STUDIES ON THE FERMENTATION OF MOLASSES BY *Clostridium acetobutylicum*

by

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ABSTRACT

The bacterium Clostridium acetobutylicum produces acetone and n-butanol from molasses in an industrial fermentation system. Although the bacterium has been cultured in liquid media it does not grow well on agar plates and requires high concentrations of hydrogen. Pretreatment of agar plates with bovine catalase improves growth on agar media. The bacteria produce an area of clearing (halo) on Potato agar plates due to butyric acid (the precursor of n-butanol) and β -amylase production. This characteristic will be used as a plate screening assay for the selection of high solvent producing mutants. A laboratory scale fermentation system was developed and detailed studies including pH, turbidity and cell morphology changes, and the details of solvent production were undertaken. The fermentation was optimized for mutant selection. The production of normal solvent yields by isolated clones is required for the mutant selection programme. Studies revealed that sporulation of the clones increased their solvent yield although solvent yields were still lower than normal. Efficient sporulation is therefore a prerequisite for clone fermentation.

The origin of the phage infection during the factory outbreak was determined and resistant clones obtained.

The presence of a bacteriocin-like toxin causing decreases in turbidity was identified during the final fermentation stage. The strain sensitivity, optimum conditions for stability as well as the kinetics of inactivation and lethality have been investigated. Preliminary characterization and purification studies indicate the proteinaceous nature of the toxin.

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CHAPTER I

GENERAL INTRODUCTION

Unless otherwise indicated, the information in this general introduction is taken from the reviews of Prescott and Dunn (1940), Rose (1961), Doelle (1975), Stanier (1958) and Beesch (1953).

1.1 The genus Clostridium

Clostridia (Clostridium. - Closter is the Greek for spindle, relating to the shape of the parental cell) are Gram-positive rods, motile with peritrichous flagella and are found in soil and the intestinal tract of man and animals. Many are pathogens eg. Clostridium botulinum (botulism), Clostridium perfringens (gas gangrene) and Clostridium tetani (tetanus). The majority are obligate anaerobes although a few grow in microaerophilic conditions. Their anaerobiosis being due to a deficiency of cytochromes for electron transport, catalases and peroxidases. Only a few produce capsules eg. C. perfringens. All produce spores which are considerably broader than the vegetative cells and therefore at sporulation the cell becomes grossly distorted. Clostridium acetobutylicum produces oval spores that are subterminally situated.

The most striking feature of the genus is the variety of anaerobic energy yielding reactions that have developed. The species of the genus Clostridium are commonly divided into a number of groups depending on their source of carbon used for metabolism. There are two main groups; those which ferment carbohydrates and have limited proteolytic properties known as the "saccharolytic clostridia", and those with mostly proteolytic activity known as the "proteolytic clostridia".

The "saccharolytic clostridia" produce a great variety of end products and are consequently further subdivided according to the end products they produce, eg. Clostridium butyricum and Clostridium lactoacetophilum produce mostly butyric acid, CO_2 and H_2; C. acetobutylicum produces mostly butanol and acetone together with CO_2 and H_2 while Clostridium butylicum produces butanol, isopropanol, CO_2 and H_2.

Crummins and Johnson (1971) analysed cell wall sugar composition, DNA homology and nutritional requirements of the genus Clostridium to determine if they could be used as a more accurate means of classifying the butyric acid producing bacteria than that used by McCoy et al. (from 1926-1930) (Section 1.2). They determined that C. butyricum could be divided into two main groups. Group I, those containing glucose as the only cell wall sugar with growth occurring on mineral salts-glucose medium supplemented with biotin. The addition

of amino acids improved growth. Group II, those containing glucose and galactose as cell wall sugars. In this group growth would not occur in a mineral salts-glucose medium with amino acids and vitamins unless yeast extract was present. Several other clostridial strains could be classified in one of the two groups and it was suggested that group I be known as C. butyricum and the second as Clostridium beijerinckii. Some strains eg. Clostridium fallax, C. acetobutylicum, Clostridium aurantibutyricum, Clostridium pasteurianum and Clostridium tyrobutyricum did not belong to either group.

Catabolism of amino acids by the "proteolytic clostidia" is carried out by the following methods.

i) Single amino acids, eg. Clostridium tetanomorphum exhibits an anaerobic oxidative deamination accompanied by the evolution of hydrogen. Arginine is metabolised by C. botulinum, tryptophan is fermented by Clostridium sporogenes and Clostridium sticklandii is able to utilize lysine as its sole carbon and energy source.

ii) Pairs of amino acids. This involves a coupled oxidation reduction reaction between suitable amino acids and is known as the Stickland reaction. Clostridia using this reaction include C. sporogenes and C. botulinum (A & B).

iii) One amino acid in combination with a non-nitrogenous compound. This metabolism is the same as the pairs of amino acids except the reaction occurs between a suitable amino acid and a non-nitrogenous compound. An

example of a Clostridium undergoing this metabolism is Clostridium propionicum.

Several species of the genus can undergo both "proteolytic" and "saccharolytic" metabolism and these include C. perfringens and C. acetobutylicum.

C. acetobutylicum is a non-pathogenic organism most closely associated with the industrial fermentation process in the production of acetone and n-butanol.

1.2 History of the acetone-butanol fermentation process

n-Butanol was discovered as a regularly occurring constituent of fusel oil by Wurtz in 1852. Pasteur however was the first to show that butanol was a direct product of fermentation. From 1876 to 1884 Fitz investigated the fermentation process of "Bacillus butylicus". The bacterium was a sporeformer producing butanol, butyric acid and small quantities of ethanol. Between 1887 and 1897 various butanol producing fermentations involving different bacterial strains were investigated. Among them Beijerinck described a species named by him Granulobacter butylicum.(Granulobacter was the term applied to microorganisms that demonstrated a blue colour with iodine and showed a distinct swelling of the cell at sporulation.) Winogradsky in 1902 reported on Clostridium pastorianum and described it as a butyric acid producer.

Acetone was first discovered as a fermentation product by Schardinger in 1905 when studying the fermentation of Bacillus macerans.

The first successful commercial process resulted from the need to synthesise synthetic rubber. This was obtained through the polymerization of isoprene and butadiene and these compounds were best prepared from isoamyl alcohol and butanol respectively. Fernbach and Weizmann in 1911, who were working on the above process, discovered bacteria that fermented potato starch, yielding acetone, ethanol and butanol. In 1912 Weizmann continued research on the fermentation and isolated an organism producing four times the amount of acetone than his previous isolate. This organism was able to ferment starches other than potato starch and was named Bacillus granulobacter pectinovorum. During 1913 and 1914 factories were established for the production of solvents by fermentation. With the advent of World War I large quantities of acetone were required for use in the manufacture of cordite, an explosive, and "dopes" for aeroplane wings. However the demands for acetone could not be met using potatoes as the raw material. Consequently, all factories were replaced with the Weizmann strain which used maize as the raw material. With the entry of the United States into the first World War the industry was established in America and by 1918 large amounts of these two solvents were being produced in both countries.

At the end of the war there was no further demand for acetone production and as butanol had never been much in demand many factories closed. Shortly afterwards a need arose for butanol in the manufacture of automobile laquers and the fermentation process was re-established. From 1926-1930 McCoy *et al*. undertook a study of the acetone-butyl alcohol organisms and suggested *C. aceto-butylicum* as a suitable name for the Weizmann strain. The motile, sporeforming butyric anaerobes were classified as a non-pathogenic group of the genus *Clostridium* and subdivided into the "butyric organisms" and the "butyl organisms."

In America during 1958 the industry was ranked second to the alcoholic fermentation as an industrial process. However, now very few factories are still operational and several reasons are given for this. Firstly, competition from butanol produced from petrochemicals severely affected the industry. Secondly, there was a steep rise in the price of molasses and thirdly, despite detailed studies, no entirely satisfactory way had been found for increasing the low yields of butanol obtained in the fermentation or, for effectively controlling the proportions of acetone and butanol that are formed.

1.3 Growth factors and solvent production requirements

Much of the early research on the fermentation was centered on finding different raw materials to ferment as well as the nutritional requirements of the bacteria, particularly C. acetobutylicum. A wide variety of raw materials were found suitable including different grains and sugar substances such as molasses, sugar syrups or sugarbeet.

Studies on the growth requirements of C. acetobutylicum showed that thiamin, riboflavin, tryptophan, nicotinic acid, pimelic acid, pantothenic acid, alanine, uracil, pyridoxin and inositol did not stimulate growth of the organism in synthetic media but, that biotin was essential. Oxford et al. (1940) demonstrated that an additional factor found in yeast extract and designated the "BY factor" was also essential for normal growth in a synthetic medium. This factor was not identical to any other known bacterial growth factor at the time and was identified in subsequent studies by Rubbo and Gillespie (1940) and Lampen and Peterson (1941) as para-aminobenzoic acid (paba). Rubbo et al. (1941) and Housewright and Kaser (9144) determined that structurally related compounds of paba could also function as growth factors for C. acetobutylicum. There appears to be some discrepancy as to the role of biotin as a growth factor. Rubbo et al. (1941) maintained that paba was the only

growth factor required and that Weizmann's biotin preparation contained trace quantities of paba, while Lampen and Peterson (1941) postulated that biotin and paba are both required and that the glucose used by Rubbo et al. (1941) in their medium contained appreciable quantities of biotin. Lampen and Peterson (1941) used strains S9 nos. 824-826 for their study which included one of the strains used by Rubbo and Gillespie (1940). However, Reyes and Mickelson (1944) found that two strains of C. acetobutylicum B_1 and B_2 grew in synthetic medium with biotin only, a third BA required paba as well. Some strains were found by Cummins and Johnson (1971) to require paba only and others studied by Davis (1942) to require neither paba nor biotin.

Oxford et al. (1940) made the significant observation that there was no strict correlation between growth and fermentation and postulated that the presence of other factors may be required for normal solvent production to take place with normal growth. Extensive research has been done on this and improving solvent yields and some of the findings are outlined below.

Rubbo et al. (1941) investigated the ability of C. acetobutylicum to ferment a synthetic glucose medium containing paba and found that normal levels of butanol were found but that acetone production was suppressed, possibly due to an "acetone factor" deficiency. A factor was isolated from yeast and was found to restore acetone

yields. The "acetone factor" did not support growth on its own and it was postulated that it was probably a coenzyme related to nitrogenous bases. The acetone factor was required in the formation of an acetic acid intermediate and the conversion of this acid to acetone. In the same study Rubbo et al. (1941) unsuccessfully investigated various modifications of wheat mash fermentations in order to obtain increased solvent yields. They did however determine that traces of copper and prolonged sterilization had an adverse effect on solvent yield.

It was found that only slight changes in the ratio of solvents resulted from the use of nitrogen in different forms, but mashes containing a deficiency of nitrogen were abnormal in the solvent yield. It was determined that no growth of B. granulobacter pectinovorum resulted when the sole source of nitrogen in a mash was ammonium salts or a single amino acid. Subsequent studies showed that a complex nitrogen supply, eg. proteins and commercial peptones, was necessary for growth of C. acetobutylicum and subsequent fermentations. The addition of a prolamine containing substance, yellow corn, was initially found to be required for normal butanol production. However, Weizmann and Rosenfeld in 1937 found that complex proteins (peptone and prolamines) were not necessary for normal butanol-acetone fermentations. Asparagine in the presence of an activator, possibly a coenzyme, produced normal levels

of solvents in semisynthetic medium. In fact, L-asparagine was found to stimulate the production of butanol by C. butyricum during starch fermentations and, Davis and Stephenson (1941) demonstrated that asparagine was essential for acetone production by C. acetobutylicum in glucose but not starch containing media. Asparagine therefore appears under certain conditions to play an essential role in solvent production. The coenzyme was postulated to function as a hydrogen carrier as well as to favour growth of the bacterium. Potassium was found by Davis (1942) to be essential for solvent production in corn mashes by C. acetobutylicum. Rosenfeld and Simon (1950) in their study of the role of pyruvate as an intermediate of solvent production suggested that both potassium and magnesium were required for the normal formation of acetone by bacterial enzymes. The presence of fluoride caused the formation of hydrogen from pyruvate to decrease and the formation of butyl products to increase with no effect on acetone production.

Ierusalimskiĭ and Semenova (1944) studied the effect of adding 4,5% beet molasses to their starch medium and showed that the fermentation proceeded abnormally with solvent production being greatly inhibited. This was due to the readily assimilable nitrogenous substances contained in molasses, which, if present in excess, accelerated bacterial growth and production of acids but inhibited the formation of solvents. They found that if 20 to 50% of the molasses was added to the mash prior to inoculation

with C. acetobutylicum and the rest 12-18h later, the normal amount of solvents was obtained. In this way the molasses content of the mash could be increased to 6,2%.

Oxford et al. (1940) determined that glucose with ammonium phosphate as the sole nitrogen source produced normal solvent yields and Lukina et al. (1972) found that fermentation using sugar beet achieved a high yield of butanol.

Davis and Stephenson (1941) studied the details of the fermentation of C. acetobutylicum in glucose medium. These investigations had previously only been carried out in starch medium. They found that when maize meal was fermented higher yields of solvents were obtained than if a 2% glucose and yeast extract medium was used. This agrees with Ierusalimskiĭ and Semenova (1941) findings discussed above. Davis and Stephenson (1941) found that frequently yields were very low (acid fermentations), whilst occassionally solvents were produced and the fermentation approached "normal". In order to convert an acid fermentation to a "normal" fermentation the addition of several growth supplements present in liver, maize and yeast was required. The exact nature of the supplements and their relationship to those mentioned above is unknown. They established that the pH optimum for glucose fermentation was pH5,7 and by removing samples during the fermentation, centrifuging and resuspending

the bacteria in fresh medium they found they could study the course of the fermentation. There was however a rapid decrease in activity of the centrifuged cell suspension and it was determined by Davis (1942) that the cells only maintained their activity as long as they were fermenting glucose. Using the centrifuged cells Davis and Stephenson (1941) established that no acetone was produced until c. 10h after inoculation and this increased to a maximum, after which it remained constant. Nakhamanovich and Yarovenko (1971) investigated the growth kinetics and kinetics of aceto-butyl fermentations of different sugars ie. arabinose, xylose, sucrose, glucose as well as starch. The growth rate was similar for all substances with xylose the lowest. Fermentative activity was also similar, and the slow fermentation of xylose was caused by the slow growth of the bacteria on xylose medium.

It was known that volatile acids, chiefly acetic and butyric acids were formed during the first phase of the fermentation, although different hypotheses were proposed for the process of solvent formation from the acids. The addition of acetic acid increased acetone yields but did not affect butanol production while the addition of butyric acid increased butanol production.

Bernhauer and Kurshner in 1935 (Nakhmanovich and Shcheblykina, 1960) showed that the efficiency of this conversion was dependent on the activity of the C. aceto-

butylicum culture used. An active culture would convert 97% of the added acetic acid while a weakened culture would only convert 30% to acetone and the remainder to ethanol. The same trend was found with the addition of butyric acid. Nakhmanovich and Shcheblykina (1959) observed that substitution of their starch fermentation with increasing concentrations of sugar (neutralized hydrolysates of corn flakes) increased the acetone yields. It was postulated that the increased acetone formation was due to the fermentation of calcium acetate formed during the production of sugar. This was confirmed by the addition of the calcium salts of acetic and butyric acids. Addition of calcium acetate increased acetone production by 20-24% while 70% of the calcium butyrate added was converted to butanol. Furthermore fermentations were activated by calcium acetate as the reactions began sooner and proceeded more rapidly in the presence of glucose and calcium acetate.

Hongo (1957a, 1957b, 1957c, and 1957d) found that the solvent ratio could be altered in C. acetobutylicum fermentations by the addition of 0,1% of the redox dye, neutral red. Normal yields of butanol were increased (25%) but those of acetone and ethanol were decreased. However, there was an overall increase in total solvent production. This effect was tested for in an isopropanol-butanol fermentation and was shown to increase the yield of butanol but decrease that of isopropanol. Neutral

red was effective in increasing the reduction of precursors to butanol and evidence was presented to support the proposal that neutral red acted as a hydrogen carrier. Janus green was the only other redox dye that was effective in slightly enhancing butanol production.

The effect of exposure to oxygen on growth and solvent production of C. acetobutylicum is discussed in Chapter II.

In 1960 Hongo patented a new organism Clostridium saccharoperbutylacetonicum (Gutcho, 1973) which produced increased solvent yields. Four times as much acetone was produced than previous isolates in a medium containing 4% glucose and ammonium sulphate as the nitrogen source. By substituting ammonium chloride as the nitrogen source and increasing the glucose concentration by 1%, a further increase in solvent yield was obtained using this bacterium.

Continuous fermentations were initially investigated in 1932. It was known that high solvent yielding strains of C. acetobutylicum were unstable and lost their ability to produce large quantities of solvents when repeatedly transferred at 24 hourly intervals. Consequently continuous cultivation of these strains did not seem possible. This was however achieved in 1958 and Finn and Nowrey (1959) demonstrated that a strain of Clostridium saccharoacetobutylicum showed less tendency to degenerate when held in exponential growth in continuous

propagation than when serially transferred at 24 hourly intervals. Continuous propagation was maintained for 14 days with little decrease in solvent production, however, towards the end of this time period, changes had occurred and the culture could only tolerate one further serial transfer after the 14 days continuous cultivation.

An interesting use of C. acetobutylicum in diagnosing tumors was proposed by Möse (1970). The C. acetobutylicum spores were injected intravenously into rats. In the absence of tumors very few spores germinated while at the site of tumors germination and multiplication occurred. As spores and vegetative cells had different antigenic characters, tumors could be identified by antibody reactions.

1.4 By-products of the fermentation

The stillage obtained after distillation of the solvents become a major by-product due to its widespread use in animal feeds. The normal dried stillage contained various vitamins eg. Riboflavin or vitamin B2 in amounts of 40-80µg g^{-1} (dry weight) and vitamin B12.

C. acetobutylicum was the first micro-organism to be found capable of producing large amounts of riboflavin and was for some time used as a commercial source of the vitamin (Rose, 1961). Yields were increased after it was discovered that the iron content of the medium

was an important factor in determining the amounts of riboflavin produced. Extensive research was undertaken to increase the riboflavin yield. Very little riboflavin is now produced commercially by this method as the fermentation industry for acetone and butanol is largely redundant and the use of Eremothecium ashbyii and Ashbya gossypii has superceded the use of C. acetobutylicum.

1.5 The biochemical pathway of solvent production

The biochemical pathway leading to solvent production in the clostridia is now fully understood. Many investigations have contributed to this. Some of the initial studies were carried out by Speakman in 1920 who differentiated the different phases and Peterson and Fred in 1932 who contributed much from their study on the fermentation of corn mashes.

The biochemical pathway for the formation of acetate, acetone, butanol, ethanol and butyric acid used by the clostridia is illustrated in Fig 1.

The formation of acetyl-CoA from pyruvate with the formation of hydrogen and CO_2 usually followed by the clostridia, occurs via a pathway that does not involve formate as the precursor of H_2 and CO_2.

Saccharolytic clostridia ferment glucose to butyric acid in preference to acetate, as the latter is a

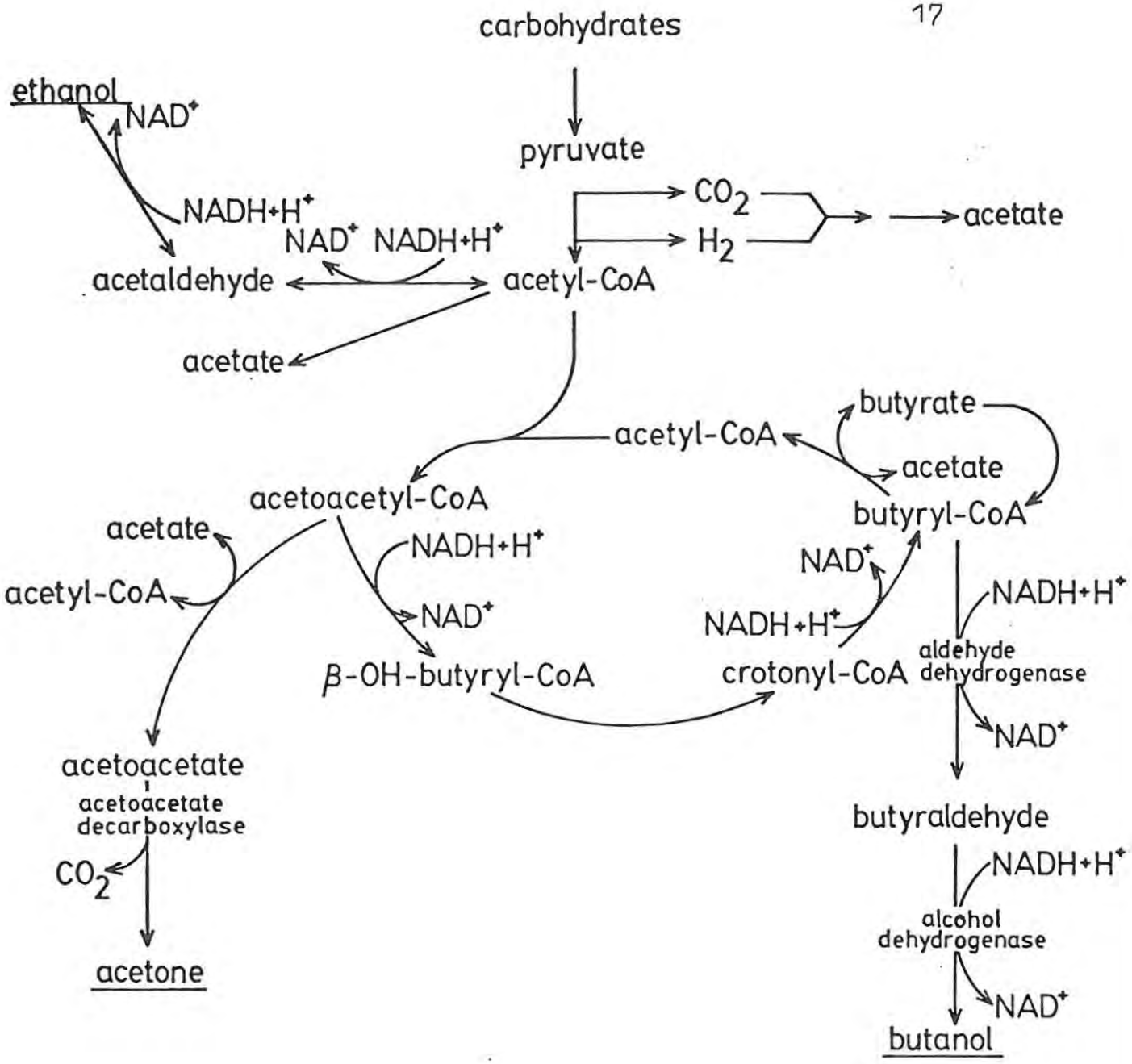


Fig 1. The formation of acetate, acetone, butanol, ethanol and butyric acid by species of the genus Clostridium (Doelle , 1975).

stronger acid and it becomes more difficult to reoxidize the $NADH^+$ as the pH drops towards the acid region. The clostridia have therefore developed a cyclic mechanism similar to that found in the propionibacteria which brings about the formation of butyric acid. A number of the saccharolytic clostridia are able to change their system favouring the production of acetone and convert the butyric acid already produced to butanol. This occurs as soon as the butyric acid production has caused the lowering of the pH of the medium to c. 5,4.

