GENETIC STUDIES ON
CLOSTRIDIUM ACETOBUTYLICUM

by

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ABSTRACT

The aim of this study involved the characterisation of the cellulolytic properties of Clostridium acetobutylicum and the development of a genetic transfer system for this organism. The production of a carboxymethyl cellulase and a cellobiase by C. acetobutylicum was demonstrated. In liquid medium the carboxymethyl cellulase was induced by molasses, and it was not repressed by glucose. Optimum carboxymethyl cellulase activity occurred at pH 4.6 and 37°C.

Optimum conditions for autolysis and autoplast formation in C. acetobutylicum were defined. Autolysis-deficient mutants which produced less autolysin than the parent strain were isolated. Growth of the P262 strain and the lyt-1 mutant was inhibited by the same concentrations of wall-inhibiting antibiotics. Electrophoresis of cell-free autolysates indicated the presence of two autolytic enzymes. Electron microscopy of autolysing cells suggest that most of the active autolysin is located at various sites along the peripheral wall of the cell.

Methods for the production and regeneration of viable C. acetobutylicum protoplasts were developed. The protoplasts were stable in sucrose solutions containing magnesium and calcium and 90% regeneration of stable protoplasts was obtained.

It was not possible to transform _C. acetobutylicum_ protoplasts with plasmid DNA from other _Clostridium_ strains or with plasmids from other Gram-positive species. Phage DNA, however, was taken up and whole phage were produced after the protoplasts infected with phage DNA were regenerated to the bacillary form. The transfection system will be utilized for the development of a vector and a transformation system for _C. acetobutylicum_.

CHAPTER 1

GENERAL INTRODUCTION

The food processing, chemical and pharmaceutical industries are the three major users of fermentation today, the food industry being the first to exploit micro-organisms to produce alcoholic beverages and fermented foods. A number of factors have been identified in a recent review on fermentation technology (Impacts of Applied Genetics, 1981) which may influence the introduction of fermentation processes to replace present chemical synthetic processes. These include:

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

Each of these factors has tended to accelerate the application of fermentation.

The first step in the development of a novel fermentation system is finding a suitable organism. Until recently this selection was confined to organisms already producing the required product. However, through genetic manipulation strains can be engineered and micro-organisms can be made to produce substances beyond their natural capacities. Before embarking on a novel fermentation process, one should consider:

1. Whether a biological process can produce a particular product; and
2. What micro-organism has the greatest potential for production and how the desired characteristics can be engineered for it (Impacts of Applied Genetics, 1981).

One should also note that despite the many genetic manipulations that are theoretically possible, there are several notable technical limitations:

1. Genetic maps - the identification and location of desired genes on various chromosomes have not been constructed for most industrially useful micro-organisms.
2. Genetic systems for industrially useful micro-organisms, such as availability of useful vectors are at an early stage of development.
3. Physiological pathways - the sequence of enzymatic steps leading from a raw material to the desired product, are not known for many chemicals. Much basic research is necessary to identify all the steps.

4. Finally, the problems are further increased when the genes in question have not yet been identified.

As a consequence of these limitations it is likely that genetic engineering will initially be applied to the development of capabilities that require the transfer of only one or a few identified genes.

Recombinant DNA methods, particularly those applied to the production of the interferons, may well represent the next great advance in clinical medicine and in the pharmaceutical industry (Aharonowitz and Cohen, 1981).

Although the majority of cloning experiments at the present time have utilized *Escherichia coli* as the host vector, this host is not particularly suited to the large scale production of enzymes, proteins or chemical feedstocks. *Clostridium acetobutylicum*, on the other hand, has potential for large scale fermentations and may be useful for the exploitation of genetic engineering. Features which make it suitable for fermentation include:

1. The organism is non-toxic and non-pathogenic.

2. Being an anaerobe, it is suitable for large scale fermentation as no expensive aeration system is required.

3. The organism is non-exacting nutritionally and can utilise a number of different cheap substrates.

4. The organism shows rapid growth under industrial conditions.

5. As a sporeformer the organism is easily maintained as a spore suspension.

6. The organism has a long history as a fermentation organism in the acetone-butanol-ethanol (ABE) process.

7. As extracellar proteins are produced during growth, the appropriate genes that influence excretion of products are present.

8. Finally, a single strain could be used for a variety of fermentations provided the development of a genetic system allowed the introduction of useful foreign genes into the strain. It is worth noting that _C. acetobutylicum_, being an anaerobe conserves _c_. 70% of the energy content of glucose fermented as solvents (Lonz and Moroira, 1980) and, therefore, any fermentation system using _C. acetobutylicum_ must want solvents as products in addition to any cloned gene product.

The most advanced applications of genetic manipulation today, in terms of technological sophistication and commercial development, are in the field of hormones. The capacity to synthesize proteins through genetic engineering has stemmed mainly from attempts to prepare human peptide hormones. A totally synthetic DNA sequence was inserted into an E. coli plasmid which led to the production of a 14 amino acid polypeptide sequence corresponding to that of somatostatin, a hormone produced in the brain (Itakura et al., 1977). The knowledge of somatostatin's amino acid sequence made the experiment possible, and the existence of sensitive assays allowed expression of the hormone to be detected. Somatostatin is one of about 20 recognised small human polypeptides that can be made without difficulty from chemical synthesis. Some small peptides that may justify the development of a biosynthetic process of production are (Impacts of Applied Genetics, 1981):

(a) A 7 amino acid sequence known as MSH/ACTH 4-10, which is reputed to influence memory, concentration and other psychological behavioural effects.

(b) Both cholecystokinin (33 amino acids) and bombesin (10 amino acids) which have been shown to suppress appetite. There is a large market for anti-obesity agents.

(c) Several hormones, such as somatostatin, which are released by nerves in the hypothalamus of the brain to stimulate or inhibit the release of hormones by the pituitary gland.

(d) Calcitonin (32 amino acids) which is currently the largest polypeptide produced by chemical synthesis for commercial pharmaceutical use. It is useful in pathologic bone disorders.

(e) Adrenocorticotropic hormone (ACTH) (39 amino acids) which promotes and maintains the normal growth and development of the adrenal glands.

Higher molecular weight polypeptides which cannot be made practically by chemical means and must be extracted from human or animal tissue can now also be manufactured by fermentation using genetically engineered bacteria, as has been demonstrated by the production of insulin, human growth hormone and various vaccines (Seeburg et al., 1977; Goeddel et al., 1979; Küpper et al., 1981). The in vitro synthesis, determination of the nucleotide sequence, and amplification in bacteria of structural gene sequences for growth hormone from mRNA of cultured rat pituitary cells have been reported (Seeburg et al., 1977). The construction and analysis of bacterial plasmids containing the gene coding for human chorionic somatomammotropin have been described (Shine et al., 1977). Promising preliminary studies have supported the use of interferon in the treatment of viral diseases such as rabies, hepatitis, varicellazoster and various herpes infections. A hybrid plasmid has been isolated containing the leukocyte interferon gene, which elicited the formation in E. coli of a polypeptide with the immunological and biological properties of human leukocyte interferon (Nagata et al., 1980). The human fibroblast interferon gene has been inserted into a thermoinducible plasmid under control of the λ phage promoter (Derynck et al., 1980). On induction antiviral activity was synthesised which closely corresponded to the physiological, immunological and biological characteristics of authentic fibroblast interferon. The construction and identification of plasmids have been described encoding the human fibroblast interferon gene that directed the efficient synthesis of two proteins (Taniguchi et al., 1980). The primary sequences corresponded to the sequence of fibroblast interferon in one case and pre-fibroblast in the other. A plasmid has been constructed which directed the high level expression in E. coli of human leukocyte interferon and showed in vivo antiviral activity (Goeddel et al., 1980). A report describing the sequences of 8 cloned human leukocyte interferon DNA's has demonstrated that the multiple human leukocyte interferon genes code for a family of homologous, yet distinct proteins (Goeddel et al., 1981). Recombinant DNA technology therefore provides an alternative method of producing large quantities of interferon using bacterial cells.

An early benefit of genetic manipulation should be in the area of vaccines where recombinant technologies may lead to the production of harmless substances capable of eliciting specific defences against various stubborn infectious diseases. Novel pure vaccines based on antigens synthesised by cloned DNA fragments have been proposed to fight diseases such as malaria, influenza and foot and mouth disease. The cloning of DNA of the major antigen of foot and mouth disease virus and its expression in *E. coli* has been described (Küpper *et al*., 1981).

The advent of cloning in *Bacillus subtilis* has led to the cloning of *B. subtilis* enzyme genes (Yoneda *et al*., 1979). *B. subtilis* is a non-pathogenic Gram-positive bacterium which excretes several extracellular proteins in large amounts. Because of the major commercial importance of these proteins *Bacillus* strains have been used for the production of a variety of enzymes (Priest, 1977) and could be tailored for the excretion of eukaryotic products. Existing methods for large-scale growth of *Bacillus* strains and isolation of proteins from such cultures may be useful for making viral or eukaryotic polypepties from *Bacillus* strains containing cloned DNA (Hardy *et al*., 1981). The development of a chimeric plasmid by Ehrlich (1978) that replicates in both *B. subtilis* and *E. coli* has greatly increased the potential of this cloning system.

Proteins cross-reacting with antibodies against either the core antigen of hepatitis B virus or the major antigen of foot and mouth disease virus can be made in B. subtilis when the appropriate viral gene is inserted into a suitable plasmid vector (Hardy et al., 1981). In the field of enzyme production, the cloning of the Bacillus amyloliquefaciens α-amylase gene into B. subtilis has been described (Yoneda et al., 1979). The authors state that by the introduction of appropriate mutations into the recipient strain the yields of α -amylase can be improved. Furthermore it is anticipated that the introduction of multiple gene copies encoding different α-amylases coupled with genes regulating excretion of the exoenzymes should further increase fermentation yields.

Genetic engineering can increase an organisms productive capability but it can also be used to construct strains with characteristics other than increased productivity. Properties such as the formation of spores that could lead to airborne spread of the micro-organism can be suppressed and the formation of harmful products can be eliminated or reduced.

Industry's basic function is to transform low-cost raw materials into end-use products of greater value. To do this via biotechnology there is a need for host organisms which could be genetically engineered to produce chemical feedstocks from low-cost substrates.

The ABE fermentation of C. acetobutylicum has current industrial interest for two major reasons: Firstly the demand for butanol and acetone from biomass fermentations has increased because of the high cost of petroleum based chemical feedstocks and the use of butanol as an intermediate for the synthesis of butyl acetate, butyl acrylate and other chemical products. Certain chemical companies may have a specific need for acetone. Secondly, the organism can produce solvents from a variety of low-cost substrates, including pentose sugars derived from biomass residues (Zeikus, 1980). Presently acetone and butanol are the only major chemical feedstocks produced in significant quantities by bacterial fermentations (Spivey, 1978; Tong, 1978).

The acetone-butanol fermentation has a long career as an industrial fermentation process. Bacterial production of butanol was first studied by Pasteur in 1861 (Ross, 1961). He was studying butyric-acid producing organisms and identified butanol as one of the products formed. This work led to the isolation of a bacterium capable of producing a mixture of acetone, butanol and ethanol by Weizman (Rose, 1961). The Weizman culture was given the name Bacillus granulobacter pectinovorum but was later renamed C. acetobutylicum. This organism yielded 10-20% solvents based on total sugar (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). During this period considerable quantities of acetone were produced, along with about twice as much butanol. The storage and disposal of the butanol presented a problem for some time until intensive studies showed that butanol could be used for the preparation of butyl acetate which was an excellent solvent for nitrocellulose lacquers. These lacquers found use as finishes in the growing automobile industry. Soon butanol became a chemical in demand and acetone was somewhat of a by-product. The demand for butanol allowed fermentation plants to reopen following their closure after the war and the use of molasses in addition to grain as a fermentation substrate was introduced. Industrial production of acetone and butanol via fermentation has not been significant for the past 20 years due to the superior economic position of petrochemical based processes (Lonz and Moroira, 1980).

The production of acetone and butanol by C. acetobutylicum follows a rather complex mechanism which leads to a number of end products namely CO_2, H_2O, H_2, acetic and butyric acids in addition to the desired acetone, ethanol and butanol. In spite of the large number of products formed, it is interesting to note that about 97% of the energy content of the glucose fermented is conserved in the products produced during the fermentation (Table 1.1).

The morphological and cytological changes which occurred in the C. acetobutylicum strain during the production of acetone,butanol and ethanol in an industrial fermentation medium were correlated with growth and physiological changes of the organism (Jones et al., 1981). It was found that the swollen cigar-shaped clostridial forms of the organism were involved in the conversion of acetic and butyric acids to neutral solvents and there was a correlation between the number of clostridial forms and the production of solvents. The production of solvents by sporulation mutants was also investigated and it was found that sporulation mutants which were unable to form clostridial forms did not produce solvents. Oligosporogenous mutants which showed reduced clostridial stage production produced intermediate levels of solvents and sporulation mutants blocked after the clostridial stage which were unable to form mature spores produced normal levels of solvents (Jones et al., 1981). The identification of a distinct

TABLE 1.1: Fractional Energy Recovery in the ABE Fermentation (Lonz and Moroira, 1980).

Fermentation Product	% of Total Energy Recovered in Product
Butyric acid	3,2
Acetic acid	4,5
Hydrogen	14,2
Ethanol	3,5
Butanol	55,1
Acetone	14,5
Acetoin	5,0
TOTAL	100,0

morphological stage associated with solvent production and the isolation of sporulation mutants which can be maintained as clostridial forms suggests the possibility of the development of novel ABE fermentation processes.

A limiting factor in the ABE fermentation is butanol toxicity. An approach to overcome this problem is to search for butanol tolerant strains or mutants. The results of Jones _et al_. (1981) indicate that enrichment or screening programmes must involve the clostridial stage and not the vegatative cell.

Lonz and Moroira (1980) reported in an economic evaluation of the ABE fermentation that the economics of producing these solvents by fermentation from high-quality molasses was unattractive when current prices of petroleum based commodities were used in the calculations. The total production costs were found to be slightly higher than the total annual income even when all the fermentation by-products were taken into account. This was mainly due to the high cost of the molasses feedstock.

Lonz and Moroira (1980) also evaluated the fermentation process using liquid whey-waste as a feedstock. The study showed that whey-waste would be economically attractive and suggested that other low-grade or waste-type feeds such as sulphite waste liquors be investigated as well. They add however, that although economically attractive, the ABE fermentation of waste-type materials has a number of drawbacks which must be addressed before any attempt for commercial production is made. The major one is that very low levels of butanol are obtained in the final fermented broth which results in the need for large size vessels for fermentation and an energy-intensive distillation recovery of solvents. Other difficulties include the need for strict anaerobic conditions, careful culture maintenance and propagation, and the problem of bacteriophage infection.

Before progress can be made in other fermentations using _C. acetobutylicum_, genetic systems must be established for _C. acetobutylicum_, so that controlled gene transfer can occur. With this end in view, we have begun a study on the genetics of _C. acetobutylicum_ and its phages.

CARBOXYMETHYL CELLULASE AND CELLOBIASE PRODUCTION BY *C. ACETOBUTYLICUM*

The production of a carboxymethyl cellulase and a cellobiase by *C. acetobutylicum* was demonstrated. In liquid medium the carboxymethyl cellulase was induced by molasses, and it was not repressed by glucose. Optimum carboxymethyl cellulase activity occurred at pH 4.6 and 37°C.

2.1 INTRODUCTION

An examination of the chemical industry by Flickinger and Tsao (1978) suggested the production of fuels, chemical feedstocks and protein supplements by fermentation rather than from non-renewable resources is now possible due to the low cost quantitative recovery of hexoses and pentoses from any cellulosic material. Cellulose being the largest renewable resource is the most logical raw material on which attempts to base our future chemical needs should be made.

Although the exact mechanism by which cellulose is degraded enzymatically is not yet known, recent research has provided some insight into the process. Many workers consider that at least three classes of enzymes are involved, namely endo-cellulases of different specificity, exo-cellulases (cellobiohydrolases) and β-D-glucosidases (cellobiases) (Mandels et al., 1976; Dekker and Lindner, 1979). Together these enzymes constitute the cellulase complex and act synergistically to degrade native cellulose to D-glucose. Enzymes hydrolysing the hemicelluloses are well characterised (Dekker and Richards, 1976) and have been classified according to their substrate specificity.

The measurement of cellulase activity is complex and no absolute unit exists that can be used as a measure for a single enzyme acting on a soluble substrate. The unit of measurement depends upon the substrate chosen, its concentration and the extent of the conversion. In an attempt to simplify this problem Mandels et al., (1976) proposed a filter paper assay which was simple, reproducible and quantitative. This substrate is readily available and is neither too susceptible nor too resistant to cellulase attack. Other substrates used include soluble carboxymethyl cellulose, Avicel and cellobiose.

Studies on the microbial degradation of cellulosic material have mainly been confined to mesophilic fungi and bacteria. Evidence suggests that high cellulolytic activity is a characteristic of many thermophiles (Cooney and Ackerman, 1975; Cooney and Wise, 1975; Romanelli *et al*., 1975).

Enzymatic hydrolysis of cellulose and cellulase production by several fungi have been studied extensively (Ghose, 1969; Mandels and Weber, 1969; Ghose and Kostick, 1970; Reese and Mandels, 1971; Mandels *et al*., 1974; Huang, 1975). It was found that the fungus, *Trichoderma viride* was a convenient source of the cellulase complex capable of total hydrolysis of native cellulose to glucose. Increases in cellulase yields have been achieved by optimising culture conditions and by the addition of a surfactant to the medium (Pathak and Ghose, 1973). Further increases in enzyme yield were obtained by mutation and selection (Mandels *et al*., 1974). In selected mutants the specific activity of the enzyme and the proportions of the components of the complex were similar to that of the wild type strain (Mandels and Sternberg, 1976). The *T. reesei* (formerly *T. viride*) QM9414 strain boosted the efficacy of the extracellular cellulases by a factor of four as compared to the wild type strain (Mandels and Andreotti, 1978). A mutant of *T. viride* isolated by a combination of ultra-violet irradiation and chemical mutagenesis which showed resistance to catabolite repression by glucose was also a more potent cellulase producer (Montenecourt and Eveleigh, 1978).

Suzuki et al., (1969) showed that a Pseudomonas flourescens strain produced cellulases constitutively. Constitutive cellulases were also demonstrated in a species of Clostridium and Cellulomonas (Hammerstrom et al., 1955). The P. flourescens strain was shown to produce two extracellular and one cell-bound cellulase component. The cellulase of Bacteroides succinogenes on the other hand was found to be cell-bound and not actively released into the culture medium (Zeikus, 1980).

Among the anaerobes, Clostridium thermocellum strains display the highest cellulolytic ability. C. thermocellum produces large quantities of extracellular cellulase which was shown to be thermally stable at 70°C and was not constitutive (Ng et al., 1977; Weimer and Zeikus, 1977; Ait et al., 1979). Weimer and Zeikus (1977) have demonstrated a growth rate dependent metabolic interaction between C. thermocellum and Methanobacterium thermoautotrophicum co-cultured on cellulose. Interestingly the supernatant cellulase of C. thermocellum differs significantly from that of T. viride in both the types of activities present and their specific activities. C. thermocellum contains both endo- and exoglucanase activities but the ratio of endoglucanase to exoglucanase is greater in *C. thermocellum* than in *T. viride* (Shinmoyo *et al*., 1979). Filter paper activity is significantly higher in *T. viride* hypercellulase mutants than in strains of *C. thermocellum* (Zeikus, 1980) while supernatant cellulases from *C. thermocellum* lack cellobiase activity, unlike that of *T. viride* (Ng *et al*., 1977) and are not as sensitive to end-product inhibition by cellobiose or glucose (Shinmoyo *et al*., 1979). Mutant strains of *C. thermocellum* have been obtained that produce higher endo- and exoglucanase activities than is found in the original wild type strain (Shinmoyo *et al*., 1979). A major component purified 170-fold from *C. thermocellum* cellulase hydrolyzed both carboxymethyl cellulose and microcrystalline cellulose and preliminary purification studies have suggested that the *C. thermocellum* cellulase is complex and the mechanism of action of the individual components is not described (Ait *et al*., 1979). Herrero and Gomez (1980) have shown that *C. thermocellum* cultures are inhibited by ethanol and other end products of cellobiose catabolism, such as lactate, acetate and butyrate. Through a selection procedure, these authors obtained *C. thermocellum* derivatives that were adapted to grow at higher ethanol concentrations than the wild type. One of these mutants was able to grow in the presence of 25g of ethanol per litre (Herrero *et al*., 1980).

In assessing the possibility of engineering microbial strains which could produce cellulase and form methane, Chakrabarty and Brown (1979) examined the feasibility of constructing a cellulolytic Escherichia coli. They showed that it was possible to transfer the cellulolytic genes, specifying cellulase enzymes from a cellulolytic P. flourescens strain to a strain of E. coli C or a restriction negative mutant of E. coli K12. The cellulolytic E. coli produced cellulases that appeared to have essentially the same properties as those of the original P. flourescens strain. Chakrabarty and Brown's work with the cellulolytic E. coli has been suspended and the cultures destroyed pending an appraisal of its biohazards (Chakrabarty and Brown, 1979).

During previous studies in this laboratory on the isolation and purification of an extracellular autolytic enzyme by Webster (1980) from a molasses fermentation medium, it was found that cellulose dialysis membranes were degraded by growing cultures of C. acetobutylicum. An investigation of the cellulolytic activity of C. acetobutylicum was undertaken (Allcock and Woods, 1981) as its potential as an industrial organism for the utilisation of industrial wastes would be enhanced if the bacterium could degrade cellulose.

2.2 METHODS

2.2.1 Materials and Media

As described in Appendix A.

2.2.2 Bacterial Strain

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

The organism C. acetobutylicum is an obligate anaerobe which is Gram-positive, spore-forming and motile with peritrichous flagella. During vegetative growth, the organism is approximately 4,0 µm x 1,0 µm and when conditions are no longer suitable for growth, the spores formed at or near the ends of the cells measure 1,5 µm x 1,0 µm. The morphology of the culture varies with the growth stage and the formulation of the medium (Jones et al., 1981).

The cellulase producing strain was C. acetobutylicum P270. Cultures were incubated at 34°C and agar plate cultures were incubated in GasPak jars. Liquid culture manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box (Forma-Scientific).

2.2.3 Production of Carboxymethyl Cellulase, Cellobiase and Filter Paper Activity in a Molasses Fermentation Medium

Filter paper activity, carboxymethyl cellulase (CMC'ase) and cellobiase activities were determined in 1,0 ml molasses fermentation medium (MFM) supernatant samples by assaying reducing sugar equivalents released in 1 h at 37°C from 2,0 ml of sodium acetate buffer, pH 4.6, containing either 60mg filter strips (Whatman No. 1) or 0,1% (wt/vol) carboxymethyl cellulose or 0,7% (wt/vol) cellobiose. Reducing sugar was estimated by the method of Nelson (1944) as modified by Somogyi (1952), using glucose as a standard. One unit of enzyme activity was defined as the quantity of enzyme required to catalyze the formation of 1 μmol of reducing sugar, expressed as glucose, per minute under the above conditions.

2.2.4 Optimisation of Assay Conditions for Measuring Carboxymethyl Cellulase Activity

The optimum pH for measuring carboxymethyl cellulase activity was determined in 0,2 M sodium acetate buffer at pH3.6 to pH 5.6 and the optimum temperature for activity at 20, 30, 37 and 50°C. The stability of the carboxymethyl cellulase at 4°C over a period of 24 h was also monitored.

