumi, 1971). Cell walls of *C. botulinum* type A and B strains were sensitive to the autolysin, whereas walls of *C. botulinum* type E and C and *C. perfringens* were resistant. The authors point out that the cell walls of *C. perfringens* contained glycine as a component of the peptidoglycan, whilst *C. botulinum* type A did not contain this amino acid.

Further studies on the *C. acetobutylicum* autolysin should also include determination of its site of action on cell walls by analysis of cell wall degradation products after autolysin action. Once it is known which linkages the autolysin hydrolyses, the enzyme may be a useful tool, along with other fully characterised lytic enzymes, for determining the structure of the peptidoglycan of *C. acetobutylicum*.

APPENDIX A

THE FORMATION OF SIMPLE FRUITING BODY-LIKE STRUCTURES ASSOCIATED WITH SPORULATION UNDER AEROBIC CONDITIONS IN *CLOSTRIDIUM ACETOBUTYLICUM*

The following work, which formed part of a general program on *Clostridium acetobutylicum*, has been published in the Journal of General Microbiology (1980) 116, 195-200.

INTRODUCTION

The only group of bacteria known to produce differentiated fruiting bodies are the myxobacteria (Dworkin, 1966; 1973). Fruiting bodies produced by the myxobacteria vary from simple mounds of cells formed by *Myxococcus* to elaborate lobed forms found in the higher myxobacteria *(Polyangiaceae)*. Within the fruiting body, the individual vegetative cells lose their gliding motility and become resting cells (myxospores) which in some species are contained within cysts, and in others occur as an undifferentiated mass. Although a number of other bacteria do show rudimentary multicellular organization (eg *Actinomycetes*; Chater & Hopwood, 1973) no other group is known to produce fruiting body-like structures.

During a study of bacteriocin production by *Clostridium acetobutylicum* (Barber *et al.*, 1979), we observed the formation of elongated macroscopic structures resembling fruiting bodies. We describe here the production and nature of these structures.

