METHODOLOGY OF CULTURE MAINTENANCE AND INOCULUM DEVELOPMENT FOR PRODUCTION OF SOLVENTS BY *CLOSTRIDIUM ACETOBUTYLICUM*

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ABSTRACT

Various methods of culture maintenance and inoculum development were evaluated for their effectiveness in conserving and improving the property of 2 strains of *Clostridium acetobutylicum*, namely NCIB 2951 and NRRL B-594, to produce solvents by fermentation of whey permeate.

The majority of the methods were effective in maintaining the viability and solventogenic property of the organism. However, since in some cases the viability was maintained but the solventogenic property was not, it is clear that the latter should be used as the index in determining the storage life and time of reprocessing of the stock culture.

The methods of culture maintenance investigated included refrigeration at 4°C in distilled water, in phosphate buffer and in Cooked Meat Medium containing glucose (CMMG); by freezing at -20°C in distilled water and in phosphate buffer; by drying in soil and by lyophilization (freeze drying); and by periodic transfer in CMMG and in whey permeate containing yeast extract.

Maintenance of the stock cultures at -20° C in distilled water was found to be the most efficient for the storage stability of both strains of organism.

The viability and the potential to produce high solvent concentrations, primarily butanol were maintained without any significant loss after 9 months and 12 months, for strain NCIB 2951 and strain NRRL B-594, respectively. The criteria important for a commercial fermentation, i.e., sugar utilization, yield and butanol production rate, remained stable during storage by this method.

It was observed that periodic transfer was a poor method as the culture lost their solventogenic property despite remaining viable.

The other preservation methods were not as satisfactory as freezing in distilled water at -20°C since the fermentation ability degenerated to some extent after 9 months of storage. Therefore, after such a period reprocessing of the stock cultures kept by these methods is necessary to revive the cultures and minimize degeneration.

The repeated use of the stock cultures was found to be deleterious and should be avoided.

The inoculum development procedure investigated to maximize fermentation efficiency included the conventional heat shocking of the stock culture; variation in the number of culture stages; use of gassing as an index of transfer time; and the use of different levels of inoculum size.

The strain differences which exist between NRRL B-594 and NCIB 2951 influenced how the inocula from these strains should be propagated prior to fermentation. Strain NRRL B-594 responded to heat shocking while strain NCIB 2951 did not. Neither ethanol nor butanol treatment of the stock cultures of the latter were advantageous.

Using a 3-stage inoculum development procedure, the fermentation efficiency of strain NRRL B-594 was improved by employing heat shocking at 80°C for 15 min in the revival stage of the stock culture. The germination factors for the spores of NCIB 2951 await identification. However, by using the presence of highly motile cells as an index in transferring from the revival stage, the inoculum development procedure resulted in a significantly higher butanol concentration value and production rate. Thus, the revival stage was the most critical.

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CHAPTER 1

INTRODUCTION

The great value of microorganisms to biotechnology is closely connected with the vast diversity of chemical transformations catalyzed by these organisms. The number of industries based on the metabolic activities of microbes is expanding year by year as new processes are developed and new microbial strains are isolated and tamed for the service of mankind. Without overstatement it may be said that the most valuable working capital of a microbiological or biotechnological institution is its collection of stock cultures of well defined microbial strains constant in their ability to produce useful compounds in high yields.

Once a superior culture has been developed it is most essential that the culture be preserved for many months or even years, in such a fashion that no physiological changes occur. Further, the culture must be grown in a way that its full biosynthetic powers are put to use. Neither variation nor population selection can be tolerated during culture preservation or during the multiple stages of inoculum propagation leading to final employment in a plant fermenter. Prevention of population changes can be a difficult problem particularly with those cultures which are genetically unstable or are heterogenous in character (Brown, 1963) and, therefore, should be a major concern of biotechnology.

The anaerobe *Clostridium acetobutylicum* is an industrially important microorganism. It has been used to carry out the traditional fermentation process to produce acetone, butanol and ethanol.

The organism can produce these solvents from a variety of low-cost substrates, including pentose sugars derived from biomass residues or from wood acid - and pre-hydrolysate (Langlykke, et al, 1948; Beesch, 1952; Compere and Griffith, 1979; Mes-Hartree and Saddler, 1982; Maddox, 1980; Maddox, 1982; Maddox & Murray, 1983; Petitdemange et al, 1983; Marchal et al, 1984). Interest in the process, otherwise known as the ABE fermentation, has been reinitiated due to the escalating cost of petroleum-based chemical feedstocks. Several studies have been done to investigate the factors controlling the fermentative production of acetone-butanol in order to increase the efficiency of the process for higher solvent yields (Abou-Zeid, et al, 1978; Baghlaf, et al, 1980; George and Chen, 1983; Bu' Lock and Bu' Lock, 1983; Long et al, 1984).

However, studies on the microbiology of the process seem to lag behind the technology aspect of the process. It has been recognized that the history of the inoculum, which involves the manner of culture maintenance and propagation, affects solvent production (Kutzenok & Aschner, 1952; Prescott and Dunn, 1959; Gapes et al, 1983). Procedure should be assessed and devised such that minimal stress is imposed upon the culture during storage or during the propagation of the inoculum from the stored source.

This work, therefore, aims to evaluate various methods of culture maintenance and inoculum development for *Clostridium acetobutylicum* to obtain maximum yields and production rates of butanol.

CHAPTER 2

PRESERVATION OF MICROORGANISMS

2.1 INTRODUCTION

A great number of methods are available for maintenance of stock culture. However, there is no universal method of preservation suitable for all cultures. In view of the marked diversity in the morphological and physiological properties of various microbial species this seems quite natural.

The preservation of microorganisms has a long history of study in microbiology although no one can safely conjecture when the first attempts at preserving cultures of mico-organisms were made. This was probably done by transferring them from their natural habitats to fresh and/or uninfected materials of the same kinds. Techniques were of necessity crude and sterility problematical. Emphasis soon shifted to extracts of animal tissues, plant parts, fruits and dung, often solidified after a fashion by the addition of gelatin. With the introduction of agar as a relativley inert gelling agent by Frau Hesse in the early 1880's, this was used increasingly to provide a solid base. Generally, natural extracts have been incorporated as nutrients and have been as varied as the microorganisms cultivated and maintained. Nutrient solutions of defined composition were then intro-duced. Whether consisting of natural extracts of mixtures compounded in the laboratory, such nutrient solutions were solidified with agar dispensed into tubes and as agar slants used for the transfer of stock cultures at regular intervals as required for the specific organism.

Most of the studies on long-term preservation of micro-organisms were conducted by microbiologists interested in maintaining only a few strains. However, several useful reviews appeared during the 1960's and 1970's when the subject was examined by Martin (1963, 1964), Clarke and Loegering (1967), Pegg (1976), Lapage et al (1970), Perlman and Kikuchi (1977) and Heckly (1978). Lapage et al (1970) wrote a general account of their own procedures and problems on the preservation practice of the National Collection of Type Cultures (NCTC), and of the National Collection of Industrial Bacteria (NCIB). The work of Perlman and Kikuchi (1977), Heckly (1978) and, most recently, that of Dietz (1981) emphasized the importance of the maintenance process in the design of an industrial fermentation process.

2.2 CRITERIA FOR PRESERVATION

Variations in the characteristics of industrially important cultures can lead to intolerable losses in the manufacture of products. Hence, the selection of procedures for the preservation of specific cultures should be performed properly. It should be based on the maintenance of viability, of functional properties which are linked to the biochemical and physiological properties, and of full genetic complement.

2.2.1 Maintenance of Viability

There have been few detailed investigations, unfortunately, on quantitative measurements of viability before and after storage. The "growth or no-growth" test has been more commonly used than the more exacting measurements of colony-forming units (cfu) on plaque forming units (pfu). Nevertheless Lapage et al (1970) estimated viability of NCTC and NCIB culture obtained from colony counts based on a modification of the method of Miles and Misra (1938).

Since preservation methods may injure cells, it is important that the medium on which the organism is recovered should be specified. The percentage of injured cells in preserved cultures has been reported to be as high as 95% (Gibson et al, 1965). Hence, cells might not grow on ordinary media, and may require special growth factors for recovery. For example, Collins (1967) found that the counts of *Vibrio* or *Pseudomonas* on blood agar were 10- to 100-fold greater than they were on nutrient agar.

2.2.2 Maintenance of Biochemical Properties

Several studies have revealed that biochemical activity is not necessarily directly related to the viability of recovered cultures. Therefore, tests on the biochemical properties of culture should be done in conjunction with viability tests. The major use of the viability tests is to indicate when cultures should be reprocessed.

2.2.3 Maintenance of Genetic Constitution

The genetic constitution of a culture is a factor that must be considered, particularly in respect to characteristics concerning product formation, sensitivities to various toxic agents, special nutritional responses, virulence and fermentation reactions. Possession of one or more of these traits by a culture is frequently the reason its preservation is desirable.

Whether selection or mutation occurs as the result of preservation methods has not been clearly demonstrated. Studies on the population change in lyophilized cultures of *Penicillium chrysogenum* and *Nocardia lurida* were suggestive as to the occurrence of selection and mutation during culture preservation and reactivation (Brown, 1963).

MacDonald (1972) observed lower penicillin production from a subpopulation of a *P. chrysogenum* preserved at 4°C but not at -196 C. A clear demonstration of selection or mutation is difficult. However, genetic changes can be maintained through gross changes in morphology e.g. pigmentation, temperature requirements and fermentation ability. Prototrophs are more stable than auxotrophic mutants, hence, it is extremely important that methods used to preserve mutants yield maximum survival and do not alter DNA (Heckly, 1978).

2.3 METHOD OF PRESERVATION

2.3.1 Subculture or Periodic Transfers on Culture Media

The most traditional method of preservation is regular subculture on agar slants. Stab or broth cultures are usually used for anaerobic microorganisms. Conventional agar slants or stab cultures of microorganisms are stored at refrigerator temperatures (ca 5°C) following growth, primarily to retard the ageing process by reducing the metabolic activity. The interval of subculturing varies from days to weeks or even months or years depending on the species.

It is a method commonly used for the maintenance of algae, filamentous fungi, and yeasts. Thousands of strains of fungi appear to be the same now as they were when isolated years ago. On the other hand, many organisms, e.g. *Fusarium*, degenerate rapidly on periodic transfer. The method is seldom employed for the preservation of bacteria or of organisms of industrial importance (Martin, 1964).

This procedure has certain obvious advantages. Transfers can be made quickly and, the purity and non-viability of the culture can be verified during its development. Hence, it is continually used by mycologists. On theoretical grounds, this appears to be the least satisfactory method of preservation due to the risks of mutation and degeneration. Furthermore, events of mislabeling, contamination, and inoculation with the wrong organism due to tedious transfer to a series of tubes are other disadvantages. The

frequency of subculture can be reduced by using a medium of minimal nutrients by layering the culture with paraffin oil or by storage at low temperature (Lapage, 1970).

2.3.2 Storage Under Oil

To increase the longevity of cultures, sterile mineral oil may be used to cover actively growing slant or stab cultures which then are subsequently stored in the cold. Although it is a simple extension of the periodic transfer method, this technique has the advantage of greatly lengthening the transfer interval. Over-layering with oil prevents dehydration and slows down metabolic activity by reducing oxygen availability.

The technique has proved useful for extending the longevity of aquatic phycomycetes (Martin, 1964) and of fungus cultures, e.g. Basidiomycetes, which are not amenable to lyophilization due to their mycelial or poor sporulation. Fennel (1962) and Raper (1963) listed the following operating principles:

1. The oil must be sterile, dry and of medicinal grade.
2. Slants must be completely covered with oil at all times.
3. Storage temperatures should be chosen carefully according to the needs of the individual organisms.
4. Transfer from oil-covered slants should be made to the same medium as that used for preservation.

Heckly (1978) has reported that fungi keep for up to 5 years under oil with only a little tendency to vary. *Pseudomonas*, *Bacillus* and *Escherichia* can survive for 3 years without changes in their morphological, cultural or physiological properties.

2.3.3 Storage in Distilled Water

Distilled water has been found suitable as a suspending medium, and in some cases survival for up to 10 years can be achieved without alteration in morphology or physiology. McGinnis et al (1974) found 93% survival of several species

of filamentous fungi, yeasts and some aerobic actinomycetes stored for 4 years at room temperature.

2.3.4 Preservation by Drying

Drying, without freezing, is a method which has taken many forms, from the simple drying of conventional slant culture of sporulating molds to some rather more complex techniques for vacuum drying.

The practice of conserving bacteria in soil or sand seems to have had a dual origin. Legume inoculants applied to seed *via* soil in 1917 were found to be still viable after 30 years. *Clostridium acetobutylicum* was first stored on sand in 1915 in Canada. *Cl. pasteurianum* was recovered in Winogradsky's laboratory from a mixture of sand and chalk after 23 years (Raper, 1963). Since then, this technique has been used for the storage of anaerobic sporeforming bacteria and fungi. Disadvantages are that quantitation is difficult and soil is a variable commodity not easily defined (Heckly, 1978).

Troloppe (1975) described a preservation method whereby bacteria, fungal spores or fungal mycelium suspended in skim milk were adsorbed onto anhydrous silica gel particles at 4^{o}C. Sixty percent of the bacteria and 36% of the fungi were still viable after storage for periods between 3 and 4 years. The procedure used was modified from the methods described by Perkins (1962) and Grivell and Jackson (1969).

Lapage (1970) reported that *Staphylococcus aureus*, dried on paper discs enclosed between two layers of plastic material, remained viable for about 6 months. Other cellulose carriers used were tufts or string. This method of drying in cellulose appears easy but quantitative determination of viability may prove difficult.

The materials and method of drying in gelatin discs have been summarized by Lapage (1970). This method does not require any specialized equipment, allowing it to be carried out in almost any laboratory. This may be applicable to a wide range of common heterotrophs but there is a lack of data on long term preservation. Small variations in storage conditions which are not readily standardized may have a marked effect on viability.

Reported viabilities of up to 5 years or more are not uncommon for a variety of fungi stored in carriers including clean sand, peat soil with $CaCO_3$, and clay and sawdust.

2.3.5 Freezing

In each of the preceding methods, storage at 4^{o} C is recommended as a means of limiting metabolic processes. The simple device of freezing cultures has, therefore, been employed to preserve vegetative cells or spores. Besides lyophilization, freezing has received considerable attention from a number of workers. Recent reports include those of Feltham (1978), Defives and Cattleau (1977), and Calcott (1978).

Freezing has become a method of choice for both short- and long-term storage of viable microorganisms. This has occurred due to improvements in refrigeration systems and the greater availability of liquid nitrogen, and most bacteria can be kept for long periods (Heckly, 1978). However, many fungi are fragile on freezing and success requires greater attention to the details of the techniques of freezing and thawing.

Lapage et al (1970) and Mazur (1963) listed the following general rules applicable to the frozen storage of living cells:

1. The rate of cooling should be low (about 1°C per minute) to about -20° C, and then as rapid as possible until storage temperature is reached.
2. The electrolyte content of the suspending medium should be kept to a minimum.
3. An adjuvant such as glycerol (10-20%v/v) or dimethlysulfoxide (10%v/v) should be added to the suspending medium, particularly with organisms other than bacteria.
4. Small volumes of cell suspensions in thin-walled containers should be used.
5. Protection may be achieved by the presence of sugars.

Heckly (1978) summarized the important factors as follows:

a. Age of culture.
It is generally accepted that cells from stationary phase culture are more resistant to damage by freezing and thawing than cells from the early or midlog phase of growth. Use of high cell densities increases survival since lysed cells tend to release cryoprotective substances.

b. Rate of freezing and thawing
Generally, slow freezing and rapid thawing yield the highest number of viable cells. As mentioned earlier, the rate of 1°C/min is widely used for cooling.

c. Storage temperature
This would depend on the sensitivity of particular microorganisms. Temperatures as low as -196° C can be reached using liquid nitrogen, and this has been demonstrated to be a very useful technique (Lapage et al, 1970). Temperatures of -20° C and -70° C are often used, but some loss of viability can be expected. However, Heckly (1978) believes that storage at -70°C is as effective as at -196°C.

Some disadvantages of using liquid nitrogen include its cost, the necessity to control both freezing and thawing rates, and the space required (Lapage et al, 1970).

d. Cryoprotective agents

The best protective additive is glycerol. Mycoplasma, fungi and bacteria that cannot survive lyophilization can be frozen in 10% (v/v) glycerol. Other protective materials can be used although the effectiveness varies with the organism. Dimethyl sulfoxide, lactose, maltose or raffinose added to 10% (v/v) glycerol increase survival of lactic and bacteria, yeast and algae (Gibson, et al, 1966; Daily and Higgens, 1973).

The nature of freezing damage has been a subject of interest for the cryobiologists. Freezing results in two types of injury, namely, the injury due to concentrated electrolyte and intracellular freezing injury. If a cellular suspension is frozen, the external medium crystallizes, releasing heat of fusion which prevents intracellular ice formation. The difference in temperature between the external water and the cells creates difference in vapour pressure. Assuming an intact cell membrane, one of two processes can occur. Either the cell interior can freeze, or water can diffuse through the membrane and freeze externally. The net result in either case will be the withdrawal of liquid water from the cytoplasm and its deposition as ice (internally in the first case, externally in the second). This is lethal. The nature of injury, whether intracellular freezing or loss of water and deposition as extracellular ice, would depend on the permeability of the cell to water, the surface area of the cell, and on the magnitude of the vapour pressure differential that is providing the driving force (Mazur 1966, 1970).

Injury during cooling and subsequent warming can be counteracted by cryoprotective agents such as glycerol. These agents are believed to act by penetrating the cell, and, diluting the intracellular electrolyte concentration.

The second way to minimize injury is to control the freezing rate. Freezing at the rate of $1^{o}C$/min is quite common, but this may require optimization for any particular organism.

2.3.6 Lyophilization

This method, also known as freeze-drying, involves freezing the organism followed by the removal of water by sublimation. Without doubt, this is the method most commonly used nowadays for the long-term conservation of microorganisms. The majority of cultures of bacteria, fungi, viruses and yeasts can be stored conveniently for long periods. Ninety five percent of all cultures consisting of approximately 4000 bacteria, 3000 fungi and 3000 yeasts in the Northern Regional Research Laboratory are maintained by this method (Martin, 1964).

The merits of lyophilization as a method for preserving most microorganisms are now quite well-established. Viability is greatly extended. Once the ampoules have been sealed, contamination is precluded. Variability is minimal. Furthermore, lyophilized cultures for distribution can be conveniently shipped without the need for special storage during shipment and upon receipt.

The two main methods of freeze-drying are a) centrifugal, in which a low-speed centrifuge is used to overcome frothing until the material is frozen and b) pre-freezing before connection of the ampoules to the vacuum system. Centrifugal freeze-dryers are manufactured by e.g. Edwards Company, London, U.K. The manifold type units used for

the second method are often the products of laboratory workshops and the designs are many and varied. The pumps used in freeze-drying must be capable of removing the large amounts of water vapour evolved which is at least at 0.01 torr. A vacuum gauge in the unit is desirable but not essential.

Lapage et al (1970) have described and discussed the different stages of freeze-drying with reference to Edwards freeze-dryer model 30P.

Excellent discussions on the factors affecting preservation by freeze-drying have appeared (Collins, 1967; Heckly, 1978). Survival after freeze-drying is influenced by the following factors:

1. Type of organism
   Organisms sensitive to lyophilization include multicellular algae, mycelial fungi, viruses and mammalian cells. However, most bacteria and yeasts remain viable for long periods when lyophilized. The resistance of different bacterial types follows the order:
   sporeformers > Gram-positive > Gram-negative

2. Age of the culture
   Well-nourished cells taken from the stationary phase are usually the most resistant cells.

3. Substrate or suspending medium
   This is possibly the most important, hence, the most studied single factor. The following materials have been suggested by various workers:
   peptone
   serum
   sodium glutamate
   milk
   glucose
   bovine plasma albumin

Mist dessicans which is 3 parts serum and 1 part broth plus 7.5% (w/v) glucose, has been commonly used as the suspending medium. The addition of sugars is to ensure retention of approximately 1% of the water content and so prevent complete dessication of the microorganisms which may be lethal. Nutrient broth and glutamic acid neutralizes toxic carbonyl radicals in the glucose and in the cells themselves.

4. Cell concentration

Generally, the higher the cell concentration, the greater is the ability to survive lyophilization.

5. Rate of freezing and drying temperature

The recommended procedure is to freeze slowly and to dry at the lowest practical temperature.

6. The residual moisture content of the dried product

This has apparently been a controversial matter among freeze-drying experts. The residual moisture content may vary from 1% to 8%, although Heckly (1978) regards the lower end of this range as preferable.

7. Storage conditions

Since small amounts of oxygen are toxic to dry bacteria, ampoules should be sealed properly. Sensitivity to oxygen is reduced by the suspending media previously mentioned.

The temperature of storage depends upon the moisture content, presence of oxygen, nature of the protective additive and nature of the organism, but storage at $4^{o}C$ or lower is adequate for most organisms.

9. Recovery method

There is no single preferred method for growing organisms recovered from the dried state. In general, as rich a medium as possible is recommended.

Injury that may occur during lyophilization includes:

1. damage to bacterial membranes

2. damage to proteins and RNA

3. breakage of DNA strands

4. mutations and increased numbers of spontaneous mutants

2.4 CULTURE COLLECTIONS AND GENE BANKS

Microorganisms used in specific fermentation processes are often obtained from public culture collections. The holdings of some of these are mentioned in Martin and Skerman's "World Directory of Collections of Cultures of Microorganisms", (1972). Kirsop (1983) listed some of the major culture collections which are particularly useful to biotechnology.

Culture collections operate to provide a comprehensive range of pure microorganisms that are of past, present or potential interest. In addition they serve as banks for genetically marked strains, genetically manipulated strains, plasmid-bearing strains and plasmids and gene fragments. However, detailed research into the molecular events which occur during preservation so that the genetic stability can be maintained is lacking.

2.5 INDUSTRIAL OR COMMERCIAL PRACTICES

Dietz (1981) presented a list of publications pertaining to maintenance of certain cultures of industrial interest. Heckly (1978) made a representative sampling of industrial maintenance methods. However, it was recognized that the details of such maintenance methods are usually well kept trade-secrets.

2.6 CONCLUSIONS

Of the methods discussed, storage in liquid nitrogen (at -196 ^{o}C) is the most suitable for all types of micro-organisms. However, the cost of operation is high. Comparable to it is storage at -70 ^{o}C. Subculture or periodic transfer is simple and effective enough for short-term preservation, while freeze-drying is economical and successful for long-term preservation.

However, it seems that there is no universal method which can be applied to all microorganisms used industrially. The morphological and biochemical properties of a culture should be determined before an optimum methodology for culture preservation can be evaluated for each specific case.

CHAPTER 3

PREPARATION AND PROPAGATION OF INOCULA

3.1 INTRODUCTION

Before a culture is used in a production fermenter it goes through a process of preparation and propagation which is referred to in this study as inoculum development. Although it has always been recognised that inoculum type and quality have a considerable effect on the course of a fermentation, very little has been written about it. Casida (1968) wrote a brief chapter on this subject in general terms, while Nakayama (1981) recognized the process as being critical to scale-up of production. Recently information on the effects of inoculum on assay and productvity was gathered by Dietz (1981).

The starting point of the fermentation process is the laboratory stock culture previously prepared by any of the methods discussed in the preceding chapter. The inoculum is typically prepared in a stepwise sequence employing increasing volume of media.

3.2 FACTORS OF IMPORTANCE

A number of factors need to be considered to ensure that inoculum development is a satisfactory process. The following is based on reports by Casida (1968) and Nakayama (1981).

3.2.1 Type of Inoculum

With bacteria, rapidly growing cultures are often preferred. Cultures preserved as spores, such as *Clostridium aceto - butylicum*, are often subjected to a heat treatment known as "heat shock" to induce spore germination and obtain vegetative growth. With filamentous microorganisms such as fungi and actinomycetes, inocula may grow as pellets or as free mycelium. The subject of pelleting has been carefully reviewed by Whitaker and Long (1973) who point out that while the mycelial form of growth is usually preferred, in some cases, e.g. citric acid production, pellets are better.

The type of inoculum dictates how inoculum build-up should be initiated. It may be necessary to pregerminate fungal spores in a special medium if there is doubt whether the spore will germinate in the normal inoculum medium. The spores are often scraped off the surface of agar slants and prepared as a suspension in a suitable diluent. Non-sporulating or poorly sporulating actinomycetes or fungi are often transferred to the inoculum medium as fragmented hyphae.

The cells adapt themselves to changing conditions during the build-up, and must reach an adequate concentration to ensure a fast rate of production in the final fermenter.

3.2.2 Size of Inoculum

All steps of the inoculum development process, except that of the initial inoculation from a stock culture, require the transfer of approximately 0.5 to 1.0 percent inoculum by volume from the preceding step in the sequence. At each step, the organisms should grow quickly and in high numbers so that the period of incubation required is relatively short. Little if any fermentation product should accumulate during the inoculum stages, because

the cells should be transferred to larger batches of media while they are still in their logarithmic growth phase and before accumulation of product would normally occur. In fact, inoculum media are usually balanced for rapid cell growth and not for product formation.

Higher levels of inocula are commonly used in the production scale and may require separation of the inoculum from the medium. It should be insured that high levels of inoculum do not concurrently cause excessive dilution of the production medium, unwanted pH changes or carry-over of unwanted nutrients or metabolic waste products.

The quality and reproducibility of the inoculum are critical factors in reproducing high yields from one production to another and in obtaining valid experimental results in smaller tanks used in research studies. For these studies, enough inoculum should be available in a single batch so that it can be split uniformly and allow each tank to receive a similar inoculum.

3.2.3 Stability of Inoculum

No physiological or genetic change should occur during the sequence of transfers of microorganisms. However, although mutation does sometimes occur, it is usually infrequent and lethal to the cell, or at least provide no selective advantage over the non-mutated cells. However, problems may occur if the organism used for the fermentation is in itself a mutant strain. Mutant cells are not always stable and the frequency of back mutation might be high. Provisions should be made such that the media used select against back-mutants. Similarly, when dealing with processes involving inducible enzymes, provision may be made to include the inducer compound in all steps of inoculum development so that the required biochemical activity is maintained.

Further, stability of the inoculum is affected by culture maintenance methods which should preserve the genetic constitution of the stock or minimize back-mutation of mutant strains.

3.2.4 Contamination

Every stage of inoculum build-up should be examined for contamination. The final fermenter stage should be completely devoid of contaminants. Phage attack of the fermentation organism should be recognised and prevented.

3.3 CONCLUSIONS

Although some generalisations can be made about procedures for inoculum development, it is a subject which has not been investigated in any great detail.

Most workers manage to devise what they regard as a satisfactory situation concerning such procedures, but it appears to remain an art rather than a science.

It is clear, therefore, that for any particular fermentation process, the procedures adopted for inoculum development can influence the final fermentation stage, and these procedures must be optimized for each individual process.

CHAPTER 4

METHODS EMPLOYED FOR CULTURE MAINTENANCE AND INOCULUM DEVELOPMENT OF *Clostridium acetobutylicum*

4.1 THE USE OF *Cl. acetobutylicum* IN ACETONE-BUTANOL-ETHANOL FERMENTATION

The availability of large quantities of fossil fuels such as gas and oil has been mainly responsible for the technological and industrial boom of the last 35 years. However, the oil crisis of 1973 marked a turning point, and the possible revival of traditional fermentation processes is now being widely investigated.

The acetone-butanol fermentation has a long career as a successful industrial fermentation process. Its history is well documented by Prescott and Dunn (1959), Ross (1961), Hastings (1978) and Compere and Griffith (1979). Butanol was first discovered as a fermentation product by Pasteur in 1861. In 1911, Strange and Graham Ltd (England) obtained the service of Perkin & Weizman and Fernbach & Schoen of Manchester University and the Pasteur Institute, respectively, to study butanol fermentation for the production of synthetic rubber. This led to Fernbach's discovery of an organism capable of fermenting potato starch to a mixture of amyl alcohol, butanol, ethanol and acetone. In 1912, Weizman, working on his own, isolated an organism which yielded 10-20% of solvents which was four times higher than that produced by Fernbach's organism (Ross, 1961; Compere and Griffith, 1978). The organism is now known as *Clostridium acetobutylicum* Weizman.

The demand for acetone sharply increased during World War I, as a feedstock for cordite and airplane wing dope production. After the war, this demand markedly decreased,

and the fermentation would have assumed little further importance had it not been found that butanol was a good solvent for the nitrocellulose lacquers used on automobiles. Eventually the solvent market became so large that chemical production was developed. At the present time, butanol finds extensive use in brake fluids, antibiotic recovery procedures, urea-formaldehyde resins, amines for gasoline additives, as esters in the protective-coatings industry (Casida, 1968) and as a cosurfactant/coagent in tertiary oil recovery from existing wells (Compere and Griffith, 1979).

4.1.2 The Organism

The acetone-butanol fermentation is carried out by anaerobic bacteria belonging to the genus *Clostridium*. Two clostridial species have been historically used in, or developed for, solvent production, namely *Clostridium acetobutylicum* Weizmann and *Cl. beijerinckii* Donker (syn. *Cl. butylicum*). They are commonly known as "butyl" organisms and produce largely neutral products such as butanol and acetone from carbohydrate fermentation (George et al, 1982; Cummins and Johnson; 1971; Prescott and Dunn; 1959). *Cl. acetobutylicum* ferment starch, hexoses or pentoses to butanol, acetone and ethanol in the general ratio of 6:3:1 (Spivey, 1978). *Cl. beijerinckii* ferments hexoses or starch and produces largely butanol and smaller quantities of isopropanol.

Various butyric acid producing Clostridia, the umbrella group to which the butyl organisms belong, have been studied for their genetic relationships through DNA homology and cell wall composition (Cummins and Johnson, 1971). However, this study did not include strains labeled *Cl. butylicum*. George et al (1983) found the classification and nomenclature of *Cl. butylicum* vague. Their study revealed additional findings on the taxonomic status of *Cl. butylicum*. Despite the appearance of *Cl. butylicum* in current literature and its listings in various collect-

ions such as the American Type Culture Collection, *Cl. butylicum* is no longer a recognized species, nor is it considered a synonym for an existing species. The name did not even appear on the 1980 approved Lists of Bacterial Names (Cummins and Johnson, 1971; George et al, 1983).

In addition to genetic and phenotypic distinctions, the two species also exhibit some physiological differences. Recent reports have suggested that a low pH value (5.2 or below) and an elevated concentration of acetate or butyrate, or both, are required by *Cl. acetobutylicum* for transition to solvent production (Gottschal and Morris, 1981 a and b; Andersch et al, 1982; Bahl et al, 1982). *Cl. beijerinckii* requires neither a change in pH nor an acidic pH for the onset of butanol production (George et al, 1983). The specific strain utilized for fermentation also has a bearing on the solvent yield of butanol (Compere and Griffith, 1979).

4.1.2.1 Morphological characteristics

Bergey's manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) describes *Cl. acetobutylicum* as straight rods measuring 0.6-0.9 by 2.4-4.7 µm. They are Gram-positive but tend to be Gram-variable with age. The vegetative cells are motile with peritrichous flagella. However, motility is absent at certain stages of fermentation. Most strains form granulose and capsules.

Division occurs by transverse fission resulting in chains of organisms. These chains tend to be very long on solid media but break apart into single cells in liquid medium during vigorous fermentation.

