1

METRO

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Services des thèses canadiennes

Ottawa, Canada
K1A 0N4

CANADIAN THESES

THÈSES CANADIENNES

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

**THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED**

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

**LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE**

Canada

DEVELOPMENT OF IMMOBILIZED CELL SYSTEMS
FOR BIOTECHNOLOGICAL PROCESSES

by

(C) Sushama (Joshi) Sontakke, B.Sc., (Honours, Microbiology), M.Sc.

A thesis submitted to the Faculty of Graduate Studies
and Research in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Department of Biology
Carleton University
Ottawa, Ontario
November 6, 1986.

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-35864-5

The undersigned hereby recommend to the Faculty
of Graduate Studies and Research acceptance of
the thesis, submitted by Sushama (Joshi) Sontakke,
B.Sc. (Honours, Microbiology), M.Sc., in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy.

.....
Chairman, Department of Biology

.....
Supervisor

.....
External Examiner

ABSTRACT

Cell immobilization permits controlled and repeated use of enzymes in biotechnological processes. The purpose of this thesis is to develop a new cell entrapment method and new applications of cell adsorption.

Microbial cells were entrapped in porous cellulose acetate aggregates. Cotton cloth segments were dipped in a cell suspension in cellulose acetate in aqueous acetone and air dried. The cell cloth was stabilized by treatment with polyethyleneimine followed by alkaline glutaraldehyde containing dithionite. L-Aspartase containing Escherichia coli cell cloth exhibited higher and stable aspartate productivity than gel-entrapped systems.

Various types of cloths were examined for adsorption of microbial cells: epichlorohydrin-triethanolamine (ECTEOA)-, polyethylenimine (PEI)-, naphthyl- and unmodified cotton cloth, cellulose acetate, and polyester. To develop immobilized cell population on cloth (cell cloth), the cloths were wetted with microbial cultures, incubated and periodically rinsed with growth media for few days. Cotton cloth was found to be a superior adsorbent.

Yeast cloths parallelly fixed in a fermentor were examined for ethanol batch fermentation from the

Jerusalem artichoke tuber hydrolysate. Circulation of media significantly increased ethanol productivity. The thickness of yeast layer and ethanol productivity increased with time until maximum productivity of 20 g of ethanol per liter fermentor per hour was obtained. The productivity remained constant for 60 days.

Six industrial bacteria formed immobilized populations on cotton cloth. The bacterial films generated free cells at significant and stable rates for at least 60 days. By retaining these films in a fermentor, the films were used as resident inocula in repeated batch fermentations. An equation for the rate of cell mass generation from the resident inocula was developed.

Contamination did not decrease the capacity of bacterial films as resident inocula for repeated batch fermentation. Since the contaminants are removed along the culture, the resident inoculum batch fermentation is resistant to contamination problems.

Using the resident inoculum method, Streptomyces secondary metabolites, thienamycin and cephalosporin C were produced at maximal levels during 20 cycles of three day batch fermentations.

A cloning vector, pBR322 was more stably maintained in E.coli immobilized on cotton cloth than in free cells. Therefore, the resident inoculum method would be useful in batch production of recombinant DNA products.

ACKNOWLEDGEMENTS

I would like to thank Dr. Hiroshi Yamazaki, my supervisor for his expert guidance, wisdom and patience. I would like to thank Drs. V.N. Iyer, A. Nasim, and A.D.E. Fraser for their valuable advices and the time they spent on my behalf. I would like to acknowledge the collaboration by Ms. K.L. Leung in early works described in Chapter 2. I thank all my other colleagues at Carleton University for friendship and advices. Special thanks are due to my parents, Sharad and Malini Joshi, my relatives Datta and Madhuri Saigaonkar and my husband Sharad, for their continuous support, understanding and patience.

My research was supported by the Natural Science and Engineering Research Council of Canada (NSERC) operating grant (A-4698) and strategic grant (G0955) to Dr. H. Yamazaki. My graduate studies have been also supported by a Carleton teaching assistantship.

TABLE OF CONTENTS

	Page
ABSTRACT	III
ACKNOWLEDGEMENTS	V
TABLE OF CONTENTS	VI
LIST OF FIGURES	X
LIST OF TABLES	XII
LIST OF ABBREVIATIONS	XIV
GENERAL INTRODUCTION	1
CHAPTER 1 CELLULOSE ACETATE ENTRAPMENT OF <u>ESCHERICHIA COLI</u> ON COTTON CLOTH FOR ASPARTATE PRODUCTION	18
ABSTRACT	19
INTRODUCTION	19
MATERIALS AND METHODS	21
Materials	21
Cellulose acetate entrapment of <u>E.coli</u>	22
PEI and glutaraldehyde treatment	22
Aspartase assay	23
RESULTS	26
DISCUSSION	36
CHAPTER 2 USE OF CATIONIC CELLULOSE FILMS FOR ETHANOL PRODUCTION IN A CYLINDRICAL FILM FERMENTOR	41
ABSTRACT	42
INTRODUCTION	42
MATERIALS AND METHODS	45

Assay of reducing sugars and ethanol	45
Yeast binding assay	46
Assay of ATP and DNA accumulation on ECTEOA-papers	46
Preparation of hydrolysate of Jerusalem artichoke tubers	47
Preparation of ECTEOA-cloth	48
RESULTS	48
DISCUSSION	64
APPENDIX	66
CHAPTER 3 IMMOBILIZATION OF YEAST ON COTTON CLOTH FOR ETHANOL PRODUCTION	67
ABSTRACT	68
INTRODUCTION	68
MATERIALS AND METHODS	69
Growth media and conditions	69
Assay of reducing sugars, ethanol and cell mass	70
Yeast colonization in a cylindrical film fermentor	71
Construction of a rectangular fixed film fermentor	72
Construction of a film stirring fermentor	72
RESULTS	77
DISCUSSION	95
APPENDIX	98

CHAPTER 4	USE OF BACTERIAL FILMS AS RESIDENT	
	INOCULA FOR REPEATED BATCH	
	FERMENTATION	99
ABSTRACT		100
INTRODUCTION		100
MATERIALS AND METHODS		101
Cloth		101
Microorganisms and growth conditions		101
Bacterial immobilization on cloth		103
RESULTS		104
DISCUSSION		108
APPENDIX		111
CHAPTER 5	RESISTANCE OF BACTERIAL FILM TO	
	CONTAMINATION DURING USE AS	
	INOCULUM IN REPEATED BATCH	
	FERMENTATION	113
ABSTRACT		114
INTRODUCTION		114
MATERIALS AND METHODS		115
Microorganisms and growth conditions		115
Preparation of <u>E.coli</u> film		115
β -galactosidase assay		116
RESULTS		116
DISCUSSION		119
CHAPTER 6	REPEATED BATCH ANTIBIOTIC	
	FERMENTATION BY STREPTOMYCETES	
	IMMOBILIZED ON COTTON CLOTH	123

ABSTRACT	124
INTRODUCTION	124
MATERIALS AND METHODS	125
Microorganisms and growth conditions	125
Cloth	125
Immobilization	125
Antibiotic production	126
RESULTS	131
DISCUSSION	138
CHAPTER 7 STABILITY OF pBR322 PLASMID IN	
IMMOBILIZED <u>ESCHERICHIA COLI</u>	
CELLS USED AS RESIDENT INOCULUM	139
ABSTRACT	140
INTRODUCTION	140
MATERIALS AND METHODS	141
Bacteria and growth conditions	141
Preparation of the <u>E.coli/pBR322</u> film	142
RESULTS	142
DISCUSSION	153
CONCLUSION	154
REFERENCES	158
LIST OF PUBLICATIONS	163

LIST OF TABLES

	Page
Table 1 Major characteristics of the three continuous flow reactors.	6
Table 2 Examples of cell entrapment systems in commercial use.	9
Table 3 Examples of cell adsorption immobilization systems.	10
Table 4 Examples of cell entrapment immobilization systems.	12
Table 5 Characteristics of cell entrapment systems described in Table 4.	14
Table 2.1 Binding of ^{35}S labelled yeast cells to film materials.	53
Table 2.2 ^{32}P incorporation into DNA and ATP in bound yeast.	54
Table 3.1 Cell mass thickening observed during colonization of <u>S.cerevisiae</u> cells.	83
Table 3.2 Ethanol production in a rectangular fixed film fermentor.	89
Table 4.1 Characteristics of bacteria immobilized on cloth.	102
Table 4.2 Specific rates of cell mass generation from bacteria immobilized on various cloth.	109
Table 5.1 <u>B.ammoniagenes</u> contamination of <u>E.coli</u>	

film cultures.	118
Table 5.2 Effect of <u>B.ammoniagenes</u> contamination on the specific activity of β -galacto- sidase in cells generated from the <u>E.coli</u> film.. . . .	120
Table 5.3 Effects of <u>B.ammoniagenes</u> contamination on the <u>E.coli</u> population on the film.. . . .	121
Table 7.1 Lag caused by 10mg/ml ampicillin in. <u>E.coli/pBR322</u> cultures grown by daily dilution and from the film in ampici- llin-free medium.	146

LIST OF ABBREVIATIONS

$A_{500, 420 \text{ or } 340}$	absorbance at 500, 420 and 340 nm respectively
ATP	adenosine 5'-triphosphate
AU	absorbance unit in ml * absorbance
$^{\circ}\text{C}$	degree centigrade
CAMP	adenosine 3',5'-cyclic monophosphate
cm	centimeter
cm ²	centimeter square
cpm	counts per minute
DNA	deoxyribonucleic acid
g	gram
h	hour
H	height
i.d.	inside diameter
kg	kilogram
l	liter
M	molar
mg	milligram
μCi	microcurie
μg	microgram
μl	microliter
min	minute
ml	milliliter
mM	millimolar
mm	millimeter

nm	nanometer
³² Pi	[³² P] orthophosphate
RNA	ribonucleic acid
rpm	revolutions per minute
³⁵ S	[³⁵ S] sulphate
sec	second
TOX	Triton 114-Omnifluor-xylene mixture
v/v	volume per volume
W	width
w/v	weight per volume
*	multiplied by

GENERAL INTRODUCTION

Biotechnology is concerned with the application of the activities of cells or enzymes to manufacturing, agricultural and service industries. Research in biotechnology should aim for economic, materialistic, industrial and environmental benefits.

Activities of cells or enzymes have been exploited not only in the production of traditional fermented foods but also in the production of organic materials required in food and pharmaceutical industries. As petroleum sources will be depleted sooner or later, efforts are now directed towards the conversion of renewable biomass to chemicals and fuels which are currently produced from petroleum sources.

Enzymes are catalysts of choice for transformation of biological compounds as they perform reactions at moderate pH and temperature with a considerable substrate specificity. Thus enzymic processes produce relatively high yields of products and depend on less energy. Fixation (immobilization) of enzymes on an inert insoluble support has markedly improved applications of enzymes in biotechnology. If an enzyme is used in free form, reuse of the enzyme requires its recovery from the reaction mixture, and stopping the reaction requires its inactivation. However, immobilized enzymes allow substrates to come into contact with enzymes for a desired period of time and then allow reaction products to be separated from the

enzymes. Because the enzyme can be reused many times, the cost of the enzymic process is lower. Another important advantage of enzyme immobilization is that enzyme stability is often greatly increased, presumably because immobilization restricts enzymes from assuming inactive conformations.

Immobilized enzymes are used in various reactors as described in Fig.1 (Diagram from Kennedy, 1985). Continuous flow stirred tank reactors, packed-bed reactors and fluidized-bed reactors permit continuous and controlled enzymic processes. Major characteristics of these three reactors are summarized in Table 1.

Enzymes in a cell can be used 'in situ' as catalysts if substrates are able to diffuse into the cell and if undesired side reactions are prevented. A cell catalyst can be immobilized and used in the reactors in the same manner as enzyme catalyst. The cell catalyst is economical as purification of enzyme is not required and may have several other advantages which are as follows:

- (1) Full supplementation of cofactor may not be necessary.
- (2) The enzyme within the cell (its natural environment) is generally more stable than free enzyme.
- (3) The living cell may be able to bring the substrate into the cell via active transport.
- (4) The living cell can regenerate the enzyme and

Fig. 1 Principal reactors used for immobilized enzymes.

Packed-bed reactor

Stirred tank reactor

Fluidized bed reactor

Table 1 Major characteristics of the three continuous flow reactors (Venkatsubramanian and Karkare, 1983)

	CSTR ^a	PBR ^b	FBR ^c
Operation	Easy	Easy	Difficult
Catalyst replacement	Easy	Difficult	Easy
Pressure drop	Low	High	Low
Mixing properties	Good	Poor	Good
Catalyst loss	High	Low	In between
Scale-up	Easy	Easy	Difficult
Cost	Low	Low	High
Reactor control	Easy	Difficult	Easy
Suitability for			
1) Product inhibited enzymes	Poor	Good	Fair
2) Substrate inhibited enzymes	Good	Poor	Fair
3) Processing of colloidal substrates	Good	Poor	Good
Backmixing with product	Yes	No	Yes
Plugging problem	No	Yes	No
Reactor size	Largest	Smallest	In between
Suitability for gas phase	Yes	No	Yes

^a Continuous-flow stirred tank reactor

^b Packed-bed reactor

^c Fluidized-bed reactor

^d low as compared to CSTR but high as compared to PBR

^e compressible small sized support can cause slow flow rates

^f between two extremes depending upon bed expansion

cofactors.

(5) The cell catalyst can carry out multistep enzyme reactions as well as single enzyme reaction.

(6) The living and reproducing cell can produce cells and thus can increase amount of biocatalyst.

Since the purpose of the present thesis is to develop new cell immobilization methods, I shall review current immobilization techniques.

Methods for Cell Immobilization.

Four major principles have been used for cell immobilization which are as follows:

(1) Covalent coupling: Cells are covalently bound to a suitable insoluble support. However, due to chemical reactions involved enzyme activity is often lost or reduced. Chemicals required for this type of immobilization are either costly, unsafe or difficult to receive regulatory approval for use. Thus this type of immobilization is seldom used in commercial processes.

(2) Cross-linking: Cells are cross linked to each other by a bifunctional reagent such as glutaraldehyde (via covalent-linkages) or polyvalent ions (via ionic bonds). Immobilized cells are then molded into pellets, chips or films. Although this system provides concentrated forms of cells, diffusion of large-sized substrates and products in these preparations is restricted. This method cannot be applied to enzymes

which are sensitive to the crosslinking agents.

(3) Entrapment: Cells are physically entrapped in a three dimensional network such as gels. However, the network tends to create diffusional resistance to movement of substrates and products and thus decreases the rate of catalysis. Furthermore, the network (particularly, gels) tends to be unstable and compressible. Further, chemical treatments are often required to overcome these difficulties. Because it permits the highest cell loading, it has been commercially mostly used for single enzyme reactions. The examples of such entrapped cell systems are shown in Table 2.

(4) Adsorption: Cells are adsorbed on a suitable support largely by hydrogen bonds, ionic and hydrophobic interactions. Since it results in cell binding at the surface, it permits freer movement of substrates and products than in cell entrapment. It is the simplest and mildest immobilization. However, its cell loading is less and the chance of cell detachment is greater than cell entrapment. Because of its mildness, it has been mostly used for multienzyme reactions, examples of which are described in Table 3.

The present thesis deals with the development of two new cell immobilization systems. Since the first one is based on cell entrapment and the second one on cell adsorption, I will review (A) entrapment and

Table 2 Examples of cell entrapment systems in
commercial use

Microorganism	Enzyme (reaction)	Entrapment method (commercial user)
<u>Escherichia coli</u>	Aspartase (Ammonium fumarate -----→aspartate)	Entrapment in k carrageenan treatment with hexanediamine and glutaraldehyde (Tanabe Seiyaku, Japan)
<u>Brevibacterium flavum</u>	Fumarase (Fumarate--→malate)	Entrapment in k carrageenan treatment with polyethyleneimine (Tanabe Seiyaku, Japan)
<u>Escherichia coli</u>	Penicillin acylase (Penicillin G----- →6 amino penicillanic acid)	Entrapment in gelatin crosslinking with glutaraldehyde (Shanghai, China)

LIST OF FIGURES

	Page
Fig.1 Principal reactors used for immobilized enzymes.	4
Fig.1.1 Standard curve for aspartic acid assay by the trinitrobenzenesulphonic acid method.	24
Fig.1.2 Effect of PEI and glutaraldehyde on stability of aspartase in <u>E.coli</u> cloths..	30
Fig.1.3 Aspartic acid production in a packed column and stirred tank reactor.	34
Fig.1.4 A network of PEI-polymerized glutaraldehyde around <u>E.coli</u> cell.	37
Fig.2.1 (A) Structure of DEAE-cellulose (B) preparation of ECTEOLA-cellulose (C) and structure of polyethyleneimine.	50
Fig.2.2 Construction of a film fermentor.	56
Fig.2.3 Ethanol production in ECTEOLA-cellulose and cellulose film fermentors.	59
Fig.3.1 Diagram of a rectangular fixed film fermentor.	73
Fig.3.2 Diagram of a film stirring fermentor.	75
Fig.3.3 Binding of <u>S.cerevisiae</u> cells to cotton cloth.	78
Fig.3.4 Colonization of ethanol producing yeasts on cotton cloth as measured by ethanol	

Table 3 Examples of cell adsorption immobilization systems

-
- 1) ECTEOLA-cellulose (Azotobacter vinelandii, 10^{11} cells/g)
 $N_2 \rightarrow NH_3$ (Sehyan and Kirwan, 1979)
 - 2) Wood chips (S. carlsbergensis, 248mg/g)
 Ethanol fermentation (Durand and Navarro, 1978)
 - 3) Polyvinylchloride chips (S. carlsbergensis, 80mg/g)
 Ethanol fermentation (Durand and Navarro, 1978)
 - 4) Dowex 1 anion exchanger (S. carlsbergensis, 21mg/g)
 Ethanol fermentation (Durand and Navarro, 1978)
 - 5) Porous silica (S. carlsbergensis, 2mg/g)
 Ethanol fermentation (Durand and Navarro, 1978)
 - 6) Porous ceramic (Acetobacter aceti, 2.6mg/g)
 Acetic acid fermentation (Ghommidh et al., 1981)
 - 7) ECTEOLA-cellulose (Aspergillus oryzae, 4×10^{10} spores/g)
 Sucrose $\rightarrow$ Glucose (Johanson and Ciegler, 1969)
-

productivity in a cylindrical film fermentor.	81
Fig.3.5 Ethanol production by <u>S.cerevisiae</u> in a rectangular fixed film fermentor.	87
Fig.3.6 Ethanol production by <u>S.cerevisiae</u> segments stirred in a film stirring fermentor.	93
Fig.4.1 Specific rate of cell mass generation from bacterial films.	105
Fig.6.1 Standard curve for thienamycin assay.	127
Fig.6.2 Standard curve for cephalosporin C assay.	129
Fig.6.3 Kinetics of accumulation of thienamycin by <u>Streptomyces cattleya</u> and cephalosporin C by <u>Streptomyces clavuligerus</u>	132
Fig.6.4 Maximal levels of antibiotics accumulated in repeated batch fermentations.	135
Fig.7.1 Effect of ampicillin on growth of <u>E.coli</u> and <u>E.coli/pBR322</u>	144
Fig.7.2 Effect of ampicillin-free growth of <u>E.coli</u> / <u>pBR322</u> on sensitivity to ampicillin.	147
Fig.7.3 Ampicillin sensitivity of cells generated from the <u>E.coli/pBR322</u> film.	150

(B) adsorption in more details.

(A) Entrapment

Currently used cell entrapment systems can be classified by the principles used in forming three dimensional networks; (1) Thermal gelation; (2) Ionotropic gelation; (3) Polycondensation; and (4) Addition polymerization.

(1) Thermal gelation: A mixture of long polymers and cells is warmed and upon cooling, the polymers form gels because the polymeric chains are entangled, forming a network in which the cells are entrapped.

(2) Ionotropic gelation: Polyionic polymers are joined by polyvalent counterions to form a network. If the gel is formed in the presence of cells, the gel entraps the cells.

(3) Polycondensation: Polyfunctional monomers are covalently joined to each other by removal of small molecules (e.g. CO_2 and H_2O) and thus form a network polymer in which the cells are entrapped.

(4) Addition polymerization: Monomers are covalently joined to each other and to cross linker to form a three dimensional network polymer in which cells are entrapped.

Reported and widely used examples for each principle of entrapment are given in Table 4 and characteristics of each example described in Table 4 are

Table 4 Examples of cell entrapment immobilization systems (Mattiason, 1983)

1) k Carrageenan

- A) Thermal +ionotropic gelation.
- B) Cell suspension in warm k carrageenan added dropwise to 2% KCl to form beads.

2) Alginate

- A) Ionotropic gelation by Ca^{++} .
- B) Cell suspension in alginate added dropwise to a $CaCl_2$ solution to form beads .

3) Chitosan

- A) Ionotropic gelation by polyphosphate ions.
- B) Cell suspension in chitosan added dropwise to a polyphosphate solution to form beads

4) Polyurethane

- A) Polycondensation, polyisocyanates condense at multiple points with each other.
- B) Cell suspension in water mixed with polyisocyanate, casted to form a film.

5) Polyacrylamide

- A) Addition polymerization.
- B) Cell suspension in acrylamide monomer, crosslinker, and initiator (ammonium persulphate) is set to form a block.

A- Principle of polymer formation.

B- Entrapment procedure

given in Table 5. Essential requirement for cell entrapment is that the size of cell should be larger than the pore size of the network. Although, cell entrapment by ionotropic gelation is most commonly used, gel structure is affected by ionic constituents of the reaction mixture. For example calcium chelating agents such as citrate or phosphate and excess monovalent ions such as K^+ , Na^+ and NH_4^+ weaken alginate gel. Bead forms of entrapped cell systems are commonly used. However, the larger beads suffer greater diffusional resistance, for transport of substrates and products, smaller beads show reduction in this problem, but they suffer destabilization of structure under high pressure or shear force.

(B) Adsorption

In nature, most microorganisms do exist in adsorbed forms such as the films formed on minerals, plant and animal surfaces. Three major factors which affect adsorption of microorganisms are:

- (1) surface characteristics of microorganisms;
- (2) surface characteristics of supports; and
- (3) nature of environment.

(1) Surface characteristics of microorganisms:

Microorganisms are regarded as macroscopic ions as their surface contains ionizable groups such as amino, carboxyl groups of surface proteins or phosphate groups

Table 5 Characteristics of cell entrapment systems described in Table 4 (Mattiason, 1983)

-
- 1) k Carrageenan
 - A) Physically not strong, strengthened with hexanediamine and glutaraldehyde.
 - B) 10^9 E.coli cells /ml of gel
 - C) Dissolves above 40°C ,
 - 2) Alginate
 - A) Greater strength than carrageenan, compressible at high flow rates
 - B) >50% (wet weight by volume) for E.coli cells
 - C) Phosphate and citrate dissolve the gel, stable at high temperature, mild method, multienzyme reactions possible
 - 3) Chitosan
 - A) Strength equivalent to alginate and similarly compressible at high flow rates.
 - B) 10 to 40% (wet weight by volume) for E.coli cells.
 - C) Excess mono or polycations dissolve gel.
mild method; multienzyme reactions possible.
 - 4) Polyurethane
 - A) Compressible at high flow rates, but stable thus suitable in stirred tank.
 - B) 2-10% (wet weight by volume) for E.coli cells.
 - C) Isocyanates react with hydroxyl and amino groups and thus may kill the cells and inactivate the enzyme.
 - 5) Polyacrylamide
 - A) Compressible at high flow rates.
 - B) 4-18% (wet weight by volume) for E.coli cells.
 - C) Toxic monomer, heat of polymerization and free radicals kill the cell and may inactivate the enzyme.
-

- A- Mechanical stability
 B- Cell loadings
 C- Comment

as in teichoic acids of Gram positive bacteria and phosphomannan of yeast. At neutral pH, most microorganisms have net negative charges (Daniels, 1972). In bacteria, net negative charges are due to excess of acidic amino acids over basic amino acids, in their surface proteins (Sheldon, 1981).

Besides ionizable groups, many microorganisms may contain carbohydrate moieties such as lipopolysaccharides and capsule in bacteria and mannan in yeast. These carbohydrate moieties are rich in hydroxyl groups and microorganisms can be adsorbed by hydrogen bonding between these hydroxyl groups and NH-, and HO-, and ~~HS~~-groups of the support. Hydrophobic groups in lipopolysaccharides (in Gram negative bacteria) and lipoproteins in bacterial surfaces can cause hydrophobic interaction with hydrophobic support. The abundance of mycolic acid on the surface of the mycobacteria, corynebacteria and nocardia make their surfaces highly hydrophobic.

Although chemical compositions of flagella and fimbriae (pili) vary from species to species, these outer structures are generally involved in adsorption. The polysaccharide slime layer found on many bacteria may also serve as a binder in adsorption (Daniels, 1972).

Surface characteristics of microorganisms may vary with their chemical and physical growth environment such

as pH, nutrients and temperature and with their growth phase, division stages and life cycle (Daniels, 1972; Johnson and Ciegler, 1969; Sheldon, 1981).

(2) Surface characteristics of supports:

Adsorption of microorganisms by ionic interaction requires the presence of opposite charges in a support. As microorganisms generally have 'net' negative charges, adsorption to anion exchangers has been more extensively studied (Daniels, 1972; Hattori and Furusaka, 1972). However, since microorganisms still carry positive charges, they can adsorb to cation exchangers (Daniels, 1972).

Hydrophobic supports have a potential for adsorbing microorganisms which have hydrophobic groups on their surface. However, immobilization by hydrophobic interaction has not yet been studied in detail.

Hydroxyl surface groups in supports such as polysaccharides and bentonite adsorb microorganisms which are rich in surface hydroxyl groups due to carbohydrates.

Although hydrogen bonds, ionic and hydrophobic interactions are individually much weaker than covalent bonds, combined interaction at multiple sites will generate a strong binding force. The density of surface charges, hydrophobic and hydroxyl groups (amount per unit surface area) determines the strength of binding and thus loading of microorganisms on the surface. For

porous supports further increase in loading can be obtained by having pore sizes large enough for cell penetration.

(3) Nature of environment:

The pH and temperature affect degree of ionization of ionizable groups at the surface of both, microorganism and support. Amphoteric surfaces of microorganisms will have net negative charges above the isoelectric pH and net positive charges below the isoelectric pH (Daniels, 1972). Increase in ionic strength of the environment may cause desorption of microorganisms adsorbed by ionic interactions. If microorganisms are adsorbed by hydrogen bonds, then hydrogen bond disrupting agents such as urea, guanidinium salts and substances of high hydroxyl contents (e.g.glycerol and sugar) can desorb the microorganisms. Higher ionic strength and higher temperature can increase hydrophobic interaction to some extent.

The purpose of this thesis is twofold (1) to develop a new cell entrapment system which is structurally stable and possesses a larger and porous surface for cell entrapment and (2) to develop new applications of cell adsorption on a film material.

CHAPTER 1
CELLULOSE ACETATE ENTRAPMENT OF ESCHERICHIA COLI
ON COTTON CLOTH FOR ASPARTATE PRODUCTION

ABSTRACT

Aspartase containing Escherichia coli cells were entrapped in cellulose acetate aggregated on cotton cloth. The enzyme activity of the cloth was determined by the newly developed simple aspartase assay. The cloth enzyme activity was stabilized by treatment with polyethylenimine and alkaline glutaraldehyde in the presence of 0.1 M sodium dithionite. A column packed with the cloth segments catalyzed 100% conversion of 1 M ammonium fumarate to aspartic acid at a space velocity of 7/h. When the same cloth segments were stirred at 100 rpm in the substrate solution, the productivity of the cloth increased 2.5 times.

INTRODUCTION

Many biotechnological processes depend on catalysis by a single enzyme. Immobilized cells containing such an enzyme can be used as a catalyst if other cellular enzymes do not interfere with the process. A notable example is production of L-aspartic acid by immobilized Escherichia coli cells containing L-aspartase.

K-carrageenan or alginate gel entrapment is now commonly used to entrap cells for such a process primarily because it is relatively simple and produces gel beads containing a high density of cells. For use in a packed bed further treatments (e.g. polyanine

followed by glutaraldehyde) are necessary to strengthen the beads and reduce compression of the bead. However, such gels are generally known to create mass transfer resistance which increases with increasing size of the substrate. Although use of beads with smaller diameter partly reduces such problems, the bead structure is destabilized.

The project in this chapter, is to develop a new cell entrapment system which is less compressible and less resistant to mass transfer than gel entrapment methods and also amenable to scale-up. Here cells will be entrapped in porous cellulose acetate aggregated onto cotton cloth.

Cellulose acetate is readily available as it is used in textile industries. It is made by partial removal of acetate from cellulose triacetate and has a degree of substitution of about 2.3 (as compared to 3 for complete substitution).

Entrapment of cells in cellulose acetate beads has been only briefly described (Marconi and Bartoli, 1981) In their method, a cell suspension is homogenised with a cellulose acetate solution in acetone. The addition of the homogenate into chilled petroleum ether coagulates the cellulose acetate into beads, entrapping the cells. However, the properties of such an entrapped cell system has not been fully described. As in other gels, transport of substrates and products would be

slower in large beads, while smaller beads generally would suffer structural instability when compressed in a packed bed reactor. Furthermore, small beads are difficult to separate from products after use in a stirred tank operation.

In this project L-Aspartase containing Escherichia coli will be used as a model system. L-Aspartase catalyzes the conversion of ammonium fumarate to L-aspartic acid as shown below:

Aspartic acid is a component of the popular sweetener, Aspartame (Sampathkumar et al., 1981).

MATERIALS AND METHODS

Materials.

Glutaraldehyde (Aldrich G400-4) was diluted to 0.5 or 1.0% (w/v) with 0.1 M Na_2HPO_4 (pH of approx. 9.5) and stored for at least 3 days at room temperature before use. This solution will be referred to as 'alkaline glutaraldehyde'. Alternatively, glutaraldehyde in 0.1 M Na_2HPO_4 was heated at 100°C for 17 h before use. This will be referred to as 'heated alkaline glutaraldehyde'. Polyethylenimine (Cordova Corcat P-600, average molecular weight of approx. 60,000) was diluted to 0.3 or 1.0% (w/v) with H_2O .

Cellulose acetate powder with a 54% acetic acid content was obtained from BDH Chemicals. Cotton flannel (pure cellulose cloth) was obtained locally, and its weight per area was 12 mg/cm². E.coli cells containing a high activity of aspartase were prepared as follows:

E.coli Crooks (ATCC 8739) was grown at 37°C to a late log phase (absorbance at 500 nm of approx.3) in ammonium fumarate (AF) medium. One liter of AF medium contains 11.6 g of fumaric acid, .12 g of MgSO₄, 0.5 mg of FeSO₄, 6.8 g of KH₂PO₄ and NH₄OH (added until a final pH of 7.3). The cells were washed twice with a washing solution which consisted of 1 mM sodium-dithionite and 1 mM MgCl₂, and 1 g of the wet cells were resuspended in 0.3 ml of the above solution.

Cellulose acetate entrapment of E.coli cells

One volume of the E.coli cell suspension was homogenized with 4 volumes of 5% (w/v) cellulose acetate in acetone. Cotton cloth segments (0.5 or 2 cm square) were dipped into the homogenate, removed and air-dried for approx.3 h. The segments (designated cell cloth) were treated with polyethyleneimine (PEI) and glutaraldehyde to stabilize aspartase as described below.

PEI and glutaraldehyde treatment

The cell cloth segments were soaked in 0.3% (w/v) PEI. After 30 min incubation at 25°C, the segments were

removed, washed with the washing solution, and soaked in a substrate solution overnight (approx. 16 h) to increase cell permeability. The substrate solution consists of 1 M fumaric acid, 1 mM $MgCl_2$, 1 mM sodium dithionite and NH_4OH (added until pH 8.5). The treated cell cloths were washed with the washing solution and assayed for aspartase.

Aspartase assay

A treated cell cloth segment (2 cm square) was shaken in 2 ml of the substrate solution at 320 rpm and $37^{\circ}C$. After 10 min a 100 μ l of sample was transferred to a 20 ml scintillation vial containing 100 μ l of 5 M NaOH. The mixture was heated at $100^{\circ}C$ for at least 10 min until dryness. The residue was suspended in 100 μ l of 5 N HCl, and heated at $100^{\circ}C$ for at least 10 min until dryness. After cooling, the residue was resuspended in 10 ml of 0.1 M/sodium borate buffer (pH 8.2). One ml of the suspension or L-aspartic acid standard was mixed with 1 ml of 1 mg/ml picrylsulfonic acid (Sigma P 5878) in 0.1 M sodium borate buffer (pH 8.2), and incubated at $50^{\circ}C$. After 1 h, 2 ml of 0.1 N HCl was added and absorbance at 340 nm was measured against the blank. A standard curve correlating absorbance to concentration of aspartic acid was constructed (Fig.1.1).

Fig.1.1 Standard curve for aspartic acid assay by the trinitrobenzenesulphonic acid method. Aspartic acid (10^{-6} mole/ml) refers to the concentration of standards. One ml of the trinitrobenzenesulphonic acid solution (1 mg/ml), heated at 50°C for 1 h, and mixed with 2 ml of .1 N HCl, and absorbance at 340 nm was measured against a blank.

RESULTS

Preparation of aspartase

E. coli B strain grown on aspartate as the sole carbon source (doubling time 5.2 h) produces a high specific activity of aspartase (10^{-5} moles of aspartate produced per min per mg protein) and formation of aspartase is subject to glucose repression (Nishimura and Kisumi, 1984). E. coli Crooks strain is resistant to glucose repression (Armstrong and Yamazaki, 1977) and grew on ammonium fumarate as the sole nitrogen and carbon sources with doubling time of 1.2 h, producing aspartase with the specific activity of 3.4×10^{-5} moles of aspartate produced per min per mg of protein.

Aspartase assay

L-Aspartic acid is commonly determined by amino acid analyzer or bioassay (Nishimura and Kisumi, 1984). I developed a simple colorimetric method for assay of multiple aspartase samples (see Methods). The assay is based on the reaction of picrylsulfonic acid with amino compounds (Satake et al., 1960). Since aspartic acid is the only amino acid formed in the aspartase reaction (confirmed by amino acid analyzer), this colorimetric method was applied after removal of NH_4^+ with hot alkali. A standard curve for this assay is linear up to 10^{-3} M and as little as 10^{-4} M is readily measurable (Fig.1.1).

Cellulose acetate entrapment

Cellulose is physically, chemically stable and relatively inexpensive. Cellulose cloth (e.g. cotton cloth) is a compressed version of fibers with open and regular structure. It can be packed into a column as segments. The resulting packed bed can be used at high flow rates without compression of the bed. If cells are entrapped in cellulose acetate aggregated onto the cellulose cloth, the resulting immobilization system may overcome the potential disadvantages of the cellulose acetate bead system.

One volume of the E.coli cell suspension (containing L-aspartase) was mixed with various volumes of 5% cellulose acetate in acetone to obtain stable homogeneous mixtures. Cotton cloth was dipped into each mixture, removed and air-dried. As acetone evaporated, cellulose acetate formed porous aggregates and adhered to the cloth because it is insoluble in the remaining water. If porosity was too great, the cells washed out during subsequent washing. Higher ratios of the cellulose acetate solution to cell suspension (such as 6:1 and 5:1) gave a smaller porosity (and thus a greater cell retention) but lowered cell loading. A 1:4 ratio of cells to cellulose acetate suspension gave the highest retention of aspartase activity on the cloth, and this ratio was used throughout to prepare entrapped E.coli cloth ('cell cloth').

When the cell cloth was immersed in the substrate (ammonium fumarate) solution, it was found to evolve bubbles (presumably CO_2) and could not be used in a packed column because the gas phase replaced the substrate solution. Since fumaric acid was used as a sole carbon source for E.coli, the cells may have induced high levels of malic enzyme which catalyzes CO_2 evolution from malic acid (derived from fumaric acid). Such a pathway depends on nicotinamide adenine nucleotide (NAD^+/NADH). I studied the effect of various detergents on bubble formation as the coenzyme may leak out when the cell permeability is increased by detergents. Although bubble formation was stopped by .1% Brij 58, it was reinitiated when the detergent was removed. Since the detergent is difficult to separate from aspartate and is relatively expensive, its continual use during aspartate production is not desirable.

Continued CO_2 evolution would depend on regeneration of NAD^+ which in turn depends on dissolved oxygen. Sodium dithionite is a strong reducing agent and an oxygen scavenger (used in textile, soap and sugar industries). The inclusion of 1 mM sodium dithionite in the substrate solution was found to completely eliminate gas evolution without affecting aspartase activity. Since this agent is soluble at pH 2.8 to 3.0 (the isoelectric point of L-aspartic acid),

it will not interfere with purification of L-aspartic acid by isoelectric point precipitation.

Fig.1.2 shows that when incubated in the substrate solution at 37°C the aspartase activity of the cell cloth decreased with a half life of approx.13 days. Since incubation in the substrate has been shown to increase permeability (Chibata et al., 1974), leakage of the enzyme from the cells may occur during prolonged incubation. To test this possibility, the cell cloth was incubated with naphthyl cloth in the substrate solution. We have previously shown that E.coli aspartase adsorbs to naphthyl cloth in a stable form (Yamazaki et al., 1986). The activity which was lost from the cell cloth during incubation was found on the naphthyl cloth. Thus I concluded that the decrease of aspartase activity was largely due to enzyme leakage.

PEI and glutaraldehyde treatment

To prevent enzyme leakage, the cell cloths were treated with glutaraldehyde in 3 different ways. Treatment with 1% glutaraldehyde in H₂O for 10 min caused more than 90% loss in enzyme activity. Since aspartase is an intracellular enzyme, glutaraldehyde must enter the cell and inactivate the enzyme. Glutaraldehyde is known to polymerize at alkaline pH and high temperatures (Rasmussen et al., 1974) and polymerized glutaraldehyde may not permeate through the

FIG.1.2 Effect of PEI and glutaraldehyde on stability of aspartase in E.coli cloths. Aspartase containing E.coli cells were entrapped in cellulose acetate aggregated onto cotton cloths as described in methods. Glutaraldehyde and PEI solutions were prepared as described in Materials. The resulting cell cloths were treated at 25°C as follows: (1) Soaking in 1% heated alkaline glutaraldehyde for 10 min; (2) soaking in 1% heated alkaline glutaraldehyde (containing 0.1 M sodium dithionite) for 10 min; (3) soaking in 1% alkaline glutaraldehyde (containing 0.1 M dithionite) for 10 min; (4) soaking in 1% PEI for 5 min and then in 1% alkaline glutaraldehyde (containing 0.1 M dithionite) for 10 min; (5) soaking in 0.3% PEI for 30 min and then in 0.5% alkaline glutaraldehyde (containing 0.1 M dithionite) for 30 min. No significant difference in aspartase activity was observed between (2) and (3) and between (4) and (5) treatments. The aspartase activities (○, untreated; ▲ for 1; △ for 2 and 3; ● for 4 and 5) are expressed in fractions of the activity of untreated cell cloth at time 0 day.

cell membrane. Fig.1.2 shows that treatment with heated alkaline glutaraldehyde slowed down the decline of aspartase activity from the cell cloth though it caused a 50% loss of the activity. However, the inclusion of 0.1 M sodium dithionite during this glutaraldehyde treatment prevented the loss of aspartase activity and stabilized the enzyme activity of the cell cloth for at least 60 days (Fig.1.2). I also found that alkaline glutaraldehyde, when stored at room temperature for at least 3 days, produced a preparation which exhibited the same stabilizing effect as heated alkaline glutaraldehyde. Thus, alkaline glutaraldehyde was subsequently used without heating.

Alkaline glutaraldehyde treated cell cloth, while satisfactory for packed bed operation, was found to be unsatisfactory for stirred tank operation, because the entrapped cells came off under strong shear forces. However, I found that soaking the cell cloth in PEI solutions prior to alkaline glutaraldehyde treatment increased the strength of the cell cloth and also caused a significant increase in its aspartase activity (Fig.1.2). No decay of the enzyme activity occurred for at least 60 days. The strength of cell entrapment could be adjusted by concentrations of these reagents as well as treatment times. Cellulose acetate entrapment or PEI and glutaraldehyde treatments did not change the optimal pH of aspartase. It remained at 8.5.

Aspartic acid production in a packed bed and a stirred tank

The cell cloth segments (0.5 cm square) were treated with PEI and glutaraldehyde and packed into a column (10 mm diameter) to a bed volume of 7 ml. The conversion of 1 M ammonium fumarate to aspartic acid was examined at various space velocities (hourly flow rates/bed volume). Fig.1.3 shows that 100% conversion was achieved at a retention time of 8.5 min, that is, a space velocity of approx. 7/h. The productivity of the cell cloth in the packed bed is 0.94 Kg of aspartic acid per liter of the cloth per h, or 0.14 g per cm² of cloth per h.

In a packed column, biocatalysts near the port of substrate entry work efficiently because of high substrate concentrations, while the catalysts farther away from the port become increasingly idle because of decreasing substrate concentrations. In a stirred tank, all the catalysts would equally share catalysis until the equilibrium reaches. To examine the efficiency of the present system in a stirred tank, the cell cloth segments were removed from the column, washed, suspended in 100 ml of the substrate solution in a beaker (63 mm diameter). The suspension was stirred at 100 rpm by means of a stirring shaft with 2 angled blades (a total dimension of 40 mm W / 15 mm H). Fig.1.3 shows that 100% conversion of ammonium fumarate to aspartic acid

Fig.1.3 Aspartic acid production in a packed column (O) and stirred tank reactor (●). After treatment with PEI⁺ and glutaraldehyde, the cell cloth segments (0.5 cm square) were packed to 7 ml bed volume in a jacketed column. When the column was equilibrated to 37°C, 10 volumes (70 ml) of the substrate solution was pumped through the column at various flow rates. The last 0.1 ml of effluent was withdrawn and assayed for aspartic acid. After the column operation, the segments were removed, washed, blotted and transferred to a beaker containing 100 ml of the substrate solution equilibrated at 37°C. The mixture was stirred at 100 rpm, and 0.1 ml samples were removed periodically and assayed for aspartic acid. Degrees of conversion (%) of 1 M ammonium fumarate (pH 8.5) were plotted against retention times (min).

was achieved after 47 min reaction. The average productivity of the cell cloth in the stirred tank is 2.4 Kg of aspartic acid per liter of cloth per h, or 0.35 g per cm² of cloth per h. Thus, in this stirred tank operation the cell cloth produced aspartic acid 2.5 times faster than in the packed bed.

DISCUSSION

I have developed a simple method for entrapping a high density of E.coli cells in cellulose acetate aggregated on cotton cloth. A large scale entrapment is feasible by dipping the cloth in a series of tanks containing cell suspension in cellulose acetate solution, PEI solution and alkaline glutaraldehyde solution. I have shown that treatment with PEI and glutaraldehyde stabilized aspartase activity. I observed that this treatment also strengthened entrapment on the cloth. Stabilization of aspartase is likely due to the prevention of enzyme leakage by formation of the network of PEI-polymerized glutaraldehyde around E.coli cell. The reactions involved in the network formation are illustrated in Fig.1.4.

An industrial system in current use (carrageenan gel entrapped E.coli cells) performs 100% conversion of 1 M ammonium fumarate at a space velocity of 2/h (Sato et al., 1979).

The present cell cloth system in a packed bed performed

Fig.1.4 A network of PEI-polymerized glutaraldehyde
around E.coli cell

Polymerized glutaraldehyde

CELL + PEI

Polymerized glutaraldehyde

the same conversion at a space velocity of 7/h. Its higher productivity may be ascribed to a large porosity of the cellulose acetate aggregate and a large surface area of the cloth. An E.coli mutant containing the higher specific activity of aspartase has been isolated recently (Nishimura and Kisumi, 1984). Using such a mutant it should be possible to produce aspartate at a space velocity even greater than 7/h. Such a high flow rate is difficult to obtain with the gel entrapment system but should be possible with the present method because of compact and porous nature of the cloth.

Stirring the cell cloth segments in a substrate solution significantly increased the productivity of the cell cloth. Since the segments can be readily separated from the product, it is feasible to repeat stirred tank production automatically. A higher stirring rate would further increase the productivity. Continual use of the cell cloth at such a rate will require stronger cell entrapment which can be achieved by increasing the concentrations of PEI and glutaraldehyde and treatment times. The present method should be applicable to immobilization of cells for other enzymes. Removal of lactose from milk products is desired by dairy industry since lactose is insoluble in concentrated milk products and 90% of the world's adults are intolerant to lactose. Kluyveromyces fragilis produces lactase which is commonly used. An attempt was made to immobilize this

yeast for lactose hydrolysis in milk. Although immobilized K.fragilis cells were found to efficiently hydrolyse lactose to glucose and galactose, the monosaccharides were further metabolized to acids and ethanol by other intracellular enzymes. I tried without success to heat inactivate these catabolysing enzymes without affecting lactase.

CHAPTER 2
USE OF CATIONIC CELLULOSE FILMS FOR ETHANOL PRODUCTION
IN A CYLINDRICAL FILM FERMENTOR

ABSTRACT

Epichlorohydrin-triethanolamine

(ECTEOLA)-cellulose films (paper and cloth) have been found to bind Saccharomyces cerevisiae cells which were able to develop metabolically active populations on the surface of the films. Unmodified cellulose films also bound the yeast but to a lesser extent. Film fermentors were constructed by coiling a double layer of the cloth and copper screen and vertically placing the resulting cartridge into a column. These film fermentors were able to convert the sugars (14%) in the hydrolysate of a Jerusalem artichoke tuber into ethanol, with 90% of the theoretical yield after 6 h of fermentation. The bound yeast produced ethanol at a specific rate of 1 g of ethanol per g of cell per hour. Immobilization of S.cerevisiae on polyethyleneimine coated cloth produced the yeast film which showed the same ethanol productivity as ECTEOLA-cloth.

INTRODUCTION

In Chapter 1, I have demonstrated the application of a single enzyme (E.coli aspartase) to a biotechnological process (aspartate production). For this, relatively harsh immobilization conditions may be used to effect immobilization of a large amounts of the cells, and cell permeabilization as long as the

stability of the enzyme is maintained. However, many biotechnological processes depend on multienzyme systems in the cell. Ethanol production by yeast is a notable example of such processes, which requires different kinds of enzymes for transformation of hexoses to ethanol. For such processes milder cell immobilization is desired to maintain maximal levels of all enzyme activities and coenzymes required by some of these enzymes. Ideally, immobilization should not reduce viability of immobilized cells since live cells can efficiently regenerate enzymes and coenzymes, thus ensuring the longevity of the immobilized cell system. Furthermore if the system allows the release of the cells reproduced from the immobilized cells, the process can use both immobilized cells and free cells as catalysts. This would be difficult for cells entrapped in gels such as carrageenan or alginate. Furthermore, the gels create mass transfer resistance to a larger or gaseous substrates and products (e.g. CO_2).

Fermentation ethanol from renewable carbohydrates is receiving attention as a future fuel and chemical feed stock. Ethanol, as a fuel and octane enhancer is non polluting and can be dehydrated to ethylene, a major feed stock for chemical industries. Its industrial production demands efficiency of fermentation and low cost of substrates.

In conventional batch ethanol production, a medium

is seeded with starter yeast cells, and ethanol production becomes significant only as cell density increases. Thus, the process is time consuming and a substantial portion of the substrate is consumed for the production of cell mass. Immobilized yeast systems not only permit reuse of cells but ~~also accelerate~~ ethanol production if the systems maintain high densities of active cells.

If yeast cells can develop dense and metabolically active populations on a film material, the resulting yeast film can be repeatedly used for ethanol production. The yeast film would have two major advantages: the cells reproduced from the film can be released and it permits freer mass transfer of substrates and products (ethanol and CO_2).

Cellulose is relatively inexpensive, physically and chemically stable. Cellulose fabric such as cotton cloth would be a suitable medium for cell immobilization as it provides a large surface area and porosity. The fabric can be chemically modified to facilitate binding of yeast cells.

The outer surface of Saccharomyces cerevisiae consists of phosphorylated mannan and proteins. Thus yeast should be able to bind to anion exchanger cellulose film through ionic interactions between phosphate and positive charges of films. Unmodified cellulose film may also be able to bind yeast cells

through hydrogen bonds between hydroxyl groups of mannan and amorphous regions of cellulose.

The present chapter demonstrates that yeast can develop dense population on anion exchange film such as epichlorohydrin-triethanolamine (ECTEOLA)-cellulose and produce ethanol efficiently from the hydrolysate of Jerusalem artichoke tubers and efficiency (rate of ethanol production) did not change for atleast 2 months.

MATERIALS AND METHODS

A haploid strain of Saccharomyces cerevisiae X2180-1B (Yeast Genetic Stock Centre) was used throughout. Yeast nitrogen base without amino acids (YNB), yeast extract and peptone were obtained from Difco Laboratories. Jerusalem artichoke tubers were obtained from the Experimental Farm, Agriculture Canada, Ottawa. ECTEOLA- paper, diethylaminoethyl (DEAE)-cellulose and Whatman papers were the products of Whatman Inc. [^{35}S] Sulphate and [^{32}P] orthophosphate were obtained from New England Nuclear Corporation.

Assay of reducing sugars and ethanol

Reducing sugars were assayed by the 3,5-dinitrosalicylic acid method, (Miller, 1959) using D-fructose as a standard. Ethanol was assayed by means

of nuclear magnetic resonance spectroscopy or gas chromatography.

Yeast binding assay

S.cerevisiae was grown overnight in a medium consisting of 0.1% YNB, 2% D-glucose and [^{35}S] sulphate (5 μCi per ml).

The labelled cells were harvested by centrifugation, washed three times with the medium without [^{35}S] sulphate and resuspended in 10 times the initial culture volume of the nonradioactive medium. Weighed (0.05 to 0.1 g) papers and beech wood shavings were shaken in the above suspension at a speed of 240 rev/min for 7 h, then washed 3 times with nonradioactive medium, and transferred to glass scintillation vials. The bound cells were dissolved in 1 ml 10% sodium dodecyl sulphate by heating at 100°C for 1 h, mixed with 10 ml Tox (Yeung et al., 1976) (a detergent-based scintillation fluid) and the radioactivity counted in a liquid scintillation counter.

Assay of ATP and DNA accumulation on ECTEOLA-papers

S. cerevisiae was grown overnight in a medium consisting of 0.67% YNB and 2% D-glucose, and then diluted 10 times with fresh medium. ECTEOLA-paper segments (1 cm * 1 cm) were placed in the diluted culture, and shaken for 7 h and then washed with fresh medium. Each segment with bound cells was incubated for

different periods of time in 1 ml of the above medium containing [^{32}P] orthophosphate (50 $\mu\text{Ci/ml}$). At various times, duplicate segments were transferred to centrifuge tubes and assayed for DNA and ATP, respectively. To assay DNA accumulation, the segment was immersed in 1.5 ml 1 M NaOH at 37°C for 18 h to remove RNA while leaving DNA. Radioactive DNA was assayed as previously described (Yamazaki and Leung, 1981). To assay ATP, the segment was immersed in 200 μl ice-chilled 5% trichloroacetic acid for 30 min and centrifuged. ATP in the supernatant was separated by two-dimensional chromatography, extracted with 1 M HCl, and counted as described previously (Yamazaki and Leung, 1981).

Preparation of Hydrolysate of Jerusalem artichoke tubers

Jerusalem artichoke tubers were scrubbed in water, sliced, mixed with half their weight of 0.3 M H_2SO_4 and autoclaved at 121°C for 20 min. The juice was separated from the pulp by filtration through cloth and adjusted to pH 5 with concentrated NH_4OH . The concentration of reducing sugars in the hydrolysates varied from 7 to 13% (using D-fructose as a standard). The hydrolysates were supplemented with D-fructose to obtain a final concentration of 14% and were used as an ethanol production medium.

Preparation of ECTEOLA-cloth

Sixty grams of NaOH was dissolved in H₂O to a final volume of 150 ml, and the solution was cooled to room temperature. Thirty-five millilitres of triethanolamine was mixed with 60 ml epichlorohydrin, and the mixture was mixed with the NaOH solution. Cotton flannel was soaked in the final mixture and left at room temperature 17 h. The modified cloth was washed sequentially with 2 M NaCl, 1 M NaOH, H₂O until pH 5, 1 M HCl, H₂O until pH 5, 1 M NaOH, H₂O until pH 5. The above acid and alkali washes were repeated once more and the cloth was finally washed with ethanol and air-dried.

RESULTS

Preliminary screening of films

First, I semiquantitatively compared the capacity of following anion exchange and unmodified cellulose film to bind S.cerevisiae cells: ECTEOLA-cellulose paper, (DEAE)-cellulose paper, Whatman No.1 and 3 MM paper and cotton cloth.

A segment (2 cm * 2 cm) of each of these films was placed into freshly inoculated yeast culture in 0.67% YNB medium containing 9% glucose and shaken in this yeast culture overnight. The films were washed with medium to remove free cells and suspended into 10 ml of fresh medium. Within 1 h ECTEOLA- and DEAE-cellulose papers showed CO₂ bubbling from their surfaces

and others after 2 h. The medium containing ECTEOLA-paper accumulated more than 2% of ethanol after 7 h.

By the criteria of CO₂ evolution and ethanol accumulation, the films can be ranked: ECTEOLA-paper > DEAE-paper > Whatman No.1 and 3 MM paper and cotton cloth. This suggests that introduction of positive charges into the cellulose indeed increases yeast binding. The differences between ECTEOLA and DEAE-derivatives can be ascribed to their three-dimensional structures. As shown in Fig. 2.1 charge on DEAE resides only on the surface of the cellulose fiber, whereas ECTEOLA has extensive (and flexible) network of positive charge. Any similar cationic network on the surface of a film would be suitable for binding of the yeast cells.

Comparison of cellulose films with beech-wood shavings

Beech-wood chips have been shown to adsorb yeasts in higher number than various supports tested (Moo-Young et al., 1980). Therefore, I compared the binding capacity of ECTEOLA-paper and Whatman No.1 paper with that of beech-wood shavings. Beech wood was shaved to produce films of a thickness (~.2mm) similar to that of the ECTEOLA-paper used. The beech wood shavings were washed with hot water and ethanol as described previously (Moo-Young et al., 1980). To compare yeast

Fig. 2.1 (A) Structure of DEAE-cellulose (B)
preparation of ECTEOLA-cellulose (C) and structure of
polyethylenimine (PEI)

A)

B)

C)

binding, ^{35}S -labelled *S. cerevisiae* cells were shaken in growth medium with either ECTEOLA-paper, Whatman No.1 paper or washed beech wood shavings for 7 h, and radioactivity bound to the films was assayed. The radioactivity was due to yeast cells since little radioactivity was released into the extracellular fluid. Table 2.1 shows that on a weight basis, ECTEOLA-paper bound ~ 70 times more yeast cells than beech wood films and ~ 1.6 times more cells than Whatman No.1 paper. This indicated that ECTEOLA is better than other materials that have been tested so far in adsorbing yeast cells.

Growth on ECTEOLA-paper

A film in the fermentor not only binds cells but also should permit the development of metabolically active yeast populations on the film. To examine the later, accumulation of yeast DNA and ATP on ECTEOLA-paper was measured by incorporation of [^{32}P] orthophosphate ($^{32}\text{P}_i$). ECTEOLA-paper segments were allowed to adsorb yeast cells during 7 h incubation and were then transferred to fresh medium containing $^{32}\text{P}_i$ and 2% D-glucose. Table 2.2 shows that $^{32}\text{P}_i$ was actively incorporated into yeast DNA and ATP during the first 17 h. The radioactivity ratio of ATP to DNA in the cells bound to the ECTEOLA-paper was similar to that in free cells. The result suggests that the bound yeasts can

Table 2.1 Binding of ^{35}S -labelled yeast cells to film materials^a

Film material	$10^{-3} \times$ Radioactivity (cpm/g material)
Beech wood shaving .	0.46
Whatman No. 1 paper	19.7
ECTEOLA-paper	31.8

^a Saccharomyces cerevisiae was labelled with [^{35}S] sulphate and shaken with the film materials for 7 h, and the radioactivity (cpm) bound to the film materials was counted as described in Materials and Methods.

Table 2.2 ^{32}P incorporation into DNA and ATP in bound yeast^a

$10^{-3} \cdot$ Radioactivity in ECTEOLA-paper		
Time after $^{32}\text{P}_i$ addition	(cpm/cm ²)	
(h)	DNA	ATP
17	19	3.8
41	22	2.0
65	25	1.2

^a Saccharomyces cerevisiae bound to ECTEOLA-paper was allowed to grow in a medium containing $^{32}\text{P}_i$ and the radioactivity (cpm) incorporated into DNA and ATP was measured at various times as described in Materials and Methods.

develop viable populations on the surface of the ECTEOLA-film. D-Glucose was exhausted from the medium within 24 h, after which time the ratio of ATP to DNA declined significantly, reflecting a slowing of metabolism, presumably due to utilization of accumulated ethanol.

When an ECTEOLA-film with yeast populations (yeast film) was rinsed and transferred to fresh medium, the medium was clear but became turbid after several hours, indicating that the yeast film can generate free cells. In a fermentor, both bound and free yeast cells should produce ethanol. However, since free cells will be removed along with products, continuation of ethanol production depends on the longevity of the yeast film.

Construction of a film fermentor

A film fermentor was constructed as follows. A rectangular sheet (7 m * 35 cm) of either ECTEOLA-paper or Whatman No.1-paper was placed on a rectangular bronze (copper alloy) screen of the same dimension. The double layers were coiled along the longer side to form a cartridge and vertically fitted into a jacketed column (28 mm internal diameter * 25 cm height) (Fig.2.2). To the bottom of the column a conical funnel was fused to facilitate removal of free cells, and the top of the column was plugged with a plastic sponge. The film fermentor was sterilized by

Fig. 2.2 Construction of a film fermentor: Coiled cartridge (A) is made from a cellulose film (B) and copper screen (C) and placed in a column. Space (D) above the film is about three times the height of the film. This much space is required to accommodate the foam formed due to CO_2 evolution during ethanol fermentation.

autoclaving. To make the yeast film, S. cerevisiae was grown in a medium consisting of 1% peptone, 1% yeast extract and 2% D-glucose and the overnight culture was diluted 10 times with the medium and poured into the film fermentor. After 5 min the culture was drained off and the attached yeast cells were allowed to grow on the film at 30°C for 7 h. The fermentor was then filled with 50 ml of an ethanol production medium so as to cover the film (7. cm height) totally and incubated at 30°C for 17 h (overnight).

To study the longevity of the yeast film in supporting ethanol production, the fermentor was drained every morning, rinsed to remove free cells, filled with the ethanol production medium and incubated at 30°C for 2 h and again filled with fresh medium. After 7 h (day time), the liquid was withdrawn and assayed for ethanol (ethanol productivity). Draining of the liquid, rinsing and filling of the fermentor was repeated twice a day.

Ethanol production in a synthetic medium

Ethanol productivity of this fermentor was first examined in a synthetic medium consisting of 0.1% peptone, 0.1% yeast extract and 15% D-glucose. Curve A of Fig. 2.3 shows that during first 6 days ethanol productivity was significantly greater in the ECTAOLA-paper fermentor than in the Whatman No.1

Fig. 2.3 Ethanol production in ECTEOLA-cellulose and cellulose film fermentors. The data in A,B and C represent percent (w/v) of ethanol accumulated during 7 h incubation at different days after zero time when Saccharomyces cerevisiae was first grown on the films. A and B, Ethanol production in the ECTEOLA-paper fermentor (▲) and Whatman No.1 paper fermentor (●) using a medium consisting of 0.1% peptone, 0.1% yeast extract and 15% D-glucose. B, Ethanol production in the above medium after the ECTEOLA-paper fermentor had been incubated for 24 h in the hydrolysate of Jerusalem artichoke tubers. C, Ethanol production in the ECTEOLA-cotton cloth fermentor (▲) and cotton cloth fermentor (○) in the tuber hydrolysate. D, Ethanol accumulated in the ECTEOLA-cotton fermentor for different periods of incubation in the tuber hydrolysate.

fermentor but both became similar after 8 days. The productivity remained at $\sim 2.4\%$ per 7 h for 20 days, which corresponds to only 30 % of the theoretical yield (Appendix) The medium used may not supply sufficient nutrients to all yeast populations on the film.

Ethanol production in a Jerusalem artichoke tuber hydrolysate

The tuber of Jerusalem artichoke (Helianthus tuberosus) is a realistic source for ethanol production in northern climates (e.g. in Canada) since the plant is well adapted to these climates, it can be grown in the fields unfit for conventional crops and produces a high yield of carbohydrates. The major carbohydrate in the tuber, inulin, is a polymer of D-fructose and can be readily hydrolysed by acid. The hydrolysate of the tubers (pH adjusted to 5) supports a good growth of S. cerevisiae, giving a doubling time < 2 h. The previous fermentor (A of Fig.2.3) was incubated in the Jerusalem artichoke tuber hydrolysate for one day, and then switched to the previous medium (0.1% peptone, 0.1% yeast extract and 15% glucose). Curve B of Fig.2.3 shows that the ethanol productivity jumped to $\sim 6.5\%$ per 7 h and then gradually decreased to 5% per 7 h in 7 days. These results suggest that continuation of ethanol production with high efficiency requires enough nutrients as available in the J.artichoke tuber

hydrolysate.

ECTEOLA-cotton cloth fermentor

ECTEOLA-paper is no longer commercially available. Since its preparation requires a series of washes which tends to destroy its film structure, I prepared ECTEOLA-cloth from cotton flannel. Its strength makes it more suitable for industrial preparation and use. I modified the previously described method (Peterson and Sober, 1956) so that no stirring or heating is required to derivatize cloth.

Two film reactors were constructed in the same way as before: one consisted of ECTEOLA-cloth and the other of unmodified cloth. The yeast films were prepared by using yeast suspension in the artichoke tuber hydrolysate. Ethanol production in the tuber hydrolysate (containing 14% D-fructose) was measured 7 h after addition of the hydrolysate. Curve C of Fig.2.3 shows that an ethanol productivity of 6.5% per 7 h was reached after 2 days in the ECTEOLA-cloth fermentor and after 3 days in the unmodified cloth fermentor. The ethanol concentration of the overnight incubation (17 h) was also ~ 6.5% which corresponds to 90% of the theoretical yield from hexose (See Appendix). Using the ECTEOLA-cloth fermentor, incubation times < 7 h gave progressively less ethanol (D of Fig.2.3).

To study the longevity of the fermentors, both the

ECTEOLA-cloth and unmodified cloth fermentors were incubated for 60 days in the Jerusalem artichoke tuber hydrolysate, which was changed twice a day (at 17 and 7 h intervals). Ethanol productivity remained unchanged for at least 60 days.

During active ethanol production, the double layers (cloth and copper screen) in the fermentors tend to trap CO_2 and as a consequence not all the area of the yeast film is exposed to the medium until the CO_2 bubbles escape. To facilitate CO_2 escape, the distance between the coiled film was widened. By shortening the coil length from 35 to 22 cm, the ethanol production rate in the ECTEOLA-cloth fermentor was increased to 6.5% per 6 h (instead of 7 h).

PEI cotton cloth fermentor

Since ECTEOLA gave encouraging results I then studied similar and more economical method for introducing cationic network onto the surface of the cotton cloth. Polyethyleneimine (PEI) coated cellulose fibers, have been used as anion exchanger for nucleotide separation (Randerath and Randerath, 1967) As shown in Fig.2.1 structure of PEI is a highly branched network containing dense cationic charges due to primary, secondary and tertiary amines. PEI almost irreversibly adsorbs to cellulose presumably due to hydrophobic interaction and ionic interaction between

the amines of PEI and the residual carboxyl group of cellulose. I have found that soaking cotton cloth in 0.1% PEI solution for 30 min results in effective coating of cloth. Resulting PEI-cloth (7 * 22) like ECTEOLA-cloth was similarly used in the cylindrical film fermentor and tested for ethanol production. Ethanol yield was 6.5% per 6 h^o as in ECTEOLA-cloth fermentor.

Ethanol productivity of bound yeast cells

During 6 h period, the medium became turbid due to generation of free cells which should also produce ethanol. To estimate ethanol production by bound yeast, the medium (containing 14% fructose) was changed every hour in the PEI-cloth and ECTEOLA-cloth fermentors. Since withdrawn medium was clear, ethanol production was largely due to the bound yeast (CO₂ bubbling was evident on the surface of film). The bound yeasts, in both fermentors produced ethanol at the rate of 16 g per l per h. This suggests that bound yeast produced substantial portion of ethanol in the fermentor. Since the films (7 cm * 22 cm) contained 0.77 g dry cell, a specific rate of ethanol production by the bound yeasts is 1 g of ethanol per g of cell per h.

DISCUSSION

I have demonstrated that Saccharomyces cerevisiae cells bind to cationic cellulose films and developed an ethanol producing population on the film. I have

constructed a small cylindrical film fermentor by coiling yeast film and placing it in a cylinder. In this fermentor I have shown that the yeast films can be used to repeat batch ethanol fermentations for at least 60 days, and that the tuber of Jerusalem artichoke uniquely available in Canada is a good source for ethanol because its hydrolysate supports continuous production of ethanol without supplementation of any nutrients. The yeast film has an advantage over the gel entrapment systems in that free cells can be freely released from the film and participate in ethanol production.

The fermentor I have constructed is useful in examining long term performance of the yeast film (as well as substrates) in producing ethanol since only a small volume of substrate solution is required and it is easy to repeat batch fermentations. Furthermore, evolving CO_2 facilitates fermentation by mixing the substrates.

The present studies have also shown that an unmodified cellulose film binds the yeast (although at a slower rate than the cationic film) and ethanol production by such an yeast film eventually reaches the same productivity by the cationic film. Thus, it is possible to use an unmodified cellulose cloth as yeast immobilization support.

APPENDIXCalculation of % theoretical yield of ethanol

$C_6H_{12}O_6$ -----	2 C_2H_5OH	+	$2CO_2$
Fructose	Ethanol		Carbon dioxide
180 g	92 g		
14 g	7.15 g		

92 g of ethanol from 180 g of fructose

(or 7.15 g ethanol from 14 g of fructose)

represents 100% theoretical yield.

% of theoretical yield can be calculated by
the formula:

$$\% \text{ of theoretical yield} = \frac{\text{Practical value obtained}}{\text{Theoretical value}} \times 100$$

Example-

When 6.5 g of alcohol is produced from
14 g of sugar then:

$$\% \text{ of theoretical yield} = \frac{6.5}{7.15} \times 100 = 90.9$$

CHAPTER 3
IMMOBILIZATION OF YEAST ON COTTON CLOTH
FOR ETHANOL PRODUCTION

ABSTRACT

Saccharomyces cerevisiae cells were immobilized on cotton cloth. The resulting yeast film cloths were placed in parallel in a rectangular fermentor which was designed for scale-up. Ethanol production from sugars in the hydrolysate of Jerusalem artichoke tubers was studied in three modes of operations: batch, circulated batch, and continuous flow. Circulated batch fermentation gave the shortest time of fermentation and accordingly the highest average ethanol productivity. When the same yeast film segments were stirred in a tank, ethanol productivity was reduced due to stripping of the yeast layer from the film.

INTRODUCTION

In Chapter 2, I have shown that Saccharomyces cerevisiae cells, developed ethanol producing immobilized populations on anion exchanger cotton cloths. I have also noted a possibility of using an unmodified cotton cloth as an immobilization support, which would be simpler and less costly than the anion exchange cloths. Since S. cerevisiae cannot utilize inulin, a major carbohydrate in the Jerusalem artichoke tubers, the hydrolyzed tuber extract containing monosaccharides (formed by inulin hydrolysis) was used as a substrate for ethanol production. However, other

yeasts such as Kluyveromyces marxianus and Kluyveromyces fragilis are known to ferment inulin as they produce inulinase (Guiraud et al, 1981; Duvnjak et al, 1981). In this chapter, three yeasts will be immobilized on unmodified cotton cloth and production of ethanol from the unhydrolyzed tuber extract by the Kluyveromyces yeasts will be compared with that from the hydrolyzed tuber extract by S.cerevisiae using the cylindrical film fermentor.

Since the cylindrical film fermentor is not amenable to scale-up, a new film fermentor ('rectangular fixed film' fermentor) has been devised and ethanol production in this fermentor will be compared with that by the film stirred in a tank.

MATERIALS AND METHODS

Saccharomyces cerevisiae X2180-1B (Yeast Genetic Stock Center) / Kluyveromyces marxianus ATCC 10606 and Kluyveromyces fragilis ATCC 36534 were used. Jerusalem artichoke tubers were obtained from the Experimental Farm, Agriculture Canada, Ottawa. Cotton flannel (cloth) was obtained locally.

Growth media and conditions

Jerusalem artichoke tubers were washed, sliced, mixed with an equal weight of water, and autoclaved for 30 min. The juice, separated from the pulp by filtration, contains 7 to 8% inulin, a major

carbohydrate in the tubers. The juice was autoclaved and used as a medium for the inulin-utilizing yeasts, K. marxianus and K. fragilis.

For S. cerevisiae which cannot use inulin, the juice was adjusted to pH 3.0 with concentrated H_2SO_4 , autoclaved for 30 min to hydrolyze the inulin, and adjusted to pH 5.0 with concentrated NH_4OH . All yeasts were grown at $30^\circ C$. In some experiments (in a film stirring fermentor), corn starch hydrolysate was used as a carbon source for ethanol production by S. cerevisiae. A starch hydrolysate was prepared by St. Lawrence Reactor Ltd., Mississauga, Ontario by means of its patented reactor; a starch solution in dilute acid (pH about 2) was heated at $149^\circ C$ for 3 min. One volume of the hydrolysate was mixed with .2 volumes of the nutrient solution which contained 3.2% K_2HPO_4 , 4% $NH_4H_2PO_4$ and 2% yeast extract, giving a final pH of 4.5 and 14.7% glucose.

Assay of reducing sugars, ethanol, and cell mass

Reducing sugars were assayed by the 3,5-dinitrosalicylic acid method (Miller, 1959) using D fructose as a standard. The reducing sugar content of growth media was determined after hydrolyzing inulin as described above.

Ethanol was assayed by gas chromatography in which a glass column of chromosorb W was used. N_2 was used as

a carrier gas and flame ionization was used as a detection method.

Cell mass weight of yeast immobilized on cloth was determined by cell extraction with formic acid followed by drying at 110°C for 1 day and weighing.

Yeast colonization in a cylindrical film fermentor

A rectangular cotton cloth was placed on a bronze screen of the same dimensions (7 cm * 15 cm). The double layer was coiled along the longer side and the coil was vertically fitted into a jacketed column (2.8 cm i.d.; 25 cm height). The average distance between the coil was 0.4 cm. The temperature of the jacket was maintained at 30°C .

Yeast colonization on cloths was initiated as follows. A late log phase yeast culture was diluted to about 4×10^7 cells/ml with fresh medium and added to the fermentor. After 1 h, the fermentor was drained and yeast was allowed to grow on the wet cloth in air for 17 h. The fermentor was then filled with fresh medium and incubated for 24 h.

Ethanol productivity of yeast cells' colonized on cloth was daily determined as follows. The fermentor was drained, refilled with fresh medium, and incubated for 2 to 3 h. After this activation period, the fermentor was drained and refilled with fresh medium. After 60 min, ethanol was assayed, from which ethanol

productivity (g ethanol produced per l of fermentor volume per hour) was calculated (see Appendix).

Construction of a rectangular fixed film fermentor

Three rectangular cloths (5 cm * 30 cm) were stretched lengthwise and mounted in parallel (0.7 cm apart) in a stainless steel stand and placed in a plexiglass container (2.2 cm width, 32 cm length, 20 cm height, inside) as illustrated in Fig. 3.1. Yeast colonization on cloth was performed as described for the cylindrical fermentor. The medium was changed once a day for 8 days until maximum ethanol productivity was attained. When ethanol productivity of the fermentor was to be determined, the fermentor was first activated by incubation in fresh medium for 2 to 3 h, drained, then filled with 285 ml fresh medium (liquid volume of the fermentor) and incubated at approximately 30°C.

Construction of a film stirring fermentor

Versatac fermentor (Pegasus) capacity of 1.5 liter and maximum working volume .5 liter was used (Fig. 3.2). The shaft having 3 marine propeller blades (4.8 cm diameter and 1 mm thickness) was used. The blades were placed 2.4 cm above the bottom of the fermentor. Fermentor contained 200 cloth segments of 1 cm square. Each segment was painted at the sides with 5% cellulose acetate solution in acetone to prevent loss of filaments from the edge and entanglement of segments

Fig.3.1 Diagram of a rectangular fixed film fermentor.
where (A) is a pump and (9) is a solenoid valve.

Fig. 3.2 Diagram of a film stirring fermentor.

- a) drainage tubing (0.8mm inside diameter)
with a screen
- b) air filter
- c) stirring shaft (30 mm diameter)
- d) medium supply tubing

during mixing. Yeast colonization was performed in a sterile fermentor as described in a rectangular fixed film fermentor. Once a day the fermentor was drained, refilled with medium to cover the segments and stirred briefly. After 8 days, ethanol productivity was measured weekly as follows: Yeast films were first activated by stirring at 150 rpm for 2 to 3 h in fresh medium. The fermentor was drained and filled with 133 ml of fresh medium. The medium was stirred at various speeds and samples (1 ml) were withdrawn via the drainage tube every hour and assayed for ethanol.

RESULTS

Colonization of yeast cells on cotton cloth

In Chapter 2, I have shown that unmodified cellulose paper binds a significant number of S. cerevisiae cells. Here I examined the binding of S. cerevisiae by using ³⁵S-labelled cells as described in Chapter 2. Fig.3.3 shows that the yeast cells also bound to the cotton cloth. The calculation of the percentage of bound cells (Fig.3.3) shows that not all cells are capable of binding to the cloth since only about 10% of the cells were found to bind even when small amount of cells was added to the cloth. This shows that the yeast generates phase variants which show high affinity for cellulose (cellulophilic variant).

Fig.3.3 Binding of Saccharomyces cerevisiae cells, to cotton cloth. ³⁵S-labelled yeast cells were prepared as described in Chapter 2. The cells were suspended at various cell densities in 2% glucose and 1% YNB. 200 μ l portions of the cell suspension was uniformly applied to cotton cloth segment (2 * 2 cm). After 1 h at room temperature, the segments were washed 5 times by vortexing in the above medium for 1 min. The radioactivity was counted as described in Chapter 2. The data shows the amount of radioactivity (cpm) 'added' to the cloth versus the amount of radioactivity (cpm) 'bound'. The percentage of the bound cells was calculated by dividing the bound radioactivity by the added activity.

of pili, flagella, lipopolysaccharides and cell walls (Simon et al., 1980). Since phase variation does not affect intracellular metabolism, it is expected that variants will exhibit the same ethanol productivity.

When the bound cells were allowed to grow on wet cloth suspended in air, the population of bound cells increased whereas the increase was slower by a factor of 2 to 3 when the cloth was immersed in the medium. The development of cell colonization on wet cloth consumes much less medium, than in liquid medium. For these reasons, yeast immobilization was performed as follows. A cloth was immersed in a yeast suspension in fresh medium for 1 h, and then suspended in air for 17 h. The cells grown in this manner were immobilized on cloth. That is, when the cloth (designated yeast film) was suspended in the medium, it did not release cells although the bound cells reproduced free cells after a few hours. Ethanol productivity of the S. cerevisiae film was studied in a cylindrical film fermentor as described in Methods. Fig. 3.4 shows that ethanol productivity of the S. cerevisiae film increased during the first 7 days of fermentor operation. Microscopic observation and determination of cell mass (Table 3.1) showed that the yeast film thickened during this period.

At the plateau the S. cerevisiae film fermentor

Fig.3.4 Colonization of ethanol producing yeasts on cotton cloth as measured by ethanol productivity in a cylindrical film fermentor. On day 0, S.cerevisiae (O), K.marxianus (Δ) and K.fragilis ($\bullet$) cells were immobilized (bound) onto cotton cloth and colonized in a cylindrical film fermentor as described in Methods. Ethanol productivity (g ethanol/l of fermentor /h) was determined daily.

Table 3.1 Cell mass thickening observed during colonization of S.cerevisiae cells on cotton cloth.^a

Day of yeast colonization	Dry cell weight (mg/cm ² cloth)
0	1.5+ 0.5
4	3.5+ 0.5
8	5.0+ 0.5

^a On day 0, S.cerevisiae cells were bound to cotton cloth and colonized in a cylindrical fermentor. The amounts of bound dry cell weight were determined as described in Methods..

Cell mass accumulated per unit area was also similar (5 mg/cm²). The longevity of the S. cerevisiae film was tested for 60 days in the cylindrical fermentor. No significant change in ethanol productivity was observed during this period.

Ethanol production has been previously studied with some Kluyveromyces species (Duynjak et al ., 1981). Fig. 3.4 also shows ethanol productivity by the films of inulin fermenting K. marxianus and K. fragilis in an unhydrolyzed extract of artichoke tubers. These yeasts colonised on cloth more slowly than S. cerevisiae and ethanol productivity at the plateau was less than that of S. cerevisiae. Since inulin can be readily hydrolyzed to fructose which is fermentable by S. cerevisiae, the use of inulin-fermenting yeasts has few advantages. Thus, only the S. cerevisiae film was used in all subsequent experiments involving rectangular fixed film and film stirring fermentors.

Construction of a rectangular fixed film fermentor

The cylindrical film fermentor has been useful in examining ethanol productivity of yeast films with relatively small amounts of the substrate in batch fermentation. However, it is not amenable to scale-up. Its scale-up by increasing its column height will effectively reduce the liquid portion of the fermentor because evolving CO₂ occupies the upper part of the

film. Enlarging the column diameter will decrease the efficiency of removal of heat generated during fermentation. Furthermore, coiling of large yeast film would be technically difficult.

A rectangular prism (' rectangular ') fixed film fermentor was designed as illustrated in Fig. 3.1. A narrow width was used to facilitate dissipation of heat generated. Rectangular cloths were stretched in parallel along the length of the fermentor. Maintaining a uniform distance between the cloth layers is easier in this fermentor than in the cylindrical film fermentor. The rectangular fermentor can be scaled-up by using multiple units in parallel or by increasing its length. Furthermore, evolving CO_2 can be easily allowed to escape and circulation of the medium is feasible.

Effect of modes of operation on ethanol production in rectangular fixed film fermentor.

The rectangular fixed film fermentor was used for ethanol production from a hydrolyzed extract of Jerusalem artichoke tubers in the following modes of operation: (1) stationary batch; (2) circulated batch; and (3) continuous flow. In both batch modes, the medium was added to the fermentor and removed after ethanol fermentation was completed. The medium was not circulated in the stationary batch mode but circulated in the circulated batch mode. In the continuous mode,

the medium was continuously fed through one end of the fermentor and removed from the opposite end. Fig. 3.5 compares ethanol production in these modes. In both batch modes, the rate of ethanol production declined with time, reflecting decreasing concentrations of the substrate. Circulation of medium in the batch fermentation yielded constantly higher rates of ethanol production until the end of fermentation. Accordingly, fermentation of sugars in the circulated batch mode was completed at 4 h as compared to about 8 h in the stationary batch mode. Maximum ethanol productivity in g ethanol per l of fermentor volume per h was 17 in the stationary batch mode and 20 in the circulated batch mode, while the ECTEOLA-cloth fermentor (Chapter 2) gave 16 in the stationary batch mode. In the continuous flow mode, similar ethanol productivity was obtained only at a high flow rate (e.g. medium retention time of 1 h) but a large proportions of the sugars was not fermented. In this mode, complete fermentation of sugars was achieved only after a retention time of 9.5 h (Fig.3.5). Table 3.2 lists average ethanol productivity for complete sugar fermentation in the three modes.

Automation of circulated batch ethanol fermentation in a rectangular film fermentor.

Retention of immobilized yeast in a fermentor permits automation of batch fermentation. I devised a

Fig. 3.5 Ethanol production by S.cerevisiae in a rectangular fixed film fermentor. S.cerevisiae was colonized in a rectangular film fermentor as described in Methods. After maximum ethanol productivity was attained, ethanol production was studied in three modes of operation : stationary batch (Δ); circulated batch ($\circ$); and continuous flow ($\bullet$). The medium used was the Jerusalem artichoke tuber hydrolysate supplemented with D-fructose to give final hexose concentration of 10%. In the circulated batch mode, the medium was circulated at a specific flow rate of 7.3 ml/min/cm². Ethanol concentration (% w/v) was plotted against time of incubation ($\circ$, Δ) in the stationary modes and medium retention times ($\bullet$) in the continuous flow mode. The broken line represents ethanol concentration when all the sugars are converted to ethanol with 90% of the theoretical yield.

Table 3.2 Ethanol production in a rectangular fixed film fermentor

Ethanol production	Mode of operation		
	Stationary batch	Circulated batch	Continuous flow
Completion time ^a	8	4	9.5
Average productivity ^b	5.5	11	4.8

^a Time (h) for conversion of all fermentable sugars to ethanol with 90% of the theoretical yield, estimated from the results in Fig. 3.5. ^b Ethanol (g) produced per liter of fermentor volume divided by completion time.

timed draining and pumping device for the rectangular fixed film fermentor. In this fermentor, circulation mode completes fermentation of 10% sugar after 4 h. Automation of 4 h batch fermentation was achieved by use of a pump (between the medium reservoir and the fermentor), a circulating pump, a two way solenoid valve (between the fermentor and the outside) and a timer connected to a valve and a pump (Fig.3.1). This device performs as follows:

- (1) addition of medium at 0 time ;
- (2) initiation of circulating pump after delivery of the medium;
- (3) after 4 h, the pump stops and the drain pipe opens;
- (4) after removal of the fermented broth, the drain pipe closes and (1) is repeated. For 60 days, the device was used to automatically repeat circulated batch ethanol fermentation of artichoke tuber hydrolysate containing 10% sugar. At 2 to 3 day intervals, the fermented broth was analyzed for ethanol. During this batch fermentation all sugars were utilized and ethanol was produced at about 90% of theoretical yield.

Film stirring fermentor

In the rectangular fixed film fermentor, mixing by circulation was found to increase ethanol production. Similar mixing may be obtained if segments of yeast film

are freely suspended and stirred. Such a fermentation can make use of a conventional fermentation tank after attaching a screen at the bottom to retain the film segments. Since such a tank is readily available, the present film fermentation can be readily applied. To examine this, a small stirred tank was constructed as shown in Fig.3.2. Since stirring loosened the edges of cloth segments and caused entanglement of the segments, variety of stirring blades were examined. Marine propallar was chosen over the other types since it affected the segments least. To prevent the loosening of the edges, the edges were fortified by painting with 5% cellulose acetate in acetone followed by drying. The fortified cloth segments were colonised by S.cerevisiae cells.

The J.artichoke tuber hydrolysate was used as a substrate so that the ethanol productivity in this fermentor can be compared with that in the rectangular fixed film fermentor. Corn starch hydrolysate was also used as a substrate since corn starch is abundant in the U.S. and there is a prospect of exporting the film fermentation technology over there.

Since the film segments would experience shear forces, the immobilized yeast cells may come off at a high stirring rate. Indeed I observed that some fraction of the colonized yeast cells came off at speeds greater than 200 rpm but little loss occurred at speeds

less than 150 rpm. However, below 150 rpm, the film segments floated away from the action of a stirring blade because formed CO_2 did not leave the film as soon as formed.

Fig.3.6 shows ethanol production in a film stirring fermentor when yeast film segments were stirred at two film densities and at two stirring rates. The rectangular fixed film fermentor (Fig.3.1) has used 1500 cm^2 of yeast film per l of the fermentor. When the yeast film segments at the same density were stirred at 150 rpm, the film stirring fermentor gave an average ethanol productivity (g/l/h) of 5 as compared to 11 in the fixed film fermentor with circulation (Fig.3.6). When the film density was increased to 2000 cm^2 per l, ethanol productivity increased to 6.5. When the yeast film segments at the same density were stirred at 200 rpm, ethanol productivity increased to 8. Since the suspension was very viscous, further increase in either film density or stirring rate would require much energy and result in loss of immobilized cells. Fig.3.6 also shows that starch hydrolysate could be used to produce ethanol in a film stirring fermentor.

Using a film density $2000 \text{ cm}^2/\text{l}$, a stirring rate of 200 rpm and timed draining and pumping devices, I was able to repeat batch ethanol production every 5 hours in the Jerusalem artichoke tuber hydrolysate and every 7 hours in the starch hydrolysate. During 2 months the

Fig. 3.6 Ethanol production by S.cerevisiae segments stirred in a film stirring fermentor. Cotton cloth segments were colonized by S.cerevisiae (as described in Methods). At 0 time, the film segments were suspended in a stirred tank (Fig. 3.2) at two different densities (area of cloth/l) of 1500 cm²/l and 2000 cm²/l, and stirred at two speeds of 150 rpm and 200 rpm. Ethanol production was examined in the Jerusalem artichoke tuber hydrolysate (JA) containing 10% sugar and in the corn starch hydrolysate (starch) containing 14.7% sugar. Ethanol concentration (%) of the fermented media was periodically assayed. The broken line represents ethanol concentration when all the sugars are converted to ethanol with 90% and 94% of theoretical yield in tuber hydrolysate and starch hydrolysate respectively.

Symbol	Substrate	Film density (cm ² /l)	Speed rpm	Ethanol productivity ^a (g/l/h)
○	JA	1500	150	5.0
△	JA	2000	150	6.5
□	JA	2000	200	8.0
●	starch	1500	150	5.5
▲	starch	2000	150	7.0
■	starch	2000	200	9.0

^a Average ethanol productivity - ethanol (g) produced per liter of fermentor volume divided by time (h) for conversion of sugars to ethanol with 90% (for JA) or 94% (for starch) of the theoretical yield.

fermentor was able to convert 10% sugars in the tuber hydrolysate and 14.7% sugars in the starch hydrolysate to ethanol with 90% and 94% of theoretical yield, respectively.

DISCUSSION

I have demonstrated that S.cerevisiae can bind and propagate on cotton cloth and the resulting film can be used for repeated batch ethanol fermentation. The use of unmodified cotton is simpler, less expensive than anion exchange cloth. Although the nature of yeast binding onto cloth is not yet known, the binding is tight: mixing (by vortexing) cannot detach bound cells. Bound yeast increases cell mass and forms a thicker layer of yeast cells. Since ethanol productivity increases during this colonization period, the whole layer of yeast cells must be responsible for ethanol production. However, the yeast layer can be destructed by strong shear force.

I have constructed a rectangular fixed film fermentor which can be scaled-up. I have shown that in this fermentor a circulated batch mode provides a faster fermentation than the continuous mode. Circulation will increase the transport of sugars to immobilized yeast cells as well as facilitate the mixing of sugars and ethanol. All the cells in the circulated batch fermentation equally share in ethanol production.

whereas in the continuous mode cells colonize downstream produce ethanol at greatly reduced rates because of the lower sugar and higher ethanol concentrations. Using a circulated batch fermentation, 4 h was required to utilize all sugars (10%) to produce ethanol with 90% of theoretical yield. This gives an average productivity of 11 g ethanol per l of fermentor volume per h (Table 3.2).

Mixing of substrates and products can be obtained by stirring the yeast film segments in a tank (a film stirring fermentor). However, I have shown that ethanol productivity is less than that in a circulated batch mode in the fixed film fermentor, presumably because a strong shear force disrupts the yeast layer reducing the amount of ethanol producing cells attached to the cloth. Another possible explanation is increased aeration by mixing may retard ethanol production. In the film stirring fermentor, production of ethanol from the starch hydrolysate did not differ from the tuber hydrolysate. Therefore the fixed film fermentor in circulated mode using the starch hydrolysate should yield 11 g of ethanol/l fermentor/h.

S. cerevisiae cells entrapped in polyester foam give approximately 5 g per l of fermentor per h (Black et al., 1984) and those entrapped in alginate gel, approximately 9 g per l per h (William and Munnecke, 1981) and 20 g per l per h (Kyowa Hakko Kogyo, 1983).

Although several S.cerevisiae immobilized systems have been described (Margaritis et al, 1981), it is not possible to compare the productivity (per unit volume of fermentor) of these systems beacuse the liquid volume fraction of the systems are not described (see Appendix) .

In the rectangular fixed film fermentor, ethanol productivity can be increased to 25 g ethanol/l fermentor/h if the distance between the films is reduced from .7 cm (in the present fermentor) to .3 cm. This productivity is comparitively more than alginate entrapped yeast cells suggesting film system permits freer movement of substrates and products. The present rectangular fermentor has a narrow width in order to facilitate heat dissipation to surrounding. However, if the circulating medium is allowed to pass through a heat exchanger, a wider fermentor (thus with larger medium capacity) can be constructed.

APPENDIX

Productivity of a reactor

Productivity used in the present study is the amount of product produced per liter of total reactor volume divided by the liquid retention time. This term is most relevant to the design of a reactor process, and thus should be used for comparison of productivity.

Productivity can then be expressed as $Pv/(TV)$ or PL/T for batch production and PF/V for continuous flow production, where

P = amount of a product per liter of liquid collected from the reactor after or during the retention time

v = liquid volume in the reactor [liter];

V = total reactor volume [liter];

F = flow rate [liter/hour];

T = retention time [hour];

L = liquid volume fraction in the reactor [v/V].

Some researchers use PF/v as productivity for continuous flow production (Margaritis et al., 1981). Since this term is equal to P/T or $PF/(VL)$, it should be multiplied by L to obtain the above productivity.

CHAPTER 4
USE OF BACTERIAL FILMS AS RESIDENT INOCULA
FOR REPEATED BATCH FERMENTATION

ABSTRACT

Six bacteria of industrial importance were found to form immobilized populations on cotton cloth. The resulting bacterial films generated free cells at significant rates which remained constant for at least 60 days. By retaining these films in a fermentor, the films could be used as resident inocula in repeated batch fermentations. An equation for the rate of cell mass generation from the resident inocula was developed.

INTRODUCTION

Commercial fermentations are commonly carried out by batch fermentation. To start a main batch fermentation, a seed culture is prepared in 2 to 3 stages of volume scale-up. To repeat a batch fermentation, a part of the fermented culture, if rich in viable cells, may be used as an inoculum for the next fermentation though it will reduce product yield. However, the fermented culture often contains substances which inhibit the growth of microorganisms. Although harvested cells can be used instead, harvest and readdition of cells add to the cost of fermentation and these steps are less amenable to automation.

In the previous chapter, I have described that the yeast film can generate cells and ethanol is produced by both immobilized and free cells. If bacteria are

immobilized on the surface of a film material and the immobilized bacteria can efficiently reproduce free cells, segments of the bacterial film can be permanently placed in a stirred tank as a resident inoculum. At the end of fermentation, the fermented culture can be separated from the bacterial film which will be retained in the tank. Use of such films as resident inoculum will permit automatic repetition of batch fermentation. The present chapter describes immobilization of several bacteria on cotton cloth and demonstrates the use of these bacterial films as resident inoculum in repeated batch production of bacterial cells.

MATERIALS AND METHODS

Cloth Cloths of polyester, acetate, triacetate and cotton (flannel) were obtained locally. Polyethylenimine (PEI) coated cotton cloth was prepared as described earlier, in Chapter 2.

Microorganisms and growth conditions

The characteristics of the bacteria used are listed in Table 4.1. E.coli Crooks and B41 were grown in 0.5% glucose in M63 medium (Miller, 1972) and other bacteria were grown in M63 supplemented with 0.1% yeast extract. The cultures were shaken at 240 rpm and 37°C for E.coli strains or 30°C for other bacteria. Cell mass density was measured by absorbance at 500 nm.

Table 4.1 Characteristics of bacteria immobilized on cloths.

Bacterium	Products	Doubling times ^a	A ₅₀₀ ^b
<u>Bacillus subtilis</u> (ATCC 6051)	Amylases and proteases	1.1	3.0
<u>Brevibacterium ammoniagenes</u> (ATCC 6872)	Nucleotides	1.25	8.8
<u>Corynebacterium glutamicum</u> (ATCC 21526)	L-lysine	1.15	7.5
<u>Escherichia coli</u> (Crooks (ATCC 8739) ^c	Inducible enzymes	1.0	6.0
<u>Escherichia coli</u> HB101 ^d	Recombinant DNA	0.8	8.5
<u>Escherichia coli</u> B41 ^e	K99 pili	1.2	5.5

^a Doubling times of free cells in h.

^b Absorbance at 500 nm of stationary cultures.

^c Because of its high cyclic AMP content (Fraser and Yamazaki, 1978), it can be used for production of inducible catabolic enzymes whose synthesis depends on cyclic AMP (e.g. L-aspartase and penicillin acylase).

^d Because it lacks modification, restriction and recombination systems (Maniatis et al., 1982), it is commonly used for hosting recombinant DNA.

^e It harbors K99 plasmid and thus produces K99 pili which can be used as a vaccine against E.coli scours in domestic animals.

(A_{500}). One absorbance unit (absorbance $\times 1$) of E.coli cultures contains approx. 5×10^{11} cells.

Bacterial immobilization on cloth

Bacterial immobilization was initiated as follows. Stationary cultures (whose A_{500} are given in Table 4.1) were diluted 1000 fold with fresh medium and added to a culture tube containing 1 cm square segments of cloth to cover the cloth. After 1 h, excess liquid was removed, and the bacteria attached to the cloth were allowed to grow on the wet cloth. After 24 h, the segments were rinsed once with fresh medium and incubated for 24 h, after which this was repeated once more. The culture tube was then filled with fresh medium and shaken at 240 rpm overnight. Next day and on the following days, the specific rate of cell mass generation from the cloth segments (bacterial films) was measured as follows: One bacterial film was vigorously washed 5 times by vortexing in 3 ml of medium followed by decantation. Each film was first activated by incubation in 1 ml of fresh medium for 2 to 3 h, washed as before, and shaken in 1 ml of fresh medium at 240 rpm. After 60 min, A_{500} was determined and the specific rate of cell mass generation from the film was calculated as described in Appendix. Every 24 h, culture liquid was replaced with fresh medium and the specific rate of cell mass generation was measured at

least once a week for 60 days.

RESULTS

The bacteria listed in Table 4.1 were used in the present study because of their current or potential use in commercial fermentations: In the previous Chapter 3, I have shown that yeasts (Saccharomyces and Kluyveromyces) develop immobilized populations when they are allowed to grow on cotton cloth moistened with growth medium. Here I demonstrate that these bacteria can be similarly immobilized. Cotton cloth was briefly immersed in a dilute bacterial suspension in fresh medium. The bacteria attached to the cloth were allowed to grow on the wet cloth, and the cloth was rinsed daily with fresh medium over 3 days. The cloth was then shaken in growth medium. Next day (first day in Fig.4.1), the cloth (bacterial film) was thoroughly washed by vigorous vortexing (to remove any loosely attached cells) and then assayed for its capacity to generate cell mass (Fig.4.1).

On the first day, the specific rates of cell mass generation from the bacterial films varied with bacteria. The rates increased with days of liquid culturing to plateau levels which varied with bacteria ranging between 3×10^{-4} and 4×10^{-4} absorbance units /h/cm². The times required to reach the plateau levels varied widely from 5 days for Brevibacterium

Fig.4.1. Specific rate of cell mass generation from bacterial films. Bacteria were immobilized on cotton cloth as described in Methods. After 3 days growth on wet cloths, the cloths were shaken in growth medium overnight. Next day (day 1 in this figure) and on the following days, the specific rates of cell mass generation from the films (absorbance units/h/cm²) were determined as described in Methods. Daily, the cloths were thoroughly washed and resuspended in fresh medium. Symbols: Bacillus subtilis (O-); Brevibacterium ammoniagenes (■); Corynebacterium glutamicum (Δ); Escherichia coli Crooks (□); E.coli HB101 (▲); E.coli B41 (●).

ammoniagenes to 11 days for Bacillus subtilis. This suggests that a large portion of cells generated during culturing does not show high affinity for cellulose but at every generation cellulophilic variants are produced. However, the rate of generation of the cellulophilic variants differs with bacterium e.g. B.ammoniagenes has a higher rate than B.subtilis.

To examine the longevity of the films as resident inocula, the films were washed daily by vigorously vortexing the films in fresh medium. The plateau rates of cell mass generation from the films did not change for at least 60 days.

In native cellulose such as cotton, hydrophilic hydroxyl groups are extensively engaged in hydrogen bonding and thus cellulose surface is largely hydrophobic. In fact, one g of cotton cloth has been shown to adsorb 4 mg of bovine serum albumin, a hydrophobic protein (Suniti Sharma, unpublished observation). If bacteria are bound to cotton cloth by hydrophobic interaction, hydrophobic cloth may provide a better adsorbent film for bacterial immobilization. Thus, the hydrophobic naphthyl cotton cloth was prepared as described previously (Sharma and Yamazaki, 1984). Although naphthyl cotton cloth adsorbed about 100 mg of bovine serum albumin, Escherichia coli Crooks strain and remaining 5 bacterial strains developed immobilized populations on naphthyl cotton cloth with the same

kinetics as on unmodified cotton cloth. The final specific rates of cell mass generation were also indistinguishable. Thus, it appears that bacterial binding site on cotton cloth was not increased by introduction of naphthyl groups.

Other cloth materials were examined for their capacity to form bacterial films. Table 4.2 shows that cotton cloth was superior as an immobilization support to polyester, acetate, triacetate and PEI-coated cotton cloths. PEI-cloth has been used to immobilize yeasts (Chapter 2). Although PEI-cotton cloth was effective in immobilizing E.coli strains, it was less effective for the other bacteria.

DISCUSSION

I have shown that several bacteria of practical value can form immobilized populations on cotton cloth. The immobilized cells could not be released even after vigorous mixing. The nature of this attachment might be hydrophobic, since residual carboxyl groups in cotton cannot attract net negative charges of bacteria and its hydroxyl groups are not readily available for hydrogen bond formation with exogenous substances. Since the resulting bacterial films can generate free cells at significant rates and can be easily retained in a fermentor, they can be used as resident inocula which would facilitate automation of repeated batch

Table 4.2. Specific rates of cell mass generation from bacteria immobilized on various cloths.

Bacterium	Specific rate (10^{-4} absorbance units/h/cm ² cloth ^a)			
	Polyester	Acetate ^b	Triacetate ^b	PEI-cotton Cotton
<u>B. subtilis</u>	0.6	0.4	0.3	1.2
<u>B. ammoniagenes</u>	0.3	0.4	0.3	0.8
<u>C. glutamicum</u>	3.0	0.1	0.2	0.7
<u>E. coli</u> Crooks	0.0	0.2	0.0	2.6
<u>E. coli</u> HB101	0.3	0.4	0.4	3.2
<u>E. coli</u> B41	0.1	0.2	0.2	3.7
				3.6

^a Bacteria were immobilized on various cloths as described in Methods. After 3 days growth on wet cloths, the cloths were shaken in growth medium which was changed daily. After 16 days of liquid culturing, the specific rates were determined as described in Methods.

^b Cellulose acetate or triacetate cloth.

fermentations. The resident inoculum method would be particularly suitable for continuous production of pathogenic bacteria (for preparation of vaccines and diagnostic antigens) as it eliminates a possible risk in preparing an inoculum culture.

I have presented an equation (Appendix) for the rate of cell mass generation which can be used to calculate the area of film required to obtain a desired cell mass in a given fermentation time. Since the films showed stable and constant rates of cell mass generation, the rate and extent of fermentation can be reproducibly controlled by varying the area of films.

APPENDIX

Rate of cell mass generation

At time 0, a bacterial film is suspended in growth medium (containing no cells). The film generates free cells at R (absorbance units/h). Free cells generated from the film double at a doubling time of D h. After time t h, absorbance of the culture is taken. Since the absorbance of the culture is proportional to cell mass density, absorbance $\times$ volume (l) of the culture (absorbance unit in l) measures cell mass present in the culture.

The rate (R) of cell mass generation is

$$R = \frac{aM}{e^{at} - 1}$$

where $a = 0.693/D$; M = cell mass (absorbance units in l) in the fermentor; t = fermentation time in h.

If the area of the film is known, the specific rate (R_s) of cell mass generation (absorbance units/h/cm²) can be calculated. If cell mass M is desired in the fermentor after time t , the area of the film required in the fermentor is R/R_s cm².

Example

If one liter of E. coli Crooks ($D = 1$ h) culture having A_{500} of 6 is desired after 8 h fermentation, $M = 6$ absorbance units and $R = 1.63 \times 10^{-2}$ absorbance units/h. Since R_s of the E. coli Crooks film is 3.5×10^{-4} absorbance units/h/cm² (Table 4.2), the fermentor

should contain $R/R_s = 47 \text{ cm}^2$ of the film. Since 1 absorbance unit of this bacterium corresponds to approx. 5×10^{11} cells, 1 l of this culture contains approx. 3×10^{12} cells. If fermentation is repeated 3 times a day, this 1 l fermentor will yield approx. 10^{13} cells per day.

CHAPTER 5
RESISTANCE OF BACTERIAL FILM TO CONTAMINATION DURING USE AS
INOCULUM IN REPEATED BATCH FERMENTATION

6

ABSTRACT

An Escherichia coli strain constitutive for β -galactosidase was immobilized onto cotton cloth. The resulting E.coli film was used as a resident inoculum in repeated batch fermentations for 30 days in the presence of Brevibacterium ammoniagenes added as a contaminant. Analysis of β -galactosidase production shows that contamination did not decrease the capacity of the film to generate E.coli cells or the E.coli population on the film.

INTRODUCTION

In the Chapter 4, I have shown that bacteria immobilized on cotton cloth (bacterial film) can generate free cells at stable and significant rates and that the film, when retained in a fermentor, can be used as resident inoculum in repeated batch fermentation. In commercial fermentation, complete sterilization of media or fermentor is often difficult or impractical and contamination may result. If continuation of fermentation depends on recycled liquid culture as inoculum, there is no mechanism to reduce introduced contamination and the fermentation may eventually have to be discontinued. In batch fermentation which uses bacterial film as inoculum, most of the contaminants in the culture will be removed along with fermented culture

at the end of fermentation. The bacterial film can be reused as inoculum for the next fermentation provided that the contaminants do not replace the original immobilized bacteria on the film. This possibility was examined in the present study.

MATERIALS AND METHODS

Microorganisms and growth conditions

Escherichia coli 3300 was obtained from the E. coli Genetic Stock Center, Yale university and is constitutive for β -galactosidase due to the lacI mutation (Pardee et al., 1959). Brevibacterium ammoniagenes ATCC 6872 is a wild type.

Bacteria were grown at 37°C with shaking at 240 rpm in M63 medium (Miller, 1972) supplemented with 0.5% glucose and 0.1% yeast extract. Growth was followed by measuring absorbance at 500 nm (A_{500}). The doubling times of the bacteria in this medium were 42 min for E. coli and 65 min for B. ammoniagenes.

Preparation of E.coli film

E. coli 3300 was allowed to grow on cotton cloth (flannel) and the resulting cloth (E. coli film) was shaken in medium (with daily changes) as described in Chapter 4. After 11 days the film was found to generate E. coli cells at a maximal rate of 1.5×10^8 cells/h cm^2 .

β -Galactosidase assay

One ml of a sample (culture, film suspension or medium blank) was mixed with one drop of toluene and shaken at 320 rpm and 30°C. After 30 min, 2 ml of 3 mM O-nitrophenyl β -galactopyranoside solution in 0.05 M sodium phosphate (pH 7.2) was mixed with the toluenized sample. After 5 min incubation at 30°C, 2 ml of the mixture was mixed with 1 ml of 1 M Na₂CO₃. Absorbance at 420 nm was measured against the blank. This was used to calculate the amount (unit) of the enzyme in one ml of the sample. One unit of the enzyme which catalyzes the hydrolysis of 10⁻⁶ mole of the substrate per min.

RESULTS

In Chapter 4, I have shown that the capacity of a bacterial film to generate free cell increases with time of incubation in medium. The times required to yield the maximal cell generation rate range from 5 days for Brevibacterium ammoniagenes to 10 days for an E. coli strain. This suggests that immobilization onto cotton cloth depends on bacterial capacity to generate cells having a high affinity toward cellulose ('cellulophilic cells'). B. ammoniagenes generates such cells more efficiently than E. coli. Suppose that immobilized E. coli cells occupy all the binding sites on cotton cloth

and the resulting film is used as a resident inoculum in the presence of a contaminant, B. ammoniagenes. Although immobilized bacteria could not be released even under vigorous mixing (Chapter 4), it is possible that during growth in liquid medium immobilized bacteria may detach from the film. If a significant detachment occurs during fermentation, cellulophilic B. ammoniagenes will attach to the vacated sites and the E. coli population in the film will decline, thus reducing the film's capacity as an E. coli inoculum.

Here, susceptibility of a bacterial film to contamination was examined by using E. coli 3300 (which constitutively produces β -galactosidase) and B. ammoniagenes (which does not produce β -galactosidase in the medium used). E. coli 3300 was allowed to fully develop an immobilized population on cotton cloth until the resulting film generated free cells at the maximum and the stable rate as described in Chapter 4. Segments of the E. coli film were shaken in medium in the absence and presence of B. ammoniagenes cells which were added as contaminants at two concentrations. After 1 day, the specific activities of β -galactosidase in free cells in the contaminated cultures were compared with that in the uncontaminated cultures to measure the degree of contamination. Table 5.1 shows that the addition of 10^{10} B. ammoniagenes cells to 50 ml of the E. coli film culture resulted in a significant decrease in the

Table 5.1 B.ammoniagenes contamination of E.coli film cultures.^a

<u>No. of</u>	<u>B. ammoniagenes</u>	<u>Specific activity of</u>
<u>cells</u>		<u>β-galactosidase</u>

0	0.58 ± 0.02
10^8	0.58 ± 0.02
10^{10}	0.29 ± 0.02

^a To each of 12 flasks were added 50 ml of medium and 2 segments ($1 \text{ cm}^2/\text{segment}$) of the E.coli film (which generated $1.5 \cdot 10^8$ cells/ h/cm^2). Three sets of 4 flasks were contaminated by B.ammoniagenes cells at 3 levels: no cells; 10^8 cells/flask; and 10^{10} cells per flask. After 1 day shaking, the degree of contamination was determined by measuring the specific activity of β -galactosidase (due to E.coli) in free cells in the cultures. The films were rinsed with medium and resuspended in fresh medium and the above experiment was repeated. The data are average (with standard deviation) of 8 determinations. The specific activity (units/absorbance unit) of β -galactosidase in the cells was calculated by dividing the amount of the enzyme per ml of culture by A_{500} of the culture.

specific activity of β -galactosidase in 1/day culture, indicating effective contamination by B. ammoniagenes.

To study the effect of extended contamination on the inoculum capacity of the E. coli film, segments of the E. coli film were shaken in medium in the absence and the presence of 10^{10} B. ammoniagenes cells per 50 ml. Every 24 h, the whole culture (grown to stationary phase) was withdrawn and replaced with uncontaminated or contaminated medium, respectively. This batch fermentation was repeated 30 times (over 30 days). Table 5.2 shows that the specific activity of β -galactosidase in cells generated by the film was not affected by the prolonged contamination. This means that the bacterial cells generated by the film largely represent β -galactosidase producing E. coli. Since the rate of cell generation of the film remained the same throughout, contamination did not decrease the capacity of the film to generate E. coli cells and produce β -galactosidase. This suggests that contamination did not affect the E. coli population on the film. Table 5.3 shows that contamination did not affect the β -galactosidase activity per unit area of the film, confirming the above conclusion.

DISCUSSION

I have shown that contamination does not decrease the capacity of bacterial film as a resident inoculum

Table 5.2 Effects of B. ammoniagenes contamination on the specific activity of β -galactosidase in cells generated from the E.coli film.^a

No. of <u>B.ammoniagenes</u> cells added per flask	Specific activity of β galactosidase	
	1st day	30th day
0	0.58 $\pm$ 0.02	0.58 $\pm$ 0.02
10^{10}	0.58 $\pm$ 0.02	0.58 $\pm$ 0.02

^a At 0-time, the E.coli film segments were suspended in medium with or without 10^{10} B.ammoniagenes cells per flask and shaken as described in Table 5.1. The films were transferred daily to fresh medium (with or without the contaminants), and shaken. On the 1st and 30th day the films were rinsed with medium, resuspended in 5 ml of medium (without the contaminant) and shaken for 2 h. The specific activity of β -galactosidase in free cells in the 2 h culture, was determined as in Table 5.1. The data are average of 8 determinations. The rate of cell generation from the film was not affected by contamination or by 30 cycles of batch fermentation.

Table 5.3 Effects of B.ammoniagenes contamination on the E.coli population on the film.^a

No. of <u>B.ammoniagenes</u> cells added per flask	Specific activity of β -galactosidase on the film after 30 days
--	---

0	0.21 $\pm$ 0.01
---	-----------------

10^{10}	0.21 $\pm$ 0.01
-----------	-----------------

a The E.coli film segments were suspended in medium with or without 10^{10} contaminant cells per flask and shaken as described in Table 5.1. The films were transferred daily to fresh medium (with or without contaminants), and shaken. After 30 days, the film segments (1 cm²) were suspended in 1 ml of medium and β -galactosidase activity was measured as described in Methods. The specific activity (units/cm²) of β -galactosidase in the film was calculated by dividing the amount of enzyme by the area of the film. This was taken as measure of the E.coli population on the film. The data are average of 8 determinations.

for repeated batch fermentation. The present data suggest that cells, once immobilized onto cotton cloth, do not detach even after prolonged use as inoculum and thus contaminants can not replace immobilized cells. Batch fermentations which use bacterial films as a resident inoculum are therefore less susceptible to contamination problems since most of the contaminants will be flushed from the fermentor and the films retained in the fermentor can generate the desired cells at an unchanged rate in subsequent batch fermentations.

CHAPTER 6
REPEATED BATCH ANTIBIOTIC FERMENTATION BY STREPTOMYCETES
IMMOBILIZED ON COTTON CLOTH

ABSTRACT

Streptomyces cattleya and Streptomyces clavuligerus were found to form immobilized populations on cotton cloth. The resulting bacterial films were used as resident inocula in repeated batch fermentation of their secondary metabolites: thienamycin and cephalosporin C. The maximal levels of antibiotics produced, remained constant during 20 cycles of 3-day batch fermentations.

INTRODUCTION

In Chapter 4, I have shown that bacteria passively immobilized on cotton cloth (bacterial film) can generate free cells at significant and stable rates. By retaining the bacterial films as resident inocula in a fermentor, I was able to repeat batch fermentation to produce bacterial cells. The present study examines the production of bacterial secondary metabolites by using models, such as, the production of thienamycin by Streptomyces cattleya and cephalosporin C by Streptomyces clavuligerus. Thienamycin is a broad spectrum β lactam antibiotic under development which shows a high resistance to β -lactamase (Kahan et al., 1979) Cephalosporin C has been used as a starting material of semi-synthetic cephalosporin derivatives. The present chapter describes use of Streptomyces films as resident inocula in repeated batch fermentation of

these antibiotics.

MATERIALS AND METHODS

Microorganisms and growth conditions

Strptomyces cattleya (ATCC 39203) and Streptomyces clavuligerus (NRRL 3585) were maintained on yeast malt extract (YME) agar (Shirling and Gottlieb, 1966). They were grown by shaking at 30°C and 280 rpm in a medium (production medium described by Monaghan and Currie, 1984) which contained per 1 l 10 g glycerol, 15 g cornsteep liquor, 10 g cottonseed meal, 15 g distiller's soluble, 0.01% $\text{CoCl}_2 \cdot \text{H}_2\text{O}$, 3 g CaCO_3 and NaOH to obtain a final pH of 7.3.

Cloth

Cotton cloth (flannel) was locally obtained, and fortified by immersing in 5% cellulose acetate in acetone and air-drying.

Immobilization

The spore (or mycelia) suspension in YME (absorbance at 500 nm of about 1) was diluted 10 times with medium and mixed with segments (1 cm square) of fortified or unfortified cloth. After 1 h, excess liquid was drained and the spores were allowed to germinate on wet cotton cloth at 30°C. After 24 h, the segments were rinsed with fresh medium and incubated for 24 h, after which this was repeated once more. The

segments were then shaken in fresh medium overnight and the resulting Streptomyces films were used as resident inocula.

Antibiotic production

Each Streptomyces film (1 cm square) was shaken in 2 ml of fresh medium for 3 days. Daily, a 1 ml culture was removed in duplicate for antibiotic assay. After 3 days, the film was washed 5 times by vortexing in 3 ml of medium followed by decantation. The washed film was shaken in 2 ml of fresh medium to start the next batch fermentation.

Antibiotic concentration was measured by standard inhibition zone assay. Paper disks (Schleicher and Schuell, 1/4 inch diameter) were soaked with appropriately diluted culture and laid on .7% agar in Penassay broth containing 10^7 cells/ml of Escherichia coli Ess which is supersensitive to lactam antibiotics (Aharnowitz and Demain, 1979). N-Formimidoyl thienamycin (Merck Sharp and Dohme) and cephalosporin C (Sigma) were used as standards for estimation of antibiotic concentrations. Standard graphs correlating antibiotic concentrations ($\mu\text{g/ml}$) to diameters of zone inhibition (mm) were constructed (Fig. 6.1) (Fig. 6.2).

Fig. 6.1 Standard curve for thienamycin assay. A paper disk (1/4 inch diameter) was dipped in each standard N formimidoyl thienamycin solution, and used for inhibition zone assay as described in Methods. Thienamycin refers to the concentration of standard; (ug/ml) and zone diameter, to the diameter (mm) of the inhibition zone.

Fig. 6.2 ~~Standard~~ curve for cephalosporin C assay. A paper disk (1/4 inch diameter) was dipped in each standard cephalosporin C solution and used for inhibition zone assay as described in Methods. Cephalosporin refers to the concentration of standard; ($\mu\text{g/ml}$) and zone diameter, to the diameter (mm) of the inhibition zone.

RESULTS

First, I studied batch antibiotic fermentation by using liquid cultures as inocula. Fig. 6.3A shows kinetics of accumulation of thienamycin by St.cattleya and cephalosporin C by St.clavuligerus when fermentation was initiated by diluting stationary phase cultures 4 times with fresh medium. After 3 days, antibiotic concentrations reached maximal values: 14 mg/l for thienamycin and 100 mg/l for cephalosporin C. It has been reported that using the same medium, a different strain of St. cattleya produces 4 mg/l of thienamycin (Kahan et al., 1979) and the same strain of St. clavuligerus produces 139 mg/l of cephalosporin C using a different medium (Aharnowitz and Demain, 1978).

Batch fermentation can be repeated by using a part of the fermented culture as an inoculum if it contains sufficient viable cells. However, it has a disadvantage in that significant amounts of the desired metabolites will be sacrificed since a large inoculum is generally required to complete antibiotic production within a short time. Furthermore, toxic substances may accumulate and reduce cell viability or antibiotic production. The above batch fermentation of thienamycin and cephalosporin C were repeated every 3 days for a total of 20 times by diluting the fermented culture 4 times with fresh medium, and antibiotic concentrations

Fig.6.3 Kinetics of accumulation of thienamycin by Streptomyces cattleya and cephalosporin C by Streptomyces clavuligerus. In part A, fermentation was started by diluting the stationary cultures 4 times with fresh medium. In part B, washed Streptomyces films (on cotton cloth) were used to start fermentation as described in Methods. The concentrations (mg/l) of thienamycin (○) and cephalosporin C (●) are plotted against fermentation time.

were measured on the 3rd day. Although the thienamycin level remained the same throughout the repeated batch fermentation, cephalosporin C level gradually declined to 50% of the original value after 20 cycles (Fig.6.4A).

Batch fermentation by *Streptomyces* films

In the Chapter 4, I have shown that bacteria form immobilized populations on cotton cloth (bacterial films) when grown on cotton cloth daily rinsed with fresh medium for 3 days. Using this method I was able to obtain the films of both *St.cattleya* and *St.clavuligerus*. Since the medium used is turbid, the optical determination of the rate of generation of mycelia, from other films was not possible. When a 1 cm square segment of *Streptomyces* film was shaken in 2 ml of medium, viscosity and turbidity visibly increased after a day; and antibiotics accumulated maximal values (14 mg/l thienamycin and 100 mg/l cephalosporin.C) after 3 days (Fig.6.3B) just as in batch fermentation by liquid inocula (Fig. 6.3A). At the end of batch fermentation, the films were removed from the culture, washed and resuspended in fresh medium to start the next batch fermentation. In the following batch fermentation, maximal antibiotic concentrations were always reached after 3 days and the maximal level remained the same throughout 20 cycles (60 days) of

Fig 6.4 Maximal levels of antibiotics accumulated in repeated batch fermentations. In part A, fermentation was repeated by diluting the fermented cultures 4 times with fresh medium. In part B, fermentation was repeated by using Streptomyces films (on cotton cloth) as resident inocula. The maximum levels (mg/l) of thienamycin (O) and cephalosporin C (●) accumulated at each cycle are shown.

repeated batch fermentation (Fig.6.4B).

Fortification of cloth by cellulose acetate

In the above fermentations, shaking was used as means of aeration and agitation. Commercial antibiotic fermentations are commonly performed in a stirred tank. In stirred tank as described in Chapter 3, problems of loosening of thread and entanglement with each other occur. Therefore, fortified cotton cloth segments (see Methods) were used and such segments were found to immobilize St. cattleya and St. clavuligerus. The resulting bacterial films were used as resident inocula as before. The kinetics and the level of antibiotic accumulation were identical to those of the bacterial films on unfortified cloth (Fig. 6.3B). The antibiotic productivity remained the same as those shown in (Fig.6.4B) throughout 20 cycles (60 days) of repeated batch fermentations.

Nonwoven cloth of polypropylene does not require the above fortification and therefore it was examined for immobilization of Streptomyces for antibiotic production. However, Streptomyces polypropylene cloth produced the same maximal level of antibiotic only after 5 day incubation (instead of 3 days in the cotton cloth).

DISCUSSION

I have shown / that streptomycetes can form immobilized populations on cotton cloth and the resulting films provide stable resident inocula in repeated batch fermentations of their secondary metabolites, antibiotics. Since this process eliminates the need for preparation of a seed culture or use of part of the culture, it economises batch antibiotic fermentation. If a fermentation tank is equipped with the screen to retain the films, removal of fermented culture and repetition of batch fermentation can be readily automated. I have also found that fortification of cotton cloth with cellulose acetate permits their use in a stirred tank.

Thus, use of bacterial films as resident inocula facilitates continuous removal of extracellular metabolites as well as free cells.

CHAPTER 7
STABILITY OF pBR322 PLASMID IN IMMOBILIZED ESCHERICHIA COLI
CELLS USED AS RESIDENT INOCULUM

ABSTRACT

E.coli cells harbouring plasmid pBR322 which confers ampicillin resistance were immobilized on cotton cloth. The resulting film was used as an inoculum in daily repeated batch culture in ampicillin-free medium. During two months, the film was able to produce cultures which showed at the late log phase little sensitivity to 10 mg/ml ampicillin. Therefore, the film can effectively be used as a resident inoculum in repeated batch production of recombinant DNA products by means of pBR322 or its derivatives as cloning vectors.

INTRODUCTION

I have previously shown that bacteria immobilized on cotton cloth (bacterial film) can generate free cells at stable and significant rates and that film segments suspended in a fermentor can be used as resident inocula for repeated batch fermentation. This type of repeated batch fermentation is less susceptible to contamination than one repeated by dilution of the previous day culture, since most contaminants will be removed with the culture at the end of the fermentation and even a heavy contamination does not contaminate or decrease the capacity of the film to generate the desired cells (Chapter 5). Its commercial potential has been demonstrated by repeated batch production of β -lactam antibiotics by the films of Streptomyces

species (Chapter 6). The method should be applicable to production of recombinant DNA products if the immobilized bacterial host can stably maintain cloning vector DNA such as plasmids. Most vector plasmids specify resistance to drugs such as antibiotics. Although a plasmid can be stably maintained in the host grown in medium containing the drug, a commercial process prefers drug-free culturing for economic reasons. It has not been shown how stably bacteria immobilized on cotton cloth can maintain a vector plasmid when the bacterial film is used as a resident inoculum in the drug-free medium. The present study describes the stability of plasmid pBR322 in Escherichia coli immobilized on cotton cloth. pBR322 and its derivatives are popular plasmids for cloning recombinant DNA because they are multicopied and possess suitable restriction endonuclease sites in ampicillin and tetracyclin resistance genes (Bolivar et al., 1977).

MATERIALS AND METHODS

Bacteria and growth conditions

Escherichia coli WA802 (met) was used as a host of the plasmid pBR322 (Bolivar et al., 1977) E.coli WA802 (here referred to as E.coli) and E.coli WA802 harbouring pBR322 (E.coli/pBR322) were obtained from Dr. Robert Watson, Agriculture Canada, Ottawa.

Bacteria were grown by shaking at 37°C and 240 rpm

in M63 basal medium (Miller, 1972) supplemented with .1% yeast extract and .5% glucose. Growth was followed by measuring absorbance at 500 nm (A_{500}) of cultures.

Preparation of the E.coli/pBR322 film

E.coli/pBR322 was grown in medium containing 50 μ g/ml ampicillin (ampicillin medium) to stationary phase (A_{500} of 3.5). The culture was diluted 1000 times with ampicillin medium and mixed with segments (1 cm square) of cotton cloth (flannel). After 1 h, excess liquid was removed and bacteria were allowed to grow on the wet cloth. Every 24 h, the cloth segments were rinsed with ampicillin medium. After 3 days, the segments were shaken in ampicillin medium which was changed daily. After 8 days, the resulting E.coli film generated cells at the maximum rate.

RESULTS

pBR322 contains the β -lactamase genes and E.coli harbouring pBR322 (E.coli/pBR322) is resistant to ampicillin. Growth of E.coli/pBR322 in the presence of ampicillin ensures maintenance of pBR322 where as its growth in the absence of ampicillin has been shown to generate cells which fail to grow on an ampicillin plate suggesting a decrease in plasmid number in the host (Wouters et al., 1980). In order to study how stably E.coli can maintain pBR322 in ampicillin-free medium, a simple measure of pBR322 content in the host was

desired. Fig 7.1 shows that 50 µg/ml ampicillin was sufficient to lyse E.coli cells without pBR322 and that E.coli/pBR322 cells grown in the presence of 50 µg/ml ampicillin exhibited total resistance to 10 mg/ml ampicillin in liquid culture. However, E.coli/pBR322 when cultured in the absence of ampicillin for longer than 21 days, exhibited a growth lag when exposed to 10 mg/ml ampicillin, though they were still totally resistant to 50 µg/ml (Fig.7.2, Table 7.1). Fig.7.2 illustrates the growth lags caused by 10 mg/ml ampicillin in 30 and 64 day cultures grown in ampicillin free medium. Table 7.1 shows that the lag time increased with duration of ampicillin-free growth. This trend suggests that the lag is related to a decrease in the number of plasmids, although the exact mechanism of lag formation is not known. Growth after the lag showed the same doubling time (48 min) as that shown by the untreated culture (Fig.7.2). Growth resumption suggests that during the lag plasmids continue to replicate to a level which generates a sufficient amount of β -lactamase so as to destroy all incoming ampicillin molecules and resume cell division. A cell having a fewer number of plasmids would then take a longer time to resume cell division. Thus, lag time was taken as a measure of plasmid content in the following experiment.

E.coli/pBR322 was immobilized on cotton cloth as described in Chapter 4. Medium containing 50 µg/ml

Fig. 7.1 Effect of ampicillin on growth of E.coli and E.coli/pBR322. Log phase cultures (A_{500} of 0.2) of E.coli and E.coli/pBR322 were prepared by growing E.coli in the absence of ampicillin and E.coli/pBR322 in the presence of 50 $\mu\text{g/ml}$ ampicillin for 20 doublings. At 0-time, ampicillin was added to E.coli ($\square$) at 50 $\mu\text{g/ml}$ and to E.coli/pBR322 ($\circ$) at 0; 50 $\mu\text{g/ml}$ and 10 mg/ml . A_{500} was followed. Growth of E.coli/pBR322 ($\circ$) at these 3 ampicillin concentrations was indistinguishable.

Table 7.1 Lag caused by 10 mg/ml ampicillin in E.coli/pBR322 cultures grown by daily dilution^a and from the film^b in ampicillin-free medium.

Growth lag (minutes) in cultures				
Days (cycles) of batch culture	Daily diluted ^a	Film generated ^b		
		20 min	3 hour	
7	0	0	0	
14	0	0	0	
21	48	0	0	
30	75	45	0	
37	135	60	0	
42	165	102	0	
50	192	144	0	
57	236	176	20	
64	288	195	40	

^a One day batch culture was repeated 64 times by daily dilution of the previous day culture with ampicillin-free medium and effect of 10 mg/ml ampicillin was examined weekly as described in Fig.7.2.

^b One day batch culture was repeated 64 times by daily transfer of the E.coli/pBR322 film to ampicillin-free medium, and effect of 10 mg/ml ampicillin was examined weekly in cultures generated from the film during the first 20 min and after 3 h shaking as described in Fig. 7.3.