The pathway of ethanol production is different in C. acetobutylicum than in yeast where ethanol is produced from pyruvate which is converted by decarboxylase to acetaldehyde and CO_2, the acetaldehyde being reduced by a NAD-linked reaction to ethanol. C. acetobutylicum forms acetyl-CoA from pyruvate which is converted by the enzyme aldehyde dehydrogenase to acetaldehyde which in turn is converted by NAD-dependent alcohol dehydrogenase to produce ethanol.

Acetone is produced in C. acetobutylicum by its specific transferase system that diverts acetoacetyl-CoA from the normal cylcic mechanism to produce acetoacetate. This is followed by the decarboxylation of acetoacetate to acetone in the acetoacetate decarboxylase dependent reaction. The diversion of the original cyclic system to form acetone (which occurs at c. pH5,4) stops further production of butyric acid. As a result of the

interruption of the cycle two steps generating NAD^+ are eliminated and some other reduction process is found ie. the production of butanol. This involves three stages. The last reaction in the cycle is reversed and the enzyme CoA-transferase transfers the coenzyme (CoA) from the acetyl group to the butyl group (Fig 1). The acetate formed is used for the generation of acetyl-CoA in the production of acetone. Butyryl-CoA may be formed by an alternative pathway if there is a deficiency in the amount of acetyl-CoA available. ATP and CoA are required for this process. The reduction of Butyryl-CoA to butyraldehyde is catalyzed by the same aldehyde dehydrogenase that reduces acetyl-CoA to acetaldehyde, the final reduction to butanol being carried out by NAD^+ linked alcohol dehydrogenase. The formation of butanol occurs after the change to the production of acetone has taken place.

1.6 Contaminants of the fermentation industry

The greatest problem in the butyl fermentation industry is the maintenance of sterile conditions to prevent contamination. There are three main types of contaminants.

i) The lactic acid bacteria. The most serious of the lactic acid bacteria contaminants is Lactobacillus luchmanii, an acid producing organism which closely resembles the clostridia in size and shape. The growth of C. aceto-

butylicum favours the development of these bacteria by hydrolysing starch to fermentable sugars and causing proteolysis of the nitrogen compounds to amino acids. If present in a fermentation the lactobacilli grow rapidly and produce acid conditions which prevents the conversion of acetic and butyric acid to solvents. In the presence of certain strains of lactic acid bacteria the characteristic gas and head of the fermentation are absent.

ii) Other bacteria and yeasts: Streptococcus lactis, certain strains of Bacillus subtilis and various yeasts have been found but their presence has been only slightly detrimental to the fermentation.

iii) Bacteriophages: These are definitely the most harmful to the fermentation industry. Phages are widely dispersed in nature and it is still unknown how the bacteriophage enters the fermentation process although several theories have been developed. The details on the characteristics of the phage infections and immunization are outlined in Chapter IV.

Kocwa (1962) suggested a means of decreasing the number of non-viral contaminants in the fermentation. It was observed that chloramphenicol and "chloromycetin water" (refuse product of antibiotic production) had no effect on C. acetobutylicum at the doses used, while it inhibited the acid producing microflora of raw material origin eg. molasses and meal. The harmful action of contamination could therefore be reduced by suitable

doses of chloromycetin water. In addition, the doses of these substances used had a positive influence on solvent production. O'Brien and Morris (1971) determined that C. acetobutylicum was sensitive to chloramphenicol on agar plates but was resistant to it in liquid cultures as the bacteria rapidly reduced and detoxified the antibiotic. This was achieved by reduction of the aryl nitro group of chloramphenicol via a ferredoxin-dependent enzymatic reaction. The same effect was found with a number of other aryl nitro-compounds.

1.7 Fermentation of molasses at National Chemical Products

National Chemical Products (N.C.P.) is situated in Germiston and its products include acetone, ethanol and butanol from the fermentation of molasses by C. acetobutylicum. The industry was founded in South Africa in 1944 and is still viable today despite closure of many factories overseas. Molasses is obtained from the various sugar mills throughout the country and serves as a cheap raw material. The stillage is air dried and used in the production of a cattle feed known as Rumavite. With the recent increase in the price of hydrocarbons, which are usually used in the production of these organic solvents, the importance of the

fermentation process is being emphasized once again. Consequently a detailed study of the fermentation process at N.C.P. was undertaken, to fully understand it and optimize laboratory scale fermentations so that mutants producing increased solvent yields could be isolated. In addition an investigation into a recent severe phage infection at the factory was carried out to prevent subsequent occurences.

CHAPTER II

GROWTH OF *Clostridium acetobutylicum* ON AGAR PLATES

2.1 Introduction

In the industrial production of acetone and butanol at N.C.P. by *C. acetobutylicum*, the clostridial spores are maintained on sterile soil and germinated by heat shocking in liquid medium. The build-up stages and final fermentation are all carried out in liquid media, and after the removal of the solvents the bacterial cells are used in the manufacture of a cattle feed called Rumavite. Consequently, the industial fermentation process is a one way liquid process in which solid media cultivation is not used. N.C.P. obtained soil spore cultures from the U.S.A. in 1944 and have never grown the bacterium on agar media. Previous attempts by N.C.P. to grow the bacterium on agar media were unsuccessful. However the growth of *C. acetobutylicum* on solid media and the isolation of pure clones is essential for phage studies and the isolation of mutants which produce higher solvent yields.

As the genus *Clostridium* is one of the more fastidious groups of bacteria (Perkins, 1964), many agar media

were tested for their ability to support bacterial growth and assay phage. Harmon and Kautter (1977) found that pretreating agar plates with catalase increased the percentage recovery of clones of different Clostridium species including C. acetobutylicum. Catalase prevents the accumulation of hydrogen peroxide (H_2O_2) which is produced as a wasteproduct of cellular metabolism when oxygen is present.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$ (Stanier et al., 1976)

Accumulation of H_2O_2 inhibits growth. Harmon and Kautter also showed that catalase overcame the inhibitory effect of high glucose concentrations in agar media.

C. acetobutylicum is not as sensitive to oxygen as some obligate anaerobes as it grew in non-reduced liquid media during the fermentation process. O'Brien and Morris (1971b) investigated the effect of oxygen on C. acetobutylicum in liquid media and found that exposure to concentrations of dissolved oxygen below 1μM had no effect on growth, utilization of glucose, or the production of acetate or butyrate. At dissolved oxygen concentrations between 40-50μM the rate of glucose consumption decreased, growth was halted and net synthesis of DNA, RNA and protein was prevented. The bacteria were deprived of energy as evidenced by the cessation of butyrate formation (but

not acetone production) and a decrease in ATP was accompanied by a simultaneous increase in the ADP content of the cell. It was also noted that there was an increase in the activity of NADH oxidase with increasing concentrations of dissolved oxygen until 1μM, above which no further increases were found. It was postulated that the bacteria detoxify exogenous oxygen with NADH oxidase but at high concentrations of oxygen the enzyme levels were insufficient and growth was inhibited. C. acetobutylicum appears to be aerotolerant and the effect of different anaerobic conditions on the growth of the bacterium in liquid or agar media was investigated.

The isolation of mutants producing high yields of solvents depends upon an efficient plate screening method for good solvent producers. It was observed that on the A_1 Potato agar medium (Detailed in Appendix B) colonies of C. acetobutylicum were surrounded by "halos" (areas of clearing of the potato agar). The characteristics of the halos were investigated to determine whether it could be used as a possible indicator of solvent production.

2.2 Materials and methods

2.21 Media

All percentage compositions are w/v and all nutrients are Difco unless stated otherwise.

All the basic media used are listed in Appendix B.

The A_2 Filtered Molasses medium was identical to the normal A_2 Molasses medium in constituents, but was filtered through cotton wool and Whatman's No 1 filter paper to remove precipitates. The 1/8 Filtered Molasses medium contained 1/8 the concentration of molasses. Brain heart infusion medium was supplemented with one of the following: 6,6 or 3,3% glucose; 6.6; 3,3; 1,6 or 0,8 % sucrose; 6.6; 3,3; 1,6 or 0,8 % molasses. Reduced media were obtained by the addition of 0,05% cysteine hydrochloride.

Actively growing liquid cultures were plated by (i) streaking (ii) spreading (iii) agar overlay technique. All aerobic and anaerobic cultures were incubated for 24-48h at 34°C which was the optimum growth temperature for solvent production.

2.22 Culture conditions

Stringent anaerobic growth conditions

Stringent anaerobic conditions were generated by (i) the GasPak (BBL) technique (ii) perfusion for 20 min or (iii) repeated evacuation and perfusion (3 or 5 cycles). The perfusion gas contained a trace of H_2 which was shown by Moodie and Woods (1973) to be suitable for the cultivation of obligate anaerobes. Plates were

incubated in anaerobic jars containing a semi-solid anaerobic indicator and any traces of oxygen were removed by a palladium catalyst. The catalyst was rejuvenated regularly by heating the pellets at 160°C for 2-4hrs and cooling in a dessicator.

Microaerophilic conditions

Microaerophilic conditions were generated by:

(i) The candle jar technique (Collins, 1964)

(ii) Petri dishes sealed with Parafilm and

(iii) Pour plates inoculated with a large inoculum

Aerobic growth conditions

Aerobic liquid cultures were incubated without aeration.

2.23 Identification

Clones were identified as C. acetobutylicum using Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

2.24 Catalase treatment

The method outlined by Harmon and Kautter (1977) was followed. Agar plates were stored in the dark and were spread with 0,1ml (500 units) purified bovine liver catalase (Miles Laboratories) 10 min before use. The stock solution of catalase was stored in the dark at 4°C.

2.25 Methods used to characterize the halos surrounding colonies on potato agar plates

(i) pH Indicator plates: A_1 Potato agar plates were supplemented with either 1,2% (v/v) Eosin-Methylene Blue or Andrades indicator.

(ii) Production of the "halos" on A_1 Potato agar plates: The production of halos on A_1 Potato agar plates was investigated by the addition of different concentrations and combinations of acetic acid, butyric acid or β-amylase to wells in the agar plates. After incubation for 22h the halos produced were compared with halos produced by C. acetobutylicum.

2.3 Results

2.31 Bacterial growth in liquid and agar media

All the liquid media associated with the fermentation process (A_1 Potato and A_2 Molasses media) and modifications of these media supported bacterial growth while Nutrient broth, Brain heart infusion broth with and without supplements did not. The bacterium did not grow on the following agar media under aerobic, microaerophilic or anaerobic conditions; A_2 Molasses media, Nutrient agar, BHI agar, Peptone Yeast Glucose medium, Peptone Yeast Molasses medium, N.C.P. Clostridium medium, Clostridial medium (O'Brien and Morris, 1971) or Potato

Dextrose agar (Oxoid).

Poor growth on A_1 Potato agar and A_1 Filtered Potato agar was obtained when the plates were incubated under anaerobic conditions generated by repeated evacuation and perfusion. However, good growth was obtained when the plates were incubated under the GasPak system. Using the GasPak system the incubation time was reduced from 48h to 18-24h. In addition, several different agar media were found to support growth under these conditions although the A_1 Filtered Potato agar was vastly superior. Since the GasPak system is expensive the use of bottled gases was investigated using different ratios of CO_2 and H_2 in order to simulate the anaerobic conditions generated by the GasPak system. Ferguson et al. (1975) showed that the level of CO_2 obtained with the GasPak system was c. 135ml per sodium bicarbonate and citric acid tablet. They found the hydrogen level attained was c. 1880ml per borohydride tablet.

The results (Table 1) indicated that by increasing the H_2 pressure from 10kPa to 20kPa and by addition of H_2O to the anaerobic jar, good bacterial growth was obtained on the A_1 Potato agar medium. Similar results were obtained using the evacuation and perfusion technique.

2.32 The effect of catalase on growth recovery on agar plates

A saline suspension of C. acetobutylicum was plated

Table 1 Growth of C. acetobutylicum on agar media under anaerobic conditions generated by perfusion of CO_2 and H_2

Time of Perfusion (min)	Approximate Rate of CO_2 flow (ℓmin^{-1})	Approximate Rate of H_2 flow (kPa)	ml H_2O in anaerobic jar	Growth
10	4,0	$<$10,0	0	- X
20	3,75	40,0	100,0	++++
20	3,75	40,0	2,0	+++
20	3,75	40,0	0	+
20	3,75	20,0	2,0	+++

X - no growth; + degree of growth.

on untreated and catalase pretreated agar plates. The viable count increased after catalase treatment (Table 2). A viable count of 6,6 x 10^6 was obtained on untreated A_1 Filtered potato agar plates while a viable count of 2,4 x 10^7 was obtained after catalase treatment (3,7-fold increase). Similar results were obtained with the other media. Furthermore the colonies on the catalase plates grew better and were markedly larger.

2.33 Development of a plate screening technique for mutant selection

Growth on A_1 Potato agar pH indicator plates

Eosin-Methylene blue indicator inhibited bacterial growth. The bacteria grew well on Andrades A_1 potato plates and the halos were stained pink, indicating a decrease in pH within the area of the halo.

Production of the halo on A_1 Potato agar plates

The characteristic halo produced by C. acetobutylicum clones when grown on A_1 Filtered Potato agar plates is shown in Plate 1.

The results shown in Table 3 indicate that the clearing of the potato agar was not due to β-amylase activity. Amylase produced an area of white precipitate surrounding the well. The area of white precipitate did not stain after the addition of iodine, indicating

Table 2 Growth of C. acetobutylicum on agar plates untreated and pretreated with bovine catalase

Agar media	Bacterial No.		Fold increase of recovery on treated plates
	Untreated plates	Pretreated plates	
A_1 Filtered Potato agar	$6,6 \times 10^6$	$2,4 \times 10^7$	3,7
A_1 Filtered Potato agar + 6% glucose.	$1,6 \times 10^6$	$9,4 \times 10^6$	5,9
A_1 Filtered Potato agar + 4% glucose	$1,4 \times 10^6$	$1,0 \times 10^7$	7,1
A_1 Filtered Potato agar + 2% glucose	$2,8 \times 10^6$	$1,6 \times 10^7$	4,8
Tryptone + 6% glucose	$5,0 \times 10^5$	$8,8 \times 10^6$	17,6
Tryptone + 4% glucose	$1,1 \times 10^7$	$5,8 \times 10^7$	5,3
Tryptone + 2% glucose	$1,1 \times 10^7$	$7,2 \times 10^7$	6,6

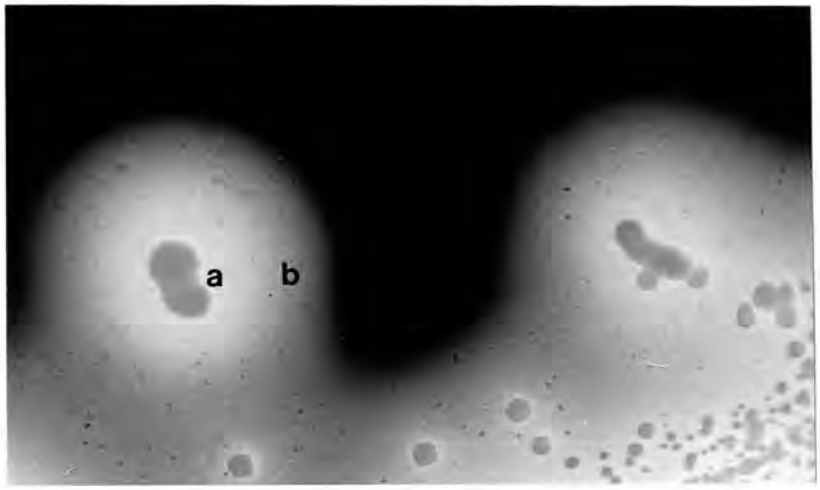


Plate 1 Halo production by two C. acetobutylicum clones on A_1 Filtered Potato agar plates. The plate was flooded with iodine to facilitate halo identification. (a) Halo due to butyric acid production and (b) halo due to β-amylase production.

Table 3 Results of clearing tests on A_1 Potato agar medium (All trails over a 22h period)

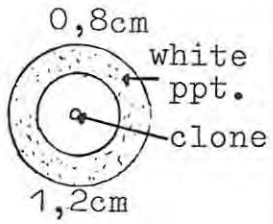
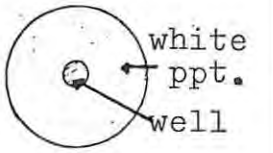
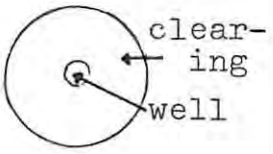
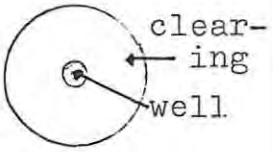
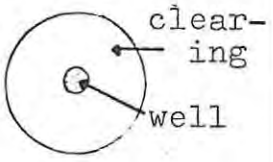
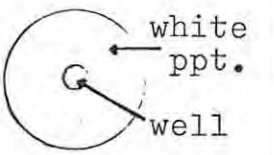
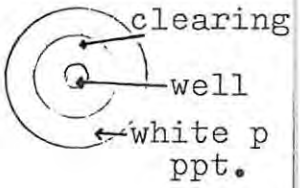
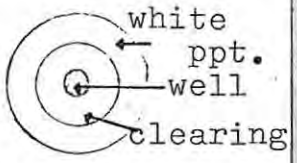
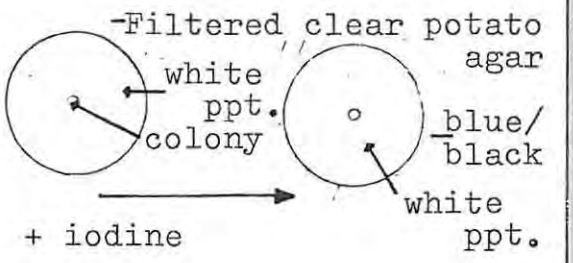
	Clearing Agent		Description of Clearing	Diam of Halo	Diagram
1.	P262 colony grown for 22h under GasPak Anaerobic conditions		Very definite clearing surrounded by white preciptiate	Varies with size of clone	
2.	Amylase 14mg/100ml		Amylase causes a halo consisting of a white ppt.	2,0cm	
3.	CH_3COOH	1M) 0,1M) 0,01M)	Clearing just visible	3,2cm 1,6cm -	
4.	$C_4H_8O_2$	1M) 0,1M) 0,01M)	Definite clearing but not as clear as that produced by P262	3,2cm 1,6cm 0,9cm	
5.	CH_3COOH + $C_4H_8O_2$	1M) 0,1M) 0,01M)	As for 3 + 4 combination of acids did not enhance clearing	2,7cm 1,2cm -	
6.	Amylase + CH_3COOH	1M) 0,1M) 0,01M)	White preciptiate Clear with whitish precipitate White precipitate similar to that formed by amylase clone	1,5cm 1,0cm 1,5cm	

Table 3 (Contd.)

	Clearing Agent		Description of Clearing	Diam of Halo	Diagram
7.	Amylase	1M)	Definite clearing	2cm	
	+	0,1M)	Clearing surrounded by white ppt.	0,9cm	
	$C_4H_8O_2$	0,01M)	Clearing surrounded by white ppt.	1,2cm	
8.	CH_3COOH	1M)	Definite clearing	2,8cm	
	+				
	$C_4H_8O_2$	0,1M)	Clearing surrounded by white ppt.	1,2cm	
	+				
	Amylase	0,01M)	White ppt.	1,4cm	
9.	P262 colony on agar medium which has been well filtered and was clear initially		White precipitate		

that the starch had been digested. Acetic acid (CH_3COOH) caused a slight clearing while butyric acid ($C_4H_8O_2$) resulted in very definite areas of clearing. The amylase precipitate remained when amylase and acetic acid were added together. The amylase and butyric acid mixture produced a clear halo surrounded by a white precipitate which most closely resembled the C. acetobutylicum colony halos. A mixture of amylase, acetic acid and butyric acid produced the same effect as the amylase and butyric acid mixture. The diameter of the clearing was proportional to the concentration of butyric acid.