They form spores which are oval, subterminal and approximately 1 µm by 1.5 µm in size. Spores are usually formed when growth conditions are no longer favourable e.g.

exhaustion of nutrients (Casida, 1968; Hastings, 1978; Ross, 1961).

The morphology of the culture varies with the strain, medium and culture stage. This variation can be used to monitor the progress of the fermentation process (Spivey, 1978). The morphological changes in *Cl. acetobutylicum* P262, the commercial strain used at National Chemical Products Ltd, Germiston, South Africa, have been studied and correlated with growth and solvent production (James et al, 1982; Long et al, 1983 and 1984).

Jones et al (1982) observed that the cells appeared as long chains with sluggish motility immediately after inoculation into the molasses fermentation medium. Motility increased after 6 hr when growth rate was maximum and rods were dividing. The chains also started to break up. After 14-18 hr, active growth ceased and motility decreased. Cells which were swollen, phase-bright and with forespores were observed before the pH break-point and are believed to be involved in the production of acetone and butanol. These are known as clostridial forms. This relationship between clostridial forms and solvent production was supported by the isolation of sporulation mutants. Such mutants either failed to form clostridial stages or produce solvents, or they formed reduced numbers of clostridial stages and produced intermediate levels of solvents.

Long et al (1983) characterized sporulation of strain P262 using a defined medium, for further studies on the regulation of solvent production and sporulation. The ability to sporulate had long been recognized to be associated with solvent production (Prescott and Dunn, 1959). Gottshal and Morris (1981b) showed that the ability to produce solvents in continuous culture is lost when there is a loss in the ability to form spores.

Optical and electron microscopic studies revealed that sporulation of P262 resembles that of other endospore-forming bacteria (Long, 1983). The process of sporulation involves the following stages (James and Young, 1969):

1. transitional stage from replicating cell to axial thread formation. The chromatin bodies change in shape and distribution, flowing one into the other, until cytologically there is a single rope or axial thread within each cell.

2. forespore development accompanied by "commitment" stage where the sporulating cell can no longer withdraw from completing a spore before entering vegetative growth.

3. cortex development.

4. coat protein deposition.

5. diaminopimelic acid and calcium accumulation.

6. spore release.

A most recent study on strain P262 sporulation revealed that the ability to form spores is not necessary for solvent production. This was demonstrated by the isolation of sporulation mutants which gave slightly higher levels of solvents than the wild type strain. The results gave strong evidence that it was the clostridial forms that were involved in butanol production rather than spore-forming ability. Further, the formation of specialized structures such as granulose and capsule is neither a function of the clostridial stage nor sporulation, and are not required for the shift to the solventogenic phase (Long et al, 1984).

Strain differences with regard to sporulation were observed between P262 and culture collection type strains (Long et al, 1983). Strain ATCC 824 showed poor sporulation on both complex and minimal media. Sporulation by ATCC

10132, NRRL 527, 592 and 593 on complex media was comparable to P262. There was no mention of clostridial forms in the culture collection strains and they generally produced lower levels of solvents than P262 from a molasses fermentation medium.

4.1.2.2 Cultural characteristics

Cl. acetobutylicum form surface colonies which are raised, circular and about 3-5 mm in diameter. The colonies appear grayish white, with translucent glossy surface and irregular margin (Buchanan and Gibbons, 1974). As with many clostridia, spreading growth is common on the surface of agar media.

Colonial variation due to differences in the degree of sporulation in individual colonies may be observed. A colony with a high proportion of spores is much more opaque than on composed largely of vegetative cells.

Abundant growth occurs in medium containing fermentable sugars.

Colonial differences also occur between normal and degenerated cells (Kutzenok and Aschner, 1952). When plated on a solid medium, cells from a normal, i.e. solvent-producing, fermentation always formed large mucoid colonies in which gas bubbles could sometimes be seen. This was irrespective of whether the inoculum consisted of spores, bacillary forms or polysaccharide material containing chiefly clostridial forms. Degenerated cells, i.e. from a non-solvent producing fermentation developed colonies which were rather flat and small with no trace of either mucoid material or gas bubbles. Microscopic examination revealed colony edges consisting of chains of bacteria arranged in a wavy pattern. Normal or healthy, mucoid colonies could be subcultured without difficulty.

Degenerated colonies could not be subcultured.

4.1.2.3 Physiological and biochemical properties

4.1.2.3.1 Biochemistry of fermentation

The biochemical steps in the formation of butyric acid and butanol are now well understood (Brock, 1979; Lenz and Moreira, 1980; Doelle, 1981; Volesky, 1981; Papoutsakis, 1984). Glucose is converted to pyruvic acid via the Embden-Meyerhof-Parnas (EMP) pathway (Fig.4.1). The breakdown of pyruvate is characteristic for clostridia and is often referred to as the 'clostridial type'. Through a phosphoroclastic reaction, pyruvate undergoes decarboxylation to form acetyl-CoA, CO_2 and H_2. The electrons and hydrogen released are not transferred to NAD^+ but are used to reduce ferredoxin. The reduced ferredoxin, FdH_2, is then reoxidized by a hydrogenase and molecular hydrogen is liberated. This switch of coenzyme is very important metabolically (Doelle, 1981). Since clostridia are obligate anaerobes, ferredoxin, with a much lower redox potential, maintains anaerobic conditions and prevents further electron transfer.

Acetyl-CoA is the precursor for all the fermentation products. An interesting feature of the pathway is the condensation of two acetyl-CoA molecules to form acetoacetyl CoA initiating a cyclic mechanism that leads to the formation of butyric acid. The reduction of acetyl-CoA to ß-hydroxybutyryl-CoA, and the formation of butyryl-CoA from crotonyl-CoA, involve the oxidation of the 2 moles of $NADH_2$ produced during pyruvate formation. The last step in the cycle is the transfer of the CoA moiety from butyryl-CoA to acetate giving acetyl-CoA and butyrate. Acetyl-CoA can reenter the cycle either as acetyl-CoA or be converted to acetate and be available for further transfer reactions.

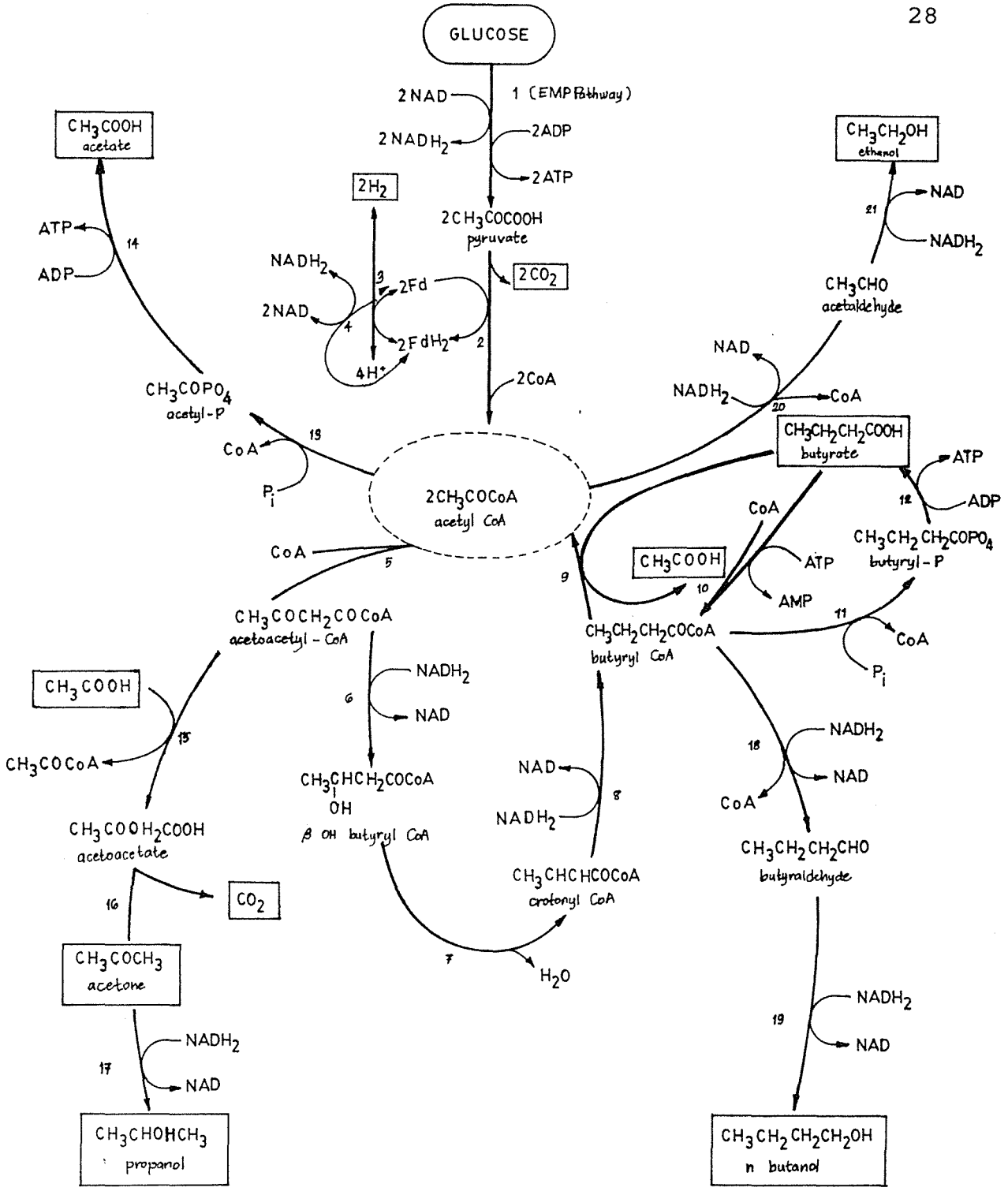


FIGURE 4.1: Biochemical pathways of glucose fermentation by butyric acid bacteria. The pathways or enzyme systems catalyzing the reactions shown are as follows: (1) phosphoenolpyruvate phosphotransferase system and the Embden-Meyerhof pathway, (2) pyruvate-ferredoxin oxidoreductase, (3) hydrogenase, (4)NADH-ferredoxin oxidoreductase, (5) acetyl-CoA-acetyl transferase, (6) L(+)-ß-hydroxybutyryl-CoA dehydrogenase, (7) L-3-hydroxyacyl-CoA hydrolase, (8) butyryl-CoA dehydrogenase, (9) CoA transferase, (10) butyryl-CoA synthetase (ATP/AMP), (11) phosphotransbutyrylase, (12) butyrate kinase, (13) phosphotransacetylase, (14) acetate kinase, (15) CoA transferase, (16) acetoacetate decarboxylase, (17) isopropanol dehydrogenase, (18) butyraldehyde dehydrogenase, (19) butanol dehydrogenase, (20) acetaldehyde dehydrogenase, and (21) ethanol dehydrogenase.

A transferase system present in *Cl. acetobutylicum* diverts acetoacetyl-CoA from the normal cyclic mechanism to produce acetoacetate which is then decarboxylated to acetone. This interruption of the cycle would be detrimental to the cell since NAD^+ cannot be regenerated. Alternative reducing sequences are, therefore, accomplished by the cell by using butyraldehyde dehydrogenase and butanol dehydrogenase in a series of reactions which finally convert the accumulated butyrate to butanol.

Cl. beijerinckii is able to reduce acetone further and produce isopropanol.

4.1.2.3.2 Course of fermentation

The typical growth and product formation profile in the batch ABE fermentation by *Cl. acetobutylicum* (Prescott and Dunn, 1959; Spivey, 1978; Volesky et al, 1981) is shown in Fig. 4.2. The whole process consists of 2 phases corresponding to the two-stage mechanism of product formation. In the first phase active growth occurs, and acetate and butyrate are formed bringing the initial pH of 6.0 to about pH 5.3. The titratable acidity correspondingly increases. This phase is known as the acidogenic phase. The second phase, called the solventogenic phase, occurs from 12 to 36-60 hr. Little growth is observed. Solvent synthesis commences as the fermentation reaches a pH break-point after which the pH rises. The decrease in titratable acidity indicates conversion of the acids to the corresponding solvents.

4.1.2.3.3 Oxygen sensitivity and Eh requirement

Cl. acetobutylicum requires anaerobic condition for growth. Its reductive metabolism leads to the creation and maintenance of a low, i.e. negative culture redox potential, Eh. Optimal growth occurs over an Eh range of -250 mV to -400 mV.

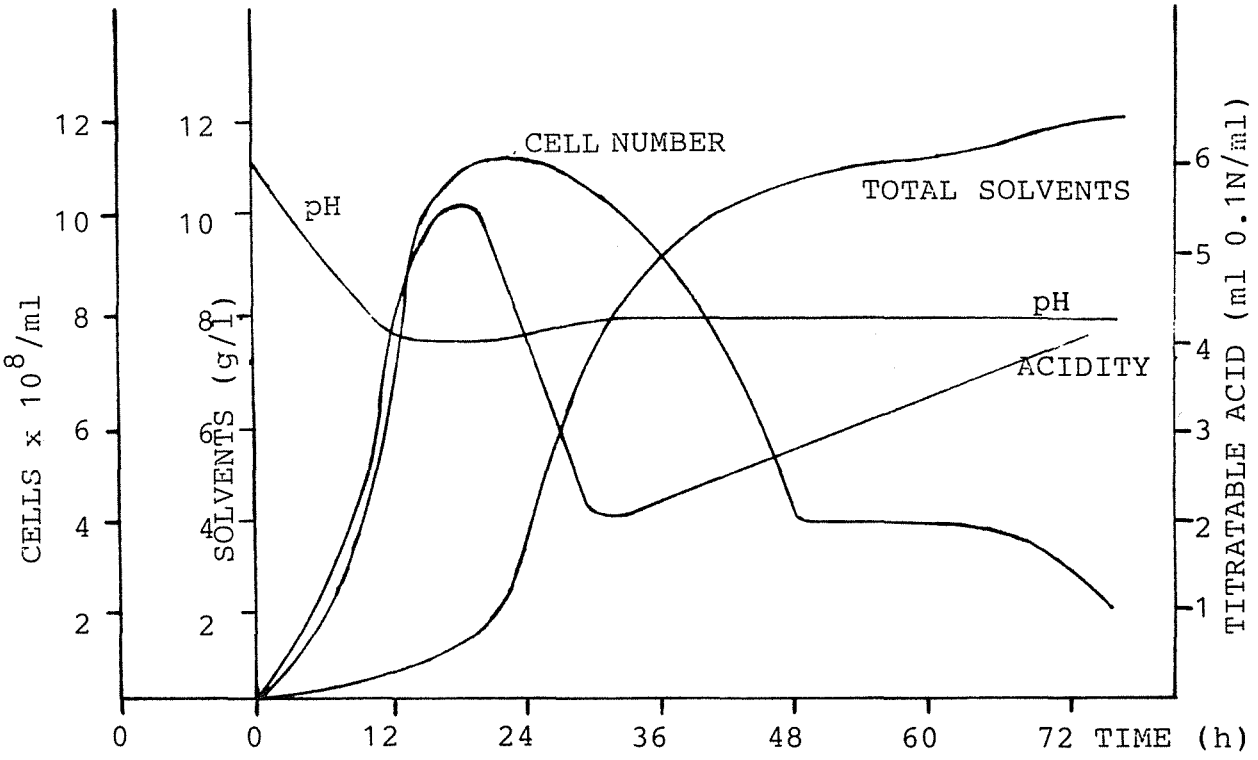


Figure 4.2: A typical profile of process parameter in the batch ABE fermentation as carried out by *Cl. acetobutylicum*.

Studies by O'Brien and Morris (1971) revealed that the dissolved O_2 concentration rather than the Eh affects growth and metabolism of *Cl. acetobutylicum*. The rates of growth, glucose consumption and production of acetate, butyrate and pyruvate remained normal in glucose and casein hydrolysate basal medium at very high Eh values but low dissolved O_2 concentration. Growth ceased when the culture was made aerobic with 40 μM dissolved O_2 but the same high Eh value.

Further, normal growth and metabolism were observed under aerated conditions consisting of Eh -50 mV and <1 μM O_2 concentration. This suggests that the organism could afford to divert a considerable part of its reducing power in which the primary electron donors generated by the organism, such as NADH, were being consumed to detoxify any exogenous oxygen. This was evident in the increase of NADH oxidase activity of the organisms as a result of aeration. Levels of intracellular ATP decreased with aeration but whether this was due to the decrease in glucose consumption rate could not be ascertained.

The effect of oxygen on the organism was reversible, with no loss of viability observed after exposures of from 4 to 6 hrs.

The germination of clostridial spores is generally less subject to O_2 inhibition than is the subsequent vegetative growth of the organism (Morris and O'Brien, 1971). However, the sensitivity to O_2 of the germination process is a function of the species, strain and composition of the medium. While some species show only delayed germination, *Cl. acetobutylicum* and *Cl. beijerinckii* require a totally anaerobic environment for spore germination.

4.1.2.3.4 Temperature and pH requirement

Cl. acetobutylicum is a mesophilic bacterium with an optimum growth temperature of 37°C (Buchanan and Gibbons, 1974). The optimal pH for growth is 6.5, while solvent production is favoured at pH 4.5 (Spivey, 1978).

4.1.2.3.5 Substrate range and nutritional requirements

Cl. acetobutylicum can utilize a wide variety of substrate owing to its unusual ability to ferment not only hexoses but also pentoses and oligosaccharides (Compere and Griffith, 1979; Mes-Hartree and Saddler, 1982; Maddox, 1982).

It is apparent from these properties that the organism could be useful for the conversion of many different liquid waste streams e.g. wood hydrolysates (Mes-Hartree and Saddler, 1982), whey filtrate (Maddox, 1980) and agricultural residues e.g. bagasse, wheat and rice straws (Marchal et al, 1984; Soni et al, 1982) for solvent production. Recent works have focused on the optimization of conversion of lignocellulosic residue to butanol (Yu, et al, 1984; Saddler et al, 1982; Saddler, et al 1983).

Biotin and p-aminobenzoic acid are necessary for the growth of some acetone-butanol organisms. These requirements may be satisfied by the use of yeast extract or other raw materials containing the essential vitamins (Prescott and Dunn, 1959). Yeast extract is also used for some strains to supply degraded protein nitrogen.

Monot et al (1982) found that Mg^{2+} and K^{+} promoted growth and solvent production, respectively. Baghlaf et al (1980) reported that on a complex medium, K_2HPO_4 is essential with an optimum concentration of 1-2 g/l. Davies (1942) also found that the growth of *Cl. acetobutylicum* on maize meal is K^{+} dependent. With ammonium acetate present, glucose could be utilized well but the reduction of the acids was supressed (Monot et al, 1982).

4.1.2.3.6 Metabolic regulation

A number of studies using batch and continuous cultures have investigated the relationship between the environmental conditions and acetone/butanol production (Prescott and Dunn, 1959; Spivey, 1979; Bahl et al, 1982a, b; Monot et al, 1982; Bu' Lock and Bu' Lock, 1983).

Gottschal and Morris (1982) reported from their experiments using turbidostat-culture that at high cell density the major products of glucose fermentation were solvents, in contrast to the essentially acidogenic fermentation when cell density was low. Monot et al (1982) reported that glucose at low concentrations of 5 and 10 g/l, in batch fermentation of synthetic medium, promoted acids but not solvents production.

When *Cl. acetobutylicum* NCIB 619 was grown in fed-batch cultures with different feeding rates it was observed that sugar utilization increased with increasing glucose flow, and that solvent production was not inhibited at high flow rates (Fond et al, 1984). Wang (1981) reported high productivities in continuous culture using high glucose and nitrogen concentrations in complex media. Bahl et al (1982b) showed that solvents are primarily produced under phosphate limitation and at pH values between 4.3 and 4.7 in continuous culture. Nitrogen limitation was suggested as another factor that may promote production of acetone/butanol, both in batch and continuous culture (Monot and Engasser, 1983a). Although poor solvent production was obtained from NH_4-limited chemostats by both Gottschal and Morris (1981b and Andersch et al (1982), other factors such as the high pH, low glucose concentration and high dilution rate may have been responsible for these observations.

The pH of the medium is recognized as an essential regulating parameter for solvent production. Bahl et al (1982a) and Nishio et al (1983) found that the optimum pH of 4.3

promotes the transition from acidogenic to solventogenic phase. High level of acetate led to rapid induction and increased concentration of acetone (Gottschal and Morris 1981a; Martin et al, 1983). The butyric acid concentration was found to be another important regulatory factor. The addition of butyric acid at a sufficiently low pH resulted in solvent formation (Gottschal and Morris, 1981a Bahl et al, 1982a; Martin et al, 1983; Monot et al, 1984).

The effects of pH and butyric acid were further elucidated by Monot et al, 1984). They established that both cell growth and solvent production are strongly pH dependent. In batch culture at pH 4.5, there was a 2-phase process corresponding with glucose and acid utilization, and with predominantly solventogenic fermentation at higher pH values; a single growth-related acetic and butyric production phase, with negligible solvent production, was observed. The inhibitory and inductive effects of butyric acid on growth and solvent production, respectively, are influenced by the concentration of the undissociated form of this metabolite. A scheme for the regulatory mechanism (Fig. 4.3) was proposed by Monot and Engasser (1983). Within this regulation pattern, ammonium ions activate cellular growth and butyric acid production. An excess of nitrogen, however, inhibits the production of solvents.

Studies have been conducted to determine the level of enzymes which are exclusively involved in reactions leading either to acid or solvent production (George and Chen, 1983; Andersch et al, 1983; Hartmanis and Gatenbeck, 1984; Hartmanis et al, 1984). However, no definitive results have been obtained that would facilitate the manipulation of enzyme synthesis to maximize production.

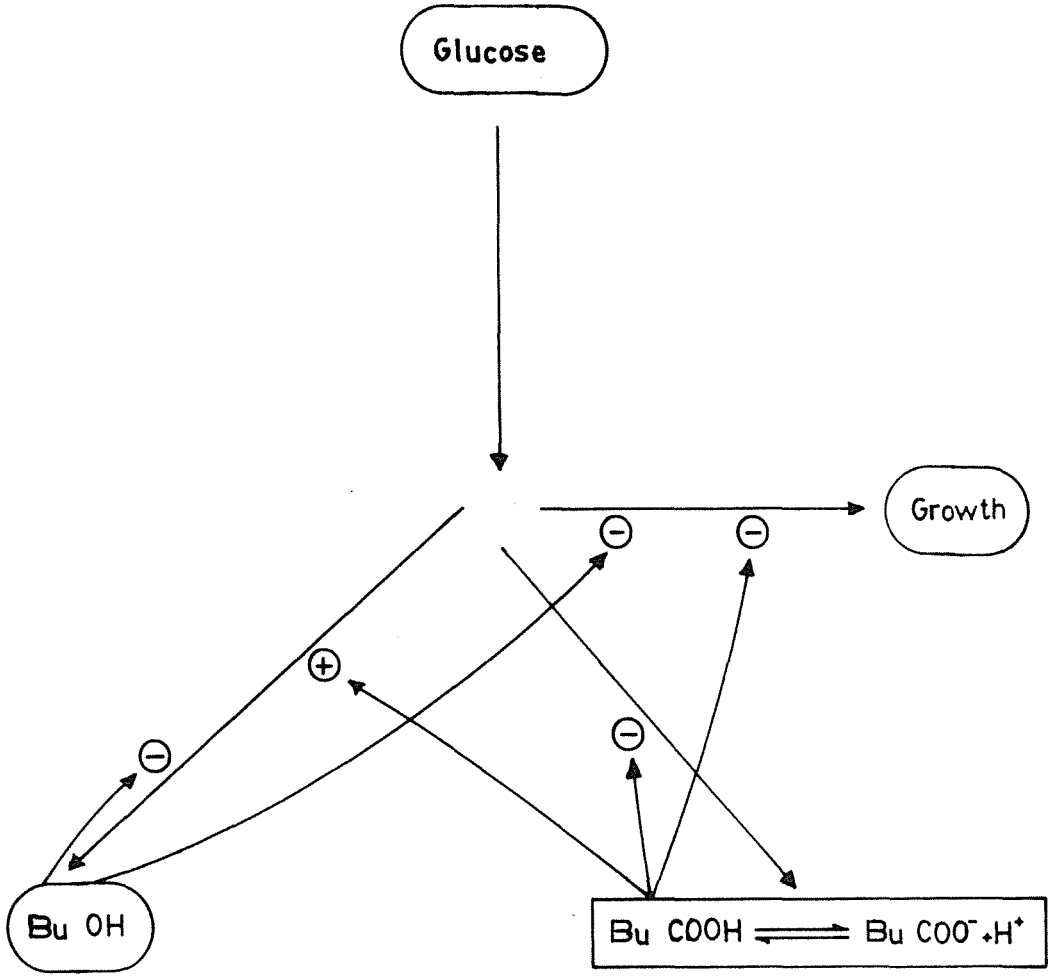


Figure 4.3: Proposed regulation of growth and metabolism of *Cl. acetobuylicum* by its metabolites, showing both positive and negative effects.

4.1.2.3.7 Butanol tolerance

The production of butanol by *Cl. acetobutylicum* is subject to end-product inhibition at butanol concentration of less than 20 g/l (Spivey, 1978; Moreira et al, 1981). Acetone and ethanol are less inhibitory (Maddox, 1982; Costa and Moreira, 1983). Various studies have suggested that butanol adversely affects the lipid bilayer of the membrane by dissolving it and increasing its fluidity. This consequently alters the membrane functionality for nutrient transport (Moreira et al, 1981; Costa and Moreira 1983). The effect of butanol on lipid composition and fluidity was further elucidated by Vollherbst-Schneck et al (1984). They found a 20-30% increase in fluidity caused by 5 to 15 g/l of added butanol. Cells were observed to shift fatty acid synthesis to higher saturated acyl chains as a homeoviscous response to butanol.

An autolysin which has an antibiotic-like activity was observed to be produced by the organism towards the end of the exponential growth phase. Lysis is effected and, hence, inhibition of solvent production (Barber et al, 1979; Webster et al, 1981; Allcock et al, 1981). Westhuizen (1982) correlated autolytic activity and butanol tolerance in a study using an autolytic mutant. This type of mutant was more resistant to butanol toxicity. Results suggested that butanol at inhibitory concentrations induced autolysis and degeneration of the clostridial forms which are involved in solvent production.

Development and isolation of mutants of *Cl. acetobutylicum* such as specific lipid synthesis mutants and autolysin-deficient mutants could overcome the limitation of butanol toxicity (Allcock et al, 1981; Vollherbst-Schneck, 1984; Moreira et al, 1981).

4.2 CULTURE MAINTENANCE

4.2.1 Introduction

The earliest observation that the inoculum affects the ABE fermentation was made by Kutzenok and Aschner (1952). These authors found that repeated subculturing resulted in the culture becoming sluggish, leading eventually to decreased solvent yield and loss of viability. However, no definitive study has since been conducted on the effect of preservation method and seed build-up on the performance of *Cl. acetobutylicum*. Thus, the next two chapters review the different methods of preservation and inoculum development procedures that have been employed for *Cl. acetobutylicum*.

4.2.2 Methods

Cl. acetobutylicum can be successfully stored by freeze-drying. The strains kept at NCIB were found to have a minimum storage life of 10 years when held at 20-25^{o}C (Lapage et al, 1970). However, since freeze drying can be laborious and expensive it is not employed in the traditional fermentation process (Spivey, 1978).

The Clostridia are usually maintained as spores rather than vegetative cells since the former are not as sensitive to oxygen or other environmental conditions. (Prescott and Dunn, 1959; Ross, 1961; Underkofler and Hickey, 1954; Raper, 1963; Spivey, 1978; Hastings, 1978; Calam, 1980). Spores are easily maintained in soil and are known to be viable for as long as 13 years. Underkofler and Hickey (1954) described the procedure in detail. A carrier composed of 49% rich loam, 49% screened, washed sand and 2% calcium carbonate was found satisfactory. Maintenance in soil was the method used in early studies (Davies and Stephenson, 1941; Sjolander and Langlykke, 1938; Beesch, 1952; Langlykke et al, 1948).

Spivey (1979) described how the 2 strains of *Cl. acetobutylicum* used then at National Chemical Products, South Africa were being maintained. The organisms were stored on a routine basis as spores. Either the spore culture or vegetative cells were inoculated into potato/glucose medium and allowed to grow at 34°C for 48 hr. A small portion of this culture was transferred to a similar medium after a heat shock treatment at 70°C for 1.5 min. The subsequent culture was then allowed to stand at 34°C for 3-4 days to induce sporulation, from which a portion was transferred to sterile sand/soil and allowed to dry. A 100% germination after several years of storage could be obtained from this soil stock. Another industrial strain, P262, was maintained as clean spores in distilled water, refrigerated at 4°C, before being used in studies to optimize solvent production (Long et al, 1984; Long et al, 1983; Jones et al, 1982; Barber et al, 1979; Westhuizen et al, 1982). Using ATCC 824, Haggstrom and Molin (1980) kept the spores frozen in a phosphate buffer. These methods using non-nutrient suspending fluids appear to be simple and economical. In contrast to these was the suspension of spores of DSM 1731 in milk medium previously boiled under a stream of oxygen-free nitrogen. The presence of milk components in maintenance medium increases viability (Heckly, 1978).

Media providing reducing substances have been used for maintenance without other anaerobic precautions being taken. Oxoid or Difco Cooked Meat Medium and ATCC liver medium have been commonly used (Compere and Griffith, 1979; Maddox, 1982; O'Brien and Morris, 1971; Allcock et al, 1981). Spores of ATCC 824 were kept in Reinforced Clostridial Medium at 4°C (Martin et al, 1983; Monot& Engasser, 1983a, b). Potato also provided reduced and enriched conditions to *Clostridium*. Baghlaf et al (1980) and Abou-Zeid et al (1978) used potato medium with the following composition:

	g/l
wet potato mash	250
glucose	5
$CaCO_3$	2
$(NH_4)_2SO_4$	1.5

for spores of an unspecified strain of *Cl. acetobutylicum*. Nishio et al (1983) suspended strains DSM 1732 and 792 in liquid potato medium for maintenance.

Fermentation substrates have been used as maintenance media, e.g. maize meal at 5% solution in tap water, for ATCC 824 (Monot et al, 1982), whey permeate for weekly transfer of NCIB 2951 (Maddox, 1980) and 4.2% corn starch for storage at -196 ^{o}C of a variety of strain including ATCC 824 (Lin and Blaschek, 1983).

Maintenance techniques involving frequent transfers have often been avoided as *Clostridium* is prone to bacteriophage attack (Ogata and Hongo, 1979). The possibility of phage attack could not be discounted in the fermentations carried out by Kutzenok and Aschner (1952) where progressive sub-culturing led to decreased solvent production. This might be true, too, with strain NRRL B-592 which underwent six passages of serial transfer in sulphuric acid whey permeate containing yeast extract (5 g/l), with heat shocking after each inoculation (Gapes, 1982). Degeneration i.e. progressive loss of solvent-producing ability, was rapid after the third transfer and by the seventh transfer very little solvent was produced in subsequent fermentation.

While a number of studies either paid no attention to the stock used or just mentioned the possible influence of the inoculum on production, there is evidence that the stock plays a role in the optimization of the process. Maddox (1983) perceived and explained that the discrepancy in the maximum butanol concentration from two studies

using the same strain NCIB 2951 was due to the differing methods of culture maintenance used. A maximum of 15 g/l was obtained when the culture was sub-cultured weekly in whey permeate containing yeast extract (5 g/l), followed by heat shocking at 70° C for 90 sec (Maddox, 1980). A lower butanol concentration, 8.6 g/l resulted from cultures maintained in Cooked Meat Medium at 4° C (Maddox, 1982). A decrease in production from 1.1 g/l to 0.6 g/l butanol was also observed with *Cl. beijerinckii* when whey permeate was inoculated with stocks that underwent two transfers and 37 transfers with heat shocking, respectively (Schoutens et al, 1984).