METHODS

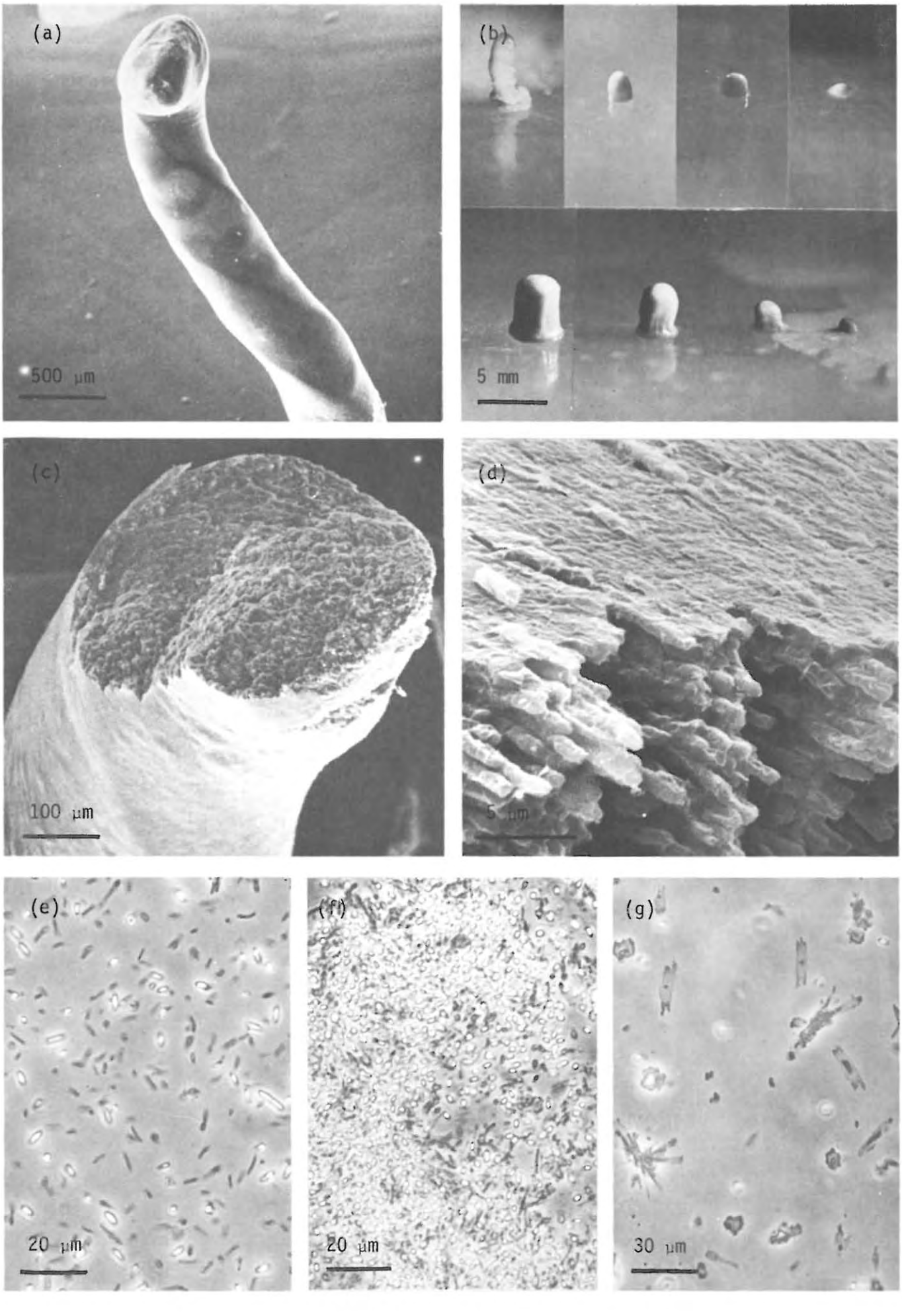
The *Clostridium acetobutylicum* strain was supplied by National Chemical Products Ltd., Germiston, South Africa. It was grown on the potato medium of Barber *et al.* (1979). Samples (0.5 g) of soil spore cultures of *C. acetobutylicum* were first heat-shocked in 3 ml 0.85% (wt/vol) NaCl at 70°C for 2 min before being inoculated into potato broth. Cultures were incubated at 34°C for 18 h and plated on potato agar. Duplicate plates were incubated in BBL GasPak jars for a further 48 h. One set of plates was then removed, and incubation was continued aerobically at 20°C. The other set was retained under anaerobic conditions at 20°C. Colonies from each set of plates were examined macroscopically and microscopically every 2 d over a period of 28 d and spore and viability counts were carried out. Wet-mounted specimens were observed with a Zeiss photomicroscope fitted with phase contrast optics. Metal-shadowed specimens were examined with a Jeol JSN U3 scanning electron microscope. Photographs of the macroscopic structures were taken with a Pentax SP 500 camera fitted with close-up rings.

RESULTS

Aerobic fruiting body-like structures

Plates containing isolated colonies of *C. acetobutylicum* were grown under anaerobic conditions for 2 d and then incubated aerobically for up to 4 weeks. Under aerobic conditions the colonies developed into unique elongated macroscopic structures which reached a height of > 10 mm over 3 to 4 weeks (Fig 1a, b). These structures only

Fig 1. Scanning electron micrographs (a to d) and photomicrographs (e to g) showing the morphology and structure of elongated fruiting body-like structures produced by *C. acetobutylicum*. (a) Elongated structure with a helical twist; (b) development of elongated structures over a 4 week period (right to left); (c,d) fractured elongated structures showing the tough pliable sheath; (e) vegetative rods and sporulating cells from the basal region of the structure; (f) trunk region showing free spores (refractile bodies) and a few cells which were either granulated or lysed in appearance; (g) sheath material dispersed in an aqueous solution showing lysed rods surrounded by extracellular refractile material.