2.4 Discussion

Growth of C. acetobutylicum on the agar medium was dependent upon the composition of gases and required a relatively high concentration of H_2. The addition of a small volume of water to the anaerobic box appeared to enhance growth.

Under these conditions several different agar media were able to support growth although the A_1 Filtered Potato agar medium was vastly superior.

The effect of pretreatment of the agar plates with catalase prior to inoculation resulted in a marked increase in clone recovery. This enhancement of growth by catalase was reported by Harmon and Kautter (1977)

and is presumably due to the removal of toxic H_2O_2. As a result of these experiments a standardized agar plate method was adopted for all future experiments. A_1 Potato agar plates were pretreated with catalase and the plates were incubated under anaerobic conditions generated by either bottled gases with a hydrogen pressure of 20kPa in the presence of a small volume of water, or, by anaerobic conditions generated by the GasPak system. Incubation was at 34°C for 18-24h.

Experiments aimed at characterizing the halo formation by C. acetobutylicum colonies on A_1 Potato pH indicator plates indicated that within the halo there is a decrease in pH of the agar. This decrease is presumably due to the production of acetic or butyric acids by C. acetobutylicum (General Introduction 1.5). Since butyric acid is a direct precursor of butanol, provided that the halos were due to butyric acid or a mixture of butyric and acetic acids, then the width of the halo could be used as an indicator of solvent production. The results suggest that the clearing within the halos is due to butyric acid production and the border of white precipitate is due to amylase production. As there is a relationship between the size of the halo and the concentration of butyric acid, the halo width could be used as an indicator of butyric acid production which

might be related to solvent yields. The relationship between halo width and solvent production needs to be established in fermentation experiments before embarking upon a mutant selection programme.

CHAPTER III

ANALYSIS OF THE FERMENTATION PROCESS

3.1 Introduction

The molasses fermentation by C. acetobutylicum for the production of acetone and n-butanol, as carried out at N.C.P., involves bacterial transfer through several different stages before inoculation into the final fermentation stage. Bacterial spores are heat shocked to stimulate germination, before transfer into the build-up stages, prior to the final fermentation inoculation. There are four build-up stages known as the A_1, A_2, B and C stages. The A_1 stage, in A_1 Potato medium, allows for initial growth of the culture following spore germination. The A_2, B and C stages involve a medium with the same low concentration of molasses (6,6%) and serve to build up a sufficiently high inoculum for the 90 000ℓ final fermentation. The final fermentation is carried out in a high concentration molasses (12,5%) medium containing a total invert sugar percentage of 6,4 (See Chapter IV). Despite the build-up stages the inoculum is still very small (0,028%).

Although routine quality control tests involving pH determinations and microscopic examinations are

carried out at N.C.P. during the final fermentation, no detailed studies of bacterial growth and solvent production have been performed. The analysis of the fermentation was therefore undertaken in order to fully understand the process. This involved the development of a laboratory scale fermentation system.

A certain percentage of fermentations fail and produce low solvent yields. The reason for these failures is not understood and poses a severe problem in the factory. As there is at present no way of detecting possible poor fermentations prior to inoculation of the final fermentation, the loss of time and media is considerable. The relationship between initial bacterial growth and solvent yields was investigated in an attempt to develop an early stage screening method to detect possible failure.

3.2 Materials and methods

3.21 Media

All the basic media used are listed in Appendix B. The A_2 Molasses medium was used for the A_2, B and C stages of the fermentation. A_1 Filtered Potato agar plates were used for all bacterial assays unless stated otherwise.

Bacteriocin assays are outlined in Chapter VI.

3.22 General Fermentation methods

All the N.C.P. factory volumes were scaled down for laboratory use. The volume of the final fermentation stage and size of inoculum were based on the N.C.P. laboratory control fermentation and not the large 90 000ℓ fermentation.

Cultures were incubated at 34°C, the optimal temperature for solvent production. All media were prewarmed before inoculation.

Heat shocking of Clostridial spores

Spores were maintained on sterile soil and heat shocked when required. A small amount (c. 0,9g) of soil plus spores was added to 3,0ml 0,85% saline in a test-tube. Heat shocking was achieved by placing the spores in a waterbath at 70-80°C for 2 min. The culture was immediately transferred to an ice-ethanol bath for 45s then incubated for at least 4h to allow spore germination before transferring to the A_1 stage.

A_1 Stage

The germinated spores (3,0ml) were transferred to 15,0ml of A_1 Filtered Potato medium and incubated for 16-18h.

A_2, B and C Stages

The entire actively growing A_1 culture was transferred to the A_2 stage which consisted of 50ml of A_2 Molasses medium in a 100ml flask. This was incubated for 6h before 16,7ml was transferred to the B stage; 350ml of medium in a 500ml flask. This was incubated for 6h before 116,7ml was transferred to the C stage; 900ml of medium in a 2ℓ round, flat-bottomed flask, which was maintained at 34°C for 10h before transfer into the final fermentation stage. Before transfer the pH of the C stage was determined and if this was $< 4,9$ the fermentation was abandoned.

Final Fermentation Stage

The final fermentation, consisted of 1,33ℓ of Control Fermenter medium (C.F.M.) in a 2ℓ round, flat-bottomed flask. Prior to inoculation with the C stage, the pH of the medium (set pH) was determined. This was normally 6,0-6,2. Media with a set pH below 5,5 were not inoculated. A sample (2,7ml) of the C stage was used to inoculate the final fermentation which was incubated for 48h before assaying the fermentation medium (butyl beer) for solvent production. During the fermentation, pH, colour, turbidity and cell morphology changes were monitored. The latter was observed either under oil immersion using a light microscope,

or, a Zeiss phase contrast microscope. At the same time a visual check for contaminants was carried out. Turbidity measurements were obtained after centrifuging a 10ml sample at 3000 rev. min^{-1} (to remove the brown colouring of the molasses) for 10min and resuspending the pellet in an equal volume of saline. Haemocytometer counts were obtained using a slide counting chamber with Thomatype ruling, and taking the average of 100 squares.

3.23 Determination of Solvent production

Solvent production was initially monitored by density determination (later abandoned) and Gas Chromatography (GC).

i) Density Determination of solvent production

This method depends on the correlation between the different concentrations of solvents. It involves the use of a pyknometer.

The weight of the empty pyknometer was accurately determined at 21°C on a Mettler five-figure balance. The pyknometer was then filled, ensuring preclusion of air bubbles, with a distilled sample of butyl beer (See below for method of distillation) and reweighed at 21°C. Care was taken to remove extraneous droplets of solution immediately prior to weighing. The pyknometer was dried at 140°C for 1h then cooled in a dry

seal dessicator before repeating the procedure outlined above until a series of precise readings was obtained. The density of the sample was determined using a hydrometer. The concentration of solvents in the sample was then read from a calibration curve. The method was abandoned in favour of the Gas Chromatography method as it was lengthy, cumbersome and inaccurate.

ii) Gas Chromatography (GC) method for solvent determination

Gas Chromatography was found to be quick and accurate and was used for routine determination of solvents.

Distillation procedure

Initially, immediately prior to GC analysis, a distillation was carried out to isolate the solvents from the butyl beer. A well-shaken sample (500ml) of the butyl beer was distilled in a flask containing 10g of $CaCO_3$ and 4 drops of defoamer (Silicolapse 5001). A 100ml volumetric flask containing 18,0ml of distilled water was used to collect the distillate and was maintained in an ice-ethanol bath until filled. The flask was well shaken and either used directly for density determination of solvent production or, following the addition of the internal standard (n-propanol), for GC assays. The n-propanol was added by removal of 1,1ml of distillate and replacement with 1,0g of n-propanol at 21°C.

Direct Injection method

The direct injection method was used to obviate the lengthy distillation procedure which would not have been suitable for the small volumes used in mutant selection. A 50,0ml sample of the well-shaken butyl beer was centrifuged on an M.S.E. bench centrifuge for 10min at 7000 rev. min^{-1}. The supernatant (20ml) was accurately measured into a standard container and 200mg of internal standard was added, ensuring minimal evaporation. A further 1/10 dilution of the sample was performed to minimize the effects of the molasses impurities before use in the GC assay. Details of operation and solvent determination using the Gas Chromatograph are outlined in Appendix A.

A comparison between the distillation and direct injection methods was carried out.

3.24 Bacterial Growth assays

Bacterial viable counts during the build-up stages of the fermentation were obtained by removing samples at the beginning and end of each stage, diluting and plating on A_1 Potato agar. Bacterial growth on agar plates from the final fermentation stage was tested on several different media. These included modifications of the final fermentation medium as well as selective clostridial media.

As poor growth at the end of the A_1 stage appeared to result in poor solvent production, viable counts at the end of this stage were obtained to determine whether good growth was indicative of good solvent production.

3.3 Results

3.31 Comparison between distilled and direct injection methods of solvent production determination

Although slight differences in solvent yield between the distillation and direct injection methods were obtained after GC analysis, they were comparable (Table 4).

3.32 Butyl beer analysis

Results of the studies of the fermentation process are illustrated in Fig 2 and 3. An average solvent yield of 16,0-17,0 $g\ell^{-1}$ was routinely obtained from the fermentation once it had been optimized for laboratory conditions. Solvent production occurred during the exponential growth (Fig 2) and reached a maximum at 36h.

All solvents were produced concurrently in the ratio of *c.* 10,0$g\ell^{-1}$ *n*-butanol, *c.* 6,4$g\ell^{-1}$ acetone and *c.* 0,6$g\ell^{-1}$ ethanol. Figs 2 and 3 illustrate a decrease

Table 4 A comparison between the distilled and direct injection methods of solvent production determination

Treatment	Solvent yield $g\ell^{-1}$			
	Exp. I	Exp. II	Exp. III	Exp. IV
Distilled	9,6	13,22	11,01	17,80
Direct injection	9,5	12,09	11,77	17,86

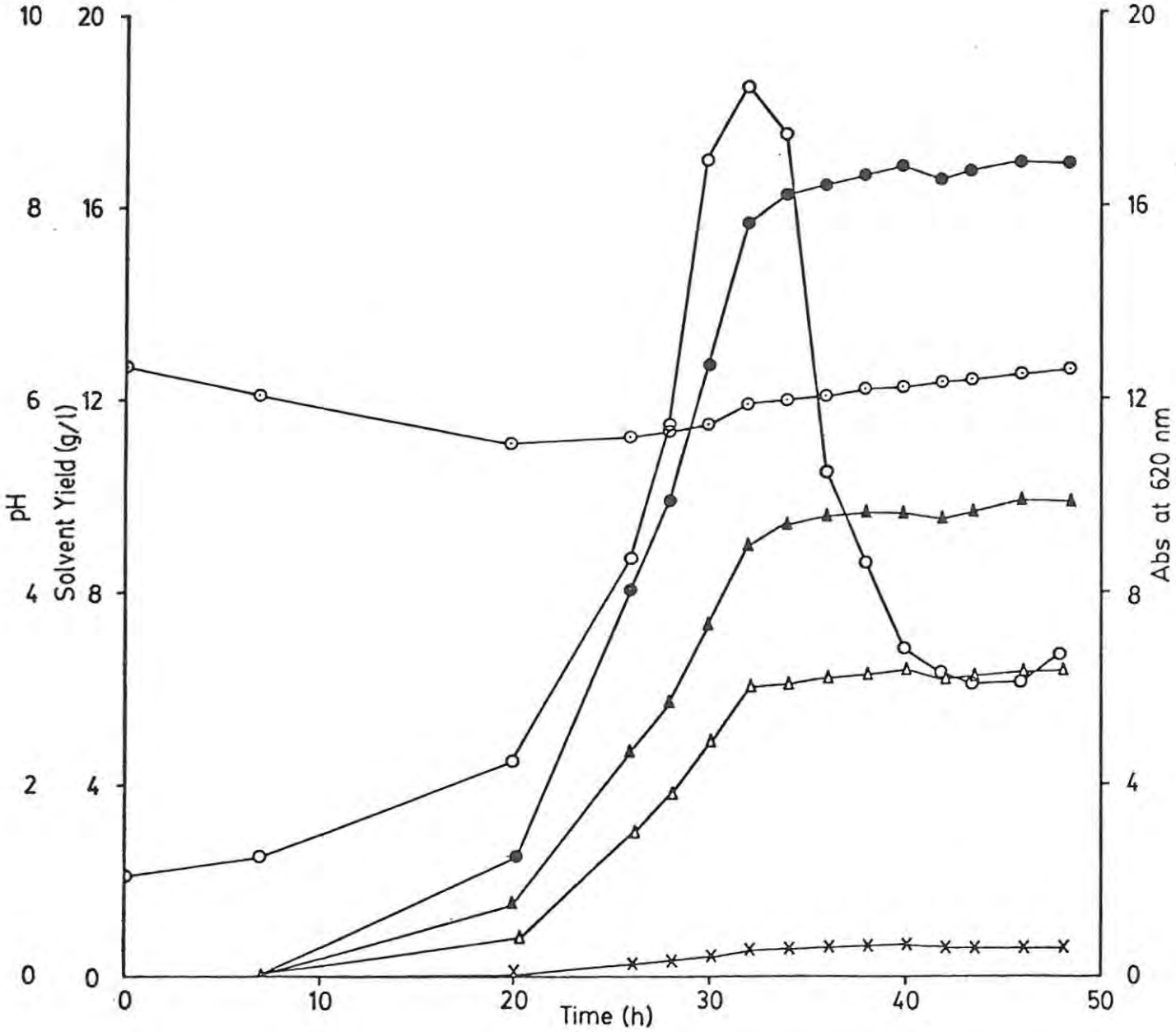


Fig 2. Detailed studies of pH changes, bacterial growth and solvent production during a C. acetobutylicum fermentation. ⊙—⊙, pH; O—O, Turbidity; ●—●, Total solvent production; ▲—▲, n-butanol production; Δ—Δ, acetone production; ×—×, ethanol production.

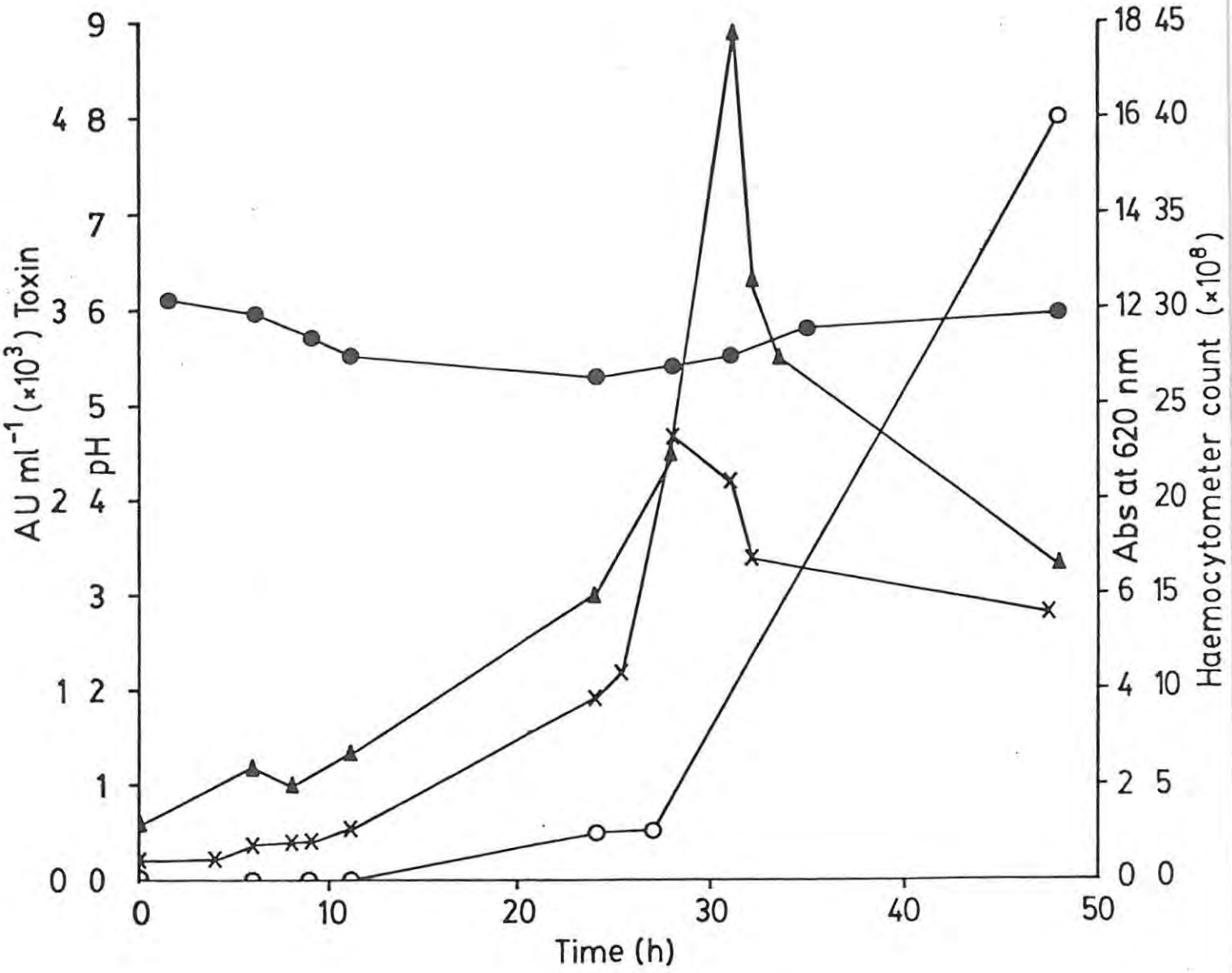


Fig 3. Detailed studies of pH changes, bacterial growth and bacteriocin-like toxin production during a C. acetobutylicum fermentation.

●—●, pH; Δ—Δ, Turbidity; ×—×, Haemocytometer counts; ○—○, Bacteriocin-like toxin production.

in pH of the fermentation during the first 16-18h when a minimum value (5,4) known as the breakpoint, was reached. This was accompanied by very little growth as indicated by both turbidity measurements and haemocytometer counts. The breakpoint was followed by a gradual increase in pH which corresponded with both solvent production and increase in turbidity measurements and haemocytometer counts. Cellular morphology changes were found to be characteristic of these different phases during the final fermentation and these are illustrated in Plate 2.

The cells were initially small rods (Plate 2a) and motile. This was followed at 5h by a gradual increase in cell length (Plate 2b) and at 7h half the culture contained long rods. At 8h the first Clostridial forms (Plate 2c and d), which are thought to be associated with solvent production by N.C.P. appeared and continued to increase, especially after the breakpoint, until the end of the fermentation. The long rods began decreasing at c. 24h with the concurrent increase in small rods. Motility increased to a maximum at the breakpoint then decreased. At 32h the numbers of small rods decreased and at this time cell debris and granulation in cells (Plate 2d) were apparent and became more prominent towards the end of the fermentation. No spores (Plate 2b) were present at the early stages although a few were seen after 48h.

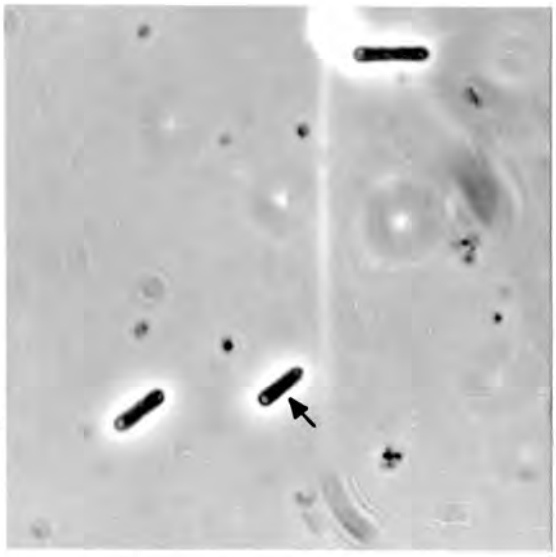


Plate 2a C. acetobutylicum small rods found at inoculation of the final fermentation stage.

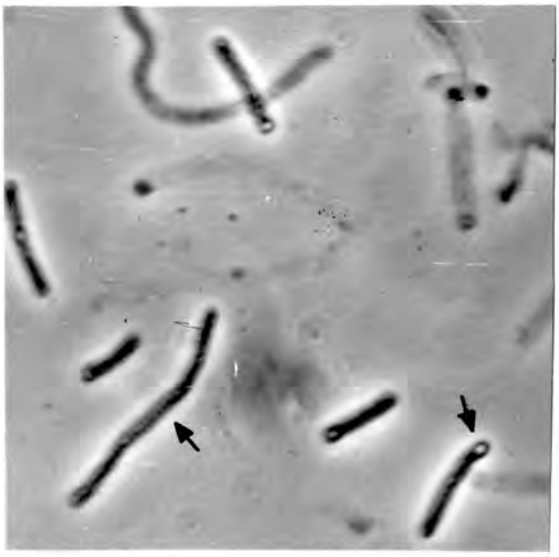


Plate 2b Increase in cell length observed 5h after inoculation and, Clostridium spores normally detected towards the completion of the fermentation.

