THESIS

This is to certify that the

dissertation entitled

FERMENTATION PROCESS IMPROVEMENT
BY MEMBRANE TECHNOLOGY

presented by

KYU HANG KYUNG

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Philipp Gerhardt
Major professor

Date 23 Feb 1983



O-12771

# FERMENTATION PROCESS IMPROVEMENT

# BY MEMBRANE TECHNOLOGY

BY

KYU HANG KYUNG

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirement
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1983

# ABSTRACT

## FERMENTATION PROCESS IMPROVEMENT BY MEMBRANE TECHNOLOGY

BY

KYU HANG KYUNG

The continuous fermentation of glucose to ethanol by cells of Saccharomyces cerevisiae ATCC 4126 "immobilized" by membrane containment was investigated. Substrate was fed into a continuous dialysate circuit and thence into a batch fermentor circuit via diffusion through the membranes of an intermediate dialyzer; simultaneously, product was withdrawn from the fermentor circuit through the dialyzer membranes into the dialysate circuit and out in the effluent. Since the fermentor was operated without an effluent, the cells essentially were immobilized and converted substrate to product by maintenance metabolism. Contrary to prior results with this novel system for the continuous fermentation of lactose to lactate by lactobacillus cells, a steady state of yeast cells in the fermentor was not obtained initially but eventually occurred by the depletion of nutrients and prevention of cell breakage, although substrate and product concentrations then became unsteady. The inherent advantages of the system were offset in the ethanol fermentation by relatively low productivity, which appeared to be limited by membrane permeability.

Mutualistic dialysis culture of Streptococcus lactis, which is valuable as a dairy starter, and Candida utilis, which is valuable as single cell protein, was investigated in a batch fermentation system. The bacterium and the yeast were inoculated into separate fermentors connected by an intermediate dialyzer, the membranes of which allowed diffusional exchange of solutes. Lactose fed into the bacterial culture was fermented to lactic acid, which was dialyzed into the yeast culture and consumed so as to relieve product inhibition of the bacterial culture. Consequently, the bacterial cell concentration more than doubled in comparison with a nondialysis control, and yeast cells were produced as byproduct. Although the acid production rate by the bacterium was much faster than the acid consumption rate by the yeast, the primary limiting factor of the process apparently was the solute exchange rate across the membrane.

## ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. Philipp Gerhardt for his help and guidance during the course of my studies and the opportunity he provided. I am further grateful for the expert advice of my guidance committee members, Dr. C. A. Reddy and Dr. Ralph N. Costilow from the Department of Microbiology and Public Health and Dr. Donald K. Anderson and Dr. Eric A. Grulke from the Department of Chemical Engineering.

I acknowledge financial support from grant DAR 79-10236 (to Dr. Philipp Gerhardt) from the U. S. National Sciene Foundation.

My gratitude is also extended to my parents, brothers and sister for their support and encouragement and to my wife Jae Pong for her patience and ever present help.

TABLE OF CONTENTS

## LIST OF TABLES

LIST OF FIGURES

# GENERAL INTRODUCTION

Dialysis is a process for separation of solute molecules by use of their unequal diffusion through a semipermeable membrane because of a concentration gradient. Reverse osmosis, electrodialysis, microfiltration and ultrafiltration also involve membranes, but all of these processes require inputs of external energy. Dialysis, on the other hand, is driven only by a solute concentration gradient across the membrane. Therefore dialysis can be most easily applied to the separation of cells from their metabolites.

When dialysis technique is applied to the growth and maintenance of living cells, it is termed 'dialysis culture'. The simplest and earliest dialysis system was made by placing a microporous porcelain filter containing a culture into a medium reservoir containing another culture to study possible microbial interactions (Frankland and Ward, 1893). Subsequently, there have been improvements in design and instrumentation to enhance the permeability and the system stability.

Dialysis fermentation can best be conducted through the introduction of a dialyzer between a culture fermentor and a dialysate reservoir, with the contents of each continuously circulated on opposite sides of the dialyzer membrane. Such a system allows diffusional exchange of nutrients and products, while confining the cells within the growth fermentor. The culture fermentor and dialysate circuits in such a system can be operated batchwise,

continuously, or in a combination of these modes (Schultz and Gerhardt, 1969). For dialysis culture to be effective for the production of cell mass or nondiffusible products, the volume of the dialysate must be large compared to that of the culture fermentor, or the dialysate must be replenishable. The permeability and area of the membrane must be sufficient to permit useful diffusion. The system can be designed for nutrients to diffuse from the reservoir to the culture and for metabolites to diffuse from the culture to the reservoir.

The advantages of using dialysis for fermentation processes are the prolongation of cell growth and the extension of the stationary phase of the growth cycle, permitting increased production of cells and their metabolites. Fermentative uses of dialysis have included, principally, the production and recovery of cells and of both diffusible and nondiffusible compounds. Dialysis has also been useful ecologically in examining interactions between microorganisms and their environment. Dialysis can also facilitate the study of medically important microbial and mammalian cells and their metabolites.

This thesis begins with a general literature review, which is the third in a cumulative effort begun by Quarles (1973) and Stieber (1979) to prepare a publishable comprehensive review article updating progress in dialysis culture since the first review on the subject published by Schultz and Gerhardt (1969). The format is the same as that of Stieber (1979) and much of the text is essentially the same as in his thesis (identified by quotation marks), with additions mainly for the period from 1979 to 1983 and in the sections on ecological and

fermentative applications. Articles published before 1969 were also included if they were not referenced in the review by Schultz and Gerhardt (1969).

The experimental parts of this thesis were undertaken with the objective to further improve the yield and productivity of cells or cell metabolites in two industrially important fermentations. Two approaches were investigated.

In the first approach, glucose was fed into a continuous dialysate circuit and thence into a fermentor circuit via the dialysis membrane to convert glucose to ethanol by Saccharomyces cerevisiae. The fermentor circuit was operated as a batch so that the cells were essentially immobilized within the fermentor circuit. The product in the fermentor circuit was continuously removed via dialysis into the continuous dialysate circuit and out in the effluent. Such a system had been designed, mathematically modelled, computer-simulated and experimentally tested with the ammonium-lactate fermentation by Stieber and Gerhardt (1981). In this process, substrate is converted into product at a high rate without appreciable expenditure of substrate for cell growth. The dialysis serves not only to yield a cell-free product but also to sterilize the substrate entering the fermentor.

In the second approach, an investigation was made of using a lactate-consuming microorganism mutualistically with lactate-producing S. lactis for the increased production of the bacterium. The lactate-consuming organism growing on the opposite side of membranes served as a biological sink to remove lactate, thus maximizing the concentration

gradient across the membrane. Candida utilis was chosen as the second microorganism because it utilizes lactate as a sole source of carbon and does not utilize lactose, and because its biomass is useful as single cell protein.

Dialysis fermentation processes in general were discussed relative to other ways for alleviating end-product inhibition and to other applications. Microfiltration was proposed as a new and possibly better way to remove products during formation and thereby to further improve industrial fermentation processes with high-value products.

## GENERAL LITERATURE REVIEW

### Dialysis Culture of Microbial and Mammalian Cells: Applications, 1969-83

## 1. MEDICAL APPLICATIONS

The dialysis culture technique has been used in many studies of a medical nature to cultivate microbial and mammalian cells and to produce their pathogenic metabolites. Purposes for which the technique is useful include separation of cells from the macromolecules of a complex medium, production and recovery of compounds such as antigens and exotoxins, extension of the period of cell growth and viability, and study of cell and tissue differentiation.

### 1.1 *In vitro*

#### 1.1.1. Microbial cells

"Rightsel et al. (1978) used dialysis culture as a method for avoiding the influence of a multiplicity of host factors in the *in vitro* evaluation of anti-leprosy drugs. *Mycobacterium lepraemurium* was grown within macrophage cultures in diffusion chambers maintained on monolayer cultures of macrophages. The method was used to evaluate the effects of three sulfone derivatives and of rifampin on the growth of the bacterium. The study revealed the effects of these drugs on the organism and demonstrated the usefulness of this method for evaluating the chemotherapeutic effects of drugs or their analogs outside the metabolic influence of the host."

In earlier studies, the bacterium had been inoculated into diffusion chambers on a complex medium containing bovine serum and mouse-brain extracts (Dhople and Hanks, 1972a, 1972b, 1973). During 17 days of incubation, with periodic renewal of the medium, both the

cell count and the energetics of the bacterium declined. Renewal of media three times a week did not sustain the energetics of this obligate intracellular microbe.

In another investigation, a double-dialysis method was used to produce antigenic material from the thermophilic actinomycete, Micropolyspora faeni that cause "farmer's lung disease" (Edwards, 1972). Nutrient medium within a dialysis tubing was equilibrated with an aqueous solution of sodium chloride, and cells of M. faeni were inoculated into the resulting dialysate. After bacterial growth, the dialysate contained dialyzable nutrients from the original nutrient medium, organisms, and products of microbial metabolism. The dialysate was next placed in other dialysis tubing, and a second dialysis was conducted against running water to remove dialyzable molecules from the bacterial cell mass and the non-dialyzable macromolecular products (e.g., M. faeni antigens).

"Neisseria gonorrhoeae of colony type $T_2$ produced good cell yields and maintained above 92% $T_2$ colony type morphology for 12 h in a modified Marbrook dialysis chamber (Ruezinsky, 1975). After incubation, however, viable cell yields were not as high as those obtained from a biphasic agar-liquid culture system (Gerhardt and Heden, 1960; Hunter and McVeigh, 1970; LaScolea et al., 1975) or from a liquid-flask culture (Ruezinsky, 1975)." The clumping and aggultination of $T_2$ gonococcal cells probably led to underestimation of the population of $T_2$ gonococcal cells.

"L-phase variants of Streptococcus faecium were inoculated on membrane filters placed on solid agar. The organisms were found to pass through filters with 0.22 μm pores (Wyrick and Gooder, 1971).

The reversion of protoplasts was also studied, and it was learned that the protoplasts could not pass through the filters and form colonies under them. In some cases, however, covering the filters with solid L-phase medium before inoculation of the protoplasts allowed colonies to grow, indicating that the three-dimensional effect of the agar was important. In similar studies, the reversion of protoplasts of Bacillus subtilis was enhanced when cell walls of the organisms were layered on membrane filters (Clive and Landman, 1970). The estimation of reversion by wall material was non-specific, since the effect was also obtained with wall preparations from other species, including Escherichia coli, pseudomonads, and yeasts."

Harris and Powell (1951) studied the actions of penicillin and lysozyme on Bacillus subtilis and Bacillus megaterium microscopically while the organisms were grown on the surface of a cellophane membrane overlying the culture medium.

The Powell microperfusion chamber was modified for the observation and manipulation of growing bacteria (Issac et al., 1975). Duxbury (1977) developed a microperfusion chamber for studying the growth of bacterial cells with phase contrast microscopy. He demonstrated use of the chamber with observations on the effect of penicillin on Arthrobacter globiformis.

Collins and Richmond (1962) studied the rate of increase in cell length of Vibrio cholerae cells between divisions using a microperfusion chamber. Vibrio cholerae was cultured in dialysis culture flasks to produce exotoxin (Bhattacharyya, 1973). Although diffusion of nutrients from the medium reservoir into the culture chamber was rate-limiting, cell density and toxin concentration were increased more than twofold."

Eight different strains of Clostridium perfringens and a portion of substrate contained in dialysis tubing were introduced into food (Willardsen et al., 1977). This method eliminated the problem of retrieving a representative portion of the population after incubation, and enabled containment of the microbe in a sample without isolation from the environment.

"When a cellophane film was placed on an agar plate and Pseudomonas aeruginosa was spread on the film (Goto et al., 1971), the film enabled easy recovery of the bacteria and extracellular slime after growth, so that the invasive role of the slime and its immune reactions could be studied."

The technique of growing bacteria on dialysis membranes spread over agar medium provided a good selection of Staphylococcus aureus strains with a satisfactory yield of essentially all the extracellular products (Baman and Haque, 1970). Although not all of them were present in concentrated form, the entire complement of products could be detected.

"Masover and Hayflick (1974) used batch dialysis cultures of T-strain mycoplasmas in observing growth and urea metabolism. This would not be possible in non-dialysis cultures because of growth inhibition caused by elevated pH and by increased ammonium ion concentration. The authors also used a continuous dialysis process, in which the reservoir medium was continuously fed and replaced. A stable, viable culture of the organisms was maintained for 10 days. Further extension of this work should result in better characterization of the nutritional requirements of T-strain mycoplasmas, which may be agents of human disease."

"Ciccarelli et al. (1977) found that dialysis culture of Clostridium botulinum yielded 30,000 mouse $LD_{50}$ of the toxin per ml -- a yield 750 times greater than that of regular broth cultures."

Untermann (1972a, 1972b) compared several dialysis techniques (Donelly et al., 1967; Casman and Bennett, 1963; Hollander, 1965) for the production of enterotoxins, and recommended the technique of Donelly et al. (1967) for the production of enterotoxin A, which is produced in very small amounts.

A diffusion chamber was used to determine the mutagenicity of test compounds or their metabolites localized in the tissues and organs of animals (Ficsor and Muthiana, 1971). A strain of Salmonella typhimurium requiring histidine was enclosed in a chamber and was exposed to blood, liver, and testes homogenates from mice previously injected with the mutagen streptozotocin. After exposure, the organism was plated in selective media to determine the frequencies of histidine-independent mutations. These increased substantially as compared to controls. This simple method should be suitable for testing other chemicals suspected of being mutagenic.

Gue et al. (1973) used dialysis to cultivate Mycoplasma pneumoniae, M. gallisepticum, M. hominis, and T-strains of mycoplasma. The primary result was a considerable increase in the final cell population: the number of mycoplasma was 100 times the quantity produced by the usual method, probably as a result of the removal of inhibitory products.

A multi-compartment dialysis membrane fermentor was constructed with vertical dialysis bags to study the growth characteristics and the secreted macromolecular products of Pasteurella haemolytica

(Himmel et. al., 1982). This type of system was preferred over a plate-and-frame system because the researchers felt that conditions approximating the growth site of bovine respiratory infections could best be reproduced with a static, "sac-type" dialysis culture.

"Dialysis tubing was used for growing Leishmania mexicana to produce antigen (Crook et al., 1969). Dialysis culture was considered the method of choice for eliminating contamination of the antigen by medium components, such as red blood cells."

A variety of chick embryo tissues infected with exoerythrocytic stages of an avian malarial parasite, Plasmodium fallax, were grown in Rose multipurpose diffusion chambers (Jensen et al., 1964). In addition, pre-erythrocytic infections were initiated in chick embryo spleen cultures by introducing Plasmodium gallinaceum sporozoites. Mature segmenters were seen within 48 h. The parasites and cells thus cultivated were easily studied using phase contrast microscopy.

Trypanosoma lewisi was cultured in the liquid phase of a culture medium containing blood agar components (protein, agar, and defibrinated rabbit blood) inside of dialysis tubing (Dusanic, 1969). Agar was necessary for cell multiplication; presumably, it acted as an absorptive sink for inhibitory products from the medium.

1.1.2 Mammalian cells

"Rose and colleagues have developed and refined equipment for in vitro dialysis growth of various types of tissue cultures (Rose, 1954; Rose et al., 1958; Rose, 1967; Rose et al., 1968; Kumegawa et al., 1968; Rose et al., 1969; Rose et al., 1970; Rose and Cattoni, 1974; Rose and Yajima, 1977, 1978; Rose et al., 1981). Their system has evolved from a single, multi-purpose chamber (Rose, 1954) to a 12-

chamber circumfusion configuration which allows control of nutrient and gas flow rates, pressure, agitation, and temperature (Rose, 1967), and to a dual unit for the rotation of 24 culture chambers with increased physical capabilities (Rose et al., 1970). The systems were designed not only for growth of tissue cells but also for differentiation. The 1970 system was tested by cultivating human gingival explants in the chambers for 5 to 6 weeks (Rose and Cattoni, 1974). The system provided micro-environments that allowed the cells to differentiate according to their genetic potential."

Spleen cells from adult guinea pigs were cultured in the Rose-type tissue culture chamber to obtain spleen cultures which retained their cellular differentiation characteristics (Robineaux, 1963). Phase contrast microscopy was used to record the evolution of the cultures; thus, the experimenter followed in vitro growth and differentiation of the cells involved in the immunological processes.

The Rose tissue culture chamber (Rose, 1954) was modified by Batzdorf et al. (1969) into a double-chamber system that could be used to study the interaction of different cells while they are physically separated by the membrane.

"Marbrook (1967) studied the primary immune response of spleen cells from unimmunized mice. Marbrook grew the cells on dialysis membranes inserted together with horse or sheep erythrocytes as antigen into a reservoir of medium. Significant numbers of antibody-producing cells were detected within 3 to 5 days. The dialysis system allowed the spleen cells to grow unshaken, so that any possible foci of antibody-producing cells could be left undisturbed. Because of its

simplicity and reproducibility, this system has been widely used and modified. (It is commonly called the Marbrook chamber)."

"In suspensions of mouse spleen cells cultivated in the Marbrook chamber, a primary immune response was obtained using a polymer of Salmonella adelaide flagellin as antigen (Diener and Armstrong, 1967). The antibody-forming cells increased in number, peaking at 4 days. Experiments showed an induction of tolerance. The important effect of this model system was that, for the first time, the primary immune response was induced using a protein antigen in vitro."

"A modification of the Marbrook chamber was used to determine the capacity of a given spleen-cell dose to produce a primary antibody response (Halshall and Makinodan, 1974). The membrane area was manipulated by changing the size of the small inner chambers."

"Mouse thoracic duct lymphocytes responded vigorously to several mitogens in a dialysis culture system similar to the Marbrook chamber (Kearney and Reade, 1974). The kinetics of activation caused by the mitogens suggested that different populations of lymphocytes were activated by different mitogens."

"A modified Marbrook chamber was used to prolong culture condition in order to achieve in vitro cell responses parallel to in vivo responses (Maizels and Dresser, 1977). Various advantages of the Marbrook chamber, such as the time-consuming preparation process and the cumbersome nature of the chamber itself, were corrected by the use of an injection-molded chamber which fit in a plastic Petri dish used as a medium reservoir. The system provided a means of cultivating mouse lymphocytes in which resting memory cells could be stimulated by antigen to differentiate into antibody-secreting cells. Cells which

secreted IgG required 6 to 10 days to develop to their full potential from the resting memory cell state."

"A miniaturized diffusion culture system holding 24 culture chambers was developed by Eipert et al. (1978). Each chamber utilized a cell suspension volume of 0.1 ml and a reservoir of 1.0 ml, one-tenth the cell suspension and reservoir volme of the standard Marbrook chamber. The anti-sheep erythrocyte response of mouse spleen cells was studied to evaluate the system, which was found to yield reliable and reproducible primary and secondary antibody responses. It was also found that a membrane filter with a 0.2 μm pore diameter (Nucleopore) yielded up to twice the response of dialysis membrane."

"An artificial capillary device consisting of two types of hollow fiber membranes in a bundle was used to perfuse both gas and medium into tissue cultures (Knazek et al., 1972; Knazek et al., 1974)." The high ratio of surface area to culture volume allowed large numbers of cells to be cultured in the relatively simple apparatus, requiring considerably less space and equipment than did existing commercial culture techniques.

A dialysis culture system was also used to cultivate normal and malignant human bone marrow cells in a liquid medium (Golde and Cline, 1973). In this in vitro diffusion chamber, cells grew in suspension and on a dialysis membrane. This system provided a convenient means for studying cell differentiation and function.

"L-strain mouse fibroblast cells were cultivated in a coil dialyzer system which removed inhibitory metabolite products and thus promoted cell growth for a limited period (Horng, 1971). Subsequent dialysis, with intermittent renewal of the reservoir medium, did not

promote growth, probably because of physical limitations of the system."

Many mature organs were maintained in an entirely synthetic medium and retained their normal histological appearance for about a week in an in vitro organ culture system (Trowell, 1959). Later, the survival of rat lymphocytes was studied using the same system (Trowell, 1963). Cells in a liquid medium were layered on a sheet of agar, which supplied nutrients and absorbed metabolites.

"In a study of primary immune reactions, spleen fragments from mice were explanted in the wall of a filter assembly placed in an organ culture dish containing tissue culture medium (Globerson and Auerback, 1965). The system permitted lymphoid maintenance, differentiation, and proliferation, which together may be necessary for the initation of immune reactions. Tissue from mice treated with either phytohemagglutinin or adjunct were stimulated to form hemagglutinins and hemolysins. Tissue from untreated mice was needed to invoke a response leading to specific splenomegaly."

The effects of granulocyte extracts on the growth of bone-marrow cells were studied in diffusion chambers by Lovhaug and Boyum (1977). The low molecular weight fraction of the extract, reduced granulopoiesis without affecting macrophage formation. The high molecular weight fraction reduced the number of granulocytes and increased the number of macrophages.

Gallicchio and Murphy (1981) pretreated a preparation of erythropoietin, (the hormone which specifically regulates erythropoiesis) by dialysis to remove non-erythropoietin small

molecular contaminants. This dialysis procedure reduced contamination by as-yet undefined substances without any loss of erythropoietin.

Madle and Obe (1977) used a dialysis bag to reduce the toxic activity of the constituents of the S-9 medium on the lymphocytes, the target cells for indirect mutagenesis. This process also made the sterilization of the test substances unnecessary. The researchers showed that cyclophosphamide caused human leukocyte chromosomes to break after *in vitro* activation with liver microsomes.

Human colorectal adenocarcinoma tumor cell lines were grown in a matrix perfusion culture system to determine the stability of the technique for synthesis of carcinoembryonic antigen (Quarles et al., 1980). High cell densities were achieved, making it possible to routinely obtain continuous high yields of the antigen over an extended period of time.

## 1.2 *In vivo*

### 1.2.1. Microbial cells

Dialysis culture techniques have been used with a variety of hosts for the cultivation of many microorganisms. The technique appears promising, especially for the culturing of fastidious, pathogenic, or slow-growing parasitic microbes.

The exoerythrocytic stages of avian malaria parasites were cultured in Algire-type diffusion chambers (Algire et al., 1954), implanted intraperitoneally within chickens, turkeys, ducks, chick embryos, and mice (Huff et al., 1960). Chickens readily encapsulated the chambers, a major limitation of the technique.

In a later study mice were immunized to *Trypanosoma gambiense* via growth in cellulose diffusion chambers (Petithory and Rousset, 1969).

15 out of 58 mice immunized via the diffusion chamber technique survived, while all 55 controls died.

"When Mycobacterium lepraemurium was cultured in cell-impermeable chambers implanted intraperitoneally in mice, a relationship was found between growth of the organism and susceptibility of the host (Rightsel and Wiygul, 1971). Another important finding was that the organism grew well in the chambers in the absence of other tissue cells; that is, living cells were not essential, and multiplication occurred in a cell-free environment." However, inclusion of macrophages in the chamber seemed to lead to better results. The organism had a generation time of 6 to 8 days (Ito and Kishi, 1972, reported a generation time of 12 to 15 days) with macrophages, and 11 days in the cell-free environments.

Mycobacterium lepraemurium grew in diffusion chambers maintained in mice, guinea pigs, and human skin cell-monolayered Petri dishes (Wiygul and Rightsel, 1971). A 28.8-fold increase in final cell number was obtained when chambers containing human embryonic skin cells were incubated in mice for 50 days. The bacterium also grew in implanted chambers containing cells from human embryonic skin -- a species other than the natural host (mice); in fact, the human skin cells enhanced growth. Chambers without skin cells gave greater yields in mice than in guinea pigs. However, diffusion chambers implanted in susceptible hosts produced greater yields of bacilli than did chambers maintained in non-susceptible hosts, such as guinea pigs. Later studies confirmed that M. lepraemurium could grow within implanted chambers in the absence of host cells (Dhople and Hanks, 1972a, 1972b, 1973).

On the other hand when M. lepraemurium, in cell-free diffusion chambers was implanted in the abdominal cavity of the mouse, no significant multiplication was seen during the 6-month observation period (Ito and Kishi, 1972).

"This model system has also been used to study the growth of Mycobacterium leprae (Rightsel and Hall, 1976). The organism was inoculated into diffusion chambers containing different mammalian cell lines. Primary suspensions of neutral elements were derived from the cerebral ventricular walls, M. leprae increased 11.3-fold after 169 days, and was infectious when inoculated into the mouse foot-pad."

"Treponema pallidum, inoculated into subcutaneous polyethylene chambers inserted in guinea pigs, increased in number by about a factor of 10 by the sixth week after infection (Rathleb, 1973). The organisms maintained their virulence throughout the period, as indicated by the fact that samples used to infect normal rabbits intratesticularly produced syphilitic orchitis."

"The effects of nitrogen and oxygen on T. pallidum were examined in chambers implanted subcutaneously in mice (Horvath et al., 1975). Compared to a control, nitrogen enhanced both motility and survival time, whereas the effect of oxygen was deleterious." It was suggested that inactivation of T. pallidum by oxygen resulted from the accumulation of oxidized metabolic products.

"Similarly, the effects of synthetic steroids (dexamethasone and oxisuran) on T. pallidum were investigated using chambers implanted in rabbits (Tight and Perkins, 1976). For a limited period, the organism multiplied to significant numbers in rabbits given dexamethasone, while it decreased rapidly and remained at low levels in a control

situation. Oxisuran appeared to have little or no effect on the growth."

Arko (1972, 1974) described a procedure for implanting polyethylene chambers subcutaneously into small laboratory animals in order to study experimental Neisseria gonorrhoeae infections. A hollow polyethylene practice golf ball (42 mm in diameter) with numerous holes was implanted in the subcutaneous tissue of rabbits. Connective tissue encapsulated the golf ball forming an artificial chamber and natural membrane. N. gonorrhoeae grew and retained virulence in the chamber, which was usable for several weeks.

Coil-shaped subcutaneous chambers were made from 0.5 mm stainless steel wire for smaller animals, such as guinea pigs, hamsters, rats, and mice (Arko, 1972, 1973, 1974). This technique allowed the repeated sampling of microorganisms growing in vivo, and raised the possibility of studying the in vivo interaction of antibodies, leukocytes, and other host factors, as well as the chemotherapeutic effects of new drugs on organisms.

Artificial vinyl cylinders were employed by Flynn and Waitkins (1973) to cultivate and study the fate of gonococci in the mouse. They showed that gonococci in urethral exudates were resistant to bactericidal effects in vitro of complement plus rabbit and human natural or immune antibodies, although, after repeated subcultures, the same strain was readily killed by these bactericidal agents However, neither type of gonococcus was totally resistant to destruction by the host's defenses.

There was a discrepancy between the survival times observed by Arko (1972) and those reported by Flynn and Waitkins (1973): Flynn and

Waitkins reported that the gonococci survived for 2 days, whereas Arko noted survival for periods up to several months. "Subsequently, Waitkins (1975) showed that when gonococci were taken into cells grown in tissue culture and then put into chambers implanted in mice, the survival time of the intracellular bacteria was extended." Waitkins explained that tissue culture cells ingest gonococci, and subsequently protect them from the action of bactericidal agents. Hafiz and McEntegart (1977) attempted to confirm Waitkin's (1975) assumption. They used Millipore diffusion chambers which allowed free access to both humoral and cellular elements. The organisms survived for 18 to 49 days when protected from cellular factors. This had not been the case when Flynn and Waitkins (1973) used Arko-type chambers.

"Utilizing another subcutaneous chamber technique, Scales and Kraus (1974) showed that the immunity developed by guinea pigs infected with N. gonorrhoeae could be transferred to normal susceptible guinea pigs." Serum from these guinea pigs was bactericidal in vitro, and protected virgin guinea pigs from in vivo challenge.

In developing an animal model for the study of gonococcus infection Buchanan and Gotschlich (1973) employed the natural chorioallantoic membrane of chick embryo. When types 1, 2, 3, and 4 gonococci were plaed on the intact chorioallantoic membrane, types 1 and 2 produced infection significantly more often than did types 3 and 4, establishing in the animal model the same correlation between colony types and infectivity observed in humans.

"Less-fastidious organisms frequently have been grown in dialysis chambers. Implanted chambers containing bursa from chicks elicited

enhanced antibody response in surgically bursectomized chickens immunized with human erythrocytes (Dent et al., 1968). Bacterial cultures were used to confirm that chambers containing the bursa were contaminated; therefore the enhanced response might have been a function of bacterial products released from contaminated chambers."

"Osebold and colleagues conducted a series of experiments on cellular immunity to Listeria monocytogenes in diffusion chambers implanted in the peritoneal cavities of mice (Osebold and DiCapua, 1968; Osebold et al., 1970a; Osebold et al., 1970b). This model system was useful for studying mechanisms of resistance to infections by facultative intracellular parasites. The researchers reported that live bacteria must contact macrophages for development of good cellular immunity. Humoral substances produced in response to diffusible antigens were not able to inactivate the organism. In several instances, implantation of chambers without bacteria increased the host's resistance to later challenge with Listeria, indicating that nonspecific resistance was associated with the presence of the foreign body. This was an important finding, for it showed that such resistance studies must be carefully interpreted to separate the role of the chamber from that of the enclosed organism. Osebold et. al., (1974) also found that tissue reactions progressed around the chambers until a chronic abscess was formed, and that pleomorphic mutants of Listeria appeared during prolonged cultivation in implanted chambers."

"Millipore diffusion chambers loaded with spores or vegetative cells of Clostridium botulinum were implanted within guinea pigs (Suzuki et al., 1971). It was shown that body fluids did not attack spores, but bacteriolytic enzymes in the fluids did attack vegetative

cells which release toxin, and polymorphonuclear leukocyte engulfment was necessary for germination and release of the spore-bound toxin."

"Gerhardt and coworkers purposely managed an ex vivo hemodialysis culture to investigate its utility as a technique for growing microorganisms entirely on nutrients from the blood stream of a living animal and yet separate from the macromolecular and cellular defense mechanisms of the blood (Quarles, 1973; Quarles et al., 1974; Gerhardt et al., 1977; Mohan et al., 1977; Belding et al., 1976). One system they used consisted of a goat, an artificial-kidney hemodialyzer, and a modular fermentor (Quarles et al., 1974). A second system consisted of a goat and a small culture chamber (3.3 ml volume) designed to allow use of various membranes and to remain attached to the neck of the animal for several weeks (Gerhardt et al., 1977). In both systems, the blood stream was shunted surgically via prosthetic tubing from a carotid artery through the hollow-fiber membranes in the hemodialyzer or to the reservoir side of the membrane in the small chamber, and back to a jugular vein. Experiments with 16 pathogenic organisms and 5 types of mammalian cells indicated that most aerobes grew well; on the other hand, obligate anaerobes did not grow. Gerhardt and colleagues studied the synergism of penicillin and gentamycin against Listeria monocytogenes (Mohan et al., 1977)."

### 1.2.2 Mammalian cells

Antibody-producing cells, blood and bone marrow cells, tumor cells, and various other cells from mammals have been cultivated in vivo within diffusion chambers. This technique is used primarily because such cells grow poorly, or not at all, in vitro, and because the technique can be used to establish the existence of humoral

influences on various cellular processes. Other uses of the technique include quantification of *in vivo* cell growth, determination of the effects of chemotherapeutic drugs and their host-mediated metabolites on the implanted cells, study of the host response to diffusible products from the cells within the chambers, and the study of cell and tissue differentiation during extended cultivation periods.

1.2.2.1 *Antibody-producing cells*

"*In vivo* diffusion chambers implanted in mice were used for the cultivation of human lymph node, spleen, bone marrow, and peripheral white blood cells to study their capacity to form antibodies against a test antigen, *Salmonella typhosa* (Gengozian, 1964). Lymph node and spleen cells were induced to actively synthesize antibodies while bone marrow and white blood cells produced negative results."