2.2.5 Induction and Repression of Filter Paper, CMC'ase and Cellobiase Activities in MFM and CBM

The induction of filter paper, CMC'ase and cellobiase activities by acid-swollen cellulose powder, Avicel, carboxymethyl cellulose, sucrose, glucose, cellobiose or xylose in MFM and clostridial basal medium (CBM) media was determined. MFM media containing decreasing concentrations of molasses were utilized to determine whether molasses or a component of molasses could induce CMC'ase, cellobiase or filter paper activity. Repression of enzyme activities was determined by supplementing MFM cultures with increasing concentrations of glucose (0,2 to 2,0% wt/vol).

2.2.6 Isolation of the Component of Molasses Responsible for Induction of CMC'ase Activity

In an attempt to isolate the component in molasses which was responsible for induction, the molasses was clarified by centrifugation at 10,000 rpm for 30 min. The supernatant and pellet fractions were dialyzed separately in running tap water for 72 h and used for the preparation of the MFM. Undialyzed fractions were also used to prepare MFM and glucose (1% wt/vol) was added to the dialyzed fractions. The media were innoculated with exponential phase cultures of *C. acetobutylicum* and the cultures were assayed for CMC'ase activity.

2.2.7 Cellulase Production by *C. acetobutylicum* on Agar Plates

In order to demonstrate cellulase activity, cultures of *C. acetobutylicum* were diluted in CBM and plated onto CBM agar plates supplemented with 0,5 to 2,0% (wt/vol) acid-swollen cellulose (CF11, Whatman) prepared by the method of Tansey (1971). The agar plates were incubated anaerobically for 5 days and viewed daily for zones of clearing around isolated clones. As a control cellulysin (Calbiochem) was added to wells made in the agar plates.

2.3 RESULTS

2.3.1 Carboxymethyl Cellulase and Cellobiase Production in the MFM

Both extracellular CMC'ase and cellobiase activities were observed in the liquid MFM (Fig. 2.1). Maximum cellobiase production occurred before the pH breakpoint (24 h) at 20 h. The maximum levels of CMC'ase activity were obtained after the pH breakpoint at 35 h. The cellobiase activity was unstable and decreased rapidly after 20 h, whereas the CMC'ase activity was relatively stable over a 37 h period. The cellobiase is a β-glucosidase since it released glucose from salicin. Localization studies involving washing and sonicating cells indicated that the cellobiase was extracellular. No activity against filter paper was detected during the fermentation.

2.3.2 Optimum pH and Temperature for the Assay of CMC'ase

The optimum pH for CMC'ase activity in the culture supernatant was pH 4.6 (Fig. 2.2). The optimum temperature for the enzyme activity was 37°C at pH 4.6 (Fig. 2.3). The activity of CMC'ase preparations was decreased by 50% after 24 h storage at 4°C.

2.3.3 Induction and Repression of CMC'ase, Cellobiase and Filter Paper Activity in MFM and CBM

No induction of CMC'ase or filter paper activity was detected in liquid CBM cultures containing acid-swollen cellulose powder, Avicel, carboxymethyl cellulose, sucrose, glucose, cellobiose or xylose. However, molasses or a component of molasses was required for induction of the CMC'ase activity as cultures containing decreasing amounts of molasses showed proportionately less CMC'ase activity (Fig. 2.4). The cellobiase did not require induction and was present in CBM cultures containing glucose. The addition of glucose to MFM

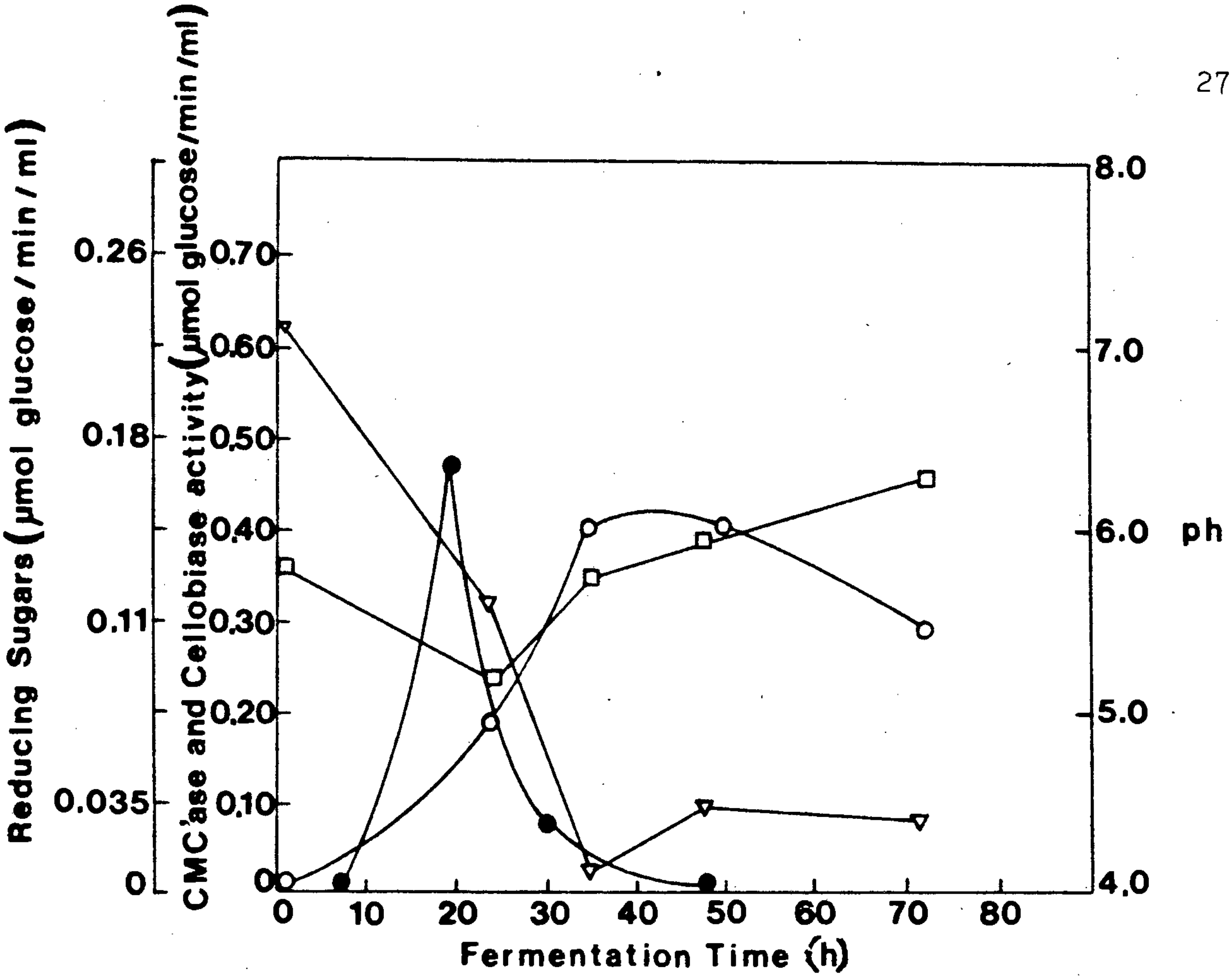

Fig. 2.1: Production of CMC'ase and cellobiase enzymes by *C. acetobutylicum* in MFM. CMC'ase activity (O); cellobiase activity (●); reducing sugars (▽); pH (□).

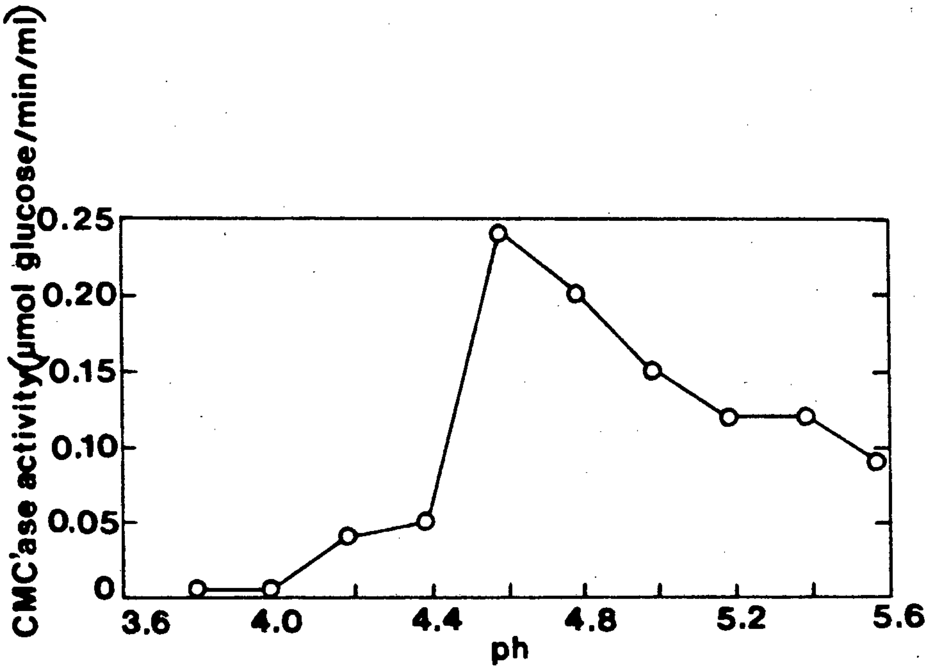

Fig. 2.2: Effect of pH on CMC'ase activity.

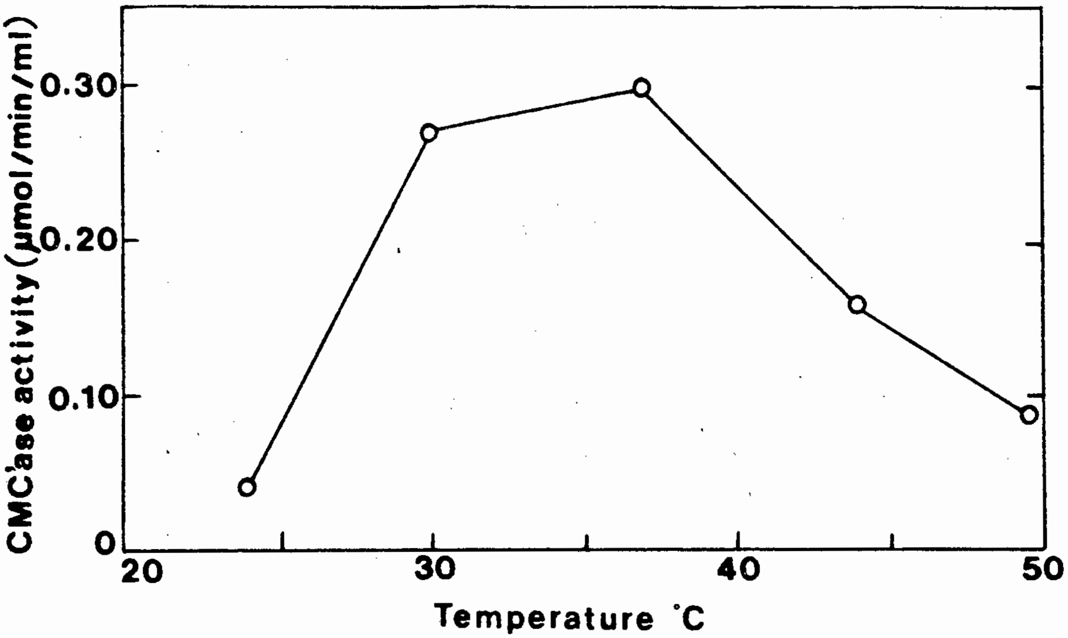

Fig. 2.3: Effect of temperature on CMC'ase activity.

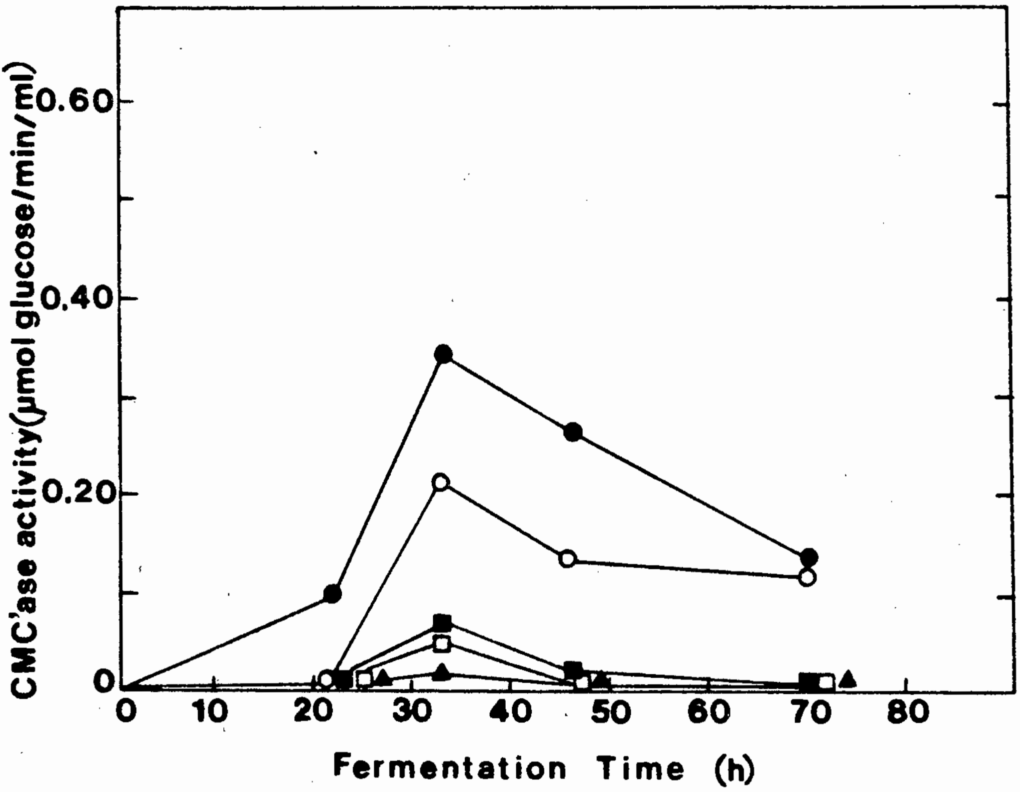

Fig. 2.4: Induction of CMC'ase by molasses. MFM contained decreasing concentrations of molasses (g/ℓ): 134 (●); 67 (O); 13,4 (■); 2,6 (□) and 0 (▲). The medium containing 13,4; 2,6 and 0 g/ℓ molasses were each supplemented with 3,6; 10 and 10 g/ℓ respectively.

cultures did not affect the CMC'ase activity, and the enzyme was not repressed by glucose.

2.3.4 Isolation of the Component of Molasses Responsible for Induction of CMC'ase Activity

The substance in molasses which was responsible for the induction was a small molecule since dialyzed molasses failed to induce CMC'ase activity. Molasses clarified by centrifugation at 10 000 rpm for 30 min induced the production of CMC'ase activity, whereas the sediment after centrifugation did not. The molasses utilized was South African final molasses and contained approximately: dry solids, 81,5%; sucrose, 36,1%; reducing sugars, 14,5%; and sulphated ash, 15%.

2.3.5 Cellulase Production by *C. acetobutylicum* on Agar Plates

The production of a cellulase by *C. acetobutylicum* was demonstrated by zones of clearing around isolated clones after 2-3 days of incubation on CBM agar supplemented with 0,5 to 2,0% (wt/vol) acid-swollen cellulose powder (Table 2.1). The clear zones on the CBM plates containing acid-swollen cellulose were not artifacts because a cellulase preparation, cellulysin, added to wells in the plates produced similar zones. The

TABLE 2.1: Cellulase Production by *C. acetobutylicum* on Agar Plates.

Conc. of Acid-Swollen cellulose (% wt/vol).	Incubation time (days)			
	2	3	4	5
	Zone radius (mm)			
0,5	1	2	3	3-4
1,0	0	1	2	3
1,5	0	0	1	2
2,0	0	0	1	2

diameter of the zones was proportional to the concentration of cellulase. A *Clostridium perfringens* strain did not produce zones of clearing on the medium. The *C. acetobutylicum* strain did not grow on CBM without glucose supplemented with either acid-swollen cellulose or Avicel as the sole carbon source.

2.4 DISCUSSION

The data from these experiments indicate that *C. acetobutylicum* produces an inducible CMC'ase and cellobiase. Both enzymes are not repressed by glucose. Production of the cellobiase activity during the fermentation occurs when the culture is actively growing and the majority of the cells are still dividing. The CMC'ase activity, however, coincides with the non-actively growing stage of the fermentation when motility has stopped and the majority of the cells have become clostridial forms which have been shown to be involved in the production of solvents (Jones *et al*., 1981). A capsule is also produced by the clostridial forms and CMC'ase activity during this period may be involved in production or degradation of the capsule. Further work needs to be done on the chemical nature of the capsule.

In comparison with other cellulolytic bacteria and fungi, the levels of CMC'ase and cellobiase activities obtained in *C. acetobutylicum* are low. The reason why acid-swollen cellulose is cleared on plates is presumably due to the CMC'ase, as no activity against crystalline cellulose could be demonstrated. The clearing may also be due to the action of an endocellulase which produces C_8 - C_{10} dextrins which are soluble. Complementation by growing a strain which utilizes dextrins such as *Clostridium thermosaccharolyticum* on the cleared acid-swollen cellulose plates could be used to test this hypothesis. However, *C. acetobutylicum* does have the genes for CMC'ase and cellobiase and the levels and activity could be improved by mutation and selection techniques (Eveleigh and Montenecourt, 1979). The cellulase required for crystalline cellulose degradation could be inserted by genetic manipulation (Eveleigh and Montenecourt, 1979).

CHARACTERISATION OF A CELL BOUND AUTOLYTIC ENZYME SYSTEM IN C.ACETOBUTYLICUM

Optimum conditions for autolysis and autoplast formation in C. acetobutylicum have been defined. Autolytic-deficient mutants which produced less autolysin than the parent strain were isolated. Growth of the P262 strain and the lyt-1 mutant was inhibited by the same concentrations of wall inhibiting antibiotics. Electrophoresis of cell-free autolysates have indicated the presence of two autolytic enzymes. Electron microscopy of autolysing cells suggest that most of the active autolysin is located at discrete sites along the peripheral wall of the cell.

3.1 INTRODUCTION

Unless otherwise stated, information on bacterial autolysins has been taken from reviews of Ghuysen and Shockman (1973), Rogers (1979) and Rogers *et al*., (1980).

One of the paradoxes inherent in microbes with cell walls is that although they have a surface completely covered with insoluble polymers, they must be able to expand during growth, and modify the surface so that division into two new individuals can take place. Obvious candidates that might modify surfaces are the autolysins but unambiguous evidence that they are necessarily involved during growth is difficult to obtain. By studying aspects of the functions of autolysins in bacterial cells, one may be able to provide more evidence in favour of this theory and it is to these aspects that this review will pay particular attention.

Autolysins and the Growth of Microbial Cells

The first bacterium to be seriously studied in the context of a possible essential role for autolytic enzymes in growth was *Streptococcus faecalis* (Shockman *et al*., 1967). This was fortunate in some ways since *S. faecalis* has a muramidase as the sole autolytic enzyme most of which was found to be concentrated in the region of the cells in which new material was being deposited during growth and division. Other evidence in favour of an essential role was the isolation of mutants of *Streptococcus faecium* with reduced autolytic activity which also had reduced growth rates (Shungu *et al*., 1979). These mutants were obtained after treatment with N-methyl-N-nitro-N-nitrosoguanidine and were selected by repeated challenges with penicillin G and cycloserine followed by further selection for resistance against lysis by the detergent Triton X-100. They were found to be highly pleitrophic - two of the three strains grew more slowly in both cultures and all three mutants in defined medium failed to reach the same optical density as the wild type indicating a different nutritional requirement. Future work is required on these mutants before emphasis can be put on the correlation between slower growth and reduced autolysin content.

It has been suggested that the role of autolysins may be to ensure that new wall is being added at the right place, rather than being added randomly over the surface of the cell (Rogers, 1970). Autolysins may accomplish this function by providing acceptor ends for dissacharide units resulting in growth, or by breaking bonds to allow realignment and rearrangement of existing peptidoglycan. Ghuysen and Shockman (1973) pointed out that only autolysins that could provide suitable additional acceptor ends (non-reducing N-acetylglucosamine) would be N-acetylmuramidases and that only a few species posess this enzyme (*S. faecalis*, *Lactobacillus acidophilus*, *Arthrobacter crystallopoietes* and *Bacillus thuringiensis*).

Cell Separation and Motility

A major approach which has been used to assess the roles of autolysins is the analysis of mutants with deficient autolytic systems. Studies of these mutants have demonstrated clearly that depriving bacterial cells of a large proportion of their autolytic activity leads to their failure to separate from each other. Two autolytic deficient mutants of *B. subtilis* (Fein and Rogers, 1976) were 90 to 95% deficient in the autolytic enzymes N-acetyl-muramyl-L-alanine amidase and endo-β-N-acetyl glucosaminidase. Although these mutants grew at normal rates compared to the wild type, they formed very long chains of unseparated cells. This altered behaviour was not due to an altered wall chemistry in the mutants as walls isolated from the mutant cells had the same chemical composition as those from the wild type. Pooley *et al*., (1972) reported a similar effect for a *S. faecalis* autolytic-deficient mutant which was shown to have a similar wall chemistry to that of the wild type but grew in chains of up to 40 cells. 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). It has been shown in *B. subtilis* and *B. licheniformis* (Ayusawa *et al*., 1975; Yoneda and Maruo, 1975; Fein and

Rogers, 1976; Fein, 1979) that autolysin-deficient mutants have also lost their flagella and are, therefore, non-motile. In _B. subtilis_ these mutants have been shown to have a pool of flagellin in the cytoplasm, suggesting that the reduced autolytic activity in some way prevents the extrusion and or organisation of the flagella from the protein subunits. In addition, motile revertants are fully autolytic. However, it is possible to argue that the lack of flagella is just one more aspect of a mutant phenotype and may not be directly connected with the absence of autolytic enzymes.

Turnover of Bacterial Wall Polymers

Studies on radioactively pre-labelled peptidoglycans in the walls of a number of bacilli and lactobacilli growing in a non-radioactive medium have shown that these organisms are in a state of flux (Boothby _et al_., 1973; Pooley, 1976). In other species such as _S. faecalis_ this is not so (Boothby _et al_., 1973), and the walls do not show turnover. Such a process would involve the formation of soluble material from the insoluble wall and this is possibly undertaken by autolysins. One report, however, (Glasser, 1973) disturbs this hypothesis in that a strain of _B. subtilis_ with a normal complement of autolysins and normal walls has a wall turnover reduced by 90%. Although little other exacting work has been reported to test such a hypothesis, the available results suggest a role for autolysins in wall turnover.

Autolysins in Transformation

Young and Spizizen (1963) reported that strains of *B. subtilis* which were highly susceptible to genetic transformation by DNA contained increased autolytic activities compared to 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 entry of negatively charged DNA molecules (Young *et al*., 1964). When competence factor (CF) was added to vegetative cells, the rate of lysis increased for both Group A *Streptococcus* (Ranhand, 1973) and *Pneumococcus* (Seto and Tomasz, 1975). Seto and Tomasz (1975) proposed that the competence factor caused some form of membrane change allowing the exit of autolytic enzymes and access to the walls. The development of the state of competence and the rate of spheroplast formation have been correlated (Lacks and Neuberger, 1975). Although not complete, this correlation suggested involvement of autolytic activity in the development of cells competent for transformation.