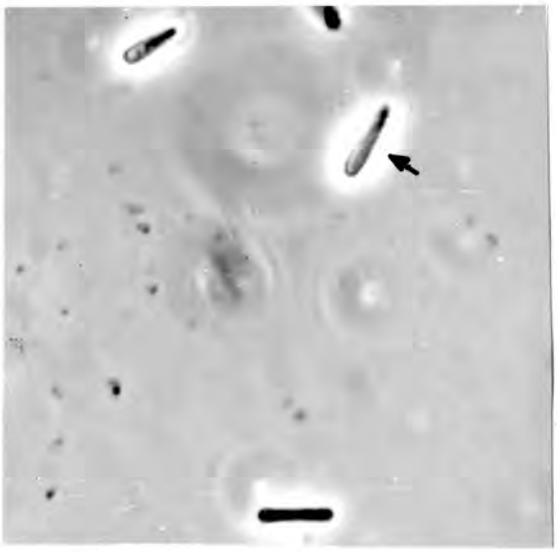


Plate 2c A possible clostridial form intermediate of C. acetobutylicum.

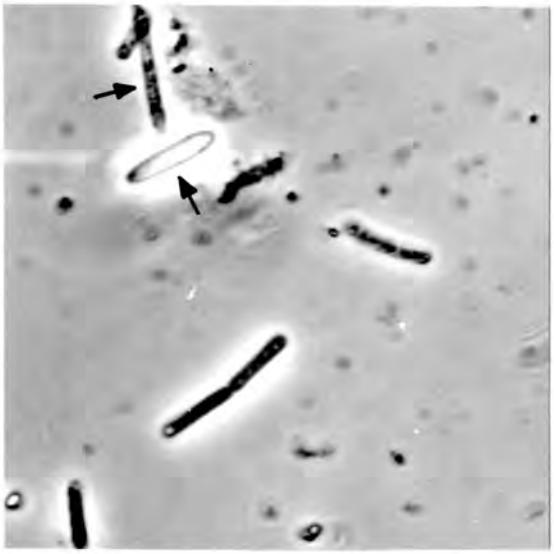


Plate 2d Clostridial form associated with high solvent production and, granular cells and cell debris prevalent towards the end of the fermentation.

Plate 2 The morphological changes present during C. acetobutylicum fermentations.

The final fermentation molasses medium is very dark and after six hours of fermentation, bubbles of CO_2 and H_2 started to appear on the surface of the medium forming a foam which proliferated until c. 24h after which time it disappeared. The foam formation was accompanied by a lightening in the colour of the molasses medium which resulted in a reddish-brown coloured butyl beer.

During a poor fermentation the increase in pH following the breakpoint was not observed and the pH continued to decrease giving a final pH of 5,0-5,2. Turbidity measurements of these fermentations (Fig 4) indicated that the degree of bacterial growth corresponded to the low solvent yield; very little bacterial growth occurred in the fermentations which yielded 4,1 and 9,6 $g\ell^{-1}$ solvents. In addition the characteristic colour and cell morphology changes observed in good solvent producing cultures were not seen in poor fermentations. Very few clostridial forms occurred. Cell debris and sporulation, associated by N.C.P. with poor fermentation, were greatly increased and were observed at an earlier stage in the fermentation.

Drastic decreases in turbidity and haemocytometer counts were observed at c. 32h in good fermentations (Fig 2 and 3). This decrease in cell number was due to the production and action of a bacteriocin-like toxin.

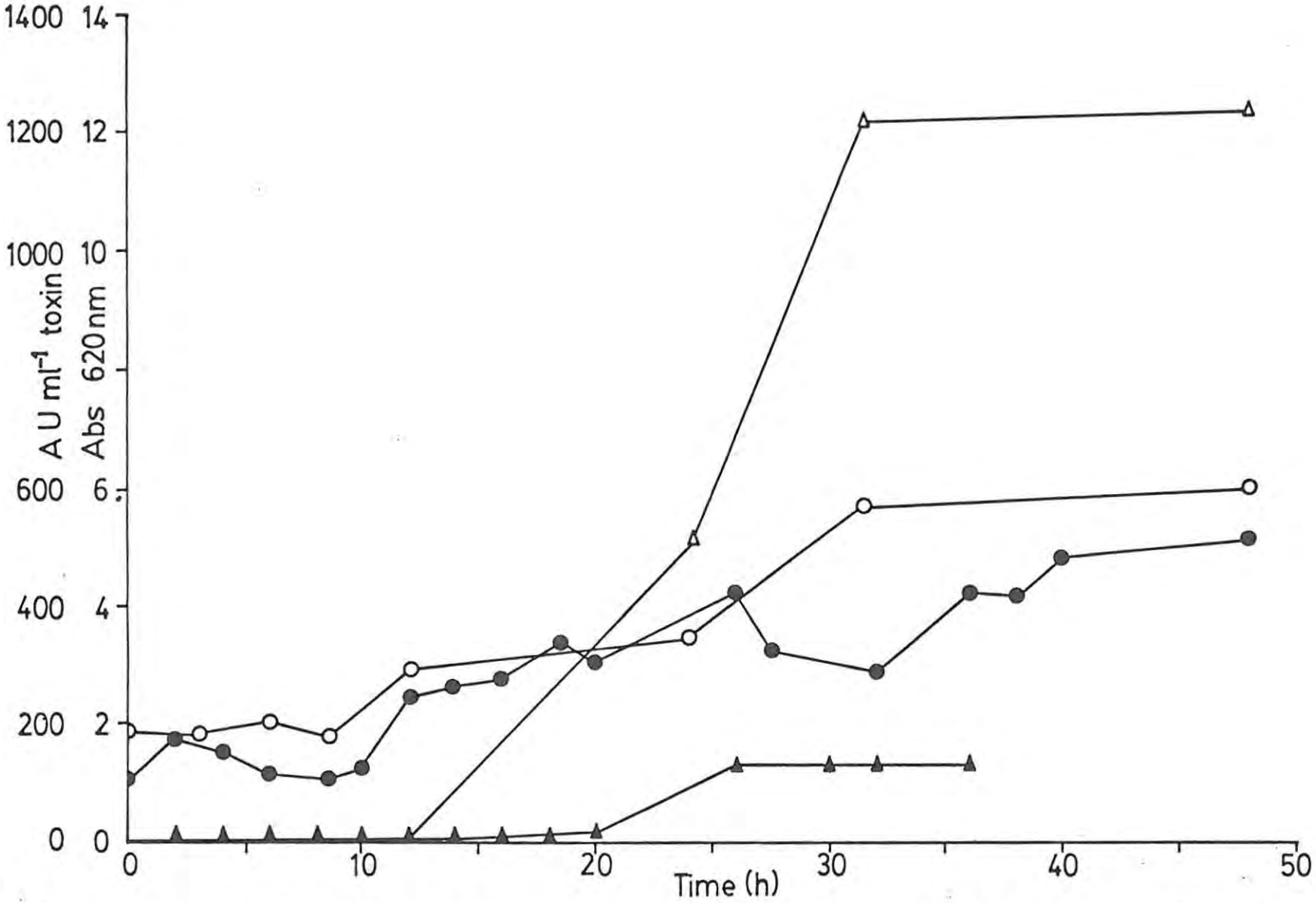


Fig 4 Bacterial growth and bacteriocin-like toxin production during C. acetobutylicum fermentations. Turbidity changes (O—O) and toxin production(Δ—Δ) during a 9,6gℓ^{-1} solvent yield fermentation. Turbidity changes (●—●) and toxin production (▲—▲) during a 4,1gℓ^{-1} solvent yield fermentation.

The toxin was also present in poor solvent producing fermentations (Fig 4). The toxin will be discussed in detail in Chapter VI.

Samples from the preliminary and build-up stages grew well when plated on A_1 Filtered Potato agar plates. There was an overall increase in cell numbers from $1,5 \times 10^5$ viable spores to $6,0 \times 10^7$ cells immediately prior to inoculation of the final fermentation medium (Fig 5). As very poor growth from the final fermentation stage occurred on all plates investigated, reliable bacterial counts were not obtained for this stage. The viable counts obtained were at least 100 fold lower than the corresponding haemocytometer counts. Improved growth was not obtained when modifications of the A_1 Potato medium, Control Fermenter medium or selective clostridial media were used.

3.33 Viable counts at the end of the A_1 stage and solvent yields

In general there was no correlation between the viable counts at the end of the A_1 stage and solvent yields (Table 5). It appears that viable counts of $6,0 \times 10^5$ ml^{-1} were required for solvent production. The highest solvent yield (16,0 $g\ell^{-1}$) was obtained with a viable count of $1,4 \times 10^6$ ml^{-1} at the end of the A_1 stage.

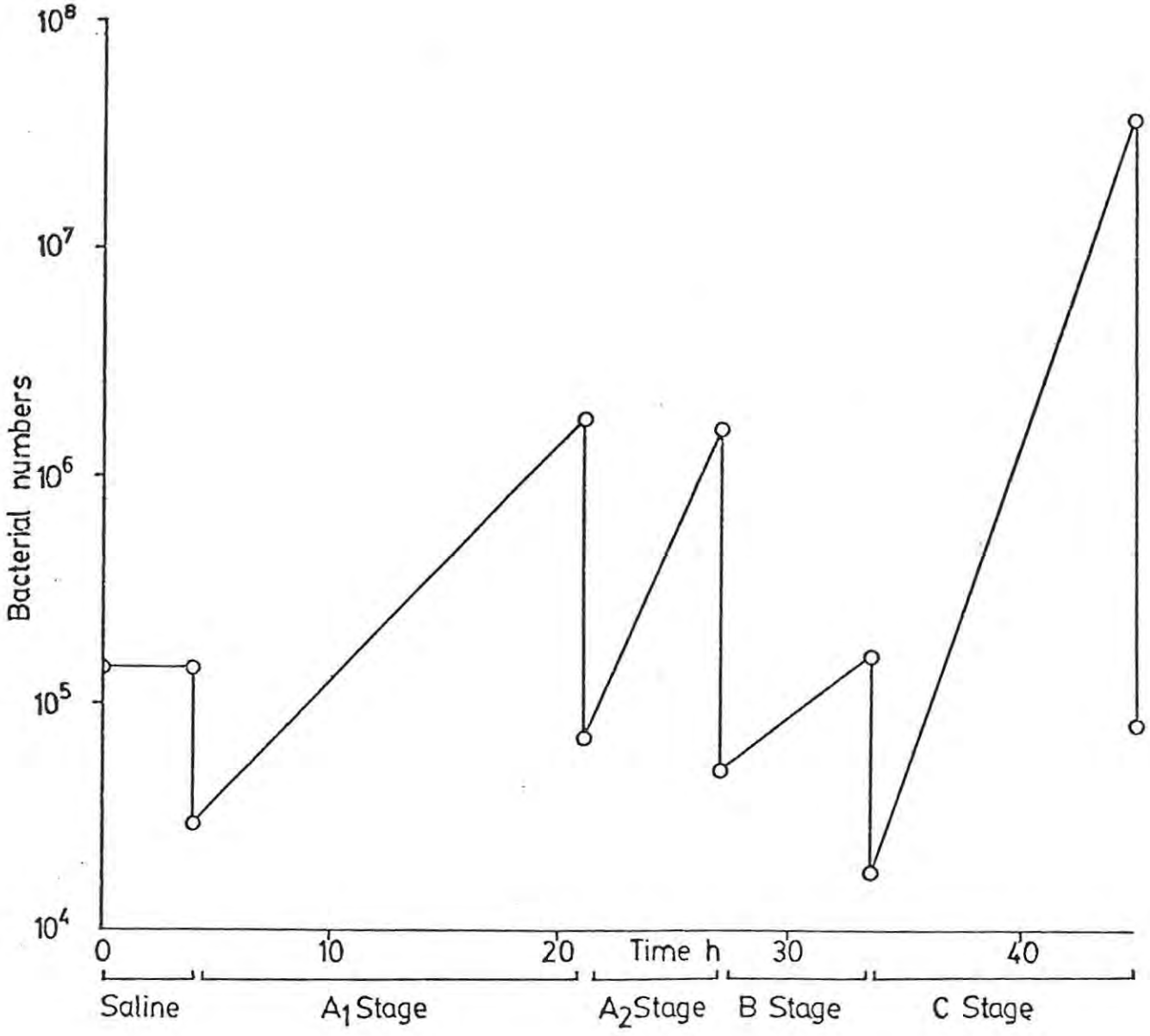


Fig 5 Bacterial growth during the A_1; A_2; B and C stages of a C. acetobutylicum fermentation.

Table 5 Viable count at the end of the A_1 stage and final solvent yields

Origin of cultures	Bacterial numbers ml^{-1} at the end of the A_1 stage	Solvent yield $g\ell^{-1}$
Spore to soil ratio (clone) 1:10	$1,5 \times 10^3$	-[x]
Spore to soil ratio (clone) 1:10	$7,5 \times 10^3$	-
Soil to spore ratio (clone) 5:10	$3,0 \times 10^5$	-
Clone C sporulated	$3,0 \times 10^5$	-
Clone A sporulated	$6,0 \times 10^5$	9,0
Clone A sporulated	$8,6 \times 10^5$	9,5
Spore to soil ratio (HSP262) 1:10	$1,0 \times 10^6$	4,0
Spore to soil ratio (HSP262) 5:10	$1,4 \times 10^6$	16,0
Mixed plate culture	$1,8 \times 10^6$	-
Clone B sporulated	$2,7 \times 10^6$	13,7
Clone B sporulated	$3,3 \times 10^6$	-
Clone C	$6,8 \times 10^6$	11,7
HSP262	$6,9 \times 10^6$	-
Clone C sporulated	$7,0 \times 10^6$	11,1
Clone C	$7,4 \times 10^6$	12,6
Clone A	$2,6 \times 10^7$	5,0
Clone C	$2,7 \times 10^7$	11,9
Clone B	$5,4 \times 10^7$	9,0
Spore to soil ratio (HSP262) 3:10	$3,0 \times 10^8$	2,0
Spore to soil ratio (HSP262) 4:10	$3,7 \times 10^8$	4,7

Table 5 cont.

Origin of cultures	Bacterial numbers ml^{-1} at the end of the A_1 stage	Solvent yield $g\ell^{-1}$
Spore to soil ratio (HSP262) 5:10	$6,8 \times 10^8$	9,6
Spore to soil ratio (HSP262) 10:10	$1,0 \times 10^9$	11,2

X (-) Fermentations abandoned due to poor growth in the C stage; (HSP262), a heat shocked stock soil culture; (Spore to soil ratio), the amount of a sporulated culture (ml) originating from either a clone (clone) or the stock soil culture (HSP262) added to 10g of sterile soil. Details of the isolation and sporulation of clones are outlined in Chapter IV.

3.4 Discussion

The direct injection and distillation methods for GC analysis of solvent production were comparable. Distillation of many small samples used for mutant screening would be impractical, and as the direct injection method wasfar easier, all subsequent solvent determinations were carried out by this method.

The solvent yields obtained in the laboratory corresponded with those produced at the N.C.P. factory. N.C.P. produce solvents in the ratio of 6:3:1; n-butanol; acetone; ethanol, while the laboratory fermentation gave approximately the same ratios of butanol: acetone but less ethanol. As the ethanol is not required by N.C.P. no attempts were made to improve ethanol production. The bacterial growth curve and the graphs of solvent production were very similar to those obtained by Peterson and Fred (1932) (Prescott and Dunn, 1940) for the fermentation of starch by C. acetobutylicum. Production of acetone was detected after c. 8h as observed by Davis (1942) in a fermentation of glucose by C. acetobutylicum. The pH study of Peterson and Fred did not however show the characteristic increase following the breakpoint demonstrated for the molasses fermentation. The latter was in agreement with the biochemical pathway (Chapter I). Presumably

the production of butyric and acetic acids resulted in the decrease in pH of the medium until the breakpoint was reached. This caused the induction of the enzyme systems responsible for solvent production. Neutral solvent production and the conversion of butyric acid to butanol, resulted in the gradual increase in pH (Doelle, 1975). No solvents were produced prior to the pH breakpoint. During poor solvent producing fermentations the coversion of butyric acid did not occur and the pH continued to decrease. This may be due to poor or no induction of the enzyme system. The reason for this low degree of induction is unknown. The fact that bacterial metabolic processes must have been taking place during the initial 12h is evidenced by the observed decrease in pH, morphology and colour changes. However the turbidity measurements and haemocytometer counts suggest that little growth occurred. This long lag phase, compared to other bacterial systems eg. Bacteroides fragilis (Burt, 1977), could be due to introduction of the bacteria into a non-reduced medium which they must render anaerobic before growth occurs. Adaption from the low molasses concentration found in the build-up stage medium to the high molasses concentration of the final fermentation medium, could also contribute to the long lag phase.

The clostridial forms, associated with solvent production, are sporulation intermediates (Gunsalus and Stanier, 1960). This could be significant for the isolation of high solvent yield mutants. If sporulation mutants could be obtained, as in Bacillus subtilis (Sonenshein and Losick, 1970), which produce clostridial forms but do not sporulate, increased solvent production may well occur.

The drastic decreases in turbidity are due to a bacteriocin-like toxin which appears to be induced as the bacteria reaches the end of exponential growth phase, and may prevent higher yields of solvent from being attained. Solvent production corresponded with the growth cycle of the bacteria until the decrease in turbidity which was accompanied by a sharp levelling off of solvent production. The isolation of bacteria which were resistant to the toxin would probably follow the normal bacterial growth pattern and consequently solvent production would increase correspondingly above present yields, until stationary phase was reached.

As the toxin has also been detected in low yield fermentations, it may be a contributary factor in fermentation failure (eg. early induction). It was for this and other reasons that the toxin was further investigated (Chapter VI).

Cells from the A_1, A_2, B and C stages grew well on A_1 Filtered Potato agar plates, but cells from the final fermentation did not grow well, even after catalase treatment. This indicates that the cells, after transfer to the final fermentation medium, are in a different and delicate physiological state throughout the final fermentation.

The extent of growth at the end of the A_1 stage can not be used as a selective method to predetermine poor solvent producing fermentations.

CHAPTER IV

STUDIES ON THE FERMENTATION PROCESS

4.1 Introduction

Various aspects of the fermentation process were investigated with a view to fully understanding the fermentation and increasing solvent yield. The amount of crude molasses required for a fermentation is dependent upon concentration of total invert sugar (T.I.S.) and is usually between 48-50%, the final fermentation medium containing c. 6,5% T.I.S. The sugar tolerance of C. acetobutylicum on agar plates and in liquid medium was determined. In addition, the effect of increasing T.I.S. concentration on solvent production was investigated to determine the concentration at which the most efficient conversion of T.I.S. to solvents occurred.

The fermentation process at N.C.P. is lengthy and, despite the scaling down of volumes for laboratory use (Chapter III), the final fermentation size was still impractical for mutant screening assays. Attempts were made to shorten the fermentation process by omitting some of the build-up stages and to decrease the size of the final fermentation to a volume suitable for large scale mutant selection.

Heat shocking of well sporulated cultures on soil is usually associated by N.C.P. with high solvent production. Experiments were therefore carried out to ensure efficient sporulation of laboratory cultures. Fermentations at N.C.P. involve heat shocking of mixed spore cultures of C. acetobutylicum. However a mutant screening technique is based on the selection of clones and it is essential that experiments are carried out to show that pure colonies can produce normal solvent yields. Solvent production was determined from both mixed cultures and isolated clones before and after sporulation.

The limiting step in the conversion of precursors to solvents is unknown, and the addition to normal fermentations of either more butyric acid, acetoacetate or the enzymes responsible for the conversion of these precursors could result in increased solvent yields eg. poor fermentations (Chapter III) may result in increased butyric acid as the pH of the medium drops lower than normal. The effect of mixing spore cultures producing poor fermentations with normal solvent producing spores was carried out to determine if higher solvent production occurred.

4.2 Materials and methods

4.21 Media

All the basic media are listed in Appendix B. The A_1 Filtered Potato agar plates were used for all bacterial assays unless stated otherwise.

4.22 General methods

The fermentation methods and solvent production analysis outlined in Chapter III were followed unless stated otherwise.

4.23 Variation of the sugar concentration in the final fermentation medium

The sugar tolerance of C. acetobutylicum was determined by monitoring bacterial growth on agar plates or in liquid medium. The agar plates were supplemented with increasing concentrations of glucose and the liquid medium with increasing concentrations of molasses (T. I.S.). The effect of increasing the initial concentration of T.I.S. on solvent production was also investigated. The T.I.S. percentage was determined by N.C.P. using the modified Lane and Eynon method (Mann and Saunders, 1967).

4.24 Variations of the fermentation process

The A_2, B and C stages of the fermentation process serve to build up an inoculum for the 90 000ℓ final fermentation stage. As these did not appear necessary for laboratory size fermentations experiments were carried out omitting the B and C stages and inoculating the final fermentation stage with an actively growing A_2 culture.

The effect on solvent production of decreasing the volume of the final fermentation to 250ml and 20ml was investigated. The different volumes of the Control Fermenter medium were inoculated from the same C stage culture.

To determine the effect of agar plate substitution during the fermentation, the A_1 Potato stage was replaced by an A_1 Filtered Potato agar plate.

4.25 Spore formation

N.C.P. method

A standard soil culture was heat shocked and, following germination, inoculated into A_1 stage medium. This was incubated for at least 48h to allow sporulation. Cultures (4,0ml) showing good sporulation were added to 12,0g of sterile soil. The cultures were kept at 50°C to dry for at least 2 days before heat shocking.

Cooked meat medium

A cooked meat medium recommended to enhance spore formation (Holdeman and Moore, 1972) was tried.

