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

A THESIS PRESENTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF TECHNOLOGY IN BIOTECHNOLOGY AT
MASSEY UNIVERSITY

NOEMI A. GUTIERREZ

1985

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.

ACKNOWLEDGEMENTS

I am deeply grateful to Dr I.S. Maddox whose assistance and support throughout the course of this study were beyond measure. I also appreciate the privilege of being able to work with him.

Acknowledgement is also extended to the following:

- Prof. R. Earle, the New Zealand Ministry of Foreign Affairs, and the University of the Philippines at Los Banos for allowing me to pursue my Masterate.

- Mrs Bacon of the Department of Microbiology and Genetics for lyophilizing my stock cultures.

- Dr M. Taylor and Mr T. Gracie for their generosity with the GLC machine.

- Messrs M. Stevens, M. Lubbers, Mrs McCutcheon and the other technical staff for sharing their competence in the laboratory.

- Christine, Sen, Albert, Fred and the other Filipino students in Massey University for their company.

- Mrs Veronica Fieldsend for typing the thesis and Mr Lemuel Diamante for doing the illustrations.

- My family for their continued support.

TABLE OF CONTENTS

Cont'd..

TABLE OF CONTENTS CONTINUED: PAGE

Cont'd..

TABLE OF CONTENTS : CONTINUED PAGE

TABLE OF CONTENTS : CONTINUED PAGE

TABLE OF CONTENTS: CONTINUED PAGE

TABLE OF CONTENTS: CONTINUED PAGE

LIST OF TABLES

LIST OF TABLES CONTINUED

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

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.