Large Scale Cell Culture Technology

Edited by Bjorn K. Lydersen

Hanser Publishers, Munich Vienna New York

Distributed in the United States of America by
Macmillan Publishing Company, New York
and in Canada by
Collier Macmillan Canada, Inc., Ontario

Editor:
Dr. Bjorn K. Lydersen, Hybritech Inc., San Diego, CA 92126

Distributed in USA by
Scientific and Technical Books
Macmillan Publishing Company
866 Third Avenue, New York, N.Y. 10022

Distributed in Canada by
Collier Macmillan Canada, Inc.
1200 Eglington Ave. E, Suite 200, Don Mills, Ontario M3C 3N1 Canada

Distributed in all other countries by
Carl Hanser Verlag
Kolbergerstraße 22
D-8000 München 80

Library of Congress Cataloging-in Publication Data

Large scale cell culture technology.
Includes index.
1. Cell culture – Industrial applications.
2. Biotechnology. I. Lydersen, Bjorn K.
TP248.25.C44L37 1987 660'.6 87-60727
ISBN 0-02-948881-8

CIP-Kurztitelaufnahme der Deutschen Bibliothek

Large scale cell culture technology / ed. by
Bjorn K. Lydersen. Munich ; Vienna ; New York :
Hanser, 1987.
ISBN 3-446-14845-0
NE: Lydersen, Bjorn K. [Hrsg.]

ISBN 3-446-14845-0 Carl Hanser Verlag, Munich, Vienna, New York
ISBN 0-02-948881-8 Macmillan Publishing Company, New York
Library of Congress Catalog Card Number 87-060727

Printed in the Federal Republic of Germany by Druckerei Appl, Wemding.

PREFACE

The use of large scale animal cell culture has become increasingly common during the 1980's due to advances in recombinant DNA and monoclonal antibody technology. Genetic engineering has provided for progress in both the understanding of how specific proteins mediate biological processes, and for methods to produce these proteins in significant quantities. A portion of these proteins can be produced by bacteria or yeast, but in many cases synthesis by animal cells is advantageous. Monoclonal antibodies also require synthesis in mammalian cells, although there has been some progress in expression in yeast and bacteria. Thus, there are many applications where a need for efficient methods of large scale animal cell culture has developed. Progress is also expected in the understanding and genetic manipulation of plant cells and this should yield more applications where large scale plant cell culture is required.

The increased need for large scale cell culture has led to many efforts to improve the traditional technology. The need for many types of animal cells to attach to surfaces, the relative sensitivity of animal cells to shear forces, and the relatively high cost of nutrients for animal cells causes the problems in cell culture to be different from those encountered in fermentation of bacteria and fungi. These problems have been the driving force for many of the recent developments and, through these recent efforts, approaches with fundamental advantages over those used in traditional fermentation have resulted.

In this book, the major approaches to systems for large scale animal and plant cell culture are explored. The first three chapters in the section on Fermentation illustrate the use of relatively standard fermentation equipment for mammalian cell protein production. Cells are grown either directly in suspension, or on the surfaces of small beads which can be readily maintained in suspension. Thus, fermentation equipment can be utilized for both adhesion dependent cells and suspension cells. In the second section, entitled Encapsulation, two approaches to enclosing cells within a matrix are described. The encapsulated cells can be maintained in a standard fermentation vessel or in a fluidized bed. Although reported successes with adherent cells are still limited, routine production from suspension cells has been accomplished with this approach. The third section on Bio-Reactors includes chapters on three different approaches to providing for high cell density perfusion reactors. Hollow fibers, porous beads in a fluidized bed, and a rigid ceramic matrix form the basis for systems applicable to both adherent cells and suspension cells. The fourth section on Plant Cell Cuture includes a chapter reviewing the potential of plant cell culture for production purposes and the special considerations required in plant cell culture. Because the use of cultured plant cells has not been as commercially successful to date as animal cell culture, the methods have not been developed to the same extent. The final section of the

book focuses on Regulatory Considerations involved in production from cell culture. Biological considerations are the most important, but the impact of the system chosen for production is also discussed.