Autolysins and the Action of Antibiotics

Antibiotics that inhibit wall synthesis are bacteriocidal, unlike most of those inhibiting protein synthesis which are bacteriostatic. The question of whether autolytic action is an essential part of killing sensitive bacteria by wall inhibiting antibiotics arose. Attempts to answer this question involved the blocking of protein synthesis to inhibit the formation of autolysins. Combinations of chloramphenicol with penicillin were not bactericidal for *E. coli* (Prestidge and Pardee, 1957) or for *Staphylococcus* (Rogers, 1967). More specific approaches to the problem were studies on autolytic-deficient mutants. *B. licheniformis* (Rogers and Forsberg, 1971) and wild type *Diplococcus pneumonia* with modified walls (Tomasz *et al*., 1970) died very much less rapidly than the unmodified wild type strains when treated with a variety of antibiotics inhibiting wall synthesis. Tomasz and Waks (1975) proposed a new hypothesis for the mechanism of penicillin-induced lysis of bacteria. They suggested that the inhibition of cell wall synthesis by any means triggered the bacterial autolytic enzymes by destabilising the endogenous complex of the autolysin inhibitor (lipoteichoic acid) and autolytic enzyme. Kitano and Tomasz (1979) showed in *E. coli* that the relative effectiveness of beta-lactams in autolysin triggering was found to parallel the effectiveness of the same drugs in causing rapid loss of viability, culture lysis and spheroplast formation.

Other areas in which autolytic enzymes are almost certainly involved are in sporulation and spore germination. Although much work has been done in these areas, little is known as to how enzymes differ from those in normal vegetative cells or exactly how and when they function in the cell differentiation process.

Autolytic enzymes are almost certianly involved in cell separation and cell growth and evidence is mounting for their involvement in other cellular functions while it remains difficult to determine whether the same enzymes are implicated in numerous cellular functions, or whether different enzymes play various roles. Processes in which autolysins may be involved all suggest that autolysin action is probably very localised and rigorously controlled. Little is known of the cellular location of the enzymes involved or the manner in which autolysin activity is regulated (Burdett, 1980). A direct approach to locate the sites of autolysin action is to examine by electron microscopy the course of lysis of intact cells suspended in buffers of suitable pH and ionic strength.

It was shown by Webster (1980) that high titres of a bacteriocin-like substance were produced by *C. acetobutylicum* in a molasses fermentation medium. The 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. 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 bacteriocin-like substance showed a broad spectrum of activity and the ability to lyse cell walls. The production of the bacteriocin-like substance was associated with lysing or autoplasting cells and it was concluded that the bacteriocin-like substance was an autolysin. Upon characterisation, the autolysin had a different mode of action from lysozyme in that it affected an interior site on the cell wall. The molecular weight of the purified autolysin was found to be 28 000 and carbohydrate staining of the autolysin showed that it was a glycoprotein.

An investigation of the cellular autolytic activity was undertaken (Allcock et al., 1981a) as it was involved in the production and regeneration of C. acetobutylicum protoplasts.

3.2 METHODS

3.2.1 Media and Materials

As described in Appendix A.

3.2.2 Bacterial Strain

The Clostridium acetobutylicum P262 strain was used. Cultures were incubated at 37°C and all manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box.

3.2.3 Production of Active and Latent Autolysin During Exponential Growth

The production of active and latent autolysin by C. acetobutylicum during exponential growth in CBM cultures was determined. Cells (5 to 10 ml) from different stages in the growth cycle were cooled on ice, harvested by membrane filtration, washed twice with 10 ml of cold double-distilled deionized water and resuspended in 4 to 6 ml of 0,04 M sodium phosphate buffer, pH 6.3. Cellular autolysis at 37°C was monitored turbidometrically at 600 nm. Trypsin (1 μg/ml) was added to the autolysing culture when total autolytic activity was measured. One unit of cellular autolysis is defined as the loss of 0,001 optical density units per h. To compare the autolysis of different samples, results are expressed in units of autolysis per 0,1 optical density of cell suspension. One unit is then equivalent to a loss of 1% of the initial turbidity per h.

3.2.4 Cellular Autolysis in Sodium Phosphate and Ammonium Acetate Buffers

The effect of ionic strength was determined by resuspending washed cells in various concentrations of sodium phosphate and ammonium acetate buffers (pH 6.3). The optimum temperature for activity was determined in 0,04 M sodium acetate buffer (pH 6.3) at 21, 34, 37, 45 and 52°C. The effect of pH on cellular autolysis was studied by resuspending harvested and washed cells in the following buffers (0,04 M): ammonium acetate pH 3.6; 4.0; 4.4; 5.0; 5.4 and 5.6 and sodium phosphate at pH 6.0; 6.3; 6.7; 7.0; 7.6 and 8.0.

3.2.5 Effect of Trypsin on Autolytic Activity

The effect of trypsin on autolysis and the release of autolysin from autolysing C. acetobutylicum cells was investigated. Cells from an exponential phase culture were autolysed in the presence and absence of trypsin (1µg/ml). Cellular autoysis was monitored turbidometrically. The activity of the autolysin towards cell walls was monitored by removing 1 ml samples from autolysing cultures and centrifuging at 11 000 rpm in a Beckman microfuge for 10 min at 4°C. Samples (500µl) of the supernatant fraction were incubated at 37°C for 1 h with freshly prepared sodium dodecyl sulfate (SDS) treated cell walls (1.0 mg/ml) from the C. acetobutylicum P262 strain. One unit of activity is defined as the loss of 0.001 optical density units per h.

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 (400ml) washed three times in cold 0,05 M phosphate buffer (pH 7.0), and then resuspended in the same buffer. The washed cells were disrupted in a Braun homogeniser with an equal quantity of glass beads (0.1mm) at full speed for 1.5 min. To remove any unbroken cells, the suspension was centrifuged at 4,000 rpm for 10 min. The resulting supernatant, containing cell walls, was pelleted by centrifugation at 18 846 x g for 30 min. The crude cell wall pellet was then washed twice in 1 M NaCl, twice in distilled water, and resuspended in 10ml 0.05 M phosphate buffer. The crude cell walls were treated with 2% (w/v)

SDS overnight at room temperature and washed 5 times with cold 0.05 M sodium phosphate buffer, pH 6.0 to remove the SDS. Cell walls prepared in this manner were resuspended in 0.05 M phosphate buffer (pH 6.0), dialysed overnight at 20°C and stored at 4°C until required.

3.2.6 Isolation of Autolytic - Deficient Mutants.

Mutants deficient in autolytic activity were isolated from exponential-phase CBM cultures ($5\mathrm{x}10^7$ cells /ml) of C. acetobutylicum p262 treated with ethyl methane sulfonate (EMS) (2.5% v/v) for 20 min at 37°C to obtain 10% survival. The washed cells were resuspended in the minimal salts medium and incubated for 18 h before diluting and plating onto CBM plates overlayed with 5 ml CBM containing autoclaved exponential phase C. acetobutylicum p262 cells. The plates were incubated at 37°C for 18 h. Colonies which produced small or no zones of lysis were selected and tested for autolytic activity.

3.2.7 Autoplast (Protoplast) Formation in C. acetobutylicum.

Autoplast formation (the spontaneous formation of protoplasts or spheroplasts) has been used by several investigators to study the autolytic systems of various bacteria (Kawata et al., 1968, Joseph and Shockman,

1974) and in particular the initiation sites of autolysis and autolysin locations (Joseph and Shockman, 1976). In C. acetobutylicum autoplasts were formed from exponential-phase cells harvested anaerobically by centrifugation, washed and resuspended at 37°C in an autoplasting buffer which contained 0.25 or 0.5 M sucrose, 25 mM $MgCl_2$, 25mM $CaCl_2$ in 0.04 M sodium phosphate buffer (pH 6.3). Trypsin (1µg/ml) was added to the buffer when required. The percentage autoplasts formed were determined by microscope counts of 8 random fields per sample. Lysis of protoplasts was determined by measuring the leakage into the supernatant fraction of material absorbing at 260 nm.

3.2.8 Effect of Cell Wall Antibiotics.

The effect of cell wall antibiotics on cellular autolysis was investigated. Penicillin (0.10µg/ml), vancomycin (2.0µg/ml), chloramphenicol (2.5µg/ml) and D-cycloserine (100µg/ml) were added to exponential-phase CBM cultures of P262 and lyt-1 cells. Growth was monitored at 600 nm and the viability of the cultures was assayed by routine plating procedures. Antibiotics were removed before plating by dilution to levels that had no detectable effect on bacterial growth in agar medium.

3.2.9 Preparation of C. acetobutylicum P262 Autolysin.

Crude C. acetobutylicum autolysin for addition as exogenous autolysin to antibiotic pre-treated cultures was prepared by harvesting exponential-phase CBM cultures as for cellular autolysis, resuspending in autolysis buffer and allowing autolysis to proceed at 37°C for 60 min. The clear lysate was utlised as the source of exogenous wild-type autolysin.

3.2.10 Isoelectric focusing and SDS-polyacrylamide Gel Electrophoresis of C. acetobutylicum Autolytic Enzymes

Isoelectric focusing and polyacrylamide gel electrophoresis (PAGE) were used to determine whether the inducible cell-free autolysin reported by Webster (1980) in molasses or sucrose-CBM media was the same as the cell - bound autolysin described above. Crude cell - free autolysin was prepared from 36 h culture supernatants (400 ml) of C. acetobutylicum P262 grown in CBM containing 15% (w/v) sucrose. The supernatant fraction collected after centrifugation at 12 061 x g for 15 min at 4°C was dialysed at 4°C against 10% (w/v) sorbitol for 6 h. To 40ml of the dialysed supernatant fraction, 10ml of Buffalyte (carrier ampholytes for isoelectric focusing) and 4.5g of Sephadex G 75 was added. The gel was allowed to swell overnight at 4°C before casting and focusing for

6 h at 25 mA and 100 V using 0.1 M NaOH and 0.1 M H_3PO_4 as wick solutions. The gel was cut into 1.7 cm slices and each fraction eluted with 5ml distilled water. The pH, protein concentration estimated by Bradfords reagent (Bradford, 1976), and autolysin activity by the well plate method (Webster, 1980) was determined in each fraction. Samples from each fraction were electrophoresed in 10% SDS-polyacrylamide gels (see Appendix A). Crude cell-bound autolysin was prepared as described in 3.2.9 above except that the clear lysate after cellular autolysis was concentrated by freeze drying before loading onto SDS-polyacrylamide gels. Crude autolysates from supernatant fractions of 36 h C. acetobutylicum P262 and lyt-1 CBM + 15% (w/v) sucrose cultures were also electrophoresed on SDS-polyacrylamide gels. After electrophoresis the gels were stained with Coomassie blue and destained in 25% (v/v) isopropanol containing 10% (v/v) acetic acid.

3.2.11 Electron Microscopy of Cellular Autolysis in C. acetobutylicum

Exponentially growing C. acetobutylicum P262 cells were harvested on membrane filters at 4°C, washed three times with 5 ml of cold distilled water, suspended in 0.04 M sodium phosphate autolysis buffer (pH 6.3), and allowed to autolyse at 37°C.

Immediatly after resuspension in phosphate buffer and after 5,15,30 and 45 min samples were removed and fixed by the addition of 4% (v/v) gluteraldehyde. Treatment with 4% gluteraldehyde halted cell autolysis in a rapid manner. Fixed cells were collected by centrifugation at 3015 x g for 10 min and allowed to stand in Kellenbergers buffer (Kellenberger et al., 1959) overnight. Cells were post-fixed in 1% $0S0_4$ for 1.5 h and washed once in Kellenbergers buffer and dehydrated in acetone before embedding in a low-viscosity resin according to the method of Spurr(1968). This method was modified to allow harvesting and washing of cells using a Beckman microfuge. Ultrathin sections were cut on a KLB ultramicrotome using a glass knife. The sections were double-stained in lead citrate and saturated uranyl acetate and viewed on a Zeiss 109 electron microscope.

3.3 RESULTS

3.3.1 Cellular Autolysis in Sodium Phosphate and Ammonium Acetate Buffers

C. acetobutylicum cells resuspended in various buffers autolysed and the concentration of sodium phosphate and ammonium acetate buffers (pH 6.3) affected the autolysis of the P262 strain (Fig. 3.1). Trypsin (1 μg/ml) increased the rate and amount of autolysis in the acetate buffer but had little effect in the phosphate buffer. As ammonium acetate is not a good buffer at pH values near neutrality sodium phosphate buffer was used in all further experiments. Autolysis of exponential phase cells in 0.04 M sodium phosphate buffer was optimal between pH 6.0 to 6.5. Above pH 6.5 and below pH 6.0 the cellular autolysis rate decreased markedly.

3.3.2 Effect of Trypsin on Autolytic Activity.

The effect of trypsin on the autolysis and the release of autolysin from autolysing *C. acetobutylicum* P262 cells was investigated. Cells from an exponential phase culture were autolysed in the presence and absence of trypsin (1 μg/ml) and the autolysis and the cell free autolysin were determined (Fig. 3.2). Trypsin enhanced the rate of autolysis and the release of the cell-free autolysin which was not sedimented by centrifugation at 11 000 rpm for 10 min. Cell-free autolysin activity assayed against SDS-treated walls reached a maximum after cellular autolysis had occurred. The optimal concentration of trypsin was 1 μg/ml (Fig. 3.3) and the rate of autolysis was not affected by higher concentrations of trypsin. Up to 1 μg/ml of trypsin there was a linear relationship between autolytic activity and trypsin concentration.

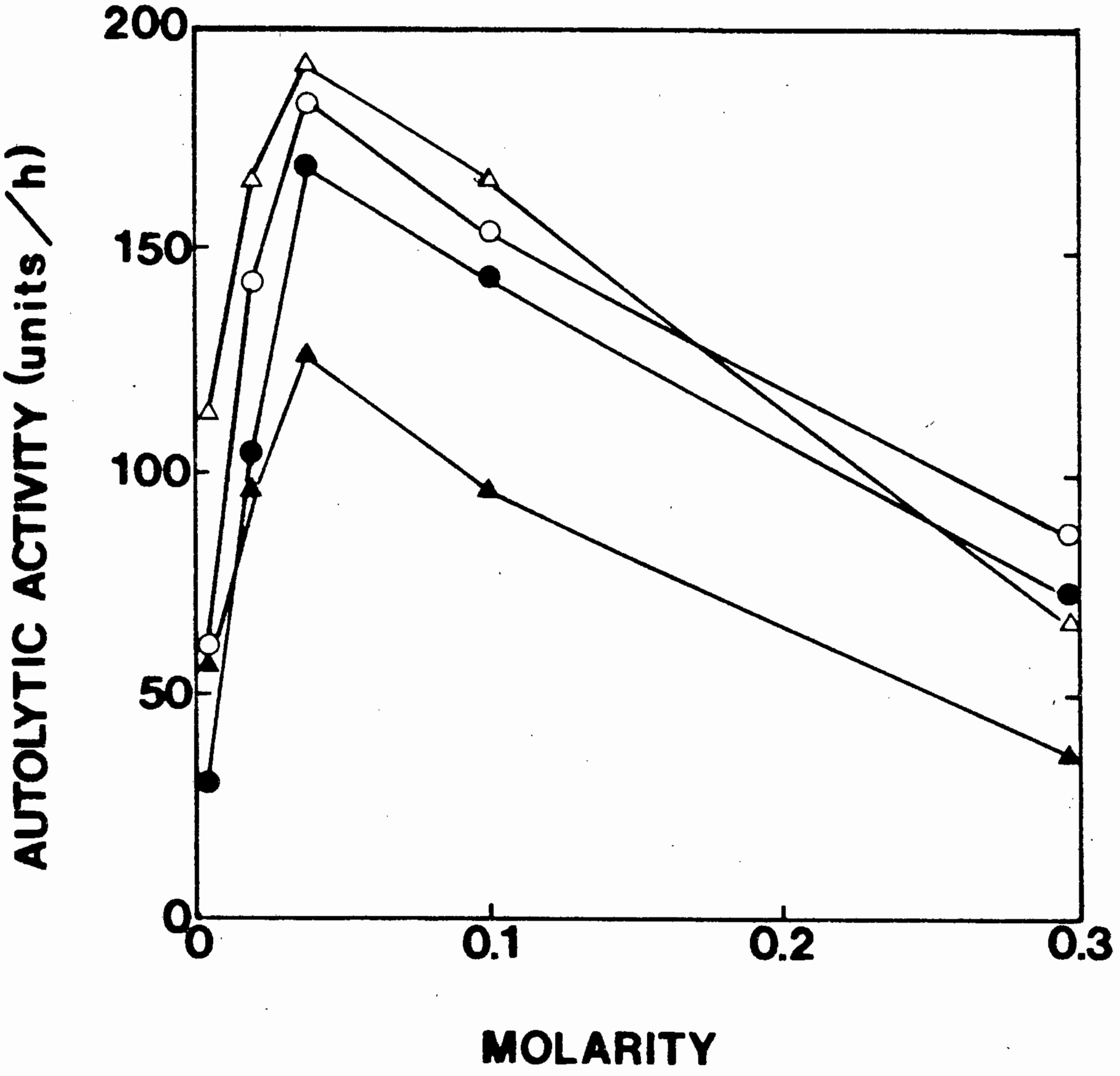

Fig. 3.1: Effect of buffer concentration on cellular autolysis in *C. acetobutylicum* P262 in the presence and absence of trypsin. Cells were resuspended in different concentrations of the following solutions at pH 6.3: sodium phosphate buffer with (O) and without (●) 1 µg/ml trypsin; ammonium acetate buffer with (Δ) and without (▲) 1 g/ml trypsin.

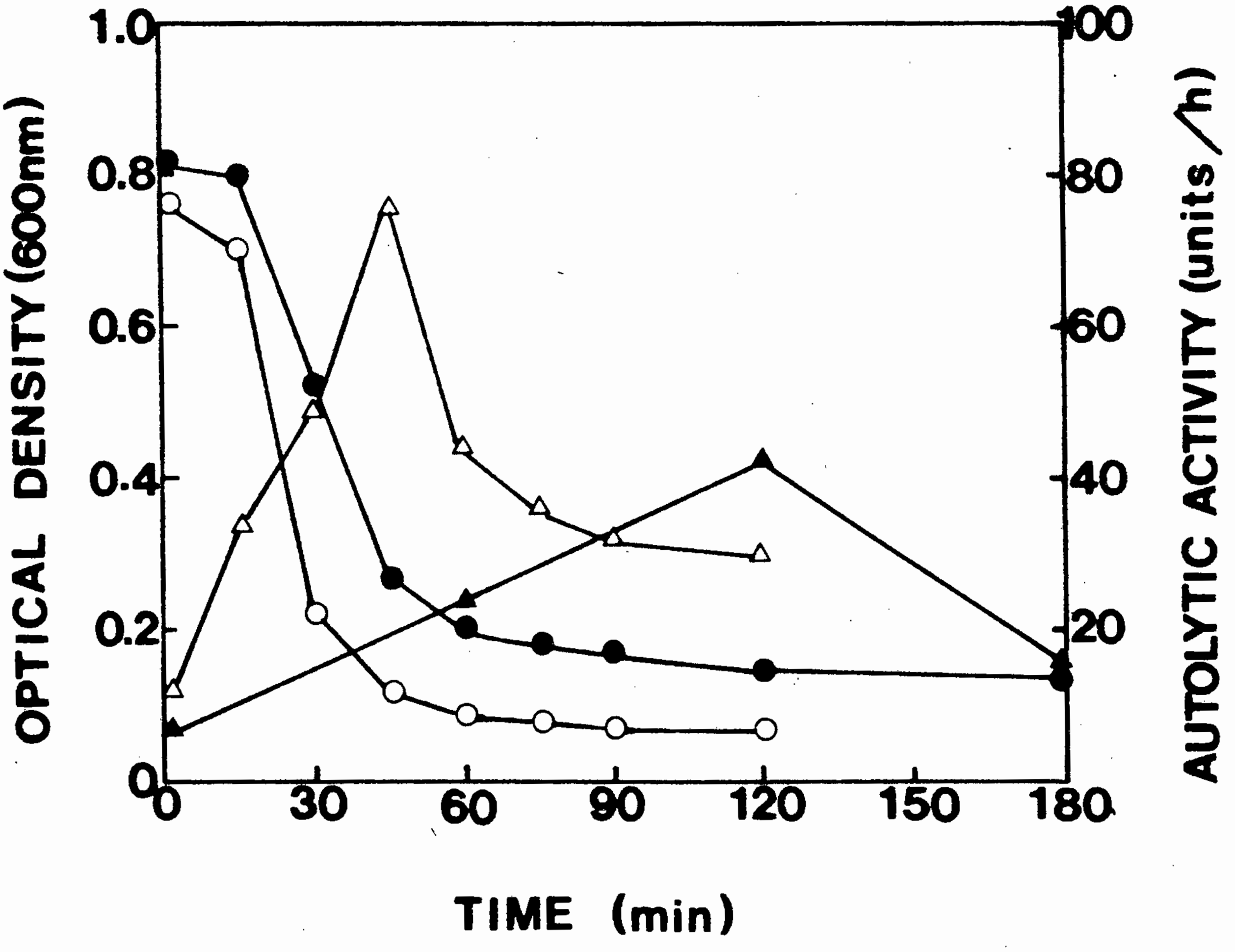

Fig. 3.2: Effect of trypsin on the autolysis and the release of autolysin from autolysing *C. acetobutylicum* P262 cells. Autolysis in the presence (O) and absence (●) of trypsin (1 μg/ml). Cell free autolysin activity in the presence (Δ) and absence (▲) of trypsin assayed against P262 SDS-treated walls.

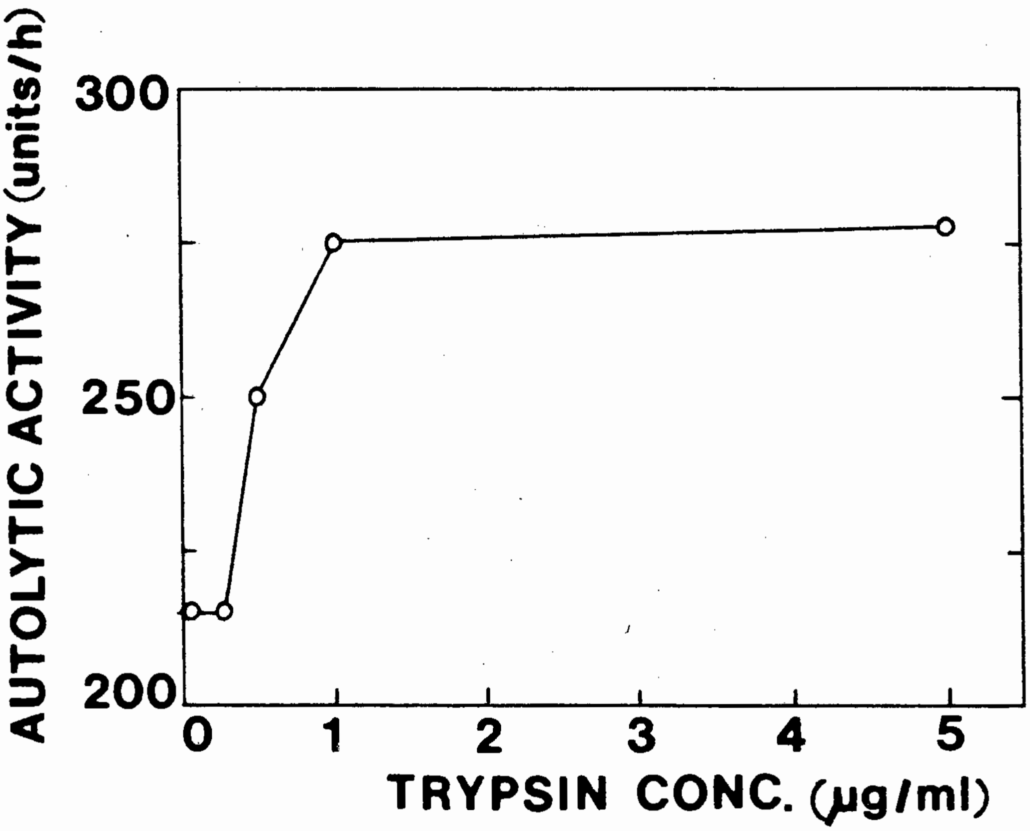

Fig. 3.3: Effect of trypsin on cellular autolysis of *C. acetobutylicum* P262.