(a)
500 µm
(b)
5 mm
(c)
100 µm
(d)
5 µm
(e)
20 µm
(f)
20 µm
(g)
30 µm

developed in isolated colonies under aerobic conditions,and crowding inhibited their development. The final shape of the macroscopic structures depended on the size of the colony at the time of transfer to aerobic conditions. Small colonies produced tall slender structures with a distinct helical twist (Fig. 1a), whereas large colonies produced shorter thicker structures.

Dissection and microscopic examination of the elongated structures indicated that they were associated with sporulation. Healthy rod-shaped vegetative cells and sporulating cells were restricted to the basal region of the structure (Fig. 1e). The trunk region contained mainly free spores (refractile bodies) and no sporulating cells were observed (Fig. 1f). There were only a few cells in the trunk region and these were either granulated or lysed in appearance.

Vegetative rods and spores within the macroscopic structure were surrounded by an extracellular substance which formed a tough pliable sheath around the colony. The difference between the surface layer (sheath) and the contents of the elongated colony was clearly seen under the scanning electron microscope in colonies which had been fractured during preparation (Fig. 1c, d). Closely packed vegetative rods near the surface appeared to be covered by a fibrous sheath (Figs. 1d and 2a).

The difference between the surface of an elongated structure and the surface of an anaerobic colony is shown in Fig. 2 (a,b). In the anaerobic colony , individual rod-shaped cells were clearly visible, whereas in the elongated structure the cells were covered by a fibrous

sheath. Material from the sheath was dispersed in aqueous solution and observed microscopically. Most of the individual rods appeared to have lysed and were surrounded by an extracellular layer of refractile material (Fig. 1g).

It was possible to scrape out the soft inner contents of an elongated structure so that only the tough pliable outer sheath remained. After the removal of the inner contents the sheath became hard and brittle. When the surface of the elongated structure was damaged by cutting away the sheath and exposing the inner contents to air, a new tough pliable sheath was rapidly reformed over the damaged area.

Preliminary studies on the chemical composition of the sheath material indicated that it was proteinaceous. It was degraded by the proteolytic enzyme Pronase (Miles-Seravac), but was unaffected by cellulysin (Calbiochem) or lysozyme (Miles-Seravac). It was soluble in sodium dodecyl sulphate but insoluble in water, hot alcohol, chloroform, ether, acetone, butanol, 2M HCl or 5M NaOH.

A solution of sheath material in sodium dodecyl sulphate absorbed ultraviolet light (280 nm) and could be assayed by the Folin-Ciocalteau test for proteins.

Aerobic development of fruiting body-like structures

The development of the elongated structures was related to the amount of sporulation which occurred within the colony. Sporulation in *C. acetobutylicum* grown on potato agar, under both aerobic and anaerobic

conditions, was dependent on the crowding of the colonies on the surface of the plates. The closer the colonies were together, the lower the proportion of cells which sporulated. Crowded colonies did not produce the elongated sporulating structures.