Preparation of sterile soil

Absorbent soil, that was rich in humus and allowed evaporation was selected. The soil was dampened, sterilized twice, then sieved (mesh size 14) while still damp. This was sterilized three times at 24 hourly intervals by placing it in a hot air oven (160°C) for 8h prior to adding c. 12g to a test-tube. A final sterilization in hot air was carried out before storing.

4.26 Fermentations using isolated colonies

A soil sample was heat shocked, grown to the end of the A_1 stage when 0,1ml was plated. Cultures were used after 18h incubation for mixed clone fermentations. Isolated clones were obtained by streaking mixed cultures. Clones were selected and restreaked to obtain a sufficiently high concentration of bacteria for direct inoculation. Both Control Fermenter agar mediumand A_1 Filtered Potato agar plates were used. The normal inoculation was large (c. 3 large loopfuls) as less than this resulted in little or no growth. The effect of increasing the inoculum size on solvent production was investigated. Inoculations from agar plates were transferred directly into duplicate A_1 stage cultures; one

was used for fermentation and solvent determination, the other for the formation of spores prior to fermentation , to determine the effect of sporulation on solvent production.

Mixed spore fermentation

The spores of poor solvent producing clones (8,1g ℓ^{-1}) or higher solvent producing clones (13,7g ℓ^{-1}) were mixed with stock culture spores, prior to heat shocking and fermentation, to determine whether solvent production could be increased.

4.3 Results

4.31 The effect of the final sugar concentration on solvent production

The results of increasing the percentage T.I.S. in the final fermentation stage are shown in Fig 6. There was an increase in yield up to 7,8% T.I.S. after which solvent production began to decrease. However, at 9,5% T.I.S. the yield was still greater than normally achieved. The percentage conversion of T.I.S. to solvents was used as a measure of the efficiency of the fermentation and at N.C.P. is usually 28-30%. In the laboratory a value of 26-27% was obtained. This low value is possibly due to the decreased volumes used.

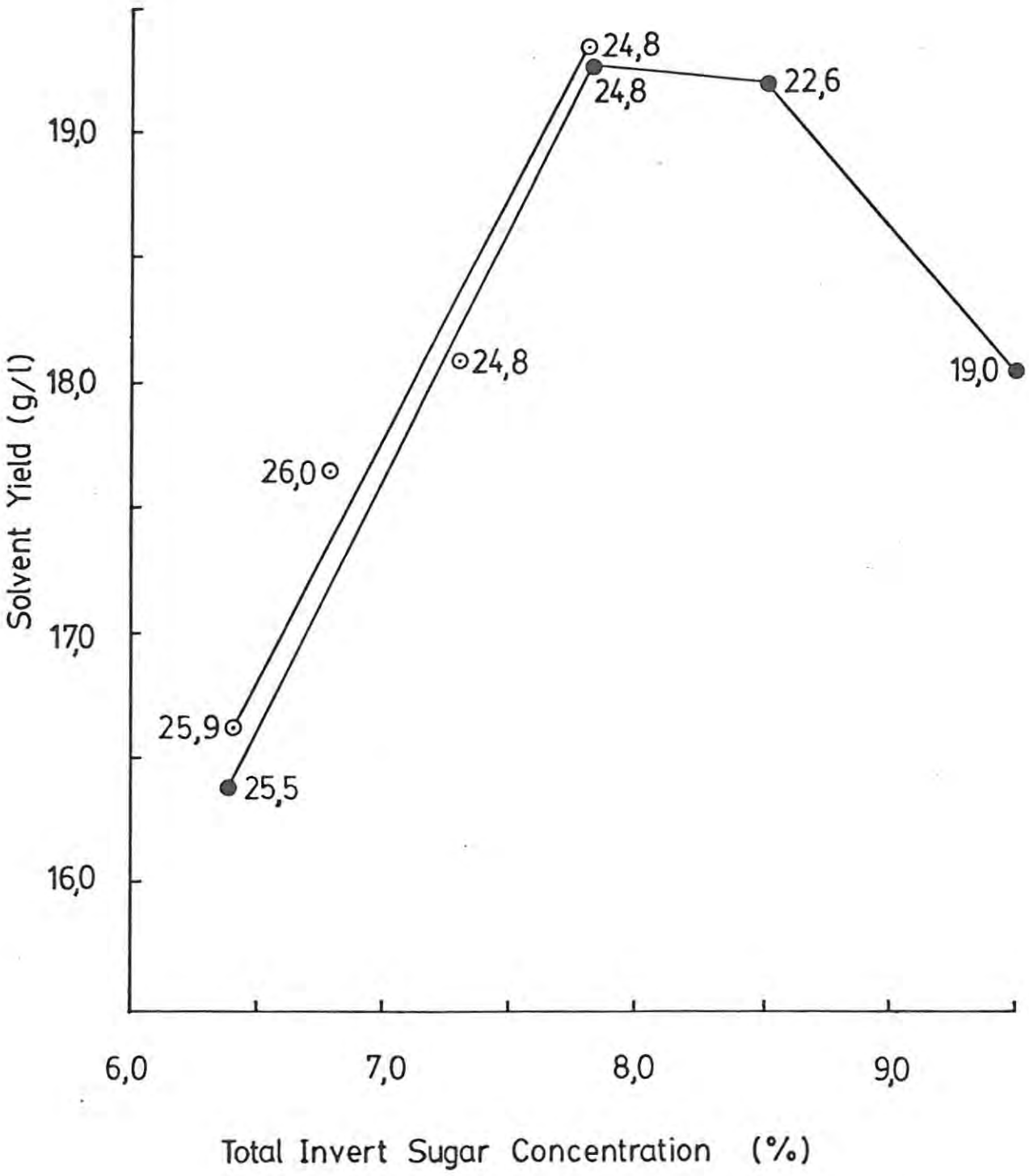


Fig 6. Changes in solvent production and percentage conversion with increasing total invert sugar concentration. ⊙—⊙, Initial experiment, ●—●, Second experiment.

Percentage conversion was calculated as follows:

$$\% \text{ Conversion} = \frac{\text{Solvent yield (\%)}}{\text{T.I.S. (\%)}} \times \frac{100}{1}$$

The values obtained (Fig 6) showed a slight decrease in efficiency up to 7,8% T.I.S. which was followed by a marked decrease at higher concentrations. Good bacterial growth occurred in the liquid medium at all T.I.S. concentrations except 9,5% while, as expected (Chapter III), little growth occurred on agar plates at the normal sugar concentrations and no growth occurred at any increased sugar concentrations.

4.32 The effect on solvent production of omitting stages and decreasing the volume of the final fermentation

Omitting two of the build-up stages of the fermentation caused a decrease in solvent production and normal levels of solvents were not obtained unless all the build-up stages were included. Substituting an agar plate for the A_1 stage resulted in no growth in the A_2 stage.

Decreasing the volume of the final fermentation caused a corresponding decrease in solvent production. Typical results (Table 6) indicate that an average yield of 4,7g ℓ^{-1} was obtained with 20,0ml cultures.

Table 6 The effect on solvent production of decreasing the size of the final fermentation

Fermentation	Yield g ℓ^{-1}
Control (1,3ℓ)	11,9
Sample No. (20,0ml)	
1	5,3
2	5,5
3	4,1
4	3,1
5	5,1
6	4,2
7	5,8
8	4,6
9	4,3

4.33 Methods of sporulation

Spore formation was induced in a freshly germinated soil culture using the N.C.P. method. Fermentation after heat shocking of these spores resulted in 16,0g ℓ^{-1} solvents being produced.

Very poor sporulation and subsequent growth was obtained using the cooked meat medium.

4.34 Solvent production from isolated clones

The production of solvents by isolated clones before and after sporulation are shown in Table 7. Mixed plate cultures or clones did not give normal solvent yields. Great variation in solvent yield occurred between colonies isolated on the same plate. Similar or higher solvent yields were obtained after the colonies were allowed to sporulate. The highest yield and percentage conversion obtained from a sporulated clone was 13,7g ℓ^{-1} and 21,7% respectively. Increasing the inoculum size from plates had no effect on solvent production.

There was very poor growth on Control Fermenter medium agar plates and when these clones were used in fermentations no solvent production occurred.

Table 7 Solvent production by isolated colonies before and after sporulation

Origin of Culture	Solvent yield g ℓ^{-1} Plate inoculum	%Conversion	Solvent yield g ℓ^{-1} Spores	%Conversion
Control	-	-	16,0	(23,3)
Mixed Culture	6,0	(9,3)	9,2	(14,3)
Clone A	5,0	(7,8)	9,0	(14,0)
Clone B	9,1	(14,2)	13,7	(21,7)
Clone C	11,8	(18,4)	11,4	(17,7)
Clone D	9,5	(14,8)	-	-
Clone E	4,5	(7,0)	-	-
Clone F	10,7	(16,6)	10,1	(15,7)

4.35 The effect of mixed spore cultures on solvent production

No significant effect was found when stock soil cultures were mixed with spores from poor solvent producers.

4.4 Discussion

The increase in solvent production obtained with increasing T.I.S. suggests that mutants with an increase in solvent production could be selected for at a T.I.S. concentration of 6,4%, and fermented normally at c. 7,8% T.I.S. This would provide additional yield with very little decrease in the new levels of conversion. The decrease in solvent production at very high concentrations of T.I.S. (9,5%) may be due to the inhibitory effect on solvent production of the assimilable nitrogenous substances found by Ierusalimskiĭ and Semenova (1944).

The build-up stages could not be omitted from the fermentation without adversely affecting solvent production. These results agree with those of Beesch (1953) who, using C. acetobutylicum in a corn mash fermentation, determined the effect of varying the number of build-up stages (from 2 to 12) on solvent production. There was a decrease in the solvent production with decreasing numbers of transfers.

The low solvent production that occurred in small volume fermentations should not affect the mutant selection programme as, in most cases, the yields were fairly consistent and could be improved by scaling up. The volume to flask ratio, and consequently the degree of aeration, may cause this adverse effect. Small volume fermentations could be used as a preliminary selection for higher solvent producing bacteria prior to scaling up.

An A_1 agar plate stage could not be substituted for the A_1 Potato stage.

Although there is no correlation between bacterial numbers in the A_1 stage and solvent production (Chapter III), good growth in this stage appears essential for the continuation of an actively growing culture through the build-up stages. Agar plate cultivation does not appear to provide sufficient actively growing cells for this purpose. As there was a trend indicating that similar or higher solvent yields were produced after sporulation, it seems essential when working with pure clones to allow them to sporulate before testing their ability to produce solvents. Mixing spore cultures does not appear to increase solvent yield.

CHAPTER V

BACTERIOPHAGES AFFECTING THE FERMENTATION PROCESS

5.1 Introduction

Bacterial viruses were independently discovered by Twart 1915 and in 1917 by d'Herelle who conferred on them the name bacteriophages "eaters of bacteria" (Stent, 1965). The first example of phage infecting a bacterial industrial process was the fermentation of C. acetobutylicum used in the production of acetone and n-butanol during the first World War (1914-1918) (Douglas, 1975). As phages were so poorly understood during this period one factory became infected to the extent that production was no longer economic. The factory was abandoned and another erected many miles away (Douglas, 1975). McCoy et al. (1944) reported a phage outbreak while testing a new fermentation process. The factory had the unusual feature of the appearance of four phage strains in little over a year during which a single species of butyl Clostridium was used. Several other bacterial dependent industries are affected by phage eg. the lactic streptococci used in cheesemaking, certain antibiotic producing actinomycetes become infected with actinophages and, several cases of viruses affecting the baking and brewing industries have been reported (Douglas, 1975).

The infection of C. acetobutylicum with phage, which usually became evident at c. 18h after inoculation (Beesch, 1953), led to the death of most of the bacteria within a few hours. The culture usually exhibited a marked decrease in the amount of gas produced, the molasses medium often appeared darker than usual and the acidity rose further than normal then remained constant.

The phage were isolated from corn mash by adjusting the pH to c. 7,0 and filtering. The phage were found to retain their virulence for an indefinite period at room temperature and could be kept for many years sealed at reduced temperatures but were inactivated by a five minute exposure to steam (Beesch, 1953). Several methods of immunization against phage were used. Resistant cultures were obtained by serial transfer through media containing phage (Patented by Legg, 1928 and Legg and Walton, 1938 in Beesch, 1953) or by placing a non-immune culture on a plot of open, unsterile soil and re-isolating the culture six months later (Patented by Hanson, 1937 in Beesch, 1953). McCoy in 1946 (Rose, 1961) devised a method of culturing the bacterium in a specific medium containing phage; 3 to 5 transfers being required to produce a resistant culture. This method also had the effect of yielding strains producing a higher proportion of acetone. Phage attacks on C. acetobutylicum were strain specific so infected fermentations were re-inoculated with another strain or an

immune strain of the same bacterium (Rose, 1961). Beesch (1953) found that any culture which had been properly immunized lost some of its solvent producing activity. The fermentation was slightly slower and a lower concentration of mash was fermented. However, the slower, less efficient fermentations of the phage resistant bacteria were usually overlooked, as once the phage had infected a sensitive culture it spread rapidly and either an immune strain, or a complete change of raw material and culture was required. Beesch (1953) determined that the addition $1,5 \times 10^{-9}$ of the phage still affected the culture.

The first template phage of C. acetobutylicum was isolated by Nippon et al. in 1955 (see Hongo et al., 1968).

The N.C.P. factory experienced a severe phage infection in June, 1975 which resulted in a two week closure for re-sterilization. The following year an investigation was undertaken to determine the source of phage infection and to produce resistant bacteria. Samples were taken from the factory environment to ascertain where the infection had originated. The phages isolated were examined under the Electron Microscope and compared with the phage present during the factory infection. Experiments were also performed to determine if the bacteria carried a prophage, induction of which could result in lysis of the culture.

5.2 Materials and methods

5.21 Media

All general media are listed in Appendix B. All agar plates and sloppy agar media for overlays were A_1 Filtered Potato medium. All plate cultures were incubated for 18-24h at 34°C.

5.22 Isolation of phage

Samples taken from the factory and the environment were stored at 4°C until required. Solid and freeze-dried samples were suspended in a small volume of T_2 buffer before use. Local sewage was also tested for phage. Samples (10µl) were spotted onto bacterial overlays seeded with C. acetobutylicum. The original culture obtained from the United States which was reportedly immune to some phages was also tested for phage sensitivity. Areas of lysis were tested for serial transfer (Adams, 1959).

5.23 Purification and Electron Microscopy

Phages were cloned by repeatedly stabbing and plating (Adams, 1959). High titre preparations were obtained using the double agar layer method outlined by Adams (1959). These cultures were purified by differential centrifugation. The phages were resuspended

in 1,0ml T_2 buffer, negatively stained with 2,0% phosphotungstic acid and examined in a Hitachi Electron Microscope.

The stability of the purified high titre preparations was determined on phage 8 after 2 and 4 weeks and on both phages 8 and 9 after one year.

5.24 Isolation of resistant strains of bacteria

The method outlined by Adams (1959) was followed. The high titre phage was diluted and plated. Colonies present within plaques were selected and tested for phage resistance. Cross-resistance studies between the different phage isolates were also carried out.

5.25 Induction of lysogens

The presence of a prophage causes resistance to related phages. Suspected lysogens of parent and resistant cultures were induced with Ultraviolet radiation (uv), mitomycin C (5µg ml^{-1}) or N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (3 or 5µg ml^{-1}). UV radiation was carried out using a Hanovia uv lamp according to the method outlined by Thomson (1973). Following radiation, cultures were grown in either A_2 Molasses or A_1 Filtered Potato medium and tested for the presence of phage. The method of Thomson (1973) was followed for the mitomycin C and NTG studies.

5.3 Results

5.31 Phage isolation

Of the 22 factory samples tested, only two samples, taken from the liquid and solid sewers between fermenters 5 and 8, contained phage (Table 8). These were designated phages 8 and 9. Seven other samples contained a lytic agent but did not show serial transfer and could have been bacteriocins. The freeze-dried sample contained phage. No phage were obtained from the sewage samples.

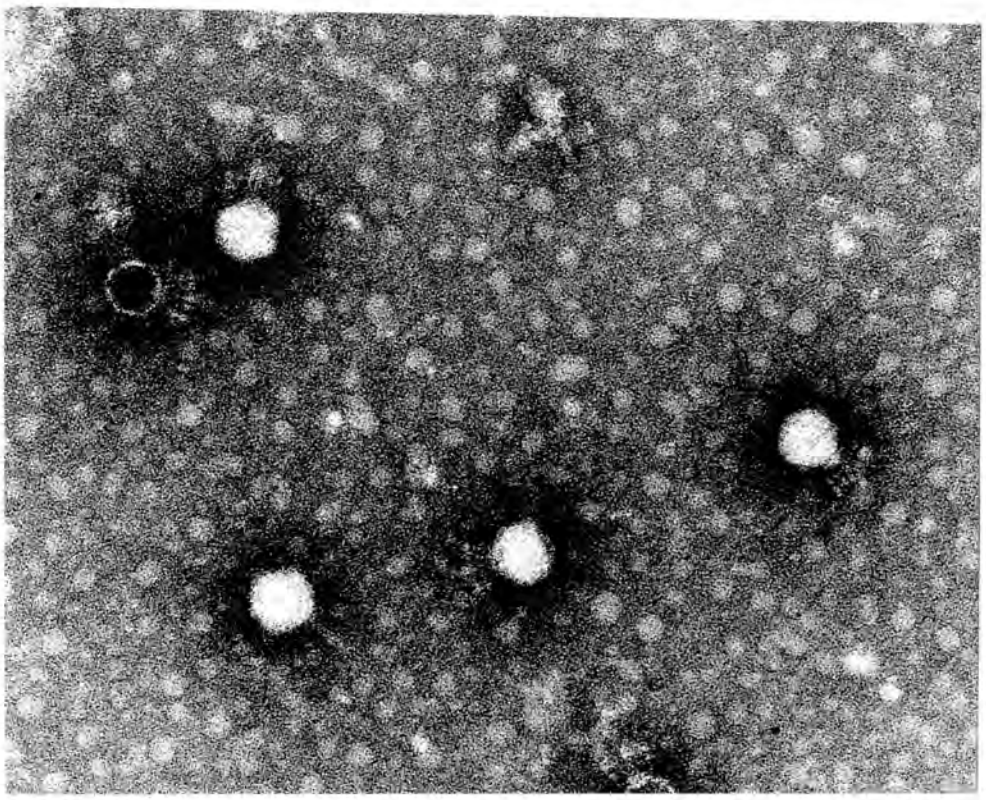
5.32 Purification and Electron Micrographs of phages

Purified high titre lysates containing $1,6 \times 10^{10}$ and $2,3 \times 10^{10}$ p.f.u. of phage 8 and 9 respectively were examined in the Electron Microscope (Plate 3). The two phages had the same morphology but were markedly different from the long tailed phage found during the factory outbreak. However, purified high titre lysates from the freeze-dried samples prepared during the phage outbreak at N.C.P. only contained a phage with the same morphology as phages 8 and 9.

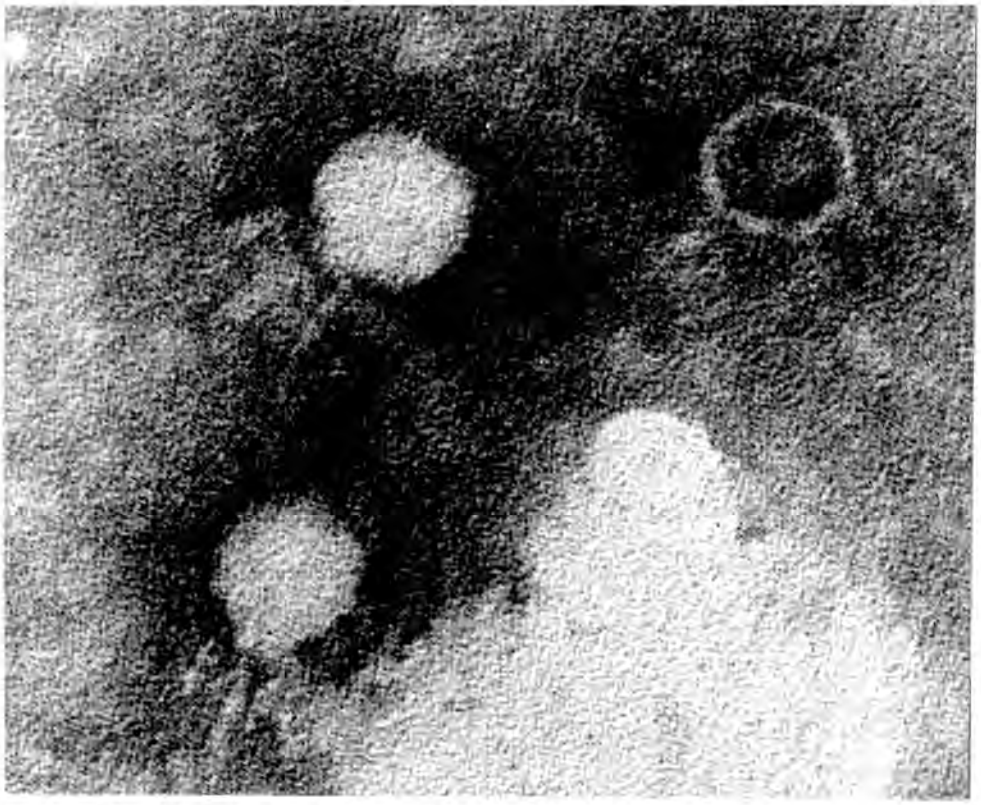
After two weeks the titre of phage 8 had decreased from $1,6 \times 10^{10}$ p.f.u. to $1,0 \times 10^{8}$ p.f.u. This phage titre remained constant after four weeks and at the end of one year both phages 8 and 9 were still viable.