4.3 INOCULUM DEVELOPMENT FOR FERMENTATION

4.3.1 Introduction

Similar to culture maintenance, the inoculum for ABE fermentation has been prepared in a variety of manner. The heat shocking regime, volume of inoculum, number of culture stages and the medium (a) in pre-fermentation stage(s) account for the differences in inoculum development.

4.3.2 Methods

Heat shocking has been conventionally employed as a means to ensure successful fermentation. This practice of heat shocking and successive subculture were commonly used to activate a culture (Prescott and Dunn, 1959; Spivey, 1978). Before the discovery of the direct effect of sublethal heating to induce spore germination, heat shocking was primarily believed to destroy the vegetative forms and the weaker spores. This left the most active spore-formers which appeared to be the most vigorous fermenters.

Heat shocking at boiling temperature was used in early works to activate soil stocks. Langlykke et al (1948) subjected the stock transferred to potato-glucose medium to 100°C for 50 sec. Beesch (1952) heat shocked the stocks in boiling water for 2 min before cooling to 30.6°C. Tubes of space suspensions from soil stock together with a few drops of fermenting medium were heated in boiling water for 10-40 sec (Kutzenok and Aschner, 1952).

For the industrial production of butanol by strains P265 and P270, the fermentation was initiated by heat shocking the spores suspended in saline at 70°C for 90 seconds followed by rapid cooling in an ice bath. This then underwent 4 culture stages before seeding the plant fermenter at a ratio of 1:3000 (v/v) (Spivey, 1978). Incubation times for each seed stage varied with the strain of organism and with the composition of the medium.

Soil stocks of P262 were activated by a heat shock of 75°C for 2 min (Westhuizen et al, 1982; Jones et al, 1982) before inoculation into the *Clostridium* basal medium (CBM) of O'Brien and Morris (1971). Exponential-phase CBM cultures were then used to inoculate the molasses fermentation medium.

Cl. acetobutylicum NCIB 8052, maintained as spores in Cooked Meat Medium, was activated by heat shocking at 80°C for 5 min. It was carried through ten vegetative transfers before inoculating the fermentation medium with a freshly activated spore inoculum (O'Brien and Morris, 1971).

Haggstrom and Molin (1980) activated ATCC 824 spores, kept in phosphate buffer, by a heat shock of 95°C for 30 min. This was followed by 2 successive transfers for sporulation in a semi-synthetic glucose medium before immobilization in calcium alginate. The immobilized spores were heat shocked in the same manner.

Spores of ATCC 824 freshly transferred to Reinforced Clostridial Medium, were subjected to 80° C for 45 min before anaerobic incubation and subsequent use in fermentation studies (Martin et al, 1983).

In place of heat shocking, spore activation can be achieved by the use of ethanol. This has been found successful for spores of clinically important Clostridia (Koransky, 1977). Krouwel et al (1981) developed a method to start a fermentor with immobilized spores of *Cl. beijerinckii*. At 50% (v/v) concentration of ethanol, sterilization of the biocatalyst particles in conjunction with spore activation was achieved.

No heat shocking procedure or ethanol treatment was noted for ATCC 824 spores kept in RCM at 4° C (Monot, 1984). Neither was used for stocks of the same strain kept at -196°C in 4.2% corn starch soluble medium (Lin and Blaschek, 1983). Another variation of inoculum start-up is apparent with regard to the number of culture stages before the actual fermentation. A single stage culture was used after vegetative growth in RCM (Mes-Hartree and Saddler, 1982) and in mixture of blackstrap molasses and corn bran flour (Abou-Zeid et al, 1978 and Baghlaf et al 1980). At least three transfers of ATCC 824 were made in synthetic medium before running the fermentation (Monot et al, 1982). There was no mention of any heat shocking treatment.

The level of inoculum commonly used is 5% v/v (Maddox, 1980 and 1982; Abou-Zeid et al, 1978; Baghlaf et al, 1980).

While some studies, particularly those performed using continuous culture, fail to report inoculum development procedures, results are now available suggesting the influence of culture treatment on solvent production. The difference in production of butanol by NCIB 2951 may be accounted for by variation in inoculum build-up employed

in two investigations by Maddox (1980, 1982). The culture which passed through 10 transfers in whey permeate with a heat shock treatment of 70°C from 90 sec at each transfer produced higher butanol concentration than the stock which passed through a single heat shock treatment and prefermentation stage. Nishio et al (1983) noted that butanol production by DSM 792 increased by about 3 times when potato medium was used instead of glucose in the prefermentation stage.

4.4 CONCLUSIONS

A variety of methods of culture maintenance and inoculum development have been applied to *Clostridium acetobutylicum*. Evidence is available that the nature of these culture treatments has a direct influence on the ability of the organism to produce solvents. A standard method for optimum production still waits to be recommended, although it should be noted that different strains might have different requirements.

CHAPTER 5

PRODUCTION OF ACETONE-BUTANOL-ETHANOL BY *Cl. acetobutylicum* FROM WHEY AND WHEY PERMEATE

5.1 STATE OF THE ART

Despite the preference of the butyl bacteria for glucose over lactose (Compere and Griffith, 1979; Abou-Zeid, 1976) and strain differences for lactose utilization, ABE production from whey and whey permeate seems to be an attractive proposition. The process can be considered a typical example of fuel production from waste materials. Whey, a by-product from casein and cheese manufacture contains about 6% total solids consisting mainly of lactose (80% of total solids), protein, non-protein nitrogen and minerals. Despite the removal of whey proteins via ultra-filtration processes, the filtrate or permeate still has a high BOD value (approx. 80,000 mg/l).

Maddox (1980) found sulphuric acid whey permeate a useful fermentation substrate. Butanol concentrations as high as 15 g/l were produced by NCIB 2951 from the permeate (53 g/l lactose) supplemented with 5 g/l yeast extract. The pH of the substrate was adjusted to 6.5 prior to sterilization and fermentation was carried out at 30^{o}C. The ratio of the ABE solvents were 1:10:1. Gapes (1982) identified the head space pressure as a factor affecting solvent production. Using larger scale fermenters under the same condition as Maddox (1980), a positive head-pressure was found necessary to obtain high yields of products at high production rates.

Conditions necessary for butanol production from whey ultrafiltrate by *Cl. beijerinckii* LMD 27.6 were established by Schoutens et al (1984). Production was compared with

those obtained from media of yeast extract containing various concentrations of lactose, glucose and/or galactose. The effect of medium composition, incubation temperature, pH and inoculum development were also investigated. Temperature was observed to be a critical factor. Higher production and sugar utilization from whey filtrate were observed when the temperature was shifted from 37° C to 30° C. The other factors did not improve fermentation at 37°C. The mean solvent productivity from whey ultrafiltrate was 2-3 times lower than that found on glucose but the overall product yields from glucose and lactose were comparable.

Schoutens et al (1984) also studied glucose and galactose mixture to determine the possibility of fermenting hydrolyzed whey permeate. The results showed that galactose could be utilized as a substrate, but that glucose was preferred.

Using *Cl. acetobutylicum* ATCC 824, Welsh and Veliky (1984) also demonstrated butanol production from acid whey. They further demonstrated that autoclaving increased solvent yields while agitation of the culture had an unfavourable effect.

5.2 POTENTIAL AND LIMITATIONS

Lenz and Moreira (1980) conducted a feasibility study on the use of liquid whey waste as feedstock for butanol production. The process was found to be more economic than using molasses feed. A discounted cash flow rate of return from 25-30% using whey places the process in a superior economic position.

However, the potential of the process can only be fully exploited once the drawbacks inherent to it can be overcome. Butanol toxicity limits the production of butanol to only about 19 g/l. The fermentation is subject to contamination either by bacteriophage or *Lactobacilli* . Further, the organism tends to degenerate and, therefore, requires a delicate method of handling and propagation. The process employs an anaerobic organism which poses technical difficulties to ascertain its physiology and to develop its genetic transfer systems. Research is still underway to identify the triggers necessary for transition from acidogenic to solventogenic phase of production.

5.3 CONCLUSIONS

Typical of any system, the production of solvents from whey and whey permeate has its own share of potentials and shortcomings. However, studies on the process and the organism are continuing in many laboratories. Findings await to be applied to allow the fermentations production of ABE solvents to once more become a commercial reality.

CHAPTER 6

MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 Media

Reinforced Clostridial Agar was purchased from BBL Microbiology Systems, Cockeysville, Maryland, U.S.A.

Yeast extract and Cooked Meat Medium were obtained from Difco Laboratories, Detroit, Michigan, U.S.A.

Merck Agar was purchased from BDH Chemicals Ltd., Palmerston North, New Zealand.

Sulphuric acid casein whey permeate was obtained from the New Zealand Dairy Research Institute, Palmerston North, New Zealand. Its preparation is described by Matthews et al (1978).

6.1.2 Sugars

Glucose was supplied by BDH Chemicals, Ltd., Palmerston North, New Zealand.

Lactose was purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

6.1.3 Chromatography Standards

Acetone, butanol and ethanol (UNIVAR grades) were from Ajax Chemicals, Sydney, Australia.

6.1.4 Gases and Other Chemicals

Hydrogen and oxygen-free nitrogen were obtained from New Zealand Industries Gases, Ltd., Palmerston North, New Zealand.

Industrial grade ammonia solution was purchased from Andrew Chemical Co. Ltd., Takapuna, New Zealand.

6.2 MEDIA PREPARATION

Commercial media were prepared prior to autoclaving according to the instructions from the manufacturer. For Reinforced Clostridial Agar (RCA), Merck agar was added to give a final agar concentration of 17 g/l. This served to minimize shattering of agar, due to gas production, and swarming of colonies.

Whey permeate agar (WPA) was prepared by adding agar at 15 g/l to whey permeate and adjusting the pH to 6.5 using aqueous ammonia prior to autoclaving.

6.3 STERILIZATION OF MEDIA, GLASSWARE AND EQUIPMENT

All media were sterilized in the autoclave at 121^{o}C for 15 min.

Pipettes, test tubes and bottles were sterilized in the hot air oven at 160^{o}C for 2 hr.

6.4 ORGANISMS

Clostridium acetobutylicum NRRL B-594 (ATCC 10132) was obtained as a freeze-dried specimen from the Northern Regional Research Centre, U.S. Department of Agriculture, Peoria, Illinois, U.S.A.

Clostridium acetobutylicum NCIB 2951 was purchased as a freeze-dried specimen from the National Collection of Industrial Bacteria, Aberdeen, Scotland.

6.5 ANAEROBIC INCUBATION

Culture for anaerobic incubation were placed in Baird and Tatlock anaerobic jars (Baird and Tatlock Ltd., Chadwell Heath, Essex, U.K.).

An anaerobic atmosphere was achieved through generation of hydrogen and carbon dioxide from a GasPak 100 disposable envelope (BBL Microbiology Systems, Cockeysville, Maryland, U.S.A.).

Check on the development of anaerobic conditions in the jar was accomplished using a BBL GasPak anaerobic indicator.

The palladium catalyst in wire gauze capsule was rejuvenated prior to use by direct flaming until red hot.

6.6 REVIVAL OF FREEZE-DRIED CULTURES

Ampoules of freeze-dried cultures were wrapped in sterile cotton wool and opened aseptically by cutting with a sharp file or diamond pencil.

The cultures were suspended in 20 ml of Cooked Meat Medium, supplemented with glucose at 10 g/l (CMMG), and incubated at 30°C until good gassing was observed.

6.7 PRODUCTION OF SPORES

6.7.1 *Clostridium acetobutylicum* NRRL B-594

Slopes of Reinforced Clostridial Agar (RCA) contained in 25 ml bottles were inoculated with the revived culture in CMMG.

The slopes were incubated anaerobically at 30° C for 4 days.

Spore fermentation was checked by Bartholemew and Mittwer's spore staining method using malachite green (Harrigan and McCance, 1966).

6.7.2 *Clostridium acetobutylicum* NCIB 2951

Slopes of Whey Permeate Agar (WPA), contained in 25 ml bottles, were inoculated with the revived culture and incubated anaerobically at 30°C for 15 days.

Spore formation was checked periodically by spore staining.

6.8 MAINTENANCE OF STOCK CULTURES

6.8.1 Storage in Distilled Water at 4°C

Sterile distilled water was pipetted onto sporulated slope cultures (7 ml/slope). Growth was aseptically dislodged with the pipette until the resultant suspension could be withdrawn. The suspension was transferred to a sterile

screw-capped test tube (16 x 100 mm) and this became the stock culture.

Stocks were kept at $4^{o}C$.

6.8.2 Storage in Phosphate Buffer at $4^{o}C$

Spore suspensions were prepared from slope culture following the addition of 0.012 M K_2HPO_4 - KH_2PO_4 buffer, pH 6.5 (7 ml/slope).

The stocks were kept in sterile screw-capped test tubes at $4^{o}C$.

6.8.3 Freezing in Distilled Water at $-20^{o}C$

Sterile distilled water containing glycerol as cryoprotective agent (30.5 ml per 100 ml of water) was used to prepare the spore suspension as described in Section 6.8.1.

Stocks in screw-capped test tubes were kept in the freezer at $-20^{o}C$.

6.8.4 Freezing in Phosphate Buffer at $-20^{o}C$

Sterile 0.012 M K_2HPO_4 - KH_2PO_4 buffer, pH 6.5, supplemented with glycerol at 30.5 ml per 100 ml buffer, was used to prepare the spore suspension as described in Section 6.8.1.

Stocks in screw-capped test tubes were kept in the freezer at $-20^{o}C$.

6.8.5 Drying in Soil

A soil mixture was prepared with the following composition:

49% garden soil
49% screened sand
2% $CaCO_3$

The mixture was sifted through a course sieve before pouring into 25 ml McCartney bottles at 3.5 g each. The bottles were plugged with cotton wool and sterilized in the autoclave in a slanting position to spread the soil in as thin a layer as possible. Sterilization was at 121° C for 1 hour for 3 consecutive days.

One ml of spore suspension in distilled water, prepared as in Section 6.8.1, was carefully mixed with the sterile soil and smeared on the side of the tube. The soil was dried at 37° C for 1 week, after which time it no longer adhered to the sides of the tube. The bottles were closed with metal lids and stored at 4°C.

6.8.6 Lyophilization

The two strains of organism were lyophilized (freeze-dried) in the Microbiology Department, Massey University, using an Edwards freeze-drying machine (G. Wilton & Co., Ltd., Auckland & Wellington, New Zealand). The suspending medium consisted of 3 parts mist dessicans in 1 part distilled water containing 75 g/l glucose and 0.33 g/l nutrient broth. The procedure used for the method is reproduced in the Appendix.

6.8.7 Storage in Cooked Meat Medium Containing Glucose

Two ml of spore suspension in sterile distilled water were transferred into 20 ml Cooked Meat Medium containing 10 g/l glucose (CMMG).

These stock cultures were then kept at 4°C.

6.8.8 Monthly Transfer in Cooked Meat Medium Containing Glucose

6.8.8.1 Transfer without heat shocking

One ml of the revived culture was transferred to 20 ml CMMG contained in a 25 ml McCartney bottle. The culture was incubated at 30° C until gassing had ceased and then stored at 4° C. Each month, a further sub-culture was made, and this was incubated and stored in the same way.

6.8.8.2 Transfer with heat shocking

One ml of the revived culture was transferred to 20 ml CMMG as above. Heat shocking was performed by placing the culture of strain NRRL B-594 and strain NCIB 2951 in a water bath at 80°C for 15 min, and at 70°C for 1.5 min, respectively, and was then immediately cooled in iced water.

The culture was incubated at 30°C until gassing had ceased and was then stored at 4° C. Each month, a further sub-culture was made, which was heat shocked, incubated and stored in the same way.

6.8.9 Weekly Transfer in Whey Permeate Containing Yeast Extract

Weekly transfer in 20 ml whey permeate containing yeast extract (5 g/l) was started using 1 ml of the revived culture. The culture was incubated at 30°C until gassing had ceased and was then held at 4° C. Each week, a sub-culture was made which was incubated and stored in the same way.

6.9 VIABILITY TESTS

Viability of the stock culture was determined periodically by pour plating in RCA. Suspensions of the lyophilized and soil stocks were prepared prior to plating by adding 4 ml of sterile distilled water to the soil stock and 1% peptone water to obtain a 1:10 dilution of the lyophilized culture. When heat shocking was done prior to plating out, aliquots of 0.1 ml from the stocks, were placed in sterile 16 x 100 mm screw-capped test tubes. Heat shocking was done by holding the tubes at 80^{o}C for 15 min for *Cl. acetobutylicum* NRRL B-594, and at 70^{o} C for 1.5 min for NCIB 2951.

6.10 FERMENTATION TESTS

A standard method was used to periodically evaluate the different preservation methods for their ability to maintain the fermentation property of the organisms. Stocks were examined for contamination by Gram staining before proceeding with the test.

6.10.1 Preparation of Inoculum

The inoculum was prepared in 3 stages prior to fermentation, using a 5% inoculum at each stage.

Except for soil and lyophilized stocks, 1 ml of the stock cultures were transferred into 20 ml of freshly autoclaved CMMG. (Alternatively, CMMG was boiled at 105^{o} C for 10 min prior to use). For the soil stock, 4 ml of sterile distilled water was added to obtain a suspension from which 1 ml was drawn out as inoculum. One ampoule of lyophilized culture was used for each test.

Stage 1: The stock was suspended in CMMG and heat shocked at 80°C for 15 min followed by cooling in iced water. The cultures were incubated at 30° C for 48 hr after which time strong gassing was observed.

Stage 2: The culture was inoculated into 20 ml CMM containing lactose at 10 g/l, (CMML), and incubated at 30°C for 48 hr.

Stage 3: The cultures were inoculated into 20 ml of fermentation medium (see below) and incubated at 30°C for 24 hr.

6.10.2 Fermentation

The fermentation medium for stocks of NCIB 2951 consisted of sulphuric acid whey permeate supplemented with yeast extract (5 g/l), (WPYE). The pH was adjusted to pH 6.5 with 1 M ammonia solution prior to autoclaving.

For NRRL B-594 the fermentation medium was further supplemented with 50 g/l of glucose, (WPYEG).

Ninety five ml of the fermentation medium, dispensed in 120 ml medicine bottles, was inoculated with 5 ml of the Stage III culture, and the fermentation was carried out at 30°C for 7 or 8 days.

Samples (5 ml) were withdrawn aseptically from the fermentation broth every 24 hours for analyses.

6.11 FERMENTER CULTURE

6.11.1 Medium and Equipment

Batch culture experiment was performed in a Microferm Laboratory Fermenter (New Brunswick Scientic Co. Ltd., New Jersey, U.S.A.) using a 2 litre glass vessel with a 1.6 l culture volume.

The fermentation medium used was sulphuric acid whey permeate containing yeast extract, (5 g/l). The pH was adjusted to pH 6.5 using aqueous ammonia prior to sterilization.

6.11.2 Sterilization

The culture vessel, containing medium, was sterilized by autoclaving at 121^{o}C for 15 min.

The gas filter, consisting of glass wool, for sterilizing inlet gas during fermentation, was sterilized in the hot air oven at 160^{o}C for 3 hours.

6.11.3 Preparation of Inoculum

The fermenter run was conducted using NCIB 2951 that had been preserved in distilled water at 4^{o}C.

The inoculum was prepared in 3 stages as in 100 ml scale fermentation (Section 6.10.1).

6.11.4 Inoculation

The culture vessel was taken out of the autoclave immediately after sterilization and mounted onto the Microferm unit while still hot (ca 85^{o}C). During cooling, oxygen-free nitrogen gas was swept across the broth surface.

After the desired temperature (30°C) was reached the vessel was inoculated using 2 20 ml Stage III cultures (Section 6.10.1).

6.11.5 Operation

Temperature was controlled at 30° C. Prior to sampling and to obtain a representative sample, agitation was switched on and nitrogen gas was swept across the broth surface. Gas outlets were blocked to force the sample out. Ten ml were drawn into a measuring cylinder to clear the sample line before taking about 8 ml sample.

6.12 Analytical Methods

6.12.1 pH Measurement

The pH of fermentation samples was measured immediately after sampling using a Metrohm pH Meter E520 (Metrohm AG, Herisau, Switzerland).

6.12.2 Total Cell Count

The total cell count was performed immediately after sampling using a standard haemocytometer under 400 x magnification. Both vegetative cells and spores, if any, were counted.

6.12.3 Analysis of Solvents

Samples frozen at 20° C were thawed and centrifuged prior to analysis to remove cells and particulate matter from the fermentation broth.

Butanol, acetone and ethanol concentrations were determined by gas-liquid chromatography (GLC) using a Varian Aerograph Model 940 (Varian Instrument Division, California, U.S.A.) equipped with a flame ionization detector. Separation took place in a stainless steel column packed with Chromosorb 101 held at 180°C. Nitrogen was used as carrier gas at a flow rate of 30 ml/min. The injector and detector temperatures were 180°C, and 3 µl of sample were injected.

Quantitation of solvents was by measurement of peak heights and reference to standard mixtures.

Error was estimated at ± 5%.

6.12.4 Analysis of Sugars

Quantitative analysis of sugars was performed using a Waters Associates Model ALC/GPC 244 high performance liquid chromatograph (HPLC) with a model 6000A solvent delivery system and a U6K Septumless injection (Waters Associates, Inc., Milford, Massachusetts, U.S.A.).

The column used was Waters Sugar-PAK 1, operated at a temperature of 90° C. Calcium acetate (20 mg/l) was used as solvent at a flow rate of 0.5 ml/min.

The detector was a Waters differential refractometer Model R401.

Samples were injected at either 25 µl or 50 µl volume depending on the sugar concentration.

Quantitation of sugars was by measuring the peak height of individual sugar and reference to the peak height of standard sugar solutions.

A ± 5% error was estimated.

6.13 DISCUSSION OF METHODS

6.13.1 Anaerobic Cultivation

The most common error associated with the use of cold-catalyst type of anaerobic jars is the size of inactive catalyst (Moore and Holdeman, 1973). This would prevent the generation of anaerobic conditions.

It is known that the catalyst can be inactivated by prolonged exposure to high humidity which is generated during incubation. Furthermore, the amount of reducing power that can be generated by the catalyst, and the requirement for such of the anaerobic strains used, are not known. Therefore, it was deemed necessary to routinely change the catalyst every 3 months, and to rejuvenate it by flaming over a bunsen burner immediately before use.

6.13.2 Viable Count

Clostridia produce gases such as CO_2 and H_2 during their metabolism. They are also motile and tend to form spreaders (spreading colonies) (Prevot, 1966; Shapton and Board, 1971). These characteristics are manifest when the organisms are cultured in agar medium. Gas production shatters the agar and spreader formed on the surface of moist agar media render the colony count inaccurate. Therefore, the agar concentration in RCA was increased to 17 g/l to alleviate these problems.

Pour plating was the method of choice for 2 major reasons. First, it affords the use of freshly autoclaved media with minimal dissolved oxygen concentration fit for anaerobiosis. Secondly, organisms which tend to spread or swarm should not be spread plated.

An experiment was performed using *Cl. acetobutylicum* NRRL B-594 to compare cell counts obtained by pour plate and spread plate techniques. Results are shown in Table 6.1.

TABLE 6.1: Comparison of viable counts of *Cl. acetobutylicum* NRRL B-594 using pour and spread plate techniques.

SAMPLE	POUR PLATE CFU*/ml	SPREAD PLATE CFU*/ml
Dist. Water, 4°C, No. 1	1.7×10^6	4.8×10^5
Dist. Water, 4°C, No. 10	2.6×10^6	1.7×10^5
Phosphate Buffer, 4°C	1.0×10^6	8.6×10^5

* CFU = colony forming units

In the samples tested, counts using pour plate were higher than spread plate by a factor of 1.2 to 3.5.

6.13.3 Obtaining Spores for Preservation

Since several authors have reported that good solvent yields are associated with spore fermentation (Gottschal and Morris, 1981; James et al, 1982; Prescott and Dunn, 1959), it was decided that stocks be laid down in the form of spores.

The method of producing spores on agar slopes followed by recovery using the technique described in Section 6.8 was developed to minimize handling and possible contamination of stocks.

Cl. acetobutylicum NRRL B-594 sporulated rapidly in RCA after 48 hour incubation. The number of spores increased after incubation for 4 days. Although no exact spore count was done, the culture approximated 20% sporulation, and was similar to that obtained on the same medium by Long et al (1983).

A number of attempts were made to obtain spores of *Cl. acetobutylicum* NCIB 2951. This strain grew well on RCA but unlike NRRL B-594, it failed to sporulate on this medium. Very poor sporulation was detected on Cooked Meat Agar (CMA) and Whey Permeate Agar containing yeast extract (5 g/l), (WPYEA). Environmental stresses such as lower incubation temperature than the optimum, aerobic incubation of vegetative cells and prolonged incubation for nutrient depletion did not increase sporulation on either CMA or WPYEA.

This led to the development of Whey Permeate Agar (WPA) which was not supplemented with yeast extract. Growth of NCIB 2951 on WPA occurred after 4 days of anaerobic incubation. Sporulation started after incubation for 6 days but cultures were left in anaerobic jars for 15 days after which good sporulation was obtained.

Relatively more spores were formed by NCIB 2951 on WPA than by NRRL B-594 on RCA.

These results indicate that there are marked differences between the 2 strains of *Cl. acetobutylicum*. The variations in the ability to grow and sporulate in different media emphasize the importance of strain differences and the necessity for further studies on the toxonomy of the genus *Clostridium* which appears to be in an unsatisfactory state (Long et al, 1983).

6.13.4 Test on Fermentation Ability

The various methods of preservation were tested for their capacity to maintain the property of the 2 strains to produce solvents. The fermentation ability was tested using whey permeate as substrate to which yeast extract (final concentration 5 g/l) was added (WPYE). Whey permeate was chosen because of its potential use as a commercial substrate (Maddox, 1980).

Preliminary trials using stocks of NRRL B-594 showed very low solvent production. One reason for this may have been the concentration of dissolved oxygen in the fermentation media which had been stored after autoclaving and then steamed at 105^{o}C for 10 min immediately before use.

Hence, the use of fermentation media immediately after autoclaving was made a cardinal rule. However, NRRL B-594 still gave poor solvent production and experiments indicated poor utilization of lactose. To assess the ability of this strain to produce solvents, therefore, the fermentation medium was modified by adding glucose at 50 g/l final concentration (WPYEG).

CHAPTER 7

RESULTS

7.1 INTRODUCTION

This chapter presents and compares the influence of different culture maintenance methods on the viability of and fermentation in whey permeate by two strains of *Cl. acetobutylicum*, NRRL B-594 and NCIB 2951.

The method of culture maintenance (preservation) used are categorized as follows:

1. Refrigeration at 4°C
   a) in distilled water
   b) in phosphate buffer
   c) in CMMG

2. Freezing at -20°C
   a) in distilled water
   b) in phosphate buffer

3. Drying
   a) in soil
   b) by lyophilization

4. Periodic transfer in culture media
   a) monthly transfer in CMMG
   b) weekly transfer in whey permeate

Simultaneously, growth and development of the inocula were investigated by devising inoculum build-up procedures to achieve maximum solvent production and yield. The fermentations were performed on the 100 ml scale. The procedures investigated included:

1. conventional heat shocking of the stock culture
2 alcohol treatment, e.g. ethanol and butanol, of the stock culture
3. variation in the number of culture stages
4. use of gassing as index of transfer time
5. higher level of inoculum.

Finally, a laboratory-scale fermentation was performed to determine whether the inoculum development method could be successfully scaled-up.

7.2 PRECISION OF THE VIABILITY AND FERMENTATION TESTS

7.2.1 Introduction

Precision is one of the most important criteria of the reliability of a method of investigation. To establish the precision of the methods chosen to determine the viability and fermenting abilities of the stock cultures, the errors inherent in the methods were assessed.

7.2.2 Viable Count

The precision of the 2 methods of viable count, namely, count with heat shocking at 80^{o}C for 15 min (VC + HS) and count without heat shocking (VC - HS) were determined by plating in 6 replicates a selected stock of NRRL B-594. A Student's t-test was also performed to determine if any significant difference exists between the two methods.

The individual counts obtained using the two methods and the statistical parameters used to assess precision are shown in Table 7.1. Both methods were found to give relatively low dispersion in counts. However, counts obtained using VC + HS showed less dispersion than those obtained from VC - HS. Based on the standard deviation,

TABLE 7.1: Precision of the viable counts with and without heat shocking

Replicates	VC - HS (CFU/ml)	VC + HS (CFU/ml)
1	2.0×10^7	7.9×10^6
2	2.3×10^7	7.9×10^6
3	1.8×10^7	6.7×10^6
4	9.1×10^6	7.8×10^6
5	2.1×10^7	7.6×10^6
6	1.6×10^7	8.4×10^6
Mean, μ	1.8×10^7	7.7×10^6
Range, R	1.4×10^7	1.7×10^6
Standard deviation, σ	4.5×10^6	5.1×10^5
Coefficient of variation, V	0.25	0.07
Variance, σ^2	2.0×10^{13}	2.6×10^{11}
Sum of squares, Q	10.0×10^{13}	13.0×10^{11}

$\hat{t}_{10\ ;\ 0.01} = 5.6^{**}$

** Significant at p = 0.01

σ, the true value of the viable counts from VC + HS at 95% probability fall within 6.7×10^6 CFU/ml to 8.7×10^6 cful ml. Counts from VC - HS are actually between 9.0×10^6 CFU/ml to 2.7×10^7 CFU/ml.

Since the coefficients of variation, V, of both methods were greater than 0.05, a triple determination was performed (Sachs, 1978) to calculate the true values of the mean, μ. At 95% confidence interval, the true value of μ for counts obtained from VC - HS and VC + HS were estimated to be within 1.7×10^7 to 2.6×10^7, and 5.6×10^6 to 8.9×10^6, respectively. V was subsequently employed to detect any sifnificant changes in viability of the stock cultures (Section 7.3).

The overall errors of the methods given as a percentage of the mean (Sachs, 1978) were calculated as 50% for VC - HS and 13% for VC - HS. Based on these values, it can be said that the 2 methods of viability test are reliable enough. However, more reproducible counts can be obtained using heat shocking.

The difference between the two methods as determined by the t-test was found to be highly significant.

7.2.3 Solvent Production

Six replicates of 100 ml scale fermentations using strain NCIB 2951 were run as described in Section 6.10 to evaluate the precision of solvent production. The concentration of the solvents at the end of the fermentations (day 8) and the results of the statistical analysis are shown in Table 7.2.

The overall error for butanol production was calculated as 22%, showing that the fermentation test is reasonably reproducible. For ethanol and acetone the errors were 38% and 100%, respectively. For the latter, this appears

TABLE 7.2: Precision of the fermentation method for solvent production

Replicates	Ethanol g/l	Acetone g/l	Butanol g/l
1	1.2	0.1	7.7
2	1.0	0.2	9.0
3	1.7	0.1	10.4
4	1.0	0.2	9.5
5	1.2	0.1	10.4
6	1.2	0.1	8.3
Mean, μ	1.2	0.13	9.2
Range, R	0.7	0.1	2.7
Standard deviation, σ	0.23	0.05	1.01
Coefficient of variation, V	0.19	0.50	0.11

to be a large error, but considering the very minor production of this solvent, and the subsequent difficulty of accurate determination, it was considered acceptable.