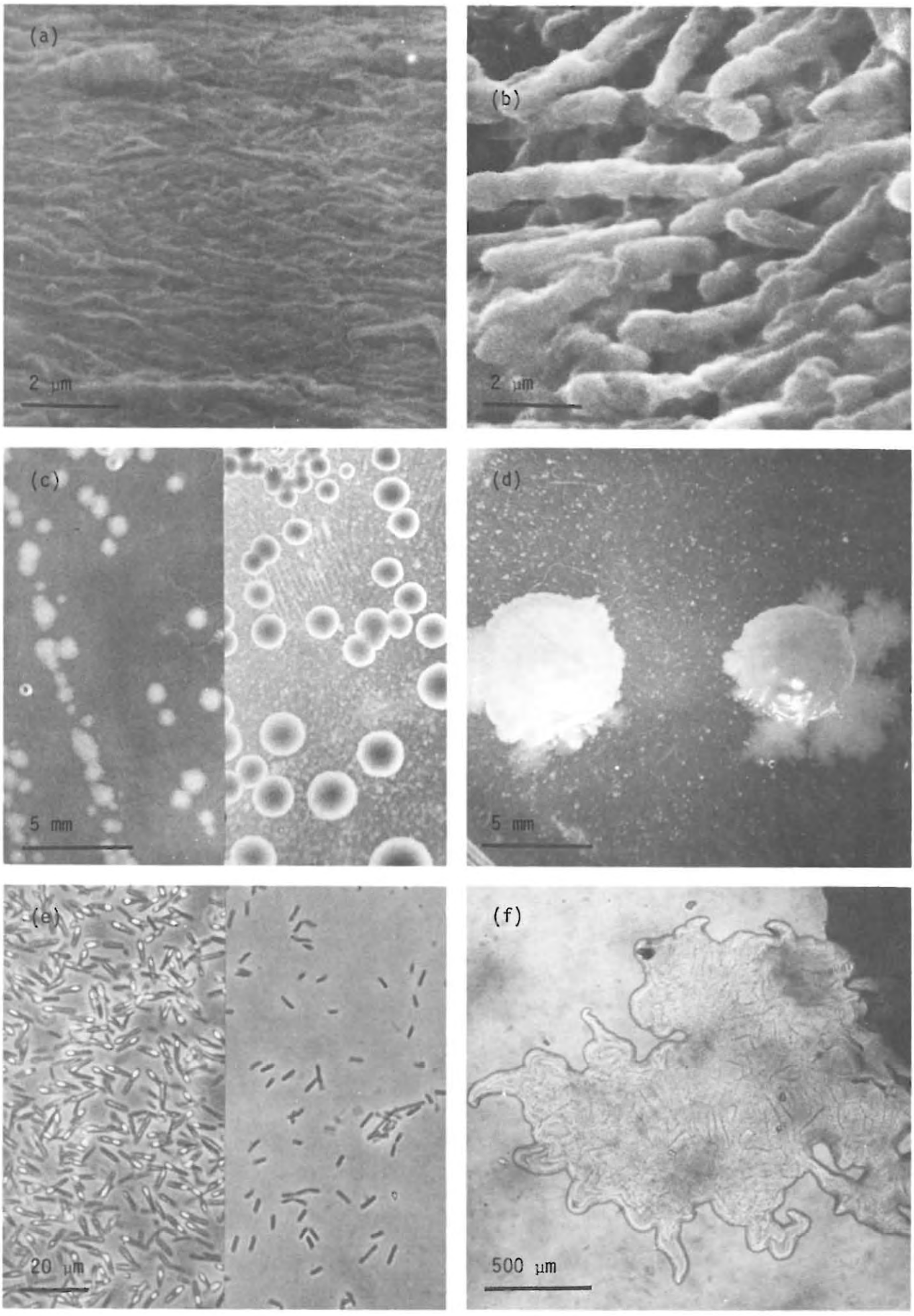
After 48 h growth under anaerobic conditions, isolated colonies were greyish white, very flat with a diffuse margin (Fig. 2c), and contained only vegetative rods. After 48 h the cells within the isolated colonies began to sporulate under both anaerobic and aerobic conditions. The area of sporulation developed from the centre of aerobic colonies and was easily visible as a thicker and more opaque zone (Fig. 2c). This zone gradually spread outwards until after 3 to 4 d the entire colony was uniformly thick and opaque. The colony continued to develop vertically and over 14 d the proportion of rods which were healthy in appearance remained constant at about 30% of the total cell population. Sporulating cells accounted for about 60% of the cells. After 14 d the number of rods and sporulating cells decreased, with a concomitant increase in the number of free spores (about 30%) and granulated or lysed rods (about 60%).