3.3.3 Autolytic Deficient Mutants

Seven autolytic deficient mutants, which produced small or no halos of lysis on the P262 indicator plates, were isolated in different experiments after EMS treatment of the P262 strain. The active and latent (trypsin-activatable) levels of the lyt mutants during mid-exponential growth were measured (Table 3.1). One of the mutants lyt-1, isolated from the first mutagenesis experiment, was chosen for further analysis.

Cellular autolysis of the lyt-1 mutant strain in the presence and absence of trypsin (1 μg/ml) was markedly reduced when compared with the P262 strain. Exponential phase lyt-1 cells showed 80,9 and 69,4 units of autolytic activity in the presence and absence of trypsin respectively. Under similar conditions, the P262 strain produced 298,0 and 278,0 units of autolytic activity in the presence and absence of trypsin respectively. The total autolytic activity of the mutant was decreased by approximately 73%. The addition of Triton X-100 (0,5% vol/vol) to autolysing cultures of the lyt-1 mutant did not result in an increase in the rate of cellular autolysis in the presence or absence of trypsin. Cell-free supernatant fractions of the lyt-1 mutant after cellular autolysis did not produce zones of inhibition against actively growing indicator strains when assayed by the well method described by Barber

TABLE 3.1: Cellular Autolytic Activity of *Lyt* Mutants.

Strain	Cell bound autolytic activity %		Reduction in total activity compared to P262 strain(%)
	Active	Latent	
P262	93,3	6,7	–
lyt-1	85,8	14,2	72,8
lyt-2	34,4	65,6	16,1
lyt-4	85,4	14,6	25,2
lyt-5	56,6	43,3	24,2
lyt-8	82,1	17,9	81,2
lyt-9	92,3	7,7	56,4
lyt-10	88,2	12,2	42,6

et al., (1979). The morphology of the *lyt*-1 mutant was altered and it formed long chains of cells in CBM medium. The mutant was motile.

3.3.4 Autolysin Activity and Growth Phase

The levels of active and latent forms of the autolysin produced by the P262 strain and the *lyt*-1 mutant were compared at different stages in the growth cycle (Fig. 3.4). Both strains showed identical growth rates in CBM medium and peaks of active and total autolysin activity were obtained during mid-exponential growth. Autolysin activity decreased sharply at the end of exponential growth phase. The autolytic activity of the *lyt*-1 mutant was markedly less than that of the P262 strain and it produced 75% and 42% lower levels of active and total autolysin respectively. At the peak of autolysin activity (during mid-exponential growth) the ratio of active:latent autolysin was 14:1 in the P262 strain and 6:1 in the *lyt*-1 mutant.

Although the growth rates of the P262 strain and the *lyt*-1 mutant were identical in CBM medium, they differed in minimal medium. The *lyt*-1 mutant had a shorter lag phase, faster growth rate and a higher cell yield in the minimal medium than the P262 strain.

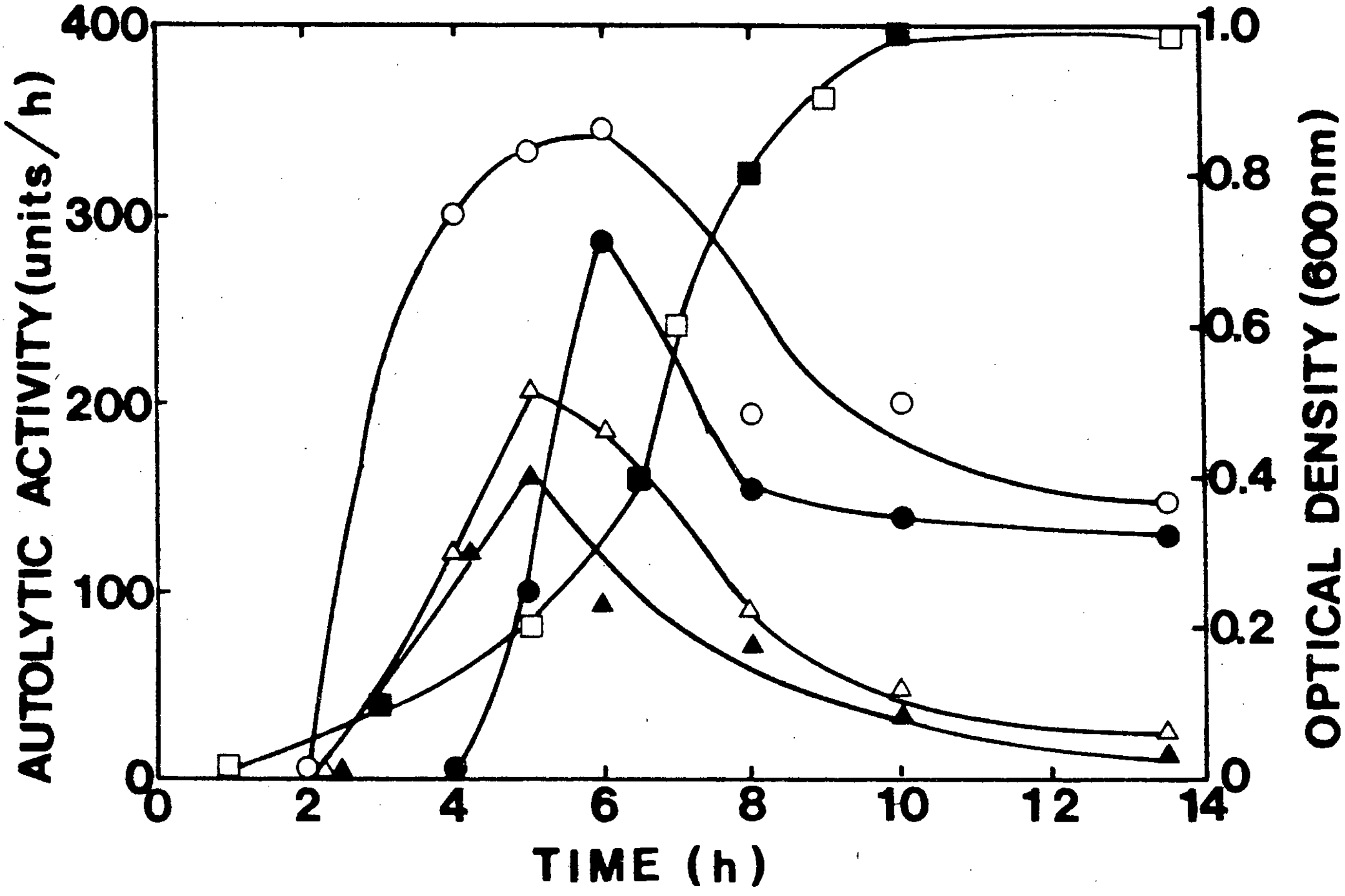

Fig. 3.4: Active and latent (trypsin-activatable) autolytic activity of *C. acetobutylicum* P262 and *lyt*-1 mutant strains during different stages in the growth cycle. Cells were removed from CBM cultures and subjected to cellular autolysis in 0,04 M sodium phosphate buffer, pH 6.3. Autolytic activity of P262 cells in the presence (○) and absence (●) of trypsin (1 μg/ml). Autolytic activity of *lyt*-1 cells in the presence (△) and absence (▲) of trypsin. Growth curve of the P262 strain (□) and the *lyt*-1 mutant (■).

3.3.5 Cell Wall Autolysin Activity

Cells from exponential CBM cultures of *C. acetobutylicum* P262 and *lyt*-1 strains were subjected to cellular autolysis in the sodium phosphate buffer. The activity of the autolysins released into the supernatant fractions was tested against SDS-treated wall preparations from each of the strains. Autolysin from the P262 strain and the *lyt*-1 mutant showed reduced activity against *lyt*-1 cell wall preparations (Table 3.2).

3.3.6 Autoplast (Protoplast) Formation

The production of autoplasts after 18 h incubation of the P262 strain in CBM was induced by the addition of sucrose (0,25 to 0,5 M) and 25% stable autoplasts were obtained (Table 3.3). The number of stable autoplasts was increased (approximately 50%) and the leakage of cellular contents was decreased by the addition of $CaCl_2$ or $MgCl_2$ (Table 3.3). These salts enhanced the stability of the autoplasts which were more spherical and phase-bright than those not treated with calcium or magnesium ions. Seventy percent autoplasts which were stable (no leakage detected) were produced when sucrose, $CaCl_2$ and $MgCl_2$ were added together. The *lyt*-1 mutant did not form autoplasts when added to CBM medium containing sucrose (0,25 M), $CaCl_2$ and $MgCl_2$.

TABLE 3.2: Effect of autolysin on SDS-treated cell walls from the *C. acetobutylicum* P262 strain and the *lyt*-1 mutant.

Autolysin source	Cell wall preparation (1 mg/ml)	[a]Autolysin activity (units)
P262	P262	91
P262	*lyt*-1	75
lyt-1	P262	45
lyt-1	*lyt*-1	33

[a] Average of five independent determinations.

TABLE 3.3: The effect of sucrose and Ca^{2+} and Mg^{2+} on the formation and stability of *C. acetobutylicum* P262 and *lyt*-1 mutant autoplasts in CBM. The % autoplasts and % leakage was determined after 18 h incubation in the different media.

Strain	Autoplasting medium	Autoplasts (%)	Leakage[a] (%)
P262	CBM + 0,25 M sucrose	25	76
P262	CBM + 0,50 M sucrose	25	72
P262	CBM + 0,50 M sucrose + 25 mM Mg^{2+}	40	36
P262	CBM + 0,50 M sucrose + 25 mM Ca^{2+}	50	24
P262	CBM + 0,50 M sucrose + 25 mM Mg^{2+} + 25 mM Ca^{2+}	70	0
lyt-1	CBM + 0,25 M sucrose + 25 mM Mg^{2+} + 25 mM Ca^{2+}	0	0

[a] Leakage was expressed as the percentage absorbance at 260 nm in the supernatant fluid; as a reference the absorbance in a completely lysed protoplast suspension following sonication for 5 min at maximum amplitude was designated as 100%.

The effect of lysozyme on the production of autoplasts was determined by adding cells to CBM medium containing 0,25 M sucrose, 25 mM $CaCl_2$, 25 mM $MgCl_2$ and 1 mg/ml lysozyme (Table 3.4). Lysozyme increased the number and enhanced the rate of stable autoplast formation. The *lyt*-1 mutant only formed autoplasts in the presence of lysozyme.

Autoplast formation and autolysin activity was inhibited by 1mM $FeCl_3$ and 1 mM $CuSO_4$. Gelatin (5%), 1 mM $MgCl_2$ and 1 mM $MgSO_4$ did not inhibit the P262 autolysin or autoplast formation.

3.3.7 Effect of Cell Wall Antibiotics

The effect of penicillin, ampicillin, vancomycin and D-cycloserine on the P262 and *lyt*-1 strains was determined (Fig. 3.5). The *lyt*-1 mutant showed the same sensitivity to penicillin, ampicillin and vancomycin as the P262 strain. Although these antibiotics inhibited exponential broth cultures, no lysis was observed. Penicillin treated cells showed a 10^5-fold loss in viability within 60 min after the addition of penicillin (Table 3.5). Similar results were obtained with ampicillin and vancomycin. The minimal inhibitory concentration of penicillin in CBM agar plates was 0,5 μg/ml for both

TABLE 3.4: Effect of lysozyme on the production of autoplasts in C. acetobutylicum P262 and lyt-1 mutant cells. Exponential phase cells in CBM (3 ml) were added to 10 ml of CBM containing 0,25 M sucrose, 25 mM Ca^{2+} and 25 mM Mg^{2+} in the presence and absence of lysozyme.

Time (min)	Autoplasts (%)			
	P262 strain		lyt-1 mutant	
	Control	Lysozyme (1 mg/ml)	Control	Lysozyme (1 mg/ml)
0	0	0	0	0
30	10	19	0	0
60	12	47	0	26
90	7	77	0	73
120	16	76	0	88
240	21	94	0	97

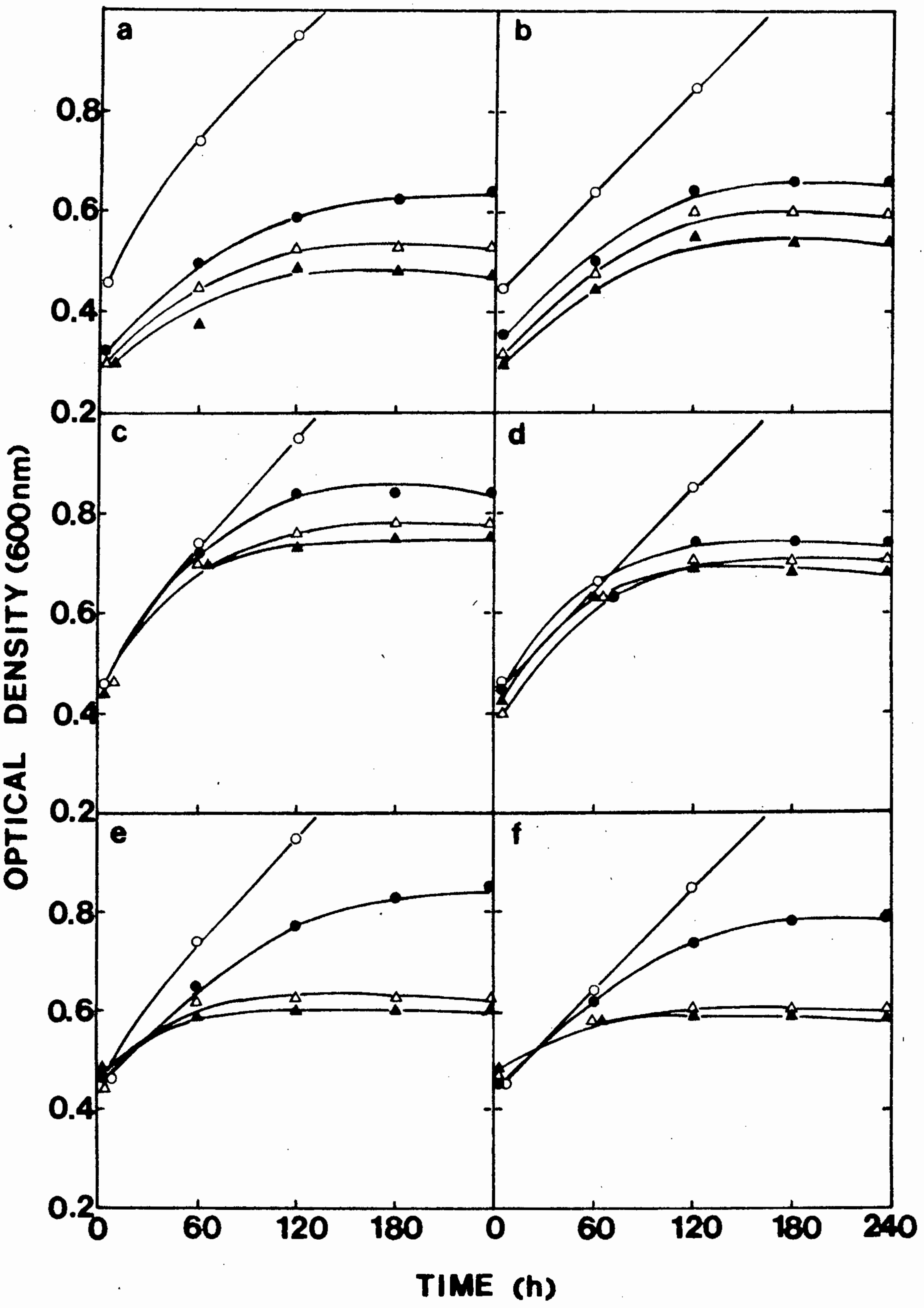

Fig. 3.5: Inhibition of C. acetobutylicum P262 and lyt-1 strains by penicillin (a,b), ampicillin (c,d) and vancomycin (e,f). Antibiotics were added to exponential phase P262 (a,c,e) and lyt-1 (b,d,f) cells at time 0. The following concentrations of antibiotics were used: 0,025 (●), 0,05 (△) and 0,1 (▲) g/ml penicillin (a,b); 0,10 (●), 0,25 (△) and 0,50 (▲) g/ml ampicillin (c,d); 0,50 (●), 1,0 (△) and 2,0 (▲) g/ml vancomycin (e,f). Control cultures without antibiotics (○).

TABLE 3.5: Effect of penicillin (0,10 μg/ml) on the viability of the *C. acetobutylicum* P262 and the *lyt*-1 mutant.

Time after addition of penicillin (min)	P262 + penicillin c.f.u. /ml	*lyt*-1 + penicillin c.f.u. /ml
0	$3{,}0 \times 10^7$	$2{,}6 \times 10^7$
60	$2{,}4 \times 10^2$	$1{,}3 \times 10^2$
120	$2{,}0 \times 10^1$	$2{,}7 \times 10^2$
180	$8{,}0 \times 10^1$	$5{,}0 \times 10^1$
330	$1{,}0 \times 10^1$	$8{,}0 \times 10^1$

the P262 and lyt-1 strains. Both strains were resistant to 100 μg/ml D-cycloserine.

The effect of penicillin pre-treatment on the autolysis of lyt-1 cells was determined. Penicillin treatment of lyt-1 cells for 1 h prior to the addition of exogenous P262 autolysin enhanced cellular autolysis in the autolysis buffer (Table 3.6) and under these conditions the lyt-1 cells showed similar rates of autolysis as the untreated P262 strain. The addition of chloramphenicol (2,5 μg/ml) 5 min before the addition of penicillin did not protect the lyt-1 cells from the increased rate of autolysis by exogenous P262 autolysin.

3.3.8 Isoelectric Focusing and SDS-PAGE of *C. acetobutylicum* Autolytic Enzymes

Cell-free autolytic supernatant fractions from 36 h CBM + sucrose cultures of *C. acetobutylicum* P262 and lyt-1 strains and autolysates following cellular autolysis of strain P262 and lyt-1 mutant CBM cultures were electrophoresed on SDS-polyacrylamide gels. The P262 cell-free fraction had a band at *c*. 28 000 M.W. which was absent in the lyt-1 cell-free sample (Figure 3.6). The fractions of autolysed cells from both the P262 and lyt-1 strains concentrated 100-fold by freeze drying contained a broad band at *c*. 28 000 M.W. Isolation of a single autolysin produced by *C. acetobutylicum*

TABLE 3.6: Effect of penicillin on the autolysis of *C. acetobutylicum* *lyt*-1 cells in the presence of exogenous wild-type autolysin.

Strain	Addition of *pen* and/or *cml*	Addition of exogenous wild-type autolysin	Autolytic activity (units)
P262	*pen*	-	134
lyt-1	*cml*	-	62
lyt-1	*pen*	-	47
lyt-1	*cml*	-	56
lyt-1	-	+	60
lyt-1	*pen*	+	138
lyt-1	*pen* + *cml*	+	120

Lyt-1 cells treated with penicillin (*pen*, 0,1 µg/ml) for 60 min were transferred to autolysis buffer and the autolytic activity determined in the presence or absence of exogenous wild-type autolysin. Chloramphenicol (*cml*, 2,5 µg/ml) was added 5 min before the addition of *pen*.

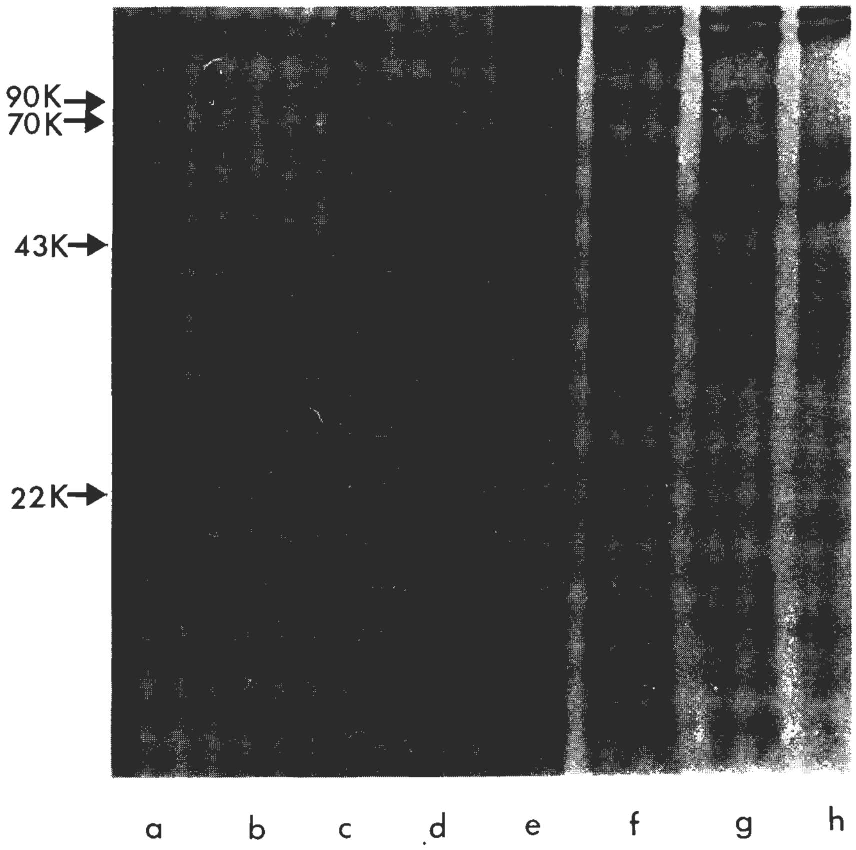

Fig. 3.6: SDS-polyacrylamide gel electrophoresis of *C. acetobutylicum* autolytic enzymes.

Samples were: lanes a and b, P262 cell-free supernatant fraction; c and d, *lyt*-1 cell-free supernatant fraction; e and f, P262 cellular autolysate; and g and h, *lyt*-1 cellular autolysate. The cellular autolysate fractions were concentrated 100-fold by freeze drying. Molecular weight markers were: transferrin (90 K); albumin (70 K); ovalbumin (43 K) and humane growth hormone (22 K).

in molasses or CBM + sucrose media by Webster (1980) was shown to have a M.W. of 28 000. The autolysin was a glycoprotein and had a different mode of action from lysozyme.

Isoelectric focusing of supernatant fractions from P262 CBM + sucrose cultures showed peaks of autolysin activity at pH 6.0 and pH 8.0 (Fig. 3.7). The fractions showing autolytic activity also contained bands at c. 28 000 M.W. on SDS-PAGE (Fig. 3.8). Two autolysins were therefore present in the cell-free supernatant fractions which migrated very closely on SDS-PAGE and had M.W. of approximately 28 000.

3.3.9 Electron Microscopy of Cellular Autolysis in *C. acetobutylicum*

The course of lysis of *C. acetobutylicum* P262 exponentially growing cells in phosphate buffer is shown in Fig. 3.9. The characteristic multibanded wall of exponentially growing cells is visible in Fig. 3.9 (a).

After 5 min in phosphate buffer sites of autolytic activity appeared along the cylindrical portion of the cell (Fig. 3.9 b). During the course of lysis the cells appeared to plasmolyse, showing retraction of the cytoplasmic membrane away from the cell wall (Figs.