As for viability tests, the coefficient of variation was subsequently used to determine significant differences in the fermentation abilities of the stock cultures.

7.2.4 Sugar Utilization, Butanol Yield and Production Rate

The results for lactose utilization and butanol yield at the end of the fermentation described in Section 7.2.3, the maximum observed butanol production rate, and the statistical parameters obtained, are shown in Table 7.3.

The R and σ for lactose utilisation were 2.3 g/l and 0.78 g/l, respectively. These results indicate that the method affords very little dispersion from the true value of lactose utilisation. A 5% overall error reveals that the method used is highly reliable.

The corresponding σ for butanol yield 4.0% and production rate (0.01 g/l.h) were quite low. The overall errors detected were 25% and 17%, respectively. The method, therefore, affords reasonably high reproducibility for these fermentation parameters.

Subsequent correction of the observed values was employed using V to assess any change in fermentation abilities of the stock cultures.

TABLE 7.3: Precision of the fermentation method for sugar utilization*, butanol yield* and maximum observed butanol production rate

Replicates	Lactose utilization g/l	Yield %	Rate g/l.h
1	28.7	26.8	0.12
2	27.2	33.1	0.12
3	28.0	37.1	0.10
4	29.3	32.4	0.10
5	28.7	36.2	0.13
6	29.5	28.1	0.12
Mean, μ	28.6	32.3	0.12
Range, R	2.3	10.6	0.03
Standard deviation, σ	0.78	4.0	0.01
Coefficient of variation, V	0.03	12.4	0.08

* at day 8 of fermentation

7.3 MAINTENANCE OF VIABILITY BY DIFFERENT PRESERVATION METHODS

7.3.1 Introduction

The different methods that have been used for the maintenance of *Cl. acetobutylicum* were evaluated for their ability to preserve the viability of the two strains of organism, namely, NCIB 2951 and NRRL B-594.

7.3.2 Results and Discussion

Before considering the results it is worthwhile to emphasize some points concerning methodology. Firstly, two methods of viability test were used for evaluation, namely, viable count without heat shocking (VC - HS) and viable count with heat shocking (VC + HS), since the stock cultures consisted of spores and vegetative cells, and no standard method was available to obtain an accurate viability measurement of Clostridia over storage. The heat shocking treatment applied for each strain of organism was adopted after a preliminary experiment on the effect of different heat treatments on their viable counts (Table 7.4). The results show the difference in heat resistance of the 2 strains of organism. More importantly, the viable counts obtained with heat shocking demonstrates the two opposing effects of heat shocking on the stock cultures:

a) activation of spore germination
b) destruction of vegetative cells

Assuming that some vegetative cells were resistant to heating, the colonies after heat shocking could be from both activated spores and vegetative forms. The heat treatments chosen were based on the highest viable count arising from the greatest degree of stimulation of spore germination and least destruction of vegetative cells. Therefore, among the treatments, heating at 80^{o}C for 15 min

and at 70°C for 1.5 min seem to be the most suitable for NRRL B-594 and NCIB 2951, respectively.

TABLE 7.4: Effect of heat shocking in the viable count of *Clostridium acetobutylicum*

Treatment	CFU/ml x 10^6	
	NRRL B-594	NCIB 2951
no heat shock	5100	1800
70°C, 1.5 min	10	1300
75°C, 2 min	26	1000
80°C, 15 min	31	39
85°C, 20 min	24	1
95°C, 30 min	12	0

Secondly, when the stock cultures were laid down, all reasonable attempts were made to ensure that each sample bottle within a particular preservation method contained an identical number of cells. Further, the methodology for sampling and heat shocking (when performed) was standardized as much as possible. Nevertheless, slight differences among "identical" samples, and in technique over a prolonged time period, are hazards which cannot be discounted when assessing results. It should also be emphasized that some bottles were sampled more than once. It is possible that the first sampling caused some disturbance of the remaining culture, e.g. thawing of frozen samples, and this had a deleterious effect on subsequent samples. Consequently, when a fresh sample bottle was used, an apparent increase in viability is possible.

On this basis, therefore, it is considered that the results should be assessed on their overall trends rather than attempting a detailed discussion of each individual data point.

7.3.2.1 *Clostridium acetobutylicum* NCIB 2951

The results of the periodic viability tests on NCIB 2951 conducted with and without heat shocking at 70°C for 1.5min are shown in Fig. 7.1. Storage by lyophilization caused only a slight reduction in the number of viable cells. This was not unexpected. Lyophilization has been acknowledged to be quite successful in maintaining thousands of cultures for long periods of time. Further, it is known that spores are inherently extremely resistant to dehydration and do survive lyophilization well (Heckly, 1978). A viability of 10% has been considered high enough for anaerobes which have been lyophilized (White et al, 1974; Phillipe et al, 1975). Factors that may have contributed to the longevity of this strain by freeze drying include the suspending medium and storage temperature. The serum used in freeze drying reduces the sensitivity of dried bacteria to oxygen, while subsequent storage at 4°C increases the survival of lyophilized cells (Heckly, 1978). The convenience of keeping the cultures in containers such as ampoules and having to use up all its contents at any one time without disturbing any remaining cultures also helps to explain this expected result. However, the variation which might occur between ampoules should not be discounted to explain a very slight loss in viability using this method.

Based on the final viability counts taken from the stock cultures, 100% viability was maintained on prolonged (9 months) storage by freezing at -20° C, refrigeration at 4°C in phosphate buffer and drying in soil. These results, again, are not entirely unexpected as a wide range of microorganisms can be successfully preserved by storing under these conditions.

The reduction in viable cells after 6 months of storage which occurred in all methods except lyophilization clearly points to a hazard imposed by these methods. Loss of

viability apparently occurred in methods where the retrieval technique caused disturbance to the remaining culture. A particular bottle was consistently sampled up to the 6th month of storage when the contents were exhausted. Reduction in viability might have been caused by withdrawal of the bottle from storage even for a minimum period of time. The thawing of frozen cultures is known to have deleterious effects.

Differences between these methods are evident by the apparent initial rate of decrease in viability. Although thawing is known to affect the number of viable cells, the decrease in viability in the stocks stored frozen in distilled water was not as much as in the same medium under refrigeration at 4^{o} C. The presence of glycerol as a cryoprotective agent possibly minimized this loss of viability.

Maintenance in CMMG seemed to be more efficient than the other methods where the organism was suspended in liquid. This may be because CMMG provides reduced condition to the vegetative cells which were present in the stock culture.

The viability of this strain was least maintained by refrigeration at 4^{o} C in distilled water. The absence of conditions which could protect both spores and vegetative cells such as salts, nutrients and reducing substances probably accounts for such results. However, it should be noted that most of the viability loss occurred during the early stages, possibly due to destruction of vegetative cells rather than spores, and the viable counts then remained reasonably constant.

Initially, the amount of vegetative cells in the total was around 70%. The results suggest that any method which involves withdrawal of small aliquots from the container carries with it the strong possibility of reducing

viability of the remaining cells. This hazard can be minimized by using retrieval procedures which do not cause any disturbance to the remaining culture such as change in temperature (e.g. thawing). It is always desirable that fresh stock cultures should be used for any purpose.

Although the stock cultures maintained by monthly transfer in CMMG, with and without heat shocking, remained viable after 9 months and 6 months of storage, respectively, all experiments which attempted to obtain colony forming units in RCA from such cultures, failed. A possible explanation for this is the combined damage of frequent transfer and lyophilization on the strain's cultural characteristic. These culture lines were started from the freshly revived lyophilized culture obtained from N.C.I.B., and probably did not contain spores (which were obtained through a methodology involving a few culture stages from the revived lyophilized culture) as the other stock cultures did, such that any injury to the organism during lyophilization might have been carried on to these culture lines and rendered the organism susceptible to mutational changes induced by frequent transfer. Consequently, there was a loss of the ability of this strain to grow in RCA.

Weekly transfer into whey permeate (containing yeast extract), followed by heat shocking at 70^{o}C for 1.5 min, did not keep this strain well. No viability measurement was performed since the culture line lost all viability before the test was conducted. Growth (as indicated by gassing) after inoculation became slower (4-5 days) as the transfer was carried out further. There was a complete loss of viability after the 5th transfer. The most likely explanation for this is that the cultures sporulated poorly in the medium, and thus the heat treatment was lethal to most cells. Then, after the 5th heat treatment all the cells were killed.

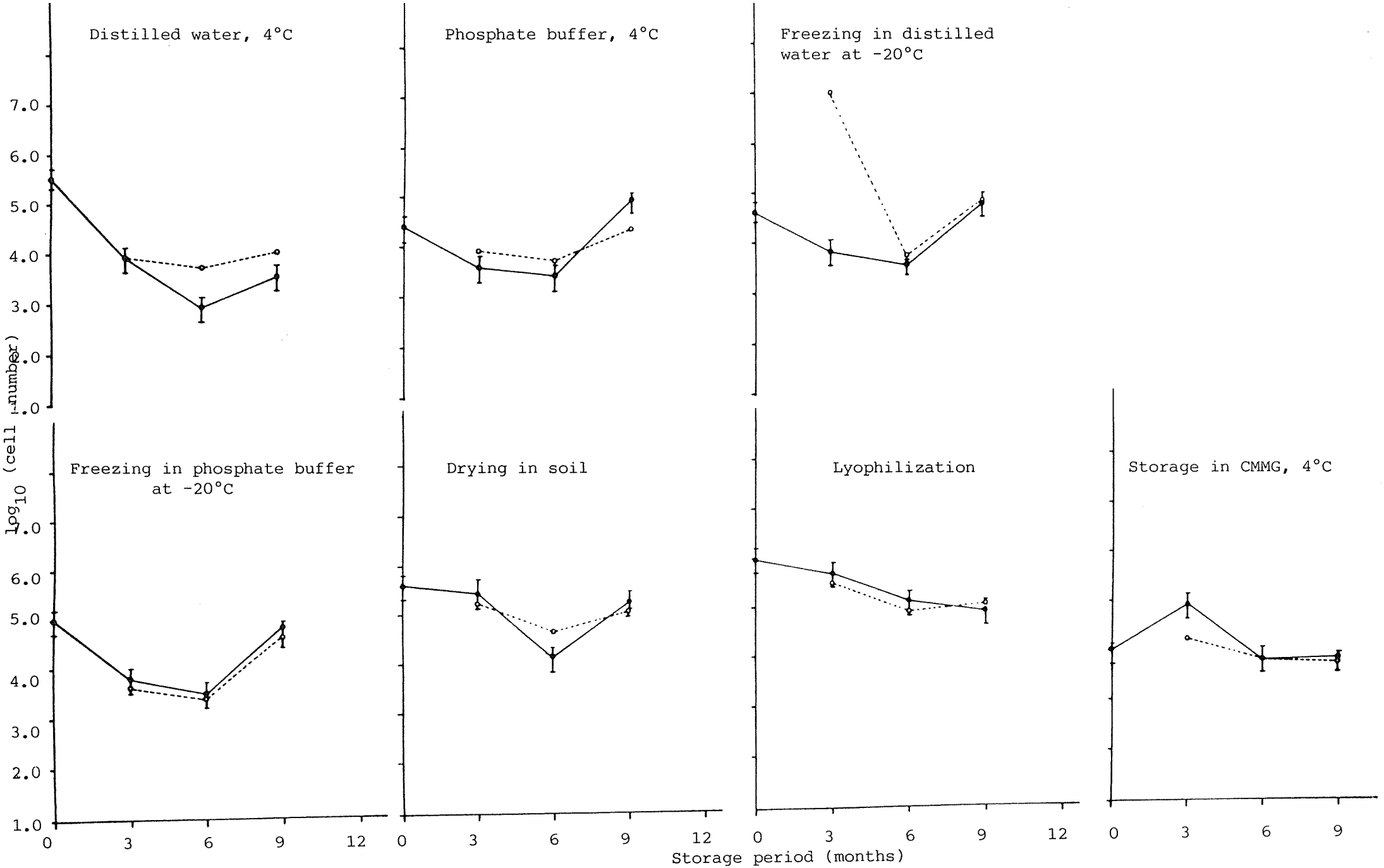


FIGURE 7.1: Maintenance of viability of *Cl. acetobutylicum* NCIB 2951 by different preservation methods
(o------o VC + HS; •——• VC - HS)

There was no real difference observed in losses of viability of the stock cultures, as assessed by the two methods of viable counts, after 9 months storage.

From the results described previously, it is apparent that a viability count which includes as heat shock gives a lower result than without a heat shock. This is almost certainly due to the destruction of some vegetative cells. Thus, after 9 months storage, it can be suggested that any reduction in viability over this period is due to loss of vegetative cells rather than spores. Assuming then, that after 9 months storage only spores remain, and given that the viable counts with and without heat shocking are roughly equal, it would appear that there is little effect of heat shocking in stimulating spore germination. However, there still remain some data points where heat shocking led to a higher viable count, and this must be considered to be due to stimulation of germination. Overall, however, in the context of the present work, the use of the heat treatment prior to the viable count does not appear to be necessary to obtain an accurate assessment of viability, at least with strain NCIB 2951.

With regard to the adequacy of the different preservation methods to maintain viability, all the methods shown in Fig. 7.1 seem to be adequate with the possible exception of storage in distilled water at $4^{o}C$.

7.3.2.2 *Clostridium acetobutylicum* NRRL B-594

Fig. 7.2 shows the viable counts of strain NRRL B-594 for the different methods of preservation.

Overall, the results, based on VC + HS, shows little loss of viability after storage for one year using the preservation methods shown. However, in all cases, except storage in CMMG and monthly transfer, the viable count after 12

months storage obtained by VC - HS was lower than that obtained by VC + HS, suggesting that for this strain of organism, heat shocking is required to assess the viability of stored cultures after they have been stored for long periods.

As with strain 2951, several points serve to explain much of the data:

a) apparent reduction in viability occurred where stock cultures were used repeatedly, and apparent increases were observed when fresh bottles were sampled.

b) initial losses of viability may have been due to losses of vegetative cells rather than spores.

c) when vegetative cells were still present, the heat shocking treatment during the viable count may have caused their destruction.

Losses of viability during the early stages, when the same bottles were repeatedly sampled, seemed worse with this strain than with NCIB 2951. It should be emphasized, however, that the ratio of spores to vegetative cells harvested for NRRL B-594 was less than for NCIB 2951 (20:80). This indicates, therefore, that vegetative cells are more susceptible to environmental stresses than are spores.

As with strain NCIB 2951, weekly transfer of this strain into whey permeate (supplemented with yeast extract and glucose), followed by heat shocking at $80^{o}C$ for 15 min, was not a satisfactory method. No viability measurement was done since there was a complete loss of viability before the first test was performed. No more viable cells were retrieved after the 6th transfer. After the 2nd weekly transfer, it took 4-5 days instead of 2 days before growth, as indicated by gassing, commenced.

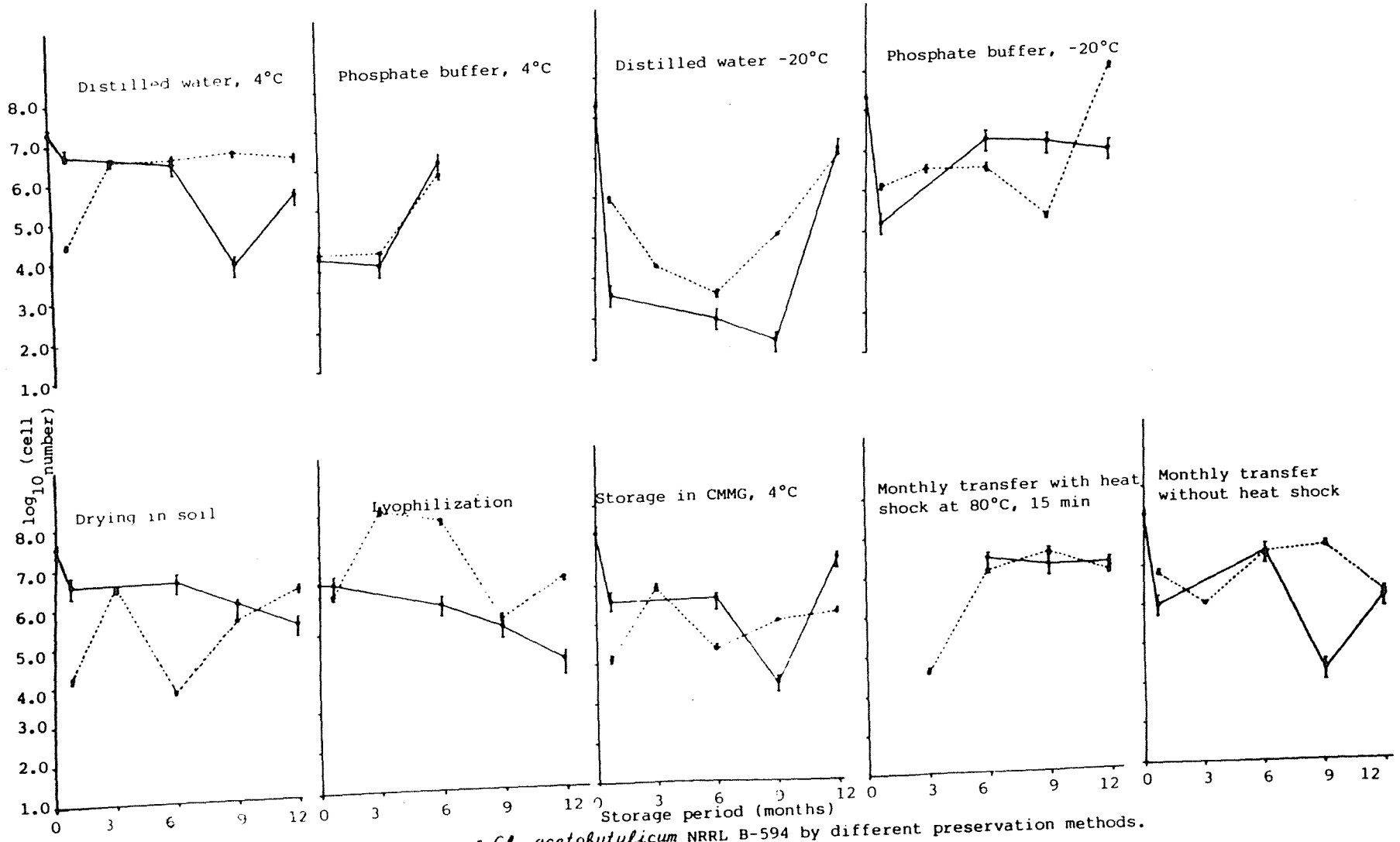


FIGURE 7.2: Maintenance of viability of *Cl. acetobutylicum* NRRL B-594 by different preservation methods. (o----o VC + HS; ●——● VC - HS)

Viability obtained by VC + HS did not follow a particular trend as observed with VC - HS. This may be due to the properties inherent in the spores of this strain that rendered them sensitive to some differences by which the heat shocking treatments were employed. The fluctuations and the increase in viability upon storage observed with the lyophilized and soil stocks can possibly be explained by relative differences in the two opposing effects of heat-shocking, i.e. stimulation of germination and destruction of vegetative cells.

The results brought to light the merits of the 2 methods of assessing the viability of strain NRRL B-594. Viable count performed with heat shocking affords a more accurate viability measurement. Viable count conducted without heat shocking can be regarded useful in monitoring the effect of handling during, rather than after, storage. Results from this test emphasize the importance of procedures in the handling of the stock cultures during storage and retrieval to minimize loss of viability.

7.3.3 Conclusions

The majority of the methods of preservation evaluated for their capacity to maintain the viability of the two strains, NRRL B-594 and NCIB 2951, were effective for prolonged storage. Differences between these methods are dictated by the physiological form (whether spores or vegetative cells) of the microorganisms at the time of storage. It is more advisable if stock cultures could be laid down purely as spores. This minimizes the necessity for provision of anaerobic condition during storage since clostridial spores are more tolerant to small amount of oxygen.

Drying in soil or by lyophilization seem to be the optimum methods for both strains as the viability remained reasonably stable during prolonged storage. Viability in liquid storage may prove satisfactory once the organism is predominantly in the spore form.

Loss of viability occurs through handling during storage and retrieval. Therefore, a stock culture should not be used repeatedly. It seems more convenient and advantageous if stock culture could be prepared in small amounts so that no remaining cultures would be wasted.

Viability is more accurately determined on the basis of plate counts of colony forming units obtained after heat shocking the stock cultures. This suggests that to obtain high concentrations of inocula for optimum performance, heat shocking of the stock culture is necessary.

However, even if viability were based on the number of organisms surviving, it is not an entirely satisfactory criterion for evaluating the effectiveness of culture preservation. High viability does not guarantee that the solvent producing ability of *Cl. acetobutylicum* is not lost during storage. Hence, it is not safe to recommend any particular preservation method at this stage.

7.4 MAINTENANCE OF FERMENTATION ABILITY BY DIFFERENT PRESERVATION METHODS

7.4.1 Introduction

An investigation into how effectively the different preservation methods maintained the fermentation ability of the two strains of *Cl. acetobutylicum* was performed using the fermentation procedure described in Section 6.10.

7.4.2 Results and Discussion

7.4.2.1 *Clostridium acetobutylicum* NCIB 2951

The fermentation test was performed using whey permeate supplemented with 5 g/l yeast extract (WPYE). The approximate lactose concentration of the substrate after autoclaving was 40 g/l. The pH after sterilization ranged from pH 5.1 to 5.8. Although the pH of the whey ultrafiltrate varied, Schoutens et al (1984) found that such variation in pH at the beginning of the fermentation did not affect butanol production by *Cl. beijerinckii*. Cell growth of this strain was completed from 1-3 days after inoculation. The major fermentation product was butanol. The typical ratio of butanol : acetone : ethanol obtained by this strain, i.e. 10:1:1 (Maddox, 1980), was observed. Maximum butanol production was obtained 7-8 days after inoculation, after which there was a decrease possibly due to ester formation.

The typical fermentation profile is represented by the stock culture frozen at -20^{o}C in distilled water for 3 months (Fig. 7.3). This profile remained consistent throughout the duration of storage. The fermentation properties which are known to accompany high solvent production such as the pH break-point and the presence of highly motile cells (Spivey, 1978) were consistently observed.

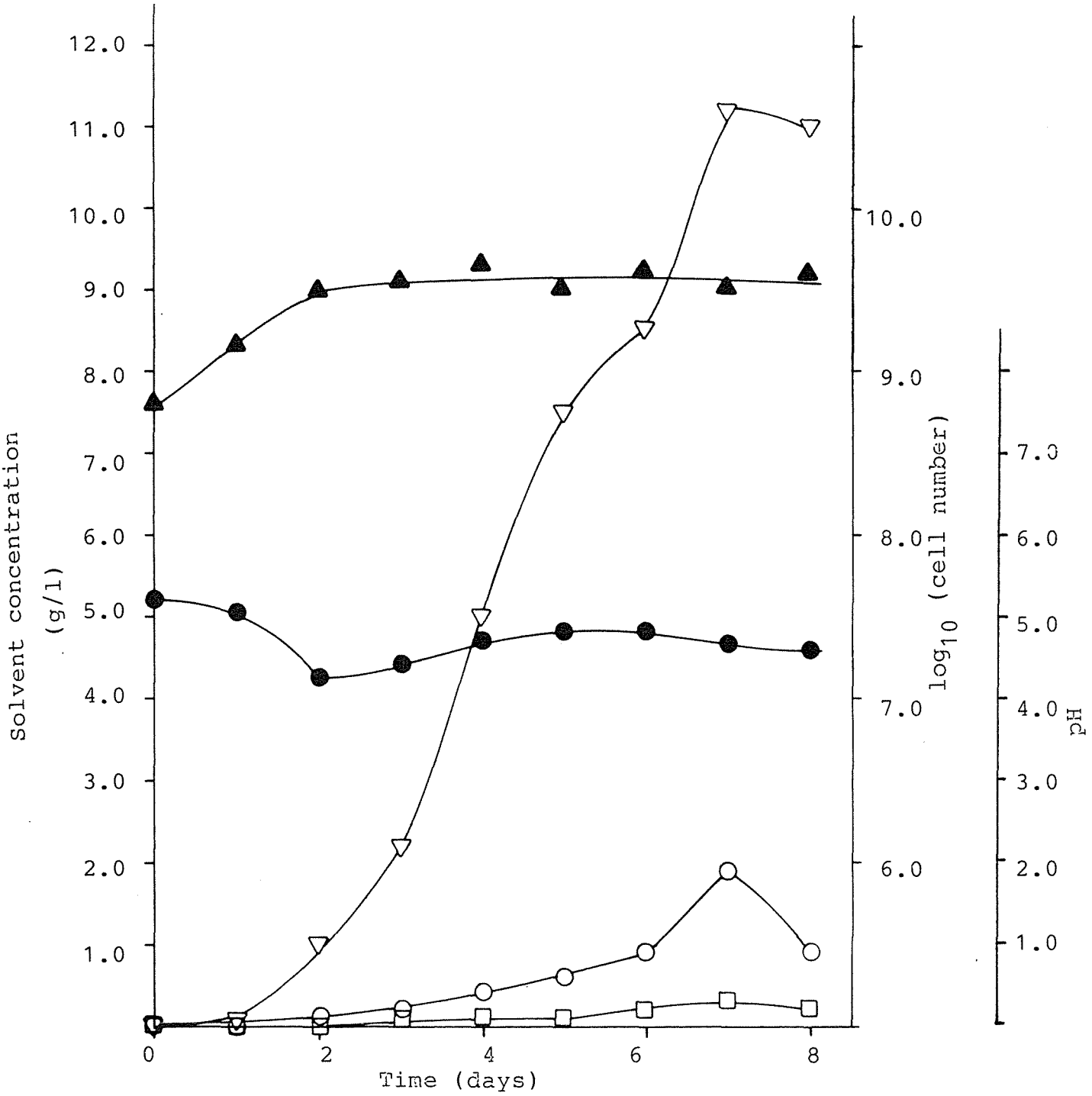


Figure 7.3: Fermentation profile of *Cl. acetobutylicum* NCIB 2951 maintained by freezing at -20°C in distilled water for 3 months. (▲ $\log_{10}$ cell number; ▽ butanol; ○ ethanol; □ acetone; ● pH).

The maximum values for solvents production, sugar utilization, and butanol production rate obtained from the fermentations carried out by the stock cultures at regular time intervals during storage are illustrated in Fig. 7.4 a-i.

It should be pointed out that the methodology of sampling was similar to that of the viability test. Further, the initial fermentation test was carried out using whey permeate media which were not freshly autoclaved. The failure of some stock cultures to produce solvents at this time led to the use of freshly autoclaved media as a cardinal rule (against boiling at 105^{o} C for 10 min, Section 6.13.4). Since, generally the production of solvents at this stage was lower than after a period of storage, this point will not be considered in evaluating the results.

Overall, the results demonstrate that the preservation methods and prolonged storage significantly influence the production of butanol but not of ethanol and acetone. Hence, attention was focused on butanol production.

Except for the stock culture maintained by monthly transfer without heat shocking, a maximum cell number was reached and maintained during the fermentation, ranging from 10^{7} to 10^{9} cells/ml, over the entire storage period (9 months). Despite the maximum cell number remaining stable, different solvent values were observed. This clearly indicates the effect of the maintenance procedure on solvent production.

Freezing the stock culture in distilled water at -20^{o} C (Fig. 7.4c) appears to be the most effective method since the maximum butanol production was maintained at 7.7 g/l to 11.2 g/l over prolonged storage. More importantly, high sugar utilization, solvent yield and maximum butanol production rate values were consistently retained during

the entire period of storage (9 months) at 36.0 g/l to 37.9 g/l, 20.3% to 31.1%, and 0.112 - 0.142 g/l.h, respectively.

The high butanol concentration values obtained by cultures preserved in nutrient-free media, e.g. distilled water and phosphate buffer (Fig. 7.4a, b, d), shortly after laying down the stocks (3 months) suggests that the solvent producing ability of this strain was not altered by the relatively mild steps involved in these methods. However, there was a significant reduction in the fermentation parameters after storage for 9 months. It must be stated here that fresh samples were used for the fermentation tests after 9 months of storage. Hence, repeated sampling of the same bottle is not a contributing factor to the poorer solvent production.

Storage in phosphate buffer seems to alter the fermentation capacity of this strain to a great extent. A sharp decrease in butanol production by the stock culture frozen at -20° C, which might be due to both repeated use of the culture and prolonged storage, was highly significant. This degeneration also emphasizes the deleterious effect of thawing.

The stock culture kept by lyophilization (Fig. 7.4f) was able to carry out relatively high butanol production during the course of storage. Except for a slight reduction in maximum butanol concentration and butanol yield values after the 6th and 9th months of storage, respectively, it is still evident that lyophilization can actually maintain consistency in the fermentation efficiency of the stock culture over the entire term of storage. The stock cultures did not show any change, upon storage, in the maximum rate of butanol production. Such consistency is comparable to that afforded by freezing at -20°C in distilled water.

Drying in soil (Fig. 7.4.e) and storage of the organism in CMMG (Fig. 7.4g) were comparable. The highest maximum butanol concentration obtained from both methods ranged from 5.1 g/l to 5.7 g/l, i.e. not considering the initial values, and this decreased considerably after 9 months of storage. There were no significant reductions in sugar utilization, yield or maximum rates of butanol production detected until after 9 months of storage. Unlike that of storage in phosphate buffer and distilled water at $4^{o}C$, the reduction in solvent production by the CMMG stock cultures was not as profound. This may possibly be due to the presence of reduced conditions which rendered protection to both spores and vegetative cells initially present in the stock culture.

Monthly transfer with or without heat shocking at $70^{o}C$ for 1.5 min was a poor maintenance procedure (Fig. 7.4h, i, respectively). Despite remaining viable, the stock culture failed to carry out fermentation after 6 months and 9 months of preservation of heat shocked and non-heat shocked culture lines, respectively. This demonstrates the risk of using such a method. Since there is an increased possibility of mutation with each sub-culture, frequent transfer are undesirable for long-term preservation of organisms (Heckly, 1978). The degeneration observed here may possibly be due to such mutation.

As discussed in the previous Chapter, these culture lines were started from freshly revived lyophilized cultures and did not have the same spore/cell mixture present as in the other stock cultures. Interestingly, these were the cultures that failed to form any colonies on RCA during the viability tests. The proposed mutation damage, therefore, did not only affect the cultural but also the biochemical property of this strain. This was also evident when there seemed to be a diauxic growth in the fermentation media when cultures maintained by monthly transfer with heat-shock were used as inocula. Normally, gassing was

observed only within 1 to 4 days after inoculation. However, vigorous gassing occurred again after 12 days of fermentation by the culture that has been preserved for 6 months.

Maintenance by weekly transfer was not assessed due to the loss of the culture line before the test was conducted. It can be predicted, though, that the same degeneration observed with monthly transfer would occur.

The results obtained with these periodic transfers (monthly and weekly) demonstrate that there is a greater risk of degeneration of the solvent producing ability when cultures are maintained by these methods. These also confirm the results from the study conducted with *Cl. butylicum* NRRL B-592 (Gapes et al, 1983). Weekly transfer of this strain in WPYE initially led to an improvement in sugar utilization and solvent concentrations but degeneration became rapid after the third sub-culture and by the sixth no solvent was detected during the fermentation.