Table 8 Presence of phage in N.C.P. factory soil and water samples

Sample No.	Sample Location	Lytic areas	Serial transfer
1	Butyl receiver tanks, adjacent to No 1 works laboratory	-	
2	Drain adjacent to butyl receiver tank	-	
3	Drain underneath CO_2 scrubber tank	-	
4	Condensate drain CO_2 lines next to scrubber	-	
5	Drain flowing into railway siding from fermentation house	-	
6	Pillars underneath No 6 fermenter	+	-
7	Wall scraping near fermenter No 7 (bottom)	-	
8	Liquid sewer between fermenters 5 and 8	+	+
9	Solid sewer between fermenters 5 and 8	+	+
10	Righthand side pillar (bottom) at fermenter No 9	-	
11	Righthand pillar, middle pipe line, (steam casing)	-	
12	Sampling bucket	+	-
13	Shoe swab after walking on floor of factory	+	-
14	Inside molasses stump (storage)	-	
15	Cooling tower (north)	-	
16	Rumavite D.C.P. No 2 spray dry	+	-
17	Condensate water	-	
18	Steep water	+	-
19	Molasses before use	+	-
20	Culture laboratory floor	-	
21	No 5 fermenter, during filling	+	-
22	Inoculating laboratory	-	



Magnification x 178000



Magnification x 450000

Plate 3 Electron micrographs (negatively stained with 2% phosphotungstic acid) showing phage isolate.

5.33 Isolation of phage resistant bacteria

Ten phage resistant colonies of C. acetobutylicum were isolated. All ten colonies were resistant to both phages as well as the phage isolated from the freeze-dried sample.

5.34 Induction of possible lysogens

No phages were produced following induction of the parent culture and resistant colonies by uv; mitomycin C and NTG.

5.4 Discussion

The Electron Micrographs and cross-resistance studies indicated that phages 8,9 and the freeze-dried isolate were all identical. Although this phage was not observed in the Electron Micrographs taken during the phage outbreak, possibly because it was present in too low a concentration to be detected, it must have been present to be in the freeze-dried sample. The long tailed phage was probably unstable and no longer viable. Phage sensitivity could be used as a routine identification tool to avoid contamination. Once the fermentation and mutation studies are complete, clones resistant to this and other phages will be obtained before handing over to the N.C.P. factory. The strain of C. acetobutylicum used in these studies does not contain a lysogen.

CHAPTER VI

STUDIES ON THE NATURE OF THE BACTERIOCIN-LIKE TOXIN PRODUCED IN *Clostridium acetobutylicum* FERMENTATIONS

6.1 Introduction

Bacteriocins are bacteriocidal substances, protein in composition, which are synthesised by certain strains of bacteria and are active against some other strains of the same or closely related species. Generally the producer strain is immune to the bacteriocin it produces (Stent, 1965).

Bacteriocins were first observed by Gratia, in 1925, who found the supernatant of *Escherichia coli* strain V was active against *E. coli* ϕ. Subsequently bacteriocins were found in many strains of *E. coli*, known collectively as colicins, other *Enterobacteriaceae* and Gram-negative and Gram-positive bacteria (Reeves, 1972).

The ability to produce bacteriocins is a hereditary characteristic of the cell (transferable in some instances) determined by cytoplasmic genes, or plasmids which are known as colicinogenic factors (Nomura, 1967). Colicins are usually classified by the scheme devised by Frédéricq according to the specificity of their adsorption to receptors on the cell wall and, are

further classified into subgroups according to the specificity of their immunity (Nomura, 1967).

Much work has been done on the colicins and specifically on the mode of action i.e. Colicins A, E_1, E_2, E_3, Ia, Ib and K, and these are usually used as models for other bacteriocins that are found. The mode of action generally follows two stages. Firstly, the physical adsorption of the bacteriocin molecule to the specific receptor where no permanent physical damage is caused. In some cases the addition of trypsin can reverse the action of the bacteriocin. Secondly, the irreversible pathological changes which are effected via specific biochemical lesions (Nomura, 1967).

The site of action of colicins E_1 and K is the energized membrane. It is proposed that colicin K de-energizes the cytoplasmic membrane and, the decrease of intracellular ATP found is due to the utilization of the ATP by the Ca^{++}, Mg^{++}- ATPase which is used to re-energize the membrane. This is accompanied by an increased permeability to Mg^{++}, Co^{++} and K^{+}. Colicin K has the secondary effect of inhibiting nucleic acid and protein synthesis (Hardy, 1975). Col A, Ia and Ib have been found to have a similar action to Col K and E_1. They affect macromolecular synthesis, cell permeability and transport systems (Reeves, 1972).

Colicins E_2 and E_3 appear to have evolved from the same ancestor but although similar in their action, in that they affect nucleic acid and subsequently protein synthesis, they have markedly different effects on sensitive cells (Hardy, 1975).

E_3 affects rRNA and consequently protein synthesis *in vivo* and *in vitro*. Action on ribosomes *in vitro* suggests that E_3, or part of it, enters the cell and brings about cleavage of a fragment 50 nucleotides from the 3' end of the 16s rRNA. It does not appear to activate the ribonuclease attached to the ribosome. E_2 on the other hand causes degradation of DNA and also inhibits cell division. The DNA appears to be degraded by the combined action of endonucleases which cause single and then double-stranded breaks, followed by an exonuclease attack. Schaller and Nomura (1976) purified E_2 free of its immunity protein, and established that E_2 has DNA endonuclease activity and that the immunity protein when attached specifically prevents this activity.

Amongst the Gram-positive bacteria relatively few of the "antagonistic substances" found closely fit the classical colicin model (Tagg *et al*, 1976). The antibiotics, analogous to the bacteriocins, produced by *Staphylococcus* spp.(Staphylococcins) have a wide variety of activity spectra against both Gram-positive

and Gram-negative bacteria. Gagliana and Hinsdill (1970) characterized a bacteriocin produced by Staphylococcus aureus. It was found to inhibit a variety of Gram-positive but not Gram-negative bacteria. Dajani and Wannamaker (1969) isolated a bacteriocidal substance in supernatant fluids of S. aureus phage type 71 that was active against β-Hemolytic Streptococci, Pneumococci and Cornyebacterium. An antibiotic produced by Bacillus cereus (cerecins) is active on other Bacillus strains and Nomura and Hosoda in 1956 (Reeves, 1972) found an autolysin specific for some strains of Bacillus subtilis and Bacillus megatarium. This substance may resemble some other bacteriocins of Gram-positive bacteria which seem to be lytic enzymes. Several species of the genus Clostridium have been shown to produce bacteriocins and are termed clostocins, clostridiocins or individual species designations e g. boticins, butyricins or either perfrinogens or welchicins (Tagg et al., 1976). One of the first reports of a Clostridium producing a bacteriocin-like toxin was that of C. perfringens by Smith in 1959 (Tagg et al., 1976). Subsequently bacteriocin production by strains of C. botulinum and related non-toxigenic strains, C. sporogenes (Reeves, 1972), C. butyricum (Clarke et al., 1975), Clostridium septicum (Schallehn and Krämer, 1976) and various non-pathogenic clostridia have been reported (Hongo et al., 1968).

The most extensively studied are the bacteriocins of C. botulinum and C. perfringens. Strains of C. botulinum belonging to toxin types A,B,C and E produce a variety of bacteriocins or bacteriocin-like substances unrelated to the type of toxin or the ability to produce toxin. Bacteriocin-like substances from strains B and E, and a non-toxic C. botulinum were shown to have structures similar to phage tails (Tagg et al., 1976). Kautter et al. in 1966 (L'au et al., 1974) described the presence of a bacteriocin produced by a non-toxic C. botulinum type E and called it boticin E. Anastasio et al. (1971) studied the properties of boticin E and found that it was active against all type E and type E related cultures, while Ellison and Kautter (1970) purified the bacteriocin. They found it was composed of two boticins and not one as originally thought. They isolated a highly active low molecular weight particle (5 000-30 000) and a higher molecular weight less active particle (4×10^7). The boticins were initially detected during exponential growth phase and the highest titres were observed in early stationary phase. They were very unstable in cell-free extracts, the instability being greatest at pH 8 at 40°C. The two boticins resembled the Staphylococcins described by Gagliana and Hinsdell (1970) and the bacteriocin-like substance produced by S. aureus phage type 71 (Dajani and Wannamaker,

1970). It was observed that the bacteriocin prevented the outgrowth of type E spores. Ellison et al. (1971) studied the mode of action of the boticins and found it was similar to that of the bacteriocin-like substance produced by S. aureus. Activity involved structural changes which consisted of condensation of nuclear material, partial loss of ribosomes, alteration of mesosomes, abortive cell division and eventual dissolution of cell contents. These effects correlated with RNA degradation and cessation of DNA and protein synthesis. The toxin was not trypsin reversible, but was rapidly inactivated by it, suggesting that either rapid penetration of the cell or rapid production of the lethal lesion occurred. Lau et al. (1974) have purified a second bacteriocin produced by a type E strain resembling the non-toxigenic C. botulinum strain. It was designated boticin P and was found to have a different activity spectrum and properties to that of the boticin E described above.

Initial studies of bacteriocins produced by C. perfringens were undertaken in 1963 by Šašarman and Antoni (Tubylewicz, 1966) who isolated four bacteriocins. Tubylewicz (1966) investigated bacteriocin production in strains of C. perfringens type A and found that, of 35 strains tested, five had bacteriocin activity. They were only active against other C. perfringens strains and not bacterial strains from other

genera. Tubylewicz (1966b) studied the properties of these bacteriocins and found that complete lysis of the culture did not occur. All were thermolabile at 55°C and maintained their activity within pH values from 4 to 10. The perfrinogen tested was completely inactivated by trypsin and papain. Subsequently Tubtylewicz (1968) purified and investigated the chemical nature of four of the bacteriocins. All were found to be proteins. Mahony and Butler (1971) and Mahony, Butler and Lewis (1971) investigated their mode of action and confirmed Tubylewicz's findings with respect to the properties of the bacteriocins. They inhibited cell wall synthesis or removed existing cell walls to form sphaeroplasts after 2h of bacteriocin addition which coincided with the decrease in optical density. The spaeroplast numbers decreased at c. 18h probably due to cell lysis. It was found that when sphaeroplasts induced by bacteriocins were plated onto sucrose containing medium L-forms developed. Viable counts suggested that cell division also ceased in bacteriocin treated cells. They did not however completely block the synthesis of DNA, RNA or proteins. Mahony (1973) compared the antibiotic sensitivity of C. perfringens and the L-forms of C. perfringens induced by the bacteriocin. It was found that there was a loss of penicillin activity in the presence of the bacteriocin which confirms that the bacteriocin acts on the cell wall allowing

L-form growth which is resistant to penicillin.

Nakamura et al. (1977) found that a mitomycin C induced lysate of C. perfringens was lytic against 50 strains of C. perfringens of type A to E, and three strains of Clostridium plagarum. The lysin was active against only two other Clostridium species. It had an optimum pH of 5,5, was thermolabile and partially inactivated by proteolytic enzymes. Due to the species specificity of the lysin it has a possible use in taxonomy, especially as the newly classified C. plagarum has the same properties as C. perfringens. Mahony in 1974 (Nakamura et al., 1977) and Uchiyama (1966) had also demonstrated a bacteriocin-like substance in the culture filtrates of C. perfringens strains, however, these were less species specific and were not induced by mitomycin C.

Hongo et al. (1968) investigated lysogeny and bacteriocin production among non-pathogenic clostridia. Five groups of bacteriocins (or clostocins), designated A,B,C,D and E respectively, were isolated. Subsequently Hongo et al. (1968b) investigated the properties of four of these bacteriocins. Clostocins A and D were inducible with ultraviolet light and were thermostable while clostocins B and C were not uv inducible and were thermolabile. All were pH stable (4-9) and partially inactivated by proteolytic enzymes. Clostocins B and

C inhibited all the species of the genus Clostridium (including C. acetobutylicum) and Bacillus spp.

During the sporulation process in Bacillus spp. several proteases are produced (Dancer and Mandelstam, 1975). Protease production appears to be a common feature of the genus Clostridium as well. Clostridium histolyticum produces a sulphydryl proteolytic enzyme called Clostripain (Ogle, Tytell, 1953; see Mitchell, 1970) while several strains of C. acetobutylicum and C. butyricum have been shown to produce weak proteinase activity with an acid pH optimum (Uchino et al., 1968). Most C. perfringens strains accumulate the exotoxins they produce during exponential growth, however, C. perfingens type A is unique in that it only releases its enterotoxin after lysis of the culture during sporulation (Duncan, 1973). C. botulinum undergoes autolysis (a general characteristic of the clostridia) in late exponential phase and in this way releases its toxin (Bonventre and Kempe, 1960). Exotoxins causing diseases have been found in most other clostridia e.g. C. tetani, C. septicum, Clostridium fallax, Clostridium novyi and Clostridium bifermentans (Davis et al., 1970). Autolysis was first recorded in C. acetobutylicum in 1941 (Manfeifelj, 1941) under conditions which encouraged the production of proteinases that were found in the supernatant. They studied the effects of various culture conditions on autolysis and proteinase activity

and found that proteinases did not appear when the medium contained a high concentration of peptone but did appear if the medium contained protein as the nitrogen source. In the presence of both proteins and peptone the proteinase activity did not appear until after the peptone had been utilized. The accumulation of proteinases by C. acetobutylicum having caseinolytic and fibrinolytic activity in synthetic glucose containing medium was observed by Egorov et al., (1971). The addition of butyric acid inhibited the biosynthesis of fibrinolytic proteinases. Egorov et al. (1972) investigated the effect of the combinations of substances with different degrees of oxidation and reduction as the carbon source on production of the fibrinolytic and caseinolytic proteases. The conditions of production differed for each type of proteinase although in both cases the lowest concentrations of proteinase were obtained in the presence of glucose.

A bacteriocin-like toxin was identified during the fermentation of molasses by C. acetobutylicum (Chapter III). Studies on the nature of the toxin were undertaken and attempts were made to purify it to enable a detailed investigation to be carried out.

6.2 Materials and methods

6.21 Media

All general media used are outlined in Appendix B. All agar plates and sloppy agar media for overlays were A_1 Filtered Potato medium unless stated otherwise.

6.22 General methods

Bacteriocin assay. Double agar layer plates seeded with 0,2ml of a dense suspension of C. acetobutylicum in the top layer were spotted with 10µl aliquots of the serially diluted cell-free sample. Plates were incubated at 34°C for 18-24h. Titres in arbitrary units (AU) were expressed as the reciprocal of the highest dilution which gave a clear zone of inhibition.

The sensitive strain was maintained on agar plates and sub-cultured daily. To obtain good bacterial lawns it was essential that these cultures were used as soon as possible after removing from anaerobic conditions. As the titre of the bacteriocin obtained was influenced by the extent of growth in the background culture, this was standardized by making a standard suspension of bacteria in saline for use in an entire series of assays.

6.23 Production and localization of the Bacteriocin-like toxin

The presence of the bacteriocin-like toxin was initially identified as areas of lysis, caused by the butyl beer on agar plates, could not be propagated by serial transfer.

Toxin production during the build-up and final fermentation stages was monitored by assaying cell-free samples for toxin activity.

The localization experiments outlined by Williams (1976) were carried out on the butyl beer. Extracellular toxin was determined by assaying cell-free extracts of butyl beer. Cell-bound toxin was determined by washing the bacterial pellet in 1M NaCl and assaying the supernatant and, intracellular toxin by sonicating the salt washed pellet for 2 min then sedimenting cell debris by high speed centrifugation and assaying the supernatant for toxin activity.

6.24 Activity spectrum of the Bacteriocin-like toxin

The inhibition spectrum of the toxin on a variety of Gram-positive and Gram-negative bacterial strains, both anaerobic and aerobic was investigated.

6.25 Stability studies

Buffer stability: The following buffers were used for stability studies over 16 days on dialysed

and non-dialysed cell-free samples: 0,01M Tris-Mg salt buffer pH 6,5; T_2 buffer pH 7,6; 0,02M Tris-$CaCl_2$ buffer pH 6,5; 0,05M Tris-Maleic acid buffer pH 6,0; 0,01M Phosphate buffer pH 6,5.

Temperature stability: Cell-free extracts of butyl beer containing toxin were maintained at different temperatures and assayed for toxin activity between 0-24h.

pH stability: pH stability was determined using 0,1M Tris-HCl buffers for pH values 7,5-10,0; 0,1M phosphate buffers for pH values 5,8-7,5 and 0,1M phosphate citrate buffers for pH values 4,0-5,8. Experiments were carried out at 4°C over 30h after which samples were serially diluted in 0,05M Tris-Maleic acid buffer pH 5,0 and assayed.

6.26 Inactivation of the toxin

DNAse, RNAse (at a final concentration of 50µg ml^{-1}), pronase, trypsin, chymotrypsinogen, papain and pepsin (all at a final concentration of 0,1mg ml^{-1}) were tested for their ability to inactivate the toxin.

The following enzyme inhibitors were used at a final concentration of 10^{-3}M: N-ethyl maleimide, p-hydroxy mercurobenzoate, dithiothreitol, ethylene-diamine tetracetic acid, phenanthroline and cysteine hydrochloride.

The effect of chloroform on the toxin was determined by mixing 50% v/v $CHCl_3$ with cell-free butyl beer, standing for 1h, centrifuging to facilitate phase separation and assaying the aqueous phase.

The effects of protein denaturing agents, sodium dodecyl sulphate, phenol and mercaptoethanol were determined at a final concentration of 1,0%.

6.27 Kinetics of Bacteriocin-like toxin lethality

The toxin was added to an exponentially growing culture of bacteria. At intervals aliquots were plated for survivors. The number of killing units (KU) of the toxin was determined from the Poissons distribution $P/P_o = e^{-n}$ (Stent, 1965).

6.28 Partial purification of the toxin

Centrifugation studies: Differential centrifugation studies were carried out on the toxin to give an indication of the size of the particle. A cell-free sample of butyl beer was centrifuged at 6400 g for 10 min, the supernatant centrifuged at 102000 g for 120 min. The procedure was repeated. The pellet and supernatant obtained after each centrifugation were assayed for toxin activity.

Sedimentation coefficient determination: The s value for the bacteriocin-like toxin was determined by

the method of Martin and Ames (1961). Sucrose gradients were prepared by successively layering 1,0 ml each of 5; 7,5; 10; 12,5 and 15% solutions of sucrose in either 0,1M Tris-HCl - 2mM $CaCl_2$ pH 7,6 for the catalase marker or 0,05M Tris-Maleic acid buffer pH 5,0 for the toxin, and allowing overnight diffusion at 4°C. As the reference marker, bovine catalase, and the toxin had different pH optima, they were centrifuged together but on separate gradients. Gradients were layered with 200µg bovine catalase or c. 0,1ml of toxin. The toxin preparation used was that obtained from an ammonium sulphate precitate. The gradients were centrifuged for 12h on a Beckman SW 50 L rotor at 36000 rev. min^{-1}, after which it was fractionated by manually collecting 5-drop fractions. Fractions were assayed for catalase activity as outlined by Martin and Ames (1961) or for lytic activity. From the relative positions of toxin and catalase (11,3 S), an estimate of s for the toxin was calculated according to the relationship:

$$\frac{\text{Distance travelled from meniscus by toxin}}{\text{Distance travelled from meniscus by catalase}} = \frac{s \text{ of toxin}}{s \text{ of catalase}}$$

Ammonium sulphate precipitation: Pilot experiments to determine the percentage saturation of ammonium sulphate required to precipitate the toxin were carried out according to the method of Woods (1966) and Welton (1974). The required amounts of ammonium sulphate

(enzyme grade) for each percentage saturation was determined by the method outlined in Dixon and Webb (1958). The precipitates were allowed to stand for either 4 or 24h before sedimentation by high speed centrifugation. The pellets were resuspended and dialysed overnight against 0,05M Tris-Maleic acid buffer pH 5,0 at 4°C.

Column chromatography: All chromatography was done at 4°C.

Cation exchange chromatography: The method based on that used by Willis et al. (1974) was followed. Carboxy methyl (CM) cellulose (0,1g) was swelled and charged before pouring the slurry into a 1,3 x 10 cm glass column. At least two column volumes of 10mM sodium acetate buffer pH 4,5 were used to condition the column. A cell-free sample of butyl beer (10ml) was adjusted to pH 4,5 and applied to the column. A step wise elution gradient 10ml of each 0,1M; 0,2M; 0,3M; 0,4M; and 0,5M NaCl in 10mM sodium acetate buffer pH 4,5 was used. The first 4ml of each solution was discarded, the rest being collected and assayed for toxin activity and for protein absorbance at 280nm.

Anion exchange chromatography: Diethyl amino-ethyl (DEAE) cellulose (1,0g) was swelled, charged and the slurry poured into a glass column and equilibrated with 10mM sodium acetate buffer at pH 4,5. Sample preparation, layering, elution with 0,1M; 0,2M; 0,3M and

0,4M NaCl in sodium acetate buffer pH 4,5 and 0,2M NaCl in 10mM sodium acetate buffer pH 5,0, and assaying was the same as that outlined for the cation exchange chromatography.