"The effects of Rauscher Leukemia Virus (RLV) on antibody production and on induction of cellular changes was studied with mouse spleen cells cultivated in diffusion chambers implanted in isogenic hosts (Borella, 1969, 1971, 1972). This was an ideal system for the study of RLV-induced immunosuppression at the cellular level. Spleen extracts from infected mice inhibited antibody formation in the spleen cells cultured in the chambers. Antigen stimulation of the infected cell culture was found to alter the cellular pathway induced by the virus."

"Makinodan and coworkers have published a series of articles on antibody-forming cells (Urso and Makinodan, 1963; Capalbo and Makinodan, 1964; Makinodan et al., 1965; Nettesheim et al., 1966; Makinodan et al., 1967; Vann, 1969; Vann and Makinodan, 1969; Sado, 1969; Sado et al., 1970; Groves et al., 1970). An implanted diffusion

chamber was used in these studies because it represented a closed system obviating the problem of cells entering or leaving the compartment being assayed. In most of the studies, the immune response of mouse spleen cells was examined, using sheep erythrocytes as antigen. Transformation of precursor cells into antibody-forming cells occurred shortly after mitosis."

1.2.2.2. Blood and Bone marrow cells

"Experimental results from use of diffusion chambers in vivo for culturing blood and bone marrow cells were reviewed in detail by Stohlman, Quesenberry, and Tyler (1973). Cells of rat, mouse, goat, rabbit, and human origin have been cultured in a variety of hosts."

2.2.2.2.1. Rat cells

Bone formation was observed in about 60% of the explants of rat bone marrow cultivated in diffusion chambers in the abdomen of the rat for 3 weeks or longer (Rosin et al., 1963). Bone formation occurred only in the presence of osteogenic cells. Petersen et al. (1974) cultured rat bone marrow in diffusion chambers implanted into the peritoneal cavities of rats and mice. Granulopoiesis was enhanced in mouse hosts and remained at a steady-state equilibrium in rat hosts, indicating the importance of host factors affecting growth in diffusion chambers. Mononuclear cells isolated from rat blood were cultured in diffusion chambers to allow study of the possible conversion of lymphocytes to macrophages (Benestad et al., 1971).

"Rasmussen and Hjortdal (1969) investigated the origin of fibroblasts in cultures of blood and blood buffy coat cells. Microscopic examination of homologous blood and buffy coat cells, cultured for 3-week periods in diffusion chambers in the peritoneum of

rats, showed that neither fibroblasts nor connective tissue fibers developed inside the chambers when contamination with extraneous connective tissue had been prevented. Consequently, the development of fibroblasts in blood and buffy coat culture was linked to contamination with connective tissue cells during sampling of the blood."

Diaphyseal fragments of previously curretted rat femurs were cultivated in vivo in diffusion chambers by Faradji et al. (1980). Histological examination of the chamber membranes revealed the presence of fibroblast-like cells. The study of the sexual chromatin demonstrated that these fibroblast-like cells, found spread all over the inner filter surfaces, were of donor origin. No cell growth was observed on the inner surfaces of the control diffusion chambers, which were free of bone explant. These results agreed well with those of Rasmussen and Hjortdal (1969).

1.2.2.2.2. Mouse cells

Boyum and Borgstrom (1970) reported a technique for determining the concentration of granulocytic stem cells by culturing mouse bone marrow cells in diffusion chambers intraperitoneally. Boyum et al. (1972) and Lovhaug et al. (1978) cultured mouse bone marrow cells in diffusion chambers implanted within the abdominal cavity of mice, which were subjected to various treatments. The researchers concluded that pre-irradiation of hosts enhanced the growth of cells in granulocytic series during the early phase of the culture period, and that hypoxia depresses granulopoiesis by depleting the pool of progenitor cells. "It was shown that the marrow cells could retain their biological activity intraperitoneally inserted within the

Algire-type chambers for 30 days (Berman and Kaplan, 1959, 1960)." Proliferating and differentiating granulocytes and macrophages were consistently present while lymphocytes from the inoculum were gradually lost during the culture period (Berman and Kaplan, 1960). In vivo, there is stimulation of granulocytopoiesis by one or more diffusible factors (Rothstein et al., 1971). After whole-body irradiation of mice, humoral substances capable of stimulating the growth of granulocytic and macrophage-like bone marrow cells in diffusion chamber cultures in vivo were observed (Beran, 1975). "Similarly, chamber marrow incubated for 6 days in mice, and stimulated with a specific antigen displayed increased eosinophilopoietic activity when compared to an unstimulated control (McGarry and Miller, 1974)." This fact was interpreted as indicating the action of a diffusible granulopoietic stimulus specific for eosinophil granulocytes.

A diffusion culture technique for mouse marrow culture was used to determine the effect of a granulocyte inhibitor on the proliferation of the pluripotent stem cell and the granulocyte progenitor cell (MacVittie and McCarthy, 1975). The early injection of inhibitor (chalone) resulted in a significantly reduced number of granulocytic progeny within the diffusion chambers, while there was no inhibition of mouse fibroblasts cultured under identical conditions.

"Diffusion chambers were used to examine the osteogenic potential of irradiated marrow (Kuralesova, 1968). The chamber was implanted intraperitoneally into mice. After 10 days, restoration of osteogenesis began to occur, and by day 15, it was close to normal."

"Algire-type diffusion chambers with Millipore or Nuclepore filters were compared for use with culturing mouse marrow (Carsten et al., 1975). Marrow proliferation and differentiation were comparable in the two types of chambers, but cell recovery was greater with the Nuclepore filters."

"Granulocytopoiesis was examined by culturing mouse bone marrow cells in diffusion chambers for up to 22 days (Marmor et al., 1975). Cells proliferated logarithmically for 7 days, after which the population remained stable for as long as 14 days, probably because diffusion became limiting i.e., the ratio of cell number to membrane area had increased to a critical point. The plateau state of the cells during this period resembled granulocyte growth in normal bone marrow."

"Mouse marrow cells at concentration of $1.1 \times 10^6$ and $1.0 \times 10^5$ cells per ml, in diffusion chambers implanted in mice, resulted in less cell growth at the higher concentration than at the lower concentration (Quesenberry et al., 1974). It was suggested that the decreased growth could reflect a type of feedback inhibition, or the release of various nucleases and proteases as cells were destroyed." An alternative theory is that high concentration may have altered the environment by limiting diffusion of nutrients, leading to decreased growth.

Sullivan et al. (1980) studied the effect of Colony Stimulating Activity (CSA) levels and irradiation-induced marrow hypoplasia upon granulopoiesis in intraperitoneal diffusion chambers in host mice. Endotoxin injections led to markedly elevated CSA level, and the CSA was shown to diffuse into the chamber, but this manipulation did not

significantly accelerate cell growth in diffusion chambers. Pre-irradiation of the host mice produced no elevation of CSA, but resulted in significant stimulation of granulopoiesis. It was concluded, therefore, that CSA elevation did not per se provide an effective stimulus for granulocyte proliferation within diffusion chambers.

"Diffusion chambers have been very useful in the study of the kinetics and regulation of myelopoiesis, in order to better understand disorders of granulocytopoiesis such as leukemia (Stohlman et al., 1973). The growth of mouse marrow (consisting of myeloid elements, macrophages, and pluripotent stem cells) was influenced by humoral factors and also by the cell concentration in the chambers (Tyler et al., 1972; Niskanen et al., 1974). However, stem cell growth has not been ruled out as the cause of the increased myelopoiesis in the chambers (Tyler et al., 1976)."

"Haematopoietic cells have inoculated and incubated in chambers, showing that mononuclear leukocytes give rise to granulocytes and macrophages which grow and differentiate; however, lymphocytes from the inoculum were gradually lost (Benestad, 1970; Breivik et al., 1971; Breivik, 1971; Benestad and Reikvam, 1975). Granulopoiesis was stimulated more in the chambers than in normal, steady-state conditions (in situ), and its rate could be manipulated (Benestad, 1972; Breivik and Benestad, 1972)."

Benestad and Toogood (1982) have maintained that cell growth must not be restricted by the diffusion capacity of chamber membranes if the diffusion cultures is to serve as a reliable assay for systemic or local peritoneal regulators of proliferation.

In a double diffusion chamber culture, bone marrow cells produced diffusible factors that prevented spleen colony-forming cells from entering the cell cycle (Benestad et al., 1978). Granulocytes and macrophages did not produce such factors. Therefore, it was concluded that diffusible factors, rather than cell-to-cell contact, appeared to be involved in the inhibition.

1.2.2.2.3. Goat cells

"Autologous bone marrow cells cultured in diffusion chambers implanted in goats provided evidence for diffusion of hematopoietic stimulators from the host into the chamber (Laissue et al., 1974, 1975). Irradiation appeared to intensify the stimulation."

"Goat erythrocytes and leukocytes maintained their numbers in an ex vivo hemodialysis culture system (Gerhardt et al., 1977)."

1.2.2.2.4 Rabbit cells

"To evaluate factors influencing granulopoiesis, Willemze et al. (1978) inserted diffusion chambers containing bone marrow cells into the peritoneal cavity of rabbits. Using rabbits as hosts allowed the experimenters to work with autologous bone marrow and up to 30 chambers in the cavity. The results showed that mature granulocytes inhibited both myeloid and erythroid cell production."

1.2.2.2.5 Human cells

"Boyum and coworkers have conducted several studies on the growth and differentiation of human bone marrow in implanted chambers (Boyum et al., 1972a, 1976; Lovhaug et al., 1978). In one study, human bone marrow cells were implanted in the abdominal cavities of mice. As with mouse bone marrow cells (Boyum et al., 1972b), both granulocytic cells and macrophages grew in the chambers (Boyum et al., 1972a). In

a more recent study, Boyum et al. (1976) found that granulopoiesis was inhibited when mature granulocytes from human blood or syngeneic mouse peritoneal fluid were added to mouse bone marrow cells. The inhibition appeared to be tissue specific and caused by a diffusible factor. Instead of granulopoiesis, there was stimulation of macrophage formation."

"Other studies have indicated that blood cells proliferate and are capable of differentiating into granulocytic, erythrocytic, megakaryotic, and macrophagic lines in diffusion chambers, establishing that the blood of normal humans contains progenitor cells (Boecker et al., 1971; Barr et al., 1975; Chikkappa et al., 1978)."

"Study of human marrow cells cultivated in diffusion chambers, implanted intraperitoneally in normal and irradiated mice, has demonstrated that the proliferation and maturation of granulopoietic cells is greatest in heavily irradiated hosts (Squires, 1975)." A linear relationship was found to exist between the number of cells inoculated and the number of cells harvested after 8 or 10 days of incubation. Autologous human bone marrow placed in subcutaneous diffusion chambers did not survive as such; there was a replacement of cell type, so that only fibroblasts and histiocytes remained (Green, 1966). This *in vivo* method was therefore not superior to the usual *in vitro* cultivation of bone marrow.

1.2.2.3 *Tumor cells*

"Using diffusion chambers implanted in rats, Laerum et al. (1973) cultivated four types of malignant cells; mammary cells of rats, melanoma cells of golden hamsters, ethylnitrosourea-induced leukemic cells of rats, and leukemic cells from two humans with untreated,

acute myeloblastic leukemia. All the cell types proliferated in the chambers for periods of 8 to 13 days. Greater proliferation of the hamster melanoma cells occurred when the cells were cultured in hamster and mouse hosts, than in two different strains of rats apparently indicating that the cells grow better in isologous than in heterologous host animals (Schieferstein and Laerum, 1974). Cells from four human tumors (carcinomas of the stomach, lung, and ovary, and adenocarcinomas of the breat) grew when placed in diffusion chambers implanted in rats and hamsters (Evgenjeva, 1970). The tumor tissues maintained their histological specificity in the chambers."

"Host irradiation did not affect the growth of HeLa cells cultured in diffusion chambers (Meck et al., 1976)." It was reasoned that diffusion culture might prove especially useful in investigations such as the assay of the kinetics and response of individual patients' tumors to therapy in a host-mediated system. This was in contrast to the stimulated growth of hemic cells observed in chambers hosted by irradiated mice and goats (Boyum et al., 1972b; Laissue et al., 1974). Various mouse tumor cells proliferated in dialysis chambers implanted in chick chorioallantois (Tucker and Owen, 1969). Although these tumor cells could be grown in vitro, an advantage of the dialysis culture is that it is easy to carry out, requires no specific tissue culture media, and may be suitable for the growth of tumor cells from a variety of species.

Transplantation-immunity reactions also were investigated with a dialysis technique, and the results showed that diffusible toxic antibody was involved in tumor transplantation rejection (Ambrose, 1969). This factor, apparently an IgG immunoglobulin, seemed to be

present only in hamsters immunized specifically to SV40 tumor specific transplants; adenovirus 31 tumor cells in diffusion chambers were not inhibited in hamsters immunized against SV40 tumor specific transplantation antigen." Green (1966) placed autologous human tumor tissue within subcutaneous dialysis chambers. The tumor tissue did not survive in any of the 10 cases studied.

"The diffusion chamber technique has been important for the study of human leukemic cells. The growth of peripheral blood cells from human leukemic patients and controls was characterized by this method (Hoelzer et al., 1974; Hoelzer et al., 1976). Most types of leukemic cells increased in number within the chambers, due mainly to proliferation of blast cells and formation of granulopoietic cells (Hoelzer et al., 1977). Growth patterns differed widely, and appeared to depend on the type of leukemia."

"In a study of whether the in vivo maturation defect in acute leukemia is due to environmental or to cellular factors, human leukemic cells were cultured in chambers implanted in the abdominal cavity of mice (Fauerholdt and Jacobsen, 1975). The defect appeared to be caused by cellular factors. Boecker et al. (1978) used bone marrow from a patient with untreated acute promyelocytic leukemia to study leukemic cell differentiation in diffusion chambers. For this leukemia microenvironmental factors were able to determine whether or not gene expression was leukemic. Boecker et al. (1975) cultivated Hodgkins cells in diffusion chambers implanted in the peritoneum of irradiated mice, and found evidence that the cells probably originate from B lymphocytes."

"Leukemic cells from the rat also have been cultivated within implanted chambers (Vilpo, 1972)." Most of the chamber cells in the cultures were chloroma cells. Although the net chloroma cell population ceased to grow after one week of culture, individual cells were still profuse after 56 days.

1.2.2.4. Other cell types

"Spleen tissues from rats, guinea pigs, and mice were enclosed in diffusion chambers and implanted in irradiated mice, resulting in increased survival and hematopoietic recovery rates for the mice (Spertzel and Pollard, 1970). The results suggested that a humoral factor was responsible for the therapeutic effect. Earlier, in a similar study, it was found that chambers containing thymus cells implanted in thymectomized mice restored radiation resistance (Schneiberg et al., 1968). Overall, the diffusion chamber technique has been very useful for studying thymic humoral influences (Athens, 1970; Levey et al., 1963; Stutman et al., 1970; Trench et al., 1966)." The restorative action observed in mice with diffusion chambers is attributed to the contribution of a humoral factor in the maturation of antibody-producing cells.

Implants of rabbit neonatal pancreas, encased in diffusion chambers led to reversal of streptozotocin-induced diabetes in rats (Gates and Lazarus, 1977). Blood glucose, plasma-insulin, and oral glucose-tolerance test results returned to normal, an indication that rabbit neonatal pancreatic implants may be feasible therapy for diabetic patients who require insulin. Implants of other non-syngeneic endocrine cells -- i.e., pituitary, thyroid, and ovary --may be useful in other hyperendocrine systems.

In view of the potential importance of this type of implantation technique in the treatment of diabetes mellitus, Theodorou and Howell (1979) studied some of the commercially available types of membrane to determine the transit time across the membranes for glucose and insulin. Although islets transplanted in this way maintained adequate long-term insulin output, they were unlikely to be able to maintain minute-by-minute regulation of blood-glucose concentrations.

"Hairless-mouse epidermal cells appeared to remain intact for the first 24 h of containment in diffusion chambers, but then significant cell loss occurred (Laerum and Boyum, 1970). In another study, cells of rabbit aortic endothelium were cultured in diffusion chambers implanted in rabbit abdominal cavities so that the biological nature of the cells could be investigated (Kitsukawa, 1969). Ring formations of two to three cells appeared often, and the cells tended to form "alveolar-like" arrangements around clusters of erythrocytes. In an experiment by Grillo and Spink (1968), liver and spleen homografts grew in diffusion chambers implanted in the abdominal cavity of newts, demonstrating DNA synthesis and cell proliferation." In another study, the histocompatibility interactions between mixed types of mouse spleen cells were investigated by use of diffusion chambers (Harrison et al., 1968); the results showed the interactions to be immunological in nature.

"Delayed hypersensitivity to 1-fluoro-2,4-dinitrobenzene (DNFB) and Mycobacterium tuberculosis was studied by implanting chambers containing peritoneal exudate cells from guinea pigs sensitized to these agents into unsensitized animals (Guthrie and Nunez, 1970); the recipients developed specific skin-test reactions to DNFB, but not to

old tuberculin or to a purified protein derivative. Lymphoid tissue was cultured in implanted chambers to test the chambers, made of 1 x 2 cm envelopes of membrane filters with 0.1 to 0.3 μm pores (Alekseeva and Yunker, 1969)." The diffusion chambers in this study were very small, to enable implantation of several chambers in a small animal at once. No thick connective tissue capsule formed around the chambers. Germain et al. (1966) grew rat and human liver tissue cells in diffusion chambers to study viral agents and drug injury. Rat tissue implanted in rats and guinea pigs was unsuccessful, while human embryonic liver cells survived well in the diffusion chambers within guinea pigs.

"Mouse macrophage polykaryons (inflammatory giant cells) cultured in diffusion chambers formed at sites of inflammation by fusion of newly arrived macrophages with macrophages already in situ, many of which exhibited chromosomal abnormalities (Mariano and Spector, 1974). The antibacterial activity in the fluid obtained from subcutaneously implanted chambers in rats was similar to that observed in serum after intramuscular injection of carbenicillin (Gardner et al., 1973). A subsequent study showed that the levels of penicillin G in the chamber fluid were also similar to the serum antimicrobial activity after intramuscular injection (Tight et al., 1975). The effect of an excess of bone mass (a hypothetical source of chalone -- a diffusible tissue-specific substance which regulates cellular growth by means of feedback inhibition) on bone formation was also studied using a diffusion chamber (Videman et al., 1978)." The DNA synthesis of the osteogenetic cells was inhibited significantly by the excess bone, suggesting the existence of chalone (Vilpo et al., 1978).

"Humans have also been used as hosts for subcutaneous diffusion chambers (Brooks et al., 1960; Green, 1966). Although autologous and homologous lymphocytes grew in such systems, the results were more satisfactory with the usual in vitro tissue culture technique (Green, 1966)" and with chambers placed intraperitoneally in animals (Brooks et al., 1960).

An interesting and possibly valuable use of diffusion culture was as an in vivo test system for mutagenic and carcinogenic assays (Huang and Furukawa, 1978). The system involved culturing human cells or Chinese hamster cells in diffusion chambers in mice. After injecting the host with a test compound, the experimenters looked for induction of mutations, sister chromatid exchanges, or chromosome aberrations in the implanted cells as indicators of mutagenicity or carcinogenicity. This system has particular merit for testing compounds which need to be metabolically activated.

## 2. ECOLOGICAL APPLICATIONS

The dialysis technique has been used in ecological studies primarily for three purposes: to examine the interbiosis between different cell populations (e.g., mutualism, commensalism, competition, or inhibition); to study a cell population sequestered in a natural environment in a way that permits easy and total cell recovery after incubation; and to characterize the *in vitro* growth of an organism, the metabolites, and the effects of the metabolites.

### 2.1 Interbiosis studies

A natural extension of the use of dialysis culture for the study of diffusible metabolic products is to bring together two or more populations of microorganisms on opposite sides of membranes so that their metabolites can interchange. The situation may be beneficial to both populations (mutualism) or to one population only (commensalism); it may be inhibitory (inhibition, antibiosis, or antagonism); or the two populations may compete with each other for common nutrients (competition). One or more of these phenomena may appear in a given pair of populations.

Frankland and Ward (1893) examined the interaction between *Bacillus liquifaciens* and *Bacillus anthrax*, expecting some antagonistic interactions between them. After 24 days of incubation, both populations of organisms were still alive, the anthrax appearing chiefly in the form of spores. Neither bacteria excreted poisonous metabolites.

"In experimentation conducted for dairy purposes, the use of a double dialysis chamber allowed the detection of an antagonistic

relationship between two types of bacteria (Collins and Tillion, 1977). Pure cultures of Streptococcus diacetilactis and Streptococcus lactis were inoculated into separate compartments of the chamber and incubated. S. diacetilactis produced a diffusible antibiotic, as indicated by the inhibited growth of S. lactis as compared to that shown in a control experiment."

Akaki (1965) found that culture filtrate of Candida utilis, when added to the growth medium, enhanced the growth of Mycotorula sp., but no enhancement of C. utilis growth was elicited by Mycotorula sp. supernatant. Akaki carried out dialysis culture experiments which gave similar results: A dialyzable product of C. utilis stimulated growth of Mycotorula sp. The stimulatory factor seemed to be biotin.

Microbial commensalism and commensalism-plus-competition were studied with four pairs of microorganisms; Proteus vulgaris with Saccharomyces cerevisiae; Acetobacter suboxydans with Saccharomyces uvarum; Lactobacillus casei with S. cerevisiae; and L. casei with S. cerevisiae (Smith, 1976). Vitamins (nicotinic acid and riboflavin), and small molecular weight incomplete oxidation product (fructose), were stimulatory; the organisms in pairs were in competition for glucose in all cases except for the second one.

"The growth of autotrophs, phototrophs, and heterotrophs in mixed cultures was studied using both dialysis and conventional methods (Pan and Umbreit, 1972b). The stimulatory and inhibitory effects of the cultures were found to be highly specific. Escherichia coli and Pseudomonas aeruginosa had essentially no effect on Nitrobacter agilis. Streptococcus faecalis caused slight inhibition, and Hydrogenomonas eutropha caused slight stimulation, of Nitrobacter.

Pseudomonas fluorescens and Saccharomyces cerevisiae enhanced the growth of Plectonema boryanum, whereas E. coli inhibited such growth. Dialysis culture showed less effect, and was considered less successful for the demonstration of the interactions, than the use of ordinary mixed cultures. It should be noted, however, that limitations such as inadequate diffusion and aeration may have influenced the results."

The effect of one alga on the growth of another varies with the media, the size and condition of the inocula, and the algae concerned. McVeigh and Brown (1954) examined the effects of two algae, Chlamydomonas chlamydogama and Haematococcus pluvialis, on each other's growth in a dialysis mixed culture. In an inorganic medium, when the sugar was used up the growth of each was stimulated by the other. Mutual inhibition was observed in the more complex media when the algae were grown together.

"A dialysis technique was used for separating free-living rhizobia from soybean cells to examine the exchange of solutes between the two cell types (Reporter and Hermina, 1975; Reporter, 1976; Bednarski and Reporter, 1976, 1978). Rhizobia of different species produced chemicals that induced soybean cells to produce other chemicals, which in turn affected acetylene reduction activity in test rhizobia."

Mixed culture interactions can now be studied via dialysis under precisely controlled conditions with commercially available equipment (New Brunswick Scientific Co., Inc., Edison, NJ), A mixed dialysis culture apparatus called "Ecologen" is mounted on a shaker to provide

efficient mixing and uniform dispersion of metabolic products. The apparatus is designed for repeated autoclaving with the medium in the growth chamber.

## 2.2 *In situ* studies

Beard and Meadowcroft (1935) used a dialysis culture technique *in situ* for examining the death rates of *Eberthella typhosa* and *Escherichia coli* under closely simulated conditions in polluted sea water. They demonstrated the reliability of the Wilson-Blair bismuth-sulfite medium for typhoid selection and for quantitative studies in polluted water. The possibility that both species could survive for at least one month was indicated. *E. coli* was shown to be sufficiently resistant to sea water to serve as an indicator of polluted conditions in such an environment.

"Hendricks and Morrison (1967) observed the growth of enteric bacteria in mountain stream water *in situ*. Dialysis tubing containing six test organisms was suspended in the stream, and samples were taken periodically. The results showed that a clear, cold mountain stream not only could maintain populations but also could support growth of enteric bacteria. The dialysis experiments did not completely simulate *in situ* conditions, however, because natural predators such as protozoa were not present."

"Several phytoplankton species have been studied using dialysis culture (Jensen et al., 1972; Prakash et al., 1973; Jensen and Rystad, 1973, 1976; Skoglund and Jensen, 1976). *In situ* use of the dialysis technique allowed the cells to accumulate trace substances from large volumes of water. In laboratory studies, the nitrogen-limited growth of diatoms (Skoglund and Jensen, 1976) and the zinc and copper

tolerance of three species of marine phytoplankton (Jensen et al., 1974; Jensen and Rystad, 1976) were examined. Generally, these laboratory dialysis sytems provided conditions which simulated those found in situ."

Algal cultures grown in dialysis bags suspended in sewage reached extremely high biomass densities, as compared to conventional batch cultures, in experiments by Dor (1975). The developing culture extracted nutrients from the surrounding sewage through the semipermeable dialysis membrane, but remained separated from the enteric microorganisms of wastewater.

"Diffusion chambers specifically designed for studying the survival of coliform bacteria, both in situ and in the laboratory under controlled conditions, were used by McFeters and Stuart (1972) and by Bissonnette et al. (1975). In experiments with well water, fecal streptococci and coliform (indicator) bacteria remained viable to a similar extent (McFeters et al., 1974)."

"An apparatus consisting of dialysis tubing suspended from a styrofoam flotation ring was built to study bacterial growth in a farm pond (Baskett and Lulves, 1974). The utility and sampling ease of the apparatus was demonstrated by inoculating Brevibacterium sp. into the tubing and monitoring its growth for 96 h. The viable population increased significantly, indicating that nutrients in the pond water necessary for growth and multiplication of the bacterium diffused through the dialysis membrane. The permeability of the membrane, however, was the limiting factor in these ecological dialysis studies. Vargo et al. (1975), while using scanning electron microscopy to study the problem, found that regenerated cellulose membranes provided an

ideal substrate for epiphytic growth *in situ*, thus influencing solute transport."

"A diffusion chamber with an internal battery-powered stirring mechanism for agitation was developed to study the survival of indicator bacteria in a marine estuary (Vasconcelos and Swartz, 1976). The stirring mechanism improved permeability and the reaction between bacteria and pollutants, and insured a uniform cell suspension for homogeneous sampling. *Streptococcus faecalis* survived longer than did *Salmonella enteritidis*, *Klebsiella pneumoniae*, and *E. coli*. In these experiments, membrane filters were employed (polycarbonate, with 0.4 μm pores), as opposed to less porous dialysis membranes."

*In situ* dialysis cage culture of diatoms was used to monitor heavy-metal pollutions in two Norwegian fjords (Eide et al., 1979). Uptake of heavy metals generally increased with increasing heavy-metal content in seawater. The test algae accumulated heavy metals from even low concentrations in the seawater, indicating the degree of pollution in cases when no reduced growth rate was observed. *In situ* cage culture of algae should be a valuable tool in the monitoring of heavy-metal pollution of marine as well as freshwater environments.

"The adverse effects of polychlorinated hydrocarbons on marine phytoplankton were examined using *in situ* dialysis by Powers et al. (1976) and Powers et al. (1977). Growth of the dinoflagellate *Exuviella baltica*, in the presence of dichlorodiphenylchloroethane, and of *Thalassiosira* sp., in the presence of polychlorinated biphenyls, was inhibited. In general, polychlorinated biphenyls at relatively low concentrations (10 μl per liter) adversely affected

certain physiological functions (chlorophyll a levels and carbon fixation per cell) of the algae."

Dialysis culture was used to estimate growth rates of phytoplankton in situ (Crumpton, 1980; Crumpton and Wetzel, in press; Owens et al., 1977; Sieburth et al., 1977). Crumpton (1980) and Crumpton and Wetzel (in press) maintained that the dialysis technique could eliminate losses due to grazing and transport, and that they were able to estimate mortality from other processes by studying changes in numbers of dead cells. The growth rate of each population could thus be estimated based on changes in the number of living and dead cells over a specified time period.

Sieburth (1979) reported that diffusion culture might be useful in measuring phototrophic as well as heterotrophic productivity, in monitoring pathogen survival, and in determining perturbations caused by pollutants.

Algae were used for the determination of nutritional limiting factors in a marine environment in situ (Berland et al., 1976). Technological details of a five-month experiment in coastal Mediterranean waters, with 10 unialgal cultures and natural phytoplankton, were discussed.

"A dialysis technique was employed to study limiting nutrients and maximum growth rates for two diatoms, Skeletonema costatum and Asterionella japonica, in tanks with running seawater (Sakshaug, 1977)." The researcher concluded that dialysis culture appeared adequate for monitoring maximum growth rates in the natural environment, and he recommended that the technique be used to

investigate potentially limiting nutrients through the study of the chemical composition of the algae.

"The distribution and survival of Aeromonas sp., which is pathogenic to many animals, were examined using dialysis in a freshwater lake that received heated effluent from a nuclear reactor (Fliermans et al., 1977; Fliermans and Gorden, 1977)." Fliermans and Gorden (1977) modified the McFeter (1972) type diffusion chamber for facilitated sampling and resistance to damage during exposure in the water. At various water depths, the survival rate of A. hydrophila was always greater when the reactor was in operation.

Dialysis bags were used to study the behavior of bacteria of enteric or terrigeneous origin when they (Vibrio cholerae, Streptococcus faecalis, Staphylococcus aureus, and Pseudomonas aeruginosa) were ejected into the sea (Bianchi and Bensoussan, 1977; Slanetz and Bartley, 1965). In experimental ecosystems, some strains of marine origin proliferated in pure culture as well as in mixed culture. The bacterial concentration grew from $10^3$-$10^4$ cells per ml to $10^5$-$10^6$ cells per ml, and subsequently stabilized near the $10^4$ cells per ml level. On the other hand, under the same experimental conditions, the concentrations of any of non-marine origin never exceeded the initial concentrations.

"Survival of enteric viruses in estuarian waters was examined using cellulose dialysis tubes (Metcalf and Stiles, 1967). Virus survival was dependent on temperature, pollution levels, and type of virus. Inactivation rates of polioviruses and coxsachieviruses were determined through dialysis in situ in the Rio Grande River;

inactivation was exponential, the rate depending primarily on the water temperature (O'Brien and Newman, 1977)."

Experimental degradation of dead phytoplanktons in seawater was studied using dialysis bags (Dumas and Bianchi, 1972). Bags containing killed planktonic materials were immersed in situ to observe changes in biomaterial concentration and bacterial population. The process developed in two steps: the first, rapid step involved was with cytoplasmic constituents and the second step resulted from cell-wall hydrolysis and the transformation of organic matter.

Laboratory-cultured cells of five marine algae were placed in dialysis sacs and grown in situ in the Mediterranean sea (Maestrini and Kossut, 1981). Only two algae (Phaeodactylum tricornutum and Thalassiosira pseudonana) took up nutrients from seawater. Enrichment bioassays demonstrated that some algae were permanently limited by phosphorus, while for a few others, nitrogen and phosphorus could be equally limiting.

Diatomic planktons were isolated from their natural environment and their fates were followed by use of dialysis chambers (Pierre, 1969). Some species that were at first few in number became abundant; others disappeared. This dialysis apparatus was useful for research in flowing waters.

"Interstitial water samplers were constructed using dialysis membranes to separate water from particular matter, including bacteria, in lake waters and sediments (Mayer, 1976; Hesslein, 1976). Using a similar sampler, Winfrey and Zeikus (1977) found that sulfate inhibited methanogenesis in a freshwater lake sediment by altering normal carbon and electron flow. In a hypereutrophic lake, anaerobic

metabolism of freshly deposited particulate organic matter initially occurred at the sediment-water interface (Molongoski, 1978)."