During the inoculum development stage of the fermentation tests, it was observed that the cultures maintained by monthly transfer and by freezing in phosphate buffer become sluggish so that longer incubation times were required for each stage.

In addition to the metabolic differences, less phase-bright cells were observed in degenerate cultures than in vigorous cultures.

Motility was observed to be more directly related to high solvent production than was the formation of clostridial forms. This property was retained in all fermentations carried out during the entire period of storage by the cultures stored at $-20^{o}C$ in distilled water. It seems, then, that this method has the capacity to retain not only cell

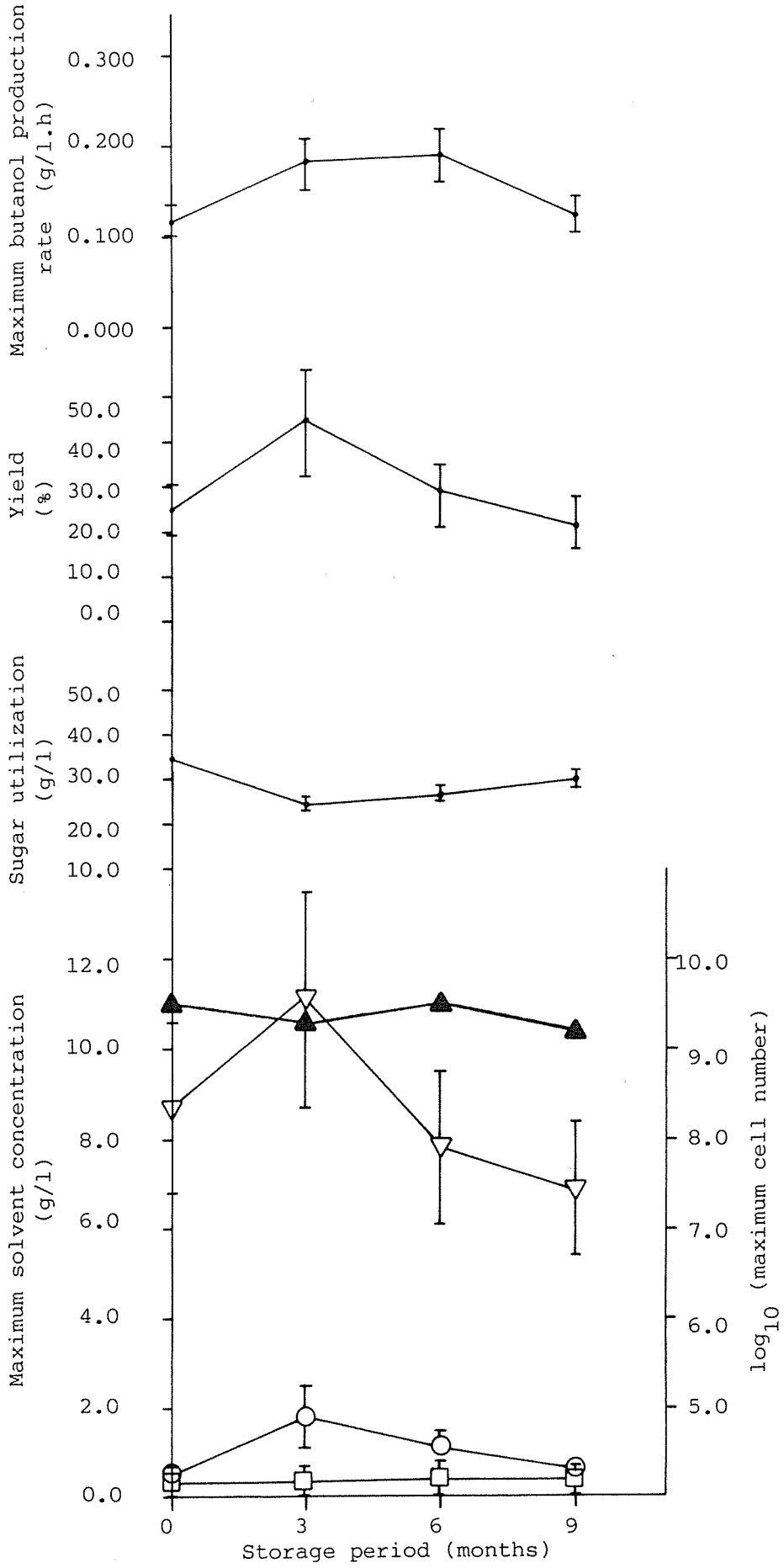


FIGURE 7.4a: Fermentation ability of strain NCIB 2951 kept under refrigeration at 4°C in distilled water (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

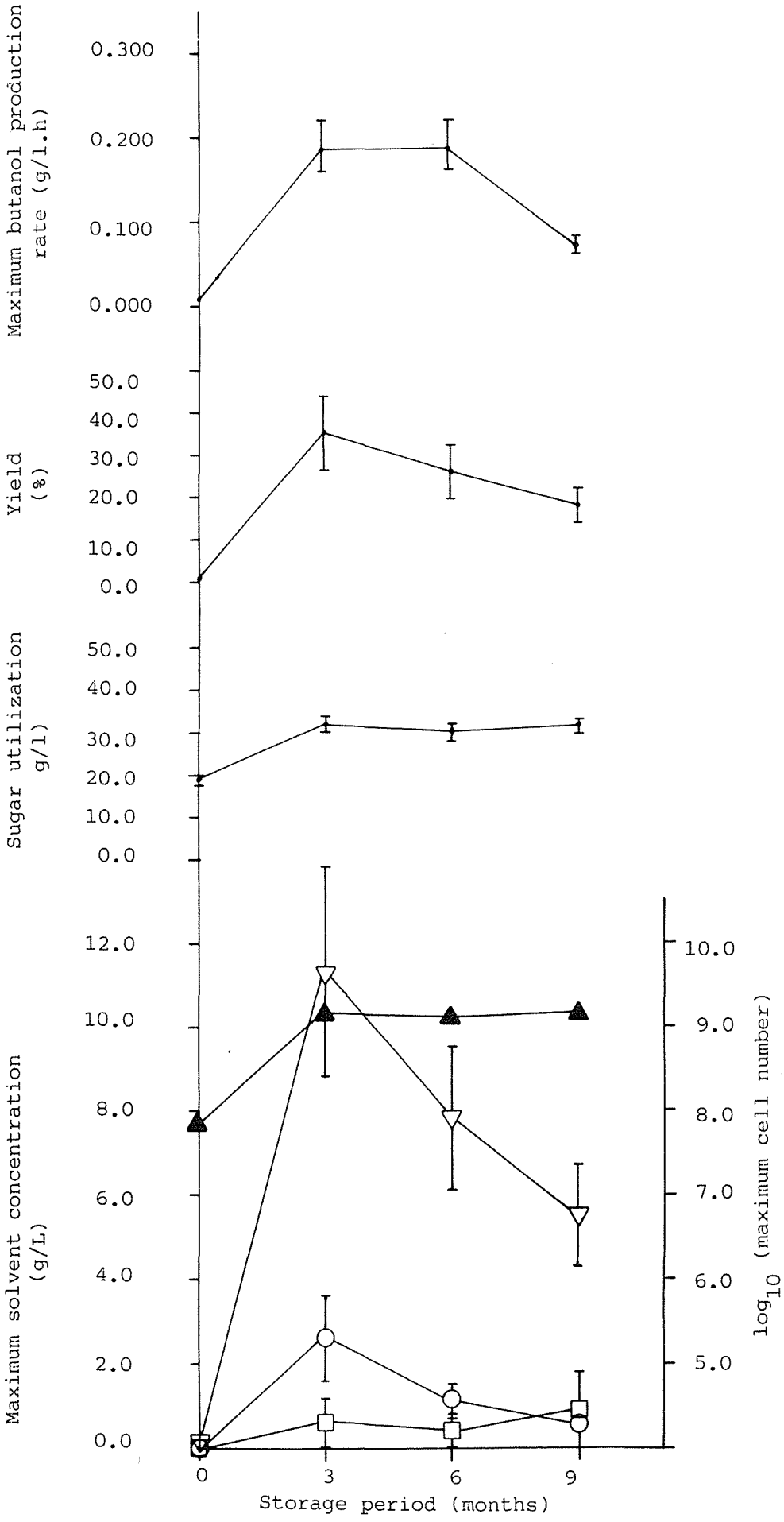


FIGURE 7.4b: Fermentation ability of strain NCIB 2951 kept under refrigeration at 4°C in phosphate buffer (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; max. acetone).

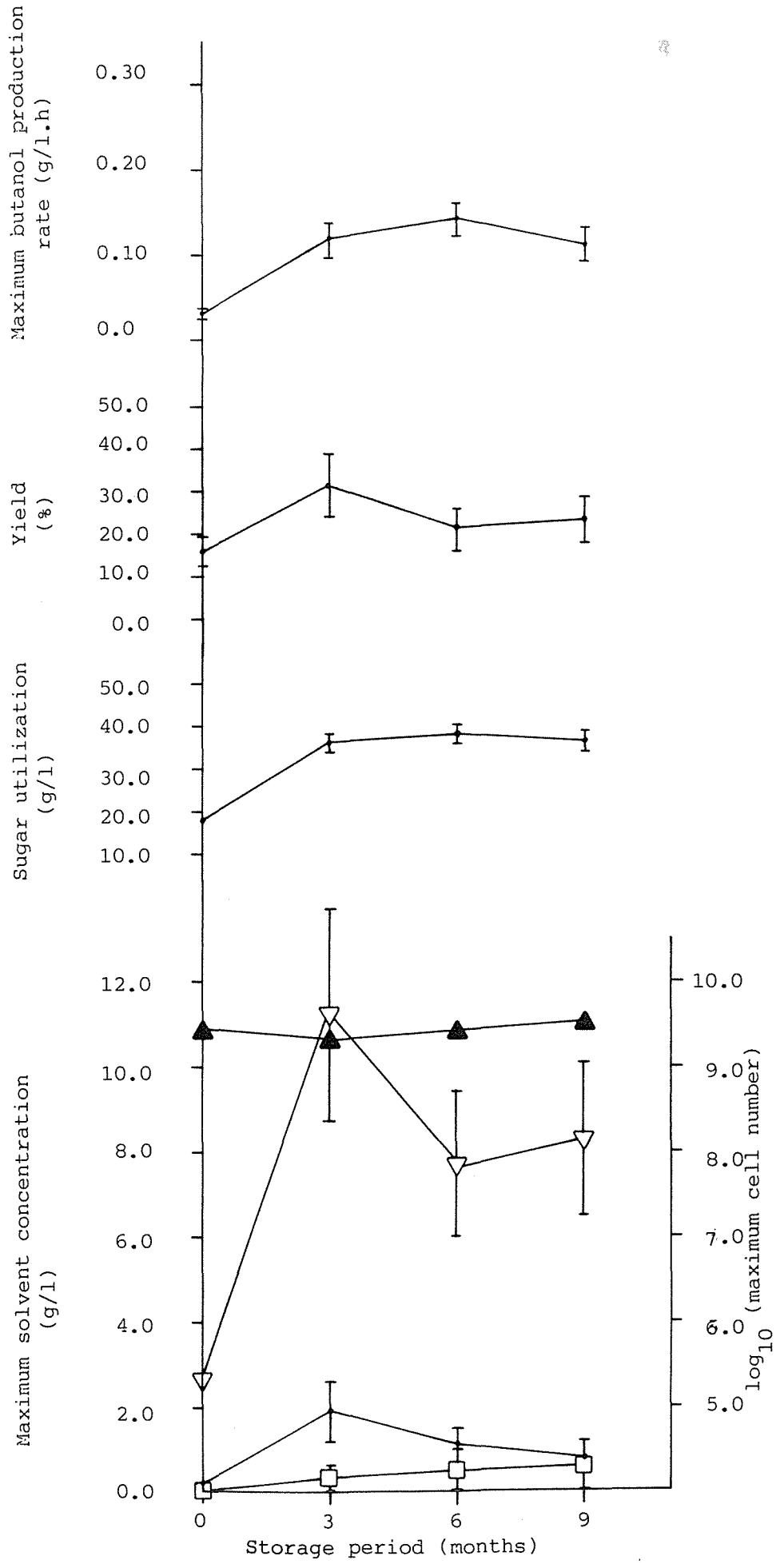


FIGURE 7.4c: Fermentation ability of strain NCIB 2951 frozen in distilled water at -20°C (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

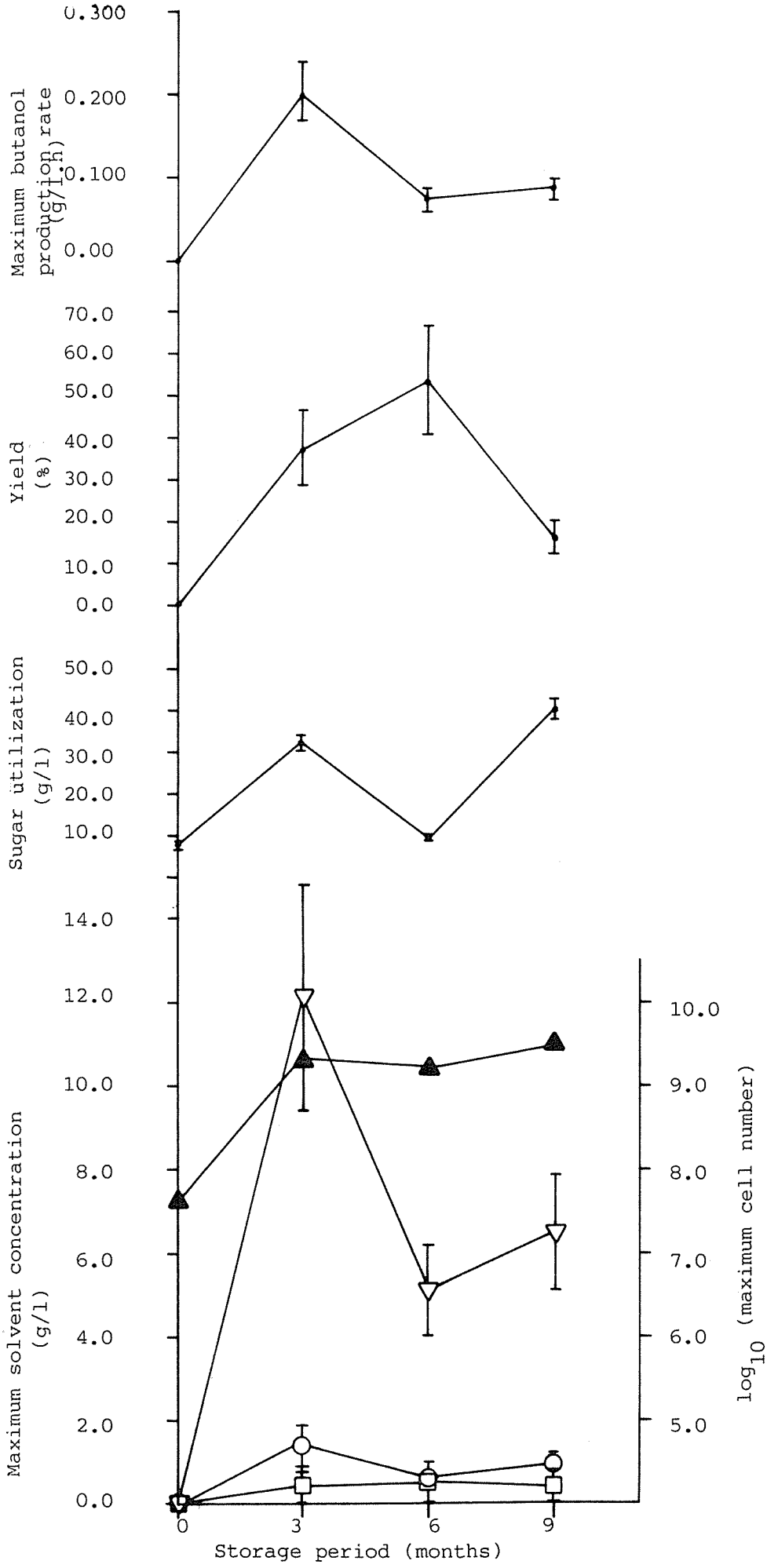


FIGURE 7.4d: Fermentation ability of strain NCIB 2951 frozen in phosphate buffer at -20°C (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

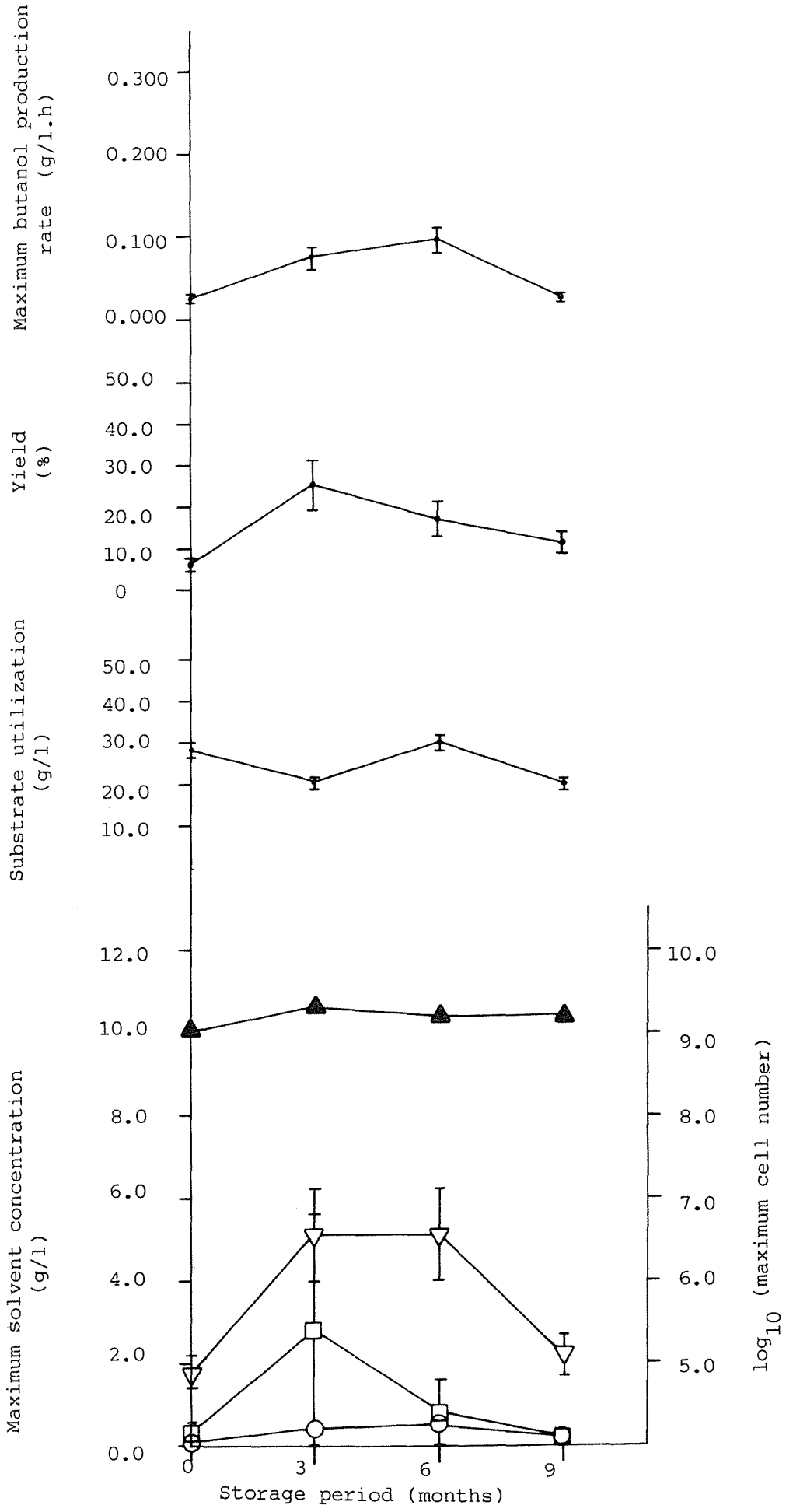


FIGURE 7.4e: Fermentation ability of strain NCIB 2951 dried in soil (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

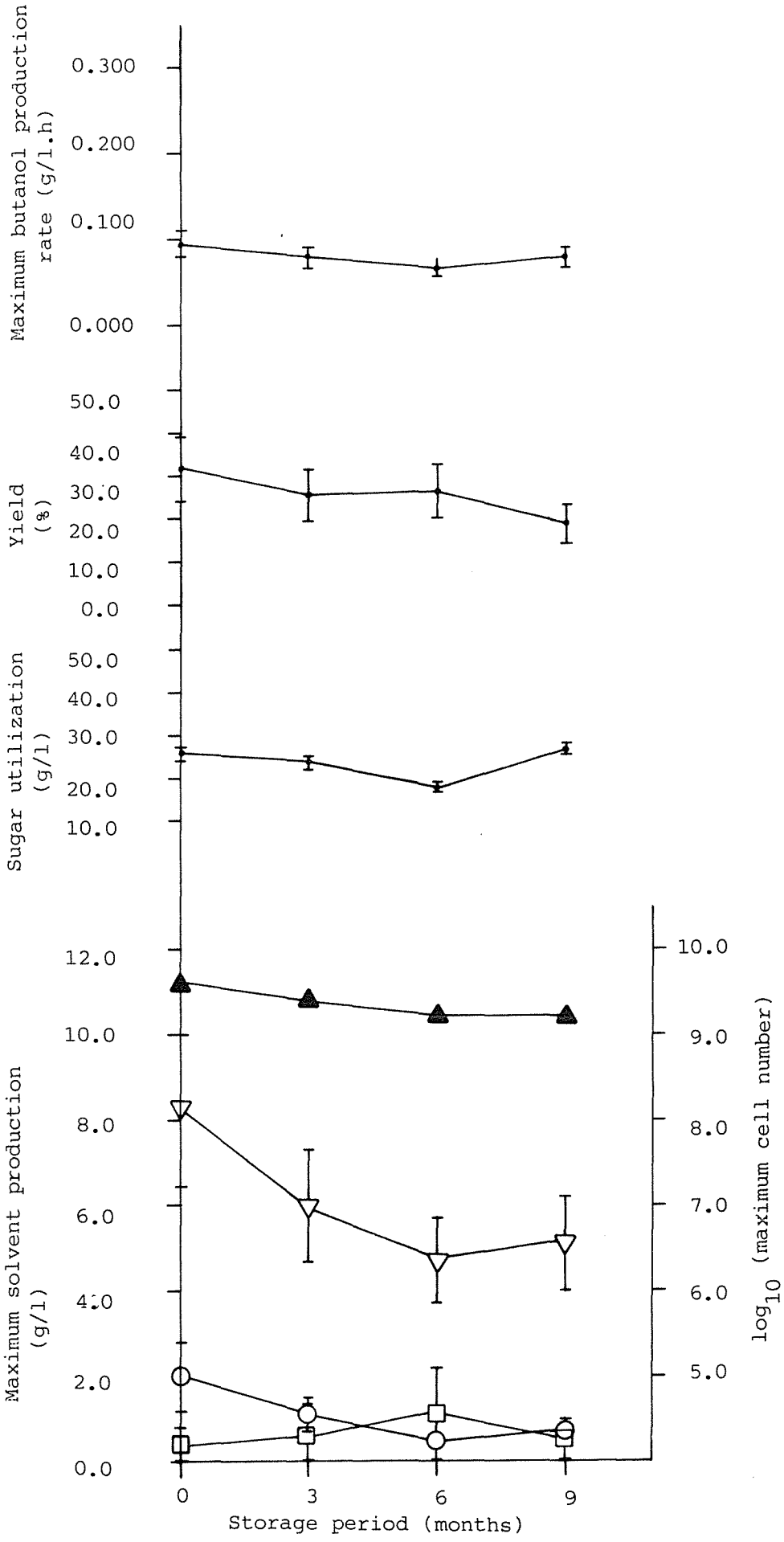


FIGURE 7.4f: Fermentation ability of strain NCIB 2951 stored by lyophilization. (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

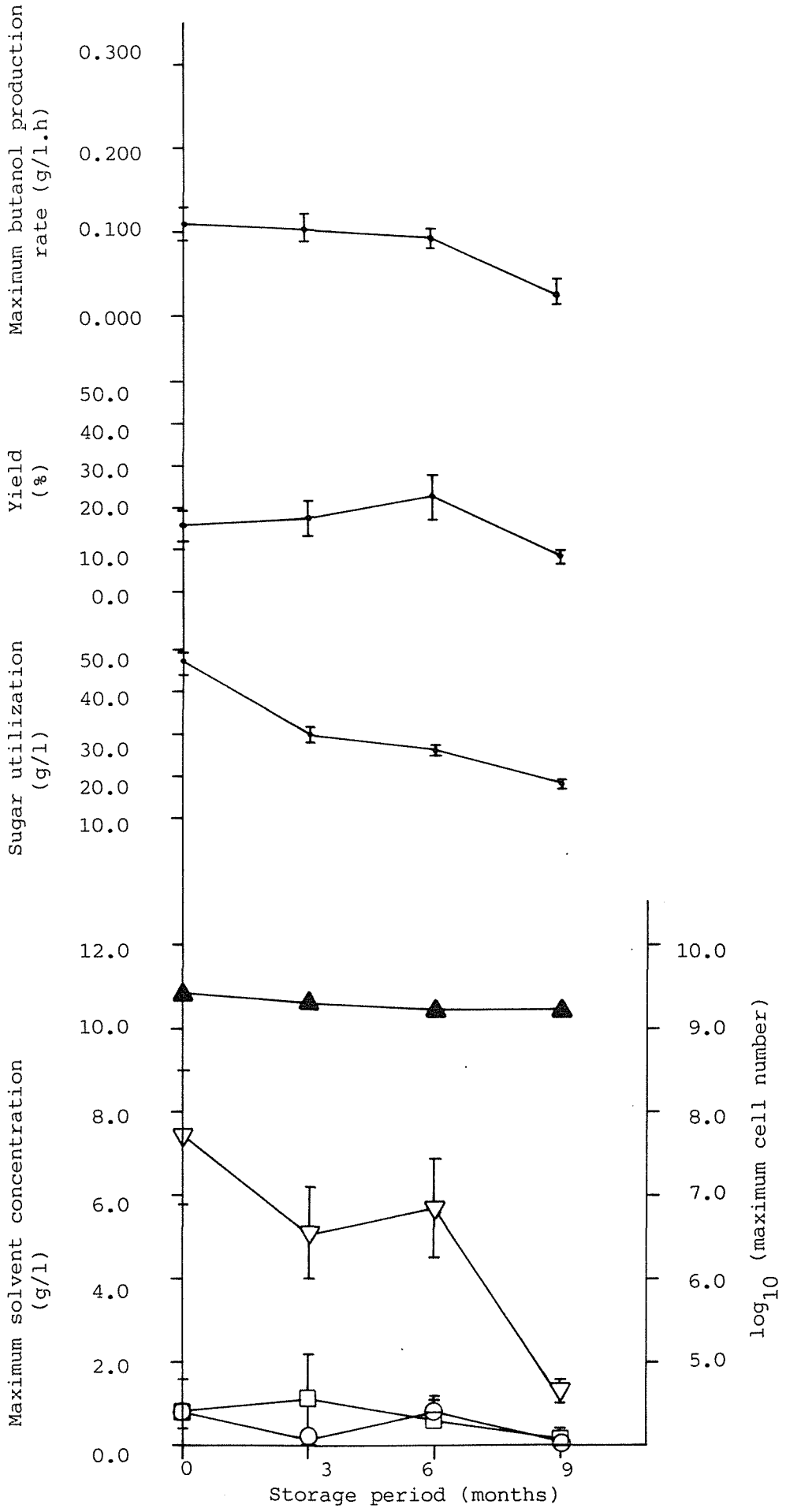


FIGURE 7.4g: Fermentation ability of strain NCIB 2951 preserved in CMMG (▲ $\log_{10}$ max. cell number, ▽ max. butanol; ○ max. ethanol; □ max. acetone)

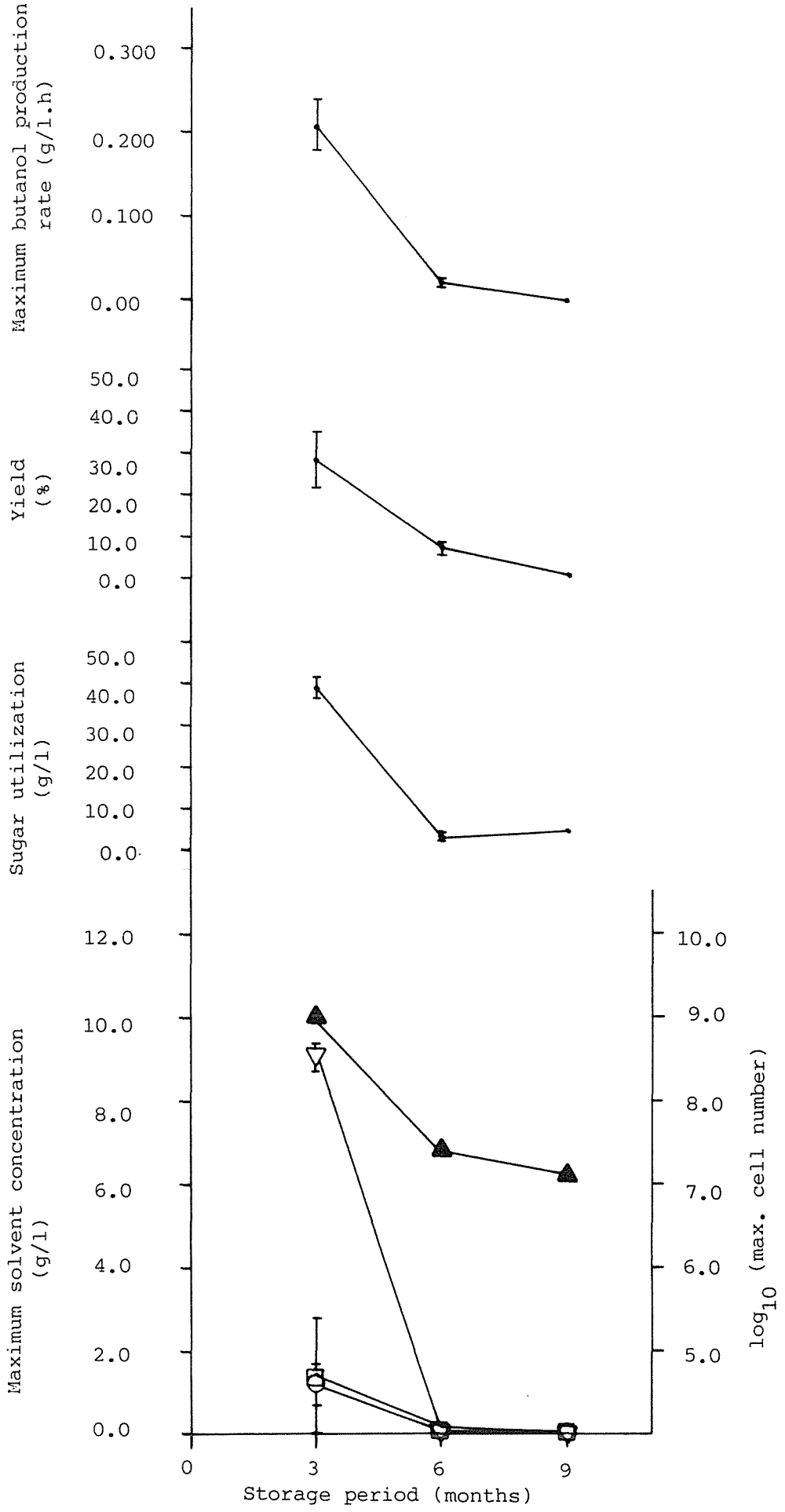


FIGURE 7.4h: Fermentation ability of strain NCIB 2951 maintained by monthly transfer with heat shocking at 70°C, 1.5 min (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol, □ max. acetone).