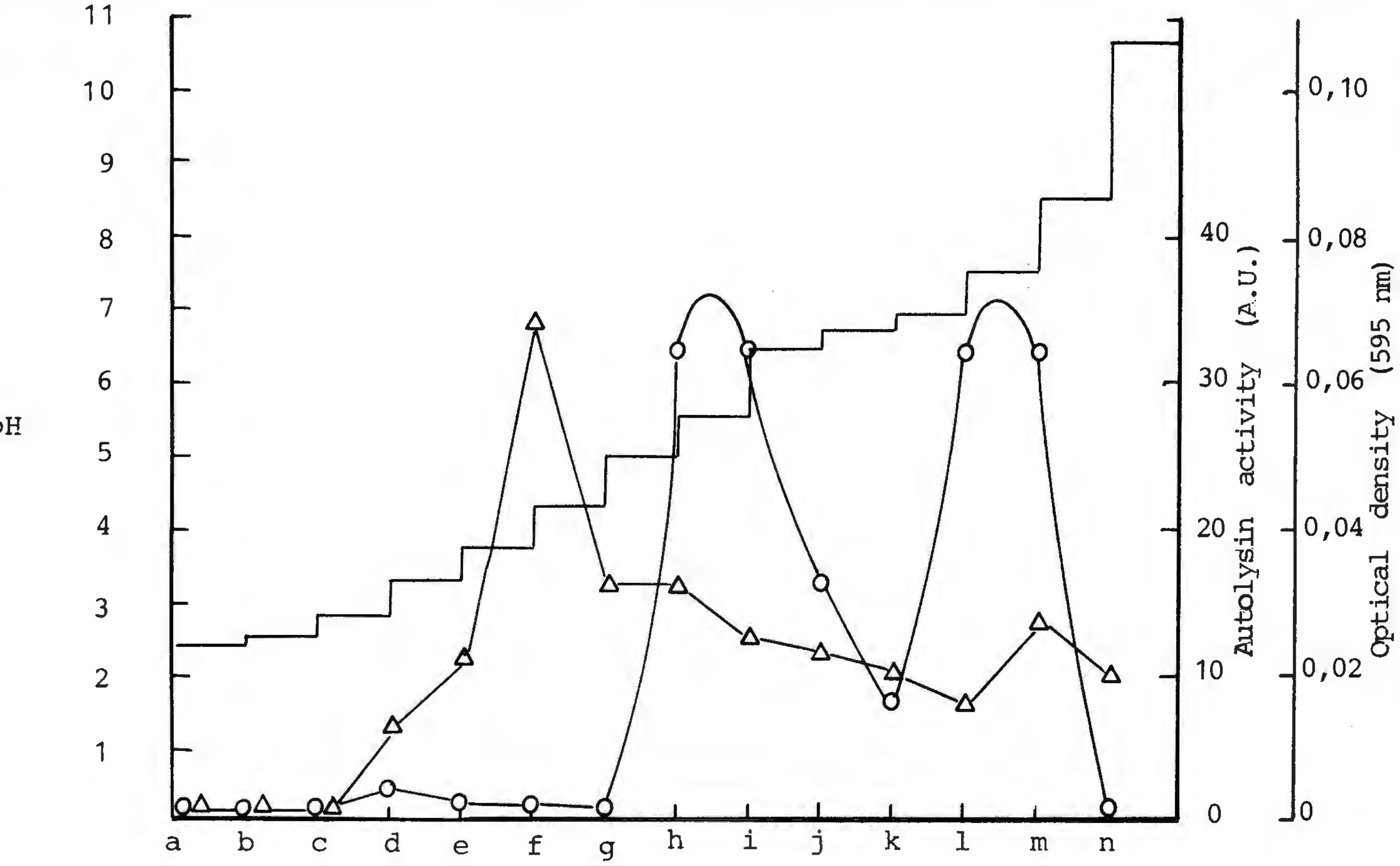

Fig. 3.7: Isoelectric focusing of C. acetobutylicum P262 supernatant fractions from CBM+ sucrose cultures. P262 culture supernatants following isoelectric focusing were divided into fractions (a-n) and assayed for autolysin activity (0) and protein concentration (Δ).

Fig. 3.8: SDS-polyacrylamide gel electrophoresis of strain P262 supernatant fractions following isoelectric focusing. Molecular weight markers (lane i) are: transferrin (90 000); albumin (70 000); ovalbumin (43 000) and humane growth hormone (22 000).

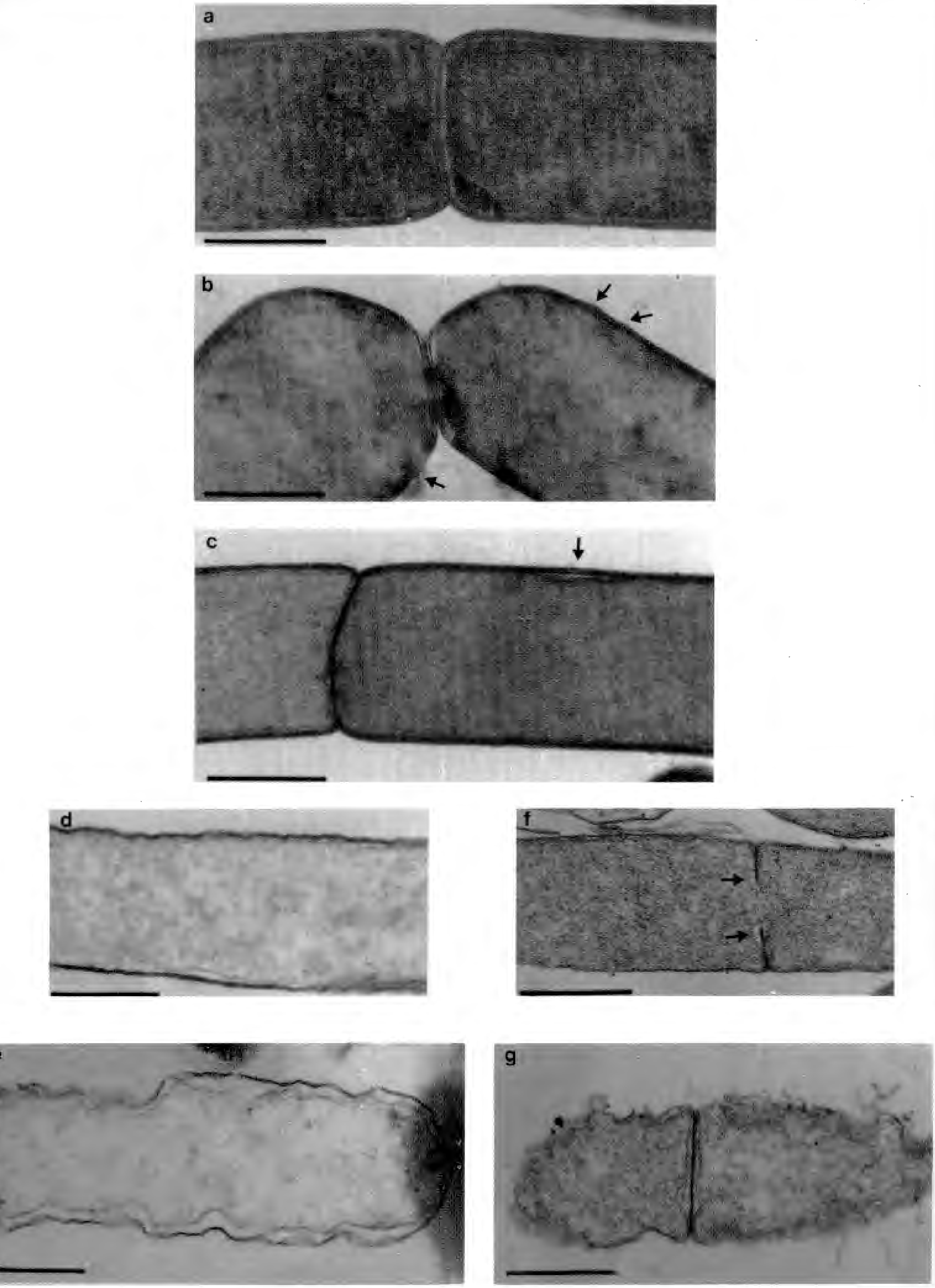

Fig. 3.9: For legend see opposite.

Figure 3.9: Longitudinal sections of *C. acetobutylicum* P262 from exponentially growing cultures in CBM immediately after resuspension in 0,04 M sodium phosphate buffer, pH 6.3 (a), and after 5 min (b), 15 min (c) and (d), 30 min (e) and (f), and 45 min (g). Arrows in (b) indicate initial sites of autolysis. Arrows in (c) and (f) indicate retraction of the cytoplasmic membrane from the cell wall. Note the presence of the cross-wall in (g). Bar markers represent 0,5 μm.

3.9 c, d, e). After 15 min in phosphate buffer two patterns of lysis were evident. The first pattern involved breakdown of the cytoplasmic membrane which resulted in leakage of the cellular contents through a limited number of sites. Leakage of the cytoplasmic contents prevented any further autolysis and the cells appeared as empty "ghosts" (Figs. 3.9 d, e). Alternatively the cytoplasmic membrane remained intact and autolytic activity continued resulting in extensive removal of the cell wall (Fig. 3.9 f) until no wall remained (Fig. 3.9 g) and the cell eventually lysed. An interesting feature of the lysis procedure was the apparent resistance of the cross-wall in dividing cells to autolysis. In the majority of dividing organisms the septal wall was often the only remaining wall visible.

During autolysis removal of the cell wall did not initiate the formation of protoplasts (swollen cells). In fact, even when the cell wall was completely removed, cells retained their original shape. The cytoplasm also underwent significant changes during lysis and seemed to "coagulate".

3.4 DISCUSSION

In common with a number of bacteria (Ghuysen and Shockman, 1973; Rogers, 1979; Rogers et al., 1980) the C. acetobutylicum P262 strain produces latent and active forms of an autolytic enzyme(s). Proteases are able to activate the enzyme(s). It appears as if two active cell-free enzymes are produced and a single autolytic glycoprotein (MW 28 000) has been purified from an industrial fermentation medium (Webster, et al., 1981) and from supernatant fractions following sucrose induced cellular autolysis (unpublished results). Autolysis of C. acetobutylicum is similar to that in S. faecalis (Pooley and Shockman, 1970) and Lactobacillus acidophilus (Coyette and Ghuysen, 1970) in that the ability of cells to autolyze decreases sharply when the cultures enter the stationary growth phase.

The autolytic-deficient mutant, lyt-1 is pleiotropic in that it produces less autolysin than the parent P262 strain and it has an altered cell wall which is more resistant to both its own and P262 autolysin. Treatment of the lyt-1 mutant with Triton X-100 which was shown to reveal the presence of cryptic autolysins in S. faecium (Shungu et al., 1980), did not increase autolytic activity. In characterising an autolytic-defective mutant of S. faecalis, Cornett et al.,(1978) indicate that it is not surprising to find pleiotropic effects from a mutation that has altered the cell envelope or a cell wall-hydrolytic enzyme(s). Autolytic-defective mutants of *B. subtilis* have been described as carrying a single mutation which results in several phenotypic alterations in addition to the loss of two (Fein and Rogers, 1976) or more (Ayusawa *et al*., 1975) detectable enzyme activities.

The *C. acetobutylicum* *lyt*-1 mutant is similar to the majority of autolytic-deficient mutants (Ghuysen and Shockman, 1973; Rogers, 1979; Rogers *et al*., 1980) which have been described in that they all possess reduced but detectable levels of autolytic activity and form long chains of cells (Tomasz, 1968; Pooley *et al*., 1972). There are only two examples of mutations altering the autolytic enzymes themselves (Fan and Beckman, 1973; Cornett *et al*., 1978). The *C. acetobutylicum* *lyt*-1 mutant differs from mutants of *S. faecium* with reduced autolytic activity which show reduced growth rates and yields in complete and minimal media (Shungu *et al*., 1979) and appears to be unusual in that it is inhibited by the same concentration of penicillin, ampicillin and vancomycin as the parental strain. Pleiotropic mutants of *B. licheniformis* (Rogers and Foresberg, 1971) and *Diplococcus pneumoniae* (Tomasz *et al*., 1970) which had walls that were modified so that they were more resistant to their own autolytic enzymes themselves, were more resistant to wall inhibiting antibiotics than the parental strains. Lysis by low concentrations of wall inhibiting antibiotics was reduced in autolytic-deficient mutants of *B. subtilis* (Fein and Rogers, 1976). In *S.faecalis* an increase in penicillin and cycloserine resistance occurred as the ability of the cells to autolyse decreased and as the wall thickened (Shockman, 1959).

Penicillin treatment of the *lyt*-1 mutant enhanced lysis upon the addition of exogenous wild-type autolysin in the autolysis buffer. A similar observation in growth medium was reported for an autolysin-defective *D. pneumoniae* mutant (Tomasz and Waks, 1975). However, the two mutants differed in that chloramphenicol did not protect the *C. acetobutylicum* *lyt*-1 cells against lysis by exogenous enzyme. The *D. pneumoniae* cells were protected by chloramphenicol and it was concluded that it protects bacteria against penicillin induced lysis by interfering with the activity of the autolytic enzyme.

The *C. acetobutylicum* *lyt*-1 mutant therefore has two interesting properties (the same sensitivity to penicillin, ampicillin and vancomycin as the wild-type parent and no protection by chloramphenicol from penicillin induced lysis) which make it suitable for further studies on the mode of action of penicillin and related antibiotics.

Pleiotropic effects of the lyt-1 mutant include increased resistance to lysis by butanol and a higher sugar tolerance (van der Westerhuizen, 1981). The ability of the lyt-1 mutant to produce solvents has been investigated and preliminary results have indicated that as autolysis is not induced by high concentrations of sucrose, it has the potential of utilizing and converting higher concentrations of molasses to solvents than the parent strain (van der Westhuizen, 1981).

Isoelectric focusing and PAGE of cell-free and cell-bound autolysates of *C. acetobutylicum* P262 cultures have indicated the presence of two autolytic enzymes of different isoelectric points. These proteins band closely together on gel electrophoresis at a position corresponding to a molecular weight of 28 000. Supernatant fractions of autolytic-deficient lyt-1 cultures lack the band at 28 000 M.W. Further work on the cell-bound autolysate needs to be done to determine whether both enzymes are present in the cell bound system.

Electron microscopic observations of autolysing cells suggest that the autolysin is located at discrete sites along the peripheral wall, and that disruption of the cross wall in dividing orgnaisms only occurs after complete degradation of the peripheral wall has occurred. This is different from the lysis of B. subtilis cells where the major site of attack is the cross-wall and lysis of peripheral walls only occurs after septal dissolution has been initiated (Burdett, 1980). During lysis in C. acetobutylicum the cells appeared to plasmolyse and the cytoplasmic membrane retracted away from the cell wall. This was accompanied by autolytic activity at discrete sites along the wall of the cell.

The cytoplasm also underwent significant changes during lysis and seemed to "coagulate". The fact that protoplasts were not formed and that cells with no cell wall retained their original shape provides evidence for this hypothesis.

Degradation of the cell wall during autolysis therefore appears to occur, not by a process of continuous thinning, but by an increase in both the size and number of gaps in the wall.

CHAPTER 4

PROTOPLAST PRODUCTION AND REGENERATION IN C. ACETOBUTYLICUM

Methods for the production and regeneration of viable C. acetobutylicum protoplasts have been developed. The protoplasts are stable in sucrose solutions containing Mg^{2+} and Ca^{2+}.

4.1 INTRODUCTION

When a wall of bacterium is partly damaged by incubation with penicillin in hypertonic media, or by treatment with lysozyme, it is ordinarily repaired once the antibiotic or enzyme is withdrawn (Altenbern, 1963; Miller et al., 1967). However, if all of the wall is stripped away and (naked) protoplasts are formed, synthesis of new wall material does not restart when penicillin or lysozyme is removed. Protoplasts kept in liquid medium enlarge but do not divide (Landman and Forman, 1969). In soft-agar containing protein, protoplasts often give rise to L-colonies which are unable to initiate wall formation through successive cell generations. Depending on the bacterial species, these L-colonies may never show reversion to the walled bacillary state, e.g., the penicillin-induced L-forms of *E. coli* mutants (Landman, 1968), or reversion may occur when the L-colonies have barely begun to grow. This occurs in lysozyme-induced protoplasts of *B. megaterium* (Kawakami and Landman, 1966). Landman and Forman (1969) found that protoplasts of *B. subtilis* prepared in a minimal medium reverted fairly synchronously 3 to 4 h after innoculation into 25% gelatin medium. These protoplasts primed by preincubation in casein hydrolysate enriched minimal medium reverted within 1 h in the gelatin medium. The priming was blocked by chloramphenicol, puromycin and actinomycin D but was not affected by penicillin, lysozyme or inhibition of DNA synthesis.

Clive and Landman (1970) developed methods for the increased reversion of protoplasts or L-bodies of *B. subtilis*. They utilized certain types of membrane filters some of which had to be extracted with 2% ethanol in water for optimal growth. Growth on the filters enhanced reversion of protoplasts to the bacillary form. These authors found that reversion was further enhanced when *B. subtilis* cell wall preparations were added to the filters. The stimulation of reversion by isolated walls was non-specific since similar stimulation could be obtained with autoclaved *B. subtilis*, *E. coli*, *Pseudomonas* and yeast cells. The stimulation of reversion probably depended upon physical contact between the naked protoplasts or L-bodies and the surface provided by the filter, walls or killed organisms.

A method for the production of wall-free protoplasts from exponential phase cells of S. faecalis in the absence of added lytic enzymes was developed by Joseph and Shockman (1974). Exponential phase cells resuspended in a hypertonic sucrose medium became osmotically fragile within 1,5 h due to the action of the native autolytic enzyme on the cell wall peptidoglycan. Maximum cell wall loss occurred after 3 to 6 h. The resultant osmotically fragile bodies were termed "autoplasts". The addition of trypsin which activated the latent form of the autolysin increased the rate of formation of autoplasts two to threefold.

De Castro-Costa and Landman (1977) demonstrated that protoplasts of B. subtilis produced a reversion inhibitory factor which blocked reversion. This inhibitor was nondialyzable and sensitive to trypsin, heat and detergent. Comparison of the autolytic behaviour of B. subtilis and the reversion inhibitory factor revealed several similarities and it was suggested that the reversion inhibitory factor was an autolysin.

Protoplast fusion using polyethylene glycol is important as a genetic recombination system. Protoplast regeneration is an important aspect of protoplast fusion. Gabor and Hotchkiss (1979) investigating the fusion of B. subtilis protoplasts increased regeneration frequencies to reach 100% by the addition of 1% bovine serum albumin to the hypertonic dilution and suspension buffers and the addition of 0,5% gelatin to the reversion media. Genetic recombination frequencies, however, did not increase correspondingly and it was concluded that regeneration of recombinant-forming cells was independently determined and not closely related to the average regeneration of the population. Kinetic studies with varying individual parental or total protoplast concentrations indicated that protoplast collision and contact was not the limiting factor determining the number of genetic recombinants obtained (Gabor and Hotchkiss, 1979).

As an initial step in the development of a genetic transfer system in C. acetobutylicum we have investigated the formation and regeneration of protoplasts (Allcock et al., 1981 b).

4.2 METHODS

4.2.1 Media and Materials

See Appendix A.

4.2.2 Bacterial Strains

C. acetobutylicum P262 and the autolytic-deficient mutant *lyt*-1 were utilised.

4.2.3 Production of Protoplasts of *C. acetobutylicum*

The production of protoplasts of *C. acetobutylicum* P262 was investigated by adding exponential phase cells to various osmotic stabilising solutions containing lysozyme (1 mg/ml). The percentage protoplasts were determined after 1 h by microscopic counts.

4.2.4 Protoplast Formation and Induction of Autoplasts of *C. acetobutylicum* by Sucrose

The induction of autoplast formation was investigated by adding exponential phase cells to CBM supplemented with 0,3 to 0,5 M sucrose. Cultures were viewed microscopically after 18 h incubation for autoplast production.

Protoplasts of <u>C. acetobutylicum</u> were prepared by preconditioning the cells by growth in CBM containing glycine (0,4% w/v) prior to adding the cells to CBM + 0,3 M sucrose (CBM + S) containing lysozyme (1 mg/ml).

4.2.5 Inhibitors of Autoplast Formation

The effect of various inhibitors on autoplast formation was investigated by adding the inhibitors to the autoplasting medium prior to innoculation with exponential phase <u>C. acetobutylicum</u> cells. After 18 h incubation cultures were viewed microscopically for the presence of autoplasts and supernatant fractions after centrifugation at 12061 x <u>g</u> for 10 min were assayed by the well plate method (Barber <u>et al</u>., 1979) for the presence of autolysins. Inhibitors tested included gelatin (0,5 to 5,0% w/v); palmitic acid (200 nmol); stearic acid (200 nmol); $MgCl_2$ (0,01 M); $CaCl_2$ (0,01 M); $FeCl_2$ (0,001 M) and $CuSO_4$ (0,001 M).

4.2.6 Osmotic Sensitivity of C. acetobutylicum Protoplasts

In order to determine the osmotic sensitivity of <u>C. acetobutylicum</u> protoplasts, various buffers were tested for their ability to lyse sensitive protoplasts without affecting unprotoplasted cells. Protoplast lysis was determined after 1 h by measuring the decrease in optical density at 600 nm or by measuring the leakage into the supernatant fraction of material absorbing at 260 nm. The stability of C. acetobutylicum protoplasts was determined by incubating washed exponentially growing cells in the phosphate autolysis buffer (see Chapter 3) containing varying concentrations of sucrose. The effect of $CaCl_2$ (25 mM) and $MgCl_2$ (25 mM) added to the autoplasting cultures was determined by measuring optical density changes at 600 nm.

4.2.7 Development of a Regeneration Medium

The effect of various physical and chemical parameters (see Table 4.1) on the regeneration of C. acetobutylicum protoplasts was determined. The number of osmotically sensitive cells and the percentage regeneration was determined by the difference between the number of colony forming units (c.f.u.) obtained on the regeneration medium after diluting the protoplast suspensions in either CBM + S or anaerobic water (pH 7.0).

4.2.8 Effect of Lysozyme on Protoplast Production and Regeneration

The effect of lysozyme on the production and regeneration of C. acetobutylicum P262 and lyt-1 protoplasts was

TABLE 4.1: Physical and Chemical Parameters Used to Determine Optimal Regeneration of *C. acetobutylicum* Protoplasts

1. Additions to the RM:(g/ℓ)

   50,0 gelatin

   4,9 $MgCl_2.6H_2O$

   3,7 $CaCl_2.2H_2O$

   8,0 bovine serum albumin

   6,0 casein hydrolysate

   5,0 agar

2. Addition of equal volumes of autoclaved *C. acetobutylicum* cells or SDS-treated autoclaved cells prior to plating.

3. Plating on membrane filters and membrane filters extracted with (2% v/v) ethanol.

4. Pre-treatment of protoplasts for 60 min with chloramphenicol (10-100 µg/ml); pronase (100-300 µg/ml) and trypsin (100-300 µg/ml) prior to plating.

5. Preconditioning by growth in CBM + S containing gelatin (12,5% w/v).

investigated. Exponential phase cells were added to CBM + S containing lysozyme (400 or 1 000 μg/ml). After 60 and 120 min incubation samples were diluted in either CBM + S or anaerobic water and plated on regeneration medium.

4.3 RESULTS

4.3.1 Production of Protoplasts of C. acetobutylicum

The production of protoplasts of C. acetobutylicum was studied by adding exponential phase cells to various osmotic stabilising solutions containing lysozyme (Table 4.2). Sucrose (0,3 to 0,5 M) and lactose (0,5 M) solutions produced 80 to 83% protoplasts after 1 h at 37°C. Other osmotic stabilisers were not as effective in the production of protoplasts by lysozyme. Preconditioning the cells by growth in CBM containing glycine (0,4% w/v) prior to adding the cells to lysozyme in CBM + S increased the rate and number of protoplast formation and 90% protoplasts were obtained within 15 min.