Molecular exclusion chromatography: Sephadex G150 was boiled in 0,05M Tris-Maleic acid buffer pH 5,0 under reflux for 6h before the slurry was poured into a Pharmacia column 1,8 x 30 cm at operating pressure. The column was stabilized overnight by passing through equilibration buffer at 18ml h^{-1}. The void volume was determined with 0,8ml dextran blue 2000 at 18ml h^{-1}. Samples were prepared by ammonium sulphate precipitation of a cell-free sample through a DEAE cellulose precolumn prior to layering 0,6ml onto the sephadex column. Fractions (2,6ml) were collected using a L.K.B. Ultrorac fraction collector. All samples were assayed for toxin activity and the absorbance at 280nm was read on a uv spectrophotometer.

6.3 Results

6.31 Production of the Bacteriocin-like toxin

As the toxin could not be propagated by serial transfer it was identified as a bacteriocin-like toxin. It was produced in the A_1 stage medium after 24h and was found to be present in the A_2, B and C stages if

incubated until the culture was in late log phase. The toxin was initially produced after 10-12h in the final fermentation stage as the bacteria entered exponential growth (Chapter III fig 3 and 4) after which production rapidly increased. The toxin was found in all butyl beer samples assayed although the titre varied considerably between fermentations. Inactivation was not due to the action of organic solvents as the calibration mixture (Appendix A) used for GC assays and, acetic and butyric acids had no effect when tested against the C. acetobutylicum strain. Producer cells were sensitive to the toxin liberated. Toxin localization studies indicated that the majority of the toxin was extracellular (2048 AU ml^{-1}) with some intracellular (512 AU ml^{-1}) and very little cell-bound (32 AU ml^{-1}).

6.32 Inhibition spectrum of the toxin

The effect of the toxin on the different bacterial strains tested are shown in Table 9. The toxin was very strain specific and only one other Clostridium strain tested, C. felsineum, showed sensitivity.

6.33 Stability of the toxin

The toxin was retained by dialysis tubing and the most stable buffer conditions were produced using a dialysed sample maintained in the Tris-Maleic acid buffer.

The results of the general temperature stability study (Table 10) and the kinetics of thermal inactivation (Fig 7) of the toxin indicate that it was thermolabile

Table 9 Spectrum of bacteriocin-like toxin activity

Organism	No. of strains	
	Tested	Sensitive
Aerobes		
Achromobacter A_2	1	0
Escherichia coli	8	0
Serratia marcescens	1	0
Salmonella typhimurium	1	0
Bacillus subtilis	1	0
Anaerobes		
Bacteroides fragilis	1	0
C. acetobutylicum	12	12
C. perfringens	16	0
C. sporogenes	2	0
C. butyricum	1	0
C. felsineum	1	1
C. septicum	1	0
C. fallax	1	0

Table 10 The effect of temperature on bacteriocin-like toxin activity (measured in AU)[X]

Time (h)	Temp°C					
	4,0	20,0	25,0	28,0	33,0	41,0
0	16	16	16	16	16	16
2	16	16	8	8	2	2
5	8	4	0	0	0	0
8	2	2	0	0	0	0
24	2	0	0	0	0	0

Note:[X] All values are low as the experiment was carried out prior to stabilization of the toxin.

Table 11 The effect of pH on bacteriocin-like toxin acivity (measured in AU)

pH	Toxin activity
4,0	16384
4,4	16384
5,0	16384
5,4	8192
5,8	8192
6,1	8192
6,5	4092
6,7	4092
7,1	4092
7,5	4092
8,0	2048
9,0	512
10,0	64

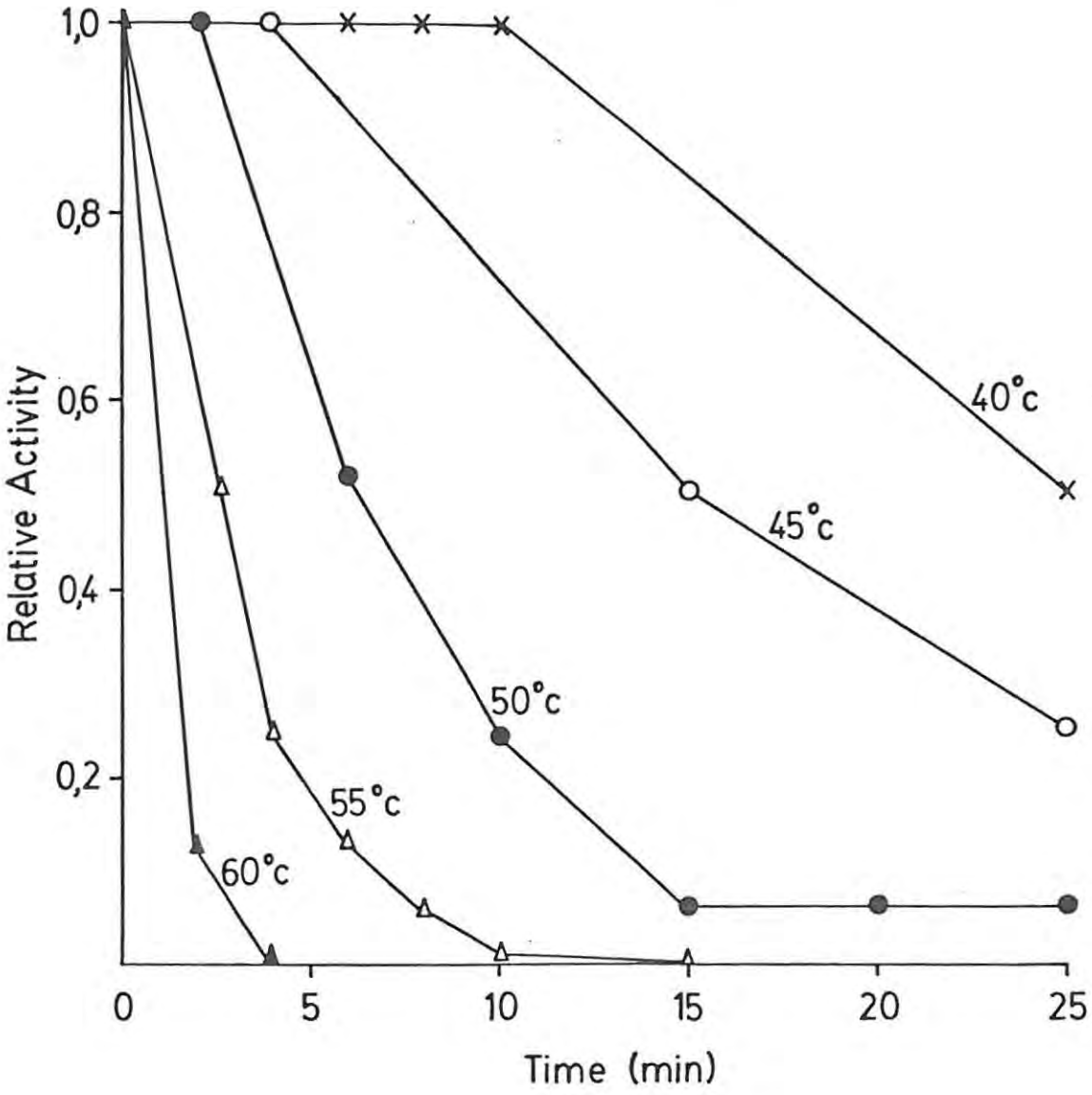


Fig 7 The kinetics of thermal inativation of the bacteriocin-like toxin.

($t_{1/2}$ = 2 min at 60°C). Unless maintained at 4°C all activity was lost. Toxin stored with cells at 4°C lost most of its activity after 2-3 weeks. The bacteriocin-like toxin was more stable in acid conditions pH 4,0-5,0 (Table 11), its activity decreasing with increasing pH.

6.34 Inactivation of the toxin

DNAse, RNAse, pronase, trypsin, chymotrypsinogen papain or pepsin did not inactivate the toxin. It was not inactivated by chloroform or N-ethyl maleimide, p-hydroxy mercurobenzoate, dithiothreitol, ethylenediamine tetracetic acid, phenanthroline or cysteine hydrochloride. It was however inactivated by the protein denaturing agents sodium dodecyl sulphate and phenol but not mercaptoethanol.

6.35 Kinetics of lethality of the toxin

The results of the kinetics of lethality of the toxin are represented in Fig 8. The killing effect did not reach a plateau but continued to decrease. The killing units after 60 min were 6 KU c.f.u.$^{-1}$.

6.36 Partial purification of the Bacteriocin-like toxin

Centrifugation studies suggested the presence of a low molecular weight particle as all the toxin activity remained in the supernatant after centrifuging at 102000 g for 120 min. Negatively stained (2% phosphotungstic acid) electron micrographs of the supernatant

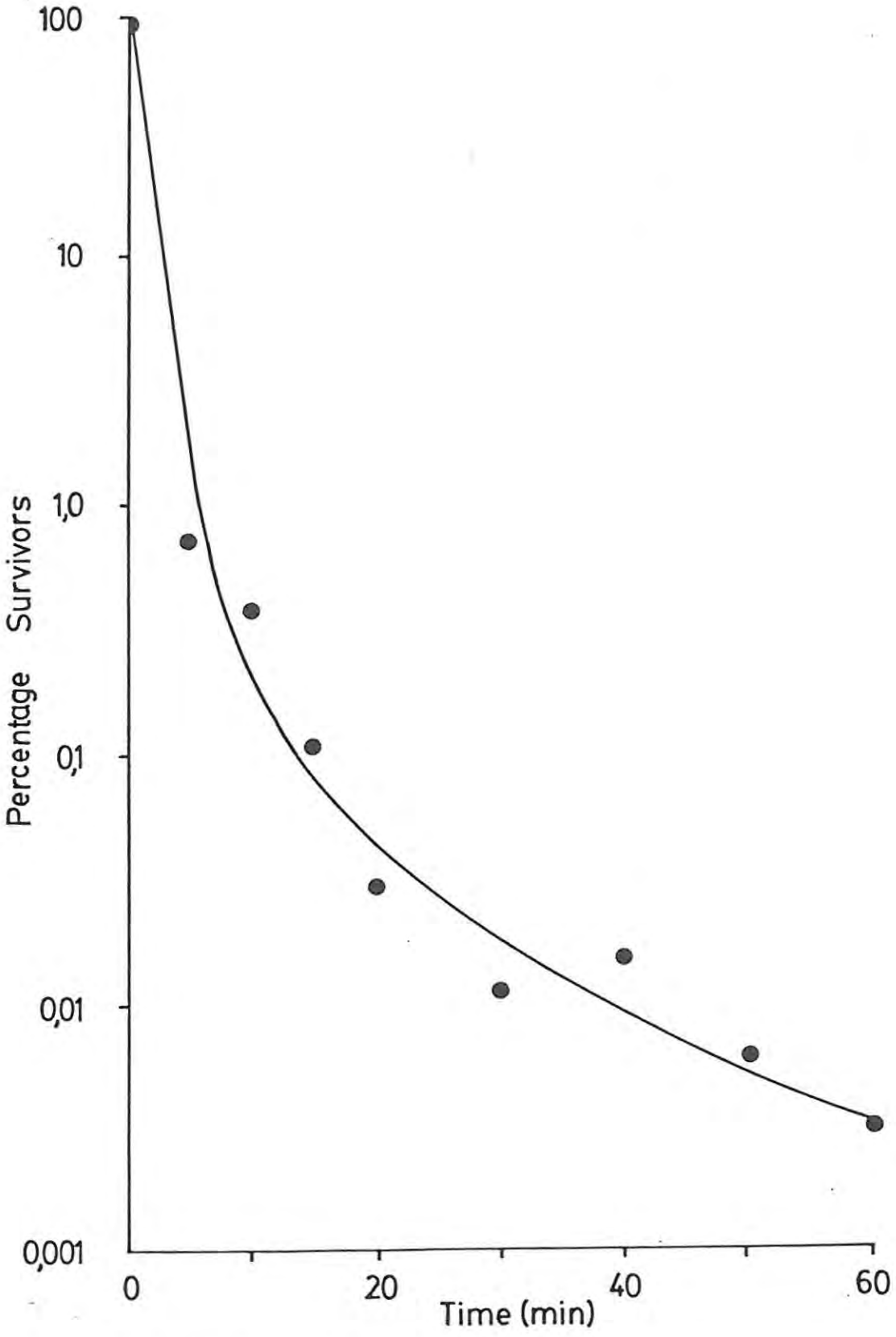


Fig 8 Kinetics of lethality of the bacteriocin-like toxin.

did not reveal any particles. The sedimentation coefficient of the toxin (Fig 9) determined by sucrose density gradient centrifugation was 5,92 S.

The ammonium sulphate precipitation studies (Table 12) indicated that the toxin was precipitated by this method. The final pellet was collected by either centrifugation or filtration. Filtration was preferred as the precipitate tended to be flocculent. A 70% saturated solution of ammonium sulphate was selected for use in future studies.

The results of the cation and anion exchange chromatography are shown in Tables 13 and 14 respectively. The cation exchange column did not retain the toxin and the activity was mainly recovered in the equilibration buffer. A slight increase in recovery occurred when the pH of the buffer was increased (Fraction 8). Conversely the anion exchange column retained the toxin. The majority of the activity was eluted with 0,2M NaCl in 10mM sodium acetate buffer pH 4,5 (Fraction 4). No protein uv spectra were obtained for any of the samples, possibly due to the presence of a contaminating pigment absorbing in the same region. The total percentage recovery obtained using the DEAE column (24,2% - Table 14), was higher than that obtained for the CM cellulose column (14,3% - Table 13). Molasses impurities and particularly the molasses pigment

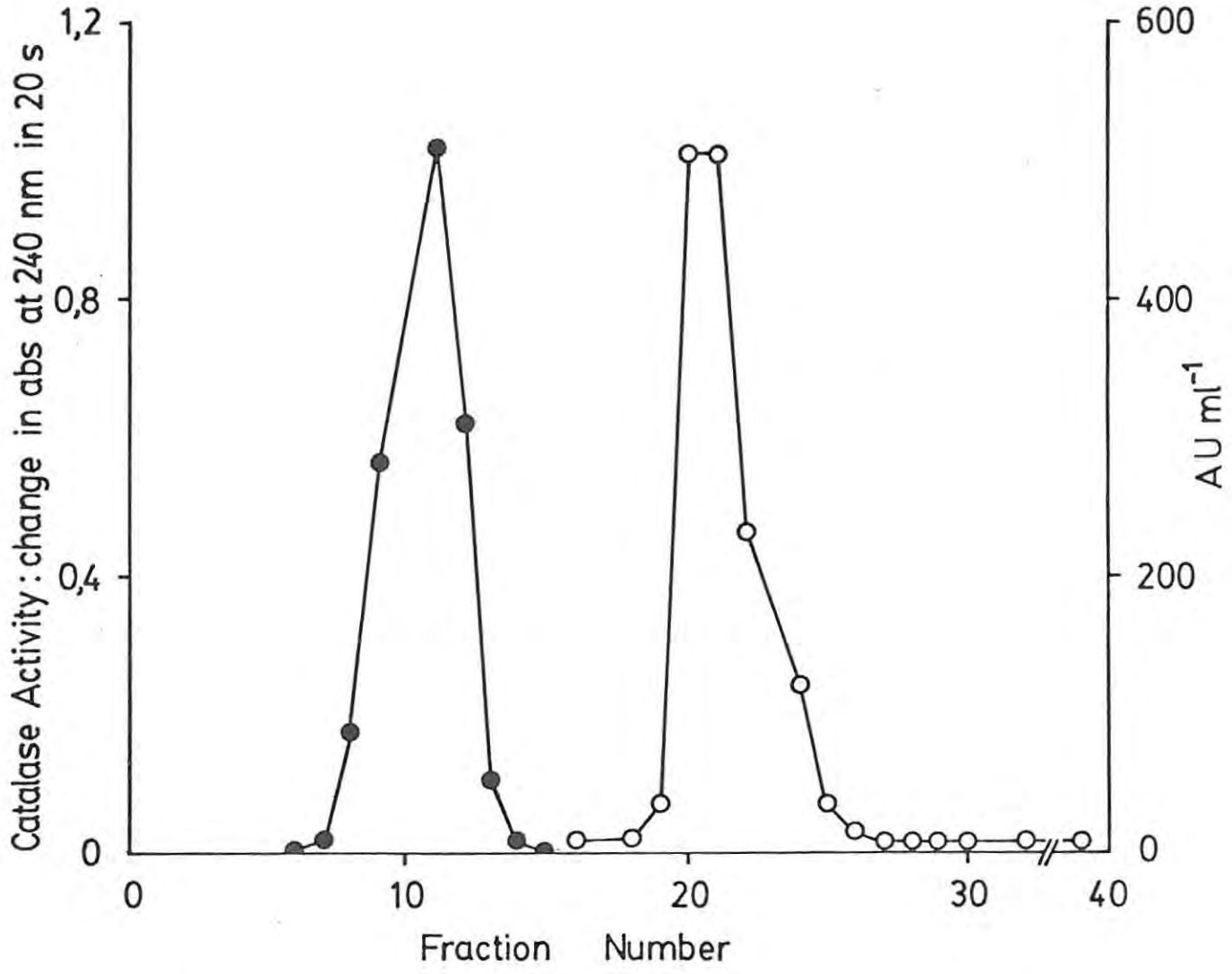


Fig 9 Estimation of the sedimentation coefficient of the bacteriocin-like toxin.

●—●, sedimentstion of bovine catalase;

○—○, sedimentation of bacteriocin-like toxin.

Table 12 Fractional $(NH_4)_2SO_4$ precipitation of bactericin-like toxin after 4 and 24h

Treatment	Time			
	4h		24h[x]	
	AU Supernatant	AU Pellet	AU Supernatant	AU Pellet
Supernatant	128		512	
50% $(NH_4)_2SO_4$	64	128	-	-
60% $(NH_4)_2SO_4$	4	128	64	512
70% $(NH_4)_2SO_4$	4	128	-	512
75% $(NH_4)_2SO_4$	4	128	4	512
80% $(NH_4)_2SO_4$	4	128	4	512
85% $(NH_4)_2SO_4$	4	128	32	512
0% $(NH_4)_2SO_4$	128	4	512	8

[x] The final pellet was filtered through a Sartorius membrane filter.

Table 13 Carboxymethyl cellulose chromatography of bactericin-like toxin

Fraction No.	Treatment	Toxin Activity AU ml^{-1}
1	Control	16264
2	Equilibration buffer	2048
3	0,1M NaCl in 10mM Sodium acetate buffer pH 4,5	64
4	0,2M NaCl in 10mM Sodium acetate buffer pH 4,5	64
5	0,3M NaCl in 10mM Sodium acetate buffer pH 4,5	8
6	0,4M NaCl in 10mM Sodium acetate buffer pH 4,5	16
7	0,5M NaCl in 10mM Sodium acetate buffer pH 4,5	-
8	0,3M NaCl in 10mM Sodium acetate buffer pH 5,0	128
	Total percentage recovery	14,3%

Table 14 Diethylaminoethyl cellulose chromatography of bacteriocin-like toxin

Fraction No.	Treatment	Toxin Activity AU ml^{-1}
1	Control	8192
2	Equilibration buffer	20
3	0,1M NaCl in 10mM Sodium acetate buffer pH 4,5	160
4	0,2M NaCl in 10mM Sodium acetate buffer pH 4,5	1280
5	0,3M NaCl in 10mM Sodium acetate buffer pH 4,5	320
6	0,4M NaCl in 10mM Sodium acetate buffer pH 4,5	160
7	0,2M NaCl in 10mM Sodium acetate buffer pH 4,0	40
	Total percentage recovery	24,2%

hindered the purification of the toxin using ammonium sulphate precipitation. The cation and anion exchange columns were found to separate most of the molasses impurities from the toxin and were therefore investigated for use as a precolumn prior to molecular exclusion chromatography. The DEAE column was selected as fraction 4 which contained most of the toxin activity was far clearer than the corresponding CM cellulose column fraction (2), although the percentage recovery of toxin for these fractions was only slightly different (15,6 and 12,6% respectively).

The sephadex column was layered with the sample eluted from the DEAE cellulose column (Fraction 4). The results obtained are shown in Fig10 and indicate that the toxin was eluted just after the void volume and did not separate very well from the contaminating molasses pigment. The total percentage recovery was only 30% of the DEAE cellulose column fraction used.

