DIALYSIS CONTINUOUS PROCESSES FOR MICROBIAL FERMENTATIONS: MATHEMATICAL MODELS, COMPUTER SIMULATIONS, AND EXPERIMENTAL TESTS

By

Robert William Stieber

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1979

ABSTRACT

DIALYSIS CONTINUOUS PROCESSES FOR MICROBIAL FERMENTATIONS: MATHEMATICAL MODELS, COMPUTER SIMULATIONS, AND EXPERIMENTAL TESTS

By

Robert William Stieber

Dialysis continuous culture processes were mathematically modeled, computer simulated and experimentally tested, and the literature was reviewed, to assess the potential for application of membrane technology to industrial fermentations. The ammonium-lactate fermentation of whey was used as a model system for the experiments because of its commercial potential. The simulated and experimental results both showed ways that dialysis continuous processes can maximize substrate utilization and produce high cell populations and cell-free metabolites at high rates. The dialysis processes were a considerable improvement over conventional continuous processes.

In one system for dialysis continuous fermentation, whey of high concentration was fed into a fermentor while the fermentor contents were dialyzed through a membrane against water to relieve the inhibitory effect of metabolites. The fermentation was modeled and a solution for the steady state was used to simulate the fermentation on a digital computer. The results showed the effects of various parameters on the process and predicted that it could be operated efficiently. To test the predictions, dried sweet-cheese whey was rehydrated to contain 230 mg of lactose per ml, charged into a 5-liter fermentor, adjusted in pH (5.3) and

temperature (44°C), and inoculated with Lactobacillus bulgaricus. The fermentor and dialysate circuits were connected, and a series of steady-state conditions was managed without sterilization or asepsis for 94 days. As time progressed, the fermentation remained homofermentative and increased in conversion efficiency, although membrane fouling necessitated dialyzer cleaning about every 4 weeks. With a retention time of 19 h, 97% of the substrate was converted into products and cells. Relative to nondialysis continuous or batch processes for the fermentation, the dialysis continuous process enabled the use of more concentrated substrate, was more efficient in the rate of substrate conversion, and additionally produced a second effluent of less concentrated but purer ammonium lactate.

The model was improved by incorporating separate terms for substrate limitation and product inhibition into the equation describing the rate of cell growth. The improved model was validated using the results from previous experiments with whole whey and also in subsequent experiments with deproteinized whey. The latter showed that the cell accumulation, cell productivity, and molar growth yield were greater than those obtained from a conventional continuous process for the fermentation and that the dialysis process could produce a continuous effluent containing 50 mg of cell-free ammonium lactate per ml. The whole whey proved to be a better substrate than the deproteinized whey.

In a second system for dialysis continuous fermentation with a prefermentor, simulations predicted that the addition would not bring improvement. In a third system with 100% recycling (feedback)

of cells, simulations predicted that the retention time would be reduced 9-fold and the conversion of substrate (deproteinized whey) to product would be increased by 25%, accomplished almost entirely by maintenance metabolism.

In a fourth system, the use of dialysis for substrate feed as well as product removal also was modeled and simulated. The simulations predicted that the feeding of substrate into the fermentor circuit via the dialysate circuit has potential for production and recovery of both cell mass and cell-free metabolite. The experimental tests indicated that the fourth system would be a useful method for immobilizing microbial cells to produce a cell-free metabolite at a rate greater than the maximum specific growth rate of the microbe, and additionally would serve to filter-sterilize the substrate.

To my Mother and Dad

and Bill, Tom, John, Pat,

Mark, Dan, Tim, and Kimberly

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Dr. Philipp Gerhardt for his encouragement, insistence on excellence, and the opportunity. I would also like to thank my guidance committee members, Dr. C. A. Reddy and Dr. Ralph N. Costilow, for their help; and especially Dr. George A. Coulman for his expertise and guidance with the mathematical modeling.

I also wish to express my appreciation to Marilyn Lautzenheiser for her excellent secretarial services.

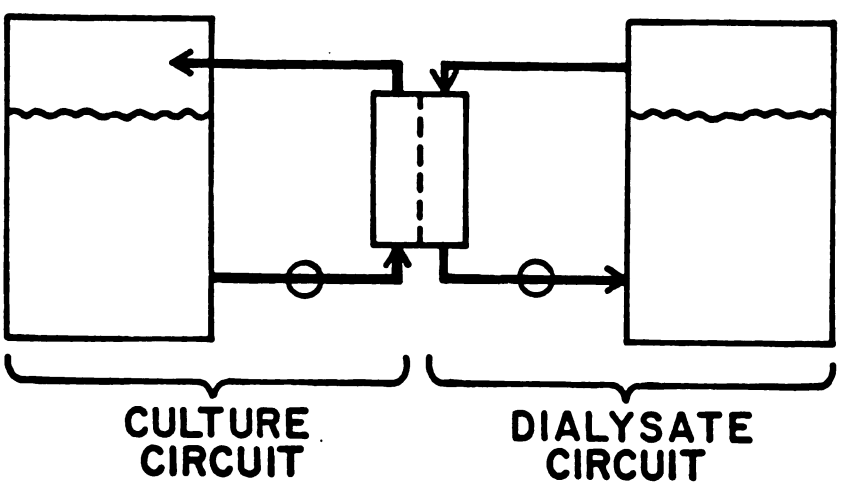
I also acknowledge the financial support from grant ENG 76-17260 of the National Science Foundation, the Department of Microbiology and Public Health of Michigan State University, and the National Institutes of Health training grant GM 01911-10.

TABLE OF CONTENTS

LIST OF TABLES

GENERAL INTRODUCTION

Of the various separation processes that involve membranes (dialysis, microfiltration, ultrafiltration, and reverse osmosis), dialysis is most easily applied to the production of microbial cells and their metabolites. Microbial cultivation can be enhanced by introducing a membrane dialyzer between a culture chamber and a dialysate reservoir with the contents of each continuously circulated on opposite sides of the membrane, enabling diffusional exchange of nutrients and products while confining the cells. The culture and dialysate circuits of such a system of dialysis culture can be operated batchwise, continuously or in a combination of these modes; and dialysis culture can be applied in a number of ways to the production of cells or metabolites from a variety of substrates. A schematic diagram of the generalized design of a dialysis culture system is as follows:



In this study, the potential for application of dialysis culture to continuous fermentation was investigated by using the ammonium-lactate fermentation of whole or deproteinized whey as a model process. The process involves the fermentative conversion of the whey lactose to lactic acid and its neutralization by ammonia. The fermentation can be managed without sterilization or asepsis as a result of the restrictive conditions of low pH, high concentration of undissociated acid, high temperature and anaerobiosis. The biochemistry of the conversion is straightforward and well described in kinetic terms. Thus, the fermentation is representative of other homofermentations, can be easily managed in the laboratory, and is amenable to mathematical analysis and computer modeling.

The substrate whey is a residue of cheese manufacture and has become a pollution problem as well as an economic and nutrient loss, so that useful disposal of whey is important. Whole whey can be processed by ultrafiltration to obtain a concentrate of milk protein of high quality and considerable market potential, but the remaining residue of deproteinized whey retains all of the lactose and a substantial disposal problem still remains. The rationale for an ammoniated organic acid fermentation is applicable also to other carbohydrate-rich agricultural residues. Ammonium salts of organic acids can be used directly as a nitrogenous feed supplement for ruminant animals, or can be converted into lactic acid for use as an industrial chemical and ammonium sulfate for use as a fertilizer. Lactobacillus cells can provide high-quality single-cell protein for animal or human consumption.

Consequently, the fermentation is useful for its practical potential as well as for fundamental study.

Dialysis can be used to improve a continuous fermentation by means of two interrelated yet different system designs. In one, the dialysate circuit is employed primarily to remove inhibitory metabolites from the culture, resulting in increased extent and rate of substrate conversion and permitting the use of a relatively high concentration of substrate. As applied to the ammonium-lactate fermentation, the substrate is fed into the fermentor circuit, the contents of which are dialyzed through the dialyzer membrane against water in the dialysate circuit. Thereby, the small molecular product (lactic acid) is removed from the immediate environment of the cells, thus relieving the inhibition by the product that normally regulates its production. As more product is withdrawn by dialysis, more substrate is consumed and more product is made; i.e., the fermentation becomes more efficient. The dialysis separation of product additionally represents a purification and recovery step.

In a second system design, the fermentor contents are dialyzed against substrate fed into the dialysate circuit and is employed primarily to obtain greatly more concentrated cell populations in the fermentor circuit. Such a design also can be used to increase the throughput of substrate for conversion purposes. The second design can be applied to fermentations that are not inhibited by metabolite products as well as to those that are. In this design, since the substrate enters the fermentor filtered through a membrane, prior sterilization of the substrate is unnecessary.

Similarly, since metabolite products leave the fermentor filtered through a membrane, the product from the dialysate circuit is free of cells. This design can be operated for the continuous production of a high concentration of cells and of a cell-free metabolite, both at high rates.

The broad goal of this study was to assess the feasibility of applying membrane technology to industrial fermentation processes. Among the several membrane techniques, dialysis was selected for study not only because of its easy application but also because of the existence of a theoretical basis for its application to bacterial culture (Schultz and Gerhardt, 1969). Continuous rather than batch operation was chosen because a continuous process generally is more productive than a batch process. Practically, continuous operation appeared desirable not only for best use of membrane technology but also because whey residue is generated and the fermentation products could be used continuously.

The specific objectives of the study were to (1) develop mathematical models of dialysis continuous processes for the ammonium-lactate fermentation, in order to define the processes and to predict by computer simulation the outcome of various modes of operation, (2) experimentally test the best predicted modes of operation by use of conventional laboratory equipment and, (3) project the simulated and experimental results in order to evaluate the potential for use of membranes in fermentations industrially.

The investigative procedure was to first develop a mathematical model of a particular dialysis continuous process design specifically

for the ammonium-lactate fermentation of whey. The fermentation was modeled as a set of equations representing material balances and rate relationships in the fermentor and dialysate circuits. The model was generalized for application to other fermentations by combining the equations and using dimensionless parameters. The generalized model was then solved for the steady state and used to simulate the ammonium-lactate fermentation on a digital computer. The results predicted the effects of changes in various material, operational and kinetic parameters. Laboratory experiments then were conducted at critical points to test the theoretical predictions. The simulated and experimental results were correlated to demonstrate the validity of the model, establish values for the bacterial metabolic constants, and evaluate the process.

This thesis was prepared as a series of articles that have been or are in process to be published separately in scientific journals. The general literature review is intended to be part of a review examining dialysis culture techniques used in studies during the ten years since the 1969 review of the subject (Schultz and Gerhardt, 1969). The materials-methods, results and discussions are presented as a series of articles on specific aspects of the study, each of which is organized in the usual journal format. For convenience of the thesis reader, the materials and methods in all of the articles are indexed in a composite table. In the thesis section on results and discussions, the articles are presented in entireties. Articles I and II describe the development of a mathematical model, simulated predictions, and experimental

tests of a dialysis continuous process for the ammonium-lactate fermentation of whole whey using the first system design. The model was improved and tested and was extended in applicability to the use of deproteinized whey (Article III) and the production of lactobacillus cells (Article IV). A nondialysis continuous process for the fermentation was modeled and experimentally tested to further validate the model and to obtain cell mass data for the dialysis studies (Article V). Dialysis continuous processes with a prefermentor or a cell-recycle system were investigated theoretically (Article VI). Dialysis substrate-feed and product-removal systems were modeled and also experimentally tested (Article VII). In the concluding thesis section, a general discussion is provided about the long-range potential for application of membrane technology to industrial fermentations.

GENERAL LITERATURE REVIEW

Dialysis Culture of Microbial and Mammalian Cells: Applications, 1969-79

1. INTRODUCTION

Dialysis is a process for separation of solute molecules by means of their unequal diffusion through a semipermeable membrane because of a concentration gradient. The process is applied to the growth and maintenance of living cells by a technique called dialysis or diffusion culture.

The principle of dialysis culture is fundamental to the growth of microbial and mammalian cells in nature. Unlike the usual batch or closed situation in the laboratory, cells free-living in an ecological niche or parasitic on or part of a host usually exist in a continuous or open situation in which the flux of nutrients or metabolites is regulated by a membrane. Metabolically active transport supplements passive diffusion in the membrane regulation of living systems, but only the passive component presently can be simulated in the laboratory cultivation of cells.

There are other passive processes that involve membranes, e.g., ultrafiltration, microfiltration, and reverse osmosis. However, dialysis is the most easily applied to the production of cells and their metabolites. To employ the technique of dialysis culture, a membrane is introduced between a culture chamber and a dialysate reservoir. For dialysis culture to be effective, the volume of the dialysate must be large compared to that of the culture, or else the dialysate must be replenishable. Further, the permeability and area of the membrane must be sufficient to permit useful diffusion, both in rate and quantity. Such a system of dialysis culture can be operated in vitro or in vivo

and batchwise, continuously, or in a combination of these modes. The system can be designed for nutrients to diffuse from the reservoir to the culture and for metabolic products to diffuse from the culture to the reservoir with the result that their respective inhibitory effects are lessened.

Specific advantages and reasons for using dialysis for cell culture include: the prolongation of cell growth, which allows the attainment of high densities of viable cells; the extension of the stationary phase of the growth cycle, thus permitting increased production of metabolites associated with this phase; the relief of product inhibition by removal or dilution of diffusible growth products, thus causing greater production; the production of metabolites free of cells and conversely the production of culture liquors free of macromolecules from the medium; the means to study and recover a cell population placed in an in situ or in vivo environment such as a river or animal system; and the capability for study of interactions between separated populations of cells.

Dialysis greatly can enhance the study of medically important microbial and mammalian cells and their metabolites, and the technique can establish the effects of certain drugs and other therapeutic agents on the cells. Dialysis has been useful ecologically to examine interactions between different microorganisms, to study an organism in its natural environment and, to characterize an organism and its metabolites in the laboratory. Fermentative uses of dialysis principally have included the production and recovery of cells and of both diffusible and nondiffusible compounds.

The history of dialysis culture can be traced to 1896 when Metchnikoff et al. (1896) inserted a collodion sac containing cholera bacteria into the peritoneum of a guinea pig to learn if the bacteria produced a diffusible toxin. This history, the generalized design, a mathematical theory, and the practical applications of dialysis culture were brought together into a comprehensive review and preview article by Schultz and Gerhardt in 1969 (Schultz and Gerhardt, 1969). In a thesis afterwards, Quarles (1973) updated their review of applications. Quesnel (1969) published a review on methods for microculture of mammalian cells, including an extensive discussion on perfusion (dialysis) chambers used for this purpose. Recently in a review on the immobilization of microorganisms, Abbott (1978) included a discussion of the fermentative uses of dialysis culture which have appeared in the literature since the review of Schultz and Gerhardt (1969). The present article reviews a few papers on dialysis culture techniques published before 1969 which had not been cited by Schultz and Gerhardt (1969) but mostly those during the last ten years.

Dialysis culture has numerous applications. References to more than 50 genera of microorganisms, including viruses, studied with dialysis culture in vitro are contained in Table 1. Many genera also have been studied with dialysis in vivo (Table 2). Mammalian cells, more than 50 types in all, have been examined with dialysis in vitro (Table 3) or in vivo within a variety of hosts, including man (Table 4). Besides the cells themselves, dialysis has been used to study both diffusible and nondiffusible metabolites (Table 5). During the last ten years, more than 200

papers have been published reporting studies that have used the dialysis technique for medical, ecological and fermentative purposes.

TABLE 1. Genera of microorganisms studied with dialysis culture in vitro

Microorganism	Selected reference
Algae:	
Asterionella	Sakshaug, 1977
Exuviella	Powers et al., 1976
Fragillaria	Jensen et al., 1977
Gonyaulax	Prakash, 1973
Navicula	Jensen et al., 1972
Nostoc (blue green alga)	Poyton and Branton, 1970
Peridinuim	Jensen et al., 1972
Phaeodactylum	Jensen and Rystad, 1973
Plectonema	Pan and Umbreit, 1972b
Prorocentrum	Jensen et al., 1972
Prototheca	Poyton and Branton, 1970
Scenedesmus	Dor, 1975
Skeletonema	Jensen and Rystad, 1973
Thalassionema	Jensen et al., 1972
Thalassiosira (*Cyclotella*)	Jensen and Rystad, 1973
Bacteria:	
Aerobacter	Hendricks and Morrison, 1967
Aeromonas	McFeters et al., 1974
Arthrobacter	Duxbury, 1977
Bacillus	Clive and Landman, 1970
Brevibacterium	Basket and Lulves, 1974
Clostridium	Willardsen et al., 1977
Escherichia	Landwall and Holme, 1977a
Hydrogenomonas	Pan and Umbreit, 1972b
Klebsiella	Anderson and Shanmugan, 1977
Lactobacillus	Friedman and Gaden, 1970
Methanomonas	Naguib, 1975
Mycobacterium	Rightsel et al., 1978
Mycoplasma	Massover and Hayflick, 1974
Neisseria	Ruzinsky, 1975
Nitrobacter	Pan and Umbreit, 1972b
Nitrosomonas	Pan and Umbreit, 1972a
Paracolobactrum	Hendricks and Morrison, 1967
Proteus	Hendricks and Morrison, 1967
Pseudomonas	Abbott and Gerhardt, 1970a
Rhizobium	Bednarski and Reporter, 1978
Salmonella	Ficsor and Muthiana, 1971
Sarcina	Basel et al., 1971
Serratia	Fogarty and Griffin, 1973
Shigella	Hendricks and Morrison, 1967
Staphlococcus	Bamen and Haque, 1970
Streptococcus	Collins and Tillion, 1977
Streptomyces	Kominek, 1975a
Thiobacillus	Borichewski, 1967
Vibrio	Bhattacharyya, 1973
Fungi:	
Candida	Pan and Umbreit, 1972b
Kluyveromyces	Lane, 1977
Micropolyspora	Edwards, 1972
Mycotorula	Aida and Yamaguchi, 1969
Saccharomyces	Pan and Umbreit, 1972b
Schizochytrium	Poyton and Branton, 1970
Thamnidium	Poyton and Branton, 1970
Thraustochytridium	Poyton and Branton, 1970
Protozoa:	
Entodinum	Nakamura and Kurihara, 1978
Eudiplodinium	Nakamura and Kurihara, 1978
Leshmania	Crook et al., 1969
Ochromonas	Poyton and Branton, 1970
Trypanosoma	Dusanic, 1969
Viruses:	
Echovirus	Metcalf and Stiles, 1967
Coxachievirus	O'Brien and Newman, 1977
Poliovirus	O'Brien and Newman, 1977

TABLE 2. Genera of microorganisms studied with dialysis culture in vivo and ex vivo

Microorganism	Host	Selected reference
Bacteria:		
Bacillus	goat	Gerhardt et al. (1977)
Bacteroides	goat	Gerhardt et al. (1977)
Clostridium	guinea pig	Suzuki et al. (1971)
Escherichia	goat	Gerhardt et al. (1977)
Haemophilus	goat	Gerhardt et al. (1977)
Listeria	mouse goat	Osebold and DiCapula (1968) Mohan et al. (1977)
Mycobacterium	mouse	Rightsel and Wigul (1971)
Neisseria	rabbit	Arko (1972)
Pseudomonas	goat	Gerhardt et al. (1977)
Serratia	goat	Gerhardt et al. (1977)
Staphylococcus	goat	Gerhardt et al. (1977)
Streptococcus	goat	Gerhardt et al. (1977)
Treponema	guinea pig goat	Rathlev (1973) Gerhardt et al. (1977)
Protozoa:		
Plasmodium	chicken, turkey, mouse, duck, chick embryo	Huff et al. (1960)
Trypansoma	mouse	Petithory and Rousset (1969)
Yeast:		
Candida	goat	Gerhardt et al. (1977)

TABLE 3. Mammalian cells and tissues studied with dialysis culture in vitro

Cell type	Source	Selected reference
bone marrow	human	Golde and Cline, 1973
fibroblasts	mouse	Horng, 1971
gingiva	human	Rose and Cattoni, 1974
lung	mouse	Rose and Yajima, 1977
lymphocytes	rat	Trowell, 1963
ovary	rat	Rose et al., 1970
pancreas	rat	Rose et al., 1970
salivary gland	rat	Rose et al., 1970
spleen	mouse	Marbrook, 1967
thoracic duct lymphocytes	mouse	Kearney and Reade, 1974
thyroid	rat	Rose et al., 1970

TABLE 4. Mammalian cells and tissues studied with dialysis culture in vivo and ex vivo

Cell type	Source	Host	Selected reference
Aortic endothelium	rabbit	rabbit	Kitsukawa, 1969
Baby kidney	hamster	goat	Gerhardt et al., 1977
Bone marrow	goat	goat	Laissue et al., 1974
Bone marrow	human	mouse	Boyum et al., 1972a
Bone marrow	mouse	mouse	Marmor et al., 1975
Bone marrow	rabbit	rabbit	Willemze et al., 1978
Bone marrow	rat	mouse	Petersen et al., 1974
Bone marrow	rat	rat	Rosin et al., 1963
Breast	human	rat, hamster	Evgenjiva, 1970
Buffy-coat	rat	rat	Rasmussen and Hjortdal, 1969
Distal radius	mouse	mouse	Vilpo et al., 1978
Epidermis	mouse	mouse	Laerum and Boyum, 1970
Erythrocytes	goat	goat	Gerhardt et al., 1977
Granulocytes	mouse	mouse	Benestad, 1970
HeLa		mouse	Meck et al., 1976
Hodgkin	human	mouse	Boecker et al., 1975
Leukemia	human	rat	Laerum et al., 1973
Leukemia	mouse	chick chorioallantois	Tucker and Owen, 1969
Leukemia	rat	rat	Laerum et al., 1973
Leucocytes	goat	goat	Gerhardt et al., 1977
Leucocytes	human	goat	Gerhardt et al., 1977
Liver	human	guinea pig	Germain et al., 1966
Liver	newt	newt	Grillo and Spink, 1968
Lung	human	rat, hamster	Eugenjiva, 1970
Lymph node	human	mouse	Gengozian, 1964
Lymphocytes	human	human	Green, 1966
Macrophage	mouse	mouse	Tyler et al., 1972
Macrophage polykaryon	mouse	mouse	Mariano and Spector, 1974
Mammary	rat	rat	Laerum et al., 1973
Melanoma	hamster	hamster, mouse	Schieferstein and Laerum, 1974
Mononuclear	rat	rat, mouse	Benestad et al., 1971
Myeloma	mouse	goat	Gerhardt et al., 1977
Myleoid elements	mouse	rat, mouse	Niskanen et al., 1974
Ovary	human	rat, hamster	Evgenjiva, 1970
Peripheral erythrocytes	human	mouse	Gengozian, 1964
Peritoneal exudate	guinea pig		Guthrie and Nunez, 1970
Pluripotent stem	mouse	mouse	Niskanen et al., 1974
Spleen	guinea pig	mouse	Spertzel and Pollard, 1970
Spleen	human	mouse	Gengozian, 1964
Spleen	mouse	mouse	Borella, 1972
Spleen	mouse	goat	Gerhardt et al., 1977
Spleen	newt	newt	Grillo and Spink, 1968
Spleen	rabbit	mouse	Urso and Makinodan, 1963
Stomach	human	rat, hamster	Evgenjiva, 1970
Thymus	mouse	mouse	Schneiberg et al., 1968

TABLE 5. Diffusible and nondiffusible products studied with dialysis culture in vitro

Product	Organism	Selected reference
Diffusible		
Cycloheximide	*Streptococcus griseus*	Kominek, 1975a
Lactic acid	*Lactobacillus* (L.) *delbrueckii*	Friedman and Gaden, 1970
Salicylic acid	*Pseudomonas fluorescens*	Abbott and Gerhardt, 1970
Urocanic acid	*Pseudomonas fluorescens*	Kan and Shuler, 1978
Nondiffusible		
Antigens		
11 untyped antigens	*Leishmania mexicana*	Crook et al., 1969
"farmer's lung disease" antigen	*Micropolyspora faeni*	Edwards, 1972
Enzymes		
Amylase	*Clostridium acetobutylicum*	Fogarty and Griffin, 1973
Lysozyme and others	*Staphlococcus aureus*	Bamen and Haque, 1970
Proteinase	*Bacillus polymyxa*	Fogarty and Griffin, 1973
Proteinase	*Sarcina* strain (coccus P)	Sarner et al., 1971
Proteinase	*Serratia marcescens*	Fogarty and Griffin, 1973
Proteinase	*Streptococcus faecalis*	Miller, 1969
Proteinase	*Streptococcus thermophilus*	Fogarty and Griffin, 1973
Protein		
Protein A	*Staphlococcus aureus* A676	Landwall, 1978
Toxin		
Botulinum toxin	*Clostridium botulinum*	Ciccarella et al., 1977
Enterotoxin	*Escherichia coli*	Landwall and Mollby, 1978
Exotoxin	*Vibrio cholerae*	Bhattacharyya, 1973
Slime	*Pseudomonas aeruginosa*	Goto et al., 1971

2. MEDICAL APPLICATIONS

2.1 In vitro

2.1.1 Microbial cells

The dialysis or diffusion culture technique has been used in many studies of a medical nature, to cultivate microorganisms and produce their metabolites. The technique was useful for several purposes which include the separation of cells from the macromolecules of a complex medium, the production and recovery of compounds such as antigens and exotoxins, and the extension of the growth and viability of cells for prolonged study.