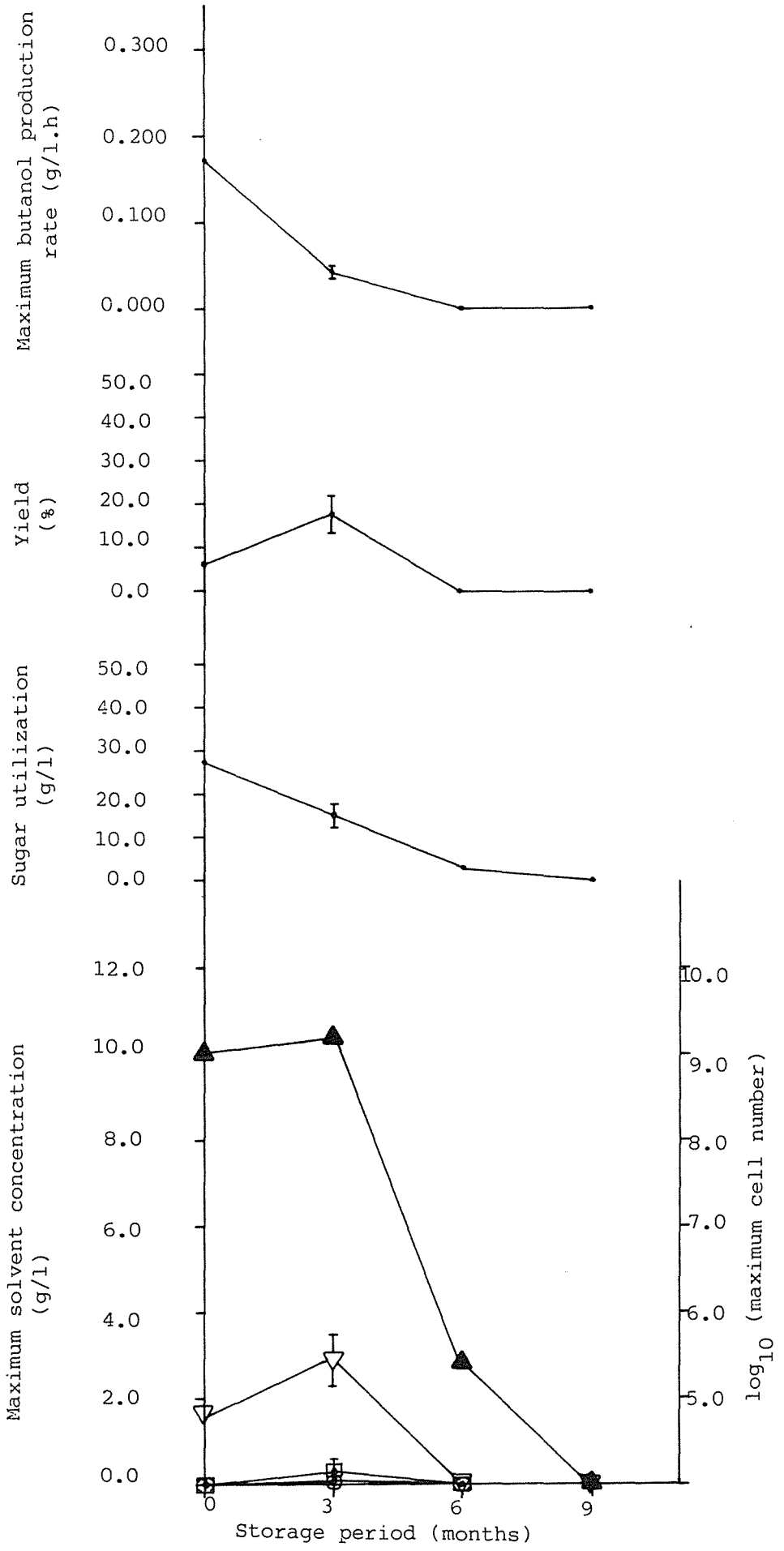


FIGURE 7.4i: Fermentation ability of strain NCIB 2951 maintained by monthly transfer without heat shock (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

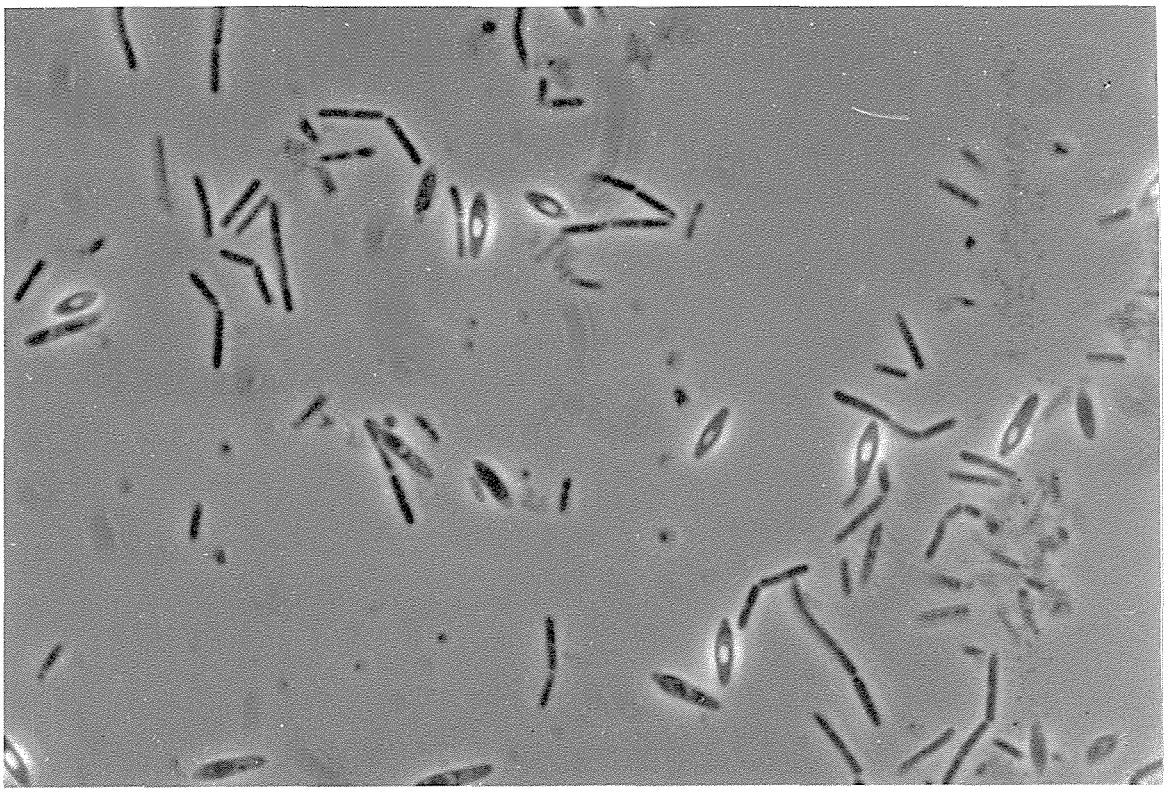


Figure 7.5: Photomicrographs of *Cl. acetobutylicum* NCIB 2951, during fermentation in WPYE, showing the cigar-shaped clostridial forms and the phase-dark clostridial forms with phase-bright maturing spores. (1250 X)

viability (Section 7.3.2.1) but also the morphological and biochemical properties related to solventogenesis. Loss of motility after prolonged storage occurred in fermentations where there was subsequent reduction in solvent production.

The formation of clostridial cells was maintained throughout the entire period of storage only by the soil culture. Fig. 7.5 shows the clostridial forms observed during fermentation carried out by the stock culture in soil. However, in contrast to previous studies (Jones et al, 1982; Long et al, 1984) this property appeared not to be directly related to high butanol production. No clostridial forms were observed in the fermentation by cultures stored frozen in distilled water where the highest maximum butanol concentration value was obtained.

Sporulation was observed only in fermentations carried out by stock cultures in soil, lyophilization, CMMG and monthly transfers. Only the soil culture formed spores during fermentation consistently throughout the storage period. Sporulation, however, did not accompany high solvent production. These results support the observation made by Long et al (1984) that solventogenesis is independent of sporulation.

7.4.2.2 *Clostridium acetobutylicum* NRRL B-594

Preliminary fermentation experiments were performed after laying down the stocks to test if this strain was capable of butanol production from whey permeate and to devise a procedure for inoculum development through which production could be best assessed (Section 7.5.2). All these preliminary experiments showed that the strain NRRL B-594 has a poor utilization of the substrate. Glucose, therefore, was added to the fermentation media to obtain reasonable levels of solvent concentration. As a consequence, an evaluation immediately after laying down the stock cultures could not be performed.

The pH of the fermentation medium, WPYEG, ranged from 5.35 to 5.8 after autoclaving. The approximate concentrations of glucose and lactose were 46 g/l and 40 g/l, respectively.

This strain completed growth one to two days after inoculation, and a similar ratio of solvents was observed as with NCIB 2951. Maximum solvent production occurred 7-8 days after inoculation. Fig. 7.6 shows the typical fermentation profile of this strain observed with the culture frozen in distilled water after 12 months of storage. Solvents are produced at about half the level as occurred with strain NCIB 2951.

Similar fluctuations in butanol production over the entire storage period of 12 months occurred as were observed with the viability of this strain (Fig. 7.7a-i). Several points may serve to explain such observations. Firstly, there were two physiological forms of the organism that went into storage, namely, the spores and the vegetative cells.

Secondly, each has its own reaction to the heat treatment to which the stock cultures were subjected prior to fermentation. Most vegetative cells are killed during heat treatment. Spores are inherently heat resistant but lose such characteristic during germination, the process of which can be activated by physical agents such as sublethal heat. However, spores may be cryptobiotically dormant, that is, spores which are more or less fully matured but not yet altered by the processes of activation and germination. The retention of cryptobiotic dormancy depends on factors that affect activation or germination or both processes. These factors are constitutive (permeability barriers, metabolic blocks, autogenous inhibitors) and exogenous (physical or chemical condition of the environment). Not all members of a sample of spores germinate simultaneously implying that the individual spores vary in their susceptibility or that some local fluctuations of the environment affect the activation or the initiation of germination

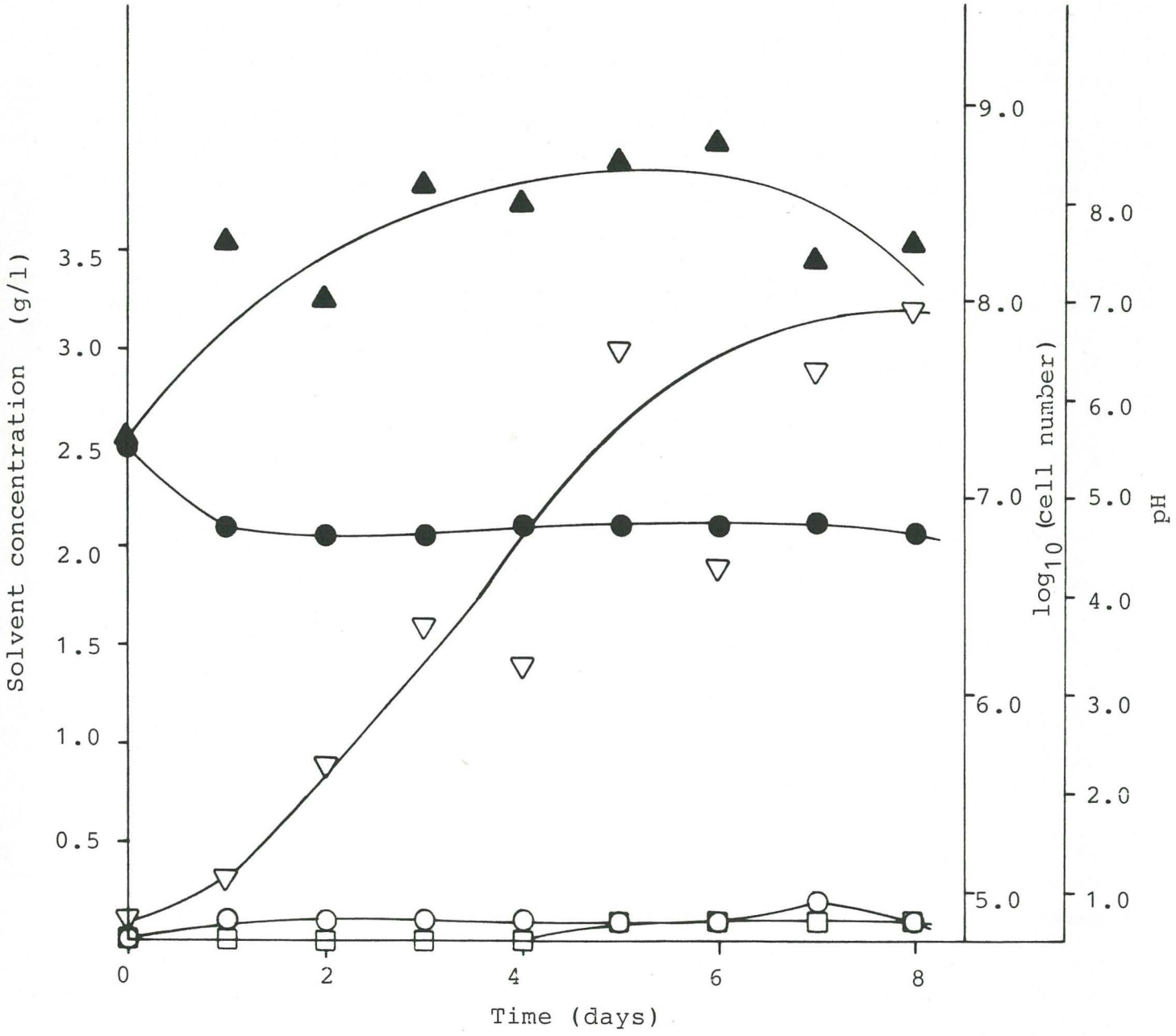


Figure 7.6: Fermentation course of strain NRRL B-594 kept frozen in distilled water at -20°C for 12 months (▲ $\log_{10}$ cell number; ▽ butanol; ◯ ethanol; □ acetone; ● pH).

of only a part of the population. The proportion that remains dormant may be so large as to be characteristic of the species. Population heterogeneity is expressed in "delayed germination" or a slow increase in colony counts. The small fraction of a spore population that remains dormant after exposure to a good germination condition has been termed "superdormant" (Lewis, 1969). The population heterogeneity, delayed germination and "superdormant" spores may be characteristics of strain NRRL B-594. The "superdormant" spores were probably activated only after 9 months of storage in soil, distilled water at -20^{o}C, CMMG and by monthly transfer with or without heat shocking as demonstrated by the results of the viability test (Section 7.3.2.2). Such activation was facilitated by the exposure of the stock culture to heating at 80^{o}C for 15 min.

Thirdly vegetative cells which outgrew from germinated spores of this strain can possibly carry out a more vigorous fermentation than vegetative cells which have not been through a sporulation stage.

As with strain NCIB 2951, production of solvents (after 3 months of storage) may have been influenced by the level of dissolved free oxygen in the fermentation media, which were not freshly autoclaved, before inoculation.

All the stock cultures remained viable and reached a maximum cell concentration during fermentation which ranged from 10^{8} to 10^{9} cells/ml, and carried out fermentation with differing levels of production over prolonged storage. Only the level of butanol production was significantly influenced by the maintenance procedure and storage, and not those of ethanol and acetone.

The typical effect of maintenance procedure on this strain is best illustrated by the results obtained from the lyophilized culture (Fig. 7.5f) where the methodology of evaluation was standard, at least from the sixth month

of storage. The highest maximum butanol production of 2.9 g/l was observed after 9 months of storage, probably when the dormancy of spores was broken. After 12 months of storage, although there was no loss in viability, the fermenting ability declined considerably. In Section 7.3.2.2, a reduction in the viability of the lyophilized culture was found after 9 months, suggesting that only the spores remained viable and that the increase in solvent production at this stage may be due to the ability of the activated spores to carry out a vigorous fermentation. The decline at 12 months of storage may indicate that the fermenting ability of this strain has a certain "shelf-life" that does not extend beyond this period and, therefore, reprocessing of the stock culture is required.

Freezing in distilled water at -20^{o}C (Fig. 7.7c) can be considered the best method for this strain, as with strain NCIB 2951. As long as a fresh stock culture could be used for each fermentation, a reasonable production could be achieved over a long storage period. High concentration values, as well as yield and maximum production rate of butanol at 2.0 - 3.2 g/l, 20.0 - 26.7%, and 0.025 - 0.029 g/l.h, respectively, were obtained and retained using this method. There was no considerable reduction in these parameters after one year of storage.

Solvents production carried out by the other stock cultures were generally poorer. The butanol producing ability of the organism was almost completely lost after 12 months of storage in methods other than storage by freezing in distilled water at -20^{o}C. However, except for the stock cultures in the periodic transfer lines and in distilled water (4^{o}C), the levels of sugar utilization, butanol yield, and maximum butanol production rate seem to have been maintained fairly well over the entire term of storage.

The deleterious effects of repeated use and thawing of stock cultures were observed with stock cultures in liquid media. Possible mutational changes imposed by frequent transfer without heat shocking (Fig. 7.7i) caused a decrease in butanol production after 6 months, and which also altered the physiology of the organism, thereby not reaching the normal period by which spore dormancy could have been broken.

Unlike that of strain NCIB 2951, there was no complete loss in the solvent producing abilities of the stock cultures of this strain maintained by monthly transfer with or without heat shocking after prolonged storage. This may be accounted for by the manner in which these culture lines were started. Prior to laying down the stocks, strain NRRL B-594 underwent more cultivation stages after retrieval from the original lyophilized specimen than did strain NCIB 2951, such that any possible injury in the lyophilized cells was not carried over to these culture lines. Consequently, the degenerative effect of frequent transfer did not become worse.

Although this strain is not highly motile, a good correlation between motility and butanol production was observed as with strain NCIB 2951. Clostridial forms and spores occurred in only some stock cultures and were not consistent features of high solvent producing cultures. However, as far as maintenance of production of clostridial forms is concerned, drying in soil may be effective.

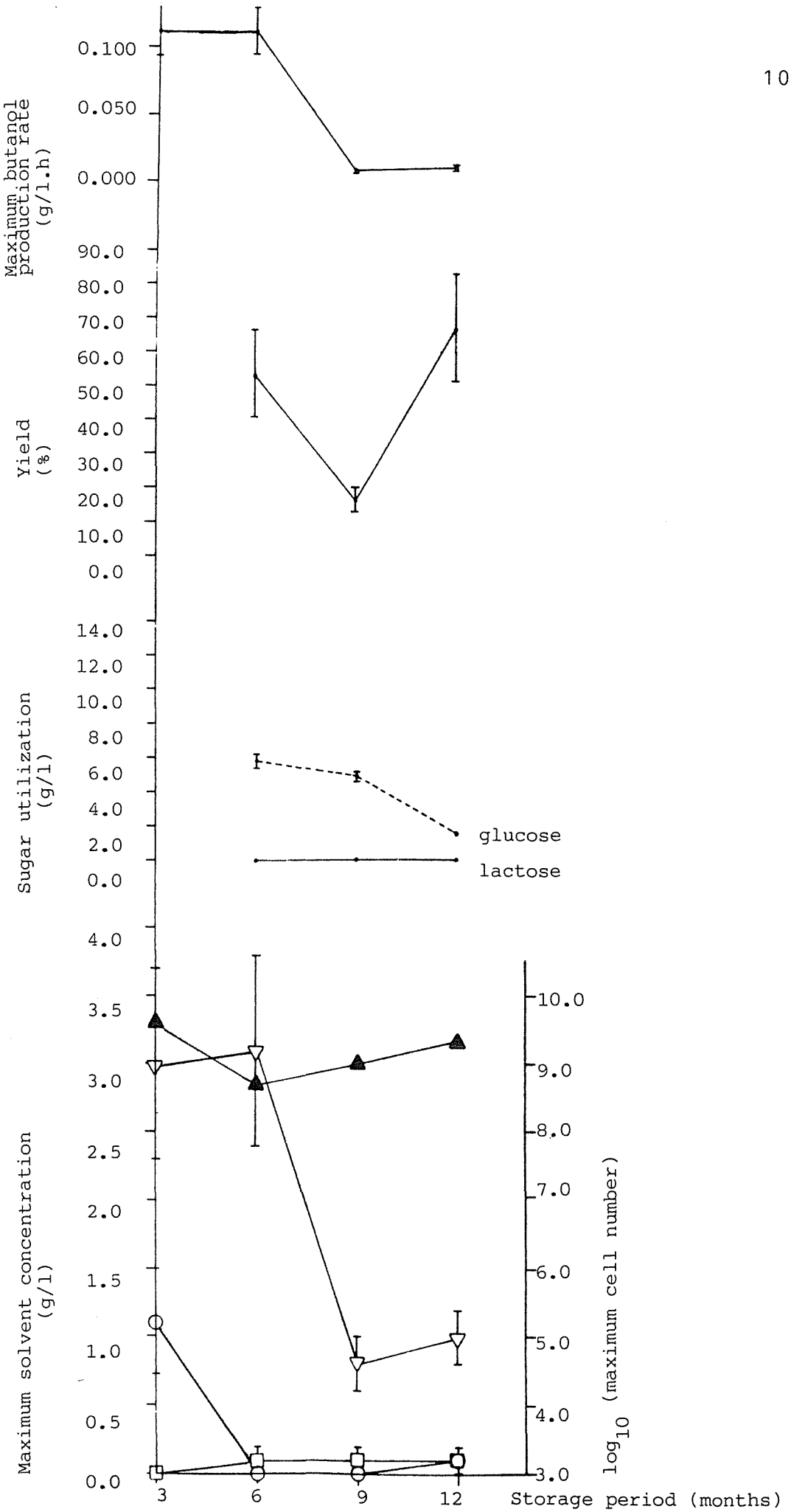


FIGURE 7.7a: Fermentation ability of strain NRRL B-594 maintained by refrigeration in distilled water at 4°C (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

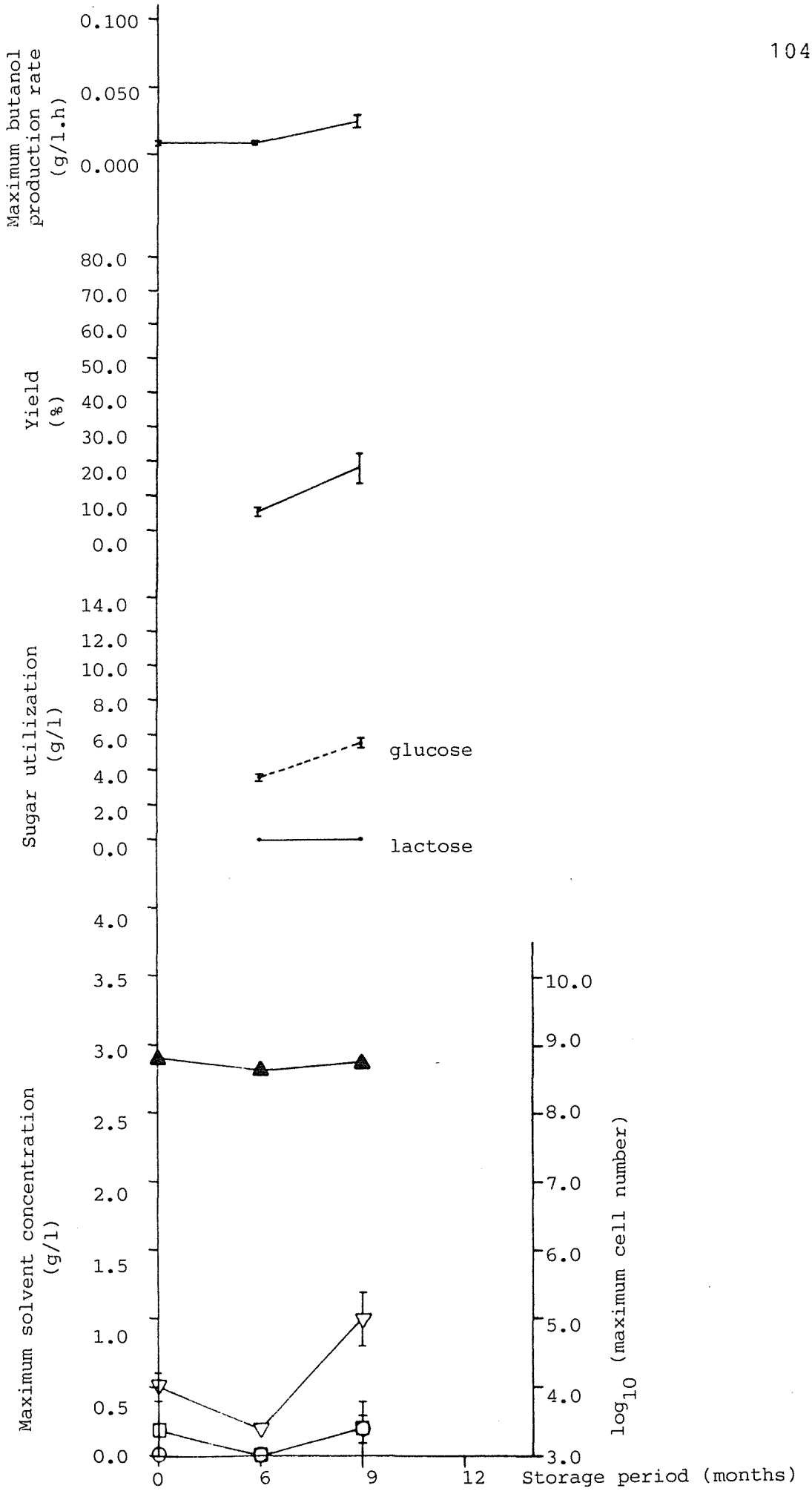


FIGURE 7.7b: Fermentation ability of strain NRRL B-594 maintained by refrigeration in phosphate buffer at 4°C (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

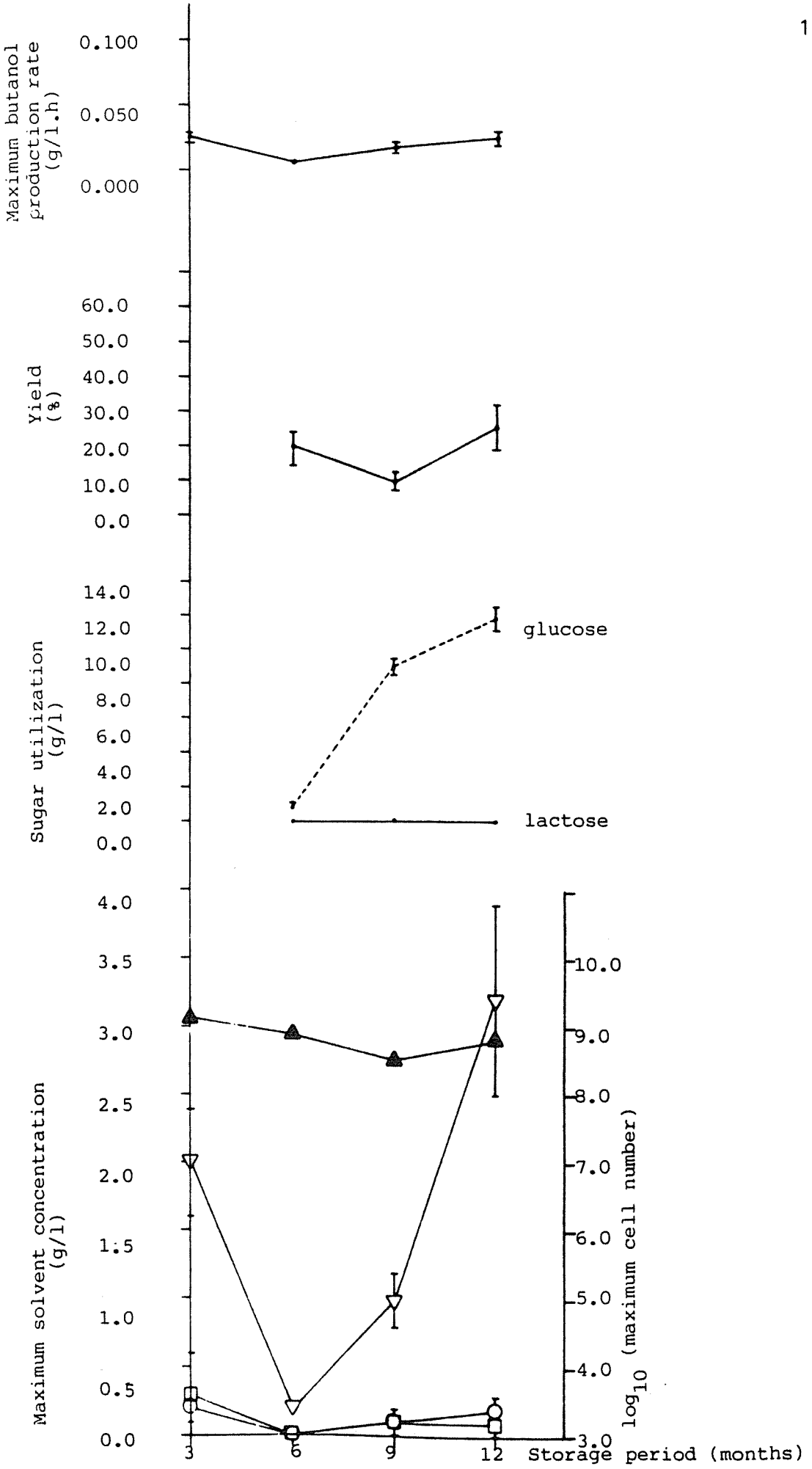


FIGURE 7.7c: Fermentation ability of strain NRRL B-594 maintained by freezing in distilled water at -20°C (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol □ max. acetone).