Photomicrographs of lyt-1 protoplasts after 1 h incubation in CBM + S containing lysozyme are shown in Fig. 4.1. Phase bright and phase dark protoplasts were visible under phase contrast optics. The phase dark

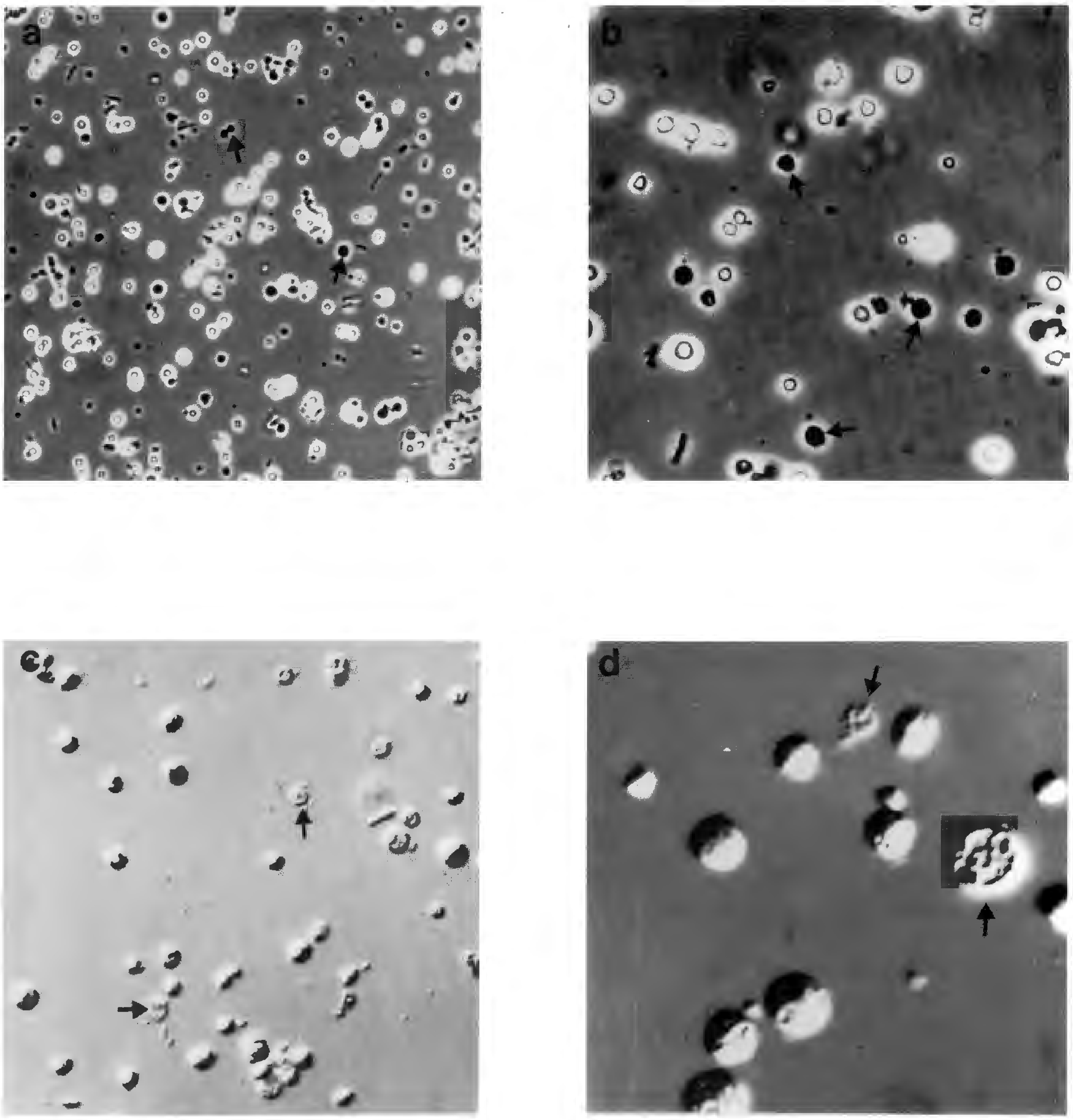

Fig. 4.1: Photomicrographs of lyt - 1 protoplasts after 1 h incubation in CBM+S containing lysozyme (1 mg/ml) taken under phase contrast (a and b) and interference contrast (c and d) optics. Magnification was (a) 160 x (b) 400 x (c) 400 x and (d) 1 000 x. Arrows indicate phase dark granular protoplasts.

protoplasts appeared very granular under interference contrast optics (indicated by arrows) and appeared to be non-viable since suspensions of protoplasts exposed to air contained predominantly phase dark protoplasts and showed low levels of regeneration.

4.3.2 Induction of Autoplasts of C. acetobutylicum by Sucrose

Induction of autoplast formation by sucrose was very rapid and cells which could not plate on CBM agar were produced within 5 min in CBM + 0,5 M sucrose (Fig. 4.2). Induction was less rapid in CBM + 0,3 M sucrose and below 0,3 M sucrose in CBM no osmotic sensitivity was observed.

4.3.3 Inhibitors of Autoplast Formation

Gelatin, palmitic acid and stearic acid which are known to be inhibitors of autolysins did not affect autoplast formation in C. acetobutylicum (Table 4.3) and cell free autolysin assayed by the well plate method was present in these cultures. The addition of $MgCl_2$ and $CaCl_2$ had a slight inhibitory effect on the number of autoplasts formed and cell free autolysin was also present in these cultures. The addition of $FeCl_2$ and $CuSO_4$ inhibited autoplast formation and only 10% autoplasts

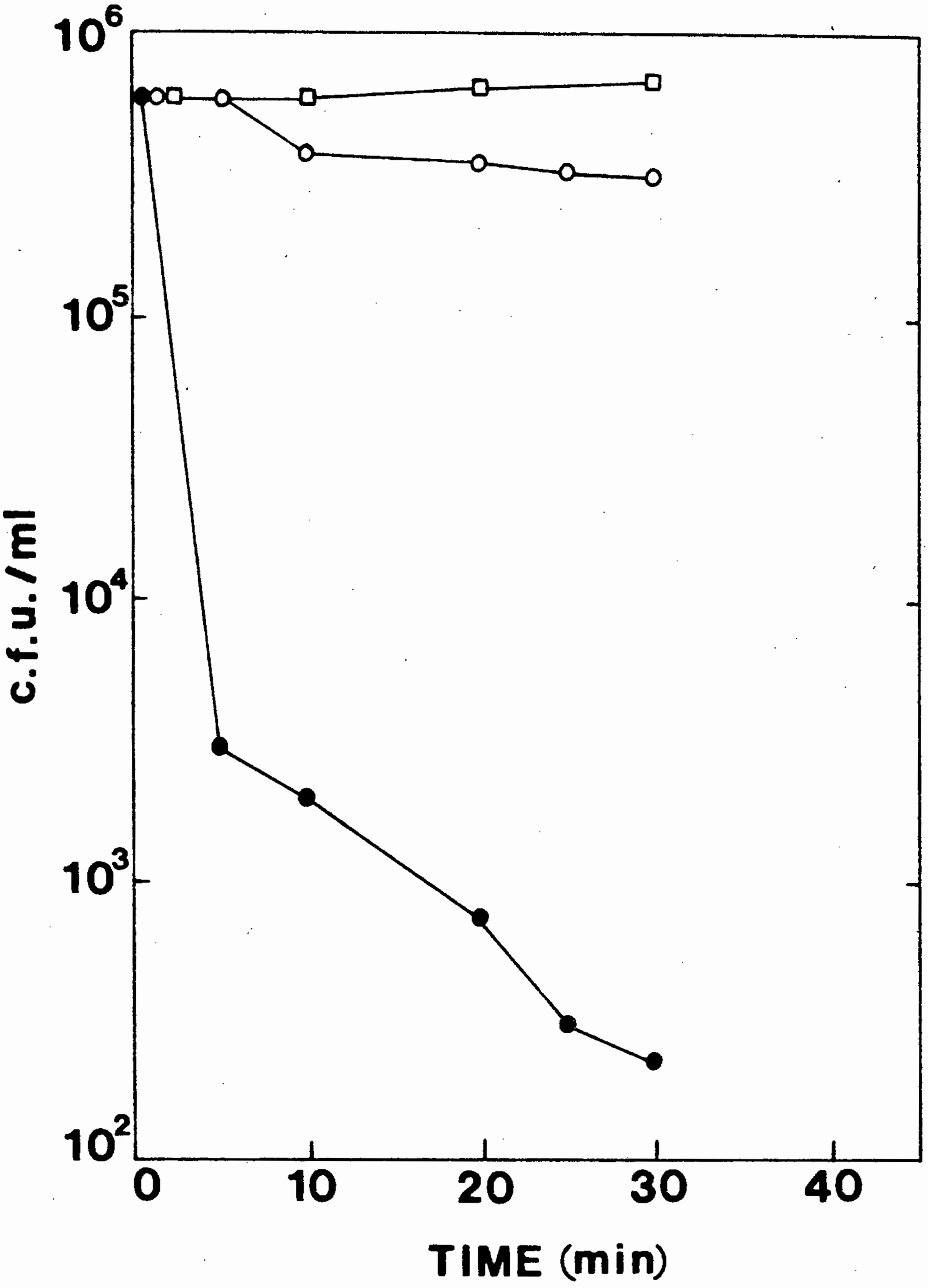

Fig. 4.2: Induction of C. acetobutylicum autoplasts by sucrose. Exponential phase cells were added to: CBM + 0,5 M sucrose (●); CBM + 0,3 M sucrose (O) and CBM (□). Samples taken during incubation were plated on CBM agar.

TABLE 4.3: Effect of Inhibitors on *C. acetobutylicum* Autoplast Formation.

Inhibitor	Concentration	[a]Autoplasts %	[b]Autolysin
No addition	-	80	+
Gelatin	0,5 to 5%	80	+
Palmitic acid	2,0 x 10^{-7} M	80	+
Stearic acid	2,0 x 10^{-7} M	80	+
$MgCl_2.6H_2O$	0,01 M	75	+
$CaCl_2.2H_2O$	0,01 M	60	+
$MgSO_4.6H_2O$	0,01 M	60	+
$CuSO_4.5H_2O$	0,001 M	12	-
$FeCl_3.6H_2O$	0,001 M	10	-

[a] Percentage autoplasts were determined after 18 h incubation in CBM + 0,5 M sucrose + inhibitor by microscopic counts of 8 random fields per sample.

[b] The presence (+) or absence (-) of autolysin was determined by the well plate method of Barber *et al*., (1979).

were present. No cell free autolysin was detected in these cultures.

4.3.4 Lysis of C. acetobutylicum protoplasts

In order to measure the degree of protoplasting and regeneration, a range of buffers was tested for their ability to cause lysis of C. acetobutylicum protoplasts without affecting the unprotoplasted cells. Ringers and TES buffers both caused significant lysis of exponentially growing cells and were unsuitable for use in regeneration experiments (Table 4.4). Anaerobic water at pH 7.0 caused $<$ 5% lysis of growing cells and resulted in 40% lysis of protoplasts in suspension over 1 h.

Autoplasts were stable in buffers containing sucrose, $MgCl_2$ and $CaCl_2$ (Fig. 4.3) and no lysis was observed during 5 h incubation. The addition of $CaCl_2$ and $MgCl_2$ to the autolysis buffer decreased the rate of autolysis. When bacterial chains were present, autolytic activity released individual cells from the chains and this could account for the increase in optical density observed during the first 30 min.

TABLE 4.4: Lysis of *C. acetobutylicum* cells and Protoplasts in Buffer Solutions.

Buffer	Exponentially Growing Cells	Protoplast Suspension
	[a]% lysis	
Ringer solution (1/4 strength)	14,3	10,0
TES (0,25 M, pH 7.0)	10,5	33,3
Anaerobic water (pH 7,0)	4,5	41,6

[a] Lysis of suspensions was determined as the % decrease in optical density at 600 nm in 1 h.

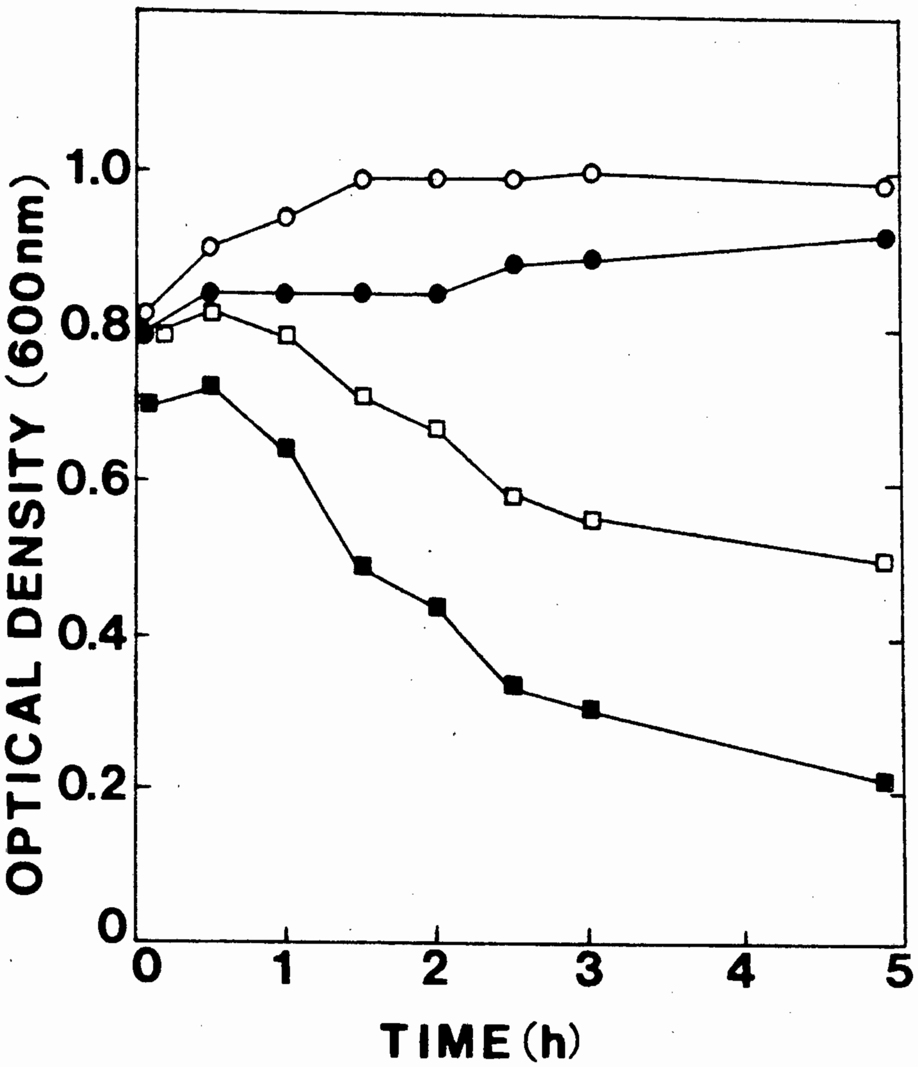

Fig. 4.3: Stability of *C. acetobutylicum* autoplasts. Exponential phase cells were added to phosphate autolysis buffer (0,04 M) containing: 0,5 M sucrose + 0,25 M $MgCl_2$ + 0,25 M $CaCl_2$ (○); 0,3 M sucrose + 0,25 M $MgCl_2$ + 0,25 M $CaCl_2$ (●); 0,25 M $MgCl_2$ + 0,25 M $CaCl_2$ (□); No addition (■).

4.3.5 Development of a Regeneration Medium (RM)

The regeneration of C. acetobutylicum protoplasts was determined using many different media and the best RM which routinely gave 80% regeneration was CBM containing (g/ℓ): casein hydrolysate, 10,0; gelatin, 50,0; $MgCl_2.6H_2O$, 5,1; $CaCl_2.2H_2O$, 3,7; and agar, 20,0. The percentage regeneration was increased 5 to 10% either by plating the protoplasts with an equal volume of autoclaved C. acetobutylicum cells or the addition of bovine serum albumin (0,8% w/v) to the RM. Techniques used to increase the percentage regeneration in other bacteria were also investigated but did not affect the ability of the protoplasts to regenerate. These techniques included plating with SDS-treated autoclaved cells, plating on membrane filters, pre-treatment of protoplasts for 60 min prior to plating with chloramphenicol, pronase or trypsin and preconditioning by growth in CBM + S containing gelatin.

Optimal stability and regeneration of protoplasts was obtained when the protoplasts were prepared in CBM + S supplemented with $MgCl_2$ (25 mM) and $CaCl_2$ (25 mM) (Table 4.5). Addition of $MgCl_2$ or $CaCl_2$ on their own increased stability of the protoplasts but decreased their ability to regenerate.

TABLE 4.5: Effect of Ca^{2+} and Mg^{2+} on the stability and regeneration of *C. acetobutylicum* protoplasts.

Protoplasting medium	c.f.u. after dilution in:		Reversion[a] %	Leakage[b] %
	CBM + S	Water		
CBM + S	$9,6 \times 10^4$	$5,5 \times 10^4$	43	72
CBM + S + 25 mM Mg^{2+}	$3,9 \times 10^5$	$3,1 \times 10^5$	21	36
CBM + S + 25 mM Ca^{2+}	$4,0 \times 10^5$	$3,3 \times 10^5$	18	24
CBM + S + 25 mM Mg^{2+}+25 mM Ca^{2+}	$3,1 \times 10^5$	$4,5 \times 10^4$	84	0

[a] Reversion was determined by the difference between the number of c.f.u. obtained on the RM after diluting the protoplast suspensions in either CBM + S or anaerobic water (pH 7.0).

[b] Leakage was expressed as the percentage absorbance at 260 nm in the supernatant fluid; as a reference the absorbance in a completely lysed protoplast suspension after sonication at maximum amplitude for 5 min was designated as 100%.

4.3.6 Effect of Lysozyme on Protoplast Production and Regeneration

The effect of lysozyme on the production and regeneration of C. acetobutylicum protoplasts was investigated (Table 4.6). Maximum regeneration (90%) of the P262 strain was obtained with 1 000 μg/ml lysozyme after 2 h incubation compared to a maximum of 69% regeneration obtained with 400 μg/ml lysozyme. With the autolytic-deficient mutant lyt-1, 76% and 90% reversion was obtained after 1 h with 400 and 1 000 μg/ml lysozyme respectively. The addition of autoclaved C. acetobutylicum cells or bovine serum albumin (0,8% w/v) to the RM enhanced reversion with the lyt-1 strain and reversion frequencies of more than 90% were obtained. Autolytic-deficient lyt-1 protoplasts in addition, required shorter incubation periods to produce protoplast suspensions capable of 90% reversion.

4.4 DISCUSSION

Methods for the production of autoplasts using the cell's own autolysin and protoplasts by the addition of lysozyme were developed in C. acetobutylicum. The production of autoplasts was unaffected by gelatin which has been shown to decrease the activity of autolysins (De Castro Costa and Landman, 1977) and low levels of

TABLE 4.6: Effect of Lysozyme on the Production and Regeneration of *C. acetobutylicum* P262 and *Lyt*-1 Protoplasts.

Strain	Protoplasting Time (min)	Lysozyme Conc. (μg/ml)	[a]Regeneration %
P262	60	400	68,7
P262	60	1 000	53,0
P262	120	400	65,0
P262	120	1 000	90,3
Lyt-1	60	400	76,1
Lyt-1	60	1 000	90,2

[a] Regeneration was determined by the difference between the number of c.f.u obtained on the RM after diluting the protoplast suspensions in either CBM + S or anaerobic water (pH 7.0).

palmitic and stearic acids which have been found to inhibit autolytic action in *S. faecalis* (Carson and Daneo-Moore, 1980). Copper and iron inhibited autoplast formation and the production of cell-free autolysin in *C. acetobutylicum*.

Sucrose induction of autoplasts and osmotic fragility in growing cells was rapid at high sucrose concentrations (0,5 M). Induction of autoplasts was not as rapid in 0,3 M sucrose but autoplasts were stable at this concentration. *C. acetobutylicum* is similar to *C. botulinum* (Kawata *et al*., 1968) and *C. saccharoperbutylacetonicum* (Ogata *et al*., 1980) in that each strain produces protoplasts when exposed to a hypertonic concentration of sucrose. *C. saccharoperbutylacetonicum* cells and protoplasts produced an inducible bacteriocin (clostocin O) after treatment with mitomycin C (Ogata *et al*., 1980).

Protoplasts can be induced to initiate wall formation in media possessing special physical characteristics and five types of environment have been found to stimulate reversion in *B. subtilis*. These include gelatin, hard agar, membrane filters, exogenous wall and heat-killed intact microorganisms (Clive and Landman, 1970). These reversion-inducing materials have little in common besides their particulate or solid state. Regeneration of *C. acetobutylicum* protoplasts was obtained on a medium containing gelatin, $CaCl_2$ and $MgCl_2$ and an increased agar content.

C. acetobutylicum lyt-1 protoplasts were similar to autolytic-deficient mutants of B. subtilis (De Castro Costa and Landman, 1977) in that they both reverted more rapidly than the standard strain.

Methods for the production and regeneration of viable C. acetobutylicum protoplasts have been developed. These techniques will be utilized for the development of host vector systems using either transformation with plasmids or transfection with phage DNA.

CHAPTER 5

TRANSFORMATION, PROTOPLAST FUSION AND TRANSFECTION OF C. ACETOBUTYLICUM PROTOPLASTS

It was not possible to transform *C. acetobutylicum* protoplasts with plasmid DNA. Phage DNA, however, was taken up by *C. acetobutylicum* protoplasts and whole phage particles were not produced until the protoplasts infected with phage DNA were regenerated to the bacillary form.

5.1 INTRODUCTION

Recently some of the barriers preventing the exchange of genetic information between different bacterial species have been overcome. New procedures allow the addition of non-homologous DNA to small replicons (plasmids or viruses) *in vitro* and the subsequent transfer of these expanded replicons into bacterial cells. The added DNA is perpetuated during cellular growth as part of the replicon (Cohen *et al*., 1973). These procedures referred to as recombinant DNA technology or the molecular cloning of genes are the result of the discovery of a set of restriction enzymes which cleave DNA at specific sites, leaving single-stranded complementary ends through which DNA from diverse sources can be joined (Hedgpeth et al., 1972; Sgaramella, 1972; Mertz and Davis, 1972; Cohen and Chang, 1973; Cohen et al., 1973), and a transformation procedure for *E. coli* (Mandel and Higa, 1970). There are a number of advantages of cloning segments of DNA (Helling and Lomax, 1979) which are summarised as follows:

1. Discrete fragments of DNA can be isolated from complex mixtures of DNA molecules which could be difficult to fractionate by conventional physical or chemical methods.

2. The cloned genes and their products may be made in large yields and high purity.

3. Genes from any orgnaism can be studied in the *E. coli* cell using the techniques available to the *E. coli* geneticist.

4. Genetic systems allowing the manipulation of small, defined segments of DNA can be established in mammals and other organisms.