In crowded colonies little growth occurred after 48 h. The colonies did not increase in size and remained thin and greyish white. After 6 d about 80% of the cells were granulated or lysed rods, the remainder being vegetative rods and sporulating cells. Only a few free spores were present after 10 d and after 21 d about 10% of the cells were free spores.

Anaerobic colony development

Under anaerobic conditions, isolated colonies produced typical flat

Fig 2. Scanning electron micrographs (a,b) and photomicrographs (c to f) showing the morphology and structure of aerobic and anaerobic colonies of *C. acetobutylicum*. (a) Surface of an aerobic elongated structure; (b) surface of an anaerobic colony; (c) greyish white flat colonies after 48 h growth under anaerobic conditions (left) and the development of the thicker central sporulating zone after 72 h (right); (d) anaerobic colonies showing the spreading fan of vegetative cells; (e) sporulating cells from the centre of anaerobic colonies (left) and vegetative rods from the fan region of an anaerobic colony (right); (f) spreading fan of an anaerobic colony.


(a)
2 μm
(b)
2 μm
(c)
5 mm
(d)
5 mm
(e)
20 μm
(f)
500 μm

spreading colonies which continued to increase in diameter over a period of 28 d. After 14 d, when the colonies were about 5 mm in diameter, large spreading fans grew out from the margins (Fig. 2d, f); these consisted entirely of vegetative rods (Fig. 2e) which contrasted with the actively sporulating cells making up the rest of the colony (Fig. 2e).

DISCUSSION

Under aerobic conditions, isolated colonies of the anaerobe *C. acetobutylicum* produced an elongated structure which contained a high proportion of sporulating cells and free spores enclosed in a tough pliable sheath. This structure seemed to be intermediate between the ordinary bacterial colony and the elaborate multicellular fruiting bodies formed by myxobacteria, and could be regarded as an example of primitive multicellular differentiation.

We have recently examined two other strains of *C. acetobutylicum* (ATCC 824 and ATCC 10132) but have not as yet observed the production of fruiting body-like structures. However, these strains do not grow as well as our strain on potato agar.

The production under aerobic conditions of an extracellular proteinaceous substance, which rapidly hardens on exposure to air to form a sheath, is interesting. An obvious suggestion is that it acts as a protective barrier against oxygen. *Clostridium acetobutylicum* and most other clostridia are fairly strict anaerobes. Free oxygen inhibits growth but *C. acetobutylicum* can grow in liquid culture in the presence of air, provided a sufficiently low oxidation reduction potential is established

in the medium (Morris & O'Brien, 1971; O'Brien & Morris, 1971). *Clostridium acetobutylicum* will not normally grow on solid media under aerobic conditions. *Clostridium histolyticum*, *C. tertium* and *C. carnis* are exceptional in being able to grow aerobically to a limited extent (Wilson & Miles, 1975), and small but visible colonies are formed on blood agar plates but no spores are produced.

Under anaerobic conditions on potato agar, *C. acetobutylicum* produced flat spreading colonies with fans of vegetative cells. This contrasts with the report of Hastings (1978),who observed that on a molasses agar (under anaerobic conditions) colonies darken slightly and grow in the form of a truncated cone, 2 to 3 mm high, with a concave top. We have only observed the production of the elongated structures, which are not truncated and do not have a concave top, under aerobic conditions.

APPENDIX B

GENERAL METHODS

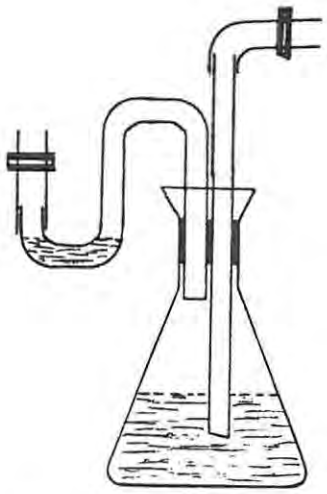
Spore preparation and germination

Spores of *C. acetobutylicum* P262 for general use were prepared from sporulating colonies off CBM plates containing 5% (vol/vol) molasses medium. The spores were washed several times in sterile distilled H_2O, and finally resuspended in the same. The spore suspension was diluted until the addition of 0.1 ml heat-shocked spores to 10 ml CBM gave an OD of 0.2 to 0.5 within 12 to 18 h, as read on a Corning colorimeter. The cell suspensions in this OD range were in exponential phase, and were used to inoculate the test media.

Spores were germinated by heat shocking at 70-75^{o}C for 2 min and cooled rapidly on ice, or in cold H_2O, for 45-60 sec.

Culture preparation for cell walls and autolysin production

Large volumes of exponential phase cells for cell wall preparations were obtained by adding 4 ml of a cell suspension at an OD of 0.6, to a 500 ml conical flask (fitted with a flushing mechanism) containing 400 ml CBM that had been perfused with H_2 and CO_2. The culture was then incubated at 37^{o}C for 9 h to obtain an OD reading of 0.5 to 0.7. The cells were harvested by centrifugation at 10 000 rev min^{-1} at 4^{o}C for 20 min using a Sorvall RC-5 centrifuge.



Anaerobic culture flask

The same procedure was carried out to obtain large volumes of autolysin in CBM containing 15% (wt/vol) sucrose, except that the culture was incubated for 36-48 h and the supernatant was collected.

MEDIA

All media was sterilised by autoclaving at 121°C for 20 min. Heat sensitive solutions were sterilised by Seitz filtering. Agar plates were made by adding 1.5% (wt/vol) agar (Difco) to broth preparations.

Factory fermentation media

A_1 Potato medium

Maggi mash (potato flakes)	40.0 g
Glucose	6.0 g

$CaCO_3$	2.0 g
Tap H_2O	1.0 ℓ

The medium was autoclaved for 15 min to solubilise the potato flakes, then filtered through three layers of cheese cloth. The filtrate was collected and dispensed before reautoclaving.

A_2,B and C Molasses medium

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
Tap H_2O	1.0 ℓ

Control fermenter medium (CFM)

Molasses	134.0 g (or amount required to give 6.5% total invert sugar)
$(NH_4)_2SO_4$	2.0 g
$CaCO_3$	1.0 g
Magou	1.0 g
Tap H_2O	1.0 ℓ

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

All factory fermentation media were used immediately after preparation, and did not require perfusion with H_2 or CO_2.

Anaerobic media

Preparation of media

Pre-reduced and anaerobically sterilised media was prepared using the methods of Hungate (1969) and Moore (1966). Most of the oxygen was driven off by heating the unsterile media. Broths were dispensed into Hungate tubes in 10 ml aliquots and perfused with H_2 and CO_2 before autoclaving. Media containing agar was autoclaved in bulk, poured into petri dishes, and stored in anaerobic jars or boxes with silica gel in muslin bags.

Clostridium basal medium agar (O'Brien and Morris, 1971)

Glucose	10.0 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.01 g
$FeSO_4.7H_2O$	0.01 g
Caesin hydrolysate	4.0 g
p-Aminobenzoic acid stock solution	1.0 ml
Thiamine HCl stock solution	1.0 ml
Biotin stock solution	1.0 ml
Distilled H_2O	1.0 ℓ

The required volume of K_2HPO_4 (c 2.5 ml) and KH_2PO_4 (c 1 ml) stock solutions were added to give a pH of 6.8-7.0. This medium was used for making CBM

well plates by addition of 1% (wt/vol) agar and normal plates by addition of 1.5% (wt/vol) agar.

Clostridium basal medium (CBM)

Glucose	10.0 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.01 g
$FeSO_4.7H_2O$	0.01 g
Caesin hydrolysate	4.0 g
p-Aminobenzoic acid stock solution	1.0 ml
Thiamine HCl stock solution	1.0 ml
Biotin stock solution	1.0 ml
Cysteine HCl	0.5 g
$NaHCO_3$	1.0 g
Yeast extract (Difco)	4.0 g
Resazurin	1.0 ml
Distilled H_2O	1.0 ℓ

Brain heart infusion (BHI)

BHI (Difco)	37.0 g
Yeast extract (Difco)	5.0 g
Distilled H_2O	1.0 ℓ

The required % of agar was added, the medium was autoclaved, and the following stock solutions were added prior to pouring:

Cysteine HCl stock solution	10.0 ml

Na_2CO_3 stock solution	20.0 ml
Hemin-menadione stock solution	10.0 ml

Semi-solid anaerobic indicator

Sodium thioglycollate	0.25 g
di-Sodium tetraborate (borax)	0.4 g
Agar	0.7 g
Resazurin stock solution	1.0 ml
Distilled H_2O	100 ml

The indicator was steamed for 30 min, dispensed into Hungate tubes in 5 ml aliquots, and perfused with H_2 and CO_2 immediately.

STOCK SOLUTIONS FOR ANAEROBIC MEDIA

All stock solutions were stored at 4^oC.

p-Aminobenzoic acid (PABA) stock solution

PABA	0.1 gm
Distilled H_2O	100 ml

Thiamine HCl stock solution

Thiamine HCl	0.1 gm
Distilled H_2O	100 ml

Biotin stock solution

Biotin	200 µg
Distilled H_2O	100 ml

K_2HPO_4 stock solution

K_2HPO_4	25.0 g
Distilled H_2O	100 ml

KH_2PO_4 stock solution

KH_2PO_4	12.5 g
Distilled H_2O	100 ml

Cysteine HCl stock solution (prepared freshly each time)

Cysteine HCl	1.0 g
Distilled H_2O	20.0 ml
Autoclaved	

Sodium carbonate stock solution

Na_2CO_3	20 g
Distilled H_2O	100 ml
Autoclaved	

Hemin-menadione stock solution

Hemin	50 gm
1N NaOH	1 ml
Distilled H_2O	100 ml
Autoclaved	
Menadione stock solution	1 ml

Menadione stock solution

Menadione	0.1 g
Ethanol (95%)	20 ml
Filter-sterilised	

Resazurin stock solution

Resazurin	20 mg
Distilled H_2O	100 ml

GENERAL STOCK SOLUTIONS

Scintillation cocktail

Primary fluor diphenyl oxazole PPO	16.7 g
Toluene	1.0 ℓ

Stored in the dark. Diluted 10 x with toluene before use.

GENERAL BUFFERS

The buffers used were those listed by Gomori (1955).

SDS-SLAB GEL ELECTROPHORESIS

(Laemmli, 1970)

Resolving gel buffer stock solution (1M Tris-HCl pH 8.8)

Tris	60.6 g
conc HCl	7.3 ml
Distilled H_2O	500 ml

Stacking gel buffer stock solution (1M Tris-HCl pH 6.8)

Tris	60.6 g
conc HCl	41.0 ml
Distilled H_2O	500 ml

Bath buffer stock solution

Tris	30.3 g
Glycine	144.1 g
SDS	10.0 g
Distilled H_2O	1.0 ℓ

Diluted x 10 before use.

Acrylamide stock solution

Acrylamide	150 g
bis acrylamide	4.0 g
Distilled H_2O	500 ml

This gives an acrylamide : bis ratio of 30 : 0.8. For 75 : 1 use only 2 g bis.

Dissociation buffer

SDS	5.0 g
Mercaptoethanol	5.0 ml
Glycerol	7.5 ml
Bromophenol blue (0.2%)	2.5 ml
1M Tris-HCl pH 6.8	6.3 ml
Distilled H_2O	28.7 ml

Slab gel formulations

Resolving gel

	6%	7%	8%	9%	10%	11%	12%	15%
Acrylamide : bis stock solution	16.0	18.7	21.4	24.0	26.7	29.4	32.0	40.0
1M Tris-HCl pH 8.8	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Distilled H_2O	29.2	26.5	23.8	21.2	18.5	15.8	13.2	5.2
10% SDS (wt/vol)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
1.5% $(NH_4)_2SO_4$ (wt/vol) prepared freshly	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
TEMED (µl)	20	20	20	20	20	20	20	20
total	80 ml							

Other % acrylamide calculation: $\frac{\%\ \text{required}}{30} \times 80 = \text{ml}$.

ml acrylamide + ml H_2O = 45.2

Stacking gel

	2.5%	3.0%	3.5%	4.0%	4.5%	5.0%
Acrylamide : bis stock solution	1.25	1.5	1.75	2.0	2.25	2.5
1M Tris-HCl pH 6.8	1.9	1.9	1.9	1.9	1.9	1.9
Distilled H_2O	10.0	9.75	9.5	9.25	9.0	8.75
80% glycerol (vol/vol)	1.0	1.0	1.0	1.0	1.0	1.0
10% SDS (wt/vol)	0.15	0.15	0.15	0.15	0.15	0.15
1.5% $(NH_4)_2S_2O_8$ (wt/vol) prepared freshly	0.7	0.7	0.7	0.7	0.7	0.7
TEMED (µl)	20	20	20	20	20	20
total	15.0 ml					

After electrophoresis the gels were removed from the plates, stained for 2 h in coomassie brilliant blue and then destained.

Staining solution

Methanol	45 ml
Glacial acetic acid	10 ml
Coomassie brilliant blue	0.2 g
Distilled H_2O	45 ml

The dye was dissolved in a small amount of methanol and filtered. Then the remaining methanol, H_2O and acetic acid were added.

Destaining solution

Methanol	450 ml
Glacial acetic acid	70 ml
Distilled H_2O	480 ml

CHEMICALS AND REAGENTS

Chromatographic chemicals

Blue dextran 2000	Pharmacia Fine Chemicals
Sephadex G-100 and G-200	" " "
DEAE-cellulose DE-52 (preswollen)	Whatman
CM-cellulose	Sigma
P11 cellulose phosphate	Whatman

Stains and indicators

Sudan black	BDH Chemicals
Amido black	Merck
Coomassie brilliant blue	"
Resazurin	BDH Chemicals

Enzymes and protein markers

Luciferin-luciferin-luciferase FLE-50	Sigma
Lysozyme	"
Pronase	Miles Laboratories

Pepsin	Merck
Proteinase K	Boehringer Mannheim
Trypsin	Difco
Cytochrome C	BDH

Vitamins

Hemin chloride	Fluka AG,
Menadione-sodium bisulphate	Merck
Biotin	"
Thiamine HCl	Sigma

Radiochemicals

^{3}H adenine (25 Ci mmol^{-1})	Amersham Radiochemical Centre
^{14}C leucine (146 Ci mmol^{-1})	" " "

Molecular weight markers

Phosphorylase b	Pharmacia Fine Chemicals
Bovine serum albumin	" " "
Ovalbumin	" " "
Aldolase	" " "
Carbonic anhydrase	" " "
Trypsin inhibitor	" " "
Chymotrypsinogen	Miles-Seravac
Myoglobin	Sigma

General chemicals and their abbreviations

Tris(hydroxymethyl)-amino-methane (Merck)	Tris
Ethylenediaminetetraacetic acid (Sigma)	EDTA
Sodium dodecyl sulphate (Hopkin & Williams)	SDS
Adenosine-5'-triphosphate (Sigma)	ATP
Trichloroacetic acid "	TCA
N,N,N',N'-Tetramethylethylenediamine	TEMED

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