Information was obtained about the matter-energy flow system of the plankton in a lake by using dialysis sacs (Toth, 1980). Matter balance figures for phytoplankton, if production was taken to be 100%, were as follows: loss to predation was 10.10%; residual mortality was 27.40%; the fraction of production appearing in actual biomass was 61.50%. Matter balance figures for bacterioplankton were: loss to predation, 10.10%; residual mortality, 67.50%; fraction appearing in actual biomass, 22.40%. Toth could not calculate respiratory energy loss because of the constant balance of physico-chemical properties inside the dialyzing sack with the open water outside the sack. In spite of this limitation, the use of dialysis sacs for hydrobiological measurements (e.g., water-quality tests, feeding experiments, estimation of turnover and production of algae, and bacterial morphological group) was validated.

### 2.3. In vitro studies

Chlorella vulgaris were cultured in collodion sacs suspended in nutrient solution that was constantly renewed (Pratt, 1944). Under non-dialysis conditions, accumulation of the growth-inhibiting substance chlorellin limits growth. When the sacs were suspended in a nutrient solution that was constantly renewed, growth improved markedly; the maximum rate and final amount of growth were more than twice those found in control cultures.

By the use of dialyzed Chlorella, two previously unknown stoichiometric light reactions were discovered (Warburg et al., 1968). The newly known reactions are the splitting of carbon dioxide from

carbon dioxide and the conversion of the carbon dioxide into the photolyte.

"Growth of the strictly autotrophic Thiobacillus thiooxidans ceased when the organism metabolized sulfur and keto acids accumulated to inhibitory levels. Dialysis led to greatly increased growth by removing inhibitory acids such as pyruvate and oxalacetate (Borichewski, 1967). A dialysis continuous fermentation was used to obtain a substantial increase in cell density of the methane-oxidizing strain M 102, confirming that the growth of the organism was inhibited by its metabolites (Naguib, 1975)."

"Dialysis culture also demonstrated that presumably obligate autotrophic bacteria grow on glucose rather than inorganic energy sources if metabolic products are prevented from accumulating in the culture environment (Pan and Umbreit, 1972a). Nitrosomonas europaea, Nitrobacter agilis, Thiobacillus denitrificans, T. neopolitanus, and T. thioparus were grown on glucose-salts media in the absence of specific inorganic energy sources. The metabolic product found to inhibit N. agilis was pyruvate acid, but the toxic product for N. europaea was not identified. Results with the Thiobacillus species indicated that pyruvate (or related keto acids) might be more inhibitory to the organism when it is growing on glucose than when it is growing on a specific nutrient."

Dialysis technique was used to study the swarming motility of Proteus mirabilis (Hoffman, 1974; Williams and Schwarzhoff, 1978). The researchers wanted to test the hypothesis that when the concentration of toxic metabolic products reaches a critical level it stimulates the formation of swarm cells, which detect and move out

away from the central colony and down the gradient in a negative chemotactic response. Dialysis of an agar medium from beneath failed to prevent swarming, and swarm cells recovered from one medium continued to swarm outward when placed on fresh medium. Thus, the hypothesis was disproven.

"A dialysis system was used to examine the energetics of nitrogen-fixation of (Nif-derepressed mutants of Klebsiella pneumonia (Andersen and Shanmugam, 1977). The system consisted of a dialysis bag (containing the inoculum and 25 ml of medium) suspended in a flask containing 250 ml of medium. The system allowed fresh medium to diffuse into the culture, and products (e.g., $NH_4^+$) to diffuse out. Results from monitoring the production of $NH_4^+$ and $H_2$ showed that nitrogenase-catalyzed $H_2$ production was a major factor in the economy of nitrogen fixation in vivo."

In vitro simulations of rumen fermentation using dialysis were studied in an experiment simulating both the removal of fermentation end-products and the flow of ingesta (Abe and Gumeno, 1973; Nakamura and Kurihara, 1978). Concentrations of volatile fatty acid and ammonia-N were maintained at levels similar to those found in situ in the rumen (Nakamura and Kurihara, 1978).

Sugiyama et al. (1978) employed a dialysis-enrichment method to detect Clostridium botulinum spores in honey. Honey was dialyzed before being inoculated, since it was felt that the high concentration of sugar was probably inhibiting the germination and growth of the bacteria.

## 3. FERMENTATIVE APPLICATIONS

Dialysis has been used in fermentation in order to enhance the production and recovery of microbial cells and their diffusible and non-diffusible products. Dialysis can improve fermentation by two interrelated approaches. In one approach, a membrane and a reservoir are employed in removing inhibitory metabolites from a fermenting culture, resulting in increases in the rate and extent of substrate conversion and permitting use of a relatively high concentration of substrate. Additionally, the dialysis separation of small molecular products represents a recovery step. A second approach is to dialyze the fermentor contents against a reservoir of substrate medium, primarily to retain cells (and non-diffusible products) in the fermentor for recovery purposes. This results in increased cell populations. Applied continuously, such a process also can allow increased throughput of substrate for conversion purposes. This approach can be applied to fermentations whether or not they are inhibited by metabolites.

### 3.1 Production of cells

Dairy starter cultures of Streptococcus and Lactobacillus sp. produce lactic acid, which inhibits their growth. To prevent this inhibition, dialysis can be used, removing lactate from the bacterial culture (Gerhardt and Gallup, 1963; Friedman and Gaden, 1970; Stieber et. al., 1977). Stieber and Gerhardt (1980) developed a mathematical model to simulate the continuous conversion of deproteinized whey into bacterial cells by a process in which fermentor contents were dialyzed through a membrane against water. Osborne (1977; Osborne and Brown, 1980) tested and patented (Osborne et al., 1975) such a fermentation

using a batch dialysis culture system. The design, based on that of Gallup and Gerhardt (1963), consisted of three components: a fermentor containing culture, a dialyzer, and a reservoir containing medium. The system provided 5 $cm^2$ of membrane area for every ml of culture, a ratio proven to be needed by the lactate-sensitive Streptococcus sp. The fermentor-to-reservoir volume ratio, was 1:15. Operation of the system with Streptococcus cremoris (Osborne, 1977) and with S. cremoris and Streptococcus lactis (Osborne and Brown, 1980) resulted in concentrations between 1 x $10^{11}$ and 1.5 x $10^{11}$ cells per ml.

Using another fully autoclavable system with membrane filters (cellulose acetate with 0.2 μm pore size), a 1:30 fermentor-to-reservoir volume ratio, and a fermentor circuit volume of 500 ml, Osborne (1977) calculated that the system could produce at least half the annual starter cultures needed in a dairy plant utilizing 100,000 gallons of milk per day for cheesemaking. The resulting starter concentrates had better activity than do conventional starters. The dialysis system also eliminated the requirement for centrifugation, which is costly, damages the cells, and can easily result in contamination.

"Another dialysis process tested for production of concentrates of Streptococcus lactis (Bergere and Hermier, 1968) was unsuccessful, probably because of the system's small membrane area in relation to the culture volume (less than 1 $cm^2$ per ml)."

Mercer (1981), combining and improving upon the innovations of several workers, developed a system for the production of Streptococcus lactis. He achieved a concentration of 6 x $10^{10}$

bacteria per ml by dialysis, compared to 1 x $10^{10}$ bacteria per ml in a non-dialyzed control. The lactic bacterium reached stationary growth phase when the viable number was approximately 1.2 x $10^{10}$ bacteria per ml, even in the dialyzed culture. The experimenter needed to clean or exchange the membranes to attain the higher cell numbers.

"Friedman and Gaden (1970) also used a batch dialysis system with three components (fermentor, reservoir, and dialyzer) to study the growth and lactic acid production of Lactobacillus delbrukii. The process revealed the specific growth rate at low lactate concentrations; this could not be determined in a conventional fermentation. The researchers also confirmed the inhibitory effect of lactate on the growth of the organism, quantified this effect and incorporated it into the mathematical model of Leudeking and Piret (1959) relating rate of growth to rate of acid production."

"As with the lactic acid bacteria, the growth of the yeast Mycotorula japonica on hexadecane or decane was improved greatly with the use of dialysis to relieve the inhibitory effects of lauric acid (Aida and Yamaguichi, 1969)."

Single-cell algal protein from sewage was produced by inoculating Scenedesmus obliquus into dialysis tubing containing tap water (300 ml) and suspending the tubing in a beaker containing 1.8 liters of sewage (Dor, 1975a, 1975b). Dialysis resulted in a three-fold increase in cell population, as compared to a control. Limits on the growth were lack of light, membrane permeability, membrane area, and agitation. Jensen (1976) tested the dialysis technique for production of algae on a large scale. A high ratio of membrane area to culture volume was found to be necessary to secure a satisfactory rate of

nutrient influx. These fermentations are promising especially because sewage wastewater is used as a nutritional medium.

"Lane (1977) investigated dialysis culture as a means for producing high concentrations of the food yeast Kluyveromyces fragilis from deproteinized whey, a residue of the dairy industry. He used a fermentor-to-reservoir volume ratio of 1:2.5 and four successive fresh reservoirs of medium during the 90-h fermentation period. The result was a biomass concentration of 90 g per liter and a residual lactose concentration of 5 to 8 g per liter in the reservoirs. In this fermentation system, dialysis resulted in a high cell concentration primarily because the culture was sequestered to a small part of the medium at any given moment, but in the long run had access to the entire medium. The principle differs from lactic acid fermentation by dialysis where dialysis serves primarily to enhance cell growth by removing inhibitory products from the culture."

"In the studies just described, substrate is supplied to the culture primarily via a membrane from a reservoir containing medium. Cell growth is limited by either the rate of diffusion of substrate into the culture, or a combination of both. Landwall and Holme (1977a, 1977b) dialyzed cultures of Escherichia coli B against a medium reservoir to supply substrate and remove toxic products. To ensure that substrate was not limiting, they also fed substrate directly into the culture fermentor. In this manner, and using a fermentor-to-reservoir ratio of 1:11, the researchers obtained biomass concentrations of 140-150 g (dry weight) per liter, as compared with 30-40 g per liter in a non-dialysis culture. The system employed by Landwall and Holme (1977a, 1977b) utilized dialysis principally to

remove toxic end-products which would decrease the molar growth yield of E. coli on glucose."

Marsot et al. (1981a, 1981b) used disposable, hollow-fiber hemodialyzers as a separate dialysis unit, coupled to a growth chamber, to cultivate Phaeodactylum tricornutum. This sytem allowed large-scale dialysis culture for a longer duration and with high biomass yields. The hollow-fiber dialysis units were independently replaceable, and the nutrient medium (seawater) was renewed via controlled hydraulic diffusion. Cell densities of up to $4 \times 10^7$ per ml were obtained.

Morandi and Valeri (1982) found that, if human diploid fibroblasts growing in a medium supplemented with fetal calf serum (FCS) was dialyzed against a large volume of medium without FCS, the growth was more rapid and the yields were more consistent. Considerable amounts of serum was saved by this approach.

### 3.2. Production of non-diffusible compounds

A variety of mammalian cells were grown in a flat-bed, hollow-fiber dialysis system (Ku et al., 1981). SV3T3 cells, baby hamster kidney cells, Vero cells, and rhesus monkey kidney cells, as well as such cell products as plasminogen activator and migration inhibitor factor, were produced. The flat-bed system displayed advantages over cartridge-type reactors for scale-up.

"To obtain concentrates of extracellular proteinase of Streptococcus faecalis var. liquifaciens, the bacterium was grown within dialysis tubing suspended in an Erlenmeyer flask containing medium (Millner, 1969). Dialysis methods for obtaining concentrates of non-diffusible enzymes are similar to those for obtaining cells.

Concentration of two proteolytic enzymes via dialysis culture also was achieved by growing a Sarcina strain (coccus P) in a dialysis bag suspended in a reservoir of medium (Bissel et al., 1971; Sarner et al., 1971), with a culture volume to reservoir volume ratio of 1:10. A concentration of $2.4 \times 10^9$ cells per ml was obtained with dialysis, as compared to $6 \times 10^8$ in conventional flasks, and the proteinases and other nondiffusible compounds were similarly concentrated. Another dialysis apparatus for producing concentrated extracellular protease and amylase consisted of two dialysis bags (Visking tubing), each protected by a perforated cylindrical glass shield and suspended in a two-liter glass vessel (Fogorty and Griffin, 1973). The apparatus was tested by cultivating Bacillus polymyxa, Serratia marcescens, Streptococcus thermophilus, and Clostridium acetobutylicum within the dialysis bags. The concentration of the enzymes in the bags was 4.8 to 22.7 times greater than that in conventional fermentation flasks."

"The production of heat-labile enterotoxin from E. coli by dialysis culture with a continuous feed of substrate was examined by Landwall and Mollby (1978). The fermentor-to-reservoir volume ratio was 1:10, and the feed (mostly glucose at 600 g per liter) was introduced into the fermentor at such a rate that the carbon source was not limiting. The dialysis process produced at least eight times more enterotoxin than did an ordinary fermentation process. Using simiar dialysis procedures, Landwall (1978) achieved a ten-fold increase in the concentration of extracellular protein A (2 g per liter) from Staphylococcus aureus A676, as compared to results from a control."

## 3.3 Production of diffusible compounds

"Abbott and Gerhardt (1970a) demonstrated that dialysis culture can be used to increase the production of a diffusible metabolite. Pseudomonas fluorescens was used to convert naphthalene to salicylic acid, which usually accumulates to inhibitory levels. A dialysis flask system with a culture volume of 150 ml and a dialysate volume of 1100 ml was used. Fermentation was conducted for 15 days with intermittent removal and replenishment of medium in the dialysate reservoir (4 times) in order to maintain low levels of product in the culture chamber. Although the salicylic acid produced was dilute, the total amount was determined to be 20 times greater than in a control without dialysis. Abbott and Gerhardt (1970b) found in a kinetic study, that maintenance metabolism accounted for 84 percent of the salicylate produced."

Tangnu and Ghose (1981) also employed dialysis to elicit improved production of salicylic acid from naphthalene by Corynebacterium renale. The total accumulated salicylic acid was 48.2 g, equivalent to a concentration of 19.3 g per liter in the fermentor. This represented 2.75 times the maximum level reached at the end of non-dialysis fermentation.

"Dialysis was also used in an attempt to alleviate product control over threonine production by an auxotroph of Escherichia coli, but inhibition, not enhancement, was observed (Abbott and Gerhardt, 1970c). This result led the researchers to conclude that depletion of $\alpha,\varepsilon$-diaminopimelic acid was the limiting factor, rather than threonine inhibition of its own synthesis.

"Kominek (1975a, 1975b, 1975c, 1975d) and Wang et al. (1981) developed and patented a dialysis extraction apparatus for production

and recovery of cycloheximide, an antifungal antibiotic, by Streptomyces griseus. As in the lactate and salicylate fermentations, cycloheximide inhibits its own synthesis; cycloheximide is also rapidly degraded in the absence of glucose. The dialysis system consisted of a 5-liter New Brunswick fermentor (3 liters of working volume) with a rubber mesh mat fastened around the baffles to support dialysis tubing ($\simeq$500 $cm^2$). The reservoir included the dialysis tubing as well as an extractor containing methylene chloride, that was connected by conduits to the tubing. The dialysate from the tubing was bubbled through the extractor to remove the cycloheximide; was then aerated to remove traces of solvent; and finally, was returned via conduits to the tubing for collection of more antibiotic. Glucose was continuously fed into the fermentor to prevent cycloheximide degradation and to ensure that the carbon source did not become limiting. By the tenth day of continuous operation, there was a two-fold increase in titer as compared to control, and 82 percent of the solids content of the extractor was cycloheximide. Therefore, use of the dialysis extraction apparatus increased the titer of the antibiotic, enhanced recovery of the antibiotic in a relatively pure form, and eliminated the need for a large reservoir. This work was the first to demonstrate the potential of dialysis for continuous production and recovery of microbial metabolism."

Hollow-fiber membrane dialyzers (Cordis-Dow artificial kidneys) were used to immobilize microbial cells for the continuous production of biochemicals (Kan and Schuler, 1978). Immobilization of the cells was achieved by containing them within the chamber (inside of the dialyzer jacket, but outside the hollow fibers), while solutes

diffused freely across the hollow-fiber membranes. The system was tested via examination of the conversion of l-histidine to urocanic acid by a heat-treated suspension of Pseudomonas fluorescens. The continuous-production experiments all lasted for at least 8 days. The rate of the reactions decreased with time, most likely as a result of degradation and denaturation of the enzyme. This type of system may have potential if microbial cells can be maintained in a living yet non-growing state. The half-life of a reactor containing such cells (disregarding membrane fouling) would be indefinite for the continuous production of a diffusible metabolite.

A dialysis process for the continuous fermentation of whey lactose to lactic acid was described mathematically (Coulman et al., 1977). The fermentation was mathematically modelled as a set of equations representing material balances and rate relationships in the fermentor and dialysate circuits. A final model was constructed by combining these equations with certain dimensionless parameters. The generalized dimensionless model was then solved for the steady state and used to simulate specific fermentation on a digital computer. Stieber et al. (1977) conducted laboratory experiments to validate the theoretical predictions. A series of steady state fermentations were managed nonaseptically for as long as 94 days and the results proved that the model allowed valid predictions.

Stieber and Gerhardt (1979) improved their mathematical model, developed previously, by incorporating separate terms for substrate limitation and product inhibition into the equation describing the rate of cell growth at steady states. The improved model was used to simulate the fermentation and the results agreed with previous

experimental tests using whole whey as the substrate. Further simulations were then made to guide experimental tests using deproteinized whey as substrate.

The generalized model was modified to simulate the outcomes of adding two systems; a prefermentor or a cell-recycling system (Stieber and Gerhardt, 1981a). Simulated results showed that the addition of nondialysis continuous prefermentor would not improve the process, but that the addition of cell recycling would greatly improve the process.

The previously developed and validated generalized mathematical model was again modified to simulate dialysate-feed systems for operating a dialysis continuous process for the ammonium-lactate fermentation (Stieber and Gerhardt, 1981b). The simulations predicted that the feeding of substrate into the dialysate circuit and thence into the fermentor circuit via dialysis would improve the production of cell mass and metabolites. An experiment was conducted to test the system in which the fermentor was operated without an effluent, thus immobilizing the cells. Experimental results showed that the system was effective in immobilizing cells for the purpose of producing a metabolite for a prolonged time. The substrate consumed by the cells is converted to product via maintenance metabolism and is sterilized by dialysis. This system, however, was apparently limited by slow rate of diffusion which led to the loss of 44% of the sugar fed without conversion.

In all of his works Stieber (Stieber, 1976; Coulman et al., 1977; Stieber et al., 1977; Stieber and Gerhardt, 1979a, 1979b, 1981a, 1981b) did not have to practise aseptic technique during his experimental period (for up to 94 days) because of low pH (5.5), the

high temperature (44 C), and the self-sterilizing characteristic of membranes.

Stieber (1976) tested Cordis-Dow artificial kidney and found that it was not suitable for continuous fermentation because the design, particularly lack of agitation in the dialyzer jacket, resulted in rapid clogging and decreased diffusion.

### 3.4 Dialysis sensors

"On-line sensors are becoming increasingly important in fermentation (Dobry and Jost, 1977)." One of the most rapidly expanding research areas related to analytical measurement is the development of potentiometric membrane electrodes with seletivity for ion, dissolved gases, and biological materials (Rechnitz, 1981). In the application of automatic instrumentation in fermentation, it is apparent that sensing elements are growing in importance (Kell, 1980).

"Schultz (abstract, 2nd Internat. Conf. Computer Appl. Ferment. Technol., Philadelphia, Pa., 1978) has used dialysis principles to develop an affinity sensor for continuous monitoring of glucose levels. The method depends on the reversible competition of glucose and a fluorescent labeled polysaccharide (FITC-dextran) for a specific binding site on a protein, concanavalin-A. Glucose from the fermentation liquor diffuses via a membrane into a probe and displaces the labeled polysaccharide from the protein. (Both the polysaccharide and the protein are impermeable to the membrane). In tests optical system was used to measure the polysaccharide, and stability studies indicated that the equilibrium reactions were stable for up to 100 h."

Zambriskie and Humphrey (1978) generated cell-free samples from a fermentation broth for on-line analysis by a dialysis process. A

baffle in a fermentor was converted into a dialyzer with the membrane separating the fermentation broth from a continuous stream of fresh water. Water leaving the dialyzer (dialysate) was channeled to a commercial glucose analyzer. A major limitation of the process is the relatively slow response time. This method and that of Schultz (1979) both are applicable for sensing compounds other than glucose".

A bioselective membrane electrode for L-glutamine was constructed by coupling living bacteria of the strain Sarcina flava as an ammonia sensor (Rechnitz et al., 1978). The researchers claimed that the electrode showed excellent sensitivity and rapid response during a useful lifetime of at least two weeks.

Hikuma et al. (1979) also constructed a microbial electrode, consisting of immobilized microorganisms, a gas permeable Teflon membrane, and an oxygen electrode, for the continuous determination of methyl and ethyl alcohols. Trichosporon brassicae was employed as a microbial electrode sensor for ethyl alcohol. The current output of the electrode sensor was essentially constant for more than three weeks.

The electrodes used by Rechnitz et al. (1978) and Hikuma et al. (1979) apparently were of limited utility because they were not autoclavable.

# EXPERIMENTAL RESULTS

ARTICLE I

# CONTINUOUS DIALYSIS PRODUCTION OF ETHANOL BY YEAST "IMMOBILIZED" IN A MEMBRANE-CONTAINED FERMENTOR

KYU H. KYUNG and PHILIPP GERHARDT,

Department of Microbiology and Public Health,

Michigan State University, East Lansing, Michigan 48824-1101

(manuscript for Biotechnology and Bioengineering)

## Summary

A dialysis continuous fermentation system was investigated as a way to relieve product inhibition in the conversion of glucose to ethanol by cells of Saccharomyces cerevisiae ATCC 4126. Substrate was fed into a continuous dialysate circuit and thence into a batch fermentor circuit via diffusion through the microporous membranes of an intermediate dialyzer; simultaneously, product was withdrawn from the fermentor circuit through the dialyzer membranes into the dialysate circuit and out in the effluent. Since the fermentor was operated without an effluent, the cells essentially were immobilized and converted glucose to ethanol by maintenance metabolism. A steady state of yeast cells in the fermentor did not occur initially but was obtained by the depletion of nitrogen and the prevention of cell breakage, although substrate and product concentrations then became unsteady. The inherent advantages of the system were offset in the ethanol fermentation by relatively low productivity, which appeared to be limited by membrane permeability.

# INTRODUCTION

Product inhibition is a major factor limiting conventional ethanol fermentation processes. In order to increase productivity significantly, it is necessary to remove the ethanol as it is formed by the cells. To accomplish this, various product-removal methods have been investigated for coupling with the fermentation, including vacuum evaporation[1,2] solvent extraction[3,4], and export by an alcohol-selective membrane[5]. However, these methods all remove ethanol selectively and leave secondary metabolites to accumulate, which also soon become toxic, reducing cell viability as well as ethanol productivity[6].

A method to relieve both primary and secondary product inhibition is nonselective export of solutes through a microporous membrane. Microfiltration driven by a hydrostatic pressure gradient is attractive, but the problem of membrane plugging by microbial cells and macromolecules has not been overcome except with complex laboratory equipment[7,8].

Nonselective dialysis driven by a solute concentration gradient across a microporous membrane also is attractive to relieve product inhibition, despite the limitation of diffusion rates, especially if managed in a continuous (steady state) fermentation system. Dialysis continuous fermentation systems have been studied extensively by Stieber, Coulman and Gerhardt[9-11] using the ammonium-lactate fermentation as a model. Substrate is fed into a continuous fermentor circuit that is dialyzed against a continuous dialysate circuit into which only water is fed. Relative to conventional nondialysis

continuous or batch processes, this process results in a higher conversion rate from more concentrated substrate, produces cells at a higher rate and concentration, and yields a dialysate effluent containing a cell-free product.

An alternative way to operate a dialysis continuous fermentation is to feed the substrate into a continuous dialysate circuit and thence into the fermentor circuit via dialysis. If the fermentor circuit is operated without an effluent (i.e., as a batch), the cells are contained and thus essentially "immobilized" within the fermentor circuit, whereas the product is continuously removed via dialysis into the continuous dialysate circuit and out in the effluent. Such a system has been modelled with the ammonium-lactate fermentation by Stieber and Gerhardt[12]. The further advantages of this process, are that substrate is converted into product without appreciable expenditure of substrate for cell growth (i.e., only by maintenance metabolism), and that substrate entering the fermentor is sterilized by membrane passage.

The purposes of the present study were to investigate further this novel dialysate-feed, immobilized-cell dialysis continuous fermentation system for relieving product inhibition in the conversion of glucose to ethanol by the yeast Saccharomyces cerevisiae.

## MATERIALS AND METHODS

### Fermentation System

Figure 1 shows a schematic of the system, which is essentially the same as System II of Stieber and Gerhardt[12]. The symbols also correspond and are listed at the end of the article. The feed into the dialysate circuit was maintained at a constant rate ($F^o_d$, 330 ml/hr) and contained the substrate at a set concentration ($S^o_d$). The dialysate circuit contained a relatively small liquid volume ($V_d$, 600 ml in total) which was continuously circulated by a pump. In the fermentor circuit, the liquid volume ($V_f$, 3600 ml in total) was maintained at a constant level. The substrate diffused into the fermentor circuit from the dialysate circuit through the dialyzer and was mostly converted into the product ($P_f$), but a residue of unused substrate ($S_f$) remained. The dialysate effluent contained the product transferred from the fermentor circuit by the dialyzer ($P_d$) and the residual substrate ($S_d$).

The experimental system was operated essentially as before[12] except that: (1) the volume of the dialysate circuit was increased by adding a small dialysate reservoir and expansion heads, which balanced the pressure between the fermentor and dialysate circuits and reduced the pulsation of circulating liquid; (2) two dialyzers were used, each with four sheets of membrane of 0.2 $\mu$m nominal pore diameter (Versapore 200; Gelman Sciences, Inc., Ann Arbor, MI) to provide a total of 2300 $cm^2$ of effective membrane area.

The fermentation was conducted at a constant pH of 4.5, at temperatures of 30°C (Experiments 1, 2 and 4) and 20°C (Experiment 3), and with a fermentor agitation rate of 300 rpm. The dialysate and fermentor liquids were circulated at a rate of 3 liters/min by two gear pumps (Maisch Metering Pump, Tuthill Pump Co., Chicago, IL) for Experiments 1-3 and by a duplex diaphram pump (Madden Corp., Elkhart, IN) for Experiment 4.

## Substrate

A semi-synthetic medium[1] was used which contained glucose (200 mg/ml), yeast extract (8.5 mg/ml), $NH_4Cl$ (1.32 mg/ml), $MgSO_4.H_2O$ (0.11 mg/ml), and $CaCl_2$ (0.06 mg/ml). The concentrations of glucose, yeast extract, and $NH_4Cl$ were changed in Experiments 2-4, as stated in the text and figure legends.

Instead of a small amount of oxygen[1], ergosterol was added and anaerobiosis was maintained[13]. A stock solution was prepared by dissolving 0.0625 g of ergosterol (Sigma Chemical Co., St. Louis , MO) and 6.25ml of Tween 80 (Sigma Chemical Co.) in ethanol to make 25ml of solution. One ml of the stock solution was added to 1 liter of the fermentation medium after sterilization of each.

## Inoculum

Saccharomyces cerevisiae (ATCC 4126) inoculum was prepared by cultivating it in 100 ml of the fermentation medium at 30° C for 24 hr on a rotary shaker. After inoculation into the fermentor, the culture

was allowed to grow batchwise aerobically for 24 hr before starting the dialysis fermentation.

## Analytical Procedures

Samples were taken two times daily from the dialysis circuit effluent and from within the fermentor circuit. Viable cell numbers were determined by the pour-plate technique on plate-count agar (Difco). Glucose was determined by the dinitrosalycilic acid method of Miller[14]. Ethanol was assayed by use of a gas chromatograph with a flame ionization detector (Varian Aerograph Series 2400, Varian Associates, Palo Alto, CA), an integrator (Varian CDS 111), and a stainless steel column (6 feet by 1/8 inch) packed with 10% SP-1000 and 1% $H_3PO_4$ on 100/120 Chromsorb WAW (Supelco, Inc., Bellefonte, PA). Cell dry weight was measured by centrifuging 2 ml fermentor samples, washing twice with distilled water, and drying at 80° C in a vacuum for 24 hr. Duplicate determinations were made in all analyses.

# RESULTS

Experiments were initiated (Fig. 2) with essentially the same fermentation system and hydraulic dilution rate (D, 0.092 $hr^{-1}$) as used by Stieber and Gerhardt[12] and with the same temperature (30°C) and medium composition as used by Cysewski and Wilke[1], except for a small amount of ergosterol instead of oxygen[13]. Unexpectedly, a steady state of cells in the fermentor was not obtained. Instead, after an adjustment period of about 2 days, the cell-mass concentration measured as dry weight ($X_f^{dw}$) increased steadily throughout the 10.5-day period (Fig. 2A). When $X_f^{dw}$ exceeded 80 mg/ml, the circulation of the fermentor liquid became restricted due to cells accumulating inside the dialyzer. Inversely, the cell-mass concentration measured as viable cell number ($X_f^{vc}$) decreased. After the adjustment period, substrate concentration in the fermentor ($S_f$) maintained a very low level (1.5 mg/ml), whereas that in the dialysate ($S_d$) increased (Fig. 2B). Product concentrations in both the fermentor ($P_f$) and in the dialysate ($P_d$) attained approximately steady states (Fig. 2C).

The means of these changing values, for a representative 7.5 day period after the adjustment period (Days 3-10.5), are shown in Table I. From these mean values, the average efficiency of substrate conversion (E) was calculated as 78.1%, and the average productivity ($r_p$) was calculated at 5.8 mg/ml hr.

Attempts then were made to obtain a steady state of cells in the fermentor through limitation of growth factors (by reduction in the concentration of yeast extract, $S_d^o$ to 2 mg/ml, Experiment 2) and

through limitation of metabolic activity (by reduction in the temperature to 20°C, Experiment 3). However, similar patterns of $X_f^{dw}$ and $X_f^{vc}$ were obtained, $S_f$ became unsteady at higher values, and Pf and $P_d$ were steady but at a lower level. From the mean values after the adjustment period, the average E was calculated as 64.8% and 55.7% and the average $r_p$ was calculated as 5.4 and 4.5 mg/ml hr for Experiments 2 and 3, respectively (Table I).

In the course of evaluating all three experiments, however, it was discovered by microscopic examination that broken yeast cells and cellular debris began to appear after about 2 days and increased thereafter. With this increase and that in $X_f^{dw}$, the viscosity of the fermentor liquid increased and solids accumulated increasingly on the separators inside the dialyzer, with resulting difficulty in maintaining fermentor volume. Consequently, it was speculated that the yeast cells, unlike bacterial cells[10-12], were being damaged by the gear pump during circulation. If so, the rise in $X_f^{dw}$ would be accounted for by the accumulation of dead cells and cellular debris.

In Experiment 4, a diaphragm pump was employed in order to prevent damage to the yeast cells. In addition, a further effort was made to suppress growth by the removal of the $NH_4Cl$, reduction of the sugar concentration (to 100 mg/ml), and yeast extract concentration (to 0.5 mg/ml). The results of these combined changes are shown in Fig. 3, Days 0-13. Despite the combined efforts, $X_f^{dw}$ still increased slightly although $X_f^{vc}$ did not decrease and actually increased slightly (Fig. 3A). After an initial adjustment period, $S_d$ became constant but $S_f$ decreased (Fig. 3B), whereas $P_f$ and $P_d$ became essentially constant

(Fig. 3C). In these first days after the adjustment period in Experiment 4, the average E was calculated as 57.1% and the average $r_p$ was calculated as 2.5 mg/ml hr (Table I).