Rightsel and coworkers used dialysis culture as a method for an in vitro evaluation of antileprosy drugs without the influence of a multiplicity of host factors (Rightsel et al., 1978). They grew Mycobacterium lepraemurium within macrophage cultures inside diffusion chambers that were maintained on monolayer cultures of macrophages. The method was used to evaluate the effect of three sulfone derivatives and rifampin on the growth of the bacterium. The results showed the effects of these drugs on the organism and demonstrated that the method may be useful for evaluating the chemotherapeutic effects of drugs or their analogs when outside the host. In earlier studies, the bacterium was inoculated into diffusion chambers on a complex medium containing bovine serum and mouse brain extracts (Dhople and Hanks, 1972a; Dhople and Hanks, 1972b; Dhople and Hanks, 1973); during 17 days of incubation with periodic renewal of the medium, both the cell count and the energetics of the bacterium declined.

A double dialysis method was used to produce antigenic material from the mold that causes "farmer's lung disease" (Edwards, 1972).

Nutrient medium within dialysis tubing was equilibrated with an aqueous solution of sodium chloride, and mycelia of Micropolyspora faeni were inoculated into the resulting dialysate. After growth, the dialysate (containing organisms, dialyzable material from the original nutrient broth, and products of microbial metabolism) was placed in other dialysis tubing and a second dialysis was conducted to separate the dialyzable molecules from the mycelium and the macromolecular products. The resultant organisms and macromolecules constitutes the antigens for further studies.

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 chamber (Ruezinsky, 1975). The chamber had two parts, one for the medium and the other for the culture, which were separated by a semipermeable membrane. After incubation, the viable cell yields were not as high as those obtained from a biphasic agar-liquid culture system (Gerhardt and Hedén, 1960; Hunter and McVeigh, 1970; LaScolea et al., 1975), which also is based on diffusion principles.

L-phase variants of Streptococcus faecium were inoculated on membrane filters placed on solid agar and the organisms were found to pass through the filters, which had 0.22 μm pores (Wyrick and Gooder, 1971). The reversion of protoplasts also was studied, with the finding that the protoplasts could not pass through the filters and form colonies under it. In some cases, however, covering the filters with solid L-phase medium before inoculation of the protoplasts gave rise to colonies, 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 organism were layered on membrane filters (Clive and Landman, 1970); the reversion by the walls was nonspecific since the effect was also obtained with wall preparations from other species.

A microperfusion chamber was designed by Poyton and Branton (1970) for many purposes including the dialysis culture of tissues and microorganisms. 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. The Powell microperfusion chamber was modified for the observation and manipulation of growing bacteria (Issac et al., 1975).

Vibrio cholerae was cultured in dialysis culture flasks to produce exotoxin (Bhattacharyya, 1973). Though diffusion of nutrients from the medium reservoir was rate-limiting, the method resulted in increased toxin yield.

A microbial population and a portion of substrate contained in dialysis tubing was introduced into food (Willardsen et al., 1977). The 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. Results using membrane-contained strains of Clostridium perfringes introduced into beef indicated that the method has potential for use in studies on various food systems.

Trypanosoma lewisi was cultured in dialysis tubing containing a medium composed of a "gel phase" of protein, agar, and defibrinated rabbit blood (Dusanic, 1969). Agar was necessary for good growth

and presumably acted as a sink to absorb inhibitory products from the medium. In addition, the system provided a good way to study amino acid utilization.

For recovery purposes, a cellophane film was placed on an agar plate and a broth culture of Pseudomonas aeruginosa was spread on the film (Goto et al., 1971). The film enabled the easy removal of the bacteria and extracellular slime after growth for study of the invasive role of the slime and its immune reactions.

Lysed blood in 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.

In search for a virulence factor, the technique of growing bacteria on dialysis membranes spread over agar medium provided a good yield of essentially all the extracellular products of Staphlococcus aureus (Bamen and Haque, 1970). Although not all of the products were present in a concentrated form, the significant finding was that the entire complement of products could be detected.

Massover and Hayflick (1974) used a batch dialysis culture of T-strain mycoplasmas to observe growth and urea metabolism. Dialysis relieved the growth inhibition of the organisms caused by elevated pH and increased ammonium ion concentration. The authors also used a dialysis continuous process in which the reservoir medium was continuously fed and replaced; the culture reservoir of 5 ml volume was operated batchwise. The process maintained a stable and viable culture of the organism 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.

Dialysis culture was used to produce toxin from Clostridium botulinum type G (Ciccarella et al., 1977). The method yielded 30,000 mouse 50% mean lethal doses of the toxin per ml, which was 750 times greater than that produced in regular broth cultures.

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). The test organism was a histidine-requiring strain of Salmonella typhimurium. The organism was enclosed in a chamber which was exposed to blood, liver, and testes homogenates from mice previously injected with the mutagen, streptozotocin. After exposure, the organism was plated in an appropriate medium to determine the frequency of histidine mutations. Frequencies increased substantially as compared to controls. The method was simple and should be suitable for testing other chemicals suspected of being mutagenic.

2.1.2 Mammalian cells

The dialysis culture technique has been used to cultivate a number of mammalian cell and tissue types in vitro for reasons similar to those for microbial cells. Ingenious variations of the technique have been particularly important for study of cell and tissue differentiation.

Rose and colleagues, over a period of 25 years, have developed and refined equipment for the 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; Rose and Yajima, 1978). Their equipment has evolved from a single multi-purpose chamber (Rose, 1954) to a 12-chamber circumfusion system which allows control of nutrient and gas flow rates, pressure, agitation and temperature (Rose, 1967) and, finally, to a dual unit for the rotation of 24 culture chambers which have increased physical capabilities (Rose et al., 1970). The systems were designed not only for growth of tissue cells but also for their differentiation. The latter system was evaluated by cultivating human gingival explants in the chambers for 5 to 6 weeks (Rose and Cattoni, 1974). Results showed that the system provided microenvironments that allowed the cells to differentiate according to their genetic potential. Fetal mouse lung and fetal rat ovarian, thyroid, pancreas, and salivary gland cultures also were maintained in a differentiating state.

The Rose tissue culture chamber (Rose, 1954) was modified by inserting a membrane to divide the chamber into two compartments (Batzdorf et al., 1969). The resulting double chamber could be used to study the interaction of different cells while the different cells are physically separated by the membrane.

Marbrook (1967) studied the primary immune response of spleen cells from unimmunized mice by growing the cells on dialysis membranes inserted in a reservoir of medium. Three to five days after exposure to sheep or horse erythrocytes, significant numbers of antibody producing cells were detected. The dialysis system had the advantage of allowing the spleen cells to grow undisturbed by agitation or mixing, so that foci of antibody-producing cells were not disturbed. The system, because of its simplicity and reproducibility, has

been widely used and modified, and is commonly called the Marbrook chamber.

Using the Marbrook chamber to cultivate suspensions of mouse spleen cells, 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 to a peak at 4 days. Experiments showed there also was an induction of tolerance. The importance of this model system is that it allowed examination of the induction of both antibody formation and immunological tolerance in a defined medium using a pure antigen. A modification of the chamber was used to determine the capacity of a given spleen-cell dose to produce a primary antibody response (Halsall 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. The pokeweed mitogen, considered to be a B-cell stimulant, caused thoracic duct lymphocytes to differentiate to plasmacytoid cells.

A modified Marbrook chamber also was used for the purpose of prolongation of culture conditions, in order to achieve a system where in vitro cell responses were parallel to in vivo responses (Maizels and Dresser, 1977). The system provided a means to cultivate 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 their full potential from being resting memory cells. It was concluded that the chamber has potential for long term experiments.

A miniaturized diffusion culture system holding 24 culture chambers was developed by Eipirt et al. (1978). Each chamber utilized a cell suspension volume of 0.1 ml and a reservoir of 1.0 ml. The anti-sheep erythrocyte response of spleen cells from mice was studied to evaluate the system, which was found to yield reliable and reproducible primary and secondary antibody responses. Also, a membrane filter of 0.2 μm pore diameter (Nuclepore) was found preferable to a dialysis membrane and yielded up to a two-fold higher response.

An artificial capillary device consisting of a bundle of hollow fiber membranes of two types was used to perfuse both gas and medium into tissue cultures (Knazek et al., 1972; Knazek et al., 1974). The capillaries also provided a matrix for cell attachment so the secreted products could be harvested without disturbing the culture. This technique of combining both nutrient-passing and gas-passing membranes in a single device allowed cells to attain tissue densities in vitro similar to those found in vivo.

A dialysis culture system also was used to cultivate normal and malignant human bone marrow cells in a liquid medium (Golde and Cline, 1973). For several weeks, normal granulocytes and macrophages proliferated and differentiated, and the entire maturational sequence of the granulocyte and macrophage series was identified. The 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.

The survival of rat lymphocytes was studied using an in vitro organ-culture system (Trowell, 1959; Trowell, 1963). The cells in a liquid medium were layered on a sheet of agar, which supplied nutrients and took up metabolites. The lymphocytes were found to have a preference for hypotonic medium. Thymus, spleen, and bone marrow of the rat and tissues from the guinea pig and the rabbit did not have this preference.

Spleen fragments from mice were explanted in the well of a Millipore filter assembly which was placed in an organ culture dish containing tissue culture medium, to study primary immune reactions (Globerson and Auerback, 1965). The system provided a method which permited lymphoid maintenance, differentiation and proliferation, which together may be necessary for the initiation of immune reactions. Tissue from mice treated with either phytohemagglutinin or adjuvant were stimulated to form hemagglutinins and hemolysins. Tissue from untreated mice was necessary to invoke a response which led to specific splenomegaly.

2.2 In vivo

2.2.1 Microbial cells

Dialysis culture techniques have been used with a variety of hosts for the cultivation of many microorganisms (Table 2). The

technique appears promising especially for the culturing of fastidious, pathogenic or slow growing parasitic microbes.

The exoerythrocytic stages of malaria were cultured in diffusion chambers implanted intraperitoneally within chickens, turkeys, ducks, chick embryos and mice (Huff et al., 1960). Chickens readily encapsulated the chambers, which is a major limitation of the technique. Another finding was that Plasmodium gallinaceum, although about 0.5 to 1.5 μm in size, could pass through 0.45 μm Millipore membranes. It was concluded that the technique should be useful for the study of problems in parasitology. In a later study with a parasite, mice were immunized to Trypanosoma gambiense via growth in cellulose diffusion chambers (Petithory and Rousset, 1969).

Relatively fastidious bacteria also have been grown in implanted chambers. Mycobacterium lepraemurium was cultured in cell-impermeable chambers implanted intraperitoneally in mice, and a relationship was found between growth of the organism and susceptibility of the host (Rightsel and Wigul, 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 yield better results. The organism had a generation time of 6 to 8 days with macrophages and 11 days in the cell-free environments. M. lepraemurium also grew in implanted chambers containing cells from a species (human embryonic skin) 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. Other studies confirmed that M. lepraemurium

can grow in the absence of host cells within implanted chambers (Dhople and Hanks, 1972a; Dhople and Hanks, 1972b; Ito and Kishi, 1972; Dhople and Hanks, 1973).

This model system also has 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. With primary suspensions of neural elements 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.

Two agents of venereal disease, Treponema pallidum and Neisseria gonorrhoeae, also have been grown in implanted dialysis chambers. T. 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 (Rathlev, 1973). The organisms maintained their virulence throughout the period as indicated by the fact that samples used to infect normal rabbits intratesticularly produced the usual syphlitic 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. Similarly, the effect of dexamethasone and oxsuran on T. pallidum was investigated using chambers implanted in rabbits (Tight and Perkins, 1976). For a limited period, the organism multiplied to significant numbers in rabbits given dexamethasone but decreased in a control situation. Oxsuran appeared to have little or no effect on the growth.

This method of using subcutaneous polyethylene chambers was developed by Arko in studies on Neisseria gonorrhoeae (Arko, 1972; Arko, 1973; Arko, 1974). It is an interesting variation of the in vivo dialysis technique. A polyethylene practice golf ball (a hollow plastic ball with numerous holes) is implanted in the subcutaneous tissue of rabbits. Encapsulation by connective tissue forms an artificial chamber and natural membrane. N. gonorrhoeae grew and retained virulence in these chambers, which were usable for several weeks. The success of this system raised the possibility of studying in vivo interactions between various factors such as antibody, leukocytes, complement and bacteria. In addition, a relatively simple system was provided for culturing virulent N. gonorrhoeae. Disadvantages to the method included the nonspecific immune response which may be elicited by the artificial chamber exclusive of bacteria, "walling off" of the chambers making diffusion minimal, and difficult sampling without sacrificing the host. It should be noted that the membrane enclosing the organisms was tissue and not an artificial membrane.

A similar method was employed by Flynn and Waitkins (1973) to cultivate and study the fate of gonococci in the mouse. Inserting vinyl cylinders under the skin provided a chamber which allowed humoral and cellular factors free access to the gonococci. However, the survival time (1 day) of the gonococci was much shorter than that observed by Arko (1972). Subsequently Waitkins (1975) showed that, when gonococci were taken into cells grown in tissue culture and then put in chambers implanted in mice, the survival time of the intracellular bacteria was extended. Using another subcutaneous

chamber technique, Scales and Kraus (1974) showed that the immunity developed by guinea pigs infected with N. gonorrhoeae can be transferred to normal susceptible guinea pigs by serum or certain serum fractions.

The chorioallantoic membrane (a natural diffusion membrane) of chick embryos was infected with gonococci to investigate differences in virulence of the four gonococcal colony types (Buchanan and Gotschlich, 1973). Type 1 and 2 organisms produced infection significantly more often than types 3 and 4, establishing in the animal model the same correlation between colony types and infectivity observed in humans. As with Arko's method, a disadvantage of this system was that sampling was difficult without sacrificing the host.

Placement of gonococci and meningocci in Millipore diffusion chambers implanted subcutaneously in mice provided a growth system which allowed diffusion of humoral factors but not of cells similarly to the method of Arko (Hafiz and McEntegart, 1977). N. gonorrhoeae grew for only a limited period. The meningcocci survived for 49 days.

Less fastidious organisms frequently have been grown in dialysis chambers. Implanted chambers containing bursa from chicks resulted in 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 and thus the enhanced response may 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 resistance mechanisms to infections by facultative intracellular parasites. They 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, sham chambers without bacteria increased the host's resistance to later challenge with Listeria, thus 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. They also found that tissue reactions progressed around the chambers to the point that a chronic abscess was formed, and that pleomorphic mutants of Listeria appeared during prolonged cultivation in implanted chambers. They further studied the relationship of antimicrobial cellular immunity to delayed hypersensitivity (Osebold et al., 1974).

By use of spores and cells of Clostridium botulinum contained in implanted chambers within guinea pigs, it was shown that body fluids do not attack spores but that bacteriolytic enzymes in the fluids attack vegetative cells which release toxin (Suzuki et al., 1971). Polymorphonuclear leukocyte engulfment was necessary for germination and release of the spore-bound toxin.

A health hazard is associated with high levels of gram negative bacteria contaminating the fluids associated with hemodialysis systems (Favero et al., 1975). The bacteria in the dialysate of the artificial kidney machines can cause pyrogenic reactions and sepsis.

Gerhardt and coworkers (Quarles, 1973; Quarles et al, 1974; Gerhardt et al., 1977; Mohan et al., 1977; Belding et al., 1976) purposely managed an ex vivo hemodialysis culture to investigate its use 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. 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 goat for 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; obligate anaerobes did not grow. They also studied the synergism of penicillin and gentamicin against Listeria monocytogenes (Mohan et al., 1977).

2.2.2 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. Mainly, this technique is used

because such cells grow poorly or not at all in vitro, and the technique can establish the existence of humoral influences on various cellular processes. Other uses of the technique include: the quantitation of in vivo cell growth, the determination of the effects of chemotherapeutic drugs and their host-mediated metabolites on the implanted cells, the 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.

2.2.2.1 Antibody-producing cells

Four human hematopoietic tissues (lymph node, spleen, bone marrow and peripheral white blood cells) were cultivated in diffusion chambers implanted in mice to determine their capacity to form antibody against a test antigen, Salmonella typhosa (Gengozian, 1964). Lymph node and spleen cells were induced for active antibody synthesis whereas bone marrow and white blood cells were negative.

The effects of Rauscher Leukemia Virus (RLV) on antibody production and induction of cellular changes was studied with a system in which mouse spleen cells were cultivated in diffusion chambers implanted in isogenic hosts (Borella, 1969; Borella, 1971; Borella, 1972). It was an ideal system for 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 the studies because it represented a closed system, resulting in no problem of cells entering or leaving the compartment being assayed. In most of their studies the immune response of mouse spleen cells was examined using sheep erythrocytes as antigens. Their later results showed that the growth of the antibody-forming cells occurs non-randomly in a "staircase" manner, and that transformation of precursor cells into antibody-forming cells occurs shortly after mitosis.

2.2.2.2 Blood and bone marrow cells

Experimental results of using diffusion chambers in vivo for culturing blood and bone marrow cells were reviewed in detail by Stohlman, Quesenberry and Tyler (1973). The cells from rat, mouse, goat, rabbit and human origin have been cultured in a variety of hosts (Table 4).

2.2.2.2.1 Rat cells

Rat marrow cells have been cultivated within diffusion chambers inserted in the abdomen of the rat (Rosin et al., 1963) and in the peritoneal cavity of the mouse and rat (Petersen et al., 1974). The former study found that bone formation occurred only in the presence of osteogenic cells. The latter demonstrated that granulopoiesis was enchanced in mouse hosts and remained at a steady-state equilibrium in rat hosts, indicating the importance of host factors on growth in diffusion chambers. Mononuclear cells isolated from rat blood were cultured in chambers to study the possible conversion of lymphocytes to macrophages (Benestad et al., 1971).

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 if contamination with extraneous connective tissue was prevented (Rasmussen and Hjortdal, 1969). Consequently, the development of fibroblasts in blood and buffy-coat culture is due to contamination with connective tissue cells during sampling of the blood.

2.2.2.2.2 Mouse cells

Granulocytopoiesis also was examined by culturing mouse bone marrow cells in diffusion chambers for extended periods of time (Marmor et al., 1975). Cells proliferated logarithmically for 7 days and then the population stabilized for as long as 14 days. This latter period probably occurred because diffusion became limiting, i.e., the ratio of cell number to membrane area increased to a critical point. The state of the cells during this period resembled granulocyte growth in normal mouse bone marrow. Earlier it was shown that the marrow cells can retain their biological activity within chambers inserted intraperitoneally for periods up to 30 days (Berman and Kaplan, 1959; Berman and Kaplan, 1960). Also, in vivo there is stimulation of granulocytopoiesis by one or more diffusible factors (Rothstein et al., 1971). Similarly, chamber marrow incubated for six days in mice and stimulated with a specific antigen had increased eosinophilopoietic activity when compared to an unstimulated control (McGarry and Miller, 1974). This was interpreted as evidence for the humoral stimulation of eosinophil granulocytopoieses. A study of mouse bone marrow colonies in agar diffusion chambers indicated

cyclophosphamide causes the elaboration of a stimulating factor acting on colony precursor cells (Gordan and Blackett, 1975). Diffusion chambers also were used to examine the osteogenic potential of irradiated marrow (Kuralesova, 1968). After 10 days of implantation, osteogenesis began to be restored and by day 15 it was close to normal. Diffusion chambers with Millipore or Nuclepore filters were compared for use by culturing murine marrow (Carsten et al., 1975). The proliferation and differentiation of the marrow were comparable in the two types of chambers but cell recovery was greater from that employing the Nuclepore filters.

Murine marrow cultured in diffusion chambers implanted in mice at concentrations of 1.1×10^6 and 1.0×10^5 cells resulted in decreased cell growth at the higher concentration as compared to that of the lower (Quesenberry et al., 1974). It was suggested that the decreased growth could be a reflection of a type of feedback inhibition or the release of various nucleases and proteases as cells were destroyed. The high concentration also may have altered the environment by limiting diffusion of nutrients, thus causing the decrease. It would be interesting to determine whether this relatively poor growth would occur if a greater membrane area was employed for the same cell number and culture volume.

Diffusion chambers have been and should continue to be very useful in the study of the kinetics and regulation of myelopoiesis for the purpose of better understanding disorders of granulocytopoiesis such as leukemia (Stohlman et al., 1973). Studies appear to show that the growth of mouse marrow consisting of myleoid elements, macrophages, and pluripotent stem cells are influenced by humoral factors and also

the cell concentration in the chambers (Tyler et al., 1972; Niskanen et al., 1974). However, stem cell growth has not been ruled out as responsible for the increased myelopoiesis in the chambers (Tyler et al., 1976).

Haematopoietic cells inoculated and incubated in chambers showed that mononuclear leucocytes 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).

2.2.2.2.3 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; Laissue et al., 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).

2.2.2.2.4 Rabbit cells

Diffusion chambers containing bone marrow cells were inserted into the peritoneal cavity of rabbits to evaluate factors influencing granulopoiesis (Willemze et al., 1978). The rabbit enabled use of autologous bone marrow and up to 30 chambers in the cavity. Experimental results showed that mature granulocytes inhibited both myeloid and erythroid cell production.

2.2.2.2.5 Human cells

Boyum and coworkers have conducted several studies on the growth and differentiation of bone marrow in implanted chambers (Boyum and Borgstrom, 1970; Boyum et al., 1972a; Boyum et al., 1972b; Boyum et al., 1976). In one study, human bone marrow cells were implanted in the abdominal cavity of mice and 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 granulopoiesis was inhibited when mature granulocytes from human blood or syngeneic mouse peritoneal fluid were added to mouse bone marrow cells. Instead, there was a stimulation of macrophage formation. The inhibition appeared to be tissue specific and caused by a diffusible factor.

Other studies indicated that blood cells proliferated and were capable of differentiating into granulocytic, erythrocytic, megakaryocytic and macrophagic lines in diffusion chambers, establishing that the blood of normal man contains progenitor cells (Boecker et al., 1971; Barr et al., 1975; Chikkappa et al., 1978).

Human marrow cells cultivated in diffusion chambers, implanted intraperitoneally in normal and irradiated mice, demonstrated that the proliferation and maturation of granulopoietic cells was greatest in heavily irradiated hosts (Squires, 1975). A linear relationship existed between the number of cells inoculated and the number of cells harvested after an 8 or 10 day period of incubation.

2.2.2.3 Tumor cells

With diffusion chambers implanted in rats, Laerum et al. (1973) cultivated four types of malignant cells: mammary cells of the rat,

melanoma cells of the golden hamster (ascitic form), leukemic cells of the rat, and leukemic cells from two humans. All the cell types proliferated in the chambers for periods of 8 to 13 days. Greater proliferation of the hamster cells occurred when the cells were cultured in hamster and mouse hosts, apparently indicating that the cells grow better in iso- and heterologous host animals (Schieferstein and Laerum, 1974). Four human tumors (carcinomas of the stomach, lung and ovary, and adenocarcinomas of the breast) were found to grow in diffusion chambers implanted in rats and hamsters (Evgenjiva, 1970). The tumors maintained their histological specificity in the chambers. This technique may have potential for evaluating the effects of chemotherapeutic drugs.

Host irradiation did not effect the growth of HeLa cells cultured in diffusion chambers (Meck et al., 1976). This observation was in contrast to the stimulated growth of hemic cells observed in chambers hosted by irradiated mice or goats (Boyum et al., 1972b; Laissue et al., 1974). Murine ascites tumor and leukemia cells proliferated in chambers implanted in chick chorioallantois (Tucker and Owen, 1969). An advantage of this system was the inability of the embryo to produce an immune reaction against transplanted tumor cells. Transplantation immunity reactions also were investigated with a dialysis technique, and the results showed that a diffusible toxic antibody was involved in tumor transplantation rejection (Ambrose, 1969). This factor appeared to be an IgG immunoglobulin and 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.

The diffusion chamber technique has been important for the study of human leukemic cells. The growth of peripheral blood cells from human leukemic and control patients was characterized by such a technique (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). The growth patterns differed widely and appeared to depend on the type of leukemia. In a study of the question of whether the maturation defect in vivo in acute leukemia is due to environmental or 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 due to 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, results showed that microenvironmental factors can determine whether or not gene expression is leukemic. They also cultivated Hodgkins cells in diffusion chambers implanted in the peritoneum of irradiated mice and found evidence that the cells probably originate from B lymphocytes (Boecker et al., 1975). Leukemic cells from the rat also have been cultivated within implanted chambers (Vilpo, 1972).

2.2.2.4 Other cell types

Spleen tissues from the rat, guinea pig and mouse, enclosed in diffusion chambers and implanted in irradiated mice, increased the survival and hematopoietic recovery rates of 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). Altogether, the diffusion chamber technique has been very useful for study of thymic humoral influences (Anthens, 1970; Levey et al., 1963; Stutman et al., 1970; Trench et al., 1966).