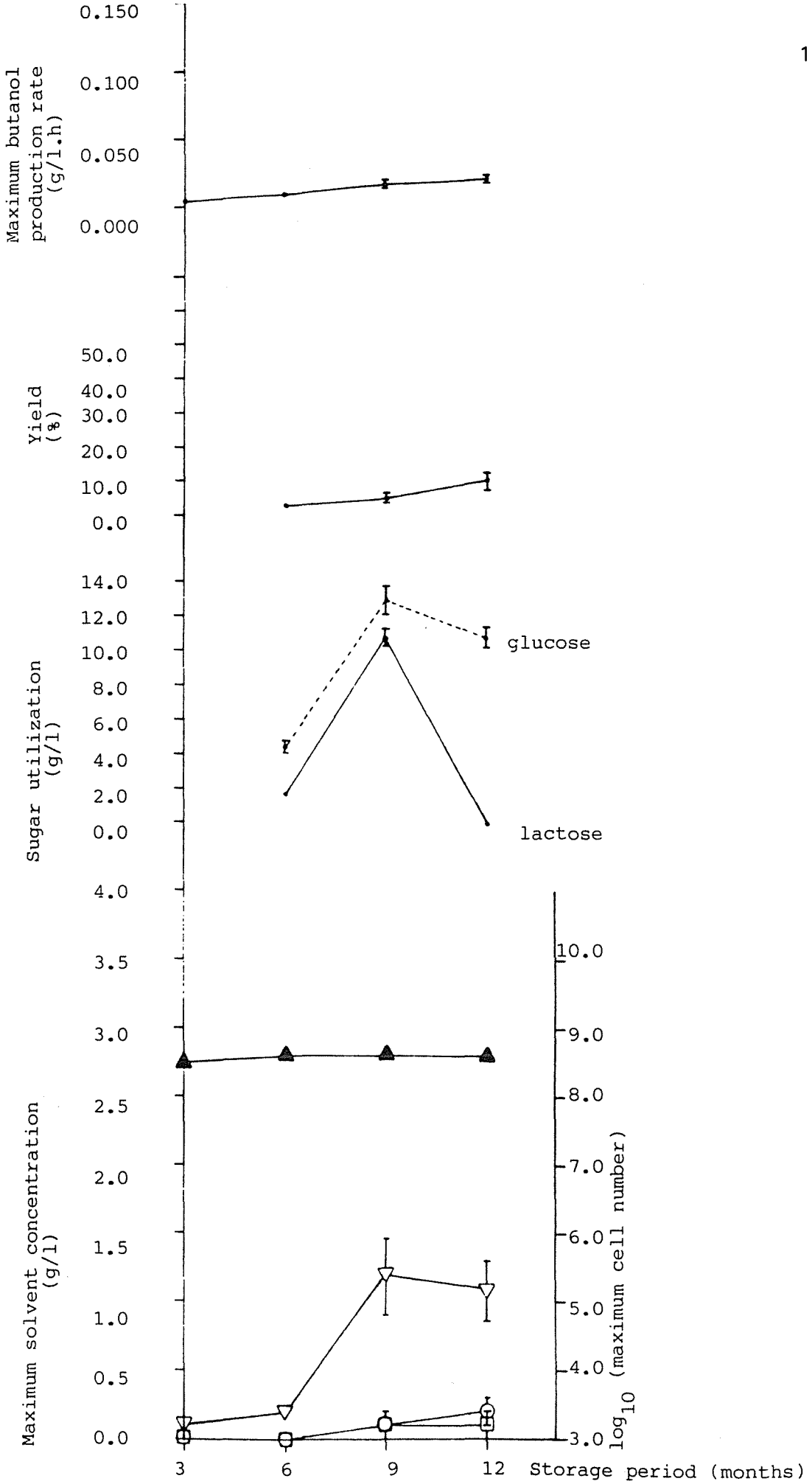


FIGURE 7.7d: Fermentation ability of NRRL B-594 maintained by freezing in phosphate buffer at -20°C (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

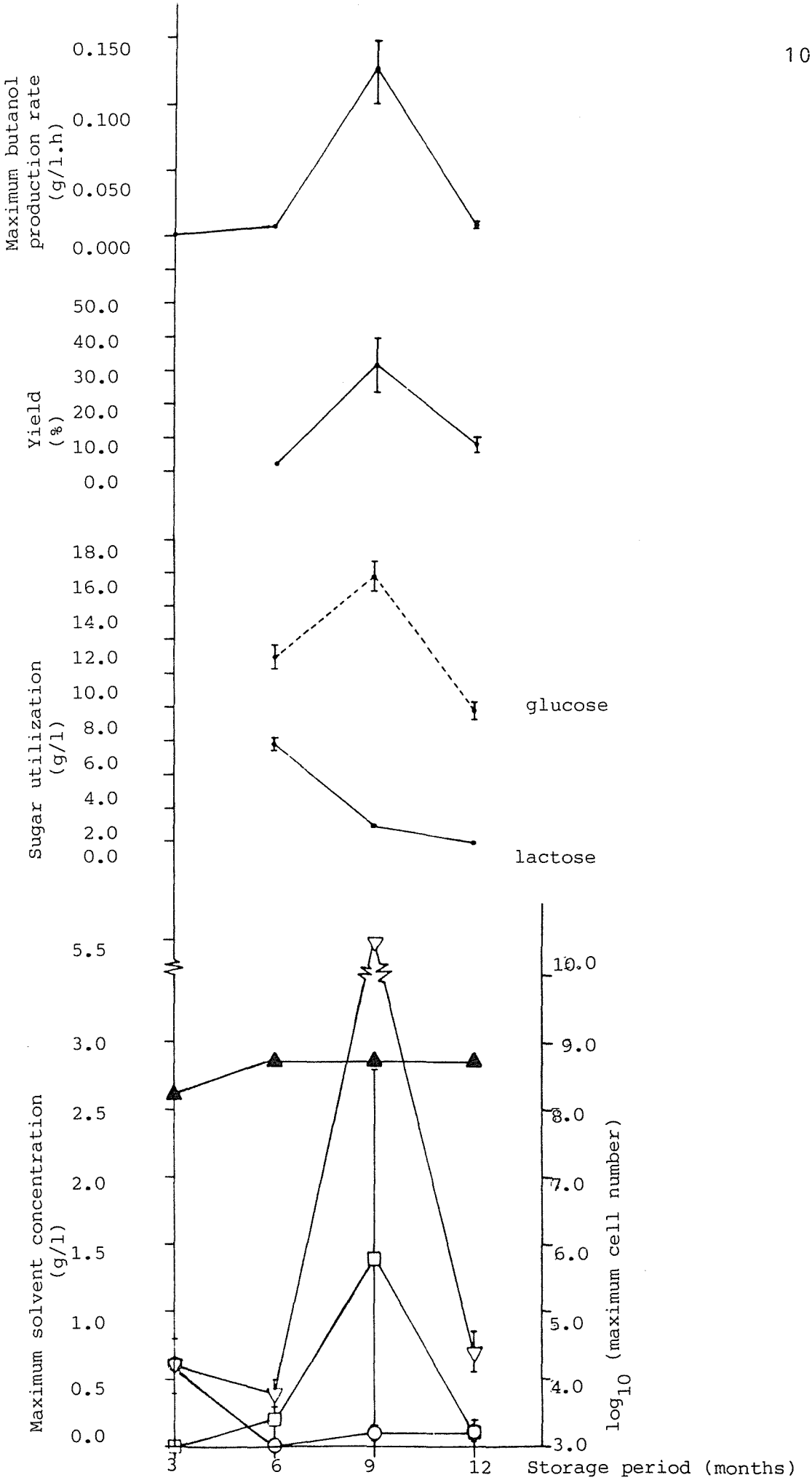


FIGURE 7.7e: Fermentation ability of strain NRRL B-594 maintained by drying in soil (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

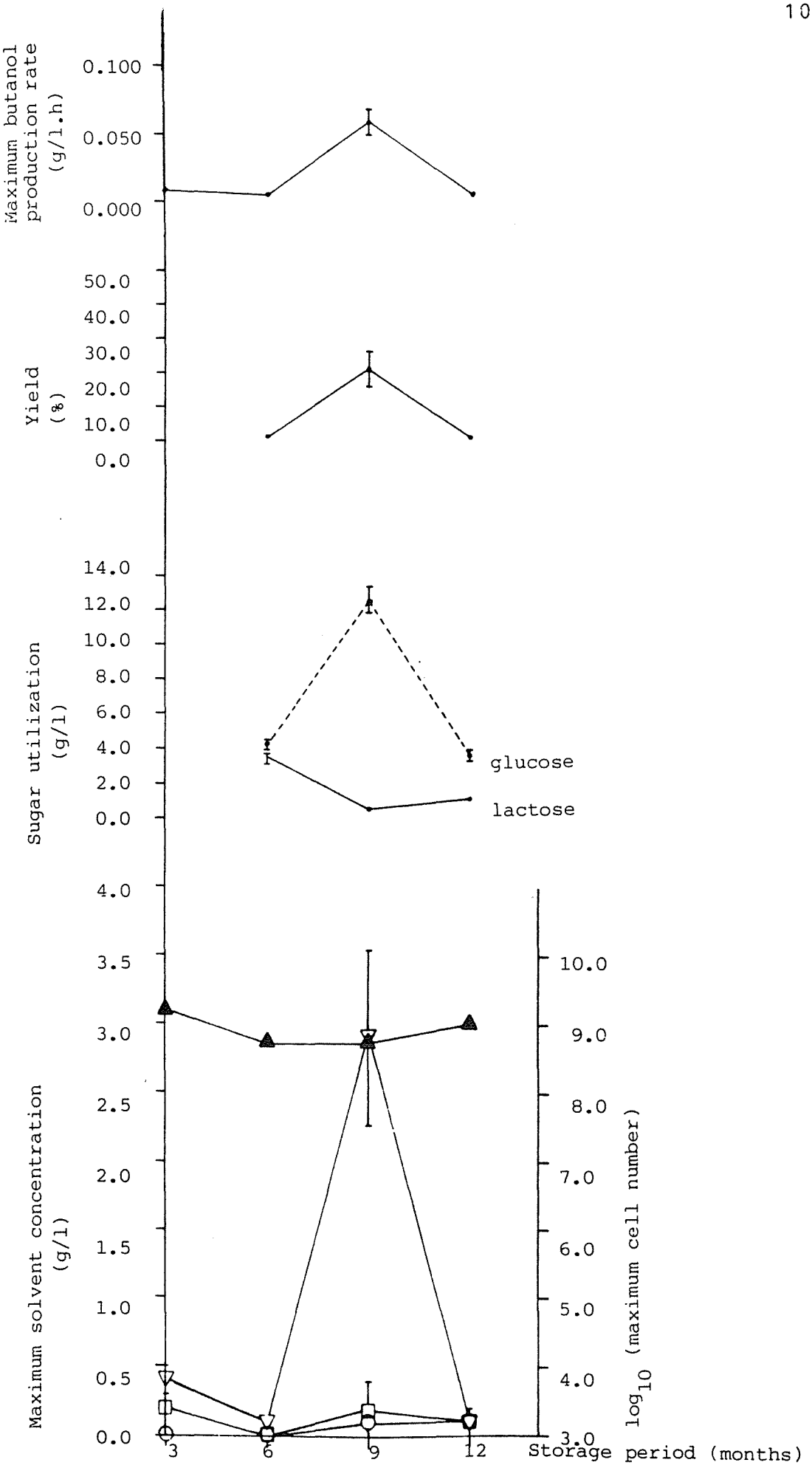


FIGURE 7.7f: Fermentation ability of strain NRRL B-594 maintained by lyophilization (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

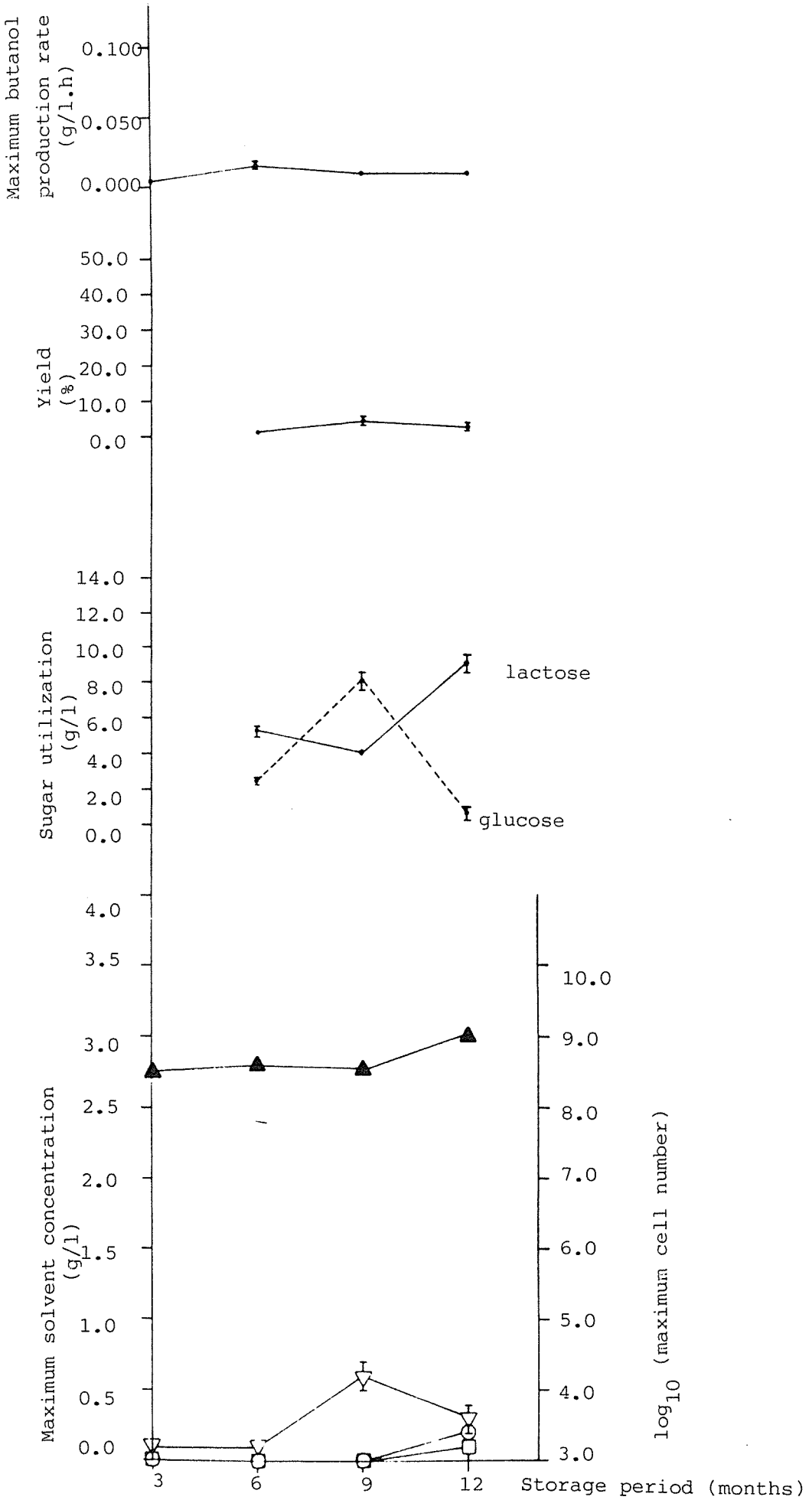


FIGURE 7.7g: Fermentation ability of strain NRRL B-594 maintained in CMMG (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

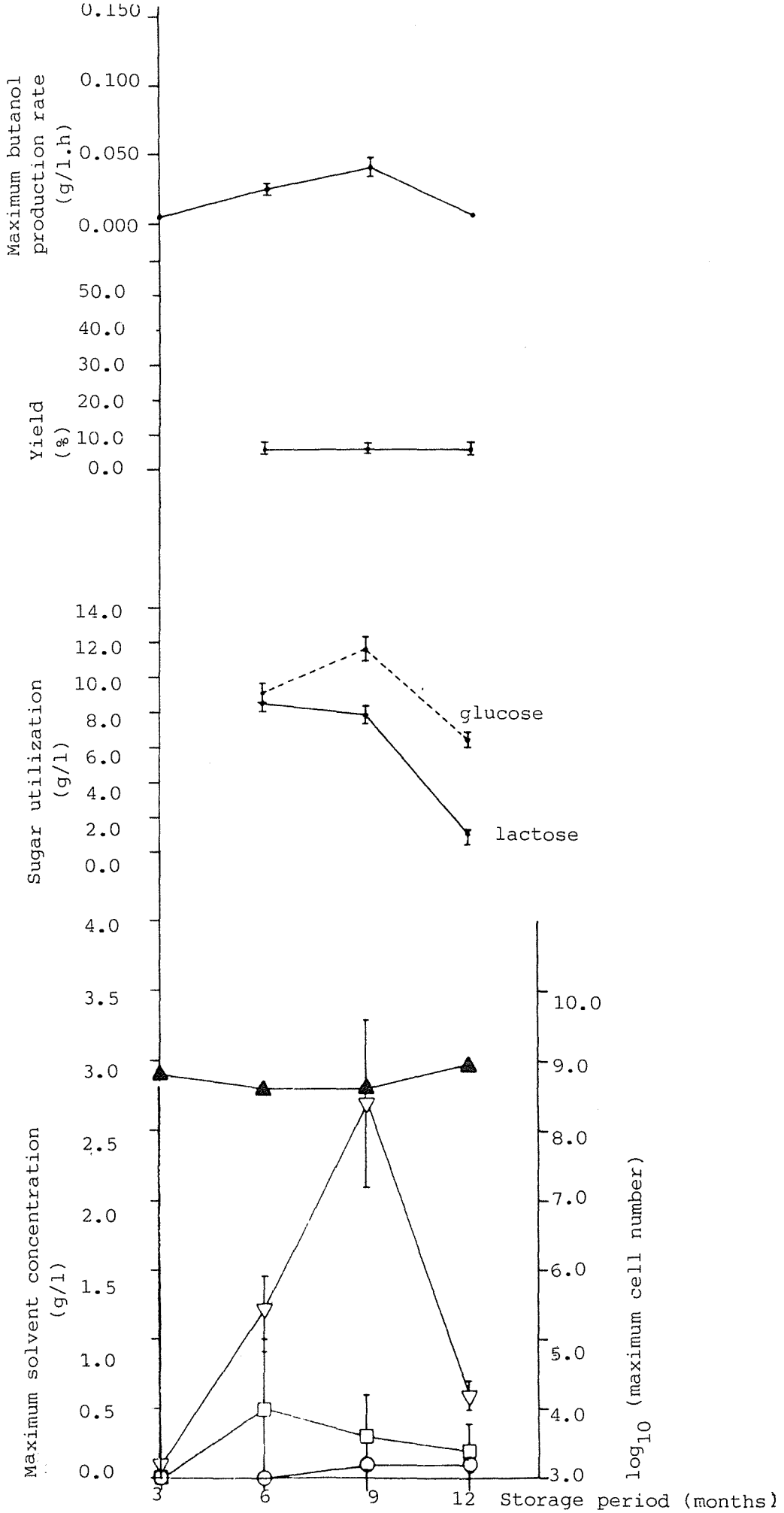


FIGURE 7.7h: Fermentation ability of strain NRRL B-594 maintained by monthly transfer with heat shocking at 80°C, 15 min. (△ $\log_{10}$ max. cell number; ▽ max butanol; ○ max. ethanol; □ max. acetone).

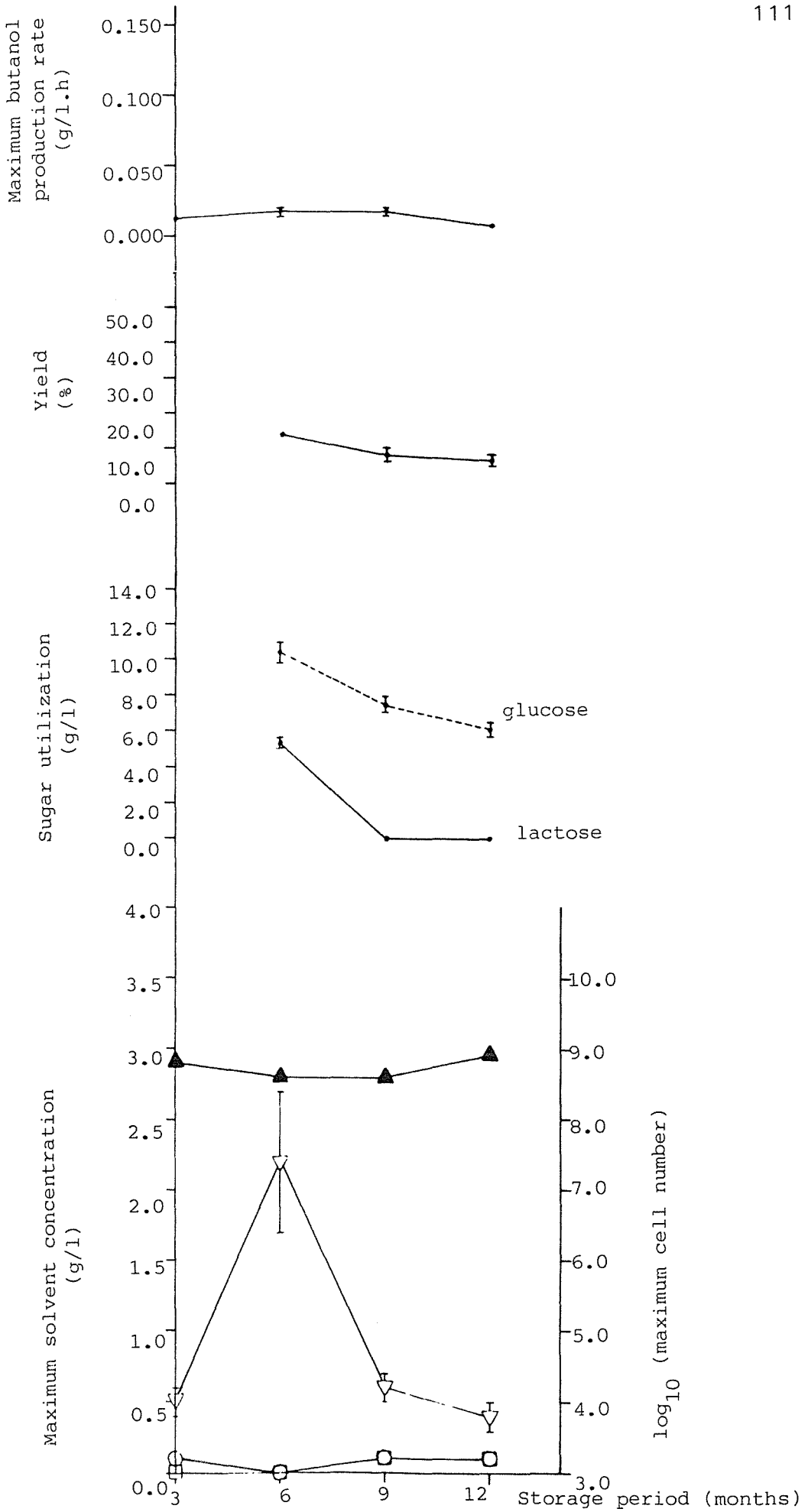


FIGURE 7.7i: Fermentation ability of strain NRRL B-594 maintained by monthly transfer without heat shocking (▲ $\log_{10}$ max. cell number; ▽ max. butanol; ○ max. ethanol; □ max. acetone).

7.4.3 Conclusions

The methods of culture maintenance and prolonged storage do have an influence on the butanol producing ability of the two strains, NCIB 2951 and NRRL B-594.

The test on fermentation capacity is a more reliable index of maintenance procedure than is viability. Some stock cultures may be viable but not necessarily solventogenic.

The physiological form of strain NCIB 2951 laid down into storage does not seem to affect solventogenesis as much as it affects viability. However, such condition is a more critical factor for both solvent production and viability of strain NRRL B-594.

Hence, upon receipt of a lyophilized culture from a culture collection, it is recommended that, prior to laying down stock cultures or testing fermentation ability, the culture be revived and propagated through several culture stages, and then stimulated to sporulate so that the spores can be collected and used as the stocks.

Among the storage methods investigated, preservation by freezing in distilled water at $-20^{\circ}C$ was the most effective for both strains of organism. Storage by this method can maintain high butanol production with a correspondingly high yield and rate of production. The strains NCIB 2951 and NRRL B-594 can be stored for 9 months and 12 months, respectively, without any significant reduction in their solventogenic properties. Culture maintenance by this method is, therefore, highly recommended for being efficient, simple and economic.

Frequent (monthly or weekly) transfer is a very poor and risky method. The organism loses its functional property despite remaining viable.

Lyophilization is not as effective as freezing of cultures in distilled water at -20°C. Although the rate of butanol production by strain NCIB 2951 is hardly affected by such storage method, a slight reduction in the level of production and yield occurred after 6 months and 9 months of storage, respectively. This method is effective only for a period of 9 months with respect to the solventogenic property of strain NRRL B-594.

To avoid degeneration of solvent producing ability, or perhaps to revive it, reprocessing of stock cultures is necessary after 9 months storage for strain NCIB 2951 if kept by refrigeration at 4°C (in distilled water, phosphate buffer, CMMG) or drying in soil.

The "shelf-life" of the solventogenic property of strain NRRL B-594 does not seem to extend beyond 9 months of storage by the methods other than freezing in distilled water. Therefore, reprocessing after this period is also necessary with respect to such methods.

The repeated use of the stock cultures does not adversely affect the fermentation capacity (degeneration) as considerably as it reduces viability. However, this practice should be completely avoided.

Degenerate cultures form less phase-bright cells than do vigorous cultures, and tend to become sluggish in growth which consequently retards the inoculum development stage of the fermentation.

Motility appears more directly related to high solvent production than does the development of clostridial forms.

Storage of cultures on soil promotes and maintains clostridial form formation during fermentation. However, the presence of such forms does not correspond to high solvent production.

7.5 INOCULUM DEVELOPMENT TO IMPROVE FERMENTATION

7.5.1 Introduction

It is necessary that a procedure for inoculum development has devised that would not only exploit the potential of the stock culture to produce the desired solvents but also to improve the yield and production rate.

Several experiments, using mainly strain NCIB 2951, were conducted and assessed on the basis of the above objectives. Since these were performed simultaneously with the evaluation of fermentation ability (Section 7.4), the stock cultures used were not necessarily the most efficient, but those where ample stocks were available.

7.5.2 Methods

7.5.2.1 Development of a Standard Procedure for Fermentation Test

Procedures were devised which generally involved the use of either glucose or lactose in the retrieval medium for the stock culture, use of lactose in the pre-fermentation stages (adaptation), and the use of a higher level of inoculum (10% v/v), with and without heat shocking at 80°C, 15 min. The heat shocking process was adopted from the experiment discussed in Section 7.3.2.

The stock culture of strain NRRL B-594 maintained in CMMG at 4°C was used for these experiments.

Method I (Use of lactose in the retrieval medium)

Stage 1: One ml of the stock was suspended in 20ml CMML and heat shocked at 80° C for 15 min followed by cooling in iced water. The culture was incubated at 30° C for 48 hr after which time gassing was observed.

Stage 2: The culture was inoculated at 1 ml to 20 ml WPYE(G).

Stage 3: Five ml of the culture were then transferred to 95 ml WPYE(G).

Method II (Use of glucose in the retrieval medium and adaptation in lactose)

Stage 1: One ml of the stock was transferred to CMMG (20 ml) followed by heat shocking at 80°C for 15 min. The culture was immediately cooled in iced water and then incubated at 30°C until gassing occurred (24 hrs).

Stage 2: The culture was transferred at 1 ml to 20 ml CMML.

Stage 3: Subsequent transfer was done at the same inoculum level into 20 ml WPYE(G).

Stage 4: Five ml of the culture were transferred to 95 ml WPYE(G).

Method III (Use of 10% v/v inoculum)

Stage 1: A higher inoculum volume, i.e. 2 ml of the stock culture, was suspended in CMML (20 ml) Heat shocking at 80°C for 15 min and subsequent cooling followed.

Stage 2: The culture was inoculated at 1 ml into 20 ml WPYE(G).

Stage 3: Five ml of the culture was transferred to 95 ml WPYE(G).

Parallel experiments were performed without heat shocking the stock culture.

The butanol production and yield from WPYE by strain NRRL B-594 are shown in Table 7.5. The highest butanol concentration was obtained using Method II + H.S. but this was not significantly greater than that using Method I + H.S. The fermentations using Method I - H.S. and II - H.S. proceeded only up to the 2nd and 3rd stage, respectively, due to very sluggish growth, suggesting that heat shocking is necessary for adequate growth and fermentation rates. Thus, the results suggest that cells from activated spores are more efficient fermenters than the original vegetative cells which, although capable of growth in the media used, were not vigorous enough to withstand several transfers and carry out fermentation.

Although butanol was produced from Method III - H.S., and this method did, in fact, perform better than III + H.S., the larger inoculum size seems deleterious to butanol production.

Due to the poor utilization of lactose in WPYE, glucose was added to the fermentation medium at 50 g/l (WPYEG) to attain a level of production at which comparison of fermentations by the different stock cultures (Section 7.4) and of inoculum development methods would be simplified.

Table 7.6 shows the effect of the different methods on butanol production and yield in WPYEG after 8 days of fermentation. Higher butanol concentration values were obtained than those from WPYE.

The production and yield of butanol using Method II + H.S. were significantly higher than those using the other methods, thus confirming that this method of inoculum development was the most effective. This suggests that Stage I culture is the most critical. The conditions that favour the growth and development of vigorous cells from the inoculum such

TABLE 7.5: Effect of the different inoculum development procedures on butanol production and yield in 100 ml WPYE by *Cl. acetobutylicum* NRRL B-594

Method	Butanol concentration (g/l)	Lactose utilization (g/l)	Yield (%)
I + H.S.	2.2 ± 0.5	2.7 ± 0.2	—
I - H.S.*	—	—	—
II + H.S.	3.0 ± 0.7	12.5 ± 0.8	24.0 ± 5.8
II - H.S.*	—	—	—
III + H.S.	0.6 ± 0.1	5.3 ± 0.3	11.3 ± 2.7
III - H.S.	1.5 ± 0.3	11.6 ± 0.7	12.9 ± 3.2

* fermentation did not proceed

TABLE 7.6: Effect of the different inoculum development procedures on butanol production and yield in 100 ml WPYEG by strain NRRL B-594

Method	Butanol concentration (g/l)	Lactose utilization (g/l)	Glucose utilization (g/l)	Yield (%)
I + H.S.	4.1 ± 0.9	3.9 ± 0.2	24.5 ± 1.5	14.4±3.5
I - H.S.	2.9 ± 0.6	4.4 ± 0.3	23.7 ± 1.4	10.3±2.5
II + H.S.	8.2 ± 1.8	4.7 ± 0.3	41.6 ± 2.5	22.2±5.3
II - H.S.	5.1 + 1.1	5.4 ± 0.3	32.6 ± 2.0	13.4±3.2
III + H.S.	2.7 ± 0.6	6.2 ± 0.4	26.1 ± 1.6	8.4±2.0
III - H.S.	0.7 ± 0.2	3.7 ± 0.2	13.1 ± 0.8	4.2±1.0

\+ H.S. with heat shocking

\- H.S. without heat shocking

as heat shocking and the presence of a more utilizable nutrient, i.e. glucose, in the retrieval medium are important factors. Data are not sufficient, however, to define the effect of conditioning or adaptation in lactose prior to fermentation.

In WPYEG, fermentation proceeded with those cultures which had not been heat shocked, possibly due to the presence of the additional sugar which promoted growth of vegetative cells and, to a little extent, germination of spores.

The use of the higher level of original inoculum was not advantageous.

Method II + H.S. was subsequently employed in assessing the fermentation capacity of the different stock cultures during storage.

7.5.2.2 Effect of the Number of Inoculum Development Stages on Subsequent Fermentation

The number of culture stages before fermentation was varied by modifying the standard procedure used for the fermentation test (Method II + H.S.).

Stock cultures of strains NRRL B-594 in soil and distilled water at 4^{o}C, and NCIB 2951 in distilled water at 4^{o}C, were used.

Inoculum Preparation. One ml of the stock culture was suspended in CMMG before heat shocking at 80^{o}C, 15 min.

This was immediately followed by cooling in iced water. The culture was then incubated at 30^{o}C until good gassing occurred.

1 - Stage: Five ml of the CMMG culture was transferred to 95 ml fermentation medium.

2 - Stage: One ml of the CMMG culture was transferred to 20 ml CMML and incubated for 48 hr at 30°C.

Five ml of the CMML culture was inoculated to 95 ml fermentation medium.

3 - Stage: Cultivation in CMML (20 ml) was carried out twice before the fermentation stage.

4 - Stage: Cultivation in CMML was carried out three times before the fermentation stage.

5 - Stage: Cultivation in CMML was carried out for three times as in the preceding method. Then, 1 ml of the culture was transferred into 20 ml of the fermentation medium and incubated for 24 hr at 30°C.

Five ml of the culture was used to inoculate 95 ml of the fermentation medium.

The solvent production by the two strains of *Cl. acetobutylicum* as influenced by the number of culture stages are shown in Table 7.7. There was a significant increase in production of butanol after 2 and 3 stages for stocks of strain NRRL B-594 in soil and in distilled water at 4°C, respectively, after which a decreasing trend was observed. Such results suggest that several stages render the culture sluggish. Considering the proportion of spores to vegetative cells, the results are not unexpected since the vegetative cells tend to be less resistant to environmental stresses than those from the outgrowth of spores.

If 2-3 culture stages were ideal for this strain, the lower butanol concentration value obtained using Method I + H.S. (Section 7.5.3.1), which consisted of 3 stages also but with the presence of lactose in the retrieval medium, can be attributed to the nature of the retrieval of the stock culture. The results, therefore, further imply that the first stage wherein the stock culture is revived or retrieved is most important. This stage should provide conditions which favour the development of vigorous cells including agents that stimulate spore germination from the stock culture, e.g. heat shocking and reduced condition; and nutrients which are easily utilizable energy sources, e.g. glucose. Glucose has been found to initiate spore germination in other species of Clostridia (Sarathchandra et al, 1977).

The results obtained with strain NCIB 2951 appear to deviate from the typical level of production. The solvent concentrations were as low as those produced by strain NRRL B-594. Except for an odd point, i.e. with 3 culture stages, there seems to be an inverse relation between production and number of cultivation stages. Based on these results, a 1-stage culture seems ideal for this strain and the vegetative cells degenerate more with an increasing number of culture stages than do those of strain NRRL B-594. Nevertheless, it is still clear that the first stage is of prime importance.

For both strains, the high solvent production obtained with the optimal number of culture stages was accompanied by a significantly high butanol yield.

TABLE 7.7: Effect of the number of culture stages on production and yield

Stock Culture	Number of stages	Maximum cell number during fermentation (log_{10})	Maximum solvent concentration (g/l)			Sugar utilization (g/l)		Yield (%)
			Ethanol	Acetone	Butanol	Glucose	Lactose	
NRRL B-594	1	8.6	0	0	0.6 ± 0.1	9.5 ± 0.6	3.2 ± 0.2	4.7 ± 1.1
(dist. water,	2	8.8	0	0.4 ± 0.4	2.6 ± 0	12.8 ± 0.8	0.9 ± 0.1	19.0 ± 4.6
4°C)	3	8.6	0	0.4 ± 0.4	3.0 ± 0.7	8.9 ± 0.5	9.4 ± 0.6	16.4 ± 3.9
	4	9.0	0	0	0.2 ± 0.04	6.6 ± 0.4	1.0 ± 0.1	2.6 ± 0.6
	5	8.9	0	0.1 ± 0.1	0.1 ± 0.02	4.3 ± 0.3	0.2 ± 0.01	2.2 ± 0.5
NRRL B-594	1	8.6	0	0	0.8 ± 0.2	7.7 ± 0.5	0	10.4 ± 2.5
(soil)	2	8.7	0	0.1 ± 0.1	2.1 ± 0.5	18.1 ± 1.1	4.1 ± 0.2	9.5 ± 2.3
	3	8.7	0	0.2 ± 0.2	1.7 ± 0.4	9.7 ± 0.6	0	17.5 ± 4.2
	4	8.6	0	0	0.2 ± 0.04	4.9 ± 0.3	0	4.1 ± 1.0
	5	8.5	0	0	0.1 ± 0.02	5.2 ± 0.3	0	1.9 ± 0.5
NCIB 2951	1	9.2	0.2 ± 0.1	0.2 ± 0.2	3.4 ± 0.7		15.4 ± 0.9	22.1 ± 5.3
(dist. water,	2	9.2	0.1 ± 0.04	0	1.5 ± 0.3		11.4 ± 0.7	13.2 ± 3.2
4°C)	3	7.5	0	0	0.1 ± 0.02		0.2 ± 0.01	-
	4	9.2	0.1 ± 0.04	0	1.3 ± 0.3		11.7 ± 0.7	11.1 ± 2.7
	5	9.1	0.1 ± 0.04	0	0.7 ± 0.2		10.0 ± 0.6	7.0 ± 1.7

7.5.2.3 Effect of different heat shocking treatments on the fermentation capacity of strain NCIB 2951

Different heat shocking conditions were employed to determine whether such treatments were necessary to improve fermentation.

The steps involved were those described for the inoculum preparation and fermentation in Sections 6.10.1 and 6.10.2, respectively, with the exception of the temperature and duration of heat shocking and interval between stages which depended on the gassing of the cultures.

The stock culture of NCIB 2951 kept in distilled water at $4^{o}C$ was used in this experiment.

The viability and fermentation of the cultures after different heat shocking conditions are shown in Table 7.8. The results indicate that heat shocking does not seem to be necessary for fermentation by this particular strain. The maximum butanol concentration values and the maximum production rates obtained with heat shocking were no higher than those obtained without any heat treatment. Further, the vegetative cells which arose from germinated spores do not appear to be more efficient fermenters than those originally present in the stock culture.

The fermentation carried out without heat shocking the stock culture can be considered efficient enough although the yield obtained from such was not the highest.

7.5.2.4 Effect of Ethanol treatment of the stock culture on fermentation by strain NCIB 2951

This experiment consisted of four treatments of the stock culture, namely, no treatment, heat shocking (as in Method II + H.S.), ethanol treatment, and ethanol followed by heat shocking treatment.

TABLE 7.8: Effect of different heat shocking treatments on the fermentation by *Cl. acetobutylicum* NCIB 2951

Treatment	Viable cells (CFU/ml x 10^2)	Maximum cell number during fermentation (log_{10})	Maximum solvent concentration (g/l) Ethanol	Acetone	Butanol	Lactose utilization (g/l)	Yield (%)	Maximum butanol production rate (g/l.h)
no heat shock	160.0	9.6	1.3 ± 0.5	0.3 ± 0.3	10.1 ± 2.2	26.1 ± 1.6	38.7 ± 9.3	0.196 ± 0.031
65.5°C, 3 min (Ross, 1961)	20.0	8.7	0.6 ± 0.2	0.3 ± 0.3	7.6 ± 1.7	16.5 ± 1.0	46.1 ± 11.1	0.133 ± 0.021
70°C, 1.5 min (Maddox, 1980)	8.0	9.3	2.0 ± 0.8	0.2 ± 0.2	7.8 ± 1.7	15.0 ± 0.9	-	0.088 ± 0.014
75°C, 2 min (Jones et al, 1982; Westhuizen et al, 1982)	20.0	9.2	1.3 ± 0.5	0.3 ± 0.3	9.8 ± 2.2	22.9 ± 1.4	42.8 ± 10.3	0.108 ± 0.017
80°C, 1 min	20.0	9.5	0.9 ± 0.3	0.3 ± 0.3	5.1 ± 1.1	16.9 ± 1.0	30.2 ± 7.2	0.046 ± 0.007
80°C, 5 min (O'Brien & Morris, 1971)	18.0	9.0	0.1 ± 0.04	0.4 ± 0.4	0.4 ± 0.1	2.1 ± 0.1	19.0 ± 4.6	0.008 ± 0.001
80°C, 15 min	26.0	9.5	0.8 ± 0.3	0.3 ± 0.3	7.1 ± 1.6	24.9 ± 1.5	28.5 ± 6.8	0.079 ± 0.013
85°C, 20 min	1.0	9.2	0.6 ± 0.2	0.4 ± 0.4	4.9 ± 1.1	19.5 ± 1.2	25.1 ± 6.0	0.083 ± 0.013
95°C, 10 min	0	-	-	-	-	-	-	-
100°C, 45 sec (Prescott & Dunn, 1959)	90.0	9.3	0.2 ± 0.1	0.1 ± 0.1	2.4 ± 0.5	4.7 ± 0.3	-	0.021 ± 0.003

For ethanol treatment, 1 ml of 50% (v/v) aqueous ethanol was mixed with 1 ml of the stock culture in a sterile screw-capped test tube and then incubated for 1 hr with occasional shaking (Koransky et al, 1978; Krouwel et al, 1981). The suspension was then centrifuged and the pellet was washed twice in sterile distilled water. The volume of the stock culture was reconstituted with sterile distilled water before inoculation.

When heat shocking was done in conjunction with the alcohol treatment, the culture was heat shocked after removal of the ethanol.

The fermentations proceeded as described in Section 6.10.2.

The stock culture kept in distilled water at $4^{o}C$ was used for this experiment.

The comparison between the fermentations obtained with ethanol treatment (with and without heat shock) and with untreated and heat shock treatment is presented in Table 7.9.

Data obtained from the heat shocking treatment confirm the observation made in Section 7.5.2.3 that there is little stimulation if any, of spore germination and that such treatment is not advantageous to fermentation.

No significant effect on the number of viable cells was observed after either heat treatment or alcohol treatment of the stock culture suggesting that the treatments involved were neither stimulatory to spore germination nor lethal to the vegetative cells of this particular strain. It becomes apparent, then, that strain has a requirement for spore germination different from those reported for other bacterial strains (Koransky et al, 1978; Keynan and Evenchik, 1969) and for NRRL B-594 (Section 7.5.2.2).

TABLE 7.9: Effect of ethanol treatment of the stock culture of strain NCIB 2951 on fermentation

Treatment	Viable cells ($CFU/ml \times 10^2$)	Maximum cell number during fermentation (log_{10})	Maximum solvent concentration (g/l) Ethanol	Acetone	Butanol	Lactose utilization (g/l)	Yield (%)	Maximum butanol production rate (g/l.h)
Untreated	8.5 ± 4.2	9.4	0.9 ± 0.3	0.4 ± 0.4	9.3 ± 2.0	41.2 ± 2.5	22.6 ± 2.5	0.125 ± 0.020
H.S.($80^{\circ}C$, 15 min)	12.0 ± 1.7	9.5	1.1 ± 0.4	0.4 ± 0.4	7.8 ± 1.7	27.0 ± 1.6	28.9 ± 6.9	0.142 ± 0.023
Ethanol (50% v/v, 1 hr)	3.0 ± 1.5	9.3	2.5 ± 1.0	0.3 ± 0.3	9.4 ± 2.1	26.0 ± 1.6	36.2 ± 8.7	0.108 ± 0.017
Ethanol (50% v/v, 1 hr) H.S.+ ($80^{\circ}C$, 15 min)	6.5 ± 0.9	9.3	1.1 ± 0.4	0.3 ± 0.3	11.1 ± 2.4	24.4 ± 1.5	45.5 ± 10.9	0.158 ± 0.025

H.S. - heat shocking

Both its spores and vegetative cells seem highly tolerant to high temperatures and the concentration of ethanol used.

Hence, no significant increase was observed in production of solvents, lactose utilization, butanol yield or production rate for any of the treatments employed.

Contrary to previous studies on the stimulatory effect of ethanol on spore germination of Clostridia (Spivey, 1978; Krouwel et al, 1981), the only important effect of ethanol in the use of this strain for solvent production could be the sterilization of any heat labile materials employed for fermentation e.g. biocatalyst particles from immobilization of cells.

7.5.2.5 Effect of butanol treatment of the stock culture of strain NCIB 2951 on fermentation

This study was conducted to explore the possibility that butanol, instead of ethanol (Section 7.5.2.4) may activate spore germination of this strain. Exposure to high butanol concentrations may also select for butanol tolerant cells.

One ml of the stock culture (kept refrigerated at $4^{o}C$ in distilled water) was mixed with 1 ml of butanol at concentrations of 10%, 30% and 50% (v/v) in a sterile screw-capped test tube. A two-phase system, i.e. between butanol and water, was observed being less distinct with 10% than the higher concentrations. Hence, frequent shaking was done during incubation of the mixture for 1 hr. The suspension was then diluted further to remove phase separation and facilitate centrifugation of the cells. The recovery of the cells and the reconstitution of the stock culture followed the procedure described for ethanol treatment (Section 7.5.2.4).

TABLE 7.10: Effect of butanol treatment of the stock culture of strain NCIB 2951 on fermentation

Treatment	Viable cells (CFU/ml x 10^2)	Maximum cell number during fermentation (log_{10})	Maximum solvent concentration (g/l) Ethanol	Acetone	Butanol	Lactose utilization (g/l)	Yield (%)	Maximum butanol production rate (g/l.h)
untreated	160.0 ± 80.0	9.3	1.9 ± 0.7	0.2 ± 0.2	12.0 ± 2.6	34.5 ± 2.1	34.8 ± 8.4	0.167 ± 0.027
10%	8.8 ± 4.4	8.7	0.7 ± 0.3	0.6 ± 0.6	5.4 ± 1.2	11.5 ± 0.7	47.0 ± 11.3	0.217 ± 0.035
30%	10.0 ± 5.0	9.2	2.7 ± 1.0	0.5 ± 0.5	16.0 ± 3.5	32.1 ± 1.9	49.8 ± 12.0	0.171 ± 0.027
50%	8.9 ± 4.4	7.6	1.2 ± 0.5	0.7 ± 0.7	1.2 ± 0.3	1.9 ± 0.1	-	0.042 ± 0.007

The fermentation proceeded as in the standard fermentation procedure (Section 6.10.2) but without any heat shocking.

Table 7.10 summarizes the results obtained with fermentations carried out by the stock cultures subjected to different concentrations of butanol. The results indicate that butanol is toxic to the cells and so it is not a chemical factor that can be used to activate germination of the spores of this strain. No significant improvement in fermentation was obtained after butanol treatment at any concentration.

7.5.2.6 Effect of transferring cultures at the onset of gassing

Transferring from one stage to another using the standard fermentation method (Section 6.10.2) was done at the onset of gassing rather than at the set incubation period. Thus, Stage 1 transfer from the CMMG culture to CMML was done after 20 hr of incubation instead of 48 hr. Transfer from the CMML culture to 20 ml WPYE (Stage 2) was performed after 24 hr rather than 48 hr. Stage 3 transfer was done after the usual interval of 24 hr.

Results (Table 7.11) show that the time at which transfer between stages is made actually has an influence on fermentation. The onset of gassing in CMMG culture took place 20 hr after inoculation while the subsequent CMML culture started gassing after 24 hr (instead of 48 hr). The most critical point appears to be the first stage. The onset of gassing of the CMMG culture coincided with the presence of highly motile cells. As previously discussed (Section 7.4.2.1), vigorous fermentation by strain NCIB 2951 was associated with motility. Spivey (1978) recognized this correlation.

TABLE 7.11: Effect of transferring culture at the onset of gassing

Treatment	Time of gassing in CMMG (hr)	Maximum cell number during fermentation (log_{10})	Maximum solvent concentration (g/l) Ethanol	Acetone	Butanol	Lactose utilization (g/l)	Yield (%)	Maximum butanol production rate (g/l.h)
Standard method	48	9.5	1.1 ± 0.4	0.4 ± 0.4	7.8 ± 1.7	27.0 ± 1.6	28.9 ± 6.9	0.142 ± 0.023
Modified method (gassing as index)	20	9.4	1.1 ± 0.4	2.8 ± 2.8	16.0 ± 3.5	27.4 ± 1.6	-	0.362 ± 0.058

Using the standard method, Stage 1 transfer was done when the vegetative cells were becoming sluggish or less motile. Whereas when transfer was performed 20 hr after incubation, highly motile cells were present. The presence of highly motile cells after heat shocking, despite their absence in the subsequent culture stages, resulted in butanol production and rate which were significantly higher than those achieved using the standard method. This implies that transfer between stages should be done when the culture is highly motile to ensure a good fermentation.

7.5.4 Conclusion

These studies have shown that inoculum development is an important aspect of fermentation by *Cl. acetobutylicum*. The manner by which this stage is undertaken has a direct bearing on the fermentation capacity of this organism.

Studies on strain NRRL B-954 showed that vegetative cells arising from spores are more efficient fermenters than those which have not undergone any sporulation. Therefore, it is important that the following should be provided during the revival of the stock cultures:

1. conditions that activate the spores in the stock culture to germinate and outgrow into vegetative cells, e.g. heat shocking.
2. nutrients that not only stimulate the outgrowth of the vegetative cells (from spores) but also enhance the growth of vegetative forms originally present in the stock culture, e.g. glucose.

More importantly, it is ideal if the stock culture consisted only of spores to ensure a vigorous fermentation.

Based on the results, the procedure for inoculum development varies with the strain of the organism. This can be accounted for by the intraspecies variation in the characteristics of spores and vegetative cells. Spores of strain NRRL B-594 are susceptible to heat, while those of strain NCIB 2951 are more resistant to heat and chemical agents such as ethanol and butanol.

Hence, heat shocking becomes necessary for the inoculum development of strain NRRL B-594 which subsequently leads to improvement in its fermentation. Neither heat shocking nor treatment with ethanol or butanol are advantageous for the inoculum development of strain NCIB 2951. The germination factors for this strain still wait to be discovered.

The time of transfer between culture stages is important, and it appears that motility should be used as the index. Highly motile cells are found to be more efficient fermenters. The revival stage (Stage 1) is the most critical and transfer from such should be done while the culture is highly motile. Such practice produces a marked increase in butanol production and rate (which is an important parameter for a commercial fermentation).

This practice remains to be of prime importance to strain NCIB 2951 while the germination factors have not been identified. Considering that the vegetative cells developing from the outgrowth of germinated spores are more efficient fermenters than those originally present in the stock culture, it can be speculated that if this practice were combined with the proper treatment to activate spore germination, even higher values would be attained.

7.6 PRODUCTION OF SOLVENTS FROM WHEY PERMEATE IN FERMENTER CULTURE

7.6.1 Introduction

A scale-up of the process utilizing the standard method adopted for the fermentation test (Method II + H.S.) was performed in a 1.6-l laboratory scale fermentation. It is necessary to obtain the most possible information during scale-up studies with the hope that this information will be valid at the production level.

7.6.2 Results and Discussion

The stock culture of strain NCIB 2951 kept by refrigeration at $4^{o}C$ in distilled water was used for this experiment. However, it was an entirely different batch to that used previously since the earlier experiments had consumed all of the original stock cultures. The study was conducted before the assessment of the preservation method could be finalized. Therefore, the choice of the stock culture was not based on consideration of the best method of preservation. The main intention of the experiment was to determine whether Method II + H.S. performed satisfactorily during a fermentation on a larger scale.

The larger scale fermentation showed cell growth at the same maximal level of 3.0×10^{9} cells/ml as the 100-ml scale. In both cases, highly motile cells were observed on the first day of fermentation, after which there was a cessation of motility. The large scale fermentation also followed the typical course of fermentation by this strain.

The results of the fermentation in WPYE obtained for the 100-ml and 1.6-l scales are summarized in Table 7.12. There was a lower solvent production obtained from this batch of stock culture than from the batches used in the previous

TABLE 7.12: Comparison of 100 ml bench and 1.6 l fermenter scale fermentations

Scale	Maximum cell number during fermentation (log_{10})	Maximum solvent concentration (g/l)			Lactose utilization (g/l)	Yield (%)	Maximum butanol production rate (g/l.h)
		Ethanol	Acetone	Butanol			
100 ml	9.5	0.2 ± 0.1	0.4 ± 0.4	3.3 ± 0.7	30.9 ± 1.8	10.7 ± 2.6	0.058 ± 0.009
1.6 l	9.5	0.6 ± 0.2	2.0 ± 2.0	5.5 ± 1.2	32.3 ± 1.9	17.0 ± 4.1	0.088 ± 0.014

experiments. The results, however, demonstrate that Method II + H.S. can be successfully adopted for a larger scale fermentation. The level of butanol and ethanol concentrations, and the maximum butanol production rate were significantly higher than those obtained on the 100-ml scale. Such fermentation appears very efficient considering that the final stage used only a 2.5% inoculum level, i.e. half of that used in the 100 ml scale. It is possibly the lower inoculum level which accounts for the efficiency in the larger scale fermentation.

7.6.3 Conclusion

It can be concluded, therefore, that the Method II + H.S. can successfully lead to solvent production in a laboratory scale fermenter.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

These studies have confirmed that the methodologies of culture maintenance and inoculum development are critical aspects of the acetone - butanol - ethanol fermentation which should not be overlooked. The solventogenic property of the two strains of *Cl. acetobutylicum*, NRRL B-594 and NCIB 2951, are profoundly influenced by the methods used. Degeneration can occur as a result of the method of preservation and prolonged storage.

The majority of the methods of preservation evaluated were effective for storage over 9 months. The fermentation capacity of the organism rather than its viability should be used as an index for the selection of the method of culture maintenance and for anticipating when to reprocess the stock cultures.

Storage by freezing in distilled water at -20°C is the most effective for the two strains of organism. There was no loss either in viability or fermentation ability after storage of strain NRRL B-594 for 1 year and of strain NCIB 2951 for 9 months. Possibly, no genetic changes occurred during such storage since the functional property of the cultures was conserved and there were no gross morphological changes observed. Therefore, this method of culture maintenance satisfies all the important criteria for preservation of micro-organisms. Factors that influence the success of this method are the use of cells/spores from mature cultures and the presence of glycerol as cryoprotectant.

More importantly, the ability of the organism to produce high butanol concentrations with correspondingly high yields and rates of production can be conserved throughout storage.

The efficiency, mildness, simplicity and economy of operation make storage by freezing at -20°C highly recommendable for a commercial fermentation process. Heckly (1978) has commented that most of the damage leading to the loss of viability or functional property of microorganisms occurs during the freezing and thawing steps and not during the storage period. Therefore, the prospects appear satisfactory for prolonged conservation of the solventogenic property during freezing at -20°C in distilled water.

Preservation by drying on soil or lyophilization, refrigeration in distilled water, phosphate buffer and CMMG, and freezing in phosphate buffer are not as satisfactory as freezing in distilled water. Degeneration using these methods occurred to some extent after 9 months of storage. If these methods are employed, reprocessing of the stock cultures after this period is necessary.

The repeated use of the stock cultures, which is a hazard to the viability and to the fermenting ability of the organism, should be completely avoided. It may be convenient and less wasteful to keep the stock cultures in small lots, e.g. 4 ml per container in case of storage in distilled water or phosphate buffer.

It is worthwhile to consider that neither laying down of stock cultures nor fermentation be initiated directly from any lyophilized culture. Revival and propagation in several culture stages are necessary to minimise any possible damage imposed by the lyophilization technique which might affect not only the morphological but the solventogenic property of the cultures.

It is not advisable to maintain the cultures by frequent (periodic) transfer as they lose their solventogenic property despite remaining viable.

For practical purposes, motility and the presence of phase-bright cells help to indicate whether the culture is degenerate or not.

The sensitivity of the organism to the method of culture maintenance is influenced by the physiological condition of the culture that went into storage - whether spores or vegetative cells. Vegetative cells are less resistant to environmental stresses and they do not survive prolonged storage as well as spores do. On the condition that the majority, if not all, of the spores can be activated to germinate and give rise to new vegetative cells, laying down the stock cultures purely as spores (rather than vegetative cells) is more advisable because of the following:

1. spores are dormant forms characterized by a lack of metabolism, and hence are more viable for extended periods.
2. spores are not sensitive to small amount of oxygen during storage which minimizes the necessity for provision of anaerobic condition.
3. vegetative cells which outgrow from spores are more vigorous fermenters than those which have not undergone sporulation.

Strain differences exist between NRRL B-594 and NCIB 2951. The vegetative cells of NCIB 2951 are more resistant to environmental stresses during storage than those of NRRL B-594. Further, both spores and vegetative cells of this strain are more resistant to heat and chemical treatments. Consequently, the two strains require different inoculum development procedures.

Spores of strain NRRL B-594 respond to heat shocking. Therefore, it is necessary to heat shock this culture during the inoculum development stage to ensure a satisfactory fermentation. Neither heat shocking nor treatment with ethanol or butanol are advantageous for strain NCIB 2951. The germination factors for this strain still wait to be identified.

During inoculum development, motility is a reliable index as to when to transfer between culture stages. Highly motile cells are found to be more efficient fermenters. The practice of transferring from the revival stage while the cells are motile remains highly important for strain NCIB 2951 while the germination factors have not been discovered.

It appears, then, that the revival stage, i.e. Stage 1 of the inoculum development process, is the most critical. At this stage, it is important that the following be considered:

1. provision of conditions that activate the spores in the stock culture to germinate and outgrow into vegetative cells e.g. heat and chemical treatment.
2. provision of nutrients that not only stimulate such outgrowth but also enhance the growth of vegetative forms present, if any, in the stock culture.
3. transfer to subsequent stage should be done while the cells are highly motile.

It can be speculated that if the most efficient stock culture, such as that kept frozen in distilled water, could be propagated for fermentation by a procedure which involves the above factors, a highly successful fermentation will be achieved.

APPENDIX

LYOPHILIZATION TECHNIQUES

ONLY LOWER CATEGORY PATHOGENS MAY BE FREEZE-DRIED.

1. Prepare ampoules; label with filter paper strip (Number of organism, date of f.d.). Plug ampoules firmly and sterilize either by dry heat or autoclaving (Dry Heat - 90 min 170^{o}C).

2. Prepare cultures on suitable media for luxuriant growth on slopes in large bijou or universal bottles.
   Aseptically transfer 3 or 4 drops of freeze-drying medium; with a sterile loop suspend the growth in this small volume of liquid to create a heavy suspension; add 1-1.5 ml of freeze-drying medium, diluting the suspension; run this additional fluid down the side of the glass container. The preparation of suspensions of pathogens must be done in the biohazard cabinet.

3. Check purity of culture prior to f.d. by streaking out f.d. suspension and by staining of culture. Check availability of liquid air desiccant: -
   1 - 2 litres required.

4. Loading of ampoules: decontaminate bench surfaces and room if possible prior to f.d. To avoid cross contamination, f.d. only 2 or 3 different organisms per batch. 48 ampoules can be f.d./batch. Use long Pasteur pipettes to load ampoules; place 5 drops (0.05 cm^3/drop) or 0.2 cm^3 into each ampoule. Avoid contaminating sides of tube with culture for containment purposes and also to prevent charring later in the procedure. Do not overload ampoules. Loading of ampoules with pathogens must be done in the biohazard cabinet.

5. Load ampoules onto freeze dryer head.

6. Grease lightly gaskets, rings, etc., on freeze dryer, also on desiccant trap.

7. Switch on machine, and pull vacuum in the desiccant trap only. Chamber valve (large black knob) is now closed, all other valves are closed so that no air is admitted to either trap or chamber.

8. Load cool trap container with liquid air.

9. Place perspex dome over ampoules and secure top fastening. (See manual for assembly scheme.)

10 Start centrifuge. (It may need a push initially).

11. Slowly open chamber valve (black knob) and allow a vacuum to pull in the chamber.

12. All other valves remain closed.

13. Allow pressure gauge to reach 760 mm, then switch on pirani gauge.

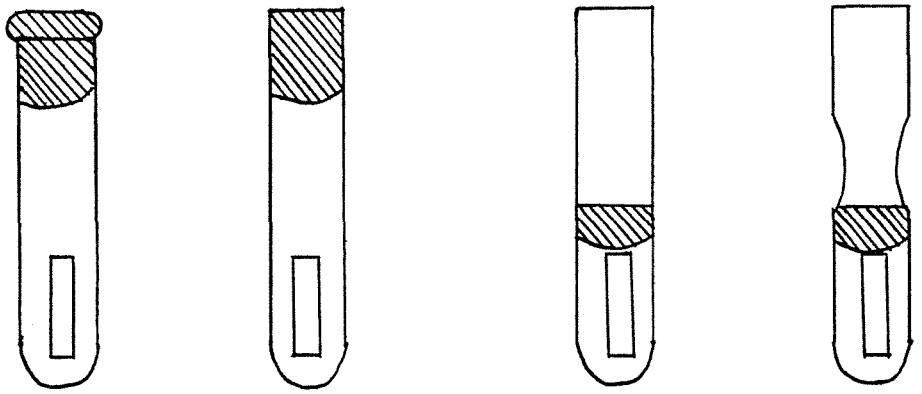
14. Centrifuge for 10-15 minutes, until suspension is frozen in ampoules.

15. Primary dry should continue until pirani gauge reads 0.1 torr(10^{-1} torr). This takes approximately $1\frac{1}{2}$ hours. Allow 2 hours for primary dry.

16. Turn off pirani gauge - do not let air into chamber until this has been done. Close chamber valve so that chamber is isolated, then slowly open chamber air valve and admit air to the chamber. Pressure will return to 0 on the gauge, and the dome can be removed.
    Remove ampoules.

(Check oil level at side of machine while machine operating)

18. (a) Cut tops off plugs, so that they are level with top of tube then push plugs down to 4cm from base of tube. (b) See diagram:



Initial (a) (b) (c)

19. Using oxygen blow torch and blue-cone flame, constrict tubes above cotton plugs (c). (No. 2 hole on Gas-oxygen burner.)

Care must be taken not to seal the constriction as a vacuum must later be pulled to the base of the tube. Constriction must not be too thick otherwise there may be difficulty in sealing off tubes later.

20. Secondary Dry. Replace tubes on freeze dryer: replace dome and fit manifold to this. Fit ampoules onto nipples of manifold firmly. Fit Durham tubes to any empty places so that a vacuum can be pulled.

21. Replace or replenish desiccant as required.

22. Close chamber air valve; slowly open chamber valve and pull a vacuum again in the chamber. When pressure reaches 760 mm, switch on pirani gauge.

N.B. If a vacuum will not pull, within 10-15 min, a check for leaks should be made. Check all tubes and ampoules on manifold as this is a common source.

23. Secondary dry for 1 - 2 hours. Close off pirani gauge.

24. Sealing off of ampoules:- Use a O_2 torch with a small flame. Carefully seal off at constriction whilst still under vacuum. Seal off from top down.

    N.B. It is essential to seal off both ends of constriction so that a vacuum remains in ampoule and also in the manifold end of the tube. Any holes pulled in the glass will cause vacuum to be lost.

25. Seal and remove all ampoules. Check for vacuum within using vacuum tester. Do not over use this, as it causes cell death if extensively used. Discard ampoules without vacuum.

26. Closing machine down. Close chamber valve, open 'admit air valve' slowly and remove manifold, dome, etc.

27. Turn Gas Ballast valve to open, run machine for 30 minutes to clean out fumes.

28. Turn off vacuum pump, admit air to cool trap and remove cool trap condenser. Clean out condenser, remove ice, etc.

FREEZE-DRYING TECHNIQUES (cont.)

*1 Freeze drying media

1) Glucose nutrient Broth

Nutrient Broth + 7.5% glucose

2) Mist desiccans

Sterile horse serum (no preservative)	300 cm^3	100 cm^3
Glucose	30 g	10 g
N. Broth	1.3 g	0.43 g
Dist. H_2O	100 cm^3	34 cm^3

3) Glucose serum

Sterile (horse) serum, no preservative	100 cm^3	50 cm^3	75 cm^3
Glucose (7.5%)	7.5 g	3.75 g	5.62 g
Sterilize by filtration	(15ml of 50%)	(7.5ml of 50%)	(11.2ml of 50%)

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