This volume focuses on the different approaches taken to large scale cell culture, with the emphasis on a description of the techniques and their practical success. It is intended to provide students and practitioners of the technology a broad view of the practical problems and the different solutions which have been developed.

B. K. Lydersen

Front cover: Scanning electron micrograph of a normal human fibroblast grown on a porous ceramic.

INTRODUCTION

The manufacture of biological products from cell cultures began about 35 years ago in the 1950's. The first major applications were in the production of viral vaccines for human and veterinary use. Initial production relied on the use of hundreds or thousands of relatively small flasks or bottles containing cells attached to the glass surface. Since virtually all of the production of viral vaccines utilized cells which required adhesion to a surface for their growth, fermentation of cells in an agitated tank was not an alternative. A major advance in production technique was the introduction of cylindrical bottles (roller bottles) placed on slowly turning rollers. This allowed for more efficient use of the glass surfaces and permitted an increase in the volume of each bottle. There were many other efforts in improving the systems for large scale cell cultures. These included the winding of plastic spirals into the roller bottles to increase the surface area per bottle and stacked plate propagators in which cultures could be consolidated into a single unit. The emphasis was on increasing the availability of surface area for cell attachment while decreasing the number of separate culture units and the volume of the containers. These innovations however did not have major impact.

The most significant development in the 1960's was the introduction of small spherical particles for the cultivation of adhesion-dependent cells. These particles, termed micro-carriers, permitted the culture of adhesion-dependent cells in relatively standard stirred tanks, making cell culture production somewhat analogous to traditional fermentation. There were many problems in the application of this technique to routine production however, and many facilities maintained the use of flasks and roller bottles into the 1980's.

The next major technological innovation was the development of hollow fiber reactors for cell culture in the 1970's. With this approach, cells are immobilized in and around a bundle of hollow fibers at a very high cell density while cell culture medium is perfused through the lumen of the fibers. Diffusion of nutrients occurs across the membrane of the fibers, but cells are prevented from mixing with the flow of medium. This approach received much attention, but was not employed in many manufacturing applications until very recently.

During the last ten years, there have been several other major innovations, including the encapsulation of cells in small beads or spheres and the immobilization of cells in different forms of porous matrices. Also, the use of cells which do not require adhesion to a surface has led to the scale-up of cultures to volumes of 1000 liters or greater in several facilities. This has been done in both traditional stirred tanks and in air-lift fermentors. At this time, there are many alternatives to choose from, and this is the subject of this book.

In addition to the changes in systems available for large scale cell culture production, there are two other major changes which have occurred during the past

ten years. Until recently, cell culture production of products intended for injection into humans or animals was limited to cells which were judged to be "normal" and without tumorigenic properties. This limited the type of cell which could be used in most cases to cells with a limited lifespan. With the advent of recombinant DNA and monoclonal antibody technology however, it became necessary to permit the use of transformed cells which have certain abnormal or tumorigenic properties, including an unlimited lifespan. The emphasis now has changed from proving that the cells used for production are "normal" to proving that the purified product does not contain potentially tumorigenic molecules.

The other major development is the success in finding alternatives to the use of relatively high concentrations of animal serum in cell cultures. The proteins and growth factors required for the growth of many cell types in culture have now been identified, resulting in cell culture media with relatively low cost and absence of adventitious agents such as mycoplasma and virus. In addition, the use of serum-free medium has made purification of cell culture-derived products easier and less expensive.

There are several challenges remaining, however. Despite the advances, cell culture production is still relatively expensive, with purified proteins costing at least $ 500/gram in most cases. Advances will come in the areas of improved cell productivity, the scale-up and perfection of methods retaining cells within the fermentor or bio-reactor, and in lower media and purification costs. These challenges are the subject of intense effort by both industrial and academic investigators, and it is judged likely that in another ten years cell culture production will be five to ten-fold more efficient than it is today.