6.4 Discussion

The bacteriocin-like toxin production is not limited to the high concentration molasses (12,5%) medium but is produced, in lower concentrations, in both A_1 stage and the 6,6% molasses concentration medium. Production occurs during the exponential growth phase and continues to increase to the end of the

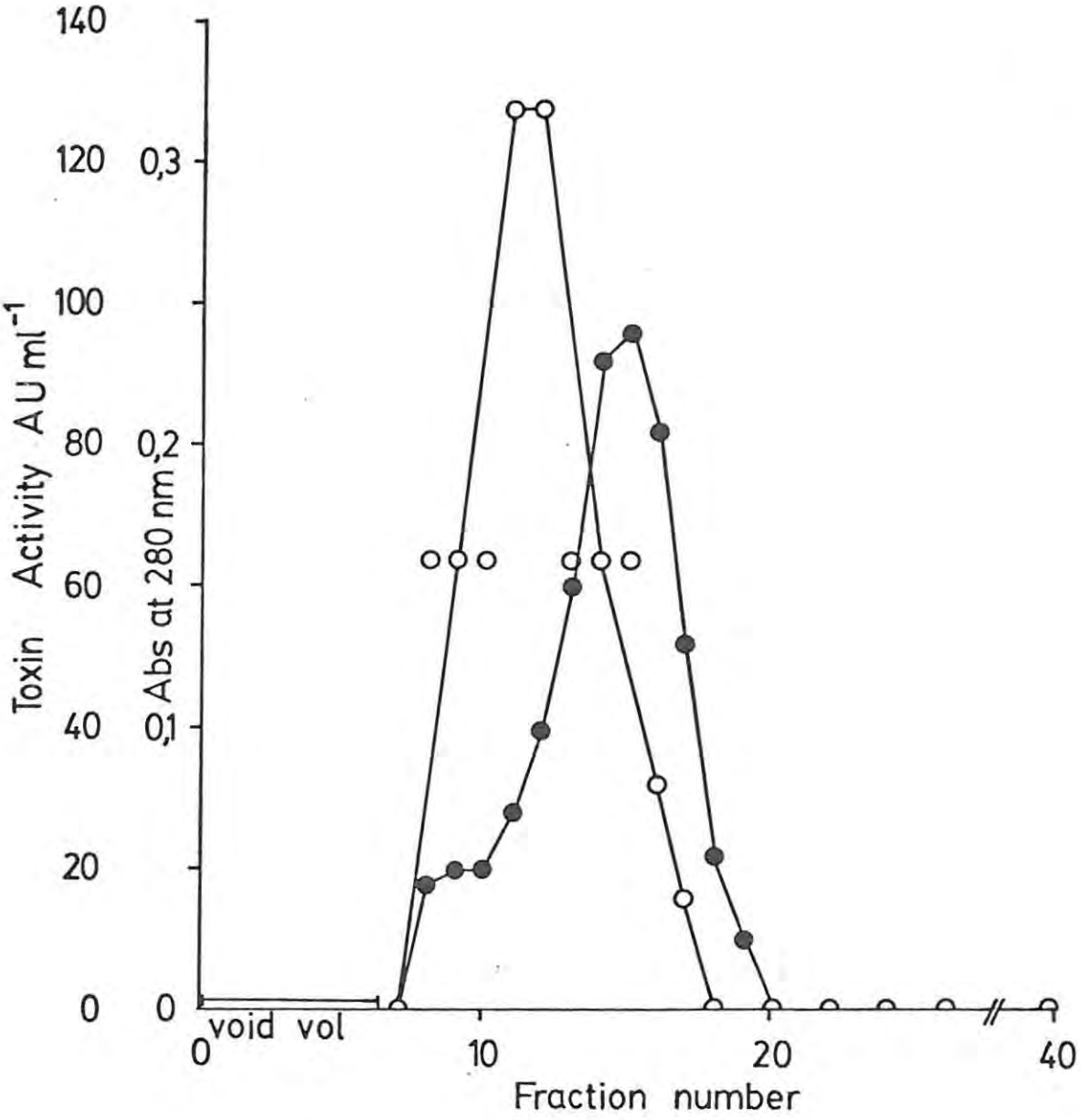


Fig 10. Molecular exclusion chromatography of Bacteriocin-like toxin. ●—● , Contaminating molasses pigment; o—o, Toxin activity.

fermentation. The toxin appears to be produced in much higher concentrations than most other bacteriocins or bacteriocin-like toxins i.e. moganocins after mitomycin C induction reached a titre of 64 AU ml^{-1} (Williams, 1976) and boticin P reached a titre of 128 AU ml^{-1} (Lau et al., 1974) while the bacteriocin-like toxin found in C. acetobutylicum has an average titre of 2048-4096 AU ml^{-1}. It has been reported that immunity is not always absolute and colicinogenic strains may be sensitive to a high concentration of their own colicin (Reeves, 1972). Immunity breakdown has also been reported in Gram-positive strains producing bacteriocins and may be widespread (Tagg et al., 1976). This could explain why the producer strains of C. acetobutylicum appear to have lost their immunity.

The specificity of the toxin may have taxonomic applications as outlined for C. perfringens (Nakamura, 1977). The one other clostridial strain sensitive to the toxin C. felsineum is closely related to C. acetobutylicum (Breed et al., 1948) and when grown in a corn or starch mash, produced yields of acetone and butanol equivalent to about one-half to three-quarters of that produced by C. acetobutylicum (Beesch, 1953).

The C. acetobutylicum toxin was unstable and thermolabile and always had to be maintained at 4°C to prevent loss of toxic activity. It was more stable in acid conditions and all further experiments were done

using the Tris-Maleic acid buffer pH 5,0. The properties and production of this toxin are very similar to those of boticin E produced by C. botulinum (Ellison and Kautter, 1970). Boticin E was initially detected during exponential growth and the highest titres were observed in early stationary phase. It was also unstable in alkaline conditions and at 40°C. The toxin has different characteristics to the bacteriocins produced by non-toxigenic clostridia which are active against C. acetobutylicum (Hongo et al., 1968). These toxins were thermolabile, pH stable (4-9), partially inactivated by proteolytic enzymes and had a wide spectrum of inhibition.

The C. acetobutylicum toxin was not inactivated by proteolytic enzymes but the following results indicate the proteinaceous nature of the particle. It was inactivated by the protein denaturing agents sodium dodecyl sulphate and phenol. The toxin did not pellet under high speed centrifugation indicative of a low molecular weight protein. It was also retained by dialysis membrane. The toxin was precipitated by ammonium sulphate which precipitates proteins, although, the toxin could be co-precipitated with another protein. Streptococcus faecium produced a bacteriocin that was also found by Brandis and van de Loo in 1965 (Reeves, 1972) to be unaffected by trypsin or a bacterial protease but behaved like a protein.

Attempts to purify the toxin were severely hindered by the impurities in the molasses medium from which it could not be separated. Attempts to remove these impurities using column chromatography caused great loss of activity and despite partial purification residual contamination precluded the use of molecular exclusion chromatography. It is essential that the toxin should be further stabilized prior to purification and if possible produced in a defined medium as used by Egorov, (1972) which would facilitate handling. Increased stability and a "cleaner" medium together with a longer sephadex column should result in toxin purification.

Preliminary studies on the mode of action of the toxin indicate it to be bacteriocidal and not bacteriolytic (Webster, 1977). The number of killing units was determined as 6 KU c.f.u.$^{-1}$. Unlike the bacteriocins produced by C. perfringens the toxin does not appear to cause sphaeroplast formation (Mahony et al., 1971). Granulation of cells is observed towards the end of log phase during C. acetobutylicum fermentations (Chapter III) but any association with toxin activity is at present unknown.

Although the bacteriocin-like toxin described has several characteristics of bacteriocins produced by other clostridia, the fact that it may be a type

of protease produced during sporulation must not be overlooked. The toxin produced by C. acetobutylicum does not appear to be the same as the proteases found by Egorov et al. (1972), as the C. acetobutylicum toxin had no proteolytic action when tested against gelatin and the lowest concentration of the proteases outlined by Egorov et al. were produced in a 1,0% glucose medium while the control fermentor medium contains 6,43% glucose. The production of the proteases appears different to the production of toxin. The protease production occurred throughout the lag phase with fibrinolytic activity decreasing markedly at the end of the exponential phase while caseinolytic activity decreased more slowly.

CHAPTER VII

SUMMARY AND CONCLUSIONS

While C. acetobutylicum grew in non-reduced liquid media a high concentration of hydrogen was required for growth to occur on agar plates. Under these conditions several different media supported growth but the A_1 Filtered Potato agar medium proved to be the best. Pre-treatment of agar plates with bovine catalase increased the percentage cell recovery as it prevented the accumulation of toxic H_2O_2. In addition growth was improved and the colonies were markedly larger.

Laboratory scale fermentations were found to produce approximately the same levels of solvents, but slightly less ethanol compared to the yields obtained by the N.C.P. factory. Detailed studies on the final fermentation revealed that solvent production; which reached a maximum 36h after inoculation, paralleled the growth curve until the late exponential growth phase. At this time the induction of a bacteriocin-like toxin caused the turbidity to decrease and a levelling of solvent production. Characteristic changes in pH during the final fermentation corresponded to those outlined in the biochemical pathway for solvent production. Distinct morphological changes in the bacterium were observed during the final fermentation stage. The

clostridial forms, sporulation intermediates, were associated with solvent production while the sporulated form was not. It was also observed that cells from the build-up stages grew well on agar media while no growth on agar media occurred with cells from the final fermentation stage. This indicated the delicate physiological state of the organism during this period.

A certain percentage of fermentations fail and produce low solvent yields. Although the extent of bacterial growth at the end of the A_1 stage appeared to be related to solvent production, this was not the case and could not be used as a method of early detection of poor solvent producing fermentations.

Conditions for large scale mutant selection were optimized. The direct injection method for solvent determination by gas chromatography was used in preference to distillation as the latter would be impractical for the many samples used in mutant selection. Alteration of the fermentation process by either omitting the B and C stages or substituting the A_1 stage with an A_1 Filtered Potato agar plate adversely affected solvent yield. Decreasing the size of the final fermentation resulted in decreased solvent yields. However, as the low yields were consistent, and could be improved by scaling up the fermentation, this may be used in the mutant selection programme as a preliminary selection for higher solvent producing bacteria.

Before a mutant selection programme could be undertaken it was essential that a mutant plate screening assay be developed. It was observed that on A_1 Filtered Potato agar plates clones produced a visible halo which was found to be due to butyric acid and β-amylase production. As there is a relationship between the concentration of butyric acid and halo diameter, and as butyric acid is the precursor of n-butanol, this may provide the necessary plate screening assay. Before mutants can be selected isolated clones capable of producing normal solvent yields must be obtained. It was found that different clones produced different solvent yields and despite these levels being increased after sporulation of the clones, normal solvent yields were not obtained. Efficient sporulation prior to heat shocking and fermentation is therefore a prerequisite for good solvent production and the most effective means of sporulation is being investigated prior to mutant selection.

Several different types of mutants may be selected. Those clones producing increased butanol yields could be isolated once the relationship between halo size and solvent production has been established. Alternatively, several mutants could be selected and used in a"dual fermentation." The first mutant producing increased solvent precursors, the second, increased levels of enzymes responsible for converting the precursors to

solvents. The isolation of sporulation deficient mutants that give rise to clostridial forms (associated with good solvent production) but do not sporulate (associated with poor solvent producing fermentations) could possibly produce increased solvent yields. Once mutants have been isolated they can be fermented at a higher T.I.S. percentage where additional solvents would be produced.

The investigation of the phage outbreak at N.C.P. revealed that the source of contamination was the liquid and solid sewers between fermenters 5 and 8. Electron Micrographs and cross-resistance studies indicated the presence of one phage type. Although this was different to the long tailed phage present during the outbreak , it was isolated from freeze-dried samples taken during this period and must have participated in the factory infection. The long tailed phage was unstable and could not be isolated from the original lyophilised samples. Resistant strains were easily obtained, therefore once the mutation studies are completed clones resistant to this and other phages will be isolated.

Studies on the nature of the bacteriocin-like toxin isolated during fermentations revealed that it was produced in several different media, although the greatest concentrations were obtained in the high concentration molasses final fermentation medium. It was present in all fermentations assayed, and may be a contributary

cause of fermentation failure. The presence of the toxin may prevent higher solvent yields from being attained. The isolation of bacteria resistant to the toxin would probably follow the normal bacterial growth pattern and consequently solvent production would increase above present yields. C. acetobutylicum is sensitive to the toxin it produces, possibly due to immunity breakdown as a result of the relatively high concentrations of toxin produced. The toxin was active against all C. acetobutylicum strains tested and one other clostridial strain, C. felsineum. As this bacterium is closely related to C. acetobutylicum the toxin may have taxonomic applications. The toxin appeared to be proteinaceous, was thermolabile and more stable in acid conditions. It had a sedimentation coefficient of 5,9S. The presence of molasses impurities hindered the purification procedure and because of this, ammonium sulphate precipitation was found unsuitable and an anion exchange precolumn which removed most of the impurities had to be employed prior to molecular exclusion chromatography. However, the toxin could not be separated from a contaminating molasses pigment which prevented purification. As large losses of toxin acitivity occurred, the toxin should be further stabilized before further purification studies are undertaken. Production of the toxin in a defined medium, to facilitate handling, and the use of a longer molecular exclusion column for better separation should result in toxin purification.

APPENDIX A

OPERATING CONDITIONS FOR GAS CHROMATOGRAPH AND PROCEDURE FOR SOLVENT DETERMINATION

A1 Operating conditions for Gas Chromatograph

A Hewlett-Packard 5830A GC was used. The operating conditions outlined below were used for the routine determination of solvent production.

Column: A 1,84m,3,18mm outside diameter, stainless steel column was packed under negative pressure with Chromsorb W/AW (80-100 mesh) coated with 15% Carbowax 20M. New columns were conditioned at 90°C for 24h with a flow of 60ml min^{-1} N_2 to remove volatile impurities. Isothermal operation was conducted at 90°C.

Sample injection: Duplicate samples (1µl) were injected using a Hamilton syringe fitted with a Chaney adaptor. The temperature of the injector was 250°C.

Sample detection: The GC was equipped with a flame ionization detector linked to a software integrator The temperature of the detector manifold was 250°C. The following parameters were used:

Chart speed	:	1,0 cm min^{-1}
Slope sensitivity	:	0 (Automatic)
Area rejection	:	1
Attenuation	:	9

Gas Flow rates

Carrier Gas: High purity nitrogen was used at a flow rate of 30 ml ml^{-1}.

Hydrogen: High purity hydrogen was used at a flow rate of 40,0 ml min^{-1}.

Air: Medical Air was used at a flow rate of 500,0 ml min^{-1}.

A2 Analysis of Gas Chromatograms

A typical gas chromatogram is shown in Fig A1. Quantitative evaluation of the peaks was performed automatically by the integrator using the internal standard method: A mixture comprising acetone, ethanol and n-butanol, with n-propanol as the internal standard, was used as the calibration solution. The integrator correlated the area under each peak in the sample chromatogram with the calibrated data, using the internal standard as a reference, and calculated the mass of the solvents in $g\ell^{-1}$.

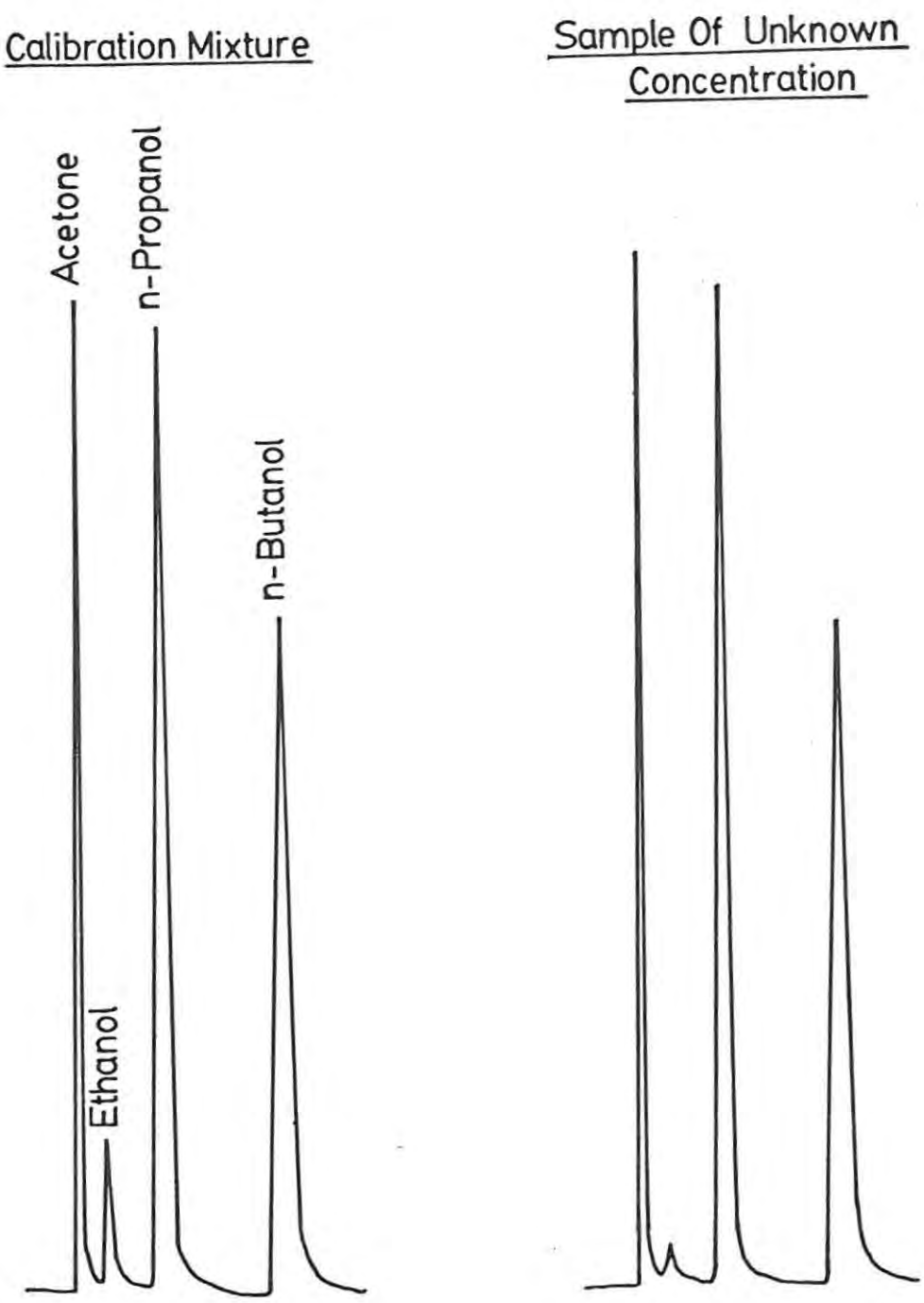


Fig A1 Gas Chromatograms of Calibration mixture (16,0gℓ^{-1}) and sample of unknown concentration. Internal standard n-Propanol.

APPENDIX B

The following media were used. Agar plates were prepared by the addition of 1,5% agar to the liquid media. Sterilization was by autoclaving for 20 min at a pressure of 15 psi unless stated otherwise.

A_1 Potato medium

Dried Maggi mash potato flakes	40,0g
Glucose	6,0g
$CaCO_3$	2,0g
H_2O (distilled)	1,0ℓ

The medium was mixed and either autoclaved or boiled for 10 min to allow breakdown of the potato flakes. This was followed by filtering, to remove large particles, through three layers of cheesecloth. The filtrate was collected and reautoclaved before use.

A_2 Molasses medium

Molasses	66,6g
$(NH_4)_2SO_4$	7,0g
$CaCO_3$	3,0g
$(NH_4)_2PO_4$	0,2g
Glucose	0,1g
H_2O (distilled)	1,0ℓ

Control Fermenter medium

Molasses	124,6g
$(NH_4)_2SO_4$	2,0g
$CaCO_3$	1,0g
Magou	1,0g
H_2O (distilled)	1,0ℓ

This quantity was determined by the amount of molasses required to give a final total invert sugar concentration of 6,4%. The sample above having a total invert sugar percentage of 50,6%.

The pH of the solution was ajusted to pH 7,0-7,3 with 1N NaOH.

Brain Heart Infusion Broth

Brain heart infusion broth	3,7g
Yeast extract	0,5g
H_2O (distilled)	100,0ml

N.C.P. Clostridium medium

Bacto peptone	8,0g
Yeast extract	3,0g
Beef extract	8,0g
Glucose	10,0g
Cysteine hydrochloride	0,5g
$MgSO_4$	1,0g
$(NH_4)_2SO_4$	1,0g
$(NH_4)_2PO_4$	0,5g
$CH_3COONa.3H_2O$	3,0g
Milk	50,0ml
H_2O (distilled)	1,0ℓ

Peptone, Yeast, Glucose medium

Peptone	10,0g
Yeast extract	10,0g
Glucose	5,0g
Resazurin solution	4,0ml
Salts solution	40,0ml
Cysteine HCl-H_2O	0,5g
H_2O (distilled)	1,0ℓ

Salts solution:

$CaCl_2$ (anhydrous)	0,2g
$MgSO_4$	0,2g
K_2HPO_4	1,0g
KH_2PO_4	1,0g
$NaHCO_3$	10,0g
NaCl	2,0g

Mix $CaCl_2$ and $MgSO_4$ in 300ml ditilled water until dissolved. Add 500ml water and adding separately, dissolve each of the remaining salts. Add 200ml distilled water and store at 4°C.

Resazurin Solution:

Dissolve 1 tablet (11mg) in 44ml of distilled water.

Peptone, Yeast, Molasses medium

This medium is identical to the above except the glucose is substituted with 66,6g of molasses.

T_2 buffer

Solution A

KH_2PO_4	7,5g
NaCl	20,0g
Na_2HPO_4	15,0g
K_2SO_4	25,0g
$CaCl_2$	0,055g
H_2O (distilled)	1,0ℓ

Store over $CHCl_3$ at room temperature. Mix 40ml of solution A with 158ml of H_2O (distilled). Add 1,0ml of sterile 4,8% $MgSO_4$ and 1,0ml of 0,2% sterile gelatin solution. Sterilize by autoclaving.

Tris-Maleic Acid buffer

Tris (2-amino-2-hydroxy methyl 1,3 propane-2-diol.)	6,0g
Maleic acid	5,8g
$MgSO_4.7H_2O$	1,0g
$Ca(NO_3)_2$	5,0g
$FeSO_4.7H_2O$	0,25mg
H_2O (distilled)	1,0ℓ

pH is adjusted with 1M NaOH to pH 5,0.

Tryptone medium

Tryptone	10,0g
Yeast extract	5,0g
NaCl	5,0g
Glucose	1,0g
H_2O (distilled)	1,0ℓ

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