A final and successful effort to obtain $X_f^{dw}$ in steady state was made by a further reduction in yeast extract concentration (to 0.2 mg/ml) in Days 13-27). Both $X_f^{dw}$ and $X_f^{vc}$ then became essentially constant (Fig. 3A). However, both $S_f$ and $S_d$ increased, and both $P_f$ and $P_d$ decreased slightly in unsteady states. In the latter period of Experiment 4, the average E was calculated as 50.9% and the average $r_p$ was calculated as 2.1 mg/ml hr (Table I).

## DISCUSSION

The investigation indicated that only with nitrogen depletion could the yeast cell mass be maintained in steady state with this dialysis continuous system for the ethanol fermentation; however substrate and product concentrations then became unsteady. This outcome was different from the results obtained when the same system was employed for the ammonium-lactate fermentation by bacteria[12]. The reasons for the difference in results are not evident, but may lie with fundamental differences between yeast and bacterial cells. One such difference was indicated by the greater susceptibility of yeast cells to breakage from a circulating pump.

The system inherently provides a novel and potentially useful method for the immobilization of microbial cells and the relief of product inhibition by membrane containment. Substrate is converted into product without appreciable expenditure of substrate for cell growth, with a greatly reduced requirement for costly nitrogen sources, and with continuous operation for extended time periods. Dialysis serves to relieve both primary and secondary product inhibition, sterilize the feed into fermentor, and withold cells from the effluent product.

In the ethanol fermentation, however, these advantages appear to be offset by relatively low conversion efficiency (E) and productivity ($r_p$). For example among the four experiments E averaged 51-78% and $r_p$ averaged 2.1-5.8 mg/ml hr. By comparison, in a typical

batch process E is about 94% and $r_p$ is about 1.8-2.5 mg/ml hr with multiple fermentors[15]. In a vacuum continuous process, $r_p$ rose to 44 mg/ml hr but declined sharply after 2 days apparently because of secondary products accumulating[6]. In a vacuum process with cell recycle, $r_p$ attained a record 83 mg/ml hr but was similarly short lived[2].

Conventional batch technology for the ethanol fermentation is antiquated and inefficient, and a number of alternative processes promise substantial improvement[15]. Despite limited success in the present effort the use of nonselective microporous membranes remains attractive, especially if managed with continuous fermentation. In a dialysis process, the limiting factor is the solute exchange capacity of the dialyzer (as indicated by the large differences between $P_f$ and $P_d$ shown in Fig. 2c and 3c). Some improvement in the present process would be obtainable by a greater membrane area or better membrane permeability. Further improvement in conversion efficiency and productivity and attainment of steady-state conditions may be obtainable by feeding substrate into the fermentor circuit and water into the dialysate circuit[9-11], but this alternative system has not yet been tested with the ethanol fermentation. Finally, driving the removal of products from cells by hydrostatic pressure - microfiltration - may yet prove to be the most effective use of membranes if the problem of plugging can be overcome, which seems likely from current industrial experience.

## ACKNOWLEDGMENT

This investigation was supported by grant DAR 79-10236 from the National Science Foundation, and was assigned journal article No. 10751 from the Michigan Agricultural Experiment Station.

## Nomenclature

| | |
|---|---|
| $D$ | hydraulic dilution rate, $F_d/V_f$ ($hr^{-1}$) |
| $E$ | extent (efficiency) of substrate conversion, $1-S_d/S^o_d$ (%) |
| $F_d$ | flow rate into and out of dialysate circuit (ml/hr) |
| $P_d$ | product concentration in dialysate circuit (mg/ml) |
| $P_f$ | product concentration in fermentor circuit (mg/ml) |
| $r_p$ | rate (productivity) of product formation (mg/ml hr) |
| $S_d$ | substrate (glucose) concentration in dialysate effluent (mg/ml) |
| $S_f$ | substrate (glucose) concentration in fermentor circuit (mg/ml) |
| $S^o_d$ | substrate (glucose) concentration in dialysate feed (mg/ml) |
| $s^o_d$ | substrate (yeast extract) in dialysate feed (mg/ml) |
| $t_f$ | temperature in fermentor circuit ($^oC$) |
| $V_d$ | volume of liquid in dialysate circuit (ml) |
| $V_f$ | volume of liquid in fermentor circuit (ml) |
| $X_f^{dw}$ | cell mass concentration as dry weight in the fermentor (mg/ml) |
| $X_f^{vc}$ | cell mass concentration as viable cell number in the fermentor (cells/ml) |

## References


1. Cysewski, G. R., and C. R. Wilke, Biotechnol. Bioeng. 19,1125 (1977).

2. Ramalingham, A., and R. K. Finn, Biotechnol. Bioeng. 19, 583 (1977).

3. Hernandez-Mena, R., J. A. Ribaud, and A. E. Humphrey, paper presented at 6th International Fermentation Symposium, London, Ontario, Canada, 20-25 July 1980.

4. Minier, M., and G. Goma, Biotechnol. Bioeng. 24, 1565 (1982).

5. Gregor, H., and T. Jeffries, Ann. New York Acad. Sci. 326, 273 (1979).

6. Mairorella, B., H. W. Blanch, and C. R. Wilke, Biotechnol. Bioeng. 25, 103 (1983).

7. Sortland, L. D., Ph.D. thesis, University of California, Berkeley, Department of Chemical Engineering (1968).

8. Margaritis, A., and C. R. Wilke, Biotechnol. Bioeng. 20, 709 (1978).

9. Coulman, G. A., R. W. Stieber, and P. Gerhardt, Appl. Environ. Microbiol. 34, 725 (1977).

10. Stieber, R. W., G. A. Coulman, and P. Gerhardt, Appl. Environ. Microbiol. 34, 733 (1977).

11. Stieber, R. W., and P. Gerhardt, Appl. Environ. Microbiol. 37, 487 (1979).

12. Stieber, R. W., and P. Gerhardt, Biotechnol. Bioeng. 23, 535 (1981).

13. Andreasen, A. A., and T.J.B. Stier, J. Cell. Comp. Physiol. 43, 271 (1954).

14. Miller, G. L., Analyt. Chem. 31, 426 (1959).

15. Mairorella, B., C. R. Wilke, and H. W.Blanch, Adv. Biochem. Eng. 20, 43 (1981).

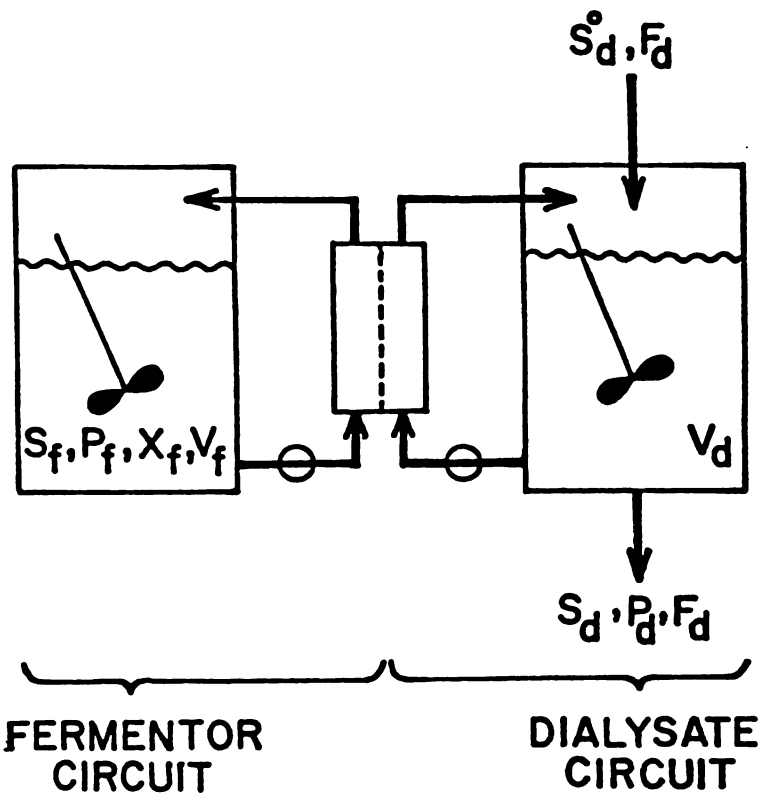
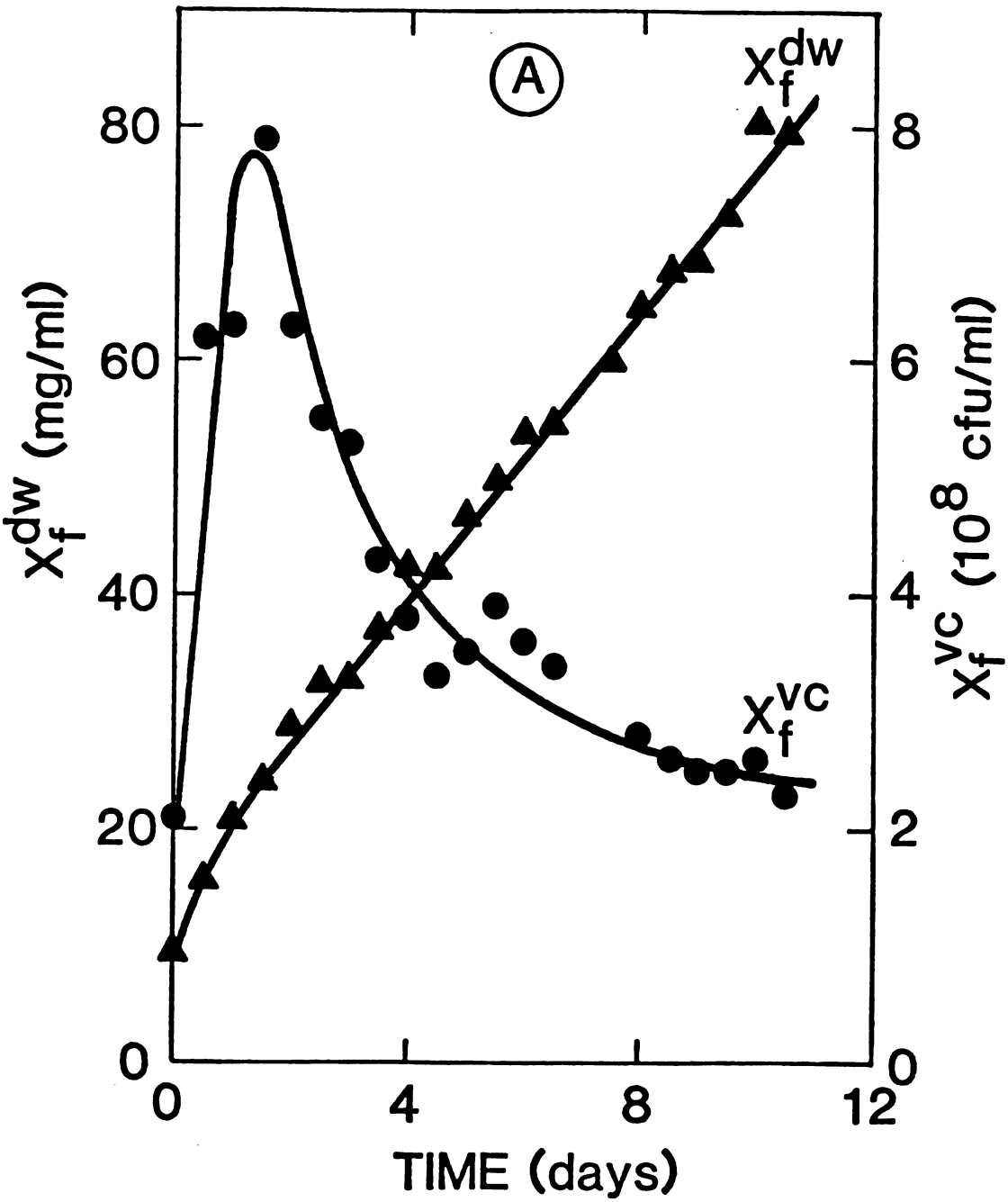
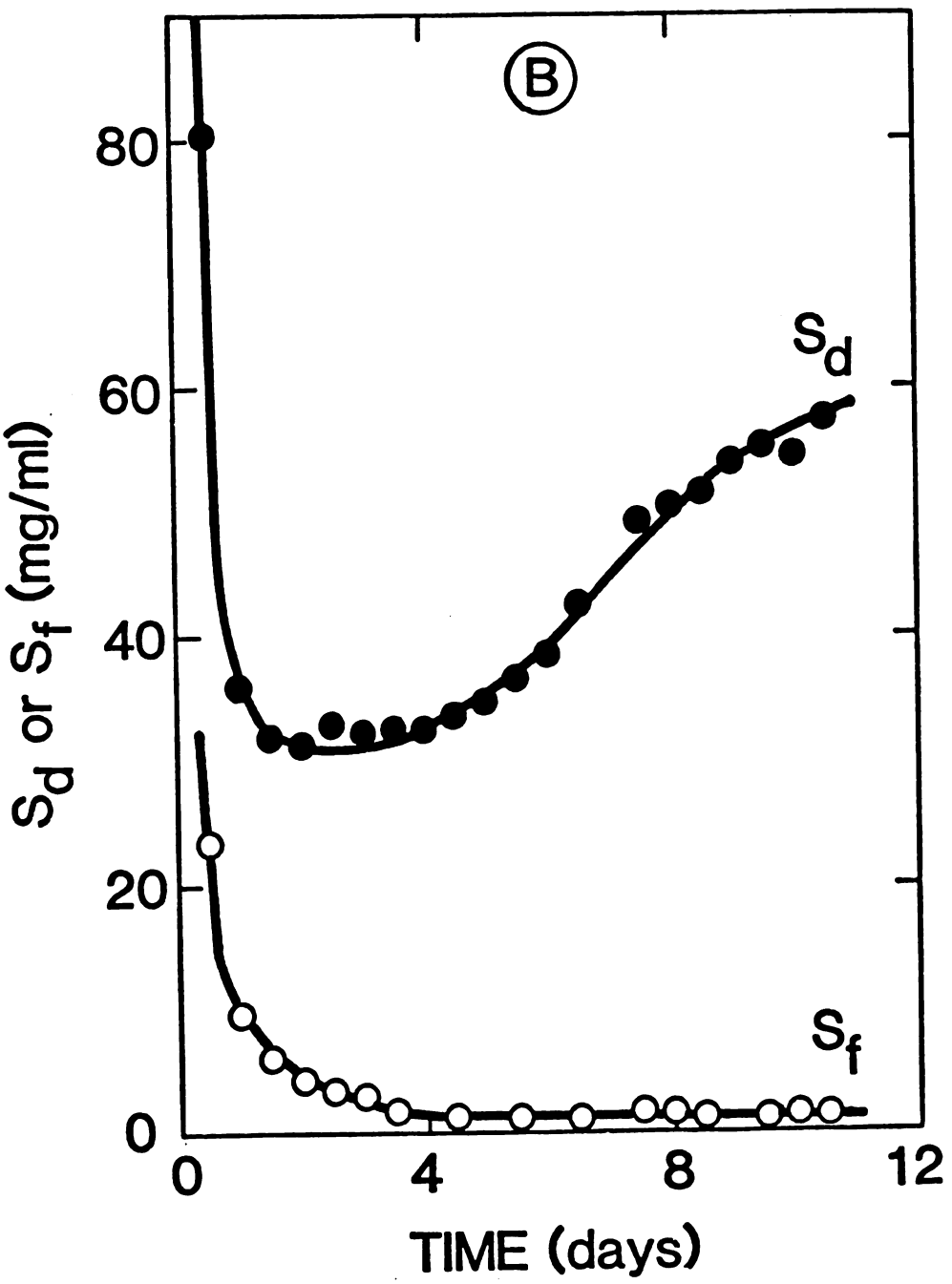



Fig. 1. Schematic of dialysate-feed, immobilized-cell system for dialysis continuous fermentation.

Fig. 2. Experiment 1: time course of dialysis continuous ethanol fermentation at 30° with complete medium. A: viable cell number ($X_f^{vc}$) and cell dry weight ($X_f^{dw}$) in the fermentor circuit. B: substrate concentrations in the dialysate ($S_d$) and fermentor ($S_f$) circuits. C: product concentrations in the dialysate ($P_d$) and fermentor ($P_f$) circuits. The linear portions of the curves were plotted by least-squares analysis.


A
$X_f^{dw}$
$X_f^{vc}$
$X_f^{dw}$ (mg/ml)
$X_f^{vc}$ ($10^8$ cfu/ml)
TIME (days)
0
20
40
60
80
0
2
4
6
8
0
4
8
12


B
80
60
40
20
0
$S_d$ or $S_f$ (mg/ml)
$S_d$
$S_f$
0
4
8
12
TIME (days)

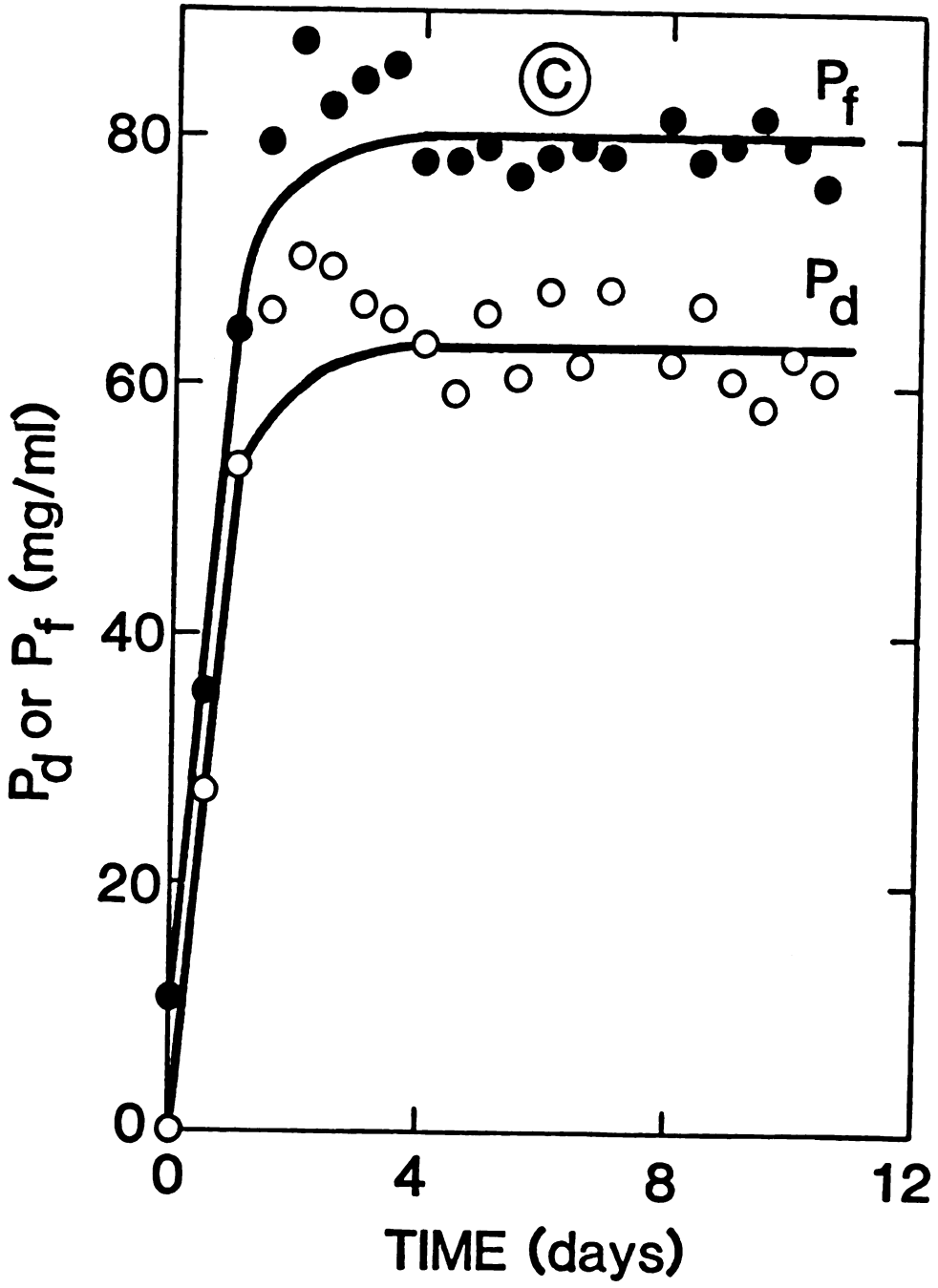
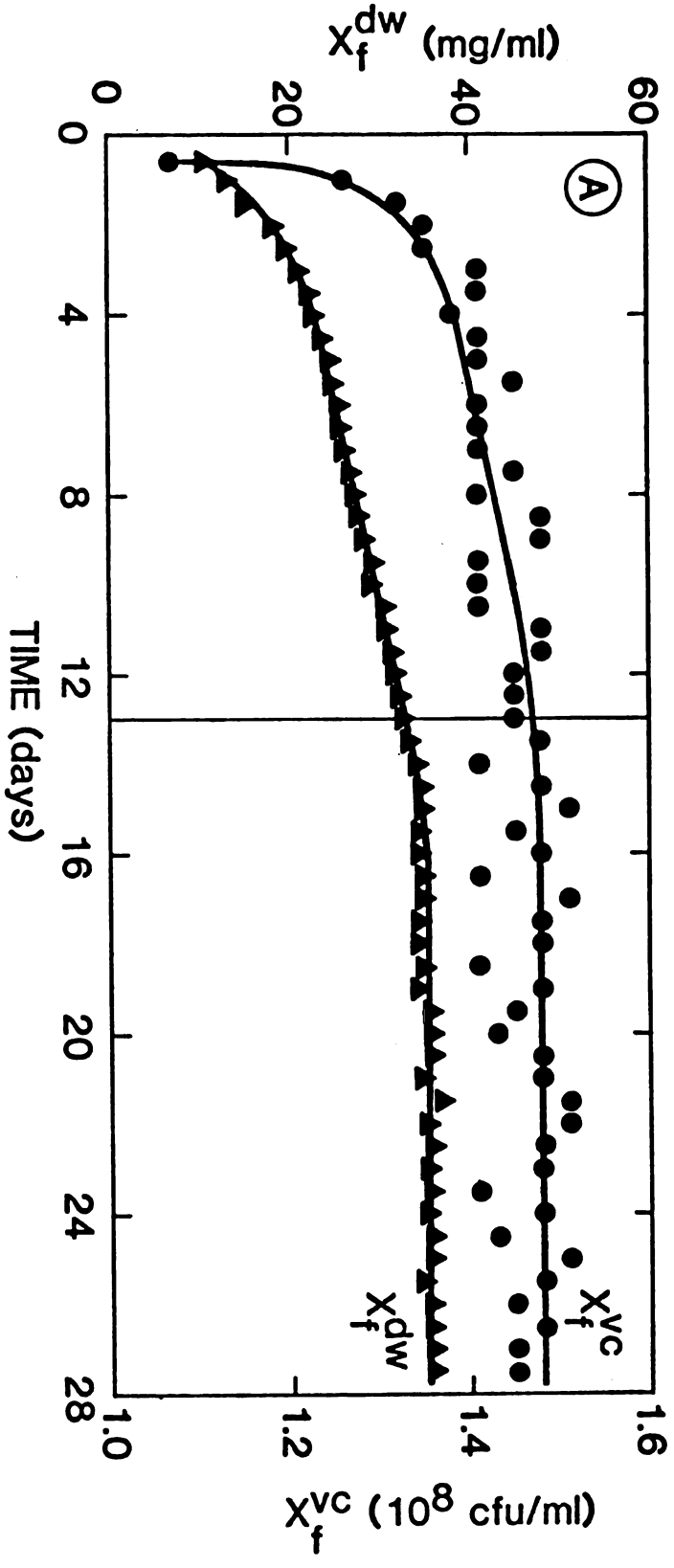
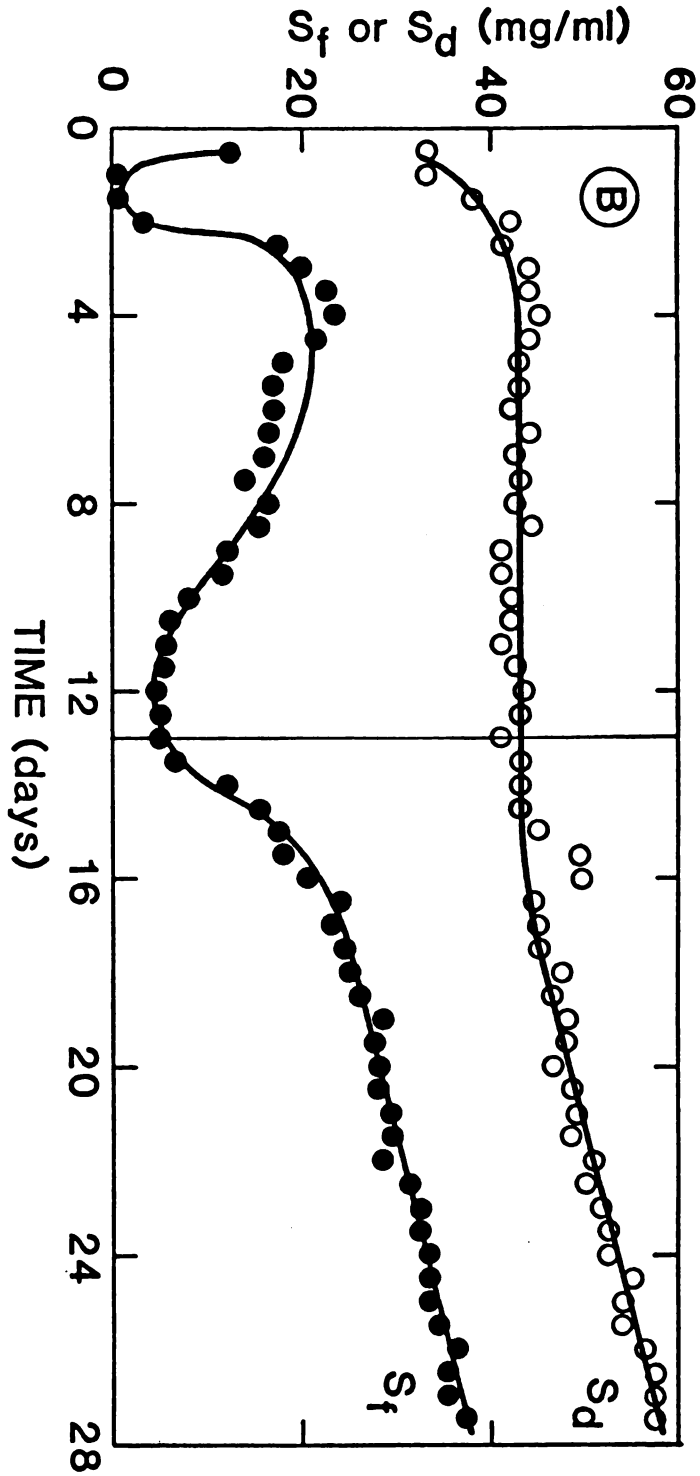

©
$P_f$
$P_d$
80
60
40
20
0
0
4
8
12
$P_d$ or $P_f$ (mg/ml)
TIME (days)

Fig. 3. Experiment 4: extended time course of dialysis continuous ethanol fermentation with deficient medium. A, B and C are plotted as in Fig. 2. Days 0-13: at 30°C, with 100 mg of glucose per ml, without $NH_4Cl$, and with 0.5 mg of yeast extract per ml. Days 13-27: as before, but with 0.2 mg of yeast extract per ml.