Although the indications are that cell culture production will continue to become more efficient, it is still perceived that production of mammalian cell proteins could be done much more efficiently if the proteins could be synthesized in bacterial or fungal cells. For several relatively simple proteins such as insulin and interferon, this has already been done. For more complex proteins including antibodies and tissue plasminogen activator, this has not yet been accomplished. If major advances are made in understanding and controlling the processing of proteins within bacterial and fungal cells, it is possible that the need for expression of mammalian cell proteins in mammalian cells could significantly decrease. At the present time, this possibility is not seen as being imminent, and it has not dampened the enthusiasm of those developing more efficient large scale cell culture technology.

B. K. Lydersen

CONTRIBUTORS

J. R. Birch
Celltech Ltd.
Slough, Berkshire
United Kingdom

P. Brodelius
Institute of Biotechnology
Swiss Federal Institute of Technology
Zurich, Switzerland

R. C. Dean, Jr.
Verax Corporation
Hanover, New Hampshire, USA

R. Fleischaker
Vista Biologicals
Carlsbad, California, USA

K. Gilbride
Damon Biotech, Inc.
Needham Heights, Massachusetts, USA

M. L. Gruenberg
Endotronics, Inc.
Coon Rapids, Minnesota, USA

M. D. Hirschel
Endotronics, Inc.
Coon Rapids, Minnesota, USA

S. B. Karkare
Verax Corporation
Hanover, New Hampshire, USA

A. C. Kenney
Celltech Ltd.
Slough, Berkshire
United Kingdom

K. Lambert
Celltech Ltd.
Slough, Berkshire
United Kingdom

A. S. Lubiniecki
Genentech, Inc.
South San Francisco, California, USA

B. K. Lydersen
Hybritech Incorporated
San Diego, California, USA

J. D. Macmillan
Department of Biochemistry and
Microbiology
Cook College
Rutgers University
New Brunswick, New Jersey, USA

L. Miller
Department of Biochemistry and
Microbiology
Cook College
Rutgers University
New Brunswick, New Jersey, USA

K. Nilsson
University of Lund
Lund, Sweden

M. Oka
Damon Biotech, Inc.
Needham Heights, Massachusetts, USA

G. F. Payne
School of Chemical Engineering
Cornell University
Ithaca, New York, USA

P. G. Phillips
Verax Corporation
Hanover, New Hampshire, USA

N. G. Ray
Verax Corporation
Hanover, New Hampshire, USA

S. Reuveny
Israel Institute for Biological Research
Ness-Ziona
Israel

P. W. Runstadler, Jr.
Verax Corporation
Hanover, New Hampshire, USA

R. Rupp
Damon Biotech, Inc.
Needham Heights, Massachusetts, USA

M. L. Shuler
School of Chemical Engineering
Cornell University
Ithaca, New York, USA

P. W. Thompson
Celltech Ltd.
Slough, Berkshire
United Kingdom

D. Velez
Department of Biochemistry and
Microbiology
Cook College
Rutgers University
New Brunswick, New Jersey, USA

L. A. Wood
Celltech Ltd.
Slough, Berkshire
United Kingdom

CONTENTS

Preface V

Introduction VII

Contributors IX

FERMENTATION

Antibody Production with Airlift Fermentors 1
J. R. Birch, K. Lambert, P. W. Thompson, A. C. Kenney and L. A. Wood

Monoclonal Antibody Production in Stirred Reactors 21
J. D. Macmillan, D. Velez, L. Miller and S. Reuveny

Microcarrier Cell Culture 59
R. Fleischaker

ENCAPSULATION

Cellular Microencapsulation for Large-Scale Production of Monoclonal Antibodies 81
R. Rupp, K. Gilbride and M. Oka

Entrapment of Cultured Cells in Agarose Beads 95
K. Nilsson

BIO-REACTORS

An Automated Hollow Fiber System for the Large Scale Manufacture of Mammalian Cell Secreted Product 113
M. D. Hirschel and M. L. Gruenberg

Continuous Cell Culture with Fluidized Sponge Beads 145
R. C. Dean, Jr., S. B. Karkare, P. G. Phillips, N. G. Ray and P. W. Runstadler, Jr.

Perfusion Cell Culture System Based on Ceramic Matrices 169
B. K. Lydersen

PLANT CELL CULTURE

Large Scale Plant Cell Culture 193
G. F. Payne, M. L. Shuler and P. Brodelius

REGULATORY CONSIDERATIONS

Safety Considerations for Cell Culture-Derived Biologicals 231
A. S. Lubiniecki

Index 249

ANTIBODY PRODUCTION WITH AIRLIFT FERMENTORS

J.R. Birch, K. Lambert, P.W. Thompson, A.C. Kenney and L.A. Wood

CONTENTS

Uses of Monoclonal Antibodies and Scale of Requirement 2

Deep Tank Fermentation of Mammalian Cells 2

Airlift Fermentors 2

Operation of Fermentors 3

Aseptic Control 4
Process Control and Automation 6
Culture Medium 7
Process Economics 7
Mixing and Mass Transfer Characteristics 8
Hydrostatic Pressure 11
Cell Growth and Antibody Production 11

Continuous Culture 13

Product Recovery 16

Summary 18

References 19

USES OF MONOCLONAL ANTIBODIES AND SCALE OF REQUIREMENT

The development of the hybridoma technique by Köhler and Milstein in 1975[1] made it possible to produce monoclonal antibodies of predefined specificity and consistent binding affinity for the first time. In the ensuing ten years interest has been developing steadily in the application of these reagents to diagnosis,[2,3] therapy[5,6] and immunopurification.[4] With the development of novel uses there has been a need to find new methods for manufacturing the substantial quantities of antibody required. The requirement is for a production process capable of producing up to a kilogram per year of certain antibodies at present but capable of scale up to produce multi-kilogram amounts in the future.

We opted to take the *in vitro* culture route as opposed to ascites culture on both economic and regulatory grounds.[7] More specifically, for engineering and economic reasons which we will develop later in this chapter, we concluded that deep tank fermentation offered the most straightforward route to scale up of antibody production.

DEEP TANK FERMENTATION OF MAMMALIAN CELLS

Mammalian cells which can be grown in suspension have been cultured in deep tank fermentor systems for many years. In general stirred tanks have been used and reactors of up to 3,000 liter capacity have been used for the production of foot and mouth disease virus vaccine from baby hamster kidney cells and more recently lymphoblastoid interferon has been produced from Namalva lymphoblastoid cells in an 8,000 liter stirred tank reactor.[8] Recently Lebherz et al.[9] have reported on the production of monoclonal antibodies from hybridoma cells grown in a 35 liter working volume stirred reactor. An alternative to the stirred tank reactor is the airlift reactor which uses gas mixtures to provide mixing. Katinger et al. (1979)[10] described the use of such reactors for the growth of Namalwa lymphoblastoid and BHK21 cells. We have described the use of airlift fermentors for the production of monoclonal antibodies from hybridoma cells.

Airlift Fermentors

The basic principle of the airlift fermentor which was first described by Le Francois (1955)[11] is illustrated in Figure 1 and is reviewed by Smart (1984)[12] and by Onken and Weiland (1983).[13] Gas mixtures are introduced into the culture from a sparge tube at the base of a central draught tube. This causes a reduction in the bulk density of the liquid in the draught tube compared with the outer zone of the

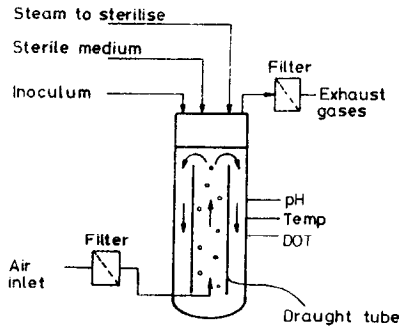

Figure 1. Principle of an airlift reactor.

vessel which sets the culture into circulation. Means of directing flow other than draught tubes can be used and are described by Onken and Weiland.[13] In addition to providing mixing the gas mixtures also supply oxygen to the culture.

It is apparent that hybridoma cells can be grown successfully in both stirred and airlift type reactors and indeed in our own laboratory we have used both types of systems in development. We are unable to show any effect of the reactor type per se on growth kinetics or specific antibody production rate. However, we concluded that airlift reactors had certain advantages which favored their use on the production scale. In the first place they have the merit of simplicity, since they lack the motors and stirrers associated with stirred reactors. This in turn helps aseptic operation since it avoids the use of shaft seals through which microorganisms might gain access to the fermentor.

Finally we find in practice that this type of fermentor has very good mass transfer characteristics especially with respect to oxygen transfer rate.

OPERATION OF FERMENTORS

To date we have gained experience with airlift fermentors of 5, 10, 30, 100 and 1,000 liters working volume. A 10,000 liter fermentor has been designed. An outline of our existing production process is given in Figure 2. An inoculum culture (ca. 1 liter) grown in roller bottles is transferred aseptically to a 10 liter inoculum fermentor. When the cell density reaches a level of 10^6/mL the contents of this vessel are transferred to a 100 liter fermentor which in turn provides the inoculum for the 1,000 liter production fermentor, a photograph of which is shown in Figure 3.

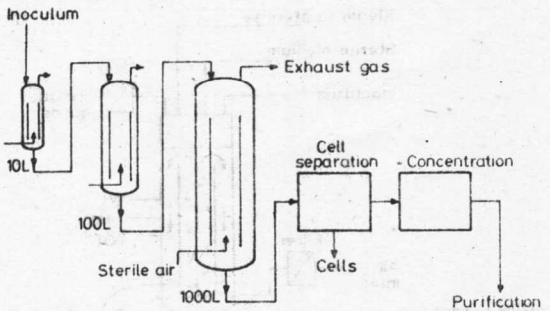

Figure 2. Flow sheet for 1000 liter scale mammalian cell culture system.

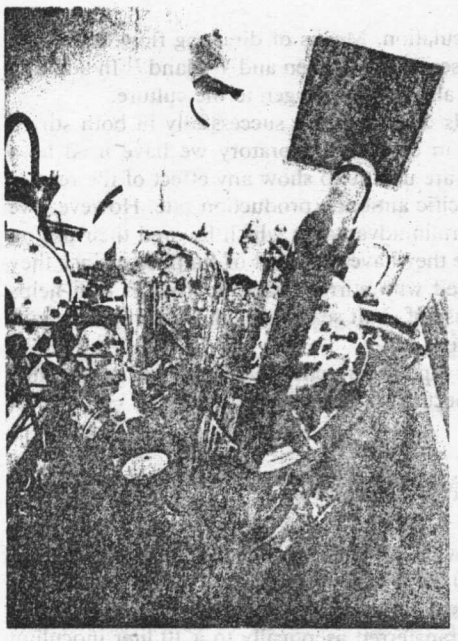

Figure 3. Photograph of 1000 liter fermentor.

Aseptic Control

Mammalian cell cultures are especially prone to microbial contamination because of the complexity of the culture medium and the long duration of the culture period which may be from one to two weeks for a batch culture to many months for a continuous culture. It is therefore essential that absolute asepsis be maintained.

Prevention of both microbial and chemical contamination may be achieved by primary or secondary containment or a combination of the two. Secondary containment permits the exposure of the product to atmosphere and hence relies on maintenance of a vigorously clean or even sterile environment by operating in class 1000 or class 100 rooms. Provision of these facilities can be extremely expensive as well as being very cumbersome and time-consuming to operate. We have therefore chosen to rely on primary containment in which the product is consistently enclosed in sealed vessels, which while requiring sophisticated process plant, may be operated in a normal environment and allows much easier operation and hence reduced operating costs. The use of deep tank fermentation is ideally suited to this approach as the techniques of fluid handling in enclosed systems are already well known. In order to ensure that the process equipment will maintain adequate primary containment it is necessary to perform various checks of the process vessels and associated pipework systems prior to sterilization. These checks include pressure testing of the fermentor and pipework and integrity testing of the filters to ensure that no leaks are present.

The production fermentors, which are stainless steel pressure vessels, are sterilized *in situ* via the air inlet line using steam at high pressure and temperature. The vessels and associated pipework are designed to be crevice-free and free draining to prevent build-up of condensate which could generate cold spots which would not be sterilized. Pipework is all welded as far as possible and incorporates sealless valves and pumps where necessary. Vessels and pipework are designed for *in situ* cleaning. Adequate cleaning is essential both to prevent chemical contamination of product and to prevent the accumulation of deposits which may otherwise prevent the attainment of sterilizing temperatures. Thorough cleansing is also necessary to prevent cross-contamination with product from previous batches. Once the fermentors have been sterilized it is essential to prevent ingress of foreign organisms. The inlet gas mixture is filter sterilized prior to sparging into the vessel. The exhaust gas passes through a condenser to remove water droplets and vapor before passing through a final sterilizing filter which prevents back contamination. Valves are arranged to prevent microorganisms growing through the valve seats by ensuring that the non-sterile side of valves can be completely drained or by using a sterile barrier incorporating two valves and a steam bleed. The culture medium is sterilized by filtration into the sterilized culture vessels after the vessels have cooled and the process sensors have stabilized. pH, temperature and dissolved oxygen sensors are then calibrated and the medium equilibrated to the optimum conditions for the growth of the cells. When serum is used in the culture medium it is heat inactivated to reduce the risk of mycoplasma infection. The functional simplicity and robustness of the equipment allied to strict adherence to Good Manufacturing Practice (principles described in reference 14) has given us a very reliable process in terms of aseptic operation. In fermentations at production scale from January 1984 to November 1985 we have had only one contamination from 114 runs. this was due to equipment failure which was readily identified and corrected.

Process Control and Automation

Growth and antibody production by an individual hybridoma cell is dependent upon its local physicochemical environment. In particular we are concerned to control pH, dissolved oxygen, temperature and appropriate nutrient concentrations. Adequate process monitoring and control is most readily achieved in a homogeneous system. This in turn requires adequate mixing of the cell culture to provide appropriate heat and mass transfer characteristics. We believe that a homogeneous system offers the best potential for process optimization since one can control the immediate environment of the cell and is the most predictable in terms of scale up characteristics. It follows from this argument that process monitoring and control and scale up are less straightforward in culture systems in which the cells are immobilized or entrapped in a non-homogeneous environment.

In our fermentations dissolved oxygen is controlled automatically by adjusting the rate of addition of air to the vessel. pH is controlled by the automatic injection of carbon dioxide in the inlet gas stream or by the addition of a sodium hydroxide solution. Temperature is controlled by the circulation of water through the vessel jacket. The water is heated or cooled as required through a heat exchanger. Foaming is not a problem at the air throughputs needed to meet the oxygen demand of the cultures and can be adequately controlled when necessary by anti-foaming agents specially chosen for this application. Cell growth is monitored by aseptically sampling the fermentor and counting cells on a hemocytometer using trypan blue to distinguish dead and viable cells. Growth may also be monitored indirectly, for instance by measuring the oxygen consumption of the fermentor. The ability to directly measure number and viability of cells in the reactor is a clear advantage over non-homogeneous culture systems.

Antibody concentration is measured by HPLC or by enzyme linked immunosorbent assay (ELISA).

In manually operated plants a major proportion of the total production cost for monoclonal antibodies is the labor input. We have therefore resorted wherever possible to automatic operation. Hence the process is controlled by a microprocessor based computer which handles all valve and pump activation sequences through cleaning, sterilization and process control to the final harvesting and subsequent recovery. Human involvement is limited to activities such as medium preparation and general process monitoring. Such reliance on automation requires that the computer is capable of extensive self checking and monitoring of plant items for correct operation. It provides extensive alarm monitoring and emergency action in the case of failure of critical plant and services.

Culture Medium

We use a proprietary culture medium optimized for production of antibody by hybridoma cells as described below. This medium has usually been supplemented with fetal calf or immunoglobulin depleted adult serum at a concentration of 2–10% v/v. There are however significant disadvantages in the use of serum on a large scale, notably cost and availability and we are increasingly using serum free medium. We have developed a serum free medium which supports the growth of all rodent hybridomas so far tested. The use of serum free medium also facilitates downstream processing and eliminates the possibility of introducing adventitious agents such as mycoplasma via serum. A number of serum free media for hybridoma culture have now been described.[15-20] However, with a few exceptions, e.g.[17] there have been few reports of the use of such media in agitated suspension culture of hybridomas.

The serum free medium which we use for production contains 1 g protein l^{-1}. This is equivalent to the protein concentration of medium containing 2% v/v serum. A reduced protein medium containing 10 mg protein l^{-1} has now been developed and is being evaluated at the pilot scale. In this medium the added protein contributes less than 10% of the protein in the final product.

Process Economics

We chose to scale up the cell culture process by increasing the unit size of the fermentor rather than the number of units. This has allowed us to benefit from economies of scale. If scale-up consists of increasing the number of units without increasing unit size then both capital and labor costs increase linearly with increase in scale. However, if scale up is addressed by increasing unit size the cap-

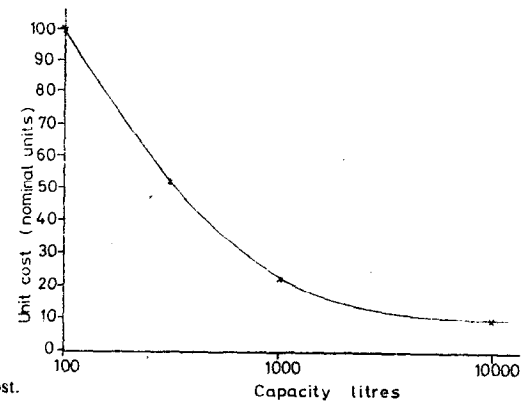

Figure 4. Effect of scale on unit cost.

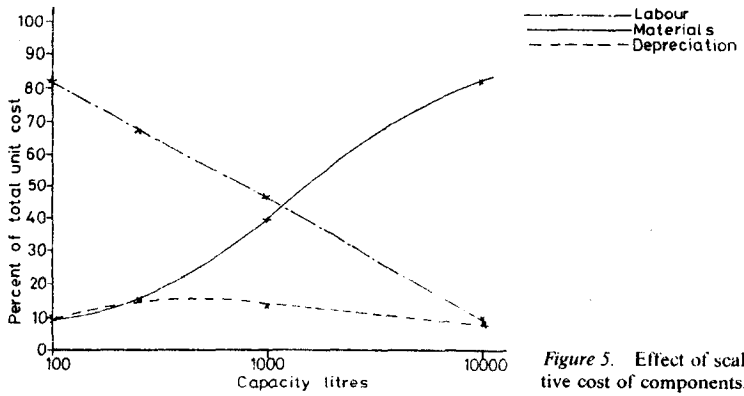

Figure 5. Effect of scale on relative cost of components.

ital cost does not increase linearly but approximately to the 0.6 power of the increase in scale. Hence a unit with ten times the capacity will cost only four times as much (and this has been our experience). This relationship as described by Perry & Chilton[21] can be more accurately applied using cost indices published in the journal *Chemical Engineering*[22] although ultimately is best supported by formal cost estimation. The labor costs required for operation are effectively independent of scale and while materials costs may increase pro rata some discount for bulk purchases may be possible. The effect of scale on unit cost of product is shown in Figure 4. In Figure 5 we show how the major components of cost vary with scale. It will be seen that as the scale increases, material costs form a greater proportion of the total production cost and increased emphasis then needs to be placed on reducing these costs.

Mixing and Mass Transfer Characteristics

As we have already discussed, we need to provide adequate mixing to ensure good mass and heat transfer in the vessel, even distribution of nutrients and cell dispersion. Of particular significance is the need to supply adequate amounts of oxygen for growth of the hybridoma cells. In many cell culture systems oxygen supply is inadequate to support high cell population densities.[23,24] This is particularly true when oxygen is supplied simply by diffusion from the surface of the culture. Problems may also occur in immobilized cell systems that generate dense masses of cells. Glacken et al.[25] have suggested that diffusional limitations through a multilayer cell pellet containing 10,000 cells may result in 40% of the cells suffering oxygen limitation, and that the limiting path length for oxygen diffusion could be 170 μm. Clearly the same limitations must exist for the diffusion into the cell mass of other essential nutrients and also for the removal of toxic or inhibitory metabolites. These considerations must impose restraints on the design of an immobilized