Fig. 7.2. Effect of ampicillin-free growth of E.coli/pBR322 on sensitivity to ampicillin. E.coli/pBR322 was grown in the presence of 50 $\mu\text{g/ml}$ ampicillin for 20 doublings, and the culture was diluted 1000 fold with ampicillin-free medium. After 24 h (first day) and every 24 h thereafter, the grown culture was similarly diluted with ampicillin-free medium and used as an inoculum for subsequent batch culture. On the 30th and 64th day, a part of log phase culture (3 h after dilution) was adjusted to A_{500} of 0.2, exposed to three concentrations of ampicillin at 0, 50 $\mu\text{g/ml}$ and 10 mg/ml , and growth (A_{500}) was measured every hour. Symbols: $\bullet$, growth after adding 0, 50 $\mu\text{g/ml}$ ampicillin to 30th and 64th day cultures (data was indistinguishable); Δ , after adding 10 mg/ml ampicillin to 30th day culture; $\blacktriangle$, after adding 10 mg/ml ampicillin to 64th' day cultures. The arrows indicate the lag periods determined by extrapolation.

ampicillin was used during immobilization. The resulting E.coli film generated cells at the rate of 1.4×10^8 cells/h/cm², and was used as an inoculum in batch culture in the absence of ampicillin. By daily transferring to ampicillin-free medium, 64 cycles of batch cultures were carried out over 64 days. At weekly intervals, at 20 min and 3 h after transferring to ampicillin-free medium, cultures were withdrawn and exposed to ampicillin. Fig. 7.3 illustrates the effects of ampicillin addition to such cultures generated from the film which had been used as an inoculum for 30 days and 64 days. While 50 µg/ml ampicillin caused no effect on growth, 10 mg/ml ampicillin caused a growth lag in some of these cultures. Table 7.1 lists the times of lag caused by 10 mg/ml ampicillin in cultures generated from the films (used as an inoculum) over 64 days. The cells generated from the films during the first 20 min shaking would closely reflect pBR322 maintained in the E.coli immobilized on cotton cloth. The lag time in the 20 min culture increased with days of batch culture in which the film was continuously used as an inoculum. However, the lag increase was significantly slower than in cultures continued by daily dilution of previous day cultures (Table 7.1). This suggests that pBR322 is more stably maintained in immobilized cells (in the film) than in free cells grown by daily dilution. However, it is more relevant to assess the pBR322

Fig. 7.3. Ampicillin sensitivity of cells generated from the E.coli/pBR322 film. The E.coli/pBR322 film was prepared as described in Methods and used as resident inoculum to generate cells in ampicillin-free medium. Every 24 h, the film was rinsed and transferred to ampicillin-free medium. This film inoculated batch culture was repeated 64 cycles (64 days). On the 30th and 64th day, the film segment (1 cm²) was first shaken in fresh medium for 2 h and then transferred to 1.0 ml fresh medium. After 20 min shaking, a portion of the culture liquid (20 min culture) was removed, mixed with 2 volumes of fresh medium, and assayed for ampicillin sensitivity. After 3 h, the second portion of the culture liquid (3 h culture) was removed, mixed with 50 volumes of fresh medium, and assayed for ampicillin sensitivity. Ampicillin was added at 0, 50 µg/ml and 10 mg/ml to the 20 min culture (Part A) and the 3 h culture (Part B), and growth (A₅₀₀) was followed. Symbols: ●, growth after adding 0 and 50 µg/ml ampicillin to the 20 min cultures from the films on the 30th and 64th day (data was indistinguishable); △, after adding 10 mg/ml to the 20 min culture from the film on 30th day; ▲, after adding 10 mg/ml to the 20 min culture from the film on the 64th day; ■, after adding 0 and 50 µg/ml to the 3 h culture from the films on the 30th and 64th day (data was indistinguishable); □, after adding 10 mg/ml to the 3 h culture from the film on the 30th day; ○, after adding 10 mg/ml to the 3 h culture from the film on the 64th day. The arrows indicate the lag periods determined by extrapolation.

content in near stationary phase cultures generated from the film since such cultures will likely be used for production of recombinant DNA or its products. The cultures generated from the film during 3 h growth were in late log phase as A_{500} was about 3 (3.5 at the stationary phase). Table 7.1 shows that when exposed to 10 mg/ml ampicillin, the 3 h culture from the film exhibited no lag until the film was used for two months. This suggests that cells divided from the immobilized cells, increase their plasmid content to the level which provides total resistance to 10 mg/ml ampicillin.

To directly assess the population of pBR322 carrying cells in the film, the film used as inoculum for 64 days was shaken in medium containing 50 μ g/ml ampicillin. The E.coli/pBR322 film generated cells in ampicillin medium at the same rate, (1.4×10^8 cells/h/cm²) as in ampicillin-free medium. This capacity remained unchanged even after 16 h treatment. In contrast, the control film of E.coli harbouring no plasmid did not generate any cells in the ampicillin medium, and the film, after 16 h treatment, generated no cells when resuspended in ampicillin-free medium. Thus, most of the immobilized E.coli/pBR322 cells maintained pBR322 to the level which provides total resistance to 50 μ g/ml ampicillin.

DISCUSSION

I have shown that the E.coli/pBR322 cells immobilized on cotton cloth (film) more stably maintain pBR322 plasmid than in cultures continued by daily dilution. Since cells generated from the immobilized cells appear to gain full plasmid content during 3 h growth before stationary phase is reached, the immobilized cells can effectively be used as a resident inoculum in repeated batch production of recombinant DNA (or its products) by means of pBR322 and its derivatives as cloning vectors.

Cultures repeated more than 21 times by daily dilution, even after 3 h growth (after dilution of the previously grown stationary phase culture) showed increasing lag upon exposure to 10 mg/ml ampicillin (Fig.7.2 and Table 7.1). This suggests that the stationary phase cells must have greatly reduced plasmid content.

CONCLUSION

Fossil carbons are nonrenewable and will deplete sooner or later. Although biomass is a renewable source, its production is limited by the availability of space as well as solar energy. Therefore, efficient and effective production and utilization of biomass is becoming important, and biotechnological investigation must continue. Biotechnology has already revolutionized our daily lives by providing substances of practical importance and economic opportunities to many nations. In light of existing competition abroad, Canada has three major strategies to consider: (1) development of new and effective processes; (2) development of processes based on resources uniquely and abundantly available in Canada and (3) improving competitiveness of existing biotechnological industries in Canada. My thesis is largely concerned with the first strategy.

Biological catalysts are ideally suitable for transformation of biomass and their immobilization permits repeated and controlled use. In fact, many commercial processes depend on immobilized cell systems most of which are based on entrapment. However, gel instability and diffusional resistance are major drawbacks of the gel systems. I have developed a new cellulose acetate entrapment system which is stable because of cotton cloth backbone; relatively inexpensive; amenable to scale-up; permits high flow rate (in column operation); and exhibits a superior catalytic performance which is likely ascribed to large

surface area and porosity of the system (Chapter 1).

Microbial cells are commonly used as catalysts of biomass transformation. Cell adsorption on film material is rarely used in such a process. It has two major potential advantages. Since the adsorbed cells are exposed to environment, transport of substrate and products are less subject to diffusional resistance than gel entrapment system. Secondly, if immobilized cell can reproduce, the new cell can be added as catalysts to the reaction mixture. I have found that cotton cloth is a good adsorbent of yeasts and bacteria. Adsorbed yeast cells develop immobilized populations on cotton cloth which produce free yeast cells. Both immobilized and free cells produce ethanol. Since immobilized cells are directly exposed to medium, ethanol production by the immobilized cells is readily enhanced by circulation of media (Chapter 2 and 3). Thus, I have demonstrated the above mentioned advantages.

Commercial batch fermentations normally require the preparation of seed culture by 2 to 3 stages of volume scale-up. I have developed ' resident inoculum ' batch fermentation which eliminated seed culture preparation. I have shown the applications of this method to produce the primary metabolites (assessed by cell mass production) (Chapter 4) secondary metabolites (assessed by antibiotic production) (Chapter 6) and production of recombinant DNA products (by production of plasmid containing cells) (Chapter 7).

Commercial fermentation is subject to contamination. In batch fermentation, which uses bacterial films as the inocula, most of the contaminants will be removed along with the fermented cultures. I have shown that contaminants do not replace the immobilized cells, and thus do not affect the capacity of the film as a resident inoculum (Chapter 5). Therefore the resident inoculum batch fermentation is resistant to contamination.

Batch fermentation can be repeated by using a part of the fermented culture as an inoculum if it contains sufficient viable cells. However, the desired metabolite will have to be sacrificed. Furthermore, toxic substances may accumulate and reduce cell viability or metabolite production. In fact, continued cephalosporin production by such a method suffer gradual decline of the antibiotic production (Chapter 6). Cells in the fermented culture can be harvested, resuspended and reused as an inoculum. However, such a cell cycling is costly and difficult to automate. If a fermentor tank is equipped with a screen at the bottom, to retain the bacterial film, then removal of fermented culture and repetition of batch fermentation can be readily automated.

Thus my thesis research has led to the development of new approaches for applications of cells in biotechnological processes.

REFERENCES

Aharonowitz, Y. and Demain, A.L. (1979) Nitrogen nutrition and regulation of cephalosporin production in Streptomyces clavuligerus. Can. J. Microbiol. 25, 61-67

Armstrong, G.D. and Yamazaki, H. (1977) Isolation and characterization of catabolite repression-resistant mutants of Escherichia coli. Can. J. Microbiol. 23, 1384-1391.

Black, G.M., Webb, C., Matthews, T.M., and Atkinson, B. (1984) Practical reactor systems for yeast cell immobilization using biomass support particles. Biotechnol. Bioeng. 26, 134-141.

Bolivar, F., Rodriguez, L.R., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Construction and characterization of new cloning vehicles II A multipurpose cloning system. Gene 2, 95-113.

Chibata, I., and Tosa, T. (1977) Transformations of organic compounds by immobilized microbial cells. Adv. Appl. Microbiol. 22, 1-27.

Chibata, I., Tosa, T., and Sato, T. (1974) Immobilized aspartase containing microbial cells: Preparation and Enzymic properties. Applied Microbiology. 27, 878-885.

Daniels, S.L. (1972) The adsorption of microorganisms onto solid surfaces. Developments in Industrial Microbiology. 13, 211-256.

Dinelli, D., Marconi, W., and Morisi, F. (1975) Fiber entrapped enzymes In: Immobilized Enzymes, Antigens, Antibodies and Peptides. H.H. Weetall, ed., 1, pp 173-243 New York, Marcel Dekker Inc.

Durand, G., and Navarro, J.M. (1978) Immobilized microbial cells. Process Biochemistry. 13, 14-23.

Duvnjak, Z., Kosaric, N., and Hayes, R.D. (1981) Kinetics of ethanol production from Jerusalem artichoke juice with some Kluyveromyces species. Biotechnol. Letters 3, 589-594.

Fraser, A.D.E., and Yamazaki, H. (1978) Construction of an Escherichia coli strain which excretes abnormally large amounts of adenosine 3',5'-cyclic monophosphate. Can. J. Microbiol. 24, 1423-1425.

Ghommich, C., Navarro, J.M., and Durand, G. (1981) Acetic acid production by immobilized Acetobacter cells. Biotechnol. Letters 3, 93-98.

Guiraud, J.P., Daurelles, J., and Galzy, P. (1981) Alcohol production from Jerusalem artichoke using yeasts with inulinase activity. Biotechnol. Bioeng. 23, 1461-1465.

Hattori, T., and Furusaka, C. (1972) Growth of bacteria on the surface of anion exchange resin. *J. Biochem.* 8, 272-283.

Jhonson, D.E., and Ciegler, A. (1969) Substrate conversion by fungal spores entrapped in solid matrices. *Arch. Biochem. Biophys.* 130, 384-388.

Kahan, J.S., Kahan, F.M., Goegelman, R., Currie, S.A., Jackson, M., Stapley, E.O., Miller, T.W., Miller, A.K., Hendlin, D., Mochales, S., Hernandez, S., Woodruff, H.B., and Birnbaum, J. (1979) Thienamycin, a new B-Lactam antibiotic. I. Discovery, Taxonomy, Isolation, and Physical properties. *J. Antibiot.* 32, 1-12.

Kennedy, J.F. (1985) Principles of immobilization of enzymes. In: *Handbook of Enzyme Biotechnology*. A.L. Wiseman, ed., pp 96-98 New York, John Wiley and Sons.

Kyowa Hakko Kogyo (1983) Brouchure Immobilization of yeast for ethanol fermentation.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) In: *Molecular cloning*. Cold Spring Harbor Laboratory, New York. 504.

Marconi, W., and Bartoli, F. (1981) Industrial applications of fiber-entrapped enzymes. *Adv. in Biotechnol.* 3, 348-354.

Margaritis, A., Bajpai, P.K., and Wallace, J.B. (1981) High ethanol productivities using small Ca alginate beads of immobilized cells of Zymomonas mobilis. *Biotechnol. Letters* 3, 613-618.

Mattiason, B. (1983) Immobilization methods. In: *Immobilized cells and organelles*, Mattiason, B. ed., 1, pp 1-10. Boca Raton, Florida.

Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31, 426-428.

Miller, J.H. (1972) In: *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, New York. 431-432.

Monaghan, R.L., and Currie, S.A. (1984) Preservation of antibiotic production by representative bacteria and fungi. *Developments in Industrial Microbiology* 26, 787-792.

Moo-Young, M., Lampley, J., and Robinson, C.W. (1980) Immobilization of yeast cells for ethanol production. *Biotechnol. Letters* 2, 541-548.

Nishida, Y., Sato, T., Tosa, T., and Chibata, I. (1979) Immobilization of Escherichia coli cells having

aspartase activity with carrageenan and locust bean gum. *Enzyme Microb. Technol.* 1, 95-98.

Nishimura, N., and Kisumi, M. (1984) Aspartase-hyperproducing mutants of Escherichia coli B. *Appl. Environ. Microbiol.* 48, 1072-1075.

Pardee, A.B., Jacob, F., and Monod, J. (1959) The genetic control and cytoplasmic expression of 'Inducibility' in the synthesis of β -galactosidase by E. coli. *J. Mol. Biol.* 1, 165-178.

Peterson, E.A., and Sober, H.A.J. (1956) Chromatography of proteins: I, Cellulose ion-exchange adsorbents. *Am. Chem. Soc.* 78, 751-755.

Randerath, K., and Randerath, E. (1967) Thin layer separation methods for nucleic acid derivatives. *Methods in Enzymology* 12, 323-327.

Rasmussen, K., and Albrechtsen, J. (1974) Glutaraldehyde. The influence of pH, temperature, and buffering on the polymerization rate. *Histochemistry*. 38, 20-39.

Sampathkumar, Dwivedi, Basant, K. (1961) US patent 4333872.

Satake, K., Okuyama, T., Ohashi, M., and Shinoda, T. (1960) The spectrophotometric determination of amine, amino acid and peptide with 2,4,6-Trinitrobenzene 1-sulphonic acid. *J. Biochem.* 47, 654-660.

Sato, T., Nishida, Y., Tosa, T., and Chibata, I. (1979) Immobilization of Escherichia coli cells containing aspartase activity with k-carrageenan. Enzyme properties and applications for L-aspartic acid production. *Biochimica Biophysica Acta* 570, 179-186.

Seyhan, E., and Kirwan, D.J. (1979) Nitrogenase activity of immobilized Azotobacter vinelandii. *Biotechnol. Bioeng.* 21, 271-281.

Sharma, S., and Yamazaki, H. (1984) Preparation of hydrophobic cloth. *Biotechnol. Letters* 6, 301-306.

Sheldon, A. (1981) The charge and pH at the surface of microbial cells. In: Chemical communication at the microbial level 1, pp 35-37, Boca Raton, Florida, CRC press Inc.

Shirling, E.B., and Gottlieb, D. (1966) Methods for characterization of Streptomyces species. *Int. J. Syst. Bacteriol.* 16, 313-340.

Simon, M., Zieg, J., Silverman, M., Mandel, G., and Doolittle, R. (1980) Genes whose mission is to jump. *Science* 209, 1370-1374

Venkatasubramanian, K., and Karkare, S.B., (1983) Process engineering considerations in the development of immobilized living cell systems. In: Immobilized cells and organelles, B.Mattiason, ed., 1, pp 133-144 Boca Raton, Florida.

Williams, D., and Munnecke, D.M. (1981) The production of ethanol by immobilized yeast cells. Biotechnol. Bioeng. 23, 1813-1825.

Wouters, J.T.M., Driehuis, F.L., Polaczek, P.J., van Oppenraay, M.L.H.A., and van Andel, J.G. (1980) Persistence of the pBR322 plasmid in Escherichia coli K 12 grown in chemostat cultures. Antonie van Leeuwenhoek 46, 353-3 62.

Yamazaki, H., and Leung, K.L. (1981) Determination of the total rates of synthesis and degradation of RNA in a bacterial culture. Can. J. Microbiol. 27, 168-174

Yeung, K.H., Chaloner-Larsson, G., and Yamazaki, H. (1976) Evidence against the involvement of adenosine 3',5'-cyclic monophosphate in glucose inhibition of B-galactosidase induction in Bacillus megaterium. Can. J. Biochem. 54, 854-865.

LIST OF PUBLICATIONS

- (1) Leung, K.L., Joshi, S., and Yamazaki, H. (1983) Adsorption of Saccharomyces cerevisiae onto cellulose and ECTEOLA-cellulose films for ethanol production. Enzyme Microb Technol. 5, 181-184
- (2) Joshi, S., and Yamazaki, H. (1984) Film fermentor for ethanol production by yeast immobilized on cotton cloth. Biotechnol. Letters 6, 797-802
- (3) Joshi, S., and Yamazaki, H. (1985) Use of bacterial films as resident inocula for repeated batch fermentation. Biotechnol. Letters 7, 753-758
- (4) Yamazaki, H., Sharma, S., and Joshi, S. (1986) Adsorption of Aspartase onto hydrophobic cloth for conversion of ammonium fumarate. Biotechnol. Letters 8, 107-110
- (5) Joshi, S., and Yamazaki, H. (1986) Cellulose acetate entrapment of Escherichia coli on cotton cloth for aspartate production. Biotechnol. Letters 8, 277-282
- (6) Joshi, S., and Yamazaki, H. Repeated batch antibiotic fermentation by streptomycetes immobilized on cotton cloth. Submitted to Biotechnological Letters.
- (7) Joshi, S., and Yamazaki, H. Resistance of bacterial film to contamination during use as inoculum in repeated

batch fermentation. Submitted to Biotechnology Letters.

(8) Microorganism Immobilization; application to U.S and Canadian patent.

END

0/2.01.88

FIN