The discovery that DNA fragments produced by EcoRI endonuclease contained single-stranded, complementary ends immediately suggested a means for joining DNA from diverse sources (Hedgepeth et al., 1972). One of the choices of a replicon to which foreign genes could be added was the coliphage λ. It is a relatively small phage and has been extensively studied genetically and biochemically. However, it was found that EcoRI cleaved λ at five points. Multiple cleavage posed a problem of how new DNA could be added to the λ genome while simultaneously retaining essential λ genes in their proper relationship. Ultimately the problem was solved by deletion of certain of the EcoRI restriction sites in non-essential genes (Rambach and Tollais, 1974; Murray and Murray, 1974). The potential difficulty of using λ as a cloning vehicle suggested that it might be simpler to find a plasmid which could serve as a cloning vehicle. In principle, the plasmid cloning vector only needs the necessary replication functions which would not be inactivated by inserting DNA at specific restriction sites in the plasmid. It is also desirable that the plasmid contain a gene by which plasmid-carrying cells could be selected from a large population of cells, most of which lacked the plasmid. Many such plasmids have been identified and characterised and a number of E. coli plasmid cloning vehicles have been constructed from a variety of naturally occurring plasmids (Cohen et al., 1973). In addition an extensive armoury of endonucleases whose recognition sequences are currently known in available. Depending upon the physical structure, incompatability group and copy number, different plasmid vehicles may vary in their applicability to a specific DNA cloning problem. In many cases, the principle aim of the cloning experiment is the insertion of a particular restriction fragment into the vehicle, its subsequent amplification and expression. A particularly useful vehicle is one that contains a single specific restriction site in the plasmid. An additional advantage is the presence of the restriction site near or within the sequence of a plasmid-coded gene that was inactivated by the insertion event (insertional inactivation), thus permitting screening or enrichment for transformants carrying the hybrid plasmid (Bernhard and Helinski, 1980).

Several *E. coli* plasmids that are useful cloning vehicles and permit the screening of an inserted fragment by insertional inactivation are pACYC 184, pBR322, pMK16, pMK20 and pMK2004 (Bernhard and Helinski, 1980). Plasmids useful for the controlled transcription of inserted prokaryotic or eukaryotic genes initiating at the promoter of the *lac* operon of *E. coli* have been constructed, e.g. pRSF2124 (Poliski *et al*., 1976) and pBGP120 (Itakura *et al*., 1977). These plasmids have been useful for the expression of fused genes consisting of the β -galactosidase gene and sequences coding for polypeptides with biological function. A similar system was used to obtain fusion proteins between β-galactosidase and the A and B chains of human insulin (Goeddel _et al_., 1979).

The Nordstrum temperature-sensitive (ts) copy number plasmids, pKN410 and pKN402 are being increasingly used as they allow the controlled production of a specific substance at a specific stage in the growth cycle. This is particularly useful for controlling the production of gene products which are lethal to the bacterium. These ts miniplasmids can increase the copy number of desired genes by 100 times and the product yield of the β-lactamase enzyme by 400 times when the temperature of the culture is raised from 30 to 35°C. A recent development regarding these plasmids is a system which utilises a ts replication plasmid unable to replicate at the higher temperature and containing the _lac_ gene. The inserted lethal gene in the ts copy number plasmid is not expressed at 30°C because of the _lac_ repressor. Towards the end of exponential phase the temperature is raised to 35°C and the ts replication plasmid is inhibited. As a result of the increased replication of the ts copy number plasmid to give 3 000 copies per cell, the _lac_ repressor is titrated out and the inserted gene is expressed.

Although transformation occurs under normal growth conditions, in a few bacteria such as *Streptococcus pneumonia*, *Bacillus*, *Haemophilus*, *Neisseria* and *Acinetobacter*, it does not occur normally in most bacteria (Helling and Lomax, 1979). The discovery that calcium treatment allowed *E. coli* cells to take up DNA from the environment was critical to the use of plasmids as cloning vectors (Mandel and Higa, 1970). The calcium procedure has been used successfully with *E. coli* (Cohen *et al*., 1972), *Salmonella* (Lederberg and Cohen, 1974), *Enterobacter* (Taketo, 1972), *Staphylococcus* (Sjöström *et al*., 1972) and *Pseudomonas* (Chakrabarty, 1977).

Prior to the discovery of transformation in *B. subtilis* by Spizizen (1958) there were no significant genetic studies in *Bacilli*. Although several plasmids indigenous to the *Bacilli* have been described (Lovett, 1973; Lovett *et al*., 1976; Berhard *et al*., 1978) much of the molecular cloning reported in *B. subtilis* has been done using plasmids of *S. aureus* which have been introduced into *B. subtilis* by transformation (Ehrlich, 1978; Gryczan *et al*., 1978). Chimeric plasmids that confer multiple antibiotic resistance have been constructed using several *S. aureus* plasmids. These include plasmids such as pBD9 and pBD64 (Gryczan *et al*., 1979). The temperature-sensitive character of plasmids such as pBD6 and pBD8 is potentially useful as a quick screeen to demonstrate that a cloned fragment is indeed carried on the plasmid, since any phenotype expressed by the fragment should be irreversibly lost after growth at the elevated temperature. Growth of ts plasmids at sub-inhibitory temperatures allows the adjustment of the plasmid copy number to intermediate levels (Dubnau *et al*., 1980). This may permit titration of cloned gene products and a study of gene dosage effects.

Investigators have isolated *B. subtilis* chromosomal, plasmid or bacteriophage DNA fragments by first cloning in *E. coli*, identifying clones carrying the desired fragment and then introducing the hybrid plasmid into *B. subtilis*. In this type of experiment, double-replicon vectors that can replicate in both *B. subtilis* and *E. coli* are very useful. Several such vectors have already been described (Kreft *et al*., 1978; Chang and Cohen, 1979). Only three *Bacillus* species are known to be transformable: *B. subtilis* (Spizizen, 1958), *B. licheniformis* (Gwinn and Thorne, 1964) and *B. amyloliquefaciens* (Coukoulis and Campbell, 1971).

A requirement for calcium ions in transformation has also been reported for Gram-positive bacteria, including *Pneumococcus* (Fox and Hotchkiss, 1957; Seto and Tomasz, 1976), *B. subtilis* (Young and Spizizen, 1963) and *Micrococcus lysodeikticus* (Kloos, 1969). Seto and

Tomasz (1976) reported that the conversion of surface-adsorbed DNA molecules to a state where they were inaccessible to exogenous DNAase required the presence of calcium ions. Magnesium ions, normally used in transformation media, could not replace the requirement for calcium which appears to be necessary for the transport of DNA molecules across the plasma membrane. Magnesium was found to stimulate the loss of surface-adsorbed DNA into the medium as a result of extracellular degradation of the DNA. It is not yet clear whether the role of calcium ions is the same in Gram-negative bacteria as that in the transformation of Gram-positive bacteria.

In _B. subtilis_ the development of competence for transformation of plasmid and chromosomal DNA follow a similar time course (Contente and Dubnau, 1979). Transformation of competent cultures with plasmid DNA is first order with respect to DNA concentration, unlike transfection with bacteriophage DNA. This suggests that a single plasmid molecule is sufficient for a successful transformation event, although 10^3 to 10^4 molecules are taken up per event (Contente and Dubnau, 1979). When linearised by cleavage at unique restriction endonuclease sites, plasmid DNA loses its ability to transform (Ehrlich, 1978). Transformation of competent cultures by covalently closed circular (CCC) plasmid DNA occurred readily in _rec E_ strains (Gryczan _et al_., 1978) and the _rec E_ product was required for both chromosomal transformation and transduction and seems to be required for the integration of chromosomal DNA (Dubnau *et al*., 1973). Canosi *et al*.,(1978) reported that plasmid transformation is due largely (if not entirely) to the presence of oligomeric forms in native plasmid DNA preparations. The uptake of oligomeric strands during uptake of duplex DNA, permits the occurrence of *rec E*-independent intracellular annealing and repair that can precisely regenerate a unit length CCC plasmid molecule (Dubnau *et al*., 1980). In contrast, when a homologous resident plasmid is present, markers carried on either linear, nicked or monomer plasmid DNA can yield transformants (Dubnau *et al*., 1980). This rescue process is *rec E*-dependent and requires homology between donor and recipient plasmids.

Bacterial autolytic enzymes have been implicated in transformation (Young *et al*., 1964; Tichy and Landman, 1969; Ranhand, 1973). This involvement in the development of competence is supported by evidence such as correlations found between competence and the rates of autolysis of cells or cell walls in *B. subtilis* (Young and Spizizen, 1963). Competence and the rate of spheroplast formation in *Pneumococcus* have been correlated (Lacks and Neuberger, 1975). Fractionation of water extracts from competent cells of *B. subtilis* yielded material showing both autolytic and competence increasing activities (Akrigg and Ayad, 1970) but they also contained a nuclease, forming single-stranded DNA. Thus the evidence for a role of autolysins in transformation is suggestive without being as yet compelling.

Fodor and Alfoldi (1976) and Schaeffer et al.,(1976) opened a broad and most promising field of genetic transfer when they reported that genetic information could be transferred by polyethylene glycol (PEG) induced fusion of protoplasts. During this procedure the cell wall, essentially a peptidoglycan layer, is removed by lysozyme treatment. PEG induced fusion of the parental protoplasts occurs under osmotic protection prior to reversion of the protoplasts to the bacillary form on selective reversion media. Alternately wall regeneration of the mixed protoplasts on a rich hypertonic agar medium can occur prior to selection by replica plating onto selective media. Dancer (1980) transferred plasmids from S. aureus to B. subtilis and between B. subtilis and several other species of Bacillus by protoplast fusion and regeneration. These plasmids replicated and were expressed normally in the Bacilli examined. Fodor et al.,(1978) in assessing the role of the individual partners in the formation of recombinants by protoplast fusion, introduced heat-inactivated protoplasts as fusion partners for the living protoplasts of B. megaterium. In the fusion process they reported that either of the parents may be rendered non-viable and yet, when fused with a viable partner, contribute to the formation of a recombinant. This may prove to be a useful method for selection when few markers or marked strains are available.

Protoplast fusion by PEG led to the use of bacterial protoplasts as hosts in PEG induced transformation (Hinen et al., 1978; Bibb et al., 1978; Chang and Cohen, 1979; Gabor and Hotchkiss, 1979; Vorobjeva et al., 1980). Protoplasts are prepared as for protoplast fusion in a hypertonic medium. Plasmid DNA is added in the presence of PEG. The mixture is diluted after 2 - 4 min at room temperature and allowed 1.5 - 2 h incubation to enable phenotypic expression of the genetic determinants prior to plating on selective reversion media. Chang and Cohen (1979) have adapted the PEG cell fusion technique to obtain efficient plasmid transformation in protoplasts prepared from non-competent cultures of B. subtilis. These can be readily regenerated to the bacillary form. Protoplasts can be transformed by the CCC monomer at about the same frequency as by dimers and higher oligomers. Linear plasmid DNA transforms protoplasts about 1% as well as do circular molecules, while nicked monomers transform as well as CCC forms (Dubnau et al., 1980).

It has been reported that restriction and modification in B. subtilis can affect transformation by plasmid DNA but not by chromosomal DNA. This effect, however, may not be an important obstacle in B. subtilis as no more than a ten-fold reduction in transformation of unmodified plasmid DNA was observed (Dubnau et al., 1980). However, in other strains/species nucleases may play an important role in preventing transformation.

The first example of intact bacterial cells being infected by phage DNA without helper phage was reported by Romig (1962), when he demonstrated transfection in B. subtilis cells that had developed competence for transformation. Földes and Trautner (1964) coined the term "transfection" to describe the infection of bacterial cells by isolated viral nucleic acid resulting in the production of complete virus particles. Prior to this, several authors (Hershey and Chase, 1952); Gierer and Schramm, 1952; Kaiser and Hogness, 1960) had succeeded in demonstrating the infectivity of protein free viral or phage RNA or DNA. Phage nucleic acid from ØX174 was found to be infective when exposed to bacterial protoplasts of various strains of E. coli (Guthrie and Sinsheimer, 1960).

A variety of techniques have been used to make bacterial cells permeable to phage nucleic acid. The most common is the manipulation of the environmental conditions during certain stages of growth of the recipient culture. In *B. subtilis* the appearance of competent cells at the end of exponential growth in minimal medium and the fact that asporogenous mutants of *B. subtilis* never attained competence, led to the conclusion that competence is brought about by a metabolic blockage (Bott and Wilson, 1968). During this competent state, the replication of DNA is arrested, making it advantageous for the cell to take up exogenous DNA for repair processes (Epstein, 1968).

Another method for making cells permeable to phage DNA which has been extensively used for *E. coli* (Guthrie and Sinsheimer, 1963; Il'yashenko, 1964), *S. aureus* (Riggs and Rosenblum, 1969) and *Streptomyces kanamyceticus* (Okanishi *et al*., 1966) is the removal of part of the cell wall resulting in the formation of protoplasts or sphaeroplasts. However, it has been suggested that some residual portion of the cell wall is required for complete adsorption and transport of DNA molecules to the interior of the cell (Tomasz, 1969). Ogata *et al*.,(1980) reported successful transfection of protoplasts of *Clostridium saccharoperbutylacetonicum* produced by sucrose-induced autolysis by phage HM2 DNA.

Plasmolysis of bacterial cells has been used successfully to increase the permeability of the cells to phage DNA. Benzinger (1977) used protamine sulphate and high concentrations of sucrose to induce plasmolysis in E. coli cells and obtained successful transfection with 0X174, λ, T_4, T_5 and P_1 DNA.

Transfection is a powerful tool which has been utilised to study many aspects of the phage-host relationship including the restriction process in Haemophilus (Stuy, 1976), recombination control in λ (Wackernagel and Radding, 1973) and the study of the initial steps of excision-repair after damage by ultraviolet light (Taketo et al., 1972).

Transformation, protoplast fusion and transfection of C. acetobutylicum protoplasts was studied with a view to developing a genetic transfer system in this organism.

5.2 METHODS

5.2.1 Media and Materials

See Appendix A.

5.2.2 Bacterial Strains

C. acetobutylicum P262, lyt-1 and P262J, an autolysin sensitive indicator strain were used.

5.2.3 Plasmid Extraction and Purification

The plasmids, their origins and the markers used are listed in Table 5.1. The method used for isolation of plasmid DNA from Bacillus and Staphylococcus strains was adapted from that of Gryczan et al., (1978). Overnight cultures (500 ml) were harvested by centrifugation at 4080 x g for 10 min and washed in 0,05 M Tris-HCl (pH 8.0) buffer and resuspended in 20 ml of 0,05 M Tris-HCl (pH 7,5) buffer containing 25% w/v sucrose and 0,1 M NaCl. Lysozyme (0,5 mg/ml final conc.) was added and the suspension was incubated at 37°C for 15 min. To the suspension 4,8 ml of 5 M NaCl, 1,2 ml of 0,05 M EDTA (pH 8.5) and 26 ml of 2% w/v SDS-0,7 M NaCl was added and the suspension was inverted once gently before standing at 4°C for 18 h. The lysate was cleared by centrifugation at 27138 x g for 60 min and the supernatant fraction adjusted to 0,3 M sodium acetate with 3,0 M sodium acetate (adjusted to pH 6.0 with glacial acetic acid). Two volumes of cold 95% ethanol were added to the lysate which was allowed to stand at -20°C for 2 h. The precipitate was collected by centrifugation

TABLE 5.1: Plasmids isolated, their origins and antibiotic markers used.

Plasmid	Host Strain	Molecular wt. x10^6	[a]Antibiotic markers	References
plP402	*C.perfringens*	41,6	Em,Cl	Brefort *et al.*,(1977)
plP401	"	35,6	Tc,Cml	
pBC16	*B. subtilis*	2,8	Tc	Kreft *et al.*, (1978)
pBD8	"	6,0	Kan,Sm,Cml	Dubnau *et al*, (1980)
pBD9	"	5,4	Kan,Em,	"
pBD64	"	3,2	Kan,Cml	"
pUB110	"	3,0	Kan,Neo	"
pl258	*S. aureus*	18,4	Em,Pc	Ruby & Novick (1975) & Ehrlich (1977)
p5177	"	2,7	Sm	
pT127	"	2,7	Tc	"
pT181	"	2,7	Tc	"
pC194	"	1,8	Cml	"
pC221	"	30,0	Cml	"
pK545	"	15,0	Kan,Neo	"
pUB101	"	14,6	Pc	"

[a] Antibiotics used were: Em, erythromycin; Cl, clindamycin; Tc, tetracycline; Cml, chloramphenicol; Kan, kanamycin; Sm, streptomycin; Neo, neomycin and Pc, penicillin.

at 3015 x g for 30 min at 4°C and resuspended in 3 ml of 0,03 M Tris-HCl (pH 7.5) - 0,05 M NaCl - 0,005 M EDTA buffer. A CsCl gradient was prepared containing ethidium bromide (0,4 mg/ml final conc.) and the refractive index was adjusted to 1,396 in Beckman SS VTi quickseal tubes and centrifuged at 42 000 rpm in a Beckman vTi-65 rotor for 17 h at 15°C. Plasmid bands were viewed under ultraviolet light, removed and extracted with two volumes of CsCl saturated isopropanol. The plasmid DNA was dialysed extensively at 4°C in 0,01 M Tris-HCl (pH 8.0) - 0,001 M EDTA and stored at -20°C until required.

The isolation of plasmid DNA from Clostridium perfringens strains was adapted from the method of Brefort et al., (1977). Late exponential phase cultures (100 ml) were harvested by centrifugation and washed once in 0,05 M Tris-HCl (pH 8.0)-0,005 M EDTA-0,05 M NaCl (TES) buffer and resuspended in 2,0 ml/TES buffer containing 25% w/v sucrose. Lysozyme (2,0 mg/ml final conc.) was added and the suspension incubated at 37°C for 30 min. Diethylpyrocarbonate (0,5% final conc.) and 0,8 ml of 0,25 M EDTA (pH 8.0) was added and the suspension incubated for a further 30 min at 37°C. To the suspension 3,6 ml of 2,0% SDS in TES buffer was added and after incubation at 37°C for 10 min the lysate was sheared by aspiration 10 to 15 times through a 5 ml pipette and centrifuged at 27138 x g for 60 min at 4°C. The supernatant fraction was collected and the plasmid DNA banded by centrifugation on ethidium bromide/CsCl gradients.

5.2.4 Conventional Transformation Procedures

(a) Calcium Treated Cells

C. acetobutylicum P262 or *lyt*-1 overnight CBM cultures were harvested by centrifugation and resuspended in fresh CBM to an optical density of 0,1 at 600 nm. The culture was diluted 10-fold in CBM and incubated at 37°C. Cells were harvested at different time intervals and washed in 0,1 M $CaCl_2$ and resuspended in an appropriate volume of 0,1 M $CaCl_2$ to give *c.* 10^9 cells/ml. In transformation experiments, 0,9 ml of these treated cells were mixed with 100 μl of plasmid DNA and incubated at 37°C for 20 min. The cells were collected by centrifugation and resuspended in 1 ml CBM and incubated at 37°C for 90 min to allow for expression before diluting and plating on selective media. In control experiments untreated cells with DNA, calcium-treated cells and DNA alone were each assayed for transformation.

(b) Conventional Transformation Assay

Cultures of the recipient strains were harvested at different optical densities and resuspended in fresh CBM to an optical density of 0,1 at 600 nm. $CaCl_2$ and $MgCl_2$ (0,05 M final conc.) were added and the cultures incubated for 90 min at 37°C. Volumes (0,4 ml) of suspensions were mixed with 50 μl plasmid DNA and the mixtures incubated for 90 min to allow expression before diluting and plating on selective media. In control experiments cells alone and DNA were each assayed for contamination.

5.2.5 Protoplast Transformation Procedure

Recipient protoplast suspensions of *C. acetobutylicum* P262 or *lyt*-1 were prepared in CBM + 0,3 M sucrose (CBM + S) as described in Chapter 4. The plasmid DNA was diluted in an equal volume of double strength CBM + S prior to use. In transformation experiments 0,5 ml of the protoplast suspension was mixed with 100 μl of the diluted DNA solution. Polyethylene glycol (1,5 ml of a 40% w/v solution in CBM + S) was added and the mixture gently mixed for 2 min before diluting with 2,5 volumes of CBM + S. The cells were collected by centrifugation and resuspended in 1,0 ml

CBM + S and incubated at 37°C for 90 min to allow for expression before diluting in CBM + S and plating on regeneration media (RM) or selected directly on RM + antibiotics. After 36 h incubation, reverted colonies on RM were harvested, diluted and plated onto selective CBM plates. In control experiments protoplasts alone and plasmid DNA were assayed for contamination.

5.2.6 Protoplast Fusion Procedure

C. acetobutylicum lyt-1 and P262 protoplasts were prepared in CBM + S as described in Chapter 4. B. subtilis and S. aureus protoplasts were prepared by the method of Gabor and Hotchkiss (1979). In fusion experiments 1,0 ml of each parental protoplast suspension were centrifuged at 1930 x g for 10 min at room temperature. The pellets were each resuspended in 0,2 ml CBM + S and 1,8 ml 40% w/v PEG in CBM + S was added and mixed by inverting gently. After 1 min 2 volumes of CBM + S was added and the suspension incubated for 90 min to allow for expression, before dilution and plating on RM media. After 36 h regeneration, colonies were harvested and plated on CBM media containing antibiotics. For measuring the osmotic sensitivity of protoplasts each suspension was diluted in anaerobic water and CBM + S and plated on RM media. In control experiments each parental protoplast suspension was assayed as described above.

5.2.7 Deoxyribonuclease (DNAase) Production in C. acetobutylicum

(a) DNAase Assay

DNAase activity in C. acetobutylicum P262, P262J and lyt-1 strains was investigated by incubating whole cell and protoplast suspensions with pBR322 DNA isolated by the method of Clewell and Helinski (1970). P262, P262J and lyt-1 cells and protoplast suspensions were prepared as described in Sections 5.2.4 (b) and 5.2.5 above. Aliquots (100 µl) of cells or protoplasts were mixed with 50 µl pBR322 DNA and incubated at 37°C for 60 min. Samples (50 µl) were removed immediately after mixing and after 60 min and the reaction stopped by the addition of 15 µl of a buffer containing 40% w/v sucrose in 0,1 M EDTA. Electrophoresis of the samples was performed using 0,7% agarose slab gels in 0,09 M Tris - 0,09 M boric acid - 0,002 M EDTA (pH 8.6) for 18 h at 25 mV. The gel was stained by soaking in a solution of ethidium bromide (0,5 µg/ml) and photographed while illuminated with a UV light source.

(b) DNAase Plate Assay

C. acetobutylicum strains were assayed for the production of DNAase by the method of Omenn and Friedman (1970).

The addition of 10 μg/ml acridine orange to 4,0% (w/v) DNAase Test Agar (Difco) in CBM before autoclaving resulted in a medium with a brilliant yellow-green fluorescence. Release and diffusion of DNAase from a colony led to a dark halo of defluorescence, as the enzyme cleaved DNA to oligonucleotides and the agar served to quench free acridine orange. Fluorescence persisted around DNAase-deficient colonies.

C. acetobutylicum P262 and lyt-1 cultures treated with EMS were screened on the DNA-acridine orange agar plates for loss of DNAase activity. EMS treatment involved exposing exponential phase CBM cultures (5 x 10^7 cells/ml) to EMS (2,5% v/v) for 20 min at 37°C to obtain 10% survival. The washed cells were resuspended in CBM and incubated for 18 h before diluting and plating onto DNA-acridine orange agar plates. Colonies showing reduced or no zones of defluorescence were tested for DNAase production using the liquid assay (5.2.7 a).

5.2.8 Bacteriophage Isolation and Assay

A bacteriophage was isolated from samples collected by Barber (1977) near the fermentation vessels at NCP, Germiston, South Africa. The phage was specific for

C. acetobutylicum P262J and was not able to infect the P262 or lyt-1 strains. The bacteriophage was structurally very similar to the DNA phage HM2 active on Clostridium saccharoperbutylacetonicum reported by Ogata et al.,(1969).

Plaque-forming titres were estimated by the sloppy-agar overlay method described by Adams (1959). This involved adding serial dilutions of the phage in T_2 buffer to warm 0,7% CBM sloppy-agar before overlaying CBM plates.