$X_f^{dw}$ (mg/ml)
0
20
40
60
A
TIME (days)
0
4
8
12
16
20
24
28
$X_f^{dw}$
$X_f^{vc}$
1.0
1.2
1.4
1.6
$X_f^{vc}$ ($10^8$ cfu/ml)


$S_f$ or $S_d$ (mg/ml)
0
20
40
60
0
4
8
12
16
20
24
28
TIME (days)
B
$S_d$
$S_f$

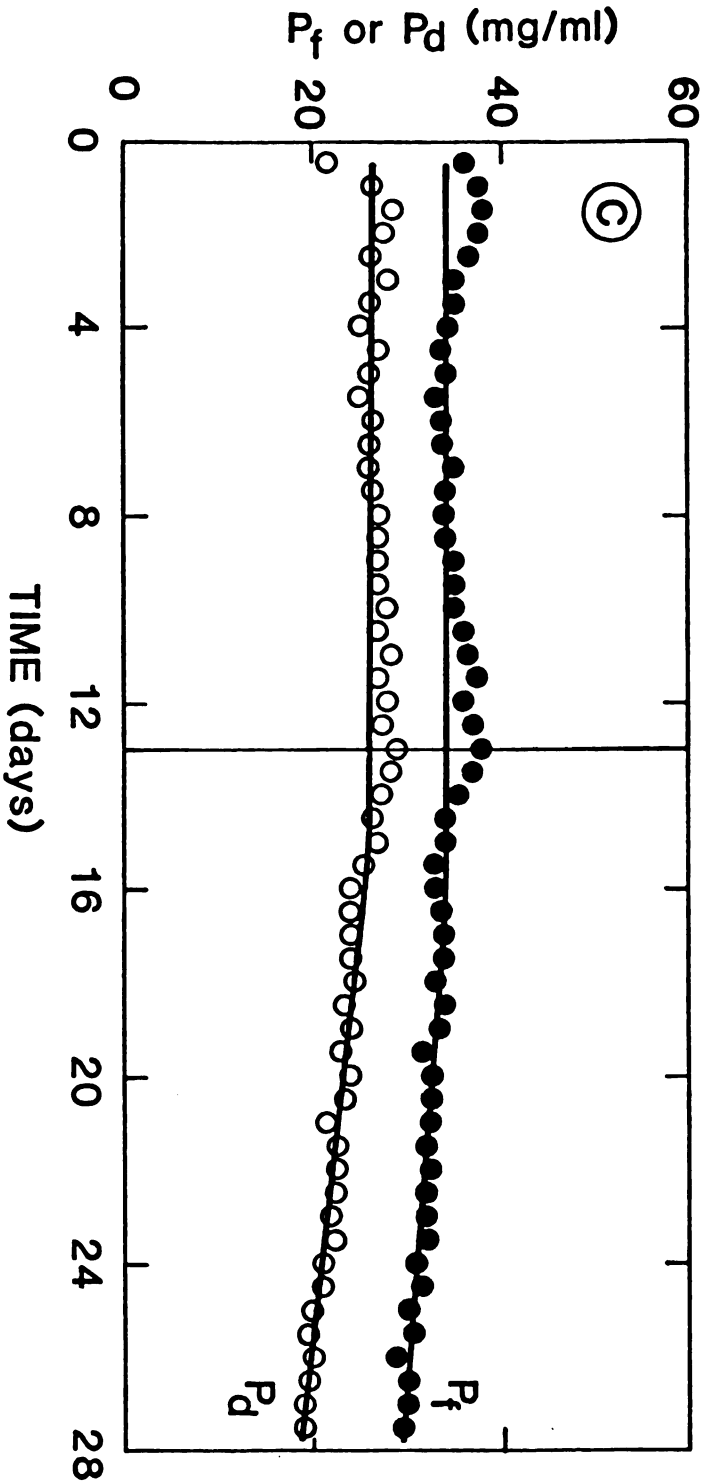

$P_f$ or $P_d$ (mg/ml)
0
20
40
60
0
4
8
12
16
20
24
28
TIME (days)
©
$P_f$
$P_d$

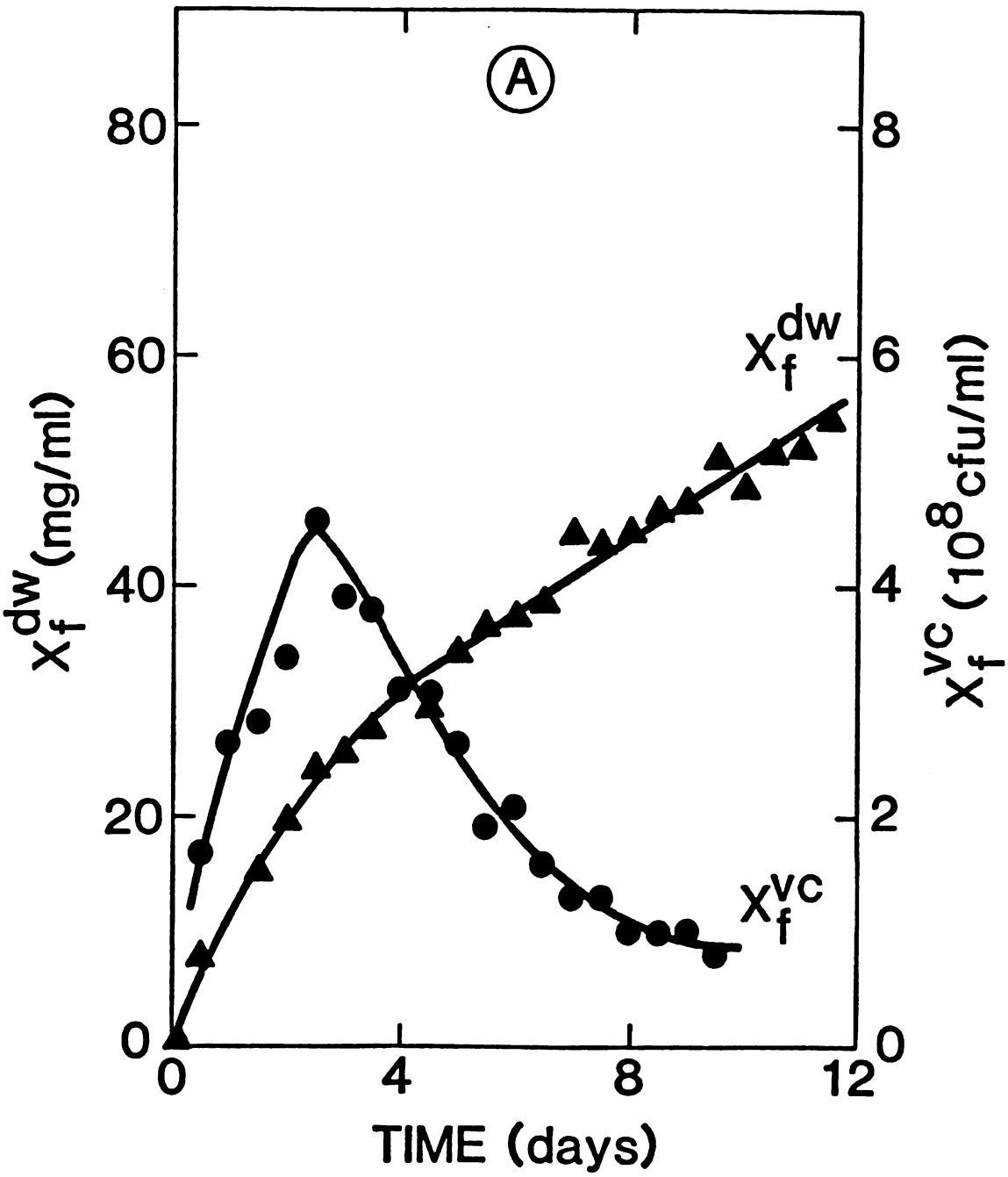
TABLE I

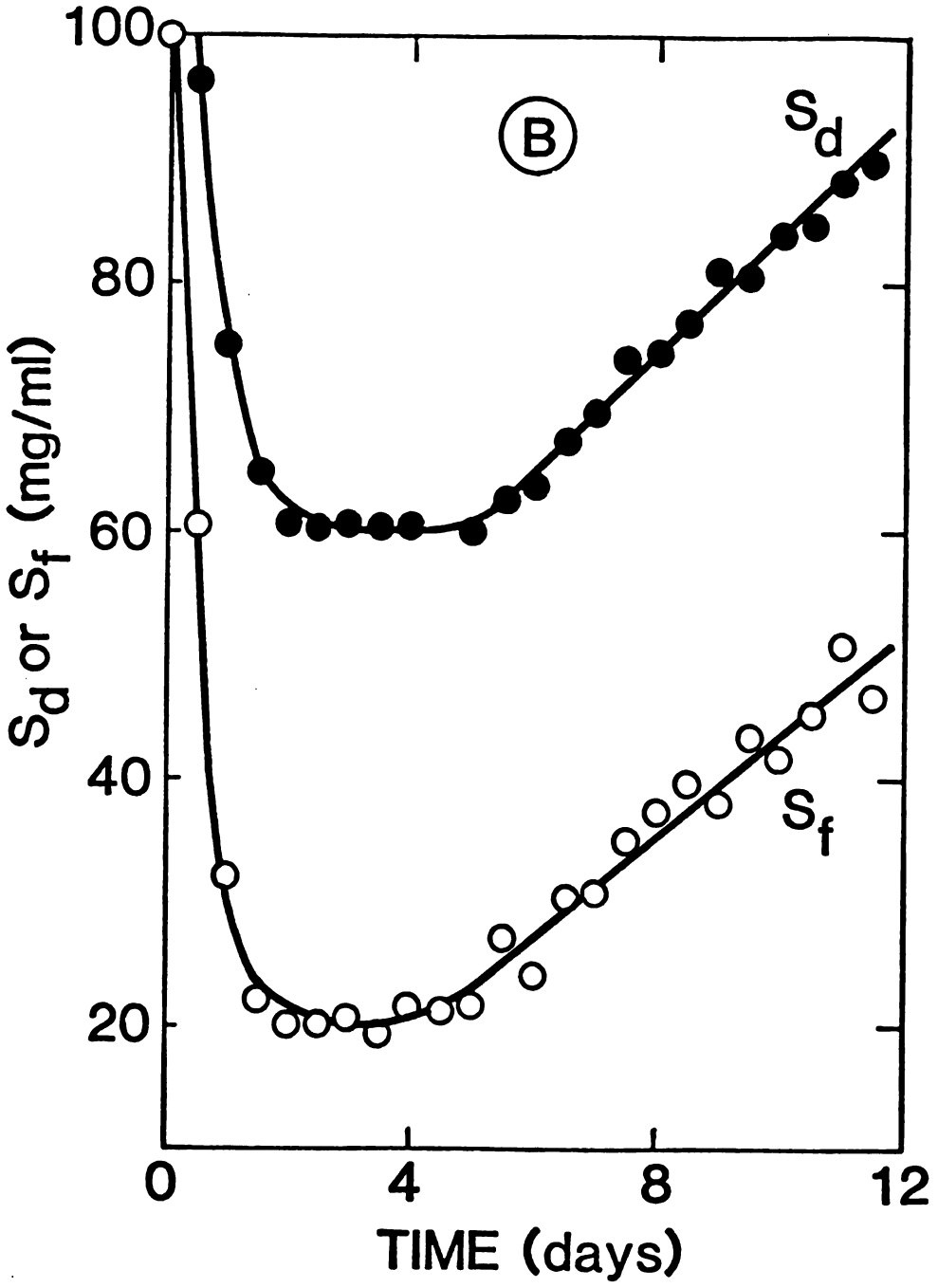
Comparative Mean Data From Four Experiments

| Exp. No. | Day | D (hr-1) | $S^o_d$ (mg/ml) | $s^o_d$ (mg/ml) | $t_f$ (°C) | $X^{dw}_d$ (mg/ml) | $X^{vc}_f$ ($10^8$ cfu/ml) | $S_f$ (mg/ml) | $S_d$ (mg/ml) | $P_f$ (mg/ml) | $P_d$ (mg/ml) | E (%) | $r_p$ (mg/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3-10.5 | 0.092 | 200 | 8.5 | 30 | 56.9 | 3.3 | 1.5 | 43.8 | 79.6 | 63.3 | 78.1 | 5.8 |
| 2 | 3-10.5 | 0.092 | 200 | 2.0 | 30 | 39.3 | 2.0 | 30.9 | 70.5 | 75.3 | 58.8 | 64.8 | 5.4 |
| 3 | 3-10.5 | 0.092 | 200 | 2.0 | 20 | 45.0 | 4.1 | 43.8 | 88.7 | 67.2 | 48.8 | 55.7 | 4.5 |
| 4 | 3-10.5 | 0.092 | 100 | 0.5 | 30 | 25.8 | 1.5 | 16.2 | 42.9 | 34.3 | 26.6 | 57.1 | 2.5 |
| | 17-24.5 | 0.092 | 100 | 0.2 | 30 | 34.9 | 1.5 | 28.8 | 49.1 | 32.5 | 22.9 | 50.9 | 2.1 |

# SUPPLEMENTAL DATA TO ARTICLE I

Fig. S-1. Experiment 2: time course of dialysis continuous ethanol fermentation at 30 C with 200 mg of glucose per ml and 2 mg of yeast extract per ml. A, B and C are plotted as in Fig. 2 in Article I.


A
80
60
40
20
0
8
6
4
2
0
0
4
8
12
TIME (days)
$X_f^{dw}$ (mg/ml)
$X_f^{vc}$ ($10^8$ cfu/ml)
$X_f^{dw}$
$X_f^{vc}$


100
80
60
40
20
0
4
8
12
B
$S_d$
$S_f$
$S_d$ or $S_f$ (mg/ml)
TIME (days)

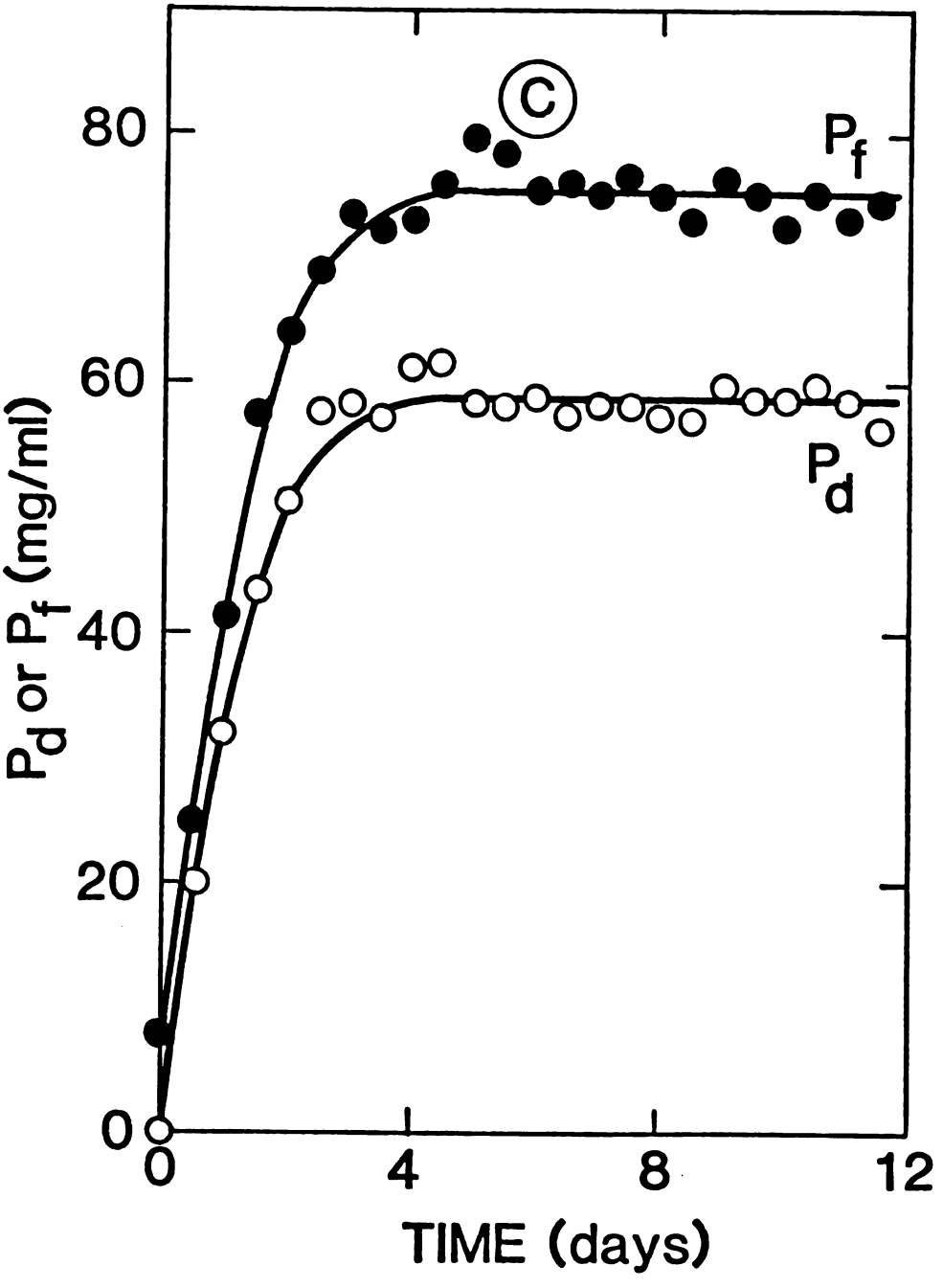
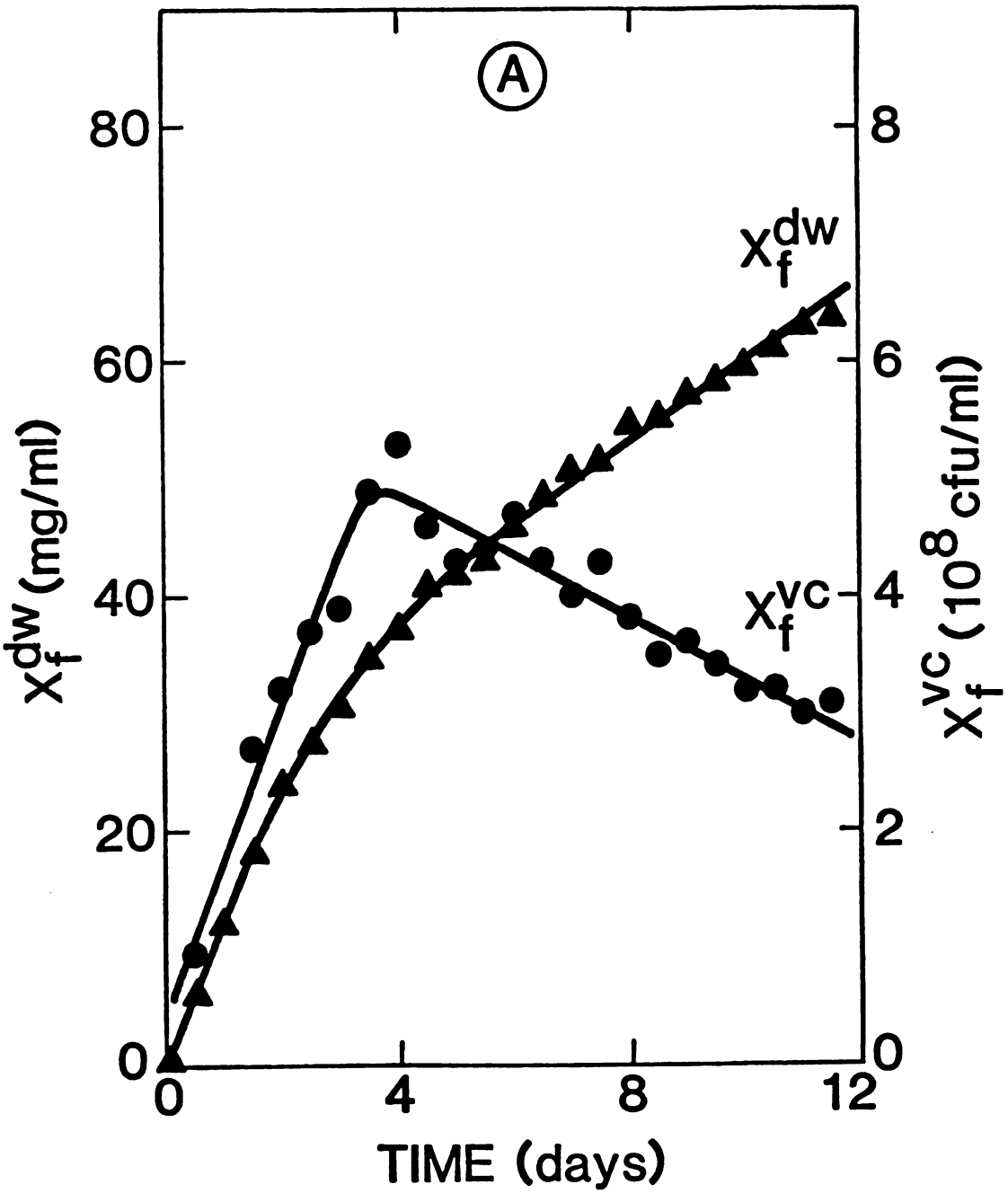
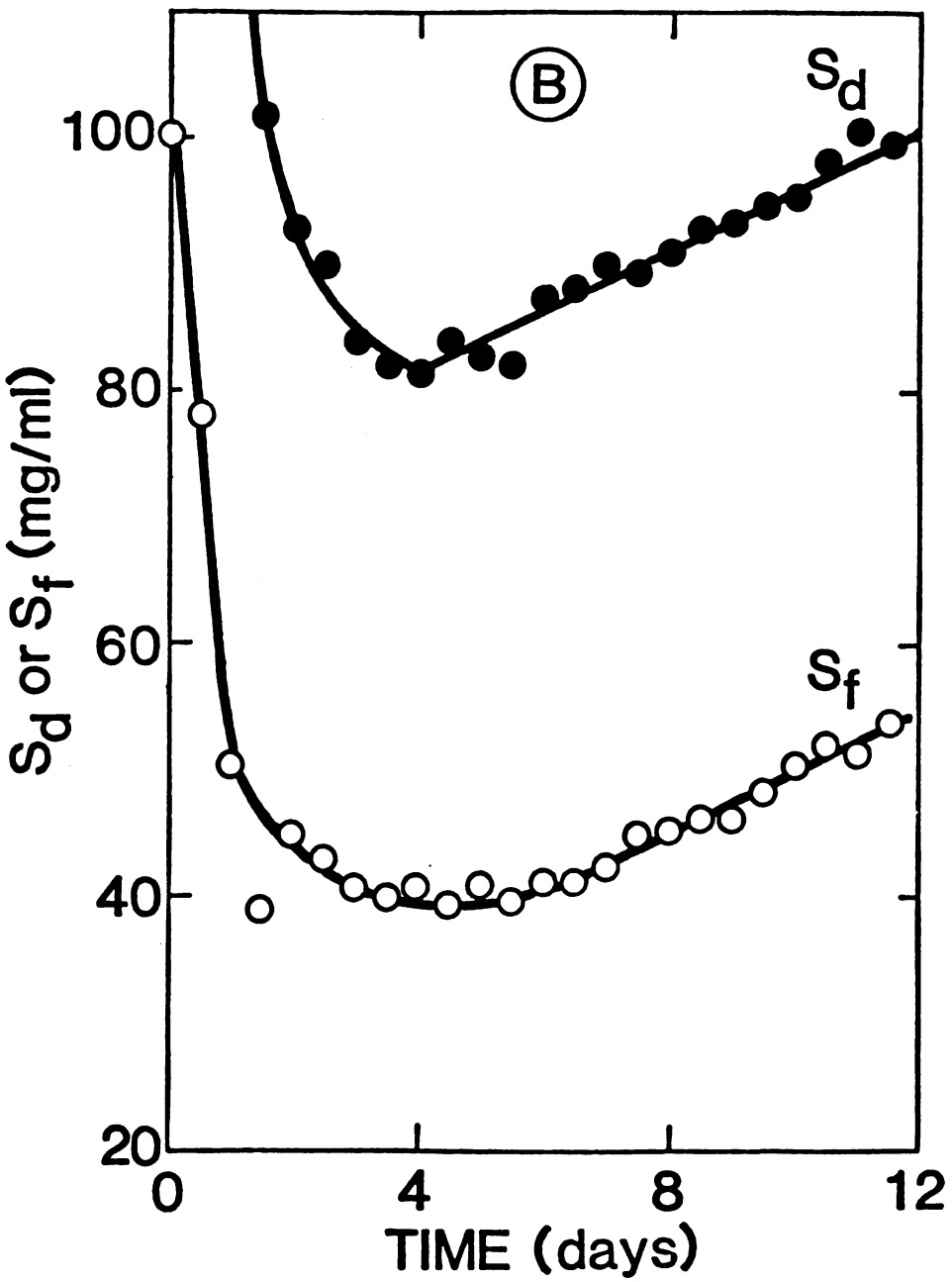
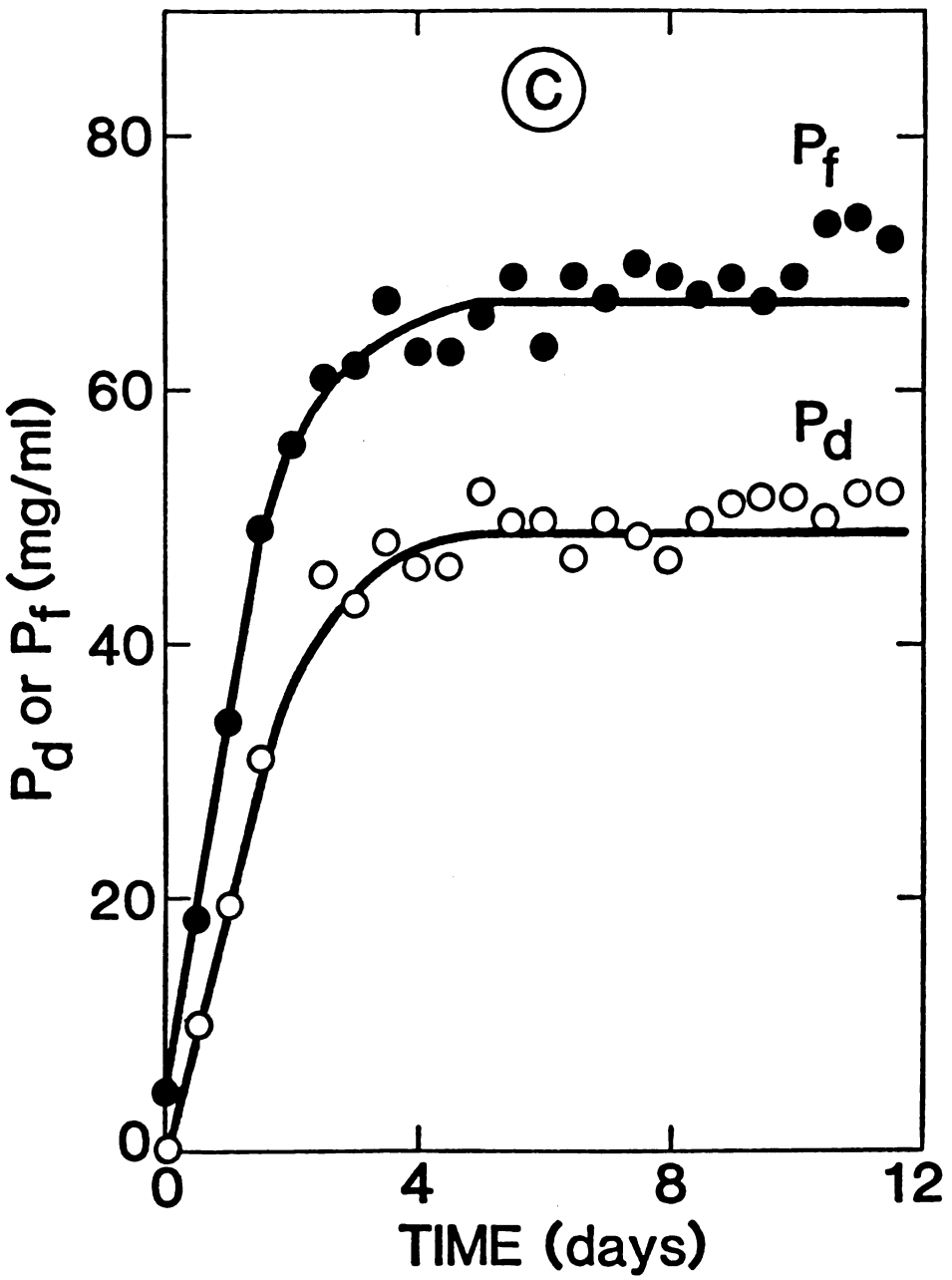

C
$P_f$
$P_d$
80
60
40
20
0
0
4
8
12
$P_d$ or $P_f$ (mg/ml)
TIME (days)

Fig. S-2. Experiment 3: time course of dialysis continuous ethanol fermentation at 20 C with 200 mg of glucose per ml and 2 mg of yeast exract per ml. A, B and C are plotted as in Fig. 2 in Article I.


A
80
60
40
20
0
8
6
4
2
0
0
4
8
12
TIME (days)
$X_f^{dw}$ (mg/ml)
$X_f^{vc}$ ($10^8$ cfu/ml)
$X_f^{dw}$
$X_f^{vc}$


B
$S_d$
$S_f$
100
80
60
40
20
0
4
8
12
$S_d$ or $S_f$ (mg/ml)
TIME (days)


©
$P_f$
$P_d$
80
60
40
20
0
0
4
8
12
$P_d$ or $P_f$ (mg/ml)
TIME (days)

ARTICLE II

# MUTUALISTIC DIALYSIS CULTURE OF *STREPTOCOCCUS LACTIS* AND *CANDIDA UTILIS*

(manuscript for *Biotechnology and Bioengineering*)

## SUMMARY

Mutualistic dialysis culture of Streptococcus lactis, which is valuable as a dairy starter, and Candida utilis, which is valuable as single cell protein, was investigated in a batch fermentation system. The bacterium and the yeast were inoculated into separate fermentors connected by an intermediate dialyzer, the membranes of which allowed diffusional exchange of solutes. Lactose was fermented to lactic acid, by S. lactis which was dialyzed into the yeast culture and was consumed by C. utilis so as to relieve product inhibition of S. lactis fermentation. Consequently, S. lactis concentration more than doubled in comparison with a nondialysis control fermentation, and yeast cells were produced as byproduct. Although the acid production rate by the bacterium was much faster than the acid consumption rate by the yeast, the primary limiting factor of the process apparently was the solute exchange rate across the membrane.

# INTRODUCTION

Streptococcus lactis is a commonly used dairy starter culture, but the cell concentration attainable in commercial production is limited by metabolic feedback inhibition from the lactic acid produced as an end-product. The yield could be increased if the lactate were removed during fermentation, thereby relieving product inhibition. Since dairy starter cultures have high economic value, there have been efforts to improve the yield of this and other lactic acid bacteria by use of dialysis culture techniques. The technique at first was successful with species of Lactobacillus (Gerhardt and Gallup, 1963, Bergere and Hermier, 1968). Osborne (1977), however, obtained a high concentration of Streptococcus cremoris cells by using a membrane area and a fermentor-to-reservoir volume ratio considerably larger than usual.

To further improve the yield and productivity of S. lactis in dialysis culture, a lactate-consuming microorganism C. utilis was used on the opposite side of the membranes to serve as a biological sink for removing the lactate and relieve feedback inhibition by lactate. Various uses of different microbial populations on opposite sides of membranes so that their metabolites can interchange ("interbiosis") have been reviewed by Schultz and Gerhardt (1969) and by Kyung (1983). Candida utilis was chosen as the second organism because it utilizes lactate as a sole carbon source and does not utilize latose, and because its biomass is useful as single cell protein. This yeast previously has been used to remove lactate from cucumber pickle brine (Stevenson et al., 1979) and sauerkraut brine (Hang et al., 1972) as a

way to reduce their biological oxygen demand in waste disposal after ordinary fermentations.

## MATERIALS AND METHODS

### Fermentation system

Figure 1 shows a schematic of the mutualistic dialysis fermentation system. The bacterium was cultivated in a conventional laboratory fermentor of 3.5 liters of working volume. The yeast was cultivated in a similar fermentor of 10.5 liters of working volume. When the yeast was not inoculated into this fermentor, the fermentation was called ordinary dialysis fermentation. The contents of each were constantly circulated through the dialyzers at rates of 1 to 3 liters per min obtained with a duplex diaphram pump (Madden Metriflow series, Madden Corp., Elkhart, IN). Air cushions (24 inch closed glass tubes) were placed between the pump heads and the dialyzer inlets to reduce the pulsation of the circulating liquid and to protect the membranes from being ruptured. Two dialyzers, each with four sheets of membrane of 0.2 μm nominal pore size (Versapore 200, Gelman Sciences, Inc., Ann Arbor, MI) were used in series to provide 2300 $cm^2$ of effective dialysis area. Pump heads and tubing leading to the air cushions and to the fermentor outlets were not autoclavable, but were sanitized with 0.5% sodium hypochlorite solution and washed three times with 3.5 liters of sterile diluted water.

Nondialysis experiments for both the bacterium and the yeast were carried out in a fermentor of 3 liters of working volume.

All fermentations were conducted at 30 C. pH was controlled at 6.0 with 30% $NH_4OH$ and 2M-HCl for the bacterium and the yeast fermentors, respectively. The yeast fermentor was agitated at 900 rpm with an aeration of 1 volume of air per volume of medium. The

bacterial fermentor was agitated at 500 rpm without aeration. Polypropylene glycol (molecular weight = 2000) was added as an antifoam whenever needed.

## Media

The growth medium for the bacterium was prepared as reported previously (Pettersson, 1975) with a few minor changes because a clear particle-free solution was necessary. 70 g of sweet dry whey powder (Galloway West Co., Fond Du Lac, WI) and 50 g of dry skim milk powder (Difco Laboratories, Detroit, MI) in 1 liter of distilled water were steamed for 30 min, cooled to room temperature, and then treated with 3 g of papain (2000 U/g, crude extract type II; Sigma Chemical Co., St. Louis, MO) sequentially at 30 C for 20 min, at 50 C for 60 min, at 75 C for 15 min, and at 95 C for 15 min. The enzyme-treated suspension was cooled to room temperature, centrifuged at 10000 x g for 20 min, and filtered through a membrane filter of 0.22 μm nominal pore size (Gelman Sciences Inc.). After the addition of 5 g of yeast extract and 0.14 g of $MnSO_4$, the medium was filter-sterilized into pre-autoclaved fermentors. The final solution was light brown in color without opalescence. Fresh medium thus prepared contained approximately 0.1% lactic acid.

Spent growth medium was used for the yeast and was prepared as follows. The bacterium was first grown batchwise in the above medium until the stationary phase was reached. The bacterial cells were removed by centrifugation (13000 x g for 20 min), and the spent medium was then filter-sterilized into pre-autoclaved fermentors as above.

## Cultures

*Streptococcus lactis* strain c-2 was obtained from Gerald D. Mercer at Miles Laboratories, Inc., Elkhart, IN. The bacterium was maintained in 10% sterile skim milk contained in a 25 ml screw-cap test tube.

*Candida utilis* strain NRRL 900 was obtained from Marguerite Dynnik at the Department of Food Science and Human Nutrition, Michigan State University. The yeast was maintained on yeast extract-malt extract-peptone-glucose agar slants. The cultures were transferred every other week to fresh medium, incubated at 30 C, and stored at 4C.

## Analytical procedures

Samples were taken at hourly intervals from the fermentor. Whenever samples were taken, optical densities were measured at 600 nm and then diluted aliquots were pour-plated on plate count agar for enumeration of the bacterium. Dry weight of the yeast was obtained from a standard curve relating dry weight and optical densities. Lactic acid was determined by use of a gas chromatograph (Series 1420, Varian Associates, Palo Alto, CA), an integrator (Model CDS 11, Varian Associates), and a stainless-steel column packed with 10% SP-1000/1% $H_3PO_4$ on 100/120 Chromsorb WAW (Supelco, Inc., Bellefonte, PA).

## Results

Growth and lactate production of *S. lactis* first were characterized in nondialysis and ordinary dialysis culture as controls. The bacterium in nondialysis culture produced $2.1 \times 10^{10}$ viable bacteria per ml and accumulated 3.5% lactate (Fig. 2). It was apparent that the growth rate of the bacterium substantially slowed down when the lactate concentration approached 1.0% (w/v).