Hairless-mouse epidermal cells in diffusion chambers appeared intact for the first 24 h, but then a significant cell loss occurred (Laerum and Boyum, 1970). Basal cells either went into differentiation or proliferation, but the latter process was outweighed by the cell loss. The differentiating cells remained as such for 7 days. Cells of rabbit aortic endothelium were cultured in diffusion chambers implanted in rabbit abdominal cavities, in an attempt to study the biological nature of the cells (Kitsukawa, 1969). The cells often showed ring formations of two to three cells, and tended to form "alveolar-like" arrangements around clusters of erythrocytes. Liver and spleen homografts in diffusion chambers implanted in the abdominal cavity of newts grew as indicated by DNA synthesis and cell proliferation (Grillo and Spink, 1968). This type of work may provide information about the factors responsible for determining the pattern of differentiation in the regenerating newt limb. The histocompatibility interactions between mixed types of mouse spleen cells was investigated by use of diffusion chambers (Harrison et al., 1968). Results showed the interactions to be immunological in nature. The 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). No thick connective-tissue capsule formed around the chambers. Human liver cells were cultivated successfully in diffusion chambers implanted in guinea pigs as an alternative to organ culture methods (Germain et al., 1966). By use of mouse macrophage polykaryons (inflammatory giant cells) cultured in diffusion chambers, it was demonstrated that the giant cells form at sites of inflammation by fusion of newly arrived macrophages with macrophages already in situ (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 (Garner et al., 1973). A subsequent study showed that therapeutic levels of penicillin G also were attainable in the chamber fluid 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 also was studied using a diffusion chamber technique (Videman et al., 1978). The DNA synthesis of the osteogenetic cells was inhibited significantly by the excess bone, suggesting the existence of a chalone. Cells of the mouse distal

radius within implanted chambers continued osteogenesis for 22 days (Vilpo et al., 1978).

Humans also have been used as hosts for diffusion chambers, subcutaneously (Brooks et al., 1960; Green, 1966). Although autologous and homologous cells grew in such systems, the results were much more satisfactory with chambers placed intraperitoneally in animals.

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

3. 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., mutalism or parasitism); to study a cell population sequestered in a natural environment, permitting easy and total cell recovery after incubation; and to characterize the in vitro growth of an organism and also the metabolites and the effects of the metabolites.

3.1 Interbiosis studies

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; Bednarski and Reporter, 1978). Rhizobia of different species were found to produce chemicals that induce soybean cells to produce other chemicals which effect the acetylene reduction activity in test rhizobia.

The growth of autotrophs, phototrophs, and heterotrophs in mixed cultures was studied with 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 the growth. Dialysis culture showed less effect and was considered less successful for the demonstration of the interactions than the use of ordinary mixed cultures. But technical limitations such as inadequate diffusion and aeration may have influenced the results.

For dairy purposes, the use of a double dialysis chamber enabled the detection of an antagonistic relationship between bacteria (Collins and Tillion, 1977). Pure cultures of Streptococcus diacetilactis and S. lactis were inoculated into separate compartments (divided by a membrane) of the chamber and incubated. S. diacetilactis produced a diffusible antibiotic, which was indicated by the inhibited growth of S. lactis as compared to that of a control experiment.

3.2 In situ studies

Hendricks and Morrison (1967) reported the first studies in which a dialysis culture technique was used in situ, for observing

the growth of enteric bacteria in mountain stream water. 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 can maintain populations but can support growth of enteric bacteria. The dialysis experiments did not wholly simulate in situ conditions, however, because natural predators such as protozoa did not have access to the bacteria.

Several phytoplankton species have been studied with dialysis culture (Jensen et al., 1972; Prakash et al., 1973; Jensen and Rystad, 1973; Skoglund and Jensen, 1976; Jensen et al., 1976). In situ use of the technique allowed the cells to accumulate trace substances from large volumes of water. Results for a "batch fermentor-continuous reservoir" dialysis system showed three growth phases (exponential, linear, and asymptotic) as mathematically predicted by Schultz and Gerhardt (1969). 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 et al., 1976) were examined. Generally, the laboratory dialysis systems provided conditions which simulated those in situ.

Diffusion chambers specifically were designed and used to study the survival of coliform bacteria both in situ and in the laboratory under controlled conditions (McFeters and Stuart, 1972; 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 (Basket and Lulves, 1974). The use and sampling ease of the apparatus was demonstrated by inoculating a Brevibacterium sp. into the tubing and monitoring its growth for 96 h. The permeability of the membranes is the limiting factor in these ecological dialysis studies. Vargo et al. (1975), while using scanning electon 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 the membrane permeability, the interaction between bacteria and pollutants and insured a uniform cell suspension for homogenous sampling. The survival studies showed that Streptococcus faecalis persisted longer than coliforms. In the experiments, membrane filters were employed (polycarbonate with 0.4 μm pores) rather than less porous dialysis membranes.

The adverse effects of chlorinated hydrocarbons on marine phytoplankton were examined with a dialysis technique in situ (Powers et al., 1976; Powers et al., 1977). The dinoflagellate Exuviella baltica in the presence of DDE was inhibited in growth for 2 to 4 days as compared to controls. 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. A dialysis technique also was employed to study limiting nutrients and maximum growth rates for two diatoms, Skeletonema costatum and Asterionella

japonica, in tanks with running sea water (Sakshaug, 1977). The distribution and survival of Aeromonas spp., which are pathogenic to many animals, were examined with dialysis in a freshwater lake that receives heated effluent from a production-nuclear reactor (Fliermans et al., 1977; Fliermans and Gorden, 1977). At various water depths, the survival of A. hydrophila always was greater when the reactor was in operation.

Survival of enteric viruses in estuarian waters was examined using cellulose dialysis tubes (Metcalf and Stiles, 1967). The virus survival was dependent on temperature, pollution levels and the type of virus. The viruses survived in the water for 56 days in the winter and 32 days in the summer. The inactivation rates of polioviruses and coxsachieviruses were determined with dialysis in situ in the Rio Grande River; the inactivation was exponential, the rate depending primarily on the water temperature (O'Brien and Newman, 1977).

A dialysis continuous culture system was developed for maintaining an in vitro culture of rumen microorganisms (Nakamura and Kurihara, 1978). For 15 days, the system maintained a concentration of ciliate protozoa, pH value, and concentrations of ammonia-nitrogen and volatile fatty acids similar to those found in situ in the rumen.

Interstitial water samplers were constructed using dialysis membranes to separate water from particulate matter, including bacteria, in lake waters and sediments (Mayer, 1976; Hesslein, 1976). With such a sampler, Winfrey and Zeikus (1977) found that sulfate inhibited methanogenesis in a freshwater lake sediment by altering normal carbon and electron flow. Anaerobic metabolism of freshly

deposited particulate organic matter initially occurred at the sediment-water interface in a hypereutrophic lake (Molongoski, 1978).

3.3 In vitro studies

Growth of the strictly autotrophic Thiobacillus thiooxidans ceases when the organism metabolizes sulfur and keto acids accumulate to inhibitory levels. Dialysis greatly increased the growth by removing the inhibitory acids (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 techniques also demonstrated that presumably obligate autotrophic bacteria will 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. neapolitanus, and T. thioparus were grown on glucose-salts media in the absence of the specific inorganic energy sources. Pyruvic acid was the metabolic product found to inhibit N. agilis, but the toxic product for N. europaea was not identified. Results with the Thiobacillus species indicated that pyruvate (or related keto acids) may be more inhibitory to the organism when growing on glucose than when growing on a specific nutrient. This work was important for it demonstrated a qualitative as well as a quantitative difference between dialysis and non-dialysis culture, and indicated that redefinition of the term "obligate autotroph" may be necessary.

Recently, a dialysis system was used to examine the energetics of nitrogen fixation of (Nif)-derepressed mutants of Klebsiella pneumonia (Anderson and Shanmugam, 1977). The system consisted of a dialysis bag (containing inoculum and 25 ml of medium) suspended in a flask containing 250 ml of medium. This system allowed fresh medium to diffuse to 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-cataloyzed H_2 production is a major factor in the economy of nitrogen fixation in vivo.

4. FERMENTATIVE APPLICATIONS

Dialysis has been applied to fermentations in order to enhance the production and recovery of microbial cells and of their diffusible and nondiffusible products. Dialysis can improve a fermentation by two interrelated yet different approaches. In one, a membrane and a reservoir are employed to remove inhibitory metabolites from a fermenting culture, resulting in increased rate and extent of substrate conversion, and permitting use of a relatively high concentration of substrate. The dialysis separation of small molecular products additionally represents a recovery step. A second approach is to dialyze the fermentor contents against a reservoir of substrate medium primarily to retain cells in the fermentor (and nondiffusible products), resulting in increased cell populations for recovery purposes. Such a process applied continuously also can allow increased throughput of substrate for conversion purposes. This second approach can be applied to fermentations not inhibited by metabolite products as well as to those that are.

4.1 Production of cells

Dairy starter cultures of Streptococcus and Lactobacillus sp. produce lactic acid which inhibits their growth. Dialysis can be used to prevent this inhibition by removing lactate from the bacterial culture (Gerhardt and Gallup, 1963; Friedman and Gaden, 1970). Osborne tested (Osborne, 1977) and patented (Osborne et al., 1975) such a fermentation using a completely batch dialysis culture system. The design was based on that of Gallup and Gerhardt (1963) and consisted of three components: a fermentor containing culture, a dialyzer, and a reservoir containing medium. The system had 5 cm^2 of membrane area for every ml of culture, which was necessary for the lactate-sensitive Streptococcus sp. The fermentor to reservoir volume ratio was 1:15. Operation of the system with Streptococcus cremoris for 24 h resulted in concentrations between 1 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 ratio and a fermentor circuit volume of 500 ml, Osborne concluded the system can produce in a 40 week year at least half the annual starter cultures necessary for a dairy plant utilizing 100,000 gallons of milk per day for cheesemaking. The resulting starter concentrates had activity better than conventional starters. The dialysis system also eliminated the requirement for centrifugation which is costly, damages the cells, and easily results in contamination.

Another dialysis process for production of concentrates of Streptococcus lactis was unsuccessful, probably because the system

had a small membrane area in relation to the culture volume (less than 1 cm^2 per ml; Bergere and Hermier, 1968).

Friedman and Gaden (1970) also used a batch dialysis system of three components (fermentor, reservoir and dialyzer) to study the growth and lactic acid production of Lactobacillus delbrueckii. The results showed the specific acid production rate was independent of the specific growth rate at low lactate concentrations; this could not be determined in a conventional fermentation. They also confirmed the inhibitory effect of lactate on the growth of the organism. Further, they quantitated the inhibition and incorporated it into the mathematical model of Leudeking and Piret (1959) relating the rate of growth to the rate of acid production.

Similarly to the lactic acid bacteria, the growth of a yeast, Mycotorula japonica, on hexadecane or decane was improved greatly by using dialysis to relieve the inhibitory effects of lauric acid (Aida and Yamaguchi, 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 raw sewage (Dor, 1975). Dialysis resulted in a 3-fold increase in the cell population as compared to a control. Limiting the growth was a lack of light, membrane permeability, membrane area and agitation. This fermentation has promise especially because the reservoir containing sewage can continuously be replenished.

Lane (1977) investigated dialysis culture as a means to produce high concentrations of a food yeast, Kluyveromyces fragiles, from

deproteinized whey, a residue of the dairy industry. He used an 8-liter fermentor, a fermentor to reservoir ratio of 1:2.5, a fermentation period of 90 h, and four successive fresh reservoirs of medium. There resulted a biomass concentration of 90 g per liter and residual lactose concentration of 5 to 8 g per liter in the reservoirs. In this fermentation system, dialysis results in a high cell concentration primarily because, at a given time, the culture is sequestered to a small part of the medium but, in the long term, the culture has access to all the medium. This principle differs from the lactic acid dialysis fermentations where dialysis primarily serves to enhance cell growth by removing inhibitory products from the culture.

In the above studies substrate mostly is supplied to the culture via a membrane from a reservoir containing medium. Cell growth is limited either by the rate of diffusion of substrate into the culture, by the accumulation of an inhibitory product 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. They ensured that substrate was not limiting by also feeding substrate directly into the culture chamber. In this manner, and using a fermentor to reservoir ratio of 1:11, they obtained biomass concentrations of 140-150 g dry wt. per liter as compared with 30 to 40 g per liter in a culture without dialysis. Their system utilized dialysis principly to remove toxic end-products which decrease the molar growth yield of E. coli on glucose.

4.2 Production of nondiffusible compounds

A dialysis method, to obtain concentrates of extracellular proteinase of Streptococcus faecalis var. liquifaciens consisted simply of growing the bacterium within dialysis tubing suspended in an Erlenmeyer flask containing medium (Millner, 1969). The dialysis methods for obtaining concentrates of nondiffusible enzymes of course are like those for obtaining cells. Concentration of two proteolytic enzymes via dialysis culture also was demonstrated 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 2.4×10^9 cells per ml was obtained with dialysis as compared to 6×10^8 in conventional flasks, and the proteinases and other nondiffusible compounds were similarly concentrated. A dialysis apparatus for producing concentrates of extracellular proteases 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 (Fogarty 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-fold greater than in conventional fermentation flasks. Considering the total bulk of medium used, however, there was no increase in the amount of enzyme produced. Mainly, the study demonstrated that dialysis can serve to benefit enzyme recovery by restricting the enzymes from the bulk of the medium.

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 ratio was 1:10 and the feed (mostly glucose at 600 g per liter) was introduced into the fermentor at a rate so that the carbon source was not limiting. The dialysis process produced at least eight times more enterotoxin than did an ordinary fermentation process. Using similar dialysis procedures, a 10-fold increase in the concentration of extracellular protein A (2 g per liter) was achieved from Staphlococcus aureus A676 as compared to results from a control (Landwall, 1978).

4.3 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 was used with a culture volume of 150 ml and a dialysate volume of 1100 ml. The flask was placed on a rotary shaker to provide aeration and mixing. The fermentation was conducted for 15 days with intermittent (4 times) removal and replenishment of medium in the dialysate reservoir in order to maintain low levels of product in the culture chamber. The rate of the fermentation increased throughout the time, suggesting that the fermentation could be improved by operating the reservoir continuously. The total amount of salicylic acid produced, though dilute, was determined to be 20-fold greater as compared to a control without dialysis. A kinetic study showed that

maintenance metabolism accounted for 84 percent of the salicylate produced (Abbott and Gerhardt, 1970b).

Dialysis also was used in an attempt to alleviate product control over threonine production by an auxotroph of Escherichia coli (Abbott and Gerhardt, 1970c). Inhibition instead of enhancement was observed. This was explained by depletion of α-ε-diaminopimelic acid as the limiting factor rather than threonine inhibition of its own synthesis.

Kominek (1975a, 1975b, 1975c, 1975d) 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; also, cycloheximide is rapidly degraded in the absence of glucose. The dialysis system consisted of a 5-liter New Brunswick fermentor (3-liter volume) with a rubber mesh mat fastened around the baffles to support dialysis tubing ($\cong$500 cm^2). The reservoir included the dialysis tubing and also an extractor, containing methyl chloride, that was connected by conduits to the tubing. Operationally, the dialysate from the tubing was bubbled through the extractor to remove the cycloheximide. The dialysate then was aerated to remove traces of the solvent, and finally was returned via conduits to the tubing for collection of more antibiotic. Glucose continuously was fed into the fermentor to prevent cycloheximide degradation and so that the carbon source did not become limiting. Results showed that, by the tenth day of continuous operation, there was a two-fold increase in titer as compared to a control. Also, 82 percent of the solids content of

the extractor was cycloheximide. Therefore, 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. Kominek concluded that this process should have application "to any fermentation product that is dialyzable and possesses a favorable distribution in a suitable water immiscible solvent." This work was the first to demonstrate the potential of dialysis for continuous production and recovery of a diffusible secondary product 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 Shuler, 1978). In this system, the cells were immobilized by containing them in the chamber volume (within the dialyzer jacket) outside the hollow fibers; while solutes freely diffused across the hollow fiber membranes. The system was tested by examining the conversion of l-histidine to urocanic acid by a heat-treated suspension of Pseudomonas fluorescens. All of the continuous production experiments lasted for at least 8 days. However, 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 nongrowing state. The half-life of a reactor containing such cells (disregarding membrane fouling) would be indefinite for the continuous production of a diffusible metabolite.

Promising experiments using a dialysis system to immobilize living microbial cells for continuous production of ammonium-lactate, were reported by Stieber and Gerhardt at a recent meeting

(Abstract, Amer. Chem. Soc. Meeting, Miami Beach, 1978). In other work (Stieber, 1976), the Cordis-Dow artificial kidney was found unsuitable for continuous fermentation use because the design and lack of agitation in the dialyzer jacket resulted in rapid clogging and decreased diffusion.

Low pressure ultrafiltration was used to separate liquid from solids in an activated sludge system (Hardt et al., 1971). The membrane flux rate was 4.5 gallons per day per ft^2. This fermentation system consistently removed 97-99 percent of the COD in the influent waste stream. During operation, biomass was monitored by oxygen uptake. The results indicated that the biomass decreased from 30 g per liter to 20 g per liter during the first six days, remaining constant thereafter (48 h). The decrease was attributed to cell lysis. Ultrafiltration was a very effective way of separating microbes from effluent, and it enabled effective control of the biomass to substrate ratio.

4.4 Dialysis sensors

On-line sensors are becoming increasingly important in the operation of fermentations (Dobry and Jost, 1977). Schultz (Abstract, 2nd Inter. Conf. Computer Appl. Fermentation Technol., Philadelphia, 1978) has used principles of dialysis to develop an affinity sensor for continuously monitoring 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. Operationally, 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. An optical system was used to measure the polysaccharide. Stability studies indicated that the equilibrium reactions involved were stable for up to 100 h.

Cell-free samples were generated from a fermentation broth for on-line analysis by a dialysis process by Zabriskie and Humphrey (1978). A baffle in a fermentor was converted into a dialyzer, the membrane of which separated 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 was the relatively slow response time. This method and that of Schultz (1979) both have application for sensing compounds other than glucose.

5. DISCUSSION

5.1 In vivo

Since Algire (1954) first used membrane filters as chamber walls, improvement in the design of in vivo dialysis systems has progressed minimally. Several problems need solutions and certain characteristics need study so that experiments are more reproducible and meaningful. Needed research areas comprise: the non-specific immune response caused by the implanted chambers; the growth of macrophages and connective tissue around the chambers, thus limiting diffusion; the lack of agitation which results in a nonhomogeneous cell suspension; the difficulty of controlling the concentration of biologically active compounds within the chambers and; the lack of knowledge concerning the diffusion capacity and rate for the chambers. Answers to some of these needs should enhance the importance of in vivo dialysis culture for study of bacterial and

blood diseases, tumors, and immune mechanisms, and for evaluation of chemotherapeutic strategies for certain of the disorders such as leukemia.

5.2 In vitro

In vitro dialysis systems for medical and ecological use usually are of small scale and are designed for a specific purpose so that the systems used are as many as the purposes. Generally, the membrane area should be relatively large compared with the culture volume and the culture should be agitated to prolong membrane utility and to provide homogeneous conditions. Membrane filters, which separate only cells from solutes, appear more useful than smaller-pored dialysis membranes.

To date, the best in vitro dialysis system for fermentative purposes is that built and used by Gerhardt and coworkers (Gerhardt and Gallup, 1965; Schultz and Gerhardt, 1969; Humphrey, 1970). The system consists of three separate components, a fermentor, a reservoir, and a dialyzer, and it can be operated batchwise, continuously or in a combination of these modes. Since the components are separate, each may be individually controlled. The dialyzer is of the plate and frame type and allows variability in membrane type and area, is sterilizable, and is designed so that there is much turbulence on both sides of the membrane, preventing membrane fouling for extended periods (Humphrey, 1970). Though the dialyzer specifically was designed for microbiological purposes, much improvement seems attainable. In recently designing a dialyzer for hemodialysis purposes, Dawids and Boe (1977) tested 27 different injection molded membrane support patterns. The patterns were judged on

several properties, e.g., compliance and rupture level of the membrane, and 15 patterns were found to be acceptable. Some of the properties were as much as 200 percent better than those of conventional patterns. Such considerations were not undertaken before (Schultz and Gerhardt, 1969).

5.3 Electrodialysis

Refinements of dialysis such as electrodialysis (Young, 1974; Johnson and Hill, 1976) and ultrafiltration (Michaels, 1968) increase the flux of certain solutes through a membrane. Electrodialysis is an electro-chemical process for separating different ionic species in solution by the use of direct-current electricity and ion-permeable membranes. The transport of ions across a membrane depends on the ability of the membrane to conduct electricity. The process separates various ions by use of different types of membranes (e.g., anion-selective, cation-selective, or multivalent-selective). Published studies of fermentation processes employing electrodialysis are few. Hongo and Iwahara (Chemical Abstracts, Vol. 88, 1978) grew Brevibacterium fulvum on a glucose medium and the fermentation product was first dialyzed and the resulting cell suspension was returned to the fermentor. The dialysate was channeled to an electrodialysis system to separate glutamic acid from the carbon and nitrogen sources, which were returned to the dialysate. The yield of glutamic acid was 51.3 percent with respect to glucose. Recently, electrodialysis was used for a L-lysine fermentation by Brevibacterium flavum (Iwahara et al., Abstract, Meeting Am. Soc. Microbiol., Honolulu, 1979). Selective removal of the L-lysine from the culture resulted in a 50 percent increase in yield as compared to a control.

5.4 Microfiltration

Ultrafiltration is a process for separating solutes of different sizes by means of their unequal passage through a membrane because of a pressure gradient. The process of using ultrafiltration to separate only microbial cells from solutes is called microfiltration. Michaels (1968) was the first to suggest and diagram a microfiltration system for continuous microbial fermentation. Sortland and Wilke (1969) and Pirt and Kurowski (1970) devised filtration methods for the retention and concentration of microbial cells in a continuous culture of limited duration. Wang et al. (1970) used an ultrafiltration cell as a fermentation and filtration device for the retention of Clostridium histolyticum cells and enzymes, and for removal of small molecular weight products. The process produced 1.55 times the enzyme units per mg of cell dry weight and 2.5 times the cell concentrations that were obtained in an ordinary batch fermentor. Fensom et al. (1974) used a microfiltration cell to retain non-growing suspensions of Agrobacterium tumefaciens as part of a continuous-flow apparatus. The process lasted about five days and was used to study the kinetics of ferricyanide reduction and 3-ketosucrose production. Vieth and Venkatasubramanian (1974) immobilized microbial cells by use of a hollow-fiber recycle reactor to eliminate several expensive steps in enzyme purification. Vera and Wang (Abstract, Meeting Am. Chem. Soc., Chicago, 1977) reported work on acetic acid production with microfiltration for cell retention. This work demonstrated that a hollow fiber filter fermentor with intermittent backflush can operate for extended periods. A heuristic indication of improved productivity also was presented.

5.5 Dialysis vs. microfiltration

The above studies as a whole indicate that microfiltration systems can improve upon dialysis systems for production of dense cell populations and recovery of cell-free metabolites. The rates would be increased, the membrane area per volume needed would be relatively small, and a large or replenishable reservoir would not be required with microfiltration. But microfiltration cannot relieve a culture from the effects of inhibitory products to as great an extent as dialysis. Thus, for a continuous fermentation which produces inhibitory and diffusible metabolites, the application of dialysis enables use of a relatively high concentration of feed substrate; microfiltration does not. Whereas there is an exchange of solutes across the membrane in a dialysis process, the flow of solutes for a microfiltration process is in one direction. Because of these process characteristics, microfiltration usually must be managed continuously; dialysis can be managed in a batch or continuous mode. Accordingly, with respect to the sterilization requirements of many microbial fermentations, dialysis may have advantage over microfiltration. As with dialysis, the major limitation of microfiltration is membrane fouling and consequently an ultrafilter also needs to be designed specifically for microfiltration use. Altogether, considerations are many in order to choose a membrane process and a mode of operation that are best suited for a microbial system.

The various membrane processes easily lend themselves to mathematical modeling and computer simulation, greatly aiding the development of a particular process. The design of a more

efficient dialyzer and a microfilter along with the use of such modeling should further advance the resource of membrane technology for cell culture and metabolite production.

ACKNOWLEDGMENT

The use of reference material, including discussion, accumulated in the thesis of John M. Quarles is appreciatively acknowledged.

MATERIALS AND METHODS

The details of materials and methods are presented in Articles I through VII. A reference index is given below (Table 6):

TABLE 6. Index of materials and methods

ARTICLE I

DIALYSIS CONTINUOUS PROCESS FOR AMMONIUM-LACTATE FERMENTATION OF WHEY: MATHEMATICAL MODEL AND COMPUTER SIMULATION

By

G. A. Coulman, R. W. Stieber, and Philipp Gerhardt

Reprinted from Appl. Environ. Microbiol. 34:725-732, 1977.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1977, p. 725-732 Vol. 34, No. 6
 Printed in U.S.A.