5.2.9 Preparation of High Titre Lysates of Phage

Phage lysates were obtained either by confluent lysis techniques on CBM agar plates or by broth lysis. Optimal yields of high titre lysates were obtained from broth lysis. C. acetobutylicum CBM plate cultures (18 h) were harvested and used to inoculate CBM broths. Growth was monitored during the exponential phase to an optical density of 0,4 at 600 nm. Phage was added at a multiplicity of infection (m.o.i.) of 0,3. Within 2 to 3 h the culture had lysed and was harvested by the addition of a few drops of chloroform followed by centrifugation at 5910 x g for 10 min. The conditions were varied in order to determine the optimal conditions for phage production. Bacterial cultures at different phases of growth were used as backgrounds for plate lysis experiments.

5.2.10 Concentration and Purification of Phage

Phage lysates (10^8 p.f.u./ml) were purified and concentrated by centrifugation and precipitation with 10% PEG as described by Yamamoto *et al*., (1970).

5.2.11 Phage DNA Extraction

Phage lysates which had been concentrated and purified were maintained at room temperature for 30 min with SDS (1% w/v final conc.). The phage nucleic acid was then extracted according to the phenol method of Sjöström *et al*.,(1972). The DNA was dialysed extensively in 0,01 M Tris - 0,001 M EDTA (pH 8.0) buffer at 4°C and stored over chloroform at -20°C until required.

5.2.12 Transfection Assay for C. acetobutylicum Protoplasts

Protoplasts of *C. acetobutylicum* P262, *lyt*-1 and P262J were prepared in CBM + S containing 0,25 M $CaCl_2$ and

$MgCl_2$ as described in Chapter 4. Protoplasts were collected by centrifugation and washed once in CBM + S containing calcium and magnesium. Volumes (1,0 ml) of the protoplast suspensions were added to 100 µl of phage DNA, mixed gently and incubated at 34°C. After 30, 60, 120 and 180 min suspensions were diluted in CBM + S and plated on RM agar plates. After 36 h, reverted colonies were scraped off the plates and re-suspended by standing in T_2 buffer for 2 h. The cells were collected by centrifugation at 3015 x g for 15 min and the supernatant fraction assayed for plaque forming units by the sloppy-agar overlay method. In control experiments protoplasts only, DNA only and DNA preincubated with 5 µg/ml DNAase for 30 min prior to adding to protoplasts were each assayed for infective centres.

5.3 RESULTS

5.3.1 Isolation of Plasmid DNA

Table 5.1 lists the range of plasmids isolated, their origins and the antibiotic markers used.

5.3.2 Transformation

It was not possible to transform *C. acetobutylicum* P262, *lyt*-1 or P262J by any of the methods used.

5.3.3 Protoplast Fusion

In control experiments using *B. subtilis* strains, fusion of pBD9 protoplasts was obtained with those of pBD64 and pBC16. Although fusion was obtained between the *B. subtilis* strains it was not possible using these methods to obtain fusion between *C. acetobutylicum* and *B. subtilis* or *S. aureus* protoplasts.

5.3.4 DNAase Production by C. acetobutylicum Strains

DNAase activity of *C. acetobutylicum* P262, *lyt*-1 and P262J cell cultures and protoplast suspensions was investigated. DNAase activity of P262 and *lyt*-1 strains was extremely rapid (Fig. 5.1) and all detectable pBR322 DNA was degraded after 60 min (lanes 4 and 8 in Fig. 5.1). Much of the plasmid DNA was already nicked in the time taken to remove and stop the time 0 samples. The P262J strain, however, produced less DNAase and after 60 min had converted most of the plasmid DNA into the linear form (Fig. 5.1). The

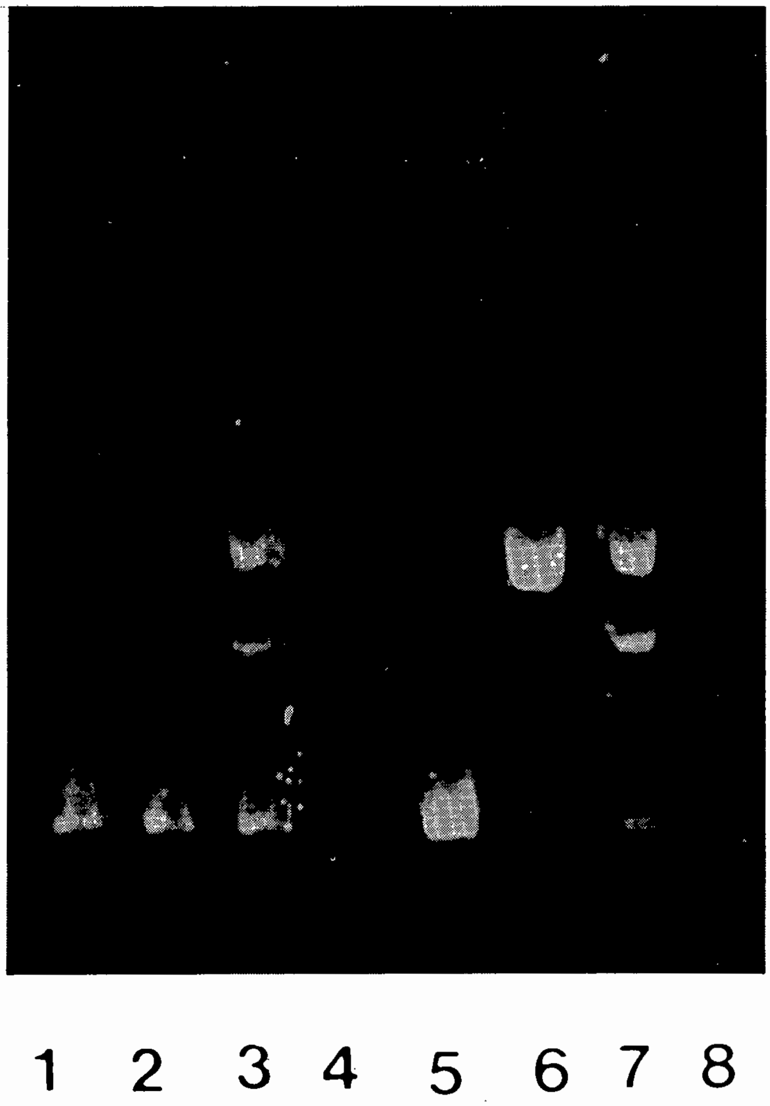

Fig. 5.1: Deoxyribonuclease activity of P262, *lyt*-1 and P262 J cells. Agarose gel electrophoresis of pBR 322 DNA incubated with:

(1) No addition - time 0
(2) No addition - time 60 min
(3) P262 cells - time 0
(4) P262 cells - time 60 min
(5) P262J cells - time 0
(6) P262J cells - time 60 min
(7) *lyt*-1 cells - time 0
(8) *lyt*-1 cells - time 60 mins

P262J strain produced the smallest halo of defluorescence on DNA-acridine orange plates and was used in all further transformation and fusion experiments. It was, however, not possible to transform this strain or to obtain fusion between protoplasts of P262J and protoplasts of B. subtilis or S. aureus by any of the methods used.

5.3.5 DNAase-Deficient Mutants

Exposure of the P262 and lyt-1 strains to EMS prior to selection on DNA-acridine orange agar plates did not yield any DNAase-deficient mutants.

5.3.6 High Titre Lysate Preparations

Phage lysates built up by the sloppy-agar overlay technique routinely had titres of $1,0 \times 10^{7}$ to $1,0 \times 10^{8}$ p.f.u./ml, while those built up by broth lysis techniques had titres of $1,0 \times 10^{9}$ to $1,0 \times 10^{10}$ p.f.u./ml under optimal conditions.

5.3.7 Concentration and Purification of Phage Preparations

PEG-precipitation and centrifugation were both effective in concentrating and purifying the phage. Centrifugation at 40 000 rpm in a Beckman 65Ti rotor for 2 h increased the phage titre 100-fold while PEG precipitation resulted in a 1 000-fold concentration.

5.3.8 Phage DNA Extraction

Phenol-extracted phage DNA showed a maximum absorption at 254 nm and a trough at 230 nm (Fig. 5.2). The ratios 258/280 and 280/230 were 1,6 to 2,0 and 1,0 to 1,2 respectively.

5.3.9 Transfection of *C. acetobutylicum* Protoplasts

Phage DNA was shown to be the infective agent in the *C. acetobutylicum* P262J transformation system (Table 5.2). It was not possible to infect P262 or *lyt*-1 protoplasts with phage DNA.

Infectivity was lost when the DNA was preincubated with DNAase before adding it to protoplasts. No infectivity was observed during the first 2 h after adding the phage DNA to the protoplasts. The reason for the eclipse period is not known.

Due to the complex nature of the regeneration medium, it was not possible to see plaques directly on this medium. Regenerated cells were removed by scraping

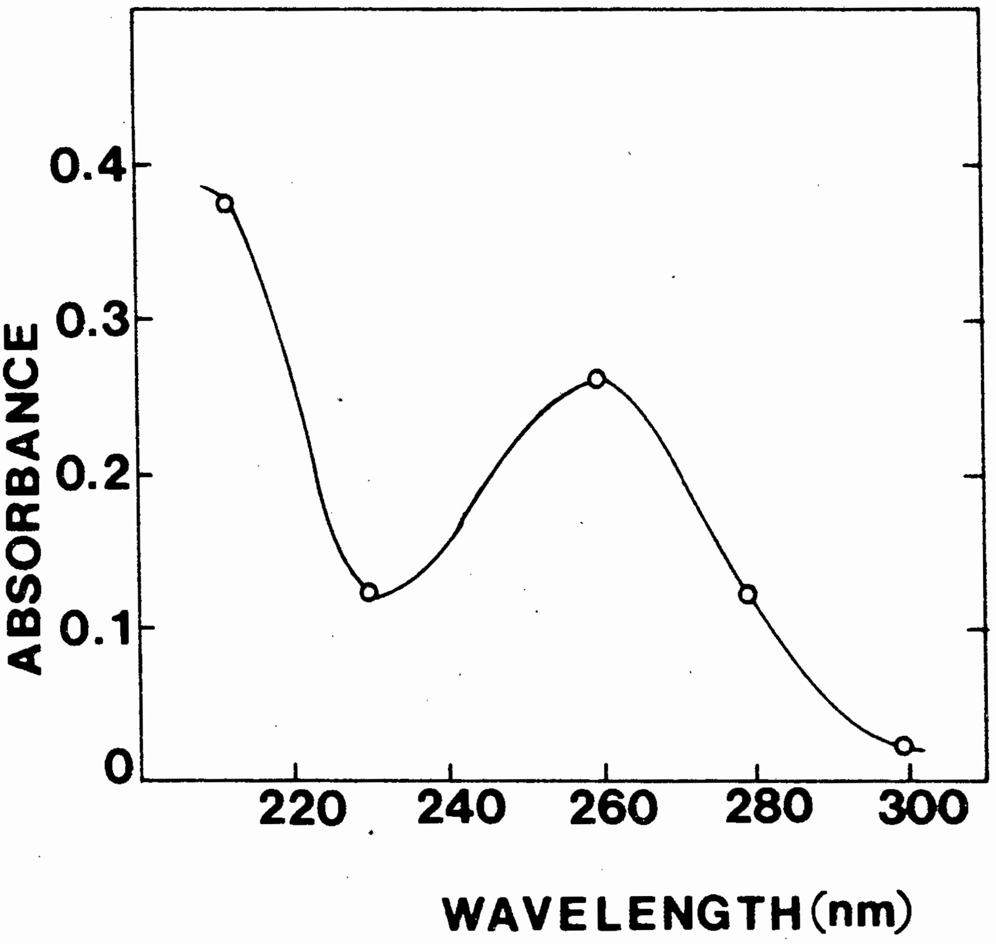

Fig. 5.2: Ultraviolet absorption spectrum of phage DNA.

TABLE 5.2: Transfection of *C. acetobutylicum* P262J Protoplasts.

Experiment	Incubation time (min)	Phage Production
DNA preincubated with 5 μg/ml DNAase for 30 min prior to adding to protoplasts	0 - 180	-
Protoplasts only	0 - 180	-
DNA + protoplasts	30	-
DNA + protoplasts	60	-
DNA + protoplasts	120	+
DNA + protoplasts	180	+
DNA spotted on assay plates		-

Protoplasts were allowed to regenerate on RM for 36 h prior to assaying for plaque forming units.

the plates and after standing in T_2 buffer for 2 h the supernatant fraction after centrifugation was assayed for infective centres.

5.4 DISCUSSION

Despite the use of protoplasts and calcium treatment to increase the permeability of recipient cells to DNA, as well as the use of different techniques and media, it was not possible to transform *C. acetobutylicum* cells with plasmid DNA. However, phage DNA was taken up by *C. acetobutylicum* protoplasts. Reasons why plasmid DNA was not taken up by *C. acetobutylicum* cells could be that the plasmid DNA was degraded during extraction and purification. Secondly, the DNA may have been restricted during uptake or it may have been taken up by the protoplasts but could not replicate or express in *C. acetobutylicum*. It is significant that transformation was not detected in *E. coli* until a *rec* B^- *rec* C^- *sbc* B^- recipient strain had been isolated (Cosloy and Oishi, 1973). It is also significant that biologically active DNA was not reproducibly obtained until an endonuclease 1 - deficient mutant strain was used as the source of transforming DNA.

During transfection experiments no phage particles were obtained unless C. acetobutylicum protoplasts infected with phage DNA were regenerated to the bacillary form. This suggests that naked phage DNA is unable to produce mature phage in protoplasts. Since it was not possible to obtain 100% protoplasts, we were unable to determine whether phage particles could replicate in protoplasts.

Transfection frequencies were not obtained since plaques were not visible on the rich regeneration medium. Experiments are presently underway to alter the regeneration medium so that plaques are directly visible and results to date using media without bovine serum albumin look promising.

Milani and Heberlein (1972) found a similar effect when they obtained no p.f.u. in the transfection mixture of competent Agrobacterium tumiefaciens cells and phage DNA plated directly. However, when these same plates were scraped, resuspended in buffer and replated, plaques were obtained. This was thought to be due to either slow replication of the phage or to a burst producing only a few phage particles. It may also reflect a requirement for growth or replication similar to that found by Romig (1962) and in Bacteriodes thetaiotaomicron described by Burt (1977).

There is also an eclipse period of 2 h in broth during which time protoplasts infected with phage DNA do not on regeneration release whole phage particles. The reason for this eclipse period is not known. Taylor and Guha (1974) studied the development of phage F1 in *Clostridium sporogenes* and reported that the phage had an eclipse period of 30 min and a latent period of 40 min during which time mRNA synthesis took place.

Factors which influence phage multiplication within the host, the effects of pH, temperature and inorganic salts have been examined by Kinoshita and Teramoto (1955) and Hongo *et al*.,(1966) for the phages of *C. acetobutylicum* and *C. saccharoperbutylacetonicum* respectively. It was shown that the optimum pH and temperature for growth of the host organisms corresponded approximately to values for the phages. Also, small amounts of calcium and magnesium has a positive effect on the multiplication of *C. acetobutylicum* and *C. saccharoperbutylacetonicum* phages.

The transfection system in *C. acetobutylicum* will be utilised in developing the phage DNA as a vector and a transformation system for this organism. This may be accomplished by isolating the origin of replication and inserting it into other plasmids. We now have a genetic system which means that *C. acetobutylicum* now has, the potential to be manipulated utilising recombinant DNA technologies.

CONCLUSIONS

The major aim of this study was to develop a genetic transfer system for *C. acetobutylicum*. Protoplasts of *C. acetobutylicum* P262J were able to take up phage DNA and whole phage were produced after regeneration of the protoplasts to the bacillary form. It was, however, not possible to transform *C. acetobutylicum* protoplasts with plasmid DNA from other Gram-positive bacteria. Future work will concentrate on developing a medium for direct expression of plaque forming units and determining whether regenerated cells are required for whole phage production.

The transfection system in *C. acetobutylicum* will be utilised in developing the phage DNA as a vector for a transformation system for this organism. This may be accomplished by isolation of the origin of replication and insertion into other plasmids. The development of a genetic system in *C. acetobutylicum* enables this important industrial microorganism to be manipulated utilising recombinant DNA technologies. This development and the demonstration that *C. acetobutylicum* produces cellobiase and carboxymethyl cellulase enzymes enhances its potential in the emerging field of biotechnology.

In developing the protoplast transformation system for C. acetobutylicum, a cellular autolytic system was characterised. The autolytic system was highly active in the exponential growth phase and active at low levels in the stationary phase. Autolysis-deficient mutants isolated after mutagenesis lacked substantial levels of autolytic enzymes and grew in long chains. One of these mutants was highly pleiotropic and four phenotypic characteristics were affected. These included reduced cell-free and cell-bound autolysin levels, an altered cell wall which was more resistant to both its own and wild-type autolysin, growth at increased sugar levels and an increased tolerance to lysis by butanol. (Initial fermentation studies have indicated that this mutant may be useful in producing higher solvent yields.) Future autolytic studies are required to fully understand the chemistry of the cell wall and the sites of action of the autolytic enzymes.

Protoplast fusion of prokaryotic organisms provides a unique opportunity for the bringing together of two complete genomes, instead of transferring fractions of DNA by transformation, transduction or conjugation. Although chromosomal protoplast fusion was not obtained between C. acetobutylicum strains, the development of a fusion technique between plasmid bearing strains would prove especially useful and C. acetobutylicum strains bearing plasmids should be sought.

APPENDIX A

GENERAL METHODS

Spore Preparation and Germination

C. acetobutylicum spores for general use were prepared from sporulating colonies off CBM plates containing 5% (v/v) molasses or sucrose. The spores were washed several times in sterile distilled water, and stored in distilled water at 4°C. The spore suspensions were diluted until the addition of 10 to 20 μl heat-shocked spores to 10 ml CBM gave an optical density of 0,2 to 0,5 within 12 to 18 h, as read on a Corning colorimeter. The cell suspensions in this optical density range were in exponential phase, and were used to inoculate the test media.

Spores were germinated by heat shocking at 70-75°C for 2 min and cooled rapidly on ice.

MEDIA

All media was sterilized by autoclaving at 121°C for 20 min. Heat sensitive solutions were sterilised by Seitz filtering. Agar plates were made by adding 1,5% (w/v) agar (Difco) to broth preparations.

Preparation of Media

Pre-reduced and anaerobically sterilized media were 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.

Molasses fermentation medium (MFM)

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.

Clostridial basal medium agar (O'Brien & 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
Casein 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 water	1,0 ℓ

The required volumes of K_2HPO_4 (c. 2,5 ml) and KH_2PO_4 (c. 1,0 ml) stock solutions were added to give a pH of 6.8 to 7.0. This medium was used for making CBM well plates by the addition of 1% (w/v) 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
Casein hydrolysate	4,0 g

p-Aminobenzoic acid stock solution	1,0 ml
Thiamine hydrochloride stock solution	1,0 ml
Biotin stock solution	1,0 ml
Cysteine HCl	0,5 g
$NaHCO_3$	1,0 g
Yeast extract	4,0 g
Resazurin stock solution	1,0 ml
Distilled water	1,0 ℓ

The required percentage 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
$NaHCO_3$ stock solution	20,0 ml

Glucose-Mineral Salts-Biotin Medium (GMB)

Glucose	1,0 g
$(NH_4)_2SO_4$	0,1 g
Salts stock solution	4,0 ml
Resazurin stock solution	1,0 ml
Biotin stock solution	2,5 ml
Cysteine HCl	0,5 g
$NaHCO_3$	2,0 g
Distilled water	92,0 ml

Stock Solutions

All stock solutions were stored at 4°C.

Salts stock solution

$CaCl_2$ (anhyd.)	0,2 g
$MgSO_4.7H_2O$	0,48 g
K_2HPO_4	1,0 g
KH_2PO_4	1,0 g
$NaHCO_3$	10,0 g
NaCl	2,0 g

p-Aminobenzoic acid stock solution

p-Aminobenzoic acid	0,1 g
Distilled water	100,0 ml

Thiamine hydrochloride stock solution

Thiamine HCl	0,1 g
Distilled water	100,0 ml

Biotin stock solution

Biotin	200 μg
Distilled water	100,0 ml

K_2HPO_4 stock solution

K_2HPO_4	25,0 g
Distilled water	100,0 ml

KH_2PO_4 stock solution

KH_2PO_4	12,5 g
Distilled water	100,0 ml

Cysteine HCl stock solution

Cysteine HCl	1,0 g
Distilled water	100,0 ml

Autoclaved

Sodium bicarbonate stock solution

$NaHCO_3$	10,0 g
Distilled water	100,0 ml

Autoclaved

Resazurin stock solution

Resazurin	20 mg
Distilled water	100,0 ml

General Buffers

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

SDS-Slab Gel Electrophoresis (O'Farrell, 1975)

Resolving gel buffer stock solution

Tris-HCl, pH 8.8	1,5 M
SDS	0,4 %

Stacking gel buffer stock solution

Tris-HCl, pH 6.8	0,5 M
SDS	0,4 %

Bath buffer stock solution

Tris base	0,025 M
Glycine	0,192 M
SDS	0,1 %

Acrylamide stock solution

Acrylamide	292 g
bis acrylamide	0,8 g
Distilled water	1000 ml

This gives an acrylamide:bis ratio of 29,2:0,8.

SDS sample buffer

Glycerol	10%
β-Mercaptoethanol	5%
SDS	2,3%
Tris-HCl, pH 6.8	0,0625 M

Slab Gel Formulations

Resolving gel

N acrylamide stock	12,0 ml
Distilled water	13,6 ml
Resolving gel buffer stock solution	8,2 ml
Ammonium persulphate 10% solution	160 μl
TEMED	18 μl
TOTAL	34,03 ml

Stacking gel

N acrylamide stock	2,0 ml
Distilled water	7,0 ml
Stacking gel buffer stock solution	3,0 ml
Ammonium persulphate 10% solution	64 μl
TEMED	14 μl
TOTAL	12,08 ml

After loading, the samples were electrophoresed at 100 V through the stacking gel and increased to 200 V through the resolving gel. After electrophoresis the gels were removed from the plates, stained for 2 h in Coomassie blue solution and then destained.

Staining solution

Propan-2-ol	25%
Glacial acetic acid	10%
Coomassie Blue	0,05%

Destaining solution

Propan-2-ol	25%
Glacial acetic acid	10%

Antibiotic stock solutions

Antibiotic powders were assumed to be self-sterilising and were dissolved in sterile distilled water and stored at -20°C until required.

Phage Buffer

T_2 buffer

Solution A

KH_2PO_4	7,5 g
NaCl	20,0 g
Na_2HPO_4 (anhyd.)	15,0 g
K_2SO_4	25,0 g
$CaCl_2$ (anhyd.)	0,055 g
Distilled water	1,0 ℓ

Store over chloroform at room temperature.

Solution B

$MgSO_4$	4,8 g
Distilled water	100,0 ml

Autoclave

Solution C

Gelatin	0,2 g
Distilled water	100,0 ml

Autoclave

To Make Up

Solution A	40,0 ml
Distilled water	158,0 ml
Solution B	1,0 ml
Solution C	1,0 ml

Autoclave at 10 lbs sq.in^{-1} for 13 min.

General Chemicals and Their Abbreviations

Tris (hydroxymethyl)-amino-methane	Tris
Ethylenediaminetetraacetic acid	EDTA
Sodium dodecyl sulphate	SDS
N,N,N',N'-Tetramethylethylenediamine	TEMED
Ethyl methane sulfonate	EMS

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of a fusion technique between plasmid bearing strains would prove especially useful and *C. acetobutylicum* strains bearing plasmids should be sought.