The bacterium in ordinary dialysis culture produced $4.0 \times 10^{10}$ viable bacteria per ml and accumulated 3.5% lactate (Fig. 3). At the end of the fermentation period about 60% of the total lactate produced was found in the reservoir.

The ability of *C. utilis* to remove lactate and to produce yeast biomass then was assessed. The yeast oxidized all of the (3.5%) lactate in the spent medium and produced 16 mg of yeast biomass per ml in about 16 hr (Fig. 4). No other nutrient seemed to be limiting.

When the bacterium was grown in mutualistic dialysis culture with the yeast, the final yield of bacterial cells was $5.2 \times 10^{10}$ per ml, which was about 2.5 times more than that in nondialysis culture. The final lactate concentration in the bacterial fermentor was 3.0%, which was lower than that in the controls. The yeast consumed the lactate so fast that by the seventh hr after inoculation no trace of lactate was found in the yeast growth fermentor. However, the yeast dry weight was still increasing from about 1 mg/ml at the seventh hr to 6.0 mg/ml at the end of fermentation. This clearly demonstrated that the yeast utilized the lactate as soon as it crossed the membrane.

The production of bacterial cells in the three systems is compared graphically in Fig. 6, and the production of yeast cells is compared in Fig. 7.

Mutualistic system produced 25% and 125% more bacterial cells compared to ordinary dialysis and nondialysis controls, respectively.

Yeast grown without dialysis showed a steady growth until lactate in the medium is exhausted. On the other hand, yeast grown mutualistically showed faster growth during the earlier half of the fermentation but cumulative cell growth fell below the level of nondialysis control after 10th hr.

## DISCUSSION

In mutualistic dialysis culture, a larger fermentor was used for the bacterium than for the yeast because the yeast had a much slower maximum specific lactate consumption rate (-0.07/hr, calculated from Fig. 3) than the maximum specific lactate production rate of the bacterium (0.77/hr, calculated from fig. 2).

While the lactate concentration increased in the bacterial growth fermentor (fig. 4), that in the yeast growth fermentor decreased rapidly to undetectable amounts. This phenomenon was originally expected to be desirable. However, the low lactate concentration in the yeast fermentor apparently was due not only to the consumption by the growing yeast but also to a slow transfer of lactate from the bacterial growth fermentor.

Solute exchange rather than lactate consumption by the yeast apparently was the limiting step in the process. The amount of the lactate permeated to the reservoir was small compared to the total lactate produced. Only 23% of the total lactate produced was found in the reservoir at the ninth hr just before growth reached the stationary phase (Fig. 4).

More than twice as many bacterial cells were produced in mutualistic dialysis culture as compared to the nondialysis control, and the yeast biomass additionally was produced. The system would be further improved by using membranes with pore sizes larger than 0.2 μm but smaller than 0.45 μm and by providing more membrane area. An attempt to employ a membrane with larger pore (0.45 μm) to improve the solute exchange rate was unsuccessful because the yeast and the

bacteria both penetrated the membrane. About 5 $cm^2/ml$ of membrane area would be needed to ensure good substrate conversion (Stieber and Gerhardt, 1981) and the production of concentrated cell populations (Osborne, 1977), whereas only 0.66 $cm^2/ml$ of membrane area was actually employed.

## References


1. Gerhardt, P., and D. M. Gallup, J. Bacteriol. 86, 919 (1963).

2. Friedman, M. R., and E. L. Gaden, Jr., Biotechnol. Bioeng. 12, 961 (1970).

3. Stieber, R. W., and P. Gerhardt, J. Dairy Sci. 63, 722 (1980).

4. Bergere, J. L., and J. Hermier, LeLait. 48, 13 (1968).

5. Osborne, R. J. W., J. Soc. Dairy Technol. 30, 40 (1977).

6 Schultz, J. S., and P. Gerhardt, Bacteriol. Rev. 33, 1 (1969).

7. Kyung, K. H. Ph.D. Thesis, Michigan State University, East Lansing, MI (1983).

8. Stevenson, K. E., D. E. Black, and R. N. Costilow, J. Food Sci. 44, 181 (1979).

9. Hang, Y. D., D. F. Splittstoesser, and R. L. Landshoot, Appl. Microbiol. 24, 1007 (1972).

10. Pettersson, H.-E., Appl. Microbiol. 29, 133 (1975).

11. Stieber, R., W., and P. Gerhardt, Biotechnol. Bioeng. 25, 535 (1981).

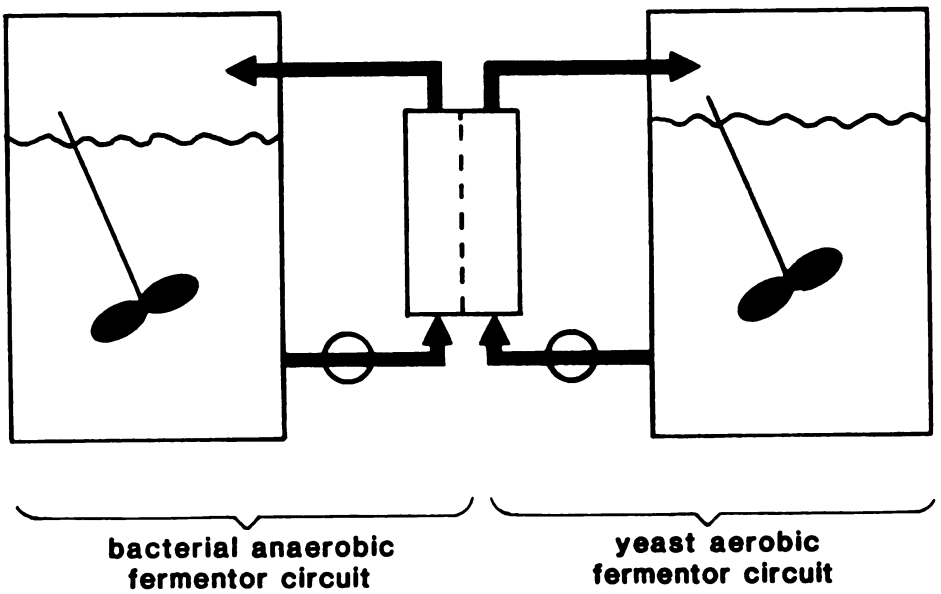



Fig. 1. Schematic of mutualistic dialysis culture system.

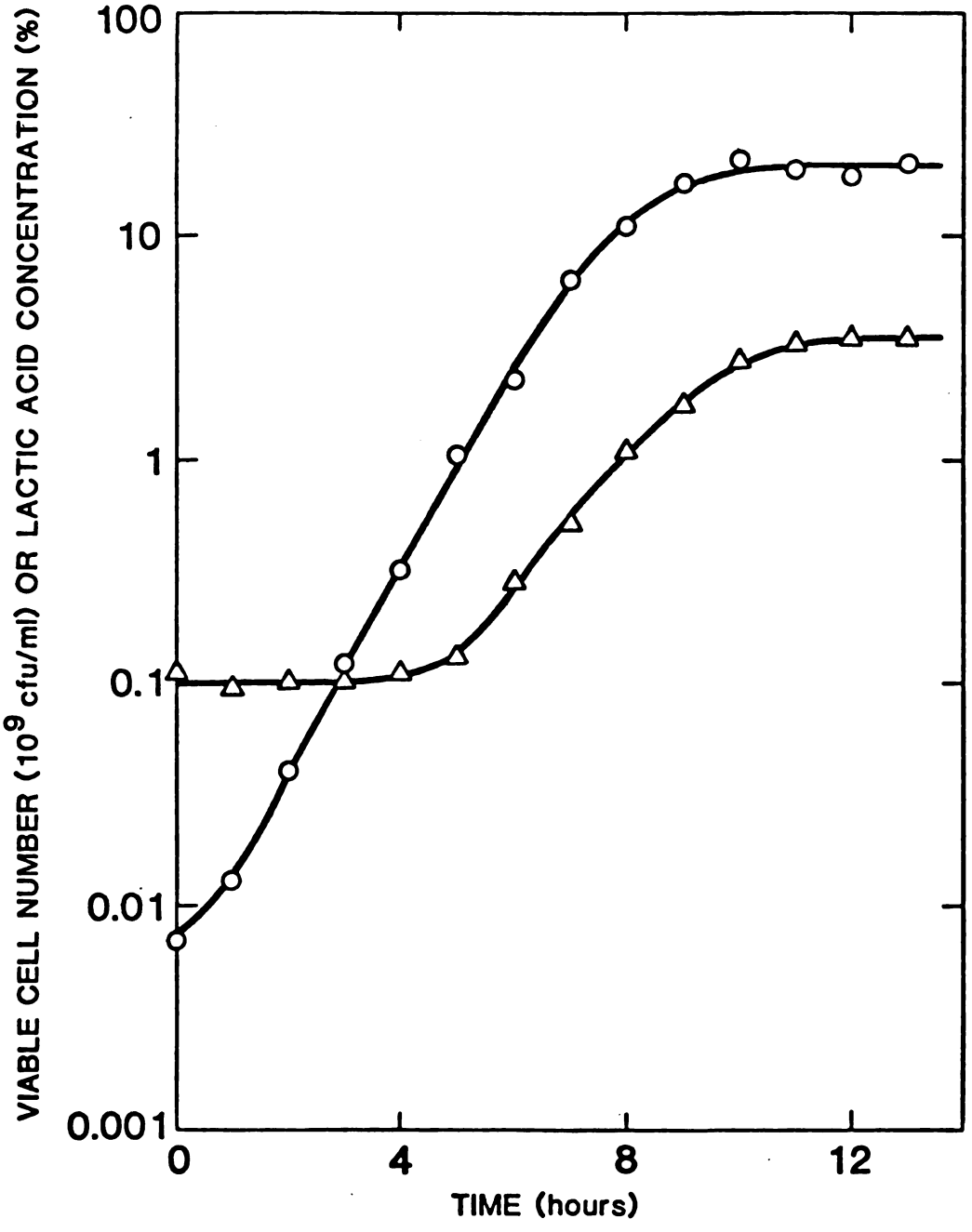



Fig. 2 Nondialysis batch culture of S. lactis: viable cell number of bacteria (O); concentration of lactate (Δ).

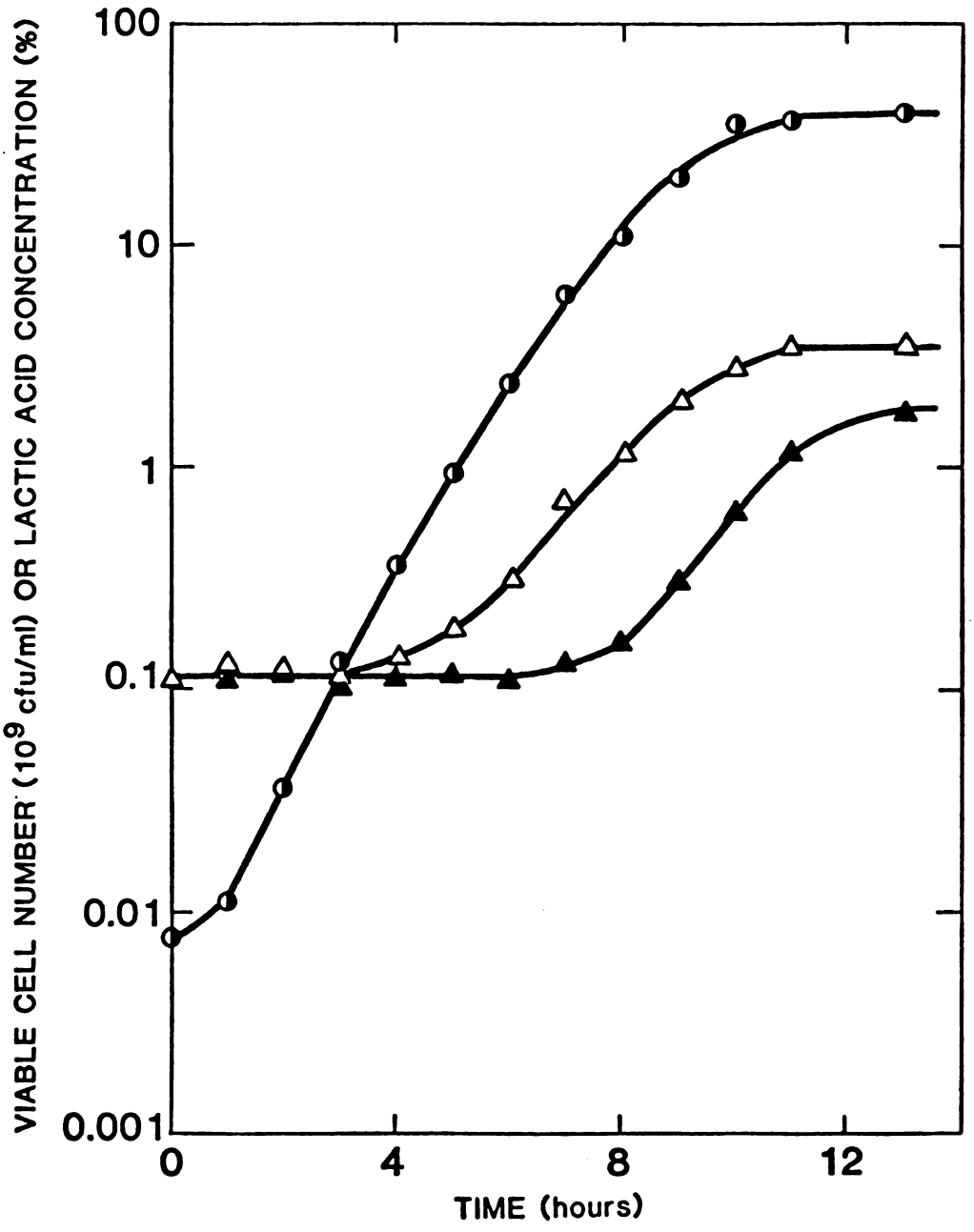



Fig. 3 Ordinary dialysis culture of S. lactis: viable cell number of bacteria (◑); concentration of lactate in the bacterial fermentor (△); concentration of lactate in the reservoir (▲).

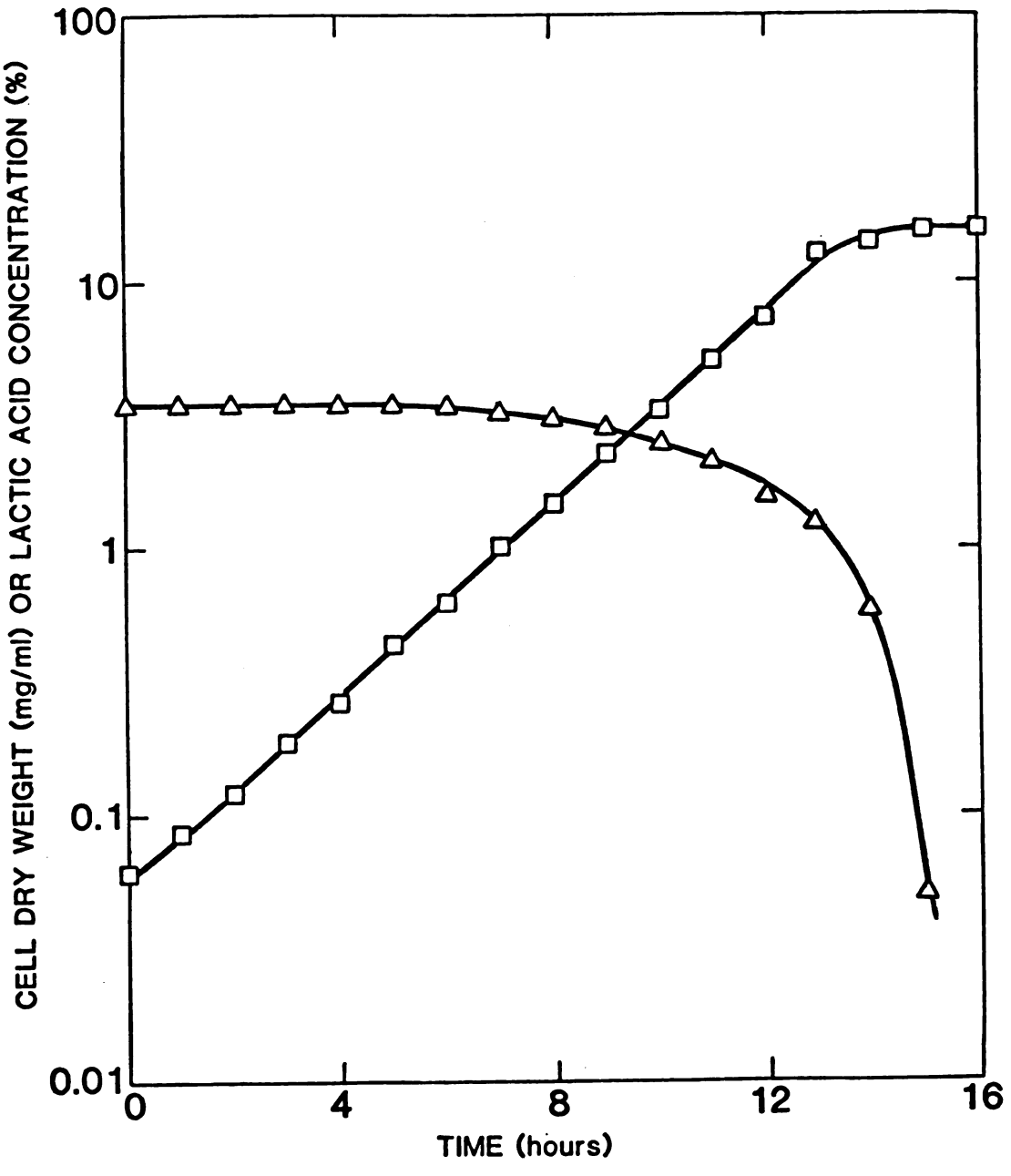



Fig. 4 Nondialysis batch culture of C. utilis: cell dry weight of yeast ( □ ); concentration of lactate ( △ ).

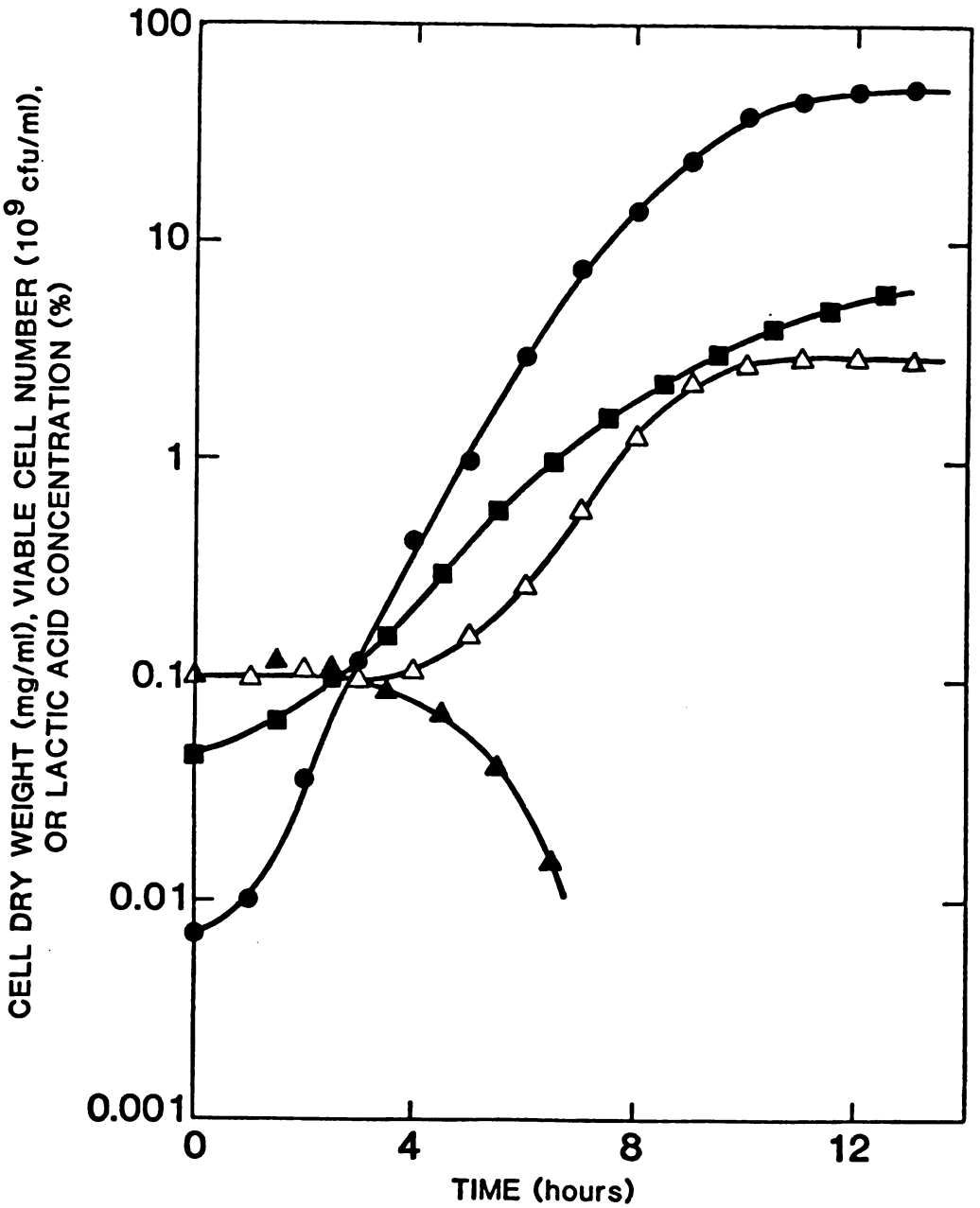



Fig. 5 Mutualistic dialysis culture of S. lactis and C. utilis: viable cell number of bacteria (●); concentration of lactate in the bacterial growth fermentor (△); cell dry weight of yeast (■); concentration of lactate in the yeast growth fermentor (▲).

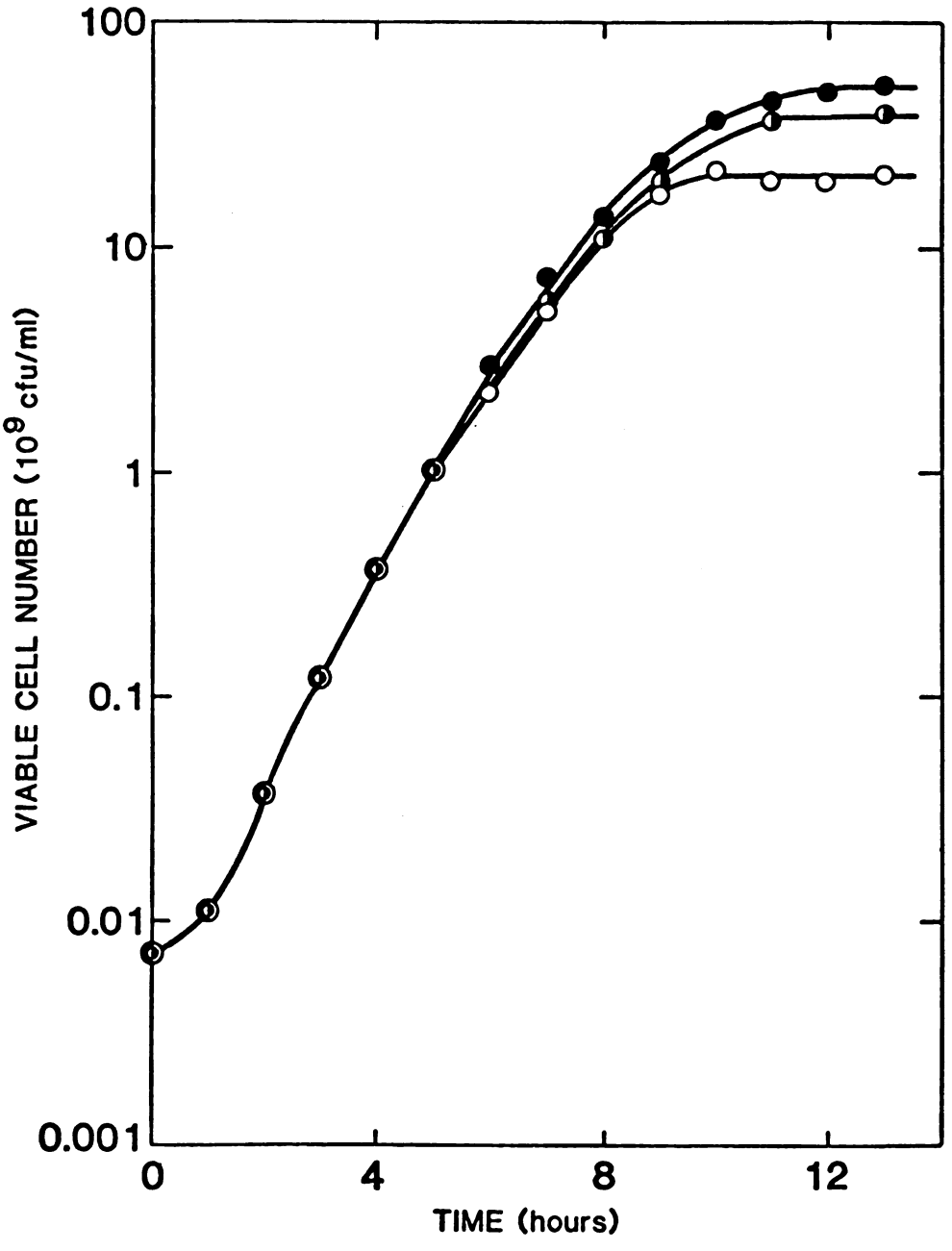



Fig. 6 Comparison of S. lactis growth as viable cell number in three systems: mutualistic dialysis culture (●); ordinary dialysis culture (◑); nondialysis culture (○); common points of the above three systems (◐).

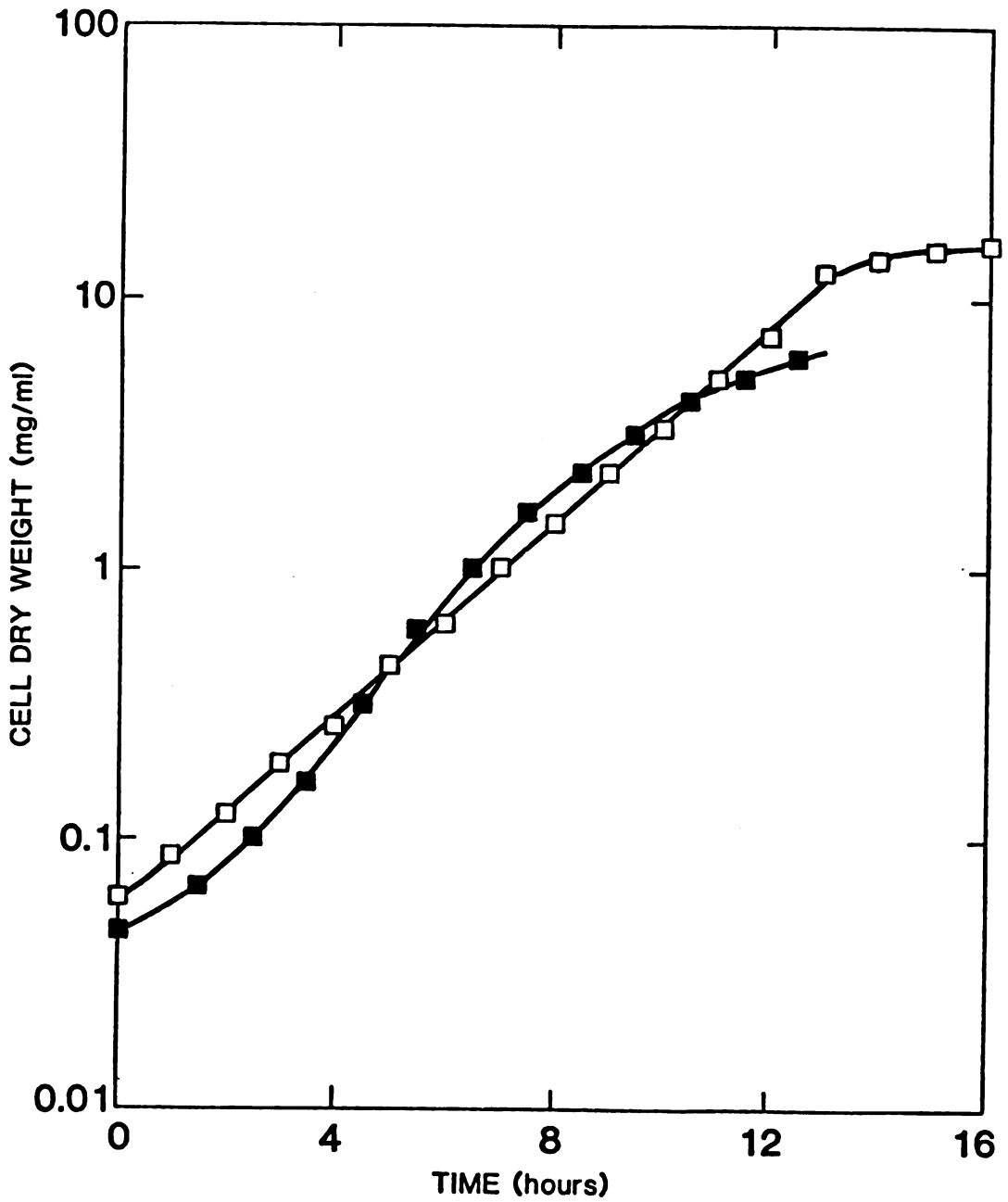



Fig. 7 Comparison of C. utilis growth as cell dry weight in two systems: mutualistic dialysis culture (■); nondialysis culture (□).

# GENERAL DISCUSSION

Fermentation processes generally are regulated by end-product inhibition or repression, which limits the concentration of the product attainable in an ordinary process. To overcome this limitation, various special fermentation techniques have been used or are under development to attain higher productivity. The engineering approaches include continuous vaporization of volatile products by vacuum, continuous extraction of the products from solution, and continuous extraction of the products from solution, and continuous diffusion or filtration of the products through a semipermeable membrane.

Several authors have expressed optimism about the use of membranes in the production and recovery of fermentation products (Fox, 1982; Gregor and Gregor, 1979; Hartline, 1979). The developments of new synthetic membranes and their applications, discussed by Fox (1982) and Gregor and Gregor (1979), are especially encouraging. Applications are apparent in biotechnology, gas separation, metal extraction, waste treatment, water purification, and many branches of food technology.

Results of earlier studies reported in the review of the literature indicate that membranes can be applied in various fields of fermentation to enhance cell growth, substrate conversion, and metabolite production. A membrane-contained fermentation has the

advantage of being able to retain microbial cells in a fermentor. This allows for increased cell population and greater throughput of substrate, and facilitates product recovery. The process can produce a cell-free metabolite at a high concentration and rate, as well as concentrated cell suspension.

In dialysis fermentations, which are driven by a solute concentration gradient, a large volume of dilute permeate is produced by either batch or continuous process modes. The large volume of permeate leads to increased expenses for product recovery when a metabolite is a product, and poses an environmental liability when cells are the product. Stieber and Gerhardt (1979, 1980) solved the problem by feeding highly concentrated substrate into the fermentor, so that the resulting permeate contained a useful concentration of metabolite. For the cycloheximide fermentation, Kominek (1975) and Wang et al. (1981) used a combination of extraction and membrane technology (dialysis) to eliminate the generation of large volumes of dilute permeate.

If the potential of dialysis in fermentation is to be realized, the solute exchange capacity of the dialyzers available at present must be improved through a substantial increase in either permeability or membrane area. Since the void space of current membrane already is large, increased membrane efficiency would have to come mainly from use of a thinner membrane; this improvement is compensated negatively by increased membrane fragility. The use of a substantially larger membrane area not only poses physical limitations but also is limited in terms of turbulence on membrane surfaces, membrane fouling, and economy.

These considerations, along with scale-up problems, indicate that full realization of the potential use of membranes in fermentation will require refinements of the dialysis process, such as electrodialysis or microfiltration, which can substantially increase solute transfer across the membranes. Microfiltration, in which solute transfer is driven by a pressure gradient, could be applied to fermentation by using conventional equipment at low capital and energy costs. Microfiltration could be used to produce a highly concentrated suspension of cells and to remove inhibitory products by bulk liquid flow, but would be limited by plugging of the membrane.

The study of microfiltration fermentation began in this laboratory initially with the author's direct involvement but now by Kevin Anderson, Philipp Gerhardt and Eric Grulke. Escherichia coli HB 101 cells are used as a model organism, because it grows in clear glucose-salts medium and is used widely in recombinant DNA research as a plasmid receptor cell.

In the novel system presently being studied, the culture is started batchwise. As cells reach the end of the exponential phase of growth, additional substrate is fed into the fermentor and microfiltration is started simultaneously. Ideally, the feed and effluent flow rate should be exponential, to meet the exponentially growing need for nutrients. Because of relatively low substrate concentrations in the feed, diffusible metabolites should not accumulate to inhibitory levels. Thus the growth of microbial cells in the fermentor should proceed at an exponential rate until the cell mass becomes unmanageable, or until the filter becomes plugged and unable to pass an adequate flow of liquid. If oxygen supply becomes

growth-limiting, first increased aeration and agitation and then enrichment with pure molecular oxygen can be supplied. A complex control system is necessary to maintain constant volume in the fermentor during microfiltration. A microfilter designed specifically for this purpose is being tested. The electronic liquid level controlling system consists of a level sensor and a level control transmitter (Drexelbrook Engineering Co.) and a current output controller (Leeds & Northrup).

Results of this new research into a microfiltration fermentation process should further realize the potential of membrane technology for application in fermentation. The capital investment in such a process would be great, but the potential for production and recovery of products appears sufficient to more than compensate for the increased expense. The process is expected to be highly efficacious for application in the burgeoning area of biotechnology that involves recombinant DNA-altered microorganisms to produce high-value products such as insulin and interferon.

## GENERAL SUMMARY

Dialysis cultivation methods for microorganisms were examined in order to assess their use to improve representative industrial fermentation processs. A comprehensive review of the literature on dialysis culture of microbial and mammalian cells for the period 1969 to 1983 was made cumulatively with those in two prior theses from this laboratory. The review covers medical and ecological as well as fermentative applications.

The continuous fermentation of glucose to ethanol by cells of Saccharomyces cerevisiae ATCC 4126 "immobilized" by membrane containment was investigated. Substrate was fed into a continuous dialysate circuit and thence into a batch fermentor circuit via diffusion through the membranes of an intermediate dialyzer; simultaneously, product was withdrawn from the fermentor circuit through the dialyzer membranes into the dialysate circuit and out in the effluent. Since the fermentor was operated without an effluent, the cells essentially were immobilized and converted substrate to product by maintenance metabolism. Contrary to prior results with this novel system for the continuous fermentation of lactose to lactate by lactobacillus cells, a steady state of yeast cells in the fermentor was not obtained initially but eventually occurred by the depletion of nutrients and prevention of cell breakage, although substrate and product concentrations then became unsteady. The inherent advantages of the system were offset in the ethanol

fermentation by relatively low productivity, which appeared to be limited by membrane permeability.

Mutualistic dialysis culture of Streptococcus lactis, which is valuable as a dairy starter, and Candida utilis, which is valuable as single cell protein, was investigated in a batch fermentation system. The bacterium and the yeast were inoculated into separate fermentors connected by an intermediate dialyzer, the membranes of which allowed diffusional exchange of solutes. Lactose fed into the bacterial culture was fermented to lactic acid, which was dialyzed into the yeast culture and consumed so as to relieve product inhibition of the bacterial culture. Consequently, the bacterial cell concentration more than doubled in comparison with a nondialysis control, and yeast cells were produced as byproduct. Although the acid production rate by the bacterium was much faster than the acid consumption rate by the yeast, the primary limiting factor of the process apparently was the solute exchange rate across the membrane.

Dialysis fermentation processes in general were discussed relative to other ways for alleviating end-product inhibition and to other applications. Microfiltratioin was proposed as a new and possibly better way to remove products during formation and thereby to further improve industrial fermentation processes with high-value products.

# LITERATURE CITED

## LITERATURE CITED


Abbott, B. J., and P. Gerhardt. 1970a. Dialysis fermentation. I. Enhanced production of salicylic acid from naphthalene by Pseudomonas fluorescens. Biotechnol. Bioeng. 12:577-589.

Abbott, B. J., and P. Gerhardt. 1970b. Dialysis fermentation. II. Kinetic analysis of salicylic acid production via cell growth and maintenance. Biotechnol. Bioeng. 12:591-601.

Abbott, B. J., and P. Gerhardt. 1970c. Dialysis fermentation. III. Anomalous inhibition of threonine biosynthesis in an auxotrophic mutant of Escherichia coli. Biotechnol. Bioeng. 12:603-613.

Abe, M., and F. Kumeno. 1973. In vitro simulation of rumen fermentation: Apparatus and effects of dilution rate and continuous dialysis on fermentation and protozoal population. J. Anim. Sci. 36:941-948.

Aida, T., and K. Yamaguchi. 1969. Studies on the utilization of hydrocarbons by yeasts. Part IV. On the dialysis culture of Mycotorula japonica and "growth-inhibitory factor" in the dialyzable material. Agr. Biol. Chem. 33:1244-1250.

Akaki, M. 1965. Studies on mixed yeast cultures in sulfite waste liquor medium. J. Ferment. Technol. (Jap) 43:365-373.

Alekseeva, G. V., and V. M. Yunker. 1969. A method of cultivating tissues in vivo in diffusion chambers. Bulletin of Exptl. Biol. Med. 67:217.

Algire, G. H., J. M. Weaver, and R. T. Prehn. 1954. Growth of cells in vivo in diffusion chambers. I. Survival of homografts in immunized mice. J. Nat. Cancer Inst. 15:493-507.

Ambrose, K. R., F. Chandler, and J. H. Coggin, Jr. 1969. Characterization of tumor-specific transplantation immunity reactions in immunodiffusion chambers in vivo. Proc. Soc. Exptl. Biol. Med. 132:1013-1020.

Andersen, K., and K. T. Shanmugam. 1977. Nitrogen fixation: determination of the ratio of formation of $H_2$ to $NH_4^+$ catalyzed by nitrogenase of Klebsiella pneumonia in vivo. J. Gen. Microbiol. 103:107-122.

Andreasen, A. A., and T. J. B. Stier. 1954. Anaerobic nutrition of Saccharomyces cerevisiae II. Unsaturated fatty acid requirement for growth in a defined medium. J. Cell.Comp. Physiol. 43:271-280.

Arko, R. J. 1972. Neisseria gonorrhoeae: experimental infection of laboratory animals. Science 177:1200-1201.

Arko, R. J. 1973. Implantation and use of a subcutaneous culture chamber in laboratory animals. Lab. Anim. Sci. 23:105-106.

Arko, R. J. 1974. An immunologic model in laboratory animals for the study of Neisseria gonorrhoeae. J. Infect. Dis. 129:451-455.

Athens, J. W. 1970. Neutrophilic granylocyte kinetics and granulocytopoiesis, p. 1143-1166. In A. S. Gordon (ed.), Regulation of hemopoiesis, Appleton-Century-Crofts, New York.

Baman, S. I., and R. Haque. 1970. Production of multivalent extracellular filtrates of Staphylococcus aureus. Can. J. Microbiol. 16:1255-1261.

Barr, R. D., J. Whang-Peng, and S. Perry. 1975. Hemopoietic stem cells in human peripheral blood. Science 190:284-285.

Baskett, R. C., and W. J. Lulves. 1974. A method of measuring bacterial growth in aquatic environments using dialysis culture. J. Fish. Res. Board Can. 31:372-374.

Batzdorf, U., R. S. Knox, S. M. Pokress, and J. C. Kennady. 1969. Membrane partitioning of the Rose-type chamber for the study of metabolic interaction between different cultures. Stain Technol. 44:71-74.

Beard, P. J., and N. F. Meadowcroft. 1935. Survival and rate of death of intestinal bacteria in sea water. Amer. J. Publ. Health 25:1023-1026.

Bednarski, M. A., and M. Reporter. 1976. Nitrogenase activity in rhizobia in vitro: evidence for auxiliary substances elaborated by cultured soybean cells. Plant Physiol. 57:103.

Bednarski, M. A., and M. Reporter. 1978. Expression of rhizobial nitrogenase: influence of plant cell-conditioned medium. Appl. Environ. Microbiol. 36:115-120.

Belding, R. C., J. M. Quarles, T. C. Beaman, and P. Gerhardt. 1976. Exteriorized carotid-jugular shunt for hemodialysis of the goat. Lab. Anim. Sci. 26:951-954.

Bell, J. F., R. K. Farrell, and J. R. Gorham. 1957. A polyvalent toxoid for botulism in mink. Am. J. Vet. Res. 18:167-170.

Benestad, H. B. 1970. Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leukocytes. Scand. J. Haemat. 7:279-288.

Benestad, H. B. 1972. Cell kinetics in diffusion chambers: survival, resumption of proliferation, and maturation rate of murine haemopoietic cells. Cell Tissue Kinet. 5:421-431.

Benestad, H. B., J. Iversen, and B. Rolstad. 1971. Macrophage proliferation in leukocyte populations from rat blood and lymph during immune responses. Scand. J. Haemat. 8:44-52.

Benestad, H. B., and A. Reikvam. 1975. Diffusion chamber culturing of haematopoietic cells: methodological investigations and improvement of the technique. Exp. Hemat. 3:249-260.

Benestad, H. B., N. G. Testa, and L. G. Lajtha. 1978. Inhibition of haemopoietic stem cell proliferation by a diffusible product of bone marrow cells. Scand. J. Haematol. 20:18-24.

Benestad, H. B., and E. O. Toogood. 1982. Diffusion chamber (DC) culturing of haemopoietic cells: A reliable assay system for regulators of proliferation? Exp. Heamtol. 10:161-171.

Beran, M. 1975. Serum levels of colony stimulating factor(s) and the growth of bone marrow cells in diffusion chambers. Cell Tissue Kinet. 8:561-568.

Bergere, J. L., and J. Hermier. 1968. La production massive de cellules de Streptocoques lactiques. LeLait 48:13-30.

Berland, B. R., D. J. Bonin, and S. Y. Maestrini. 1976. De l'emploi concomitant d'enceintes dialysantes et de tests biologiques pour la d'etermination des facteurs nutritionnels limitant la production primarire des eaux marine. Ann. Inst. Oceanogr. (Paris) 52:45-55.

Berman, I., and H. S. Kaplan. 1959. The cultivation of mouse bone marrow in vivo. Blood 14:1040-1046.

Berman, I., and H. S. Kaplan. 1960. The functional capacity of mouse bone marrow cells growing in diffusion chambers. Exp. Cell Research 20:238-239.

Bhattacharyya, F. K. 1973. Dialysis-culture production of Vibrio cholerae exotoxin and its precipitation with zinc acetate. J. Med. Microbiol. 6:499-504.

Bianchi, A. J. M., and M. G. Bensoussan. 1977. Non-marine bacteria in dialysis bags, in sea water. Mar. Pollut. Bullet. 8:282-283.

Bissell, M. J., R. Tosi, and L. Gorini. 1971. Mechanism of excretion of a bacterial proteinase: factors controlling accumulation of the extracellular proteinase of a Sarcina strain (coccus P). J. Bacteriol. 105:1099-1109.

Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart. 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. 29:186-194.

Black, S. H. 1966. Enhanced growth of Bordetella pertussis in dialysis culture. Nature 209:10105-106.

Boecker, W. R., A. Boyum, A. L. Carsten, and E. P. Cronkite. 1971. Human bone marrow (HBM) and blood (HPB) stem cell kinetics. Blood 38:819.

Boecker, W. R., D. K. Hossfeld, W. M. Gallmeir, and C. G. Schmidt. 1975. Clonal growth of Hodgkin cells. Nature 258:235-236.

Boecker, W. R., S. Ohl., D. K. Hossfeld, and C. G. Schmidt. 1978. Differentiation of auer-rod positive leukemic cells in diffusion chamber culture. Lancet 1:267-269.

Borella, L. 1969. The suppressive effect of Rauscher leukemia virus on the secondary antibody response of spleen cells cultured in cell-impermeable diffusion chambers. J. Immunol. 103:185-195.

Borella, L. 1971. Regulation of IgM memory expression by spleen cells cultured in diffusion chambers. Immunol. 20:289-298.

Borella, L. 1972. The immunosuppressive effects of Rauscher leukemia virus (RLV) upon spleen cells cultured in cell-impermeable diffusion chambers. III. Inhibition of RLV-induced cell pathways by antigenic stimulation with hemocyanin. J. Immunol. 108:45-53.

Borichewski, R. M. 1967. Keto acids as growth-limiting factors in autotrophic growth of Thiobacillus thiooxidans. J. Bacteriol. 93:597-599.

Boyum, A., and R. Borgstrom. 1970. The concentration of granulocytic stem cells in mouse bone marrow, determined with diffusion chamber technique. Scand. J. Haemat. 7:294-303.

Boyum, A., W. Boecker, A. L. Carsten, and E. P. Cronkite. 1972. Proliferation of human bone marrow cells in diffusion chambers implanted into normal or irradiated mice. Blood 40:163-173.

Boyum, A., A. L. Carsten, O. D. Laerum, and E. P. Cronkite. 1972. Kinetics of cell proliferation of murine bone marrow cells cultured in diffusion chambers: effect of hypoxia, bleeding, erythropoietin injections, polycythemia, and irradiation of the host. Blood 40:174-188.

Boyum, A., D. Lovhaug, and W. R. Boecker. 1976. Regulation of bone marrow cell growth in diffusion chambers: the effect of adding normal and leukemic (CML) polymorphonuclear granulocytes. Blood 48:373-383.

Breivik, H. 1971. Haematopoietic stem cell content of murine bone marrow, spleen, and blood. Limiting dilution analysis of diffusion chamber cultures. J. Cell. Physiol. 78:73-78.

Breivik, H., and H. Benestad. 1972. Regulation of granulocyte and macrophage formation in diffusion chamber cultures of mouse haematopoietic cells. Exp. Cell Research 70:340-348.

Breivik, H., H. B. Benestad, and A. Boyum. 1971. Diffusion chamber and spleen colony assay of murine haematopoietic stem cells. J. Cell. Physiol. 78:65-72.

Brooks, J. R., S. H. Sturgis, and G. J. Hill. 1960. An evaluation of endocrine tissue homotransplantation in the millipore chamber: with a note on tissue adaptation to the host. Ann. N.Y. Acad. Sci. 87:482-500.

Buchanan, T. M., and E. C. Gotschlich. 1973. Studies on gonococcus infection. III. Correlation of gonococcal colony morphology with infectivity for the chick embryo. J. Exper. Med. 137:196-200.

Capalbo, E. E., and T. Makinodan. 1964. Doubling time of mouse spleen cells during the latent and log phases of primary antibody response. J. Immunol. 92:234-242.

Carsten, A. L., A. D. Chanana, G. Chikkappa, E. P. Cronkite, and S. Ohl. 1975. An improved diffusion chamber (DC) for culture of hemopoietic cells. Proc. Soc. Exp. Biol. Med. 150:107-109.

Casman, E. P., and R. W. Bennett. 1963. Culture medium for the production of staphylococcal enterotoxin A. J. Bacteriol. 86:18-23.

Chikkappa, G., A. L. Carsten, A. D. Chanana, and E. P. Cronkite. 1978. Culture of normal human blood cells in diffusion chamber systems. I. Granulocyte survival and proliferation. Exp. Hemat. 6:28-36.

Ciccarelli, A. S., D. N. Whaley, L. M. McCroskey, D. F. Gimenez, V. R. Dowell, Jr., and C. L. Hatheway. 1977. Cultural and physiological characteristics of Clostridium botulinum type G and the susceptibility of certain animals to its toxin. Appl. Environ. Microbiol. 34:843-848.

Clive, D., and O. E. Landman. 1970. Reversion of Bacillus subtilis protoplasts to the bacillary form induced by exogenous cell wall, bacteria and by growth in membrane filters. J. Gen. Microbiol. 61:233-243.

Collins, E. B., and A. W. Tillion. 1977. Detection of antagonistic and symbiotic relationships among bacteria by diffusion chamber procedure. J. Dairy Sci. 60:387-393.

Collins, J. F., and M. H. Richmond. 1962. Rate of growth of Bacillus cereus between divisions. J. Gen. Microbiol. 28:15-33.

Coulman, G. A., R. W. Stieber, and P. Gerhardt. 1977. Dialysis continuous process for ammonium-lactate fermentation of whey: Mathematical model and computer simulation. Appl. Environ. Microbiol. 34:725-732.

Crook, R. H., L. V. Scott, and R. A. Patnode. 1969. Characterization of antigens from Leishmania mexicana grown in dialysate culture. J. Parasitol. 55:977-981.

Crumpton, W. G. 1980. Differential effects of growth and loss processes in controlling natural phytoplankton populations. Ph.D. thesis. Michigan State University, East Lansing, Michigan.

Crumpton, W. G., and R. G. Wetzel. 1983. Effects of differential growth and mortality in the seasonal succession of phytoplankton populations in Lawrence Lake, Michigan. Ecology (in press).

Cysewski, G. R., and C. R. Wilke. 1977. Rapid ethanol fermentation using vacuum and cell recycle. Biotechnol. Bioeng. 19:1125-1143.

Dent, P.B., D. Y. E. Perey, M. D. Cooper, and R. A. Good. 1968. Nonspecific stimulation of antibdy production in surgically bursectomized chickens by bursa-containing diffusion chambers. J. Immunol. 101:799-805.

Dhople, A. M., and J. H. Hanks. 1972a. Fate of Mycobacterium lepraemurium in diffusion chambers incubated in vitro and in vivo. Int. J. Lepr. 40:210.

Dhople, A. M., and J. H. Hanks. 1972b. The energetics (ATP) of Mycobacterium lepraemurium in diffusion chambers incubated in vitro and in vivo. Int. J. Lepr. 40:465-466.

Dhople, A. M., and J. H. Hanks. 1973. Energetics (adenosine 5' - triphosphate) of Mycobacterium lepraemurium in diffusion chambers incubated in vitro and in mice. Infect. Immun. 8:907-910.

Diener, E., and W. E. Armstrong. 1967. Induction of antibody formation and tolerance in vitro to a purified protein antigen. Lancet ii:1281-1285.

Dobry, D. D., and J. L. Jost. 1977. Computer applications to fermentation operations, p. 95-114. In D. Perlman (ed.), Annual reports on fermentation processes, Vol. 1, Academic Press, Inc., New York.

Donnelly, C. B., J. E. Leslie, L. A. Black, and K. H. Lewis. 1967. Serological identification of enterotoxigenic staphylococci from cheese. Appl. Microbiol. 15:1382-1387.

Dor, I. 1975. Dialysis culture for production of algal food from sewage. Proceedings of the Sixth Scientific Conference at the Israel Ecology Society, Tel Aviv.

Dor, I. 1975. High density, dialysis culture of algae on sewage. Water Res. 9:251-254.

Dumas, R., and A. Bianchi. 1972. Modifications des constituents cellulaires au cours de la d'egradation du phytoplancton par les bacteries etude en enceinte dialysante. Tethys 4:27-36.

Dusanic, D. G. 1969. Cultivation of Trypanosoma lewisi in a dialysate medium. I. Amino acid alterations during growth. Comp. Biochem. Physiol. 30:895-901.

Duxbury, T. 1977. A microperfusion chamber for studying the growth of bacterial cells. J. Appl. Bacteriol. 43:247-251.

Edwards, J. H. 1972. The double dialysis method of producing farmer's lung antigens. J. Lab. Clin. Med. 79:683-688.

Eide, I., A. Jensen, and S. Melsom. 1979. Application of in situ cultures of phytoplankton for monitoring heavy metal pollution in two Norwegian fjords. J. Exp. Mar. Biol. Ecol. 37:271-286.

Eipert, E. F., L. Adorini, and J. Couderc. 1978. A miniaturized in vitro diffusion culture system. J. Immunol. Methods 2:283-292.

Evgenjeva, T. P. 1970. Heterotransplantation of human cancers to animals by means of diffusion chambers. Europ. J. Cancer 6:533-535.

Faradji, A., J. P. Bergerat, and F. Oberling. 1980. Hemopoietic bone culture in diffusion chambers. Biomed. 33:75-77.

Fauerholdt, L., and N. Jacobsen. 1975. Cultivation of leukemic human bone marrow cells in diffusion chambers implanted into normal and irradiated mice. Blood 45:495-501.

Ficsor, G., and E. Muthiani. 1971. A microbial assay for detecting chemical mutagens in tissue homogenates. Mut. Res. 12:335-337.

Fliermans, C. B., and R. W. Gorden. 1977. Modification of membrane diffusion chambers for deep-water studies. Appl. Environ. Microbiol. 33:207-210.

Fliermans, C.B., R. W. Gorden, T. C. Hazen, and G. W. Esch. 1977. Aeromonas distribution and survival in a thermally altered lake. Appl. Environ. Microbiol. 33:114-122.

Flynn, J., and S. A. Waitkins. 1973. Survival of Neisseria gonorrhoeae in an artificial subcutaneous cavity of the mouse. Brit. J. Vener. Dis. 49:432-434.

Fogarty, W. M., and P. J. Griffin. 1973. A device for production of microbial extracellular enzymes in concentrated form. J. Appl. Chem. Biotechnol. 23:401-406.

Fox, J. F. 1982. Membrane development slowed by weak economy. Chem. Eng. News 8:7-12.

Frankland, P. F., and H. M. Ward. 1893. Second report to the Royal Society Water Research Committee. The vitality and virulence of Bacillus anthracis and its spores in potable waters. Proc. Royal Soc. London. 53:164-317.

Friedman, M. R., and G. L. Gaden, Jr. 1970. Growth and acid production by Lactobacillus (L.) delbrueckii in a dialysis culture system. Biotechnol. Bioeng. 12:961-974.

Gallicchio, V. S., and M. J. Murphy, Jr. 1981. Effect of membrane dialysis and filtration-sterilization on erythropoietic activity. The Yale J. Biol. Med. 54:249-254.

Gallup, D. M., and P. Gerhardt. 1963. Dialysis fermentor systems for concentrated culture of microorganisms. Appl. Microbiol. 11:506-512.

Gardner, W. G., R. B. Prior, and R. L. Perkins. 1973. Fluid and pharmacological dynamics in a subcutaneous chamber implanted in rats. Antimicrob. Agents Chemother. 4:196-197.

Gates, R. J., and N. R. Lazarus. 1977. Reversal of streptozotocin-induced diabetes in rats by intraperitoneal implantation of encapsulated neonatal rabbit pancreatic tissue. Lancet 2:1257-1259.

Gengozian, N. 1964. Heterotransplantation of human antibody-froming cells in diffusion chambers. Ann. N.Y. Acad. Sci. 120 (Part 1):91-118.

Germain, G. S., H. Schneider, and E. E. Muirhead. 1966. Multiple access diffusion chamber and the cultivation of liver cells. Exp. Cell Res. 43:493-505.

Gerhardt, P., and D. M. Gallup. 1963. Dialysis flask for concentrated culture of microorganisms. J. Bacteriol. 86:919-929.

Gerhardt, P., and C.-G Hedén 1960. Concentrated culture of gonococci in clear liquid medium. Proc. Soc. Exp. Biol. Med. 105:49-51.

Gerhardt, P., J. M. Quarles, T. C. Beaman, and R. C. Belding. 1977. Ex vivo hemodialysis culture of microbial and mammalian cells. J. Infect. Dis. 135:42-50.

Globerson, A., and R. Auerbach. 1965. Primary immune reactions in organ cultures. Science 149:991-993.

Golde, D. W., and M. J. Cline. 1973. Growth of human bone marrow in liquid culture. Blood 41:45-57.

Gordon, M. Y., and N. M. Blackett. 1975. Stimulation of granulocytic colony formation in agar diffusion chambers implanted in cyclophosphamide pretreated mice. Br. J. Cancer 32:51-59.

Goto, S., S. Enomoto, Y. Takahashi, and R. Motomatsu. 1971. Slime production by Pseudomonas aeruginosa. I. Conditions for slime production by the cellophane plate method. Japan. J. Microbiol. 15:317-324.

Green, I. 1966. Experiences with the use of microdiffusion chamber techniques in man. Int. Arch. Allergy 29:434-446.

Gregor, H. P., and C. D. Gregor. 1978. Synthetic-membrane technology. Scientific Amer. 239:112-128.

Gregor, H. P., and T. W. Jeffries. 1979. Ethanolic fuels from renewable resources in the solar age. Ann. New York Acad. Sci. 326:273-287.

Grillo, R. S., and D. A. Spink. 1968. Experiments with tissue homografts enclosed in diffusion chambers. Oncology 22:227-239.

Groves, D. L., W. E. Lever, and T. Makinodan. 1970. A model for the interaction of cell types in the generation of hemolytic plaque-forming cells. J. Immunol. 104:148-165.

Gue, P. T., G. Shabinskii, and N. Kh. Gong. 1973. Vyrashchivanie mikoplazm metodom dializa. Vestn Akad. Med. NAUK SSSR 28:14-17.

Guthrie, R. K., and W. J. Nunez. 1970. Passive transfer of hypersensitivity by cells enclosed in membrane chambers. Am. Rev. Resp. Dis. 102:398-402.

Hafiz, S., and M. G. McEntegart. 1977. The survival of gonococci and meningococci in subcutaneous diffusion chambers in mice. J. Med. Microbiol. 10:37-42.

Hallander, H. O. 1965. Production of large quantities of enterotoxin B and other staphylococcal toxins on solid media. Acta Path. Microbiol. Scand. 63:299-305.

Halshall, M. K., and T. Makinodan. 1974. Analysis of the limiting-dilution assay used for estimating frequencies of immunocompetent units. Cell. Immunol. 11:456-465.

Hang, Y. D., D. F. Splittstoesser, and R. L. Landshoot. 1972. Sauerkraut waste: a favorable medium for cultivating yeast. Appl. Microbiol. 24:1007-1008.

Harris, N. K. and E. O. Powell. 1951. A culture chamber for the microscopical study of living bacteria with some observations on the spore-bearing aerobes. J. Roy. Microbiol. Soc. 71:407-420.

Harrison, G. A., J. J. T. Owen, an M. A. Ritter. 1968. Interaction of mouse spleen cells in diffusion chambers. Nature 219:302-303.

Hartline, F. F. 1979. Lowering the cost of alcohol. Science 206:41-42.

Hendricks, C. W., and S. M. Morrison. 1967. Multiplication and growth of selected enteric bacteria in clear mountain stream water. Water Res. 1:567-576.

Hernandez-Mena, R., J. A. Ribaud, and A. E. Humphrey. 1980. Demonstration of process feasibility for continuous extraction of alcohols from fermenting systems. Abstracts of the Sixth International Fermentation Symposium, London, Ontario.

Hesslein, R. H. 1976. An in situ sampler for close interval pore water studies. limnol. Oceanogr. 21:912-914.

Hikuma, M., T. Kubo, T. Yasuda, I. Karube, and S. Suzuki. 1979. Microbial electrode sensor for alcohols. Biotechnol. Bioeng. 21:1845-1853.

Himmel, M. E., P. J. Walker, L. H. Lauerman, and P. G. Squire. 1982. A novel dialysis fermentor design and its application to the production of a cytotoxin from Pasteurella haemolytica. Biotechnol. Lett. 4:97-102.

Hoelzer, D., E. G. Harriss, M. Slade, and E. Kurrle. 1976. Growth of in vitro colony-forming cells from normal human peripheral blood leukocytes cultured in diffusion chambers. J. Cell. Physiol. 89:89-100.

Hoelzer, D., E. Kurrle, U. Ertl, and A. Milewski. 1974. Growth of human leukaemic peripheral blood cells in diffusion chambers. Europ. J. Cancer 10:579-589.

Hoelzer, D., E. Kurrle, H. Schmucker, and E. B. Harriss. 1977. Evidence for differentiation of human leukemic blood cells in diffusion chamber culture. Blood 49:729-744.

Hoffman, P. S. 1974. The swarming of Proteus mirabilis: Formation of swarm cells and swarming. M.S. thesis. Iowa State University, Ames, Iowa.

Horng, C. C. 1971. Dialysis culture of mammalian cells. M.S. thesis. Michigan State University, East Lansing, Michigan.

Horvath, I., R. J. Arko, and J. C. Bullard. 1975. Effects of oxygen and nitrogen on the character of T. pallidum in subcutaneous chambers in mice. Brit. J. Vener. Dis. 51:301-304.

Huang, C. C., and M. Furukawa. 1978. Diffusion chamber cultures for mutagenic and carcinogenic assays. Mutation Res. 53:201-202.

Huff, C. G., A. B. Weathersby, A. C. Pipkin, and G. H. Algire. 1960. The growth of exoerythrocytic stages of avian malaria within diffusion chambers in different hosts. Exp. Parasitol. 9:98-104.

Humphrey, H. E. B. 1970. Dialysis culture of bacteria: dialyzer design, nutrient transfer, growth, and dialysis aeration. Ph.D. Thesis. Michigan State University, East Lansing, Michigan.

Hunter, K. M., and I. McVeigh. 1970. Development of a chemically defined medium for growth of Neisseria gonorrhoeae. Antonie van Leeuwenhoek 36:305-316.

Issac, L., P. G. Leonard, and G. C. Ware. 1975. Modification of the Powell culture chamber for the observation and micro-manipulation of growing bacteria. Lab. Pract. 24:667-669.

Ito, T., and Y. Kishi. 1972. Application of diffusion chamber technic to the cultivation of Mycobacterium lepraemurium. I. In vivo studies. Int. J. Lepr. 40:118-129.

Jensen, A. 1976. Dialysis cultures in integrated aquaculture, p. 143-149. In D. O. Devik (ed.), Harvesting polluted waters. Plenum Publishing Corp., N. Y.

Jensen, A., and B. Rystad. 1973. Semi-continuous monitoring of the capacity of sea water for supporting growth of phytoplankton. J. Exp. Mar. Biol. Ecol. 11:275-285.

Jensen, A., and B. Rystad. 1976. Heavy metal tolerance of marine phytoplankton. II. Copper tolerance of three species in dialysis and batch cultures. J. Exp. Mar. Biol. Ecol. 22:249-256.

Jensen, A., B. Rystad, and S. Melsom. 1974. Heavy metal tolerance of marine phytoplankton. I. The tolerance of three algal species to zinc in coastal sea water. J. Exp. Mar. Biol. Ecol. 15:145157.

Jensen, A., B. Rystad, and L. Skoglund. 1972. The use of dialysis culture in phytoplankton studies. J. Exp. Mar. Biol. Ecol. 8:241-248.

Jensen, D. V., C. G. Huff, and T. Shiroishi. 1964. The use of Rose multipurpose chambers and dialysis membranes in the cultivation of exoerythrocytic stages of avian malarial parasites. Amer. J. Trop. Med. Hyg. 13:653-658.

Kan, J. K., and M. L. Shuler. 1978. Urocanic acid production using whole cells immobilized in a hollow fiber reactor. Biotechnol. Bioeng. 20:217-230.

Kearney, J. F., and P. C. Reade. 1974. The kinetics of mouse thoracic duct lymphocyte activation by mitogens in vitro. Aust. J. Exp. Biol. Med. Sci. 52:21-31.

Kell, D. B. 1980. The role of ion-selective electrodes in improving fermentation yields. Process Biochem. 15:18-23, 29.

Kitsukawa, H. 1969. In vitro cultivation of rabbit aortic endothelium and its biologic nature. J. Kurume. Med. Assoc. 32:1439-1511.

Knazek, R. A., P. M. Gullino, and W. R. Kidwell. 1974. Cell culture on semipermeable tubular membranes. U.S. Patent 3,821,087.

Knazek, R. A., P. M. Gullino, P. O. Kohler, R. L. Dedrick. 1972. Cell culture on artificial capillaries: an approach to tissue growth in vitro. Science 178:65-67.

Kominek, L. A. 1975. Cycloheximide production by Streptomyces griseus: alleviation of end-product inhibition by dialysis-extraction fermentation. Antimicrob. Agents Chemother. 7:861-863.

Kominek, L. A. 1975. Cycloheximide production by Streptomyces griseus: control mechanisms of cycloheximide biosynthesis. Antimicrob. Agents Chemother. 7:856-860.

Kominek, L. A. 1975. Process for producing cycloheximide. U.S. Patent 3,915,803.

Kominek, L. A. 1975. Dialysis process and apparatus. U.S. Patent 3,915,802.

Ku, K., M. J. Kuo, J. Delente, B., S. Wildi, and J. Feder. 1981. Development of a hollow-fiber system for large-scale culture of mammalian cells. Biotechnol. Bioeng. 23:79-95.

Kumegawa, M., M. Cattoni, and G. G. Rose. 1968. Electron microscopy of oral cells in vitro. III. In situ embedding of cultures in chambers of the circumfusion system. Texas Reports on Biology and Medicine 26:205-214.

Kuralesova, A. I. 1968. Osteogenic potential of irradiated bone marrow transplanted in diffusion chambers. Bull. Exp. Biol. Med. 66:1279-1281.

Laerum, O. D., and A. Boyum. 1970. The separation and cultivation of basal and differentiating cells from hairless mouse epidermis. J. Investi. Dermatol. 54:279-287.

Laerum, O. D., A. Gruneisen, and M. F. Rajewsky. 1973. Proliferative properties of malignant cell populations cultured in intraperitoneal diffusion chambers. Europ. J. Cancer 9:533-541.

Laissue, J., A. D. Chanana, E. P. Cronkite, D. D. Joel, and M. Pavelec. 1974. Culture of autologous bone marrow (BM) in diffusion chambers: effect of host irradiation. Federation Proc. 33:598.

Laissue, J. A., A. D. Chanana, E. P. Cronkite, D. D. Joel, and M. Pavelec. 1975. Culture of autologous bone marrow in diffusion chambers: effect of host irradiation. Blood 45:417-425.

Landwall, P. 1978. Dialysis culture for the production of extracellular protein A from Staphylococcus aureus A676. J. Appl. Bacteriol. 44:151-158.

Landwall, P., and T. Holme. 1977a. Influence of glucose and dissolved oxygen concentrations on yields of Escherichia coli B in dialysis culture. J. Gen. Microbiol. 103:353-358.

Landwall, P., and T. Holme. 1977b. Removal of inhibitors of bacterial growth by dialysis culture. J. Gen. Microbiol. 103:345-352.

Landwall, P., and R. Mollby. 1978. Production of Escherichia coli heat-labile enterotoxin in fermentor dialysis culture. J. Appl. Bacteriol. 44:141-149.

Lane, A. G. 1977. Production of food yeast from whey ultrafiltrate by dialysis culture. J. Appl. Chem. Biotechnol. 27:165-169.

LaScolea, L. J., Jr., M. J. Dul, and F. E. Young. 1975. Stability of pathogenic colony types of Neisseria gonorrhoeae in liquid culture by using the parameters of colonial morphology and deoxyribonucleic acid transformation. J. Clin. Microbiol. 1:165-170.

Levey, R. H., N. Trainin, and L. W. Law. 1963. Evidence for function of thymic tissue in diffusion chambers implanted in neonatally thymectomized mice. Preliminary report. J. Natl. Cancer Inst. 31:199-217.

Lovhaug, D., and A. Boyum. 1977. Regulation of bone marrow cell growth in diffusion chambers: The effects of granulocyte extracts. Cell Tissue Kinet. 10:137-146.

Lovhaug, D., A. Boyum, and T. Kristiansen. 1978. Culture of hematopoietic cells in diffusion chambers, p. 175-192. In Golde, D. W., M. J. Cline, D. Metcalf, and C. F. Fox (eds.), Heamtopoietic cell differentiation. Academic Press, New York, N.Y.

Luedeking, R., and E. L. Piret. 1959. A kinetic study of the lactic acid fermentation: batch process at controlled pH. J. Biochem. Microbiol. Technol. Eng. 1:393-412.

MacVittie, T. J., and K. F. MaCarthy. 1975. The influence of a granulocytic inhibitor(s) on hematopoiesis in an in vivo culture system. Cell Tissue Kinet. 8:553-559.

Madle, S., and G. Obe. 1977. In vitro testing of an indirect mutagen (cyclophosphamide) with human leukocyte cultures: Activation with liver microsomes and use of a dialysis bag. Mutation Res. 56:101-104.

Maestrini, S. Y., and M-G. Kossut. 1981. In situ cell depletion of some marine algae enclosed in dialysis sacks and their use for the determination of nutrient-limiting growth in Ligurian coastal waters (Mediterranean sea). J. Exp. Mar. Biol. Ecol. 50:1-19.

Maiorella, B., H. W. Blanch, and C. R. Wilke. 1983. By-product inhibition effects on ethanolic fermentation by Saccharomyces cerevisiae. Biotechnol. Bioeng. 25:103-121.

Maiorella, B., C. R. Wilke, and H. W. Blanch. 1981. Alcohol production and recovery. Adv. Biochem. Eng. 20:43-92.

Maizels, R. M., and D. W. Dresser. 1977. Conditions for the development of IgM- and IgG-antibody-secreting cells from primed mouse splenocyte in vitro. Immunol. 32:793-801.

Makinodan, T., I. Hoppe, T. Sado, E. E. Capalbo, and M. R. Leonard. 1965. The suppressive effect of supraoptimum doses of antigen on the secondary antibody-forming response of spleen cells cultured in cell-impermeable diffusion chambers. J. Immunol. 95:466-479.

Makinodan, T., P. Nettesheim, T. Morita, and C. J. Chadwick. 1967. Synthesis of antibody by spleen cells after exposure to kiloroentgen doses of ionizing radiation. J. Cell. Physiol. 69:355-366.

Marbrook, J. 1967. Primary immune response in cultures of spleen cells. Lancet ii:1279-1281.

Margaritis, A., and C. R. Wilke. 1978. The rotorfermentor. I. Description of the apparatus, power requirement, and mass transfer characteristics. Biotechnol. Bioeng. 20:709-726.

Mariano, M., and W. G. Spector. 1974. The formation and properties of macrophage polykaryons (inflammatory giant cells). J. Path. 113:1-19.

Marmor, J. B., J. L. Russell, A. M. Miller, and S. H. Robinson. 1975. Modulation of murine granulocyte proliferation in diffusion chamber cultures. Blood 46:39-50.

Marsot, P., R. Fournier, and C. Blais. 1981a. Culture a dialyse: emploi de fibres creuses dialysantes pour la culture massive de phytoplancton. Can. J. Fish. Aquat. Sci. 38:905-911.

Marsot, P., R. Fournnier, and C. Blais. 1981b. Un nouveau procede de culture contnue a dialyse pour le phytoplancton. Biotechnol. Lett. 3:689-694.

Masover, G. K., and Hayflick. 1974. Dialysis culture of T-strain mycoplasmas. J. Bacteriol. 118:46-52.

Mayer, L. M. 1976. Chemical water sampling in lakes and sediments with dialysis bags. Limnol. Oceanogr. 21:909-912.

McFeters, G. A., G. K. Bissonnette, J. J. Jezeski, C. A. Thomson, and D. G. Stuart. 1974. Comparative survival of indicator bacteria and enteric pathogens in well water. Appl. Microbiol. 27:823-829.

McFeters, G. A., and D. G. Stuart. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. Appl. Microbiol. 24:805-811.

McGarry, M. P., and A. M. Miller. 1974. Evidence for the humoral stimulation of eosinophil granulocytopoiesis in in vivo diffusion chambers. Exp. Hemat. 2:372-379.

McVeigh, I., and W. H,. Brown. 1954. In vitro growth of Chlamydomonas chlamydogama Bold and Haematococcus pluvialis Flotow EM. Willie in mixed cultures. Bull. Torrey Bot. Club 81:218-233.

Meck, R. A., A. L. Carsten, and J. J. Kelsch. 1976. Growth of HeLa cells in diffusion chamber cultures in vivo. Cancer Res. 36:2317-2320.

Metcalf, T. G., and W. C. Stiles. 1967. Survival of enteric viruses in estuary waters and shellfish, p. 439-448. In G. Berg (ed.), Transmission of viruses by the water route, Wiley Interscience, New York.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analyt. Chem. 31:426-428.

Millner, S. N. 1969. Apparatus for preparation of bacterial extracellular enzymes. Appl. Microbiol. 17:639-640.

Minier, M., and G. Goma. 1982. Ethanol production by extractive fermentation. Biotechnol. Bioeng. 24:1565-1579.

Mohan, K., R. C. Gordon, T. C. Beaman, R. C., Belding, D. Luecke, C. Edmiston, and P. Gerhardt. 1977. Synergism of penicillin and gentamicin against Listeria monocytogenes ex vivo hemodialysis culture. J. Infect. Dis. 135:51-54.

Molongoski, J. J. 1978. Sedimentation and anaerobic metabolism of particulate organic matter in the sediments of a hypereutrophic lake. Ph.D Thesis. Michigan State University, East Lansing, Michigan.

Morandi, M., and A. Valeri. 1982. Continuous dialysis of cultures of human diploid cells (MRC 5) grown on microcarriers. Biotechnol. Lett. 4:465-468.

Murphy, R. A., and R.-U. Haque. 1974. Large-scale production of staphylococcal delta hemolysin by the dialysis membrane technique. Can. J. Microbiol. 20:1061-1063.

Naguib, M. 1975. Overall metabolic regulations in cultures of the obligate methane-oxidizing strain M 102, p. 203-212. In Microbial growth on $C_1$-compounds, Tokyo, Japan.

Nakamura, F., and Y. Kurihara. 1978. Maintenance of a certain rumen protozoal population in a continuous in vitro fermentation system. Appl. Environ. Microbiol. 35:500-506.

Nettesheim, P., T. Makinodan, and C. J. Chadwick. 1966. Improved diffusion chamber cultures for cytokinetic analysis of antibody response. Immunol. 11:427-439.

Niskanen, E., W. S, Tyler, M. Symann, F. Stohlman, Jr., and D. Howard. 1974. The effect of neutropenia on the cell cycle of granulocyte precursors in an in vivo culture system. Blood 43:23-31.

O'Brien, R. T., and J. S. Newman. 1977. Inactivation of polioviruses and coxsackieviruses in surface water. Appl. Environ. Microbiol. 33:334-340.

Osborne, R. J. W. 1977. Production of frozen concentrated cheese starters by diffusion culture. J. Soc. of Dairy Technol. 30:40-44.

Osborne, R. J. W., and J. V. Brown. 1980. Properties of single-strain cheese starter bacteria grown in diffusion culture. J. Dairy Res. 47:141-150.

Osborne, R. J. W., A. T. Miles, and N. Tilley. 1975. Preparation of concentrated starter cultures. U.S. Patent 3,911,140.

Osebold, J. W., and R. A. DiCapua. 1968. Cellular immunity of mice infected with Listeria monocytogenes in diffusion chambers. J. Bacteriol. 95:2158-2164.

Osebold, J. W., P. M. Outteridge, and L. D. Pearson. 1970a. Mutation of Listeria monocytogenes after prolonged in vivo survival in diffusion chambers. Infect. Immun. 1:209-211.

Osebold, J. W., P. M. Outteridge, L. D. Pearson, and R. A. DiCapua. 1970b. Cellular responses of mice to diffusion chambers. I. Reactions to intraperitoneal diffusion chambers containing Listeria monocytogenes and to bacteria-free chambers. Infect. Immun. 2:127-131.

Osebold, J. W., L. D. Pearson, and N. I. Medin. 1974. Relationship of antimicrobial cellular immunity to delayed hypersensitivity in listeriosis. Infect. Immun. 9:354-362.

Owens, O. V. H., P. Dresler, C. C. Crawford, M. A. Tyler, and H. H. Seliger. 1977. Phytoplankton cages for the measurement in situ of the growth rates of mixed natural populations. Chesapeake Sci. 18:325-333.

Pan, P., and W. W. Umbreit. 1972a. Growth of obligate autotrophic bacteria on glucose in a continuous flow-through apparatus. J. Bacteriol. 109:1149-1155.

Pan, P., and W. W. Umbreit. 1972b. Growth of mixed cultures of autotrophic and heterotrophic organisms. Can. J. Microbiol. 18:153-156.

Petersen, B. H., Meyer, H. Tjernshaugen. 1974. Kinetics of rat bone marrow cells cultured in diffusion chambers: effect of heterologous implantation and irradiation of the host. Scand. J. Haemat. 13:39-47.

Petithory, J., and J. Rousset. 1969. Immunization of mice against a homologous strain of virulent trypanosomes living in diffusion chambers. Bull. Soc. Pathol. Exotique 58:1049-1053.

Pettersson, H.-E. 1975. Studies on batch production of bacterial concentrates from mixed species lactic starters. Appl. Microbiol. 29:133-140.

Pierre, J.-F. 1969. Etude experimentale du comportement in situ dune population diatomique maintenue en enceinte dialysante. Hydrobiol. 33:364-368.

Powers, C. D., R. G. Rowland, and C. F. Wurster. 1976. Dialysis membrane chambers as a device for evaluating impacts of pollutants on plankton under natural conditions. Water Res. 10:991-994.

Powers, C. D., R. G. Rowland, H. B. O'Connors, Jr., and C. F. Wurster. 1977. Response to polychlorinated biphenyls of marine phytoplankton isolates cultured under natural conditions. Appl. Environ. Microbiol. 34:760-764.

Prakash, A., L. Skoglund, B. Rystad, and A. Jensen. 1973. Growth and cell-size distribution of marine planktonic algae in batch and dialysis cultures. J. Fish. Res. Board Can. 30:143-155.

Pratt, R. 1944. Studies on Chlorella vulgaris. IX. Influence on growth of chlorella of continuous removal of chlorellin from the culture solution. Amer. J. Bot. 31:418-421.

Quarles, J. M., Jr. 1973. Hemodialysis culture of bacteria. Ph.D. Thesis. Michigan State University, East Lansing, Michigan.

Quarles, J. M., N. G. Morris, and A. Leibovitz. 1980. Carcinoembryonic antigen production by human colorectal adenocarcinoma cells in matrix-perfusion culture. In Vitro 16:113-118.

Quarles, J. M., R. C. Belding, T. C. Beaman, and P. Gerhardt. 1974. Hemodialysis culture of Serratia marcescens in a goat-artificial kidney-fermentor system. Infect. Immun. 9:550-558.

Quesenberry, P., E. Niskanen, M. Symann, D. Howard, M. Ryan, J. Halpern, and F. Stohlman, Jr. 1974. Growth of stem cell concentrates in diffusion chambers (DC). Cell Tissue Kinet. 7:337-348.

Quesnel, L. B. 1969. Methods of microculture p. 365-425. In J. R. Norris and D. W. Ribbons (eds.), Methods in microbiology, Vol. 1, Academic Press, New York.

Ramalingham, A., and R. K. Finn. 1977. The vacuferm process: A new approach to fermentation alcohol. Biotechnol. Bioeng. 19:583-589.

Rasmussen, P., and O. Hjortdal. 1969. In vivo culture of blood cells. II. The origin of fibroblasts in blood and blood buffy coat cultures studied by diffusion chamber implants in the peritoneal cavity of rats. Acta Anat. 72:476-486.

Rathlev, T. 1973. Cultivation of pathogenic Treponema pallidum in chambers surgically implanted in experimental animals. Acta Path. Microbiol. Scand. Section B 81,2:269-271.

Rechnitz, G. A. 1981. Bioselective membrane electrode probes. Science 214:287-291.

Rechnitz, G. A., T. L. Riechel, R. K. Kobos, and M. E. Meyerhoff. 1978. Glutamine-selective membrane electrode that uses living bacterial cells. Science 199:440-441.

Reporter, M. 1976. Synergetic cultures of Glycine max root cells and rhizobia separated by membrane filters. Plant Physiol. 57:651-655.

Reporter, M., and N. Hermina. 1975. Acetylene reduction by transfilter suspension cultures of Rhizobium japonicum. Biochem. Biophys. Res. Commun. 64:1126-1133.

Rightsel, W. A., and M. F. S. Hall. 1976. Experiences on attempts at cultivation of M. leprae in diffusion chambers containing various tissue cultures and maintained for long periods in mice. Int. J. Lepr. 44:535-536.

Rightsel, W. A., M. F. Sawyers, and J. H. Peters. 1978. Comparative effects of sulfones and rifampin on growth of Mycobacterium lepraemurium in macrophage diffusion chamber cultures. Antimicrob. Agents and Chemother. 13:509-513.

Rightsel, W. A., and W. C. Wiygul. 1971. Growth of Mycobacterium lepraemurium in cell-impermeable diffusion chambers. Infect. Immun. 3:127-132.

Robineaux, R. 1963. The culture of spleen cells under a dialysis membrane. Ann. New York Acad. Sci. 113:947-953.

Rose, G. 1954. A separable and multipurpose tissue culture chamber. Texas Reports on Biology and Medicine 12:1074-1083.

Rose, G. G. 1967. The circumfusion system for multipurpose culture chambers. I. Introduction to the mechanics, techniques, and basic results of a 12-chamber (in vitro) closed circulatory system. J. Cell. Biol. 32:89-112.

Rose, G. G., and M. Cattoni. 1974. Human gingiva cultivated in circumfusion systems. Archs. Oral. Biol. 19:113-123.

Rose,G. G., M. Kumegawa, and M. Cattoni. 1968. The circumfusion system for multipurpose culture chambers. II. The protracted maintenance of differentiation of fetal and newborn mouse liver in vitro. J. Cell. Biol. 39:430-450.

Rose, G. G., M. Kumegawa, H. Nikai, M. Bracho, and M. Cattoni. 1970. The dual-rotary circumfusion system for mark II culture chambers. I. Design, control, and monitoring of the system and the cultures. Microvasc. Res. 2:24-60.

Rose, G. G., M. Kumegawa, H. Nikai, M. Cattoni, and F. Hu. 1969. The HFH-18 mouse melanoma in roller tube, chamber, and circumfusion system cultures. Cancer Res. 29:2010-2033.

Rose, G. G., C. M. Pomerat, T. O. Shindler, and J. B. Trunnell. 1958. A cellophane-strip technique for culturing tissue in multipurpose culture chambers. J. Biophys. Biochem. Cytol. 4:761-764.

Rose, G. G., and T. Yajima. 1977. Fetal mouse lung in circumfusion system cultures. In Vitro 13:749-768.

Rose, G. G., and T. Yajima. 1978. Terminal bronchiolar-alveolar (TB-A) units in circumfusion system cultures. In Vitro 14:557-590.

Rose, G. G., A. Yamasaki, and C. J. Mahan. 1981. Bone induction in vitro. I. Human gigival fibroblast cell lines versus tooth matrix. J. Periodental Res. 16:344-357.

Rosin, A., H. Freiberg, and G. Zajicek. 1963. The fate of rat bone marrow, spleen and periosteum cultivated in vivo in the diffusion chamber with special reference to bone formation. Exp. Cell Res. 29:176-187.

Rothstein, G., E. H. Hugl, C. R. Bishop, J. W. Athens, and H. E. Ashenbrucker. 1971. Stimulation of granulocytopoiesis by a diffusible factor in vivo. J. Clin. Investigation 50:2004-2007.

Roy, T. B., H. W. Blanch, and C. R. Wilke. 1982. Lactic acid production by Lactobacillus delbreuckii in a hollow fiber fermentor. Biotechnol. Lett. 4:483-488.

Ruezinsky, L. L. 1975. Dialysis culture of Neisseria gonorrhoeae. M.S. Thesis, Michigan State University, East Lansing, Michigan.

Sado, T. 1969. Functional and ultrastructural studies of antibody-producing cells exposed to 10,000 R in millpore diffusion chambers. Int. J. Radiat. Biol. 15:1-22.

Sado, T., E. H. Perkins, and T. Makinodan. 1970. Staircase rise in the antibody-forming cell population in secondary response. J. Immunol. 105:642-652.

Sakshaug, E. 1977. Limiting nutrients and maximum growth rates for diatoms in Narragansett Bay. J. Exp. Mar. Biol. Ecol. 28:109-123.

Sarner, N. Z., M. J. Bissell, M. DiGirolamo, and L. Gorini. 1971. Mechanism of excretion of a bacterial proteinase: demonstration of two proteolytic enzymes produced by a Sarcina strain (Coccus P). J. Bacteriol. 105:1090-1098.

Scales, R. W., and S. J. Kraus. 1974. Development and passive transfer of immunity to gonococcal infection in guinea pigs. Infect. Immun. 10:1040-1043.

Schieferstein, G., and O. D. Laerum. 1974. Diffusion chamber culture of hamster melanoma cells in vivo. Culture characteristics in iso- and heterologous host animals. Arch. Derm. Forsch. 249:131-140.

Schneiberg, K., A. Jonecko, and W. Bartinikowa. 1968. Thymus--a hematopoietic organ? III. Influence of thymus-bearing chambers on the surviving and the hemopoietic system of mice after a whole-body irradiation with x-rays. Folia Haematol. 89:265-282.

Schultz, J. S., and P. Gerhardt. 1969. Dialysis culture of microorganisms: design, theory, and results. Bacteriol. Rev. 33:1-47.

Sieburth, J. McN. 1979. Seamicrobes. Oxford University Press, New York, N.Y.

Sieburth, J. McN., K. M. Johnson, C. M. Burney, and D. M. Lavoie. 1977. Estimation of in situ rates of heterotroph using diurnal changes in dissolved organic matter and growth rates of picoplankton in diffusion culture. Helgolander Wiss. Meeresunter. 30:565-574.

Skoglund, L., and A. Jensen. 1976. Studies on N-limited growth of diatoms in dialysis culture. J. Exp. Mar. Biol. Ecol. 21:169-178.

Slanetz, L. W., and C. H. Bartley. 1965. Survival of fecal streptococci in sea water. Health Lab. Sci. 2:142-148.

Smith, B. S. 1977. Microbial interaction in membrane-separated cultures. Master's thesis, University of Toronto, Toronto, Ontario.

Sortland, L. D. 1968. The kinetics of dense culture fermentations. Ph.D. thesis, University of California, Berkeley, CA.

Sortland, L. D., and C. R. Wilke. 1969. Growth of Streptococcus faecalus in dense culture. Biotechnol. Bioeng. 11:805-841.

Spertzel, R. O., and M. Pollard. 1970. Heterotransplanted spleen tissues in diffusion chambers: therapeutic benefits to irradiated mice. J. Reticuloendothelial Soc. 8:1-12.

Squires, D. J. P. 1975. The growth of human bone marrow in diffusion chambers. Brit. J. Haematol. 29:89-97.

Stevenson, K. E., D. E. Black, and R. N. Costilow. 1979. Aerobic fermentations of pickle process brine by Candida utilis. J. Food Sci. 44:181-185.

Stieber, R. W. 1976. Nonaseptic continuous dialysis fermentation of concentrated whey to produce ruminant feedstuff and lactic acid. M.S. Thesis, Michigan State University, East Lansing, Michigan.

Stieber, R. W. 1979. Dialysis continuous processes for microbial fermentations: mathematical models, computer simulations, and experimental tests. Ph.D. thesis, Michigan State University, East Lansing, Michigan.

Stieber, R. W., G. A. Coulman, and P. Gerhardt. 1977. Dialysis continuous process for ammonium-lactate fermentation of whey: experimental tests. Appl. Environ. Microbiol. 34:733-739.

Stieber, R. W., and P. Gerhardt. 1979a. Dialysis continuous process for ammonium-lactate fermentation: Improved mathematical model and use of deproteinized whey. Appl. Environ. Microbiol. 37:487-495.

Stieber, R. W., and P. Gerhardt. 1979b. Mathematical models and computer simulations of dialysis and non-dialysis continuous processes for ammonium-lactate fermentation. Biotechnol. Bioeng. Symp. No. 9, 137-138.

Stieber, R. W., and P. Gerhardt. 1980. Production of Lactobacillus cells by dialysis continuous fermentation of deproteinized whey. J. Dairy Sci. 63:722-730.

Stieber, R. W., and P. Gerhardt. 1981a. Dialysis continuous ammonium-lactate fermentation: simulated and experimental dialysate-feed, immobilized-cell systems. Biotechnol. Bioeng. 23:535-549.

Stieber, R. W., and P. Gerhardt. 1981b. Dialysis continuous process for ammonium-lactate fermentation: simulated pre-fermentor and cell-recycling systems. Biotechnol. Bioeng. 23:523-534.

Stohlman, F., Jr., P. J. Quesenberry, and W. S. Tyler. 1973. The regulation of myelopoiesis as approached with in vivo and in vitro techniques. Prog. Hematol. 8:259-297.

Stutman, O., E. J. Yunis, and R. A. Good. 1970. Studies on thymus function. I. Cooperative effect of thymic function and lymphohemopoietic cells in restoration of neonatally thymectomized mice. J. Exp. Med. 132:583-600.

Sugiyama, H., D. C. Mills, and L-J. C. Kuo. 1978. Number of Clostridium botulinum spores in honey. J. Food Prod. 41:848-850.

Sullivan, R., K. S. Zuckerman, and P. J. Quesenberry. 1980. The role of colony stimulating activity in modulating murine diffusion chamber granulopoiesis. Br. J. Haematol. 44:365-374.

Suzuki, J. B., R. Booth, A. Benedik, and N. Grecz. 1971. Pathogenesis of Clostridium botulinum type A: study of in vivo toxin release by implantation of diffusion chambers containing spores, vegetative cells, and free toxin. Infect. Immun. 3:659-663.

Tangnu, S. K., and T. K. Ghose. 1981. Environmental manipulations in salicylic acid fermentation. Process Biochem. 16:24-27.

Theodorou, N. A., and S. L. Howell. 1979. An assessment of diffusion chambers for use in pancreatic islet cell transplantation. Transplantation 27:350-353.

Tight, R. R., and R. L. Perkins. 1976. Treponema pallidum infection in subcutaneous polyethylene chambers in rabbits. Infect. Immun. 13:1606-1612.

Tight, R. R., R. B. Prior, R. L. Perkins, and C. A. Rotilie. 1975. Fluid and penicillin G dynamics in polyethylene chambers implanted subcutaneously in rabbits. Antimicrob. Agents Chemother. 8:495-497.

Toth, L. G. 1980. The use of dialyzing sacks in estimation of production of bacterioplankton. Arch. Hydrobiol. 89:474-482.

Trench, C. A. H., J. W. Watson, F. C. Walker, P. S. Gardner, and C. A. Green. 1966. Evidence for a humoral thymic factor in rabbits. Immunol. 10:187-191.

Trowell, O. A. 1959. The use of mature organs in a synthetic medium. Exp. Cell Res. 16:118-147.

Trowell, O. A. 1963. The optimum concentration of sodium chloride for the survival of lymphocytes in vitro. Exp. Cell Res. 29:220-224.

Tucker, D. F., and J. J. T. Owen. 1969. Growth of tumour cells in diffusion chambers on the chick chorioallantois. Europ. J. Cancer 5:591-596.

Tyler, W. S., E. Niskanen, F. Stohlman, Jr., J. Keane, and D. Howard. 1972. The effect of neutropenia on myeloid growth and the stem cell in an in vivo culture system. Blood 40:634-645.

Tyler, W. S., F. Stohlman, Jr., M. Chovaniec, and D. Howard. 1976. Effect of a congenital defect in hemopoiesis on myeloid growth and the stem cell (CFU) in an in vivo culture system. Blood 47:413-421.

Untermann, F. 1972a. Verfahren zur Kultivierung von Staphylokokken fur die Gewinnung groβerer Enterotoxinmengen Zbl. Bakt. Hyg., 1. Abt. Orig. A 219:426-434.

Untermann, F. 1972b. Cultivation methods for the production of staphylococcal enterotoxins on a bigger scale. Zbl. Bakt. Hyg., 1. Abt. Orig. A 219:426-434.

Urso, P., and T. Makinodan. 1963. The roles of cellular division and maturation in the formation of precipitating antibody. J. Immunol. 90:897-907.

Vann, D. C. 1969. In vitro antibody synthesis by diffusion chamber cultures of spleen cells. II. Effect of increased levels of free antibody. j. Immunol. 102:451-456.

Vann, D. C., and T. Makinodan. 1969. In vitro antibody synthesis by diffusion chamber cultures of spleen cells. I. Methods and effect of 10,000 r on antibody synthesis. J. Immnol. 102:442-450.

Vargo, G. A., Hargraves, and P. Johnson. 1975. Scanning electron microscopy of dialysis tubes incubated in flowing seawater. Marine Biol. 31:113-120.

Vasconcelos, G. J., and R. G. Swartz. 1976. Survival of bacteria in seawater using a diffusion chamber apparatus in situ. Appl. Enviro. Microbiol. 31:913-920.

Videman, T., L. Vilpo, and J. A. Vilpo. 1978. Inhibition of osteogenesis by bone mass in diffusion chamber cultures. Exp. Path. Bd. 15:182-184.

Vilpo, J. A. 1972. Proliferation, survival and malignancy of chloroleukemia cells grown in diffusion chambers in vivo. Ann. Med. Exptl. Biol. Fenn. 50:168-174.

Vilpo, J. A., T. Videman, and L. Vilpo. 1978. An experimental model of osteogenesis: a closed in vivo culture system using diffusion chambers. Clin. Orthop. 135:291-294.

Waitkins, S. A. 1975. Effect of tissue culture cells in promoting prolonged survival of N. gonorrhoeae in artificial subcutaneous cavities of mice. Brit. J. Vener. Dis. 51:376-381.

Wang, H. Y., L. A. Kominek, and J. L. Jost. 1981. On-line extraction fermentation processes. p. 601-607. In Moo-Young, M., C. W. Robinson, and C. Vezina (eds.), Advances in biotechnology, vol. 1. Pergamon Press.

Warburg, O., G. Krippahl, and A. Lehmann. 1968. Dialysierte Chlorella, ein neus Versuchsmaterial zur Untrersuchung der Photosynthese. Z. Naturforsch. 23b:1076-1079.

Willardsen, R. R., F. F. Busta, and C. E. Allen. 1977. Dialysis technique for containment of microbial populations inoculated into food systems. Appl. Environ. Microbiol. 34:240-241.

Willemze, R., R. I. Walker, J. C. Herion, and J. G. Palmer. 1978. Marrow culture in diffusion chambers in rabbits. I. Effect of mature granulocytes on cell production. Blood 51:21-31.

Williams, F. D., and r. H. Schwarzhoff. 1978. Nature of the swarming phenomenon in Proteus. Ann. Rev. Microbiol. 32:101-122.

Winfrey, M. R., and J. G. Zeikus. 1977. Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. Appl. Environ. Microbiol. 33:275-281.

Wiygul, W. C., and W. A. Rightsel. 1971. Growth of Mycobacterium lepraemurium in diffusion chambers containing human embryonic skin cells and in cell-free chambers. J. Gen. Microbiol. 68:375-380.

Wyrick, P. B., and H. Gooder. 1971. Growth of streptococcal protoplasts and L-colonies on membrane filters. J. Bacteriol. 105:646-656.

Zabriskie, D. W., and A. E. Humphrey. 1978. Continuous dialysis for the on-line analysis of diffusible components in a fermentation broth. Biotechnol. Bioeng. 20:1295-1301.