Dialysis Continuous Process for Ammonium-Lactate Fermentation of Whey: Mathematical Model and Computer Simulation†

G. A. COULMAN,[1]†† R. W. STIEBER,[2] AND PHILIPP GERHARDT[2]*

Departments of Chemical Engineering[1] and of Microbiology and Public Health,[2] Michigan State University, East Lansing, Michigan 48824

Received for publication 8 April 1977

A mathematical model was developed to describe a dialysis process for the continuous fermentation of whey lactose to lactic acid, with neutralization to a constant pH by ammonia. In the process, whey of a relatively high concentration is fed into the fermentor circuit at a relatively low rate so that the residual concentration of lactose is low. The fermentor effluent contains ammonium lactate, bacterial cells, and residual whey solids and could be used as a nitrogen-enriched feedstuff for ruminant animals. Only water is fed into the dialysate circuit at a relatively high rate. The dialysate effluent contains purified ammonium lactate and could be converted to lactic acid and ammonium sulfate for industry. The fermentation was specifically modeled as a set of equations representing material balances and rate relationships in the two circuits. Dialysis continuous fermentations, in general, were modeled by combining these equations and by using dimensionless parameters. The generalized model was then solved for the steady state and used to simulate the specific fermentation on a digital computer. The results showed the effects of various material and operational and kinetic parameters on the process and predicted that it could be operated efficiently.

Whey utilization is a large and worsening problem to the dairy industry. American cheesemakers annually discard about 10^7 metric tons of cheese whey because of its limited market value. Since about half of the solids originally in milk are left in whey, its discard is an environmental burden as well as an economic and nutrient loss.

A potential solution to this problem lies with the conversion of whey into feedstuff for ruminant animals, accomplished by the bacterial fermentation of whey lactose into lactic acid and its neutralization to a constant pH by ammonia. The primary purpose of the fermentation is to trap ammonia as ammonium lactate, thereby greatly increasing the nitrogen content of whey and decreasing the toxicity of ammonia. After condensation, the product is stable and can be used effectively as a ruminant feed supplement. The background of this development has been reviewed by Keller and Gerhardt (4) and Reddy et al. (7).

Practical usefulness is dependent on the ability to conduct the fermentation as cheaply and efficiently as possible. It can be conducted homofermentatively without sterilization or asepsis because of the restrictive fermentation conditions of low pH (5.3 to 5.8), high concentration of undissociated acid, high temperature (44°C), and anaerobiosis. The fermentation can be conducted either in batches (7) or continuously (4), the latter process resulting in increased conversion efficiency with time.

A further increase in efficiency appeared possible by the application of dialysis (i.e., the separation of solute molecules by their unequal diffusion through a semipermeable membrane because of a concentration gradient) with the fermentation (1, 2, 8; P. Gerhardt and D. M. Gallup, U.S. Patent 3186917, June 1965). Thereby, the small molecular products are removed from the immediate environment of the bacterial cell (and ultimately from the intracellular enzyme site), thus relieving the feedback inhibition by a product that normally regulates its production. As more product is withdrawn by dialysis, more substrate is consumed and more product is made; i.e., the fermentation becomes more efficient. As the cell population attains high density, the substrate is increasingly converted into product by maintenance rather than by growth metabolism, thus also improving

† Journal article 7986 from the Michigan Agricultural Experiment Station.

†† Present address: Department of Chemical Engineering, Cleveland State University, Cleveland, OH 44115.

fermentation efficiency. The dialysis separation of much of the small molecular products additionally represents a purification step towards alternative uses, such as the conversion of ammonium lactate into lactic acid for use as an industrial chemical and into ammonium sulfate for use as a fertilizer (6). On the other hand, the application of dialysis increases cost, complicates operation, and eventually reduces efficiency because of membrane fouling. The potential advantages must compensate the disadvantages if a dialysis process is to be useful.

In our investigations, the goal was to assess the feasibility of applying membrane technology to the fermentation. Continuous operation appeared desirable not only for the best use of membrane technology but also because waste whey is generated and the fermentation products could be used more or less continuously. Among the several membrane techniques, dialysis appeared desirable for the initial study because of its simplicity and the existence of a theoretical basis for its application to bacterial culture (8).

In the studies reported in this article, our objective was to develop a mathematical model of a dialysis continuous process specifically for the ammonium-lactate fermentation of whey, but generalized with dimensionless parameters so that the model could be applied to other fermentations. The resulting set of equations was solved for the steady state and used to simulate the specific fermentation, on a digital computer. The experimental tests are reported in a companion article (9).

MATHEMATICAL MODEL

Design of fermentation system. Figure 1 shows a schematic of a completely continuous dialysis system designed for the ammonium-lactate fermentation of whey. The symbols correspond to those used previously (4, 8) and are listed in Table 1. The system differs from the usual dialysis culture systems (see Fig. 8 in reference 8) following an earlier idea to dialyze a fermentation against a stream of water (see Fig. 3 in P. Gerhardt and D. M. Gallup, U.S. Patent 3186917, June 1965). This provides the greatest concentration gradient possible for dialysis and exploits the greater permeation rate of the product (lactate) rather than that of the substrate (lactose), thus maximizing efficiency of the fermentation.

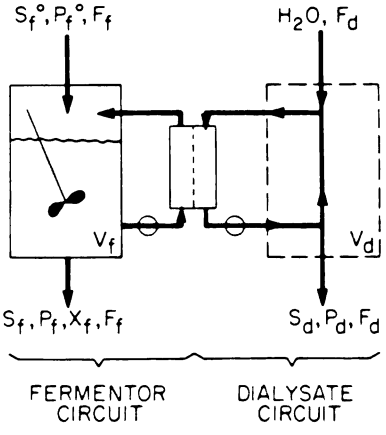


FIG. 1. *Schematic of dialysis continuous fermentation system. Symbols are described in Table 1.*

The feed into the fermentor circuit contains the substrate (lactose) in relatively high concentration (S_f^o) and is maintained at a relatively low rate of flow (F_f) consistent with a minimal concentration of unused substrate in the fermentor effluent (S_f). The substrate is converted essentially only to a single product (lactic acid) that remains in the fermentor effluent (P_f). A small concentration of the product may preexist in the whey feed (P_f^o). The liquid volume in the fermentor (V_f) and the cell population (X_f) are maintained at constant levels. The fermentor contents are thoroughly mixed and are continuously circulated through one side of the dialyzer. The addition of ammonia solution is not shown because it does not enter into the modeling.

The usual reservoir vessel for dialysate is eliminated. Instead, the dialysate circuit consists only of the tubing, pump, and dialysate side of the dialyzer and contains a relatively small volume (V_d). The feed into the dialysate circuit consists only of water and is maintained at a relatively high flow rate (F_d) consistent with a very low substrate concentration (S_d) and a practically useful product concentration (P_d) in the dialysate effluent.

For purposes of mathematical modeling, the assumptions are made that high mixing and circulation rates insure homogeneity throughout the system, liquid turbulence and excess membrane surface insure insignificant fouling of the dialyzer membranes for a practically useful period, bacterial metabolic rates remain constant, the volume of the dialysate circuit is negligible relative to that of the fermentor, the rate of ammonia-solution addition is negligible relative to that of substrate feed, and pressures are equalized between both circuits.

Material-balance equations. A set of material-balance equations was developed analogous to those in previous dialysis-culture theory (see equations 21, 22, 23, 51, and 52 in reference 8). The equations were formulated in general as follows: input + production = output + accumulation.

Equations for the substrate, product, and cell

TABLE 1. *Glossary of mathematical symbols*

Symbol	Description	Units
A_m	Area of membrane available for dialysis	cm^2
F_d	Flow rate into and out of dialysate circuit	ml/h
F_f	Flow rate into and out of fermentor circuit	ml/h
K_p	Product inhibition constant	mg/ml
K_s	Michaelis-Menten saturation constant	mg/ml
P_d	Product concentration in dialysate circuit	mg/ml
P_f^o	Product concentration in fermentor feed	mg/ml
P_f	Product concentration in fermentor circuit	mg/ml
P_{mp}	Permeability of membrane to product	mg/cm^2-h
P_{ms}	Permeability of membrane to substrate	mg/cm^2-h
r_g	Rate of cell growth	mg/ml-h
r_p	Rate of product formation	mg/ml-h
$-r_s$	Rate of substrate utilization	mg/ml-h
S_d	Substrate concentration in dialysate circuit	mg/ml
S_f^o	Substrate concentration in fermentor feed	mg/ml
S_f	Substrate concentration in fermentor circuit	mg/ml
t	Time	h
V_d	Volume of liquid in dialysate circuit	ml
V_f	Volume of liquid in fermentor circuit	ml
X_f	Cell-mass concentration in fermentor circuit	mg/ml
α	Substrate/cell ratio	mg/mg
β	Specific maintenance rate	h^{-1}
γ	Product/substrate ratio	mg/mg
μ_m	Maximum specific growth rate of cells	h^{-1}
T_f	Cell retention time in fermentor circuit	h

mass in the fermentor circuit are as follows:

$$F_f S_f^o + V_f r_s = F_f S_f + P_{ms} A_m \cdot (S_f - S_d) + V_f \frac{dS_f}{dt} \quad (1)$$

$$F_f P_f^o + V_f r_p = F_f P_f + P_{mp} A_m \cdot (P_f - P_d) + V_f \frac{dP_f}{dt} \quad (2)$$

$$V_f r_g = F_f X_f + V_f \frac{dX_f}{dt} \quad (3)$$

The symbols in these and subsequent equations are described in Table 1.

For the dialysate circuit, the corresponding equations are:

$$P_{ms} A_m (S_f - S_d) = F_d S_d + V_d \frac{dS_d}{dt} \quad (4)$$

$$P_{mp} A_m (P_f - P_d) = F_d P_d + V_d \frac{dP_d}{dt} \quad (5)$$

The terms $P_{ms}A_m(S_f - S_d)$ and $P_{mp}A_m(P_f - P_d)$ are based on Fick's law and describe the diffusion of substrate and product, respectively, across the dialyzer membrane.

To establish maximal retention times for the substrate, product, and cells in the fermentor, equations 1, 2, and 3 can be modified as follows:

$$\frac{F_f S_f^o}{F_f + P_{ms} A_m} + \frac{V_f}{F_f + P_{ms} A_m} r_s = -\frac{P_{ms} A_m}{F_f + P_{ms} A_m} S_d + S_f + \left[\frac{V_f}{F_f + P_{ms} A_m}\right] \frac{dS_f}{dt} \quad (6)$$

$$\frac{F_f P_f^o}{F_f + P_{mp} A_m} + \frac{V_f}{F_f + P_{mp} A_m} r_p = -\frac{P_{mp} A_m}{F_f + P_{mp} A_m} P_d + P_f + \left[\frac{V_f}{F_f + P_{mp} A_m}\right] \frac{dP_f}{dt} \quad (7)$$

$$\frac{V_f}{F_f} r_g = X_f + \left[\frac{V_f}{F_f}\right] \frac{dX_f}{dt} \quad (8)$$

Maximal retention times can be calculated from the terms within brackets in the above equations. If no bacteria existed in the fermentor, the differential equations would be linear and the actual retention times would equal the maximal retention times. From these times, the period necessary to reach a new steady state after the occurrence of a step change in an input variable can be estimated. From a change or start-up, the system will have moved 95% to a new steady state in three retention time periods,

and the system will reach steady state in a small fraction of the expected duration of continuous operation before membrane fouling becomes excessive. Consequently, the study was restricted to steady-state behavior, without integrating the equations for transient behavior.

Calculations of the terms within brackets in the above equations also show that the cell retention time is always longer than that of the substrate or product. The long cell retention time and short product retention time enable a high rate of substrate conversion in dialysis continuous fermentation.

Rate-relationship equations. The term for the rate of substrate utilization includes terms for both cell growth and maintenance (5):

$$-r_s = \alpha r_g + \beta X_f \tag{9}$$

The rate of product formation is proportional to that of substrate utilization (4):

$$r_p = -\gamma r_s \tag{10}$$

Although substrate limitation was not considered significant in a previous modeling of (nondialysis) continuous whey fermentation (4), the higher substrate conversion rates resulting from dialysis may cause substrate concentrations to occur at limiting levels. Consequently, the term for cell growth rate includes terms for substrate limitation as well as product inhibition (3):

$$r_g = \mu_m \left(\frac{S_f}{K_s + S_f}\right)\left(\frac{1}{1 + P_f/K_p}\right) X_f \tag{11}$$

Generalized model. The above equations for rate relationships were combined with those for material balances and the variables were defined in dimensionless parameters (Table 2) to obtain a generalized model for dialysis continuous fermentation.

The resulting equations for the fermentor circuit are as follows:

$$\frac{d\bar{S}_f}{dt} = \Bigg[-(1 + \Pi)\bar{S}_f - \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f}\right)\left(\frac{1}{1 + \bar{P}_f/\bar{K}_p}\right) + \beta \mathrm{T}_f/\alpha\right]\bar{X}_f + \Pi\bar{S}_d + 1\Bigg]/\mathrm{T}_f \tag{12}$$

$$\frac{d\bar{P}_f}{dt} = \Bigg[-(1 + R\Pi)\bar{P}_f + \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f}\right)\left(\frac{1}{1 + \bar{P}_f/\bar{K}_p}\right) + \beta \mathrm{T}_f/\alpha\right]\bar{X}_f + R\Pi\bar{P}_d + \bar{P}_f^o\Bigg]/\mathrm{T}_f \tag{13}$$

$$\frac{d\bar{X}_f}{dt} = \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f}\right)\left(\frac{1}{1 + \bar{P}_f/\bar{K}_p}\right) - 1\right]\bar{X}_f/\mathrm{T}_f \tag{14}$$

where $\mathrm{T}_f = V_f/F_f$.

For the dialysate circuit, the corresponding equations are:

$$\frac{d\bar{S}_d}{dt} = \left[-(1 + \phi\Pi)\bar{S}_d + \phi\Pi\bar{S}_f\right] F_d/V_d \tag{15}$$

$$\frac{d\bar{P}_d}{dt} = \left[-(1 + \phi R\Pi)\bar{P}_d + \phi R\Pi\bar{P}_f\right] F_d/V_d \tag{16}$$

Generalized steady-state solution. The time derivatives of the generalized model were set at zero to obtain a generalized solution for the steady state.

TABLE 2. *Glossary of dimensionless parameters*

Type	Symbol and definition	Description
Material parameters	$\bar{P}_d = P_d/\gamma S_f^o$	Product factor in dialysate circuit
	$\bar{P}_f = P_f/\gamma S_f^o$	Product factor in fermentor circuit
	$\bar{S}_d = S_d/S_f^o$	Substrate factor in dialysate circuit
	$\bar{S}_f = S_f/S_f^o$	Substrate factor in fermentor circuit
	$\bar{X}_f = \alpha X_f/S_f^o$	Cell factor in fermentor circuit
Operational parameters	$R = P_{mp}/P_{ms}$	Ratio of product/substrate membrane permeabilities
	$\Pi = P_{ms}A_m/F_f$	Membrane permeability factor
	$\phi = F_f/F_d$	Flow-rate ratio
Kinetic parameters	$\bar{K}_s = K_s/S_f^o$	Michaelis-Menten saturation factor
	$\bar{K}_p = K_p/\gamma S_f^o$	Product-inhibition factor
	$\theta = \mu_m \mathrm{T}_f$	Time factor
Conversion-efficiency parameter	$E = 1 - \bar{S}_f - \bar{S}_d/\phi$	Fraction of substrate converted to product

Equations 15 and 16 were solved for the dimensionless substrate and product factors in the dialysate circuit ($\bar{S}_d$ and $\bar{P}_d$) in terms of $\bar{S}_f$ and $\bar{P}_f$, respectively:

$$\bar{S}_d = \phi\pi_s\bar{S}_f \quad (17)$$

$$\bar{P}_d = \phi\pi_p\bar{P}_f \quad (18)$$

where $\pi_s = \Pi/(1 + \phi\Pi)$ and $\pi_p = R\Pi/(1 + \phi R\Pi)$.

Equations 12 and 13 were combined to yield an equation in terms of $\bar{S}_f$, $\bar{P}_f$, $\bar{S}_d$, and $\bar{P}_d$. The further substitution of equations 17 and 18 resulted in an expression only in terms of $\bar{S}_f$ and $\bar{P}_f$. Equation 14 was used to obtain another expression in terms of $\bar{S}_f$ and $\bar{P}_f$. These two expressions were combined to obtain one (quadratic) equation for $\bar{S}_f$ and another for $\bar{P}_f$:

$$\bar{S}_f^2 + \left[K_s + \frac{\bar{K}_p(1 + \pi_p)(\theta - 1) - 1 - P_f^o}{1 + \pi_s}\right]\bar{S}_f - \bar{K}_s\left[\frac{\bar{K}_p(1 + \pi_p) + 1 + P_f^o}{1 + \pi_s}\right] = 0 \quad (19)$$

$$\bar{P}_f = \bar{K}_p\left[\theta\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f} - 1\right] \quad (20)$$

Equation 19 has two real and distinct roots, one positive and one negative. The positive root is the only physically valid solution.

Equation 14 was used to eliminate the nonlinear term in equation 12, and the resulting expression was solved for $\bar{X}_f$ as follows:

$$\bar{X}_f = \frac{1 - \bar{S}_f + \Pi(\bar{S}_d - \bar{S}_f)}{1 + \beta T_f/\alpha} \quad (21)$$

Equations 17 to 21 comprise a generalized steady-state solution for substrate, product, and cells in the fermentor and the dialysate circuits of the system. This solution was found to be stable locally.

COMPUTER SIMULATION

The generalized mathematical model was used to simulate the dialysis continuous process for the fermentation of whey lactose into ammonium lactate. The model was programmed on a digital computer. Because the simulations were performed before actual experimental fermentations were conducted, approximations of various conditions were used as shown in Table 3, unless otherwise stated.

The dimensionless parameters that were manipulated in the simulations are included in Table 2. The time factor (θ), the flow-rate ratio (ϕ), and the membrane permeability factor (Π) each can be adjusted experimentally. The time factor (θ) was selected as the primary dimensionless variable because it included the important design parameter V_f/F_f and eliminated the need for specific values of μ_m. The permeability ratio (R) characterizes the molecular sensitivity of the membrane. The Michaelis-Menten saturation factor ($\bar{K}_s$) and the product-inhibition factor ($\bar{K}_p$) are the only parameters that are characteristic of a specific system, i.e., the ammonium-lactate fermentation of whey, but are generally relative to feed concentration. Therefore, the model could be applied to other systems without difficulty.

The simulations were made first with the two main experimental operating parameters, cell retention time in the fermentor circuit (T_f) and flow rate into and out of the dialysate circuit (F_d). To obtain values for T_f from the time factor (θ), the maximum specific growth rate (μ_m) was set at 0.27 h^{-1} (see Fig. 5 in reference 4). Figure 2 shows the simulated effects of changes in T_f on the concentration of substrate in the fermen-

TABLE 3. *Values used in computer simulations*

Symbol[a]	Value
$\bar{K}_p$	0.2
$\bar{K}_s$	0.001
P_f^o (mg/ml)	0
R	3.0
S_f^o (mg/ml)	230
γ	0.9
Π	10

[a] Symbols are described in Tables 1 and 2.

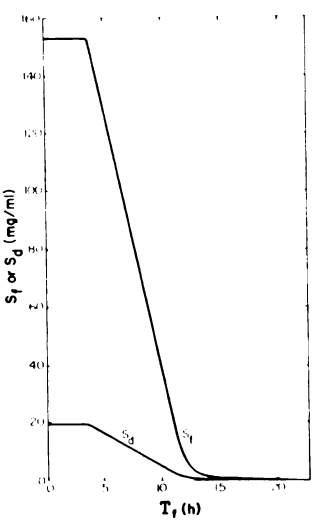


FIG. 2. *Simulated effects of changes in cell retention time (T_f) on substrate concentrations in the fermentor circuit (S_f) and the dialysate circuit (S_d) at constant flow rates, when $\phi = 0.25$ and $\Pi = 0.575$.*

tor circuit (S_f) and the dialysate circuit (S_d) at constant flow rates. Below a critical threshold value of Υ_f, the values of S_f and S_d remain constant because of cell washout. Above the threshold, S_f and S_d decrease as Υ_f increases. In each circuit, a plateau region is reached at which only marginal improvements occur with further increases in Υ_f. The simulations predicted that satisfactorily low levels of residual substrate (<5 mg/ml) could be attained by use of a reasonably short retention time in the fermentor circuit (<20 h). As expected, the general shape of the curves was the same as that for the nondialysis continuous process (see Fig. 3 in reference 4).

Figure 3 shows the simulated effects of changes in the dialysate flow rate (F_d) on the concentration of substrate in the fermentor circuit (S_f) and the dialysate circuit (S_d) at constant cell retention time. Both of the dependent variables decrease as F_d increases, and satisfactorily low levels of residual substrate apparently could be attained in both circuits with a reasonably low flow rate in the dialysate circuit. In both of the simulations, the concentration of residual substrate (rather than the concentration of accumulated product) was used as a measure of fermentation conversion efficiency because of greater accuracy and precision in the experimental analysis for lactose than for lactate. Furthermore, substrate use is more applicable in a generalized model because there are often several products, but there is usually only one substrate.

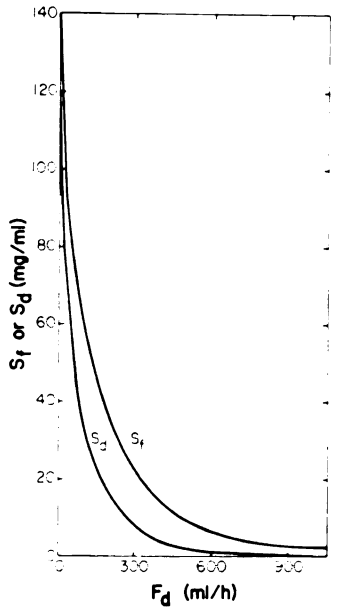


FIG. 3. *Simulated effects of changes in dialysate flow rate (F_d) on substrate concentrations in the fermentor circuit (S_f) and the dialysate circuit (S_d) at constant cell retention time (Υ_f = 13 h), when* Π = *0.575.*

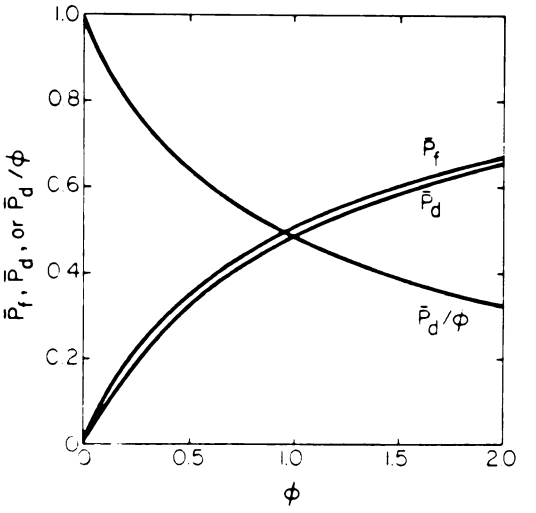


FIG. 4. *Simulated effects of changes in the flow-rate ratio (ϕ) on the product factor in the fermentor circuit ($\bar{P}_f$) and the dialysate circuit ($\bar{P}_d$) and on the dialysate product yield ($\bar{P}_d/\phi$), when E = 0.995.*

Generalized simulations then were made to illustrate the interrelationships of the dimensionless parameters. Figure 4 shows the simulated effects of changes in the flow-rate ratio (ϕ) on the product factors in the fermentor circuit ($\bar{P}_f$) and the dialysate circuit ($\bar{P}_d$) and on the dialysate product yield ($\bar{P}_d/\phi$), which represents the fraction of substrate remaining as product in the dialysate effluent. As ϕ increases, $\bar{P}_f$ and $\bar{P}_d$ increase and $\bar{P}_d/\phi$ decreases. However, to achieve a preselected level of efficiency (E = 0.995), the time factor (θ) must be adjusted for changes in ϕ (Fig. 5); i.e., Υ_f must be increased with increasing changes in ϕ to maintain a low level of substrate in both circuits. In the ammonium-lactate fermentation, it is desirable to have high values of $\bar{P}_f$, $\bar{P}_d$, and $\bar{P}_d/\phi$. Since θ varies with ϕ, a compromise probably would be made considering cost of the facility and relative marketability of the two effluents.

Figure 5 also shows the effect of changes in the product-inhibition factor ($\bar{K}_p$) on the dialysis continuous fermentation. The smaller the value of $\bar{K}_p$, the more effective dialysis becomes.

The effect of changes in the membrane permeability factor (Π) on the product factor in the fermentor circuit ($\bar{P}_f$) and the dialysate circuit ($\bar{P}_d$) at three permeability ratios (R) is shown in Fig. 6. Each of the curves reaches a plateau region where a further increase in Π does little to decrease $\bar{P}_f$ or increase $\bar{P}_d$. A high Π is needed

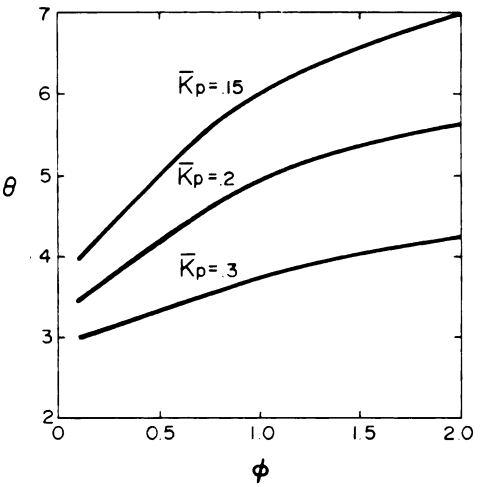


FIG. 5. *Simulated effects of changes in the flow-rate ratio (ϕ) on the time factor (θ) at three values of the product-inhibition factor ($\bar{K}_p$), when $E = 0.995$.*

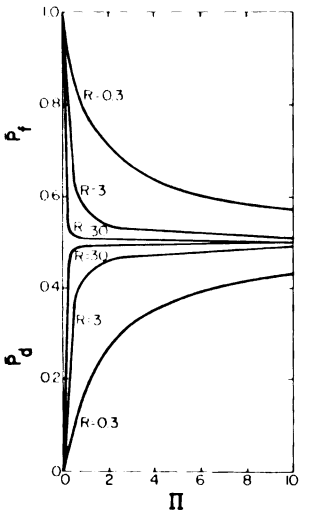


FIG. 6. *Simulated effects of changes in the membrane permeability factor (Π) on the product factor in the fermentor circuit ($\bar{P}_f$) and the dialysate circuit ($\bar{P}_d$) at three permeability ratios (R), when $\phi = 1.0$ and $E = 0.995$.*

only when R is low, and a Π value greater than 2.0 probably would prove unnecessary in the ammonium-lactate fermentation.

The effect of changes in the time factor (θ) on the conversion efficiency parameter (E) at three membrane permeability factors (Π) is shown in Fig. 7. As θ increases, E increases until a plateau region is reached. The relationship illustrates the tradeoff between the two parameters in designing the system.

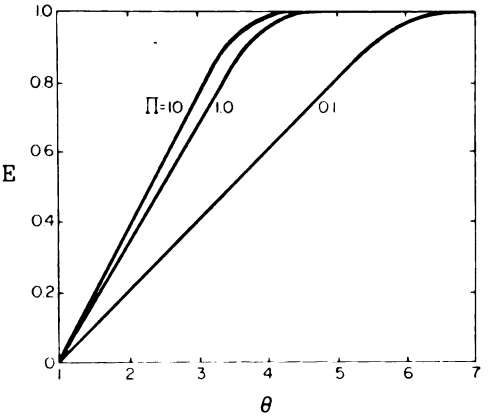


FIG. 7. *Simulated effects of changes in the time factor (θ) on the conversion efficiency parameter (E) at three membrane permeability factors (Π), when $\phi = 1.0$.*

DISCUSSION

Mathematical modeling and computer simulation provide powerful tools for predicting the results to be expected in a fermentation process. Laboratory experiments then need be conducted only to validate the predictions by using a relatively limited number of changes at preselected critical points. The experimental results in turn are used to establish constants and perhaps to indicate additional terms in the mathematical equations. By this process of successive theoretical prediction and experimental validation, the model becomes increasingly accurate and useful to predict how the fermentation should be conducted for accomplishing objectives.

The foregoing generalized model and specific simulations clearly predict that a dialysis continuous process for the ammonium-lactate fermentation of whey can be operated efficiently, enabling the rapid and almost complete conversion of a high concentration of substrate into high concentrations of product and cells in one effluent and an adequate concentration of purified product in another effluent. The stimulations also project the effects of changes in various parameters on the process and the regions for experimental validation.

Although generalized, the mathematical model was developed within certain specifications related to the steady-state conversion of substrate primarily into a product that exerts feedback inhibition. Transient as well as steady-state solutions sometimes are obtained from a model by integration, but this consideration was unnecessary for the present fermentation.

Cell yield rather than product yield sometimes is more important than in the present fermen-

tation. The generalized expression for cells in the fermentor circuit (equation 21) could be simulated but would require establishing values for α and β, which depend on the organism used. In equation 21, the cell population term ($\bar{X}_f$) increases as the term $\beta\Upsilon_f/\alpha$ decreases. Thus, in situations where substrate utilization is more a function of growth (α) than of maintenance (β), the cell population would increase with retention time (Υ_f). Beyond a critical retention time, however, maintenance supersedes growth and then the cell population would actually decrease. In a previous mathematical model of dialysis continuous culture, X_f was predicted to increase with Υ_f (i.e., with decreasing dilution rate); however, the theory did not consider values of $\Upsilon_f >$ 10 h (see p. 21–25 in reference 8). Such considerations and simulations of cell yield would be useful if the conversion of whey were designed to produce single-cell protein rather than ammonium lactate as the primary product or if the process were modeled to produce dairy starter cultures. The generalized nature of the model enables such alternative applications.

ACKNOWLEDGMENTS

This work was supported by a grant from the Office of Research Development of Michigan State University and by grant ENG 76-17260 from the National Science Foundation.

LITERATURE CITED

1. **Friedman, M. R., and E. L. Gaden, Jr.** 1970. Growth and acid production by *Lactobacillus (L.) delbrueckii* in a dialysis culture system. Biotechnol. Bioeng. **12**:961–974.
2. **Gerhardt, P., and D. M. Gallup.** 1963. Dialysis flask for concentrated culture of microorganisms. J. Bacteriol. **86**:919–929.
3. **Humphrey, A. E.** 1972. The kinetics of biosystems: a review. Adv. Chem. Ser., no. 109, p. 630–650.
4. **Keller, A. K., and P. Gerhardt.** 1975. Continuous lactic acid fermentation of whey to produce a ruminant feed supplement high in crude protein. Biotechnol. Bioeng. **17**:997–1018.
5. **Marr, A. G., E. H. Nilson, and D. J. Clark.** 1963. Maintenance requirement of *E. coli*. Ann. N.Y. Acad. Sci. **102**:536–548.
6. **Poznanski, S., K. Kornacki, Z. Smietana, J. Rymaszewski, A. Surazynski, and W. Chojnowski.** 1973. Ny metode til fremstilling af maelkesyre ved hjaelp af ionbytter. Ein neues Verfahren zur Milchsauregewinnung unter Verwendung von Ionenaustauschern. Nord. Mejeri-Tidssk. **39**:167–170.
7. **Reddy, C. A., H. E. Henderson, and M. D. Erdman.** 1976. Bacterial fermentation of cheese whey for production of a ruminant feed supplement rich in crude protein. Appl. Environ. Microbiol. **32**:769–776.
8. **Schultz, J. S., and P. Gerhardt.** 1969. Dialysis culture of microorganisms: design, theory, and results. Bacteriol. Rev. **33**:1–47.
9. **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.

ARTICLE II

DIALYSIS CONTINUOUS PROCESS FOR AMMONIUM-LACTATE FERMENTATION OF WHEY: EXPERIMENTAL TESTS

By

R. W. Stieber, G. A. Coulman, and Philipp Gerhardt

Reprinted from Appl. Environ. Microbiol. 34:733-739, 1977.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1977, p. 733-739 Vol. 34, No. 6
 Printed in U.S.A.

Dialysis Continuous Process for Ammonium-Lactate Fermentation of Whey: Experimental Tests†

R. W. STIEBER,[1] G. A. COULMAN,[2]†† AND PHILIPP GERHARDT[1]*

Departments of Microbiology and Public Health[1] and of Chemical Engineering,[2] Michigan State University, East Lansing, Michigan 48824

Received for publication 8 April 1977

Laboratory experiments were conducted to validate theoretical predictions describing a dialysis continuous process for the fermentation of whey lactose to ammonium lactate, in which the fermentor contents are poised at a constant pH by adding ammonia solution and dialyzed through a membrane against water. Dried sweet-cheese whey was rehydrated to contain 230 mg of lactose per ml, supplemented with 8 mg of yeast extract per ml, charged into a 5-liter fermentor without sterilization, adjusted in pH (5.3) and temperature (44°C), and inoculated with *Lactobacillus bulgaricus*. The fermentor and dialysate circuits were connected, and steady-state conditions were established. A series of such conditions was managed nonaseptically for 94 days to study the process and to demonstrate efficiency and productivity. As time progressed, the fermentation remained homofermentative and increased in conversion efficiency, although membrane fouling necessitated dialyzer cleaning about every 4 weeks. With a retention time of 19 h, 97% of the substrate was converted into products. Relative to nondialysis continuous or batch processes for the fermentation, the dialysis continuous process enabled the use of more concentrated substrate, was more efficient in the rate of substrate conversion, and additionally produced a second effluent of less concentrated but purer ammonium lactate.

In a companion article (3), we have provided background, rationale, mathematical modeling, and computer simulation of a dialysis continuous process for the ammonium-lactate fermentation of whey. In this article, we report on experimental tests of the simulation-predicted modes of operation, studies of the kinetics and parameters, validation of the mathematical model, and demonstration of the greatly increased efficiency obtainable by this process as compared with nondialysis batch and continuous processes for the fermentation (5, 8).

MATERIALS AND METHODS

Inoculum. *Lactobacillus bulgaricus* 2217 (Chris Hanson's Laboratory, Milwaukee, Wis.), the inoculum for the fermentation, was selected (8) on the basis of its high rate of acid production in the pH range of 5.0 to 6.0. The culture was grown at 44°C for 8 to 12 h in a sterile medium of 10% (wt/vol) skim milk powder and 90% tap water, stored at 4°C, and transferred weekly to fresh medium.

Substrate. Dried sweet-cheese whey (Galloway West Co., Fond du Lac, Wis.), used as the fermentation substrate, was rehydrated to contain 230 mg of lactose per ml and was supplemented with 8 mg of yeast extract per ml. The reconstituted whey was made up in 7.5-liter batches without sterilization and was held in a stirred, chilled (6°C) reservoir during use.

Dialysis continuous fermentation system. Figure 1 shows a diagram of the experimental dialysis continuous fermentation system. The fermentation was conducted continuously by metering the whey from the feed reservoir into the fermentor and removing overflow into the product reservoir, thus maintaining a constant volume in the fermentor. The fermentor contents were mixed thoroughly by stirring and baffling. Nitrogen was continuously purged into the fermentor to force overflow into the reservoir. Concentrated (58%) NH_4OH solution was automatically fed into the fermentor to maintain a constant pH. Concurrently, the fermentation was dialyzed continuously by circulating the fermentor contents through the dialyzer. Solutes diffused through membranes of the dialyzer to a circulating stream of dialysate, into which tap water was metered. The dialysate effluent was removed into another product reservoir.

The fermentation was conducted in a 5-liter modular fermentor with automatic temperature control (Microferm model MF 105, New Brunswick Scientific Co., New Brunswick, N.J.) and automatic pH control (model pH-22, New Brunswick Scientific). Contents of the fermentor circuit (1.4 to 4.0 liters) were maintained at a temperature of 44°C ± 0.5°C and at pH 5.3 ± 0.05. The plate-and-frame dialyzer (see Fig. 5 in reference 9) consisted of two stainless-steel plates

† Journal article 7985 from the Michigan Agricultural Experiment Station.

†† Present address: Department of Chemical Engineering, Cleveland State University, Cleveland, OH 44115.

bolted to compress a series of silicone-rubber gaskets, sheet stainless-steel frames, molded silicone-rubber separators, and regenerated cellulose membranes cut from Visking tubing (25-cm flat-width, Union Carbide Corp., New York, N.Y.). Eight membranes, providing 2,300 cm^2 of effective area, were used in the dialyzer. Peristaltic tubing pumps (models T8 and T6S, Sigmamotor, Inc., Middleport, N.Y.) and polyurethane tubing were used to meter flow rates of the whey to the fermentor and of the water to the dialyzer. Maisch gear pumps (model HQDCC, Tuthill Pump Co., Chicago, Ill.) were used to circulate the fermentor contents and water dialysate through the dialyzer. Flow rates were determined twice daily for all inputs and outputs of the system. Operation of the system occasionally was interrupted for 5 to 10 h for cleaning of the dialyzer or fermentor or for installation of a new component in the system. After 76 days the operation was suspended for 6 days and then was resumed for 18 more days. During inoperative periods, the fermentor-circuit contents were stored at 4°C.

Samples of the dialysate and fermentor circuits were taken at 12-h intervals from the dialysate effluent and from a glass "T" inserted in the tubing between the fermentor and dialyzer, respectively. Steady-state data were determined from samples taken at five times the cell retention time or at 48 h after changing a parameter. Lactose in the samples was determined by the colorimetric method of Dubois et al. (4) as modified by Montgomery (6). Lactic acid was determined by a gas chromatographic procedure (1). The concentration of residual lactose rather than lactic acid was preferred as a measure of fermentation conversion efficiency because of better accuracy and precision.

The symbols used in this article have been defined in the companion paper (3).

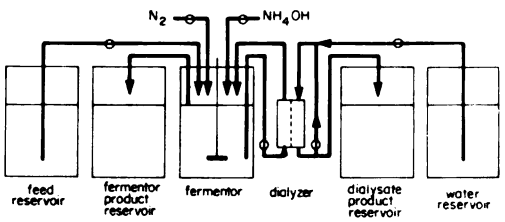


FIG. 1. *Diagram of the experimental dialysis continuous fermentation system.*

RESULTS

Effect of fermentor cell retention time on conversion efficiency. The results of experimental variation in the cell retention time (T_f) affecting the residual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) showed that the whey was readily fermented below the desired level of 5 mg/ml (Fig. 2), as predicted by the computer simulation (see Fig. 2 in reference 3). The results also showed the effect of changes in T_f on the process during two different periods, in each of which a point was reached where an increase in T_f did little to decrease S_f and S_d.

Culture adaptation. The conversion efficiency increased substantially as time progressed (Fig. 2), i.e., the culture at days 50 to 75 was able to reduce the residual lactose in the fermentor to the desired level with a shorter retention time (T_f = 18 h) than did the culture at days 19 to 24 (T_f = 22 h). A more efficient bacterial population evolves also during pro-

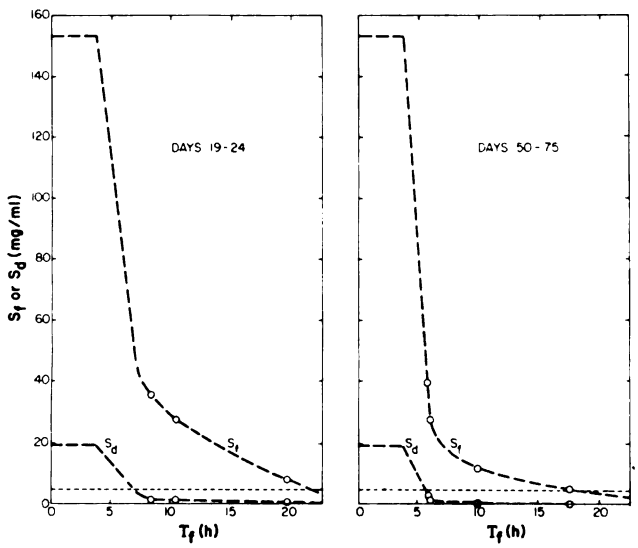


FIG. 2. *Effects of changes in cell retention time (T_f) on residual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) during two periods of dialysis continuous fermentation. T_f was adjusted by changing V_f. F_f and F_d were maintained essentially constant (ϕ = 0.27 for days 19 to 24 and 0.29 for days 50 to 75). The objective was to attain <5 mg of residual lactose per ml, as indicated by the dashed abscissa. Interpretations of the curves were based on computer simulations (see Fig. 2 in reference 3).*

longed nondialysis continuous fermentation of whey, as reported by Keller and Gerhardt (5). In neither study was it determined whether the change resulted from adaptation, mutation, replacement by a contaminant, or symbiosis with a contaminant. Whatever the change, it improved the fermentation.

This culture change can be shown in kinetic terms. Assume that the fermentor product concentration (P_f) is small in relation to the product inhibition constant K_p (i.e., $P_f/K_p = 0$), and recall that the maximal specific growth rate (μ_m) is the reciprocal of T_f at steady-state conditions. Then the rate-of-growth equation used to describe bacterial growth in the mathematical model (equation 11 in reference 3) can be rearranged and a Lineweaver-Burk plot can be constructed, with realization that the saturation constant (K_s) so determined reflects both substrate limitation and product inhibition. The results (Fig. 3) indicated that μ_m increased from 0.18 h^{-1} in the earlier period to 0.27 h^{-1} in the later period. The K_s (20.8 mg/ml), determined from Fig. 3, was the same before and after the adaptation, indicating noncompetitive inhibition kinetics. Although the maximal concentration of the product tolerated by the culture did not change, the culture was able to grow and metabolize at a faster rate after adaptation than before.

Effect of dialysate flow rate on conversion efficiency. Figure 4 shows the results of experimental variation in the flow rate of water through the dialysate circuit (F_d) affecting the residual lactose in the fermentor circuit (S_f) and dialysate circuit (S_d), with the cell retention time held constant. The results confirmed the computer-simulated prediction (see Fig. 3 in reference 3). The desired 5-mg/ml level of S_f was attained when F_d was greater than 800 ml of water per h.

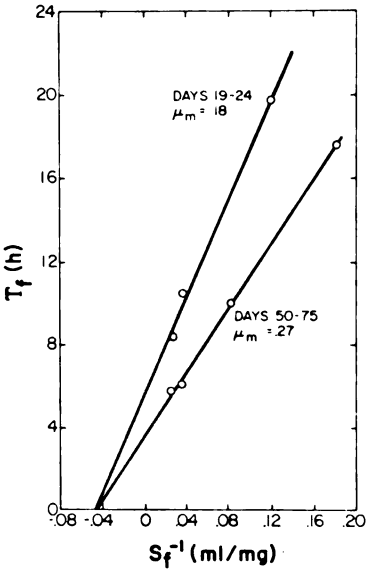


FIG. 3. *Lineweaver-Burk plot of the data in Fig. 2. The maximal specific growth rates of the culture (μ_m) were determined from the slopes of the regressions. The Michaelis-Menten saturation constant was determined from the abscissa intercept common to both regressions (K_s = 20.8 mg/ml).*

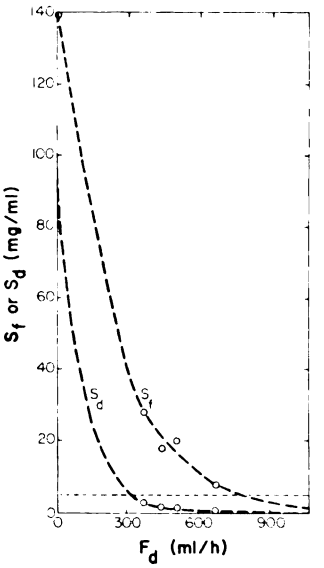


FIG. 4. *Effects of changes in dialysate flow rate (F_d) on residual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) during days 40 to 63, with cell retention time held constant (T_f = 16.4 h). The objective was to attain <5 mg of residual lactose per ml, as indicated by the dashed abscissa. Interpretations of the curves were based on computer simulations (see Fig. 3 in reference 3).*

Figure 5 shows the concentration of accumulated lactate in the fermentor circuit (P_f) as a function of the dialysate flow rate (F_d) and of the flow-rate ratio $\phi(F_f/F_d)$. As F_d increased and ϕ decreased (i.e., as the rate of dialysis increased), P_f first increased to a maximum (F_d = 370 ml/h, ϕ = 0.68) and then decreased because of dilution by water from the dialysate circuit. At these levels of F_d and ϕ, the culture withstood a much greater concentration of lactate (P_f = 116 mg/ml) than that without dialysis (i.e., P_f = 82 mg/ml when F_d = 0 ml/h).

Figure 6 shows the residual lactose concentration (S_f) correlated with the accumulated lactate concentration in the fermentor circuit (P_f), with constant cell retention time. The desired 5-mg/ml level of S_f was attained when P_f was less than 60 mg/ml.

Figure 7 shows the effect of the dialysate flow rate (F_d) on the sum of the lactate and lactose

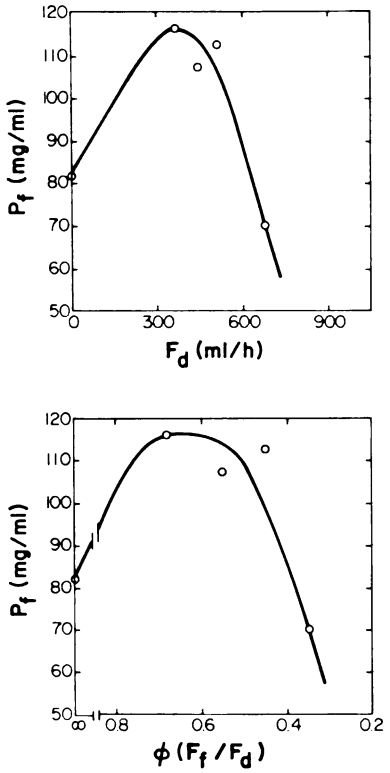


FIG. 5. *Effects of changes in dialysate flow rate (F_d) and flow-rate ratio (ϕ) on accumulation of lactate in the fermentor circuit (P_f), with cell retention time held constant (Υ_f = 16.4 h).*

concentrations in the fermentor circuit ($P_f + S_f$), the points for which correspond to those in Fig. 6. The sum decreased proportionally as F_d increased. Diffusion of the solutes across the membrane accounted for some of the decrease. However, net osmosis of water from the dialysate into the fermentor circuit accounted for most of the decrease, as indicated by an increase in flow rate of the fermentor effluent relative to that of the fermentor feed. As a result of the increased osmosis with increased dialysate flow rate, the concentrations of lactose and lactate both decreased. The former decrease is desirable and the latter is not. Consequently, a trade off in the regulation of F_d is required in designing the system.

Dialyzer dependability. The preceding experimental results were all obtained during operation of the system for 76 days. A week later, operation was resumed for an additional 18 days. Although the dialysate compartments of the dialyzer stayed clean throughout the total 94 days of operation, the fermentor compartments became fouled and needed cleaning after about 4 weeks of operation. However, the same membranes were used throughout.

Product quality. Samples from the fermentor circuit were regularly analyzed by gas chromatography not only to determine the concen-

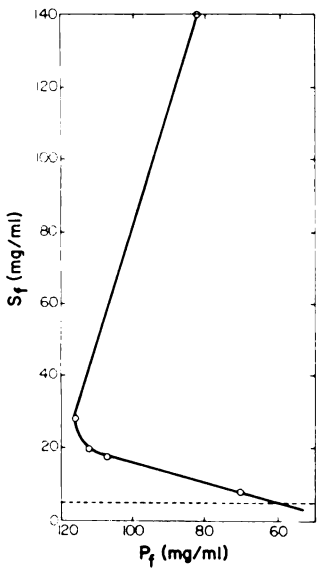


FIG. 6. *Effects of changes in lactate accumulation (P_f) on residual lactose in the fermentor circuit (S_f), with cell retention time held constant (Υ_f = 16.4 h). The objective was to attain <5 mg of residual lactose per ml, as indicated by the dashed abscissa.*

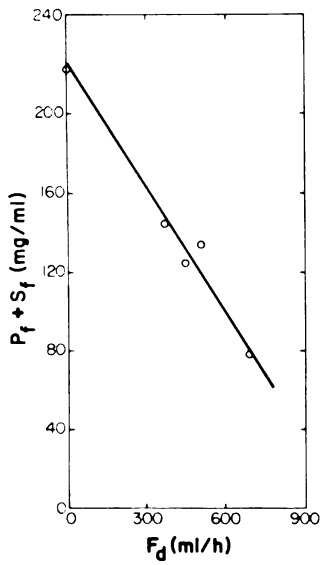


FIG. 7. *Effects of changes in dialysate flow rate (F_d) on the sum of the lactate and lactose concentrations in the fermentor circuit (P_f + S_f), with cell retention time held constant (Υ_f = 16.4 h).*

tration of lactic acid but also to monitor the possible presence of atypical metabolic products. The results indicated that the fermentation remained homofermentative, i.e., only negligible amounts of products other than lactic acid were found.

Operation at nearly optimal conditions. A dialysis continuous fermentation next was operated at nearly optimal conditions ($T_f = 19$ h, $\phi = 0.4$, $S_f < 5$ mg/ml) to demonstrate the efficiency and productivity of the process. Due to inadvertent changes in speeds of the metering pumps controlling flow rates into the fermentor and dialysate circuits, fluctuations occurred in the concentration of residual lactose during the 18 days of operation (days 77 to 94). During this period, a mean of 35% of the fermentor effluent represented water that osmosed from the dialysate circuit, i.e., a mean 77 ml of water per h entered the fermentor from the dialysate circuit (Table 1).

From material-balance data for operating periods when the parametric conditions were nearly optimal (Table 1), the equivalent substrate concentration in the fermentor-feed stream (S_f'') was calculated by using a conservation-of-mass equation. The mean calculated value (223 mg/ml) agreed well with the mean experimental value (230 mg/ml) for S_f''. With allowance for substrate incorporated into bacterial cells, the results confirmed that no significant portion of the substrate was lost to products other than lactic acid.

Validation of the mathematical model. The experimental results were used to validate the mathematical model for the fermentation (3). The values used for the computer simulations are listed in Table 2. The values for ϕ and Π were calculated directly from the experimental results. By successive approximations, the values for μ_m and K_s (determined in Fig. 3) and an exaggerated K_p proved best for correlating the simulations with the experimental data. The high value for K_p eliminates the effect of the product inhibition term in the mathematical model. Consequently, the Michaelis-Menten saturation constant (K_s) reflects not only substrate limitation but also product inhibition, as shown also by Contois (2). The dilution of the fermentor contents caused by the net osmotic transfer of water from the dialysate circuit was taken into account by assuming that the water instead entered the fermentor with the feed stream. Accordingly, since the dilution of the fermentor contents averaged 35%, S_f'' was taken as 150 instead of 230 mg/ml.

Figure 8 shows the computer-simulated curves obtained by using the mathematical model (3) and the values in Table 2 to describe the correlation between one important operating condition and the conversion efficiency, e.g., T_f versus S_f and S_d. The curves fit closely with the superimposed points of experimental results and thereby demonstrated the validity of the mathematical model.

DISCUSSION

The results obtained with the dialysis continuous process at nearly optimal conditions can be compared with the best results obtained with the nondialysis continuous and batch processes for the fermentation (5, 8; Table 3). Relative to the other two processes and with similar conversion efficiency, the present process enabled the use of more than three times the concentration of substrate at a similar retention time and thus was more efficient in the rate of substrate conversion; it was similarly efficient in the percent-

TABLE 2. *Values used for computer simulation of fermentation*

Days	K_p (mg/ml)	K_s (mg/ml)	S_f^o (mg/ml)	μ_m (h^{-1})	Π	ϕ
19–24	1,000	20.8	150	0.18	0.665	0.27
50–76	1,000	20.8	150	0.27	0.615	0.29

TABLE 1. *Material-balance data for operation of the dialysis continuous fermentation system at nearly optimal conditions*

Day	T_f (h)	ϕ	S_f (mg/ml)	S_d (mg/ml)	P_f (mg/ml)	P_d (mg/ml)	F_f^{o*} (ml/h)	F_f^* (ml/h)	F_d (ml/h)	S_f^{o**} (mg/ml)
79	20.9	0.40	2.3	0.4	65	25	110	189	478	217
81	20.4	0.36	2.0	0.3	60	22	124	193	531	183
85	18.5	0.40	2.0	0.5	79	25	131	214	527	226
86	18.8	0.39	2.3	0.4	72	24	135	210	533	203
89.5	16.9	0.38	3.3	0.4	94	24	150	234	613	243
93.5	18.5	0.33	3.5	0.2	110	23	142	214	640	267
Mean	19.0	0.38	2.6	0.4	80	24	132	209	554	223

* F_f^o, Flow rate of feed into the fermentor circuit. $F_f - F_f^o$ represents the net osmotic transfer of water from the dialysate to the fermentor circuit.

** Calculated from the formula: $S_f^o = (F_f S_f + F_f P_f + F_d S_d + F_d P_d)/F_f^o - P_f^o$, where $P_f^o = 9$ mg/ml.

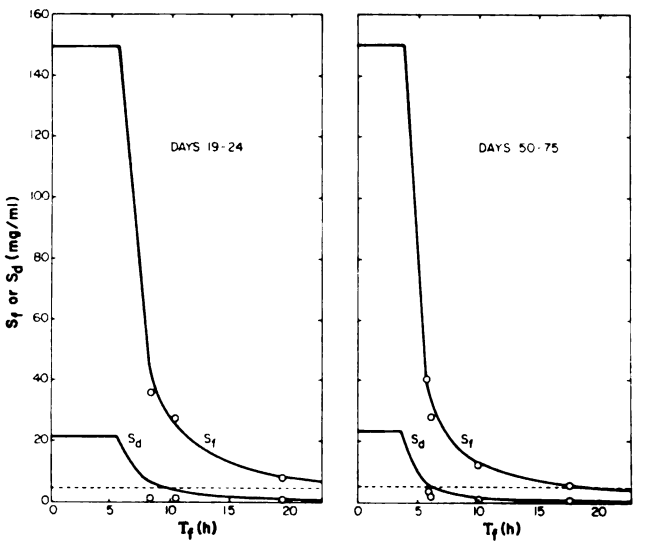


FIG. 8. *Computer-simulated effects of cell retention time (T_f) on residual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) during two periods of dialysis continuous fermentation. The curves were plotted by use of the mathematical model (3) and the values in Table 2. The points were replotted from Fig. 2 to demonstrate the close fit between the experimental results and the computer simulations. These can be compared with the preliminary simulations (see Fig. 2 in reference 3).*

TABLE 3. *Comparison of results obtained at nearly optimal conditions with three processes for the fermentation*

Process	Lactose in whey substrate (mg/ml)	Cell retention time (h)	Rate of lactose conversion (mg/ml-h)	Conversion of lactose into products (%)	Lactose in fermentor product (mg/ml)	Lactate in fermentor product (mg/ml)	Lactose in dialysate product (mg/ml)	Lactate in dialysate product (mg/ml)
Nondialysis								
Batch (reference 8)	70	16[a]	4.3	98	1.0	75		
Two-stage continuous (reference 5)	50	31	1.6	98	1.0	55		
Dialysis								
Continuous	230	19	11.7	97	2.6	80	0.4	24

[a] Required fermentation time for the batch process.

age conversion of substrate into products and in the low concentration of residual substrate and the high concentration of accumulated product in the fermentor; and it additionally yielded a second effluent containing less concentrated but purer ammonium lactate.

A dialysis culture is able to tolerate more lactic acid and thus ferment more lactose than a nondialysis culture (Fig. 5). The metabolic activity of the culture in the ammonium lactate fermentation apparently is inhibited mainly by the concentration of undissociated lactic acid (see discussion in reference 5), which in turn is dependent on the pH, temperature, and concentration of salts of the acid. Since the dialysis membrane is more permeable to the ammonium than the lactate ions (results not shown), dialysis may shift the equilibrium from the undissociated to the dissociated acid by removing the cations to a greater extent than the anions from the fermenting culture. This may explain why the culture tolerated more acid under dialysis than nondialysis conditions.

The substrate for the dialysis continuous fermentation was greatly concentrated. The cost of concentrating may be offset by lesser transportation costs, stabilization of the whey against spoilage, smaller holding capacity needed for operation of the process, and the eventual need to concentrate the products for use as feedstuff.

The use of further concentrated substrate fed into the system might be accomplished by use of a two-stage fermentation, for the same reasons that this proved more effective in the nondialysis

continuous process (5). The present system would need to be modified only by adding a continuous prefermentor, without dialysis. The conversion in this prefermentor would be relatively less affected by the greater concentration of substrate. However, since contamination is controlled mainly by a high concentration of undissociated lactic acid, a lower pH would need to be maintained in the prefermentor than in the dialyzed fermentor. Such a two-stage continuous process also might be effective if it were desirable to accomplish more complete conversion of lactose to lactic acid.

The cell retention time (Υ_f) required to ferment the whey in the dialysis continuous process might be reduced by decreasing the value for the flow-rate ratio (ϕ). However, this manipulation would cause the product solids in the effluents to become more dilute. By increasing ϕ and the permeability factor (II), the dialysate product concentration could be increased considerably (perhaps doubled) without decreasing the rate of the fermentation, as predicted by computer simulation (results not shown). However, this improvement was not validated experimentally.

The dialysis continuous fermentation yielded two product streams. The fermentor effluent containing ammonium lactate, bacterial cells, and whey proteins may be used as ruminant feedstuff. The dialysate effluent containing ammonium lactate and little else may be used as feedstuff or may be converted into industrial lactic acid and ammonium sulfate, for which it is important that the lactate is relatively pure and highly concentrated. A reasonable goal for both effluents would be concentrations of at least 50 mg of lactate per ml, which is similar to that produced by the nondialysis batch and continuous processes. A low residual lactose concentration (<5 mg/ml) is also necessary because lactose may crystallize when storing the product and form a troublesome sludge. These product goals appear readily attainable.

A side effect of the dialysis continuous process was the large osmotic influx of water from the dialysate into the fermentor, diluting its contents. A more permeable membrane might lessen the problem by allowing greater diffusion of solute and thus reducing the differences in solute concentration on each side of the membrane. Greater membrane area also might help if turbulence were maintained at the membrane surfaces. Calculations of the osmotic pressure due to solutes indicated that the net pressure resulting from the accumulated lactate is greater by a factor of at least 50 than that resulting from the residual lactose. To alleviate the problem, membranes with greater permeability to lactate might be selected or a greater atmospheric pressure might be maintained on the fermentor circuit than on the dialysate circuit.

A dialysis membrane is permeable to small molecules and ions, but not to cells and to macromolecules such as proteins. The difference in osmotic pressure is much greater in this situation, especially at low macromolecular concentrations, than that predicted by the Van't Hoff equation (7). The reason for this "Donnan effect" is that ions are at a lesser concentration on the macromolecular side of the membrane, thus causing an increase in ions of opposite charge. Removal of proteins from the whey before fermentation might eliminate most of the Donnan effect, thus also serving as a way to alleviate the problem of osmotic influx of water diluting the fermentor contents. This appears to be a practical way to improve the process, because increasingly in industry the valuable proteins are being recovered from waste whey by ultrafiltration. The resulting ultrafiltrate remains as a troublesome lactose-rich waste product, but it should be ideal as a substrate for dialysis continuous fermentation into ammonium lactate.

ACKNOWLEDGMENTS

This work was supported by a grant from the Office of Research Development of Michigan State Unviersity and by grant ENG 76-17260 from the National Science Foundation.

LITERATURE CITED

1. **Carlsson, J.** 1973. Simplified gas chromatographic procedure for identification of bacterial metabolic products. Appl. Microbiol. **25**:287–289.
2. **Contois, D. E.** 1959. Kinetics of bacterial growth: relationship between population density and specific growth rate of continuous cultures. J. Gen. Microbiol. **21**:40–50.
3. **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.
4. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
5. **Keller, A. K., and P. Gerhardt.** 1975. Continuous lactic acid fermentation of whey to produce a ruminant feed supplement high in crude protein. Biotechnol. Bioeng. **17**:997–1018.
6. **Montgomery, R.** 1961. Further studies of the phenol-sulfuric acid reagent for carbohydrates. Biochim. Biophys. Acta **48**:591–593.
7. **Moore, W. J.** 1962. Physical chemistry, 3rd ed., p. 759–761. Prentice Hall, Englewood Cliffs, N.J.
8. **Reddy, C. A., H. E. Henderson, and M. D. Erdman.** 1976. Bacterial fermentation of cheese whey for production of a ruminant feed supplement rich in crude protein. Appl. Environ. Microbiol. **32**:769–776.
9. **Schultz, J. S., and P. Gerhardt.** 1969. Dialysis culture of microorganisms: design, theory, and results. Bacteriol. Rev. **33**:1–47.
10. **Simon, E. W., and H. Beevers.** 1952. The effect of pH on the biological activity of weak acids and bases. New Phytol. **51**:163–189.

ARTICLE III

DIALYSIS CONTINUOUS PROCESS FOR AMMONIUM-LACTATE FERMENTATION: IMPROVED MATHEMATICAL MODEL AND USE OF DEPROTEINIZED WHEY

By

R. W. Stieber and Philipp Gerhardt

Reprinted from Appl. Environ. Microbiol. 37:487-495, 1979.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1979, p. 487-495 Vol. 37, No. 3
0099-2240/79/03-0487/09$02.00/0

Dialysis Continuous Process for Ammonium-Lactate Fermentation: Improved Mathematical Model and Use of Deproteinized Whey†

R. W. STIEBER AND PHILIPP GERHARDT*
Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

Received for publication 5 December 1978

Separate terms for substrate limitation and product inhibition were incorporated into an equation describing the rate of cell growth for the steady-state fermentation of lactose to lactic acid with neutralization to a constant pH by ammonia. The equation was incorporated into a generalized mathematical model of a dialysis continuous process for the fermentation, developed previously, in which the substrate is fed into the fermentor and the fermentor contents are dialyzed through a membrane against water. The improved model was used to simulate the fermentation on a digital computer, 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 the substrate. Dried cheese-whey ultrafiltrate was rehydrated with tap water to contain 242 mg of lactose per ml, supplemented with 8 mg of yeast extract per ml, charged into a 5-liter fermentor without sterilization, adjusted in pH (5.5) and temperature (44°C), and inoculated with an adapted culture of *Lactobacillus bulgaricus*. The fermentor and dialysate circuits were connected, and a series of steady-state conditions was managed nonaseptically for 71 days. The fermentation of deproteinized whey relative to whole whey, with both highly concentrated, resulted in similar extents of product accumulation but at a lesser rate.

Whey utilization continues to be a problem for the dairy industry. Whey can be processed by pressure filtration through semipermeable membranes to obtain protein concentrates (3). Forty to 70% of the original protein is recovered (10, 15). The protein has many commercial uses because of its high nutritional quality (20, 23, 24). However, the capital costs of production are high and large volumes of lactose-containing ultrafiltrate are left as residue, which is nearly as much an environmental burden and an economic and nutrient loss as the whole whey. Thus, feasibility of the process is dependent upon use of the deproteinized but lactose-rich ultrafiltrate.

A potential solution to this problem lies with the conversion of whey ultrafiltrate into feedstuff for ruminant animals, accomplished by the bacterial fermentation of the lactose into lactic acid and its neutralization to constant pH by ammonia (7). A background for this development exists in studies of the fermentation using whole whey as the substrate. The fermentation can be managed as a batch process (18), continuous process (11), or dialysis continuous process (4, 22). The latter process relative to the nondialysis processes enables the use of more concentrated substrate, is more efficient in the rate of substrate conversion, and additionally produces a dialysate effluent of less concentrated but purer ammonium lactate.

In the studies reported here, a rate expression for bacterial growth was developed containing separate terms for substrate limitation and product inhibition. The expression was incorporated into a mathematical model of the dialysis continuous process generalized with dimensionless parameters so that it could be widely applied (4). The resulting improved set of equations was employed to simulate the fermentation on a digital computer, and the results were verified using previous experimental results with whole whey as the substrate (22). Further simulations were then made to guide experimental tests in which deproteinized whey was used as the substrate. The simulated and experimental results were used to compare the relative value of the process applied to the two substrates.

MATHEMATICAL MODEL

Growth rate theory. An expression describing the rate of cell growth, previously used in a mathematical

† Journal article no. 8608 from the Michigan Agricultural Experiment Station.

model of the dialysis continuous ammonium-lactate fermentation (see equation 11 in reference 4) is as follows:

$$r_g = \mu_m\left(\frac{S_f}{K_s + S_f}\right)\left(\frac{1}{1 + P_f/K_p}\right)X_f \quad (1)$$

The symbols in this and subsequent equations are described in Table 1.

In the studies of Stieber et al. (22), an exaggerated K_p value (1,000 mg/ml) was used to correlate the simulation with the experimental results. The high value for K_p eliminates the effect of the product inhibition term, and equation 1 is reduced to the following equation:

$$r_g = \mu_m\left(\frac{S_f}{K_s + S_f}\right)X_f \quad (2)$$

This equation is based on classical Michaelis-Menten kinetics and adequately describes the rate of cell growth for the fermentation. However, the separate effects of substrate limitation and product inhibition cannot be determined. Moreover, equation 2 is based on an enzyme-substrate-product relationship which implies that there is no product inhibition (21).

These restrictions were considered, and the following competitive relationship was proposed:

$$X_f + S_f \xrightarrow{k_1} X_fS_f \xrightarrow{k_2} X_fP_f \underset{k_4}{\overset{k_3}{\rightleftharpoons}} X_f + P_f \quad (3)$$

where k_1, k_2, k_3, and k_4 are rate constants. Once the cells (X_f) obtain substrate (S_f), it is metabolized to product (P_f). P_f cannot be converted to S_f. Further, since $k_4 \neq 0$, the cells are inhibited by P_f (i.e., they cease obtaining substrate). In equation 3, assuming there is an adequate concentration of cells and substrate, the rates of substrate utilization and product formation depend mostly on the concentration of product in the environment of the cells.

An expression describing the rate of cell growth was obtained from equation 3 by a steady-state approach (21):

$$r_g = \mu_m\left(\frac{S_f}{K_s + S_f + K_pP_f}\right)X_f \quad (4)$$

where $\mu_m = k_2k_3/(k_2 + k_3)$, $K_s = k_2k_3/[k_1(k_2 + k_3)]$, and $K_p = k_2k_4/[k_1(k_2 + k_3)]$. The substrate-limitation con-

TABLE 1. *Glossary of mathematical symbols*

Symbol	Description	Units
A_m	Area of membrane available for dialysis	cm^2
F_d	Flow rate into and out of dialysate circuit	ml/h
F_f°	Flow rate into fermentor circuit	ml/h
F_f	Flow rate out of fermentor circuit	ml/h
K_p	Product-inhibition constant	mg/mg
K_s	Substrate-limitation constant	mg/ml
P_d	Product concentration in dialysate circuit	mg/ml
P_f°	Product concentration in fermentor feed	mg/ml
P_{mp}	Permeability of membrane to product	mg/cm^2-h
P_{ms}	Permeability of membrane to substrate	mg/cm^2-h
r_g	Rate of cell growth	mg/ml-h
r_p	Rate of product formation	mg/ml-h
$-r_s$	Rate of substrate utilization	mg/ml-h
S_d	Substrate concentration in dialysate circuit	mg/ml
S_f°	Substrate concentration in fermentor feed	mg/ml
S_f	Substrate concentration in fermentor circuit	mg/ml
t	Time	h
V_d	Volume of liquid in dialysate circuit	ml
V_f	Volume of liquid in fermentor circuit	ml
X_f	Cell-mass concentration in fermentor circuit	mg/ml
α	Substrate/cell ratio	mg/mg
β	Specific maintenance rate	h^{-1}
γ	Product/substrate ratio	mg/mg
E	Efficiency of lactose conversion	%
μ_m	Maximum specific growth rate of cells	h^{-1}
T_f	Cell-retention time in fermentor circuit	h

TABLE 2. *Glossary of dimensionless parameters*

Type	Symbol and definition	Description
Material parameters	$\bar{P}_d = P_d/(\gamma S_f^\circ)$	Product factor in dialysate circuit
	$\bar{P}_f = P_f/(\gamma S_f^\circ)$	Product factor in fermentor circuit
	$\bar{P}_f^\circ = P_f^\circ/(\gamma S_f^\circ)$	Product factor in fermentor feed
	$\bar{S}_d = S_d/S_f^\circ$	Substrate factor in dialysate circuit
	$\bar{S}_f = S_f/S_f^\circ$	Substrate factor in fermentor circuit
	$\bar{X}_f = \alpha X_f/S_f^\circ$	Cell factor in fermentor circuit
Operational parameters	$R = P_{mp}/P_{ms}$	Ratio of product/substrate membrane permeabilities
	$\Pi = P_{ms}A_m/F_f$	Membrane permeability factor
	$\phi = F_d/F_f$	Flow-rate ratio
Kinetic parameters	$\bar{K}_s = K_s/S_f^\circ$	Substrate-limitation factor
	$\bar{K}_p = \gamma K_p$	Product-inhibition factor
	$\theta = \mu_m T_f$	Time factor

stant (K_s) and the product-inhibition constant (K_p) express the relationships between the actual steady-state concentrations of the various cell, substrate, and product states.

Generalized model. The design of the fermentation system, the assumptions for purposes of modeling, the material balance equations, and the rate equations for substrate utilization and product formation were the same as those used previously (4). The rate equations and equation 4 were combined with the material balance equations, and the variables were defined in dimensionless parameters (Table 2) to obtain a generalized model for dialysis continuous fermentation.

The resulting equations for the fermentor circuit are as follows:

$$\frac{d\bar{S}_f}{dt} = \left[-(1 + \Pi)\bar{S}_f - \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) + \frac{\beta T_f}{\alpha}\right]\bar{X}_f + \Pi\bar{S}_d + 1\right]/\tau_f \quad (5)$$

$$\frac{d\bar{P}_f}{dt} = \left[-(1 + R\Pi)\bar{P}_f + \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) + \frac{\beta T_f}{\alpha}\right]\bar{X}_f + R\Pi\bar{P}_d + \bar{P}_f^o\right]/\tau_f \quad (6)$$

$$\frac{d\bar{X}_f}{dt} = \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) - 1\right]\bar{X}_f/\tau_f \quad (7)$$

where $T_f = V_f/F_f$ and is an operational parameter.

For the dialysate circuit, the corresponding equations are:

$$\frac{d\bar{S}_d}{dt} = \left[-(\Pi + \phi)\bar{S}_d + \Pi\bar{S}_f\right]F_f/V_d \quad (8)$$

$$\frac{d\bar{P}_d}{dt} = \left[-(R\Pi + \phi)\bar{P}_d + R\Pi\bar{P}_f\right]F_f/V_d \quad (9)$$

This generalized model improves upon that developed previously (see equations 12 to 16 in reference 4).

Generalized steady-state solution. The equations of the generalized model were rearranged and combined and the time derivatives were set at zero to obtain a generalized solution for the steady state, comparable to that developed previously (see equations 17 to 21 in reference 4).

Equations for the substrate, product, and cell mass in the fermentor circuit are as follows:

$$\bar{S}_f = \frac{\bar{K}_s\pi_p + \bar{K}_p + \bar{K}_p\bar{P}_f^o}{\pi_p(\theta - 1) - \pi_s\bar{K}_p} \quad (10)$$

$$\bar{P}_f = \frac{\bar{K}_s\pi_s + \theta - 1 + \bar{P}_f^o(\theta - 1)}{\pi_p(\theta - 1) - \pi_s\bar{K}_p} \quad (11)$$

$$\bar{X}_f = \frac{1 - \bar{S}_f + \Pi(\bar{S}_d - \bar{S}_f)}{1 + \beta T_f/\alpha} \quad (12)$$

where $\pi_s = \Pi[\Pi/(\phi + \Pi) - 1] - 1$, and $\pi_p = 1 + R\Pi[1 - R\Pi/(\phi + R\Pi)]$.

For the dialysate circuit, the corresponding equations are:

$$\bar{S}_d = \Pi\bar{S}_f/(\phi + \Pi) \quad (13)$$

$$\bar{P}_d = R\Pi\bar{P}_f/(\phi + R\Pi) \quad (14)$$

Equations 10 to 14 thus comprise a generalized steady-state solution for substrate, product, and cells in the fermentor and dialysate circuits of the system.

COMPUTER SIMULATIONS

Previous experimental results with whole whey (22) were used in digital-computer simulations to validate the improved mathematical model. The values used are listed in Table 3. The values for ϕ and Π were calculated directly from the experimental results. The values for μ_m, K_s, and K_p were determined by successive curve fitting of the simulated and experimental results.

A side effect of the dialysis process was a large osmotic influx of water from the dialysate into the fermentor, diluting its contents. The dilution was accounted for by assuming that the diluting water entered the fermentor with the feed stream rather than from the dialysate and by correcting S_f^0 accordingly.

Figure 1 shows the computer-simulated curves obtained by using the steady-state solution with the values in Table 3 to describe the correlation between one important operating parameter and the conversion efficiency, e.g., T_f versus S_f and S_d. The curves all fit closely with the superimposed points of the previous experimental results with whole whey (22).

TABLE 3. *Values used for computer simulations of previous fermentations with whole whey (22)*

Figure (days)	μ_m (h^{-1})	$\bar{K}_p$	$\bar{K}_s$	T_f (h)	ϕ	Π	R
1 (19–24)	0.145	0.6	0.0004		3.7	0.66	3.0
1 (50–75)	0.25	0.6	0.0004		3.4	0.62	3.0
2 (40–63)	0.25	0.6	0.0004	16		0.59	3.0

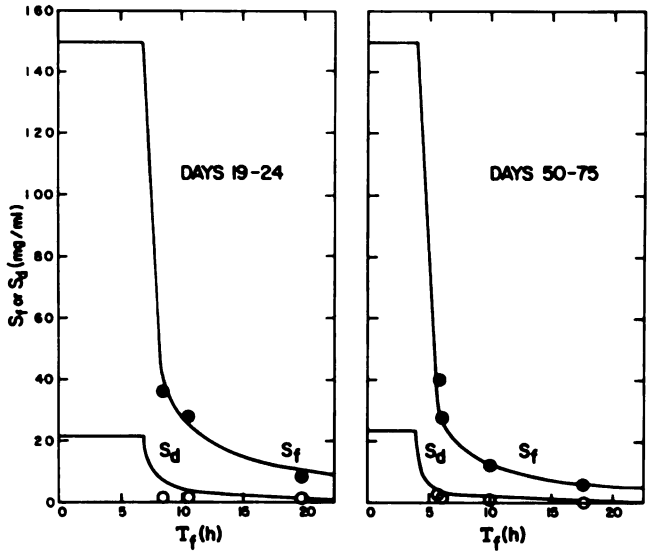


FIG. 1. *Computer-simulated effects of cell-retention time (T_f) on residual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) during two periods of dialysis continuous fermentation. The curves were plotted by use of the steady-state solution with the values in Table 3. The points were replotted from Fig. 2 in reference 22 to demonstrate the close fit between the experimental results and the computer simulations.*

A similar comparison between the simulated and the experimental results was also made for a second operating parameter, e.g., F_d versus S_f and S_d (Fig. 2). Although not fitting as closely to the experimental points, the simulation curves described the trends well. The discrepancy mostly was caused by decreased permeability of the membranes.

Altogether, the results demonstrated the validity of the mathematical model, which then was used to guide experimental tests for the ammonium-lactate fermentation of deproteinized whey. The improved model predicted the same effects of changes in the various parameters on the process and the same regions for experimental tests as did the previous model (4).

MATERIALS AND METHODS

Inoculum. The inoculum culture was obtained from the fermentor effluent (day 94) of a previous dialysis continuous fermentation (22) originally started with *Lactobacillus bulgaricus* 2217 (Chris Hanson's Laboratory, Milwaukee, Wis.).

Substrate. Dried deproteinized cheese whey (prepared by ultrafiltration of whole sweet-cheese wheys by Stauffer Chemical Co., Rochester, Minn.) was rehydrated to contain 242 mg of lactose per ml and was supplemented with 8 mg of yeast extract per ml. The reconstituted whey was made up in 7-liter batches without sterilization, stored at 4°C, and held in a stirred, heated (60°C) reservoir to keep the lactose in solution.

Dialysis continuous fermentation system. The experimental dialysis fermentation system was conducted continuously at a temperature of 44°C and a pH of 5.5 with essentially the same equipment as used previously (22). However, plunger-type reciprocating pumps (type " P", Bran and Lubbe, Inc., Evanston, Ill.) were used to meter the whey and water, and the pH was regulated with a different automatic device (model pH-40, New Brunswick Scientific Co., New Brunswick, N. J.). The circulation rates through the dialyzer, for both the fermentor and dialysate circuits, were 2 liters per min. Operation of the system was interrupted once, for 3 weeks, during which the fermentor-circuit contents were stored at 4°C.

Analytical procedures. Samples from the dialysate and fermentor circuits were taken at 12-h intervals from the dialysate effluent and from a glass "T" inserted in the tubing between the fermentor and dialyzer. Steady-state data were determined from samples taken at five times the cell retention time or 48 h after changing a parameter. Lactose in the samples was determined by the colorimetric method of Morris (14). Lactic acid was determined by use of a gas chromatograph (series 1420, Varian Associates, Palo Alto, Calif.) with an integrator (model CDS 111, Varian Associates), using a stainless-steel column (6 feet by ⅛ inch [ca. 1.83 m by 0.32 cm] outside diameter) packed with 10% SP-1000/1% H_3PO_4 on 100/120 Chromosorb WAW (Supelco, Inc., Bellefonte, Pa.). Samples were prepared by the procedure of Holdeman and Moore (9). Lactic acid also was determined by the colorimetric method of Pryce (17). Specific conductance was measured with a conductivity bridge (model RC-16B2, Beckman Instruments, Inc., Cedar Grove, N. J.).

RESULTS

Cell-retention time versus conversion efficiency. The results of experimental variation in the cell-retention time (T_f) affecting the resid-

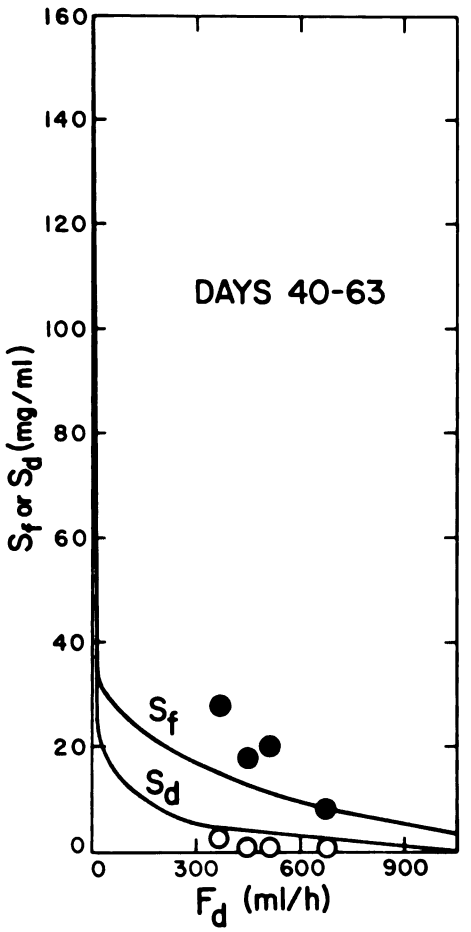


FIG. 2. *Computer-simulated effects of dialysate flow rate (F_d) on residual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) with cell-retention time held constant (T_f = 16 h). The curves were plotted by use of the steady-state solution and the values in Table 3. The points were replotted from Fig. 4 in reference 22 to demonstrate the fit between the experimental results and the computer simulations.*

ual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) showed that the lactose concentrations in both circuits decreased with increased T_f (Fig. 3). All of the curves reached a point where an increase in T_f did little to decrease the lactose concentration.

Dialysis rate versus conversion efficiency. The flow-rate ratio (ϕ) is an operational parameter used to manipulate the rate of dialysis. Results of experimental variation in ϕ affecting the residual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) are also shown in Fig. 3. The lactose concentrations were less with increased dialysis (ϕ = 2.5) than with decreased dialysis (ϕ = 1.0). The effect was mostly on S_d.

Cell-retention time versus lactate concentration. Figure 4 shows the results of variation in T_f affecting the lactate concentrations in the fermentor circuit (P_f) and in the dialysate circuit (P_d). The lactate concentrations increased to a maximum with increasing T_f.

Dialysis rate versus lactate concentration. Figure 4 also shows the results of the flow-rate ratio affecting P_f and P_d. ϕ had little effect on P_f. However, P_d was much greater with decreased dialysis (ϕ = 1.0) than with increased dialysis (ϕ = 2.5). Values for the dialysate product yield ($\bar{P}_d\phi$, which represents the fraction of substrate leaving as lactate in the dialysate effluent) were similar at either value of ϕ.

Lactose conversion and lactate productivity. Figure 5 shows that the lactate productivity decreased with increased T_f, whereas the percentage of lactose converted to product increased with increased T_f. High lactate productivity and high lactose conversion along with high lactate concentrations are all desirable.

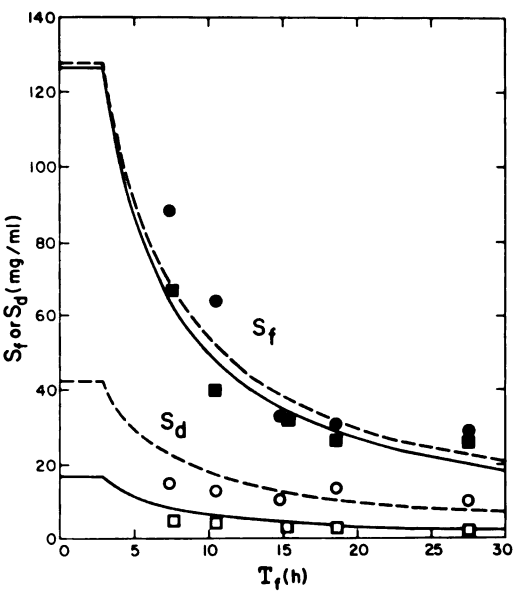


FIG. 3. *Computer-simulated effects of cell-retention time (T_f) on residual lactose in the fermentor circuit (S_f) and the dialysate circuit (S_d) at two flow-rate ratios (ϕ) and during two time periods of dialysis continuous fermentation. The curves were plotted by use of the steady-state solution and the values in Table 5. The points are experimental data and demonstrate the fit between the experimental results and the computer simulations. The dashed curves and circle points were obtained at ϕ = 1.0 and during days 26 to 36. The smooth curves and square points were obtained at ϕ = 2.5 and during days 40 to 49.*

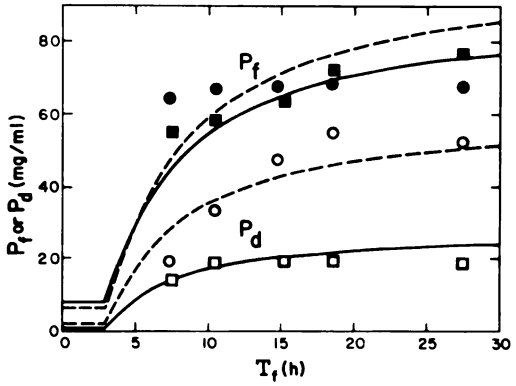


FIG. 4. *Computer-simulated effects of cell-retention time (T_f) on accumulated lactate in the fermentor circuit (P_f) and the dialysate circuit (P_d) at two flow-rate ratios (ϕ) and during two time periods of dialysis continuous fermentation. The curves were plotted by use of the steady-state solution and the values in Table 5. The points are experimental data and demonstrate the fit between the experimental results and the computer simulations. The dashed curves and circle points were obtained at ϕ = 1.0 and during days 26 to 36. The smooth curves and square points were obtained at ϕ = 2.5 and during days 40 to 49.*

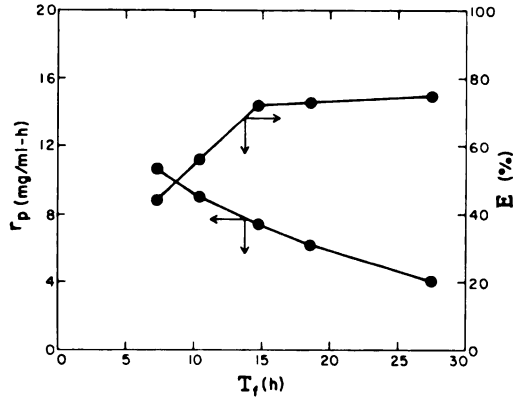


FIG. 5. *Experimental effect of changes in cell-retention time (T_f) on lactose conversion (E) and lactate productivity (r_p) at a flow-rate ratio (ϕ) of 1.0. Percent lactose conversion = $[1 - (F_f S_f + F_d S_d)/(F_f^0 S_f^0)] \times 100$. Lactate productivity = $(P_f F_f + P_d F_d - P_f^0 F_f^0)/(T_f F_f)$.*

Consequently, trade-offs in the regulation of T_f are required in designing the system for practical use.

Product/substrate ratio. Results of experimental variation in T_f and ϕ showed that these operational parameters had little effect on the product/substrate ratio (γ, results not shown). Consequently, for purposes of modeling the ammonium-lactate fermentation, a fixed value was assigned to γ (0.96). The ratio was calculated from r_p/r_s, where values for r_p were obtained from Fig. 5 and where $r_s = (S_f^0 F_f^0 - S_f F_f - S_d F_d)/(T_f F_f)$.

Dialyzer dependability. The preceding experimental results all were obtained during uninterrupted operation of the system for 56 days. After a hiatus of three weeks, operation was resumed for an additional 15 days. The fermentor and dialysate compartments of the dialyzer stayed clean throughout the total 71 days of operation. However, the membrane surfaces in the fermentor compartments were fouled with a film of debris after the 56 days, and fresh membranes were used for the final 15 days.

Product quality. Samples from the fermentor circuit were regularly analyzed by gas chromatography, not only to determine the concentration of lactic acid but also to monitor the possible presence of atypical metabolic products. The results indicated that the fermentation remained homofermentative, i.e., only negligible amounts of products other than lactic acid were found.

Prolonged steady-state operation. The dialysis continuous fermentation was operated at set steady-state conditions (T_f = 27.2, ϕ = 2.5) during the 15-day period to determine other characteristics of the process (Table 4). The mean rate of ammonium-lactate production was 4.7 mg/ml-h and the lactose conversion was 81%.

By day 58 of the fermentation, the residual lactose levels were very low, but then the levels increased with time. The lactose increase was possibly caused by a buildup of inhibitory substance in the fermentor circuit resulting from membrane fouling, as seen by the greater differences between S_f and S_d and between P_f and P_d as time progressed.

During the fermentation, a mean of 36.5% of the fermentor effluent represented water which osmosed from the dialysate circuit, i.e., a mean of 56.4 ml of water per h ($F_f - F_f^0$ in Table 4) entered the fermentor from the dialysate circuit.

From material-balance data (Table 4), the equivalent substrate concentration in the fermentor-feed stream (S_f^0) was calculated by use of a conservation-of-mass equation. The mean calculated value (247 mg/ml) agreed well with the mean analytical value (242 mg/ml) for S_f^0. Thus, the results confirmed that no significant portion of the substrate was lost to products other than lactic acid.

Validation of the mathematical model. The values shown in Table 5 were used to correlate the experimental and simulated results. The values for ϕ and γ were calculated directly from the experimental data. The values for μ_m, K_s, and K_p were obtained by successive curve fitting of simulated with experimental results of

TABLE 4. *Material-balance data for operation of the dialysis continuous fermentation system at steady-state conditions*

Day	T_f (h)	ϕ	S_f (mg/ml)	S_d (mg/ml)	P_f (mg/ml)	P_d (mg/ml)	F_f^o (ml/h)	F_f (ml/h)	F_d (ml/h)	S_f^o (mg/ml)[a]
	25.5	2.29	77.0	16.3	22.5	9.4	104.4	164.4	376.8	245.1
57	24.2	2.26	33.0	8.8	55.0	23.6	88.4	172.8	390.0	314.5
	25.7	2.36	12.6	2.6	61.5	31.2	85.2	163.2	385.8	293.7
58	28.2	2.69	6.7	0.5	65.0	29.6	84.7	148.8	400.2	265.6
	27.4	2.62	7.5	0.4	68.0	28.0	94.2	153.0	400.8	239.8
59	26.4	2.46	10.0	1.1	61.0	29.4	93.2	158.4	389.4	245.1
	27.4	2.60	15.0	1.2	67.0	25.2	97.0	153.0	398.4	233.4
60	27.9	2.66	15.8	1.4	65.0	27.0	101.3	150.0	399.0	227.9
	27.5	2.62	17.5	2.1	62.5	26.2	96.5	152.4	399.6	238.8
61	27.0	2.53	17.5	2.7	73.0	25.6	101.4	155.4	393.0	243.8
	27.0	2.53	21.7	2.8	68.0	22.0	102.9	155.4	393.6	224.3
62	26.9	2.53	25.9	2.6	65.0	22.0	100.3	156.0	394.2	233.0
	26.3	2.46	22.2	2.8	66.5	25.0	102.2	159.0	391.8	240.1
63	27.1	2.55	21.2	2.5	67.0	24.6	94.7	154.8	395.4	253.2
	27.7	2.61	18.4	2.2	67.5	22.8	99.4	151.2	394.8	224.9
64	27.6	2.61	19.7	2.2	69.0	22.6	97.1	151.8	396.0	235.2
	27.6	2.58	21.3	2.1	74.5	24.6	100.0	151.8	391.2	244.4
65	27.2	2.53	22.9	2.1	69.0	26.6	98.0	154.2	389.4	256.5
	27.4	2.53	23.6	2.0	80.0	23.6	100.0	153.0	387.6	253.1
66	27.7	2.58	22.9	2.0	74.0	23.4	95.0	151.2	389.4	254.5
	27.8	2.59	24.9	1.9	80.0	22.0	97.2	150.6	390.0	254.4
67	28.2	2.61	24.3	1.8	83.5	19.0	96.9	148.8	388.8	244.5
	27.9	2.60	24.2	1.6	75.5	21.2	104.0	150.0	390.6	225.1
68	27.8	2.58	23.1	1.5	75.5	20.2	99.5	150.6	388.2	229.8
	27.6	2.53	27.7	2.1	69.5	25.4	↓	151.8	384.6	251.0
69	27.5	2.54	26.6	2.0	72.5	22.6	99.4	152.4	386.4	243.8
	27.2	2.49	27.5	2.2	79.0	21.0	↓	154.2	384.6	251.4
70	27.0	2.47	27.5	2.3	77.5	23.6	107.6	155.4	384.6	240.1
	27.5	2.53	27.7	1.8	83.5	21.4	98.4	152.4	385.8	259.6
71	27.3	2.52	28.6	2.2	77.5	23.0	99.0	153.6	387.6	259.9
Mean	27.2	2.54	23.2	2.7	69.2	23.7	97.9	154.3	390.9	247.0

[a] $S_f^o = (F_fS_f + F_fP_f + F_fX_f + F_dS_d + F_dP_d)/F_f^o - P_f^o$, where $P_f^o = 9.1$ mg/ml.

TABLE 5. *Values used for computer simulations of experimental fermentations with deproteinized whey*

Figure (days)	μ_m (h^{-1})	$\bar{K}_p$	$\bar{K}_s$	ϕ	Π	R	γ
3 (26–36)	0.35	2.2	0.0004	1.0	0.50	3.0	0.96
3 (40–49)	0.35	2.2	0.0004	2.5	0.40	3.0	0.96
4 (26–36)	0.35	2.2	0.0004	1.0	0.50	3.0	0.96
4 (40–49)	0.35	2.2	0.0004	2.5	0.40	3.0	0.96

a nondialysis continuous fermentation (unpublished results). The value for Π was obtained similarly with the experimental results of the dialysis continuous fermentation. S_f^o was taken as 160.3 mg/ml instead of 237.5 mg/ml (which was obtained from material balances of the experimental data) because a mean 32.5% of the fermentor contents represented water which osmosed from the dialysate circuit.

Figure 3 shows the computer-simulated curves obtained by use of the steady-state solution with the values in Table 5 to describe the relation between the two principle operating parameters (T_f and ϕ) and the conversion efficiency (S_f and S_d). Figure 4 shows the relation between the same operating parameters and the accumulation of product (P_f and P_d). The curves in both figures fitted well with the superimposed points of experimental results and thereby further demonstrated the validity of the mathematical model. Moreover, the kinetic constants (μ_m, K_s, and K_p) used for the simulations in Fig. 3 and 4 were obtained from the results of nondialysis continuous fermentations, adding to the validation of the model.

Process evaluation by use of the mathematical model. The experimental and simulated results were correlated to evaluate the fermentation process. The values for μ_m (0.35 h^{-1}), K_s (0.0004), and K_p (2.2) were the same in both time periods, indicating no change in the bacterial culture from days 26 to 49. The values

for $\bar{K}_s$ and $\bar{K}_p$ also showed that the fermentation was not affected by substrate limitation but was greatly limited by increasing concentrations of product. The values for Π (0.5 at days 26 to 36; 0.4 at days 40 to 49) showed that the permeability of the membrane decreased as the fermentation progressed. Calculations of Π using a P_{ms} of 0.06 mg/cm^2-h showed that Π should have been about 0.9. Thus, by 4 weeks into the fermentation, the permeability factor decreased by 50%. Coulman et al. (4) showed that a Π of 2.0 should be used for the process to obtain suitable relief from product inhibition.

Process monitoring. On-line sensors are becoming increasingly important in the operation of fermentations (16). In the present process, the dialysate effluent is a relatively pure solution of ammonium lactate and should be measurable by its electrical conductivity. Figure 6 illustrates the positive correlation between the concentration of ammonium lactate and the conductance in the dialysate. Thus, a conductivity bridge could be readily used for the on-line monitoring of product concentration. Moreover, if coupled with the model, this parameter could indicate other parameters of the fermentation, e.g., cell concentration in the fermentor and membrane permeability in the dialyzer.

DISCUSSION

Various models have been used to describe the growth of lactic acid bacteria (4–6, 8, 11–13, 19). Although these models correlate with experimental results, they have only been used for fermentations of very low substrate and product concentrations, they contain equations or terms which have no biological basis, or they lack a strong product-inhibition effect. Recently, Aborhey and Williamson (1) developed a model which appeared useful, but the model was complicated for our purposes because it incorporated the concept of intracellular substrate concentration. Since acid production cannot continue once all of the substrate has been utilized, Rogers et al. (19) suggested improving Luedeking and Piret's (13) rate equation for acid production by incorporating a substrate-limitation term into the maintenance term. Their simulation results agreed with experimental results, but the K_s (0.371 mg/ml) used for the simulations was high and probably represents a product-inhibition effect (2, 11, 22). A better way to improve the equation would be to incorporate a product-inhibition term as well as a substrate-limitation term.

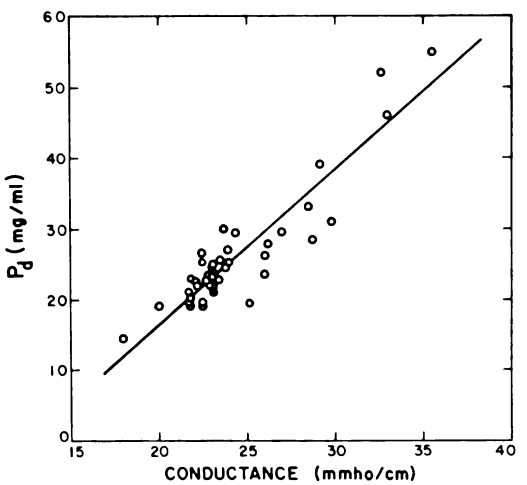


FIG. 6. *Regression correlation between concentration of ammonium-lactate (P_d) and electrical conductance in the dialysate circuit.*

The model developed in the present study is simple and correlated well with experimental tests of dialysis and nondialysis (unpublished) continuous processes for the ammonium-lactate fermentation over a wide range of operating parameters with high substrate and product concentrations. Specifically, the model agreed with experimental results of substrate utilization (Fig. 1 to 3), product formation (Fig. 4), and cell-mass accumulation (results not shown). The model also contains substrate-limitation and product-inhibition terms which have a biological basis. Moreover, the values used for these terms are realistic. To date, the model has only been correlated with steady-state data. The major shortcoming of the model is that the product-inhibition effect is not strong enough at lactate concentrations greater than 70 mg/ml. As suggested above, the incorporation of a product-inhibition term into the maintenance term may provide a solution. Another cause of the problem is that, since a dialysis culture is able to tolerate greater lactate concentrations than is a nondialysis culture (22), there may be an unknown dialyzable factor which also has an inhibition effect on the lactic acid fermentation.

The mean product/substrate ratio of the ammonium-lactate fermentation was determined as 0.96 mg/mg. Thus, 96% of the lactose utilized was converted to ammonium-lactate and 4% was incorporated into bacterial cells. On a substrate efficiency basis, the fermentation thus would be more useful for production of ammonium-lactate than for bacterial cells.

The dialysis continuous process has been used to ferment whole whey (22) and deproteinized whey. Both fermentations are most efficient when using a T_f of about 20 h, i.e., further increases in T_f do little to decrease S_f and S_d (compare Fig. 1 and 3). A comparison of the use of these substrates at similar conditions is shown

TABLE 6. *Comparison of wheys used for production of ammonium-lactate by dialysis continuous fermentation*

Substrate	S_f^o (mg/ml)	T_f (h)	ϕ	E (%)	S_f (mg/ml)	P_f (mg/ml)	S_d (mg/ml)	P_d (mg/ml)
Whole whey (22)	230	19	2.5	97	2.6	80	0.4	24
Deproteinized whey	242	27	2.5	81	23.2	69	2.7	24

in Table 6. The fermentation of whole whey relative to deproteinized whey occurred at a greater fermentation rate and resulted in more complete substrate conversion. The fermentor contents of both processes were similarly diluted by a net osmosis of water from the dialysate circuit. Both fermentations were similarly efficient in the percent conversion of lactose into ammonium-lactate only and in the high concentration of accumulated ammonium-lactate in the fermentor. The present study also showed that a high concentration of cell-free ammonium lactate (50 mg/ml) could be maintained in the dialysate effluent (Fig. 4).

The mathematical model showed the permeability factor to be as low as 0.4. Consequently, the fermentation could be improved considerably (e.g., more efficient conversion of substrate and greater lactate concentration in the dialysate effluent) with use of a more permeable membrane. Greater conversion of substrate could also be had by increasing the cell-mass concentration in the dialysis fermentor. This might be accomplished by recycling the cells or by employing a nondialysis prefermentor which is optimized for cell production and the effluent from which flows into the dialysis fermentor, which is optimized for conversion efficiency and product accumulation. Such fermentation systems can be easily modeled before conducting experimental tests.

ACKNOWLEDGMENT

This work was supported by grant ENG 76-17260 from the National Science Foundation.

LITERATURE CITED

1. **Aborhey, S., and D. Williamson.** 1977. Modelling of lactic acid production by *Streptococcus cremoris* HP. J. Gen. Microbiol. **23**:7-21.
2. **Contois, D. E.** 1959. Kinetics of bacterial growth: relationship between population density and specific growth rate of continuous cultures. J. Gen. Microbiol. **21**:40-50.
3. **Coton, S. G.** 1976. Recovery of dairy waste, p. 221-231. *In* G. G. Birch, K. J. Parker, and J. T. Worgan (ed.), Food from waste. Applied Science Publishers, Barking, England.
4. **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.
5. **Driessen, F. M., J. Ubbels, and J. Stadhouders.** 1977. Continuous manufacture of yogurt. I. Optimal conditions and kinetics of the prefermentation process. Biotechnol. Bioeng. **19**:821-839.
6. **Friedman, M. R., and E. L. Gaden, Jr.** 1970. Growth and acid production by *Lactobacillus (L.) delbrueckii* in a dialysis culture system. Biotechnol. Bioeng. **12**: 961-974.
7. **Gerhardt, P., and C. A. Reddy.** 1978. Conversion of agroindustrial wastes into ruminant feedstuff by ammoniated organic acid fermentation: a brief review and preview. Dev. Ind. Microbiol. **19**:71-78.
8. **Hanson, T. P., and G. T. Tsao.** 1972. Kinetic studies of the lactic acid fermentation in batch and continuous cultures. Biotechnol. Bioeng. **14**:233-252.
9. **Holdeman, L. V., and W. E. C. Moore.** 1975. Anaerobe laboratory manual, 3rd ed., p. 113-114. V.P.I. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va.
10. **Horton, B. S., R. L. Goldsmith, and R. R. Zall.** 1972. Membrane processing of cheese whey reaches commercial scale. Food Technol. **26**:30-35.
11. **Keller, A. K., and P. Gerhardt.** 1975. Continuous lactic acid fermentation of whey to produce a ruminant feed supplement high in crude protein. Biotechnol. Bioeng. **17**:997-1018.
12. **Lee, I. H., A. G. Fredrickson, and H. M. Tsuchiya.** 1976. Dynamics of mixed cultures of *Lactobacillus plantarum* and *Propionibacterium shermanii*. Biotechnol. Bioeng. **18**:513-526.
13. **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.
14. **Morris, D. L.** 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science **107**:254-255.
15. **Nielsen, I. K., A. G. Bundgaard, O. J. Olsen, and R. F. Madsen.** 1972. Reverse osmosis for milk and whey. Process Biochem. **7**:17-20.
16. **Perlman, D. (ed.).** 1977. Annual reports on fermentation processes, vol. 1, p. 95-114. Academic Press, Inc., New York.
17. **Pryce, J. D.** 1969. A modification of the Barker-Summerson method for the determination of lactic acid. Analyst **94**:1151-1152.
18. **Reddy, C. A., H. E. Henderson, and M. D. Erdman.** 1976. Bacterial fermentation of cheese whey for production of a ruminant feed supplement rich in crude protein. Appl. Environ. Microbiol. **32**:769-776.
19. **Rogers, P. L., L. Bramall, and I. J. McDonald.** 1978. Kinetic analysis of batch and continuous culture of *Streptococcus cremoris* HP. Can. J. Microbiol. **24**:372-380.
20. **Schingoethe, D. J.** 1976. Whey utilization in animal feeding: a summary and evaluation. J. Dairy Sci. **59**: 556-570.
21. **Segel, I. H.** 1975. Enzyme kinetics, p. 18-37. John Wiley and Sons, New York.
22. **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.
23. **Wingerd, W. H.** 1970. Lactalbumin as a food ingredient. J. Dairy Sci. **54**:1234-1236.
24. **Wingerd, W. H., S. Saperstein, and L. Lutwak.** 1970. Whey protein concentrate has excellent nutritional properties. Food Technol. **24**:758-764.

ARTICLE IV

PRODUCTION OF LACTOBACILLUS CELLS BY DIALYSIS CONTINUOUS FERMENTATION OF DEPROTEINIZED WHEY

By

R. W. Stieber and Philipp Gerhardt

Production of Lactobacillus Cells by Dialysis Continuous Fermentation of Deproteinized Whey[1]

R. W. STIEBER and PHILIPP GERHARDT

Department of Microbiology and Public Health,
Michigan State University,
East Lansing, Michigan 48824

ABSTRACT

A previously developed mathematical model was used to computer-simulate the continuous conversion of deproteinized whey into bacterial cells by a process in which the fermentor contents are poised at a constant pH by the addition of ammonia and are dialyzed through a membrane against water. The simulations predicted the fermentation and dialysis parameters for experimental test. Dried deproteinized cheese whey was rehydrated with tap water to contain 242 mg lactose/ml, supplemented with 8 mg yeast extract/ml, charged into a 5-liter fermentor without sterilization, adjusted in pH (5.5) and temperature (44°C), and inoculated with an adapted culture of Lactobacillus bulgaricus. The fermentor and dialysate circuits were connected and a series of steady-state conditions was managed for 71 days without sterilization or asepsis. Cell accumulation, cell productivity and molar growth yield were greater than those obtained from an ordinary continuous fermentation for the production of Lactobacillus cells.

---

[1]Journal article number 8962 from the Michigan Agricultural Experiment Station.

INTRODUCTION

The cells of Lactobacillus and other lactic-acid genera apparently are safe and acceptable for human use: these bacteria have been consumed in large amounts regularly for lifetimes by the various nationalities of people who use fermented milks as an integral part of their diet. Furthermore, the cells of Lactobacillus bulgaricus, for example, contain protein of high nutritional quality (Erdman et al., 1977). These cells have been used in the continuous manufacture of yogurt milk (Lewis, 1969; Driessen et al., 1977a and 1977b), and also can be immobilized and used to hydrolyze lactose in foods for intake by people who cannot digest the sugar (Ohmiya et al., 1977). However, the production of L. bulgaricus may be most useful as a model for the production of cheese starter cultures for the dairy industry.

Such cell-production processes can be managed batchwise (Marshall and Earle, 1975; Reddy et al., 1976), continuously (Keller and Gerhardt, 1975; Cox and MacBean, 1977; Stieber and Gerhardt, 1979b), and dialysis-continuously (Coulman et al., 1977; Stieber et al., 1977; Stieber and Gerhardt, 1979a). Of these the dialysis continuous process should be the most useful because it enables the use of concentrated substrate, is efficient in the rate of substrate conversion, and produces a useful cell-free permeate (Stieber et al., 1977). Moreover, preliminary theoretical study indicated that the cell mass in a continuous process is directly proportional to the feed substrate concentration that can be metabolized; therefore, the cell yield of a dialysis continuous process should be much greater than the yield obtained from an ordinary continuous process. Recently, Osborne (1977) used a dialysis batch process to produce concentrated cheese starter cultures

of Streptococcus, obtaining concentrations of 10^{11} cells per ml. A dialysis batch process also was used for production of yeast of up to 90 g dry weight per liter from deproteinized whey (Lane, 1977). However, dialysis continuous operation appeared desirable for producing Lactobacillus cells because waste whey is generated and the fermentation products could be used more or less continuously, and a continuous process generally is much more productive than a batch process (Pirt, 1975).

Deproteinized whey was used as substrate in the present study. It contains essentially all of the carbon and low molecular-weight nitrogen sources that are in milk or whole whey. Albumins and other milk proteins are removed and concentrated by ultrafiltration of the whey after making cheese from the milk (Coton, 1976) because the resulting protein is of high nutritional quality and has commercial value (Wingerd, 1970; Wingerd et al., 1970; Schingoethe, 1976; De Boer et al., 1976; Morr, 1976; Delaney, 1977; Mathur and Shahani, 1979). The residual deproteinized whey, however, represents an environmental burden and an economic and nutrient loss for which a practicable use needs be found. We first considered use of this dairy residue as a substrate for the fermentative production of ammonium-lactate (Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979b), which can be used as a nitrogen-rich feed supplement for ruminant animals (Gerhardt and Reddy, 1978; Juengst, 1979).

The present study was undertaken to determine the feasibility of producing cells of lactic-acid bacteria in general and of L. bulgaricus in particular from deproteinized whey. The usefulness of a dialysis continuous fermentation was examined and compared with the prior

results of an ordinary (nondialysis) continuous fermentation. As in our previous studies in this general area (Coulman et al., 1977; Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979b) the investigative procedure was to use a mathematical model to simulate, on a digital computer, the outcome to be expected and the parameters to be studied. The theoretical predictions then were tested experimentally. Both the theoretical and experimental results demonstrated the potential advantages and disadvantages of this unusual type of fermentation process.

THEORY

System Design. Figure 1 shows a schematic of the dialysis continuous fermentation system design. The symbols correspond to those used previously and are listed in Table 1.

The feed into the fermentor circuit contains the substrate (lactose) in relatively high concentration (S_f^o) and is maintained at a relatively low rate of flow (F_f) consistent with a minimal concentration of unused substrate in the fermentor effluent (S_f). The substrate is converted essentially only to cells (X_f) and product (lactic acid, P_f) which both remain in the fermentor effluent. A small concentration of the product preexists in the feed (P_f^o). The liquid volume in the fermentor (V_f) is maintained at a constant level. The addition of ammonia solution is not shown because it did not enter into the theoretical considerations.

The system does not have a reservoir vessel for the dialysate. Instead, the dialysate circuit consists only of tubing, pump and the dialysate side of the dialyzer, and contains a relatively small volume (V_d) which is continuously circulated. The feed into the dialysate

circuit consists only of water and is maintained at a relatively high flow rate (F_d), consistent with a very low substrate concentration (S_d) and a practically useful product concentration (P_d) in the dialysate effluent.

This system provides the greatest concentration gradient possible for dialysis and, thus, much of the product is removed from the immediate environment of the cells, relieving the inhibition of cell growth by the product. As more product is withdrawn by dialysis, more substrate is consumed and more cells are formed.

Mathematical Model. A mathematical model previously was developed for the dialysis continuous fermentation (Stieber and Gerhardt, 1979a). The generalized solution of the model for steady-state conditions is as follows:

$$S_f = \frac{K_s\pi_p + K_p(\gamma S_f^o + P_f^o)}{(\theta - 1)\pi_p - \gamma K_p \pi_s} \tag{1}$$

$$P_f = \frac{\gamma K_s \pi_s + (\theta - 1)(\gamma S_f^o + P_f^o)}{(\theta - 1)\pi_p - \gamma K_p \pi_s} \tag{2}$$

$$X_f = \frac{S_f^o - S_f + \Pi(S_d - S_f)}{\alpha + \beta\theta/\mu_m} \tag{3}$$

$$S_d = \Pi S_f/(\phi + \Pi) \tag{4}$$

$$P_d = R\Pi P_f/(\phi + R\Pi) \tag{5}$$

where $\pi_s = \Pi(\Pi/(\phi + \Pi) - 1) - 1$, $\pi_p = 1 + R\Pi(1 - R\Pi/(\phi + R\Pi))$.

Computer Simulated Predictions. The steady-state solution was used to simulate the effect on cell mass (X_f) of feed substrate concentration (S_f^o) and three operational parameters: cell-retention time (T_f), flow-rate ratio (ϕ), and permeability factor (Π). Because the fermentation was simulated before actual experiments were conducted, approximations of various conditions were used (Table 2). The values for μ_m, K_s, K_p, γ, R, and P_f^o were obtained from Stieber, Coulman and Gerhardt (1977). Values for α and β were obtained from Keller and Gerhardt (1975). Values for ϕ and Π were chosen arbitrarily. A value of 150 mg/ml for S_f^o was used because previous work (Stieber et al., 1977) indicated that dilution of the fermentor contents occurs in the dialysis process.

The results in Fig. 2 show the simulated effect of changes in feed substrate concentration on the cell mass in the fermentor circuit at constant cell retention time and constant rate of dialysis. The cell mass is proportional to the substrate concentration that can be metabolized by the cells. Thus, the dialysis process is most productive for cell production at high substrate concentrations. For these results, it was assumed that all of the substrate was metabolized, i.e., all the lactose in the whey was converted to lactic acid and cells.

Fig. 2 also shows the effect of changes in the product-inhibition factor (K_p) on the dialysis continuous fermentation. The smaller the value of K_p, the greater the cell mass.

The cell-retention time ($T_f = V_f/F_f$) is an operational parameter which is varied to change the average length of time a cell remains in the fermentor. Fig. 3 shows the simulated effects of changes in T_f on

cell mass in the fermentor circuit. At first, with increasing retention time where growth metabolism (α) predominated, the cell mass increased to a maximum. But beyond a critical retention time, maintenance metabolism (β) superseded that of growth and then the cell mass decreased.

The flow-rate ratio is an operational parameter used to manipulate the rate of dialysis ($\phi = F_d/F_f$). Simulated results of variation in the flow-rate ratio affecting the cell mass also are shown in Fig. 3. The cell mass increased only a little with a four-fold increase in the flow-rate ratio (i.e., with a greatly increased rate of dialysis).

Fig. 4 emphasizes that the overall effect of changes in the flow-rate ratio on the cell mass is small. In fact, the cell mass for a nondialysis continuous process ($\phi = 0$) is only slightly less than the cell mass for a continuous process with a high rate of dialysis ($\phi = 4$), when both processes are operated at the same feed substrate concentration. Consequently, the primary advantage of a dialysis continuous process over an ordinary continuous process for production of cells is that the former enables the efficient use of concentrated substrate when an inhibitory and dialyzable product is formed.

The permeability factor is an operational parameter which is a measure of the diffusion capacity of the dialysis process ($\Pi = P_{ms}A_m/F_f$). Simulated results of variation in the permeability factor affecting the cell mass also are shown in Fig. 4. The cell mass increased a little with decreased permeability factor at low flow-rate ratios, but almost no change occurred at high flow-rate ratios.

EXPERIMENTAL MATERIALS AND METHODS

Inoculum. The inoculum culture was obtained from the fermentor effluent (day 94) of a previous dialysis continuous fermentation (Stieber et al., 1977) originally started with L. bulgaricus 2217 (Chris Hanson's Laboratory, Milwaukee, Wisconsin).

Substrate. Dried deproteinized cheese whey (prepared by ultrafiltration of whole sweet-cheese wheys by Stauffer Chemical Company, Rochester, Minnesota) was rehydrated to contain 242 mg of lactose per ml and was supplemented with 8 mg of yeast extract per ml. The reconstituted whey was made up in 7.0-liter batches without sterilization, stored at 4°C, and held in a stirred, heated (60°C) reservoir to keep the lactose in solution.

Dialysis Continuous Fermentation System. The experimental dialysis fermentation system was conducted continuously at a temperature of 44°C and a pH of 5.5 and operated with essentially the same equipment and in the same manner as previously (Stieber et al., 1977), with only minor changes (Stieber and Gerhardt, 1979a). Operation of the system was interrupted once for three weeks, during which the fermentor-circuit contents were stored at 4°C.

Analytical Procedures. Samples of the dialysate and fermentor circuits were taken at 12 h intervals from the dialysate effluent and from a glass "T" inserted in the tubing between the fermentor and dialyzer, respectively. Steady-state data were determined from samples taken at five times the cell retention time or 48 h after changing a parameter. Since the whey was deproteinized by ultrafiltration, optical density measurements (700 nm) could and were made on samples as an index of cell concentration. Protein was precipitated from

samples with hot 5% trichloroacetic acid and measured by the method of Lowry et al. (1951), with bovine albumen as the standard. Dry weight measurements were made on samples by centrifuging cells from 5 ml samples, washing the cells twice with distilled water, and drying the cells at 105°C for at least 18 h.

EXPERIMENTAL RESULTS

Effect of Cell-Retention Time. The results of experimental variation in the cell-retention time affecting the cell concentration in the fermentor circuit showed the same form of curves (Fig. 5) as predicted by the computer simulation (Fig. 3). The curve shapes were similar for the three indexes of cell concentration (dry weight, protein, and optical density).

Effect of Flow-Rate Ratio. Fig. 5 also shows the results of experimental variation in the flow-rate ratio (ϕ) affecting the cell concentration. The results confirmed the simulation (Fig. 3) predicting that the cell concentration would increase with increasing flow-rate ratio.

Cell Productivity. The results of experimental variation in the cell-retention time affecting the cell productivity of the process showed the cell productivity decreased with increased retention time (Fig. 6). The results also showed that the cell productivity was greater with increased flow-rate ratio than decreased flow-rate ratio (ϕ). However, the degree to which the flow-rate ratio affected the cell productivity depended on the cell-retention time.

Molar Growth Yield. Fig. 7 shows the results of changes in the cell-retention time affecting the molar growth yield (Y_{ATP}), which is

the number of grams dry weight of cells produced per calculated mole of ATP generated from catabolism. The molar growth yield decreased with increased retention time. Thus, as the retention time increased, the bacteria increasingly used the energy obtained from catabolism for maintenance and other nongrowth associated purposes.

The results of variation in the flow-rate ratio (ϕ) affecting the Y_{ATP} also are shown in Fig. 7. The molar growth yield was greater with increased flow-rate ratio than with decreased flow-rate ratio. But the degree to which the ratio affected the Y_{ATP} depended on the cell-retention time.

Prolonged Steady-State Operation. A dialysis continuous fermentation next was operated at a steady-state (T_f = 27.2, ϕ = 2.5) to observe the cell mass behavior over an extended period. No cyclic behavior was observed (Table 3). The cell mass decreased during the first 5 days of the fermentation and then gradually increased for the final 10 days. The decrease occurred in the period when the bacterial population was adjusting to continuous culture at a relatively slow rate of growth after having reached a high population in a batch culture, which was necessary to start the fermentation. The increase may have been due to decreased membrane permeability in this period (see p. 492 in reference Stieber and Gerhardt, 1979a), which would confirm the trend predicted by the computer simulation shown in Fig. 4.

Correlation of Simulated and Experimental Results. The simulated results were correlated with the experimental results to evaluate the metabolic constants of the model, demonstrate the validity of the model, and evaluate the nature of bacterial metabolism in the process.

The values used for the computer simulations are listed in Table 5. The values for ϕ and Π were calculated directly from the experimental results. The values for μ_m, K_s, K_p, γ, R, and P_f^o were shown to be valid previously (Stieber and Gerhardt, 1979a). The values for α and β were obtained by successive curve fitting of the simulated results with the experimental results. S_f^o was taken as 160.3 mg/ml instead of 237.5 mg/ml (which was obtained from material balances of the experimental data) because a mean 32.5 percent of the fermentor contents represented water which osmosed from the dialysate circuit (Stieber and Gerhardt, 1979a).

In Fig. 8 are shown the computer-simulated curves obtained by using the mathematical model (Stieber and Gerhardt, 1979a) together with the values in Table 4 to show the effect of cell-retention time on accumulation of cell mass. The curves fitted closely with the superimposed points of experimental results and thereby demonstrated the validity of the model.

The relative importance of growth and maintenance metabolism in the fermentation was calculated from knowledge of the values for α and β (Keller and Gerhardt, 1975). The calculations showed that, at a cell-retention time of 7.6 h and flow-rate ratio of 2.5 where it was experimentally shown that the cell productivity was greatest (Fig. 6), 77.4 percent of the substrate utilization was due to maintenance metabolism and 22.6 percent was due to growth metabolism. Further calculations showed that, as the cell-retention time increased, energy requirements for maintenance increased and those for growth decreased.

DISCUSSION

The experimental results confirmed the computer-simulated prediction that the cell mass accumulation of a dialysis continuous process is greater than that obtained from an ordinary (nondialysis) continuous process for the ammonium-lactate fermentation. The rationale is that the cell mass accumulation is directly proportional to the feed substrate concentration which can be metabolized by the cells. The degree of proportionality depends on the magnitude of the inhibition effect by products. In the ammonium-lactate fermentation of whey there is a strong product-inhibition effect. Thus, dialysis is necessary to relieve the inhibition effect by lactic acid. As more product is withdrawn by dialysis, the growth rate of cells becomes higher and more substrate is consumed, which enables the use of concentrated substrate with the resultant high cell mass concentrations. In a fermentation with no inhibition effect by product, the present dialysis continuous process would be unnecessary because an ordinary continuous process then could accomplish the efficient use of concentrated substrate.

Figure 2 shows that, in a fermentation for biomass, it is paramount to select an organism with a small product inhibition constant. At a given rate of dialysis and feed substrate concentration, an organism with a small product inhibition constant will attain a higher cell mass concentration than one with a high inhibition constant. The magnitude of the metabolic constants α and β of an organism also have a considerable effect on cell mass. The simulated predictions of cell mass (Figs. 2 to 4) were much greater than the experimental results of cell mass (Fig. 5) because the values for α and β used to make the

predictions were relative values and not absolute values (Keller and Gerhardt, 1975). This comparison demonstrated the considerable effect of α and β on the cell mass.

The experimental results confirmed the computer-simulated prediction of a maximum cell mass at an intermediate cell-retention time (Fig. 5). Since the cell productivity was maximum at the shortest cell-retention time managed, a trade-off exists in the regulation of the cell-retention time.

The best results of the dialysis continuous process were compared with those of a nondialysis continuous process for producing cells of L. bulgaricus (Table 5). The dialysis process produced more than twice the mass of cells per volume, was more than twice as productive for cells (mg/ml-h), and was more efficient energywise (Y_{ATP}) than was the nondialysis process over a wide range of cell-retention times. Further, the dialysis process used more than twice the concentration of feed substrate (160.3 mg lactose per ml) than did the nondialysis process (74.6 mg lactose per ml). Comparisons of cell mass accumulation with concentration of feed substrate agreed with the simulated predictions (Fig. 2).

In Table 6 results are shown which compare the percentages of growth and maintenance metabolism of the culture when grown at similar cell-retention times and different rates of dialysis, including nondialysis. Interestingly, the percentage of growth metabolism decreased and the percentage of maintenance metabolism increased with increased dialysis (increased ϕ). Also, the specific maintenance rate (β) increased, whereas the substrate/cell ratio (α) of the culture decreased, with increased dialysis.

The present dialysis continuous process is useful for production of a relatively high cell mass accumulation in a fermentation where metabolic products inhibit microbial growth. The process may be especially applicable to the production of starter cultures in the dairy industry. The continuous operation would allow automated process control, would not require repetitive subcultures and inoculations, and would yield a uniform culture. The dialysis operation would enable the use of concentrated substrate and result in a great accumulation of cell mass. Thus the final product would need less concentration, which subjects the cells to the mechanical stress of centrifugation. The major disadvantages of using the present dialysis process for cell production are equipment complexities and cost, membrane fouling (Stieber and Gerhardt, 1979a), and the concurrent production of large volumes of dilute permeate. An economical process probably would entail production of a permeate of value, e.g., in the present study the ammonium-lactate permeate may have value as a nitrogenous-feed supplement for ruminants or may be converted to lactic acid for chemical use and ammonium sulfate for use as fertilizer. Yet higher cell accumulations in this system should easily be attained by use of a more permeable membrane, a greater membrane area, a greater rate of dialysis (ϕ), and a higher concentration of feed substrate.

ACKNOWLEDGEMENT

This work was supported by grant ENG 76-17260 from the National Science Foundation.

REFERENCES

1 Coton, S. G. 1976. Recovery of dairy waste. In G. G. Birch, K. J. Parker, and J. T. Worgan (eds.), Food from Waste, Applied Science Publishers, Barking, England, p. 221.

2 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.

3 Cox, G. C., and R. D. MacBean. 1977. Lactic acid production by Lactobacillus bulgaricus in supplemented whey ultrafiltrate. Australian J. Dairy Technol. 32:15-22.

4 DeBoer, R., J. N. DeWit, and J. Hiddink. 1977. Processing of whey by means of membranes and some applications of whey protein concentrate. J. Soc. Dairy Technol. 30:112-120.

5 Delaney, R. A. M. 1976. Composition, properties and uses of whey protein concentrates. J. Soc. Dairy Technol. 29:91-100.

6 Driessen, F. M., J. Ubbels, and J. Stadhouders. 1977. Continuous manufacture of yogurt. I. Optimal conditions and kinetics of the prefermentation process. Biotechnol. Bioeng. 19:821-829.

7 Driessen, F. M., J. Ubbels, and J. Stadhouders. 1977. Continuous manufacture of yogurt. II. Procedure and apparatus for continuous coagulation. Biotechnol. Bioeng. 19:841-851.

8 Erdman, M. D., W. G. Bergen, and C. A. Reddy. 1977. Amino acid profiles and presumptive nutritional assessment of single-cell protein from certain lactobacilli. Appl. Environ. Microbiol. 33:901-905.

9 Gerhardt, P., and C. A. Reddy. 1978. Conversion of agroindustrial wastes into ruminant feedstuff by ammoniated organic acid fermentation: a brief review and preview. Dev. Ind. Microbiol. 19:71-78.

10 Hargrove, R. E., F. E. McDonough, D. E. LaCroix, and J. A. Alford. 1976. Production and properties of deproteinized whey powders. J. Dairy Sci. 59:25-33.

11 Juengst, F. W., Jr. 1979. Use of total whey constituents--animal feed. J. Dairy Sci. 62:106-111.

12 Keller, A. K., and P. Gerhardt. 1975. Continuous lactic acid fermentation of whey to produce a ruminant feed supplement high in crude protein. Biotechnol. Bioeng. 17:997-1018.

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

14 Lewis, P. M. 1967. A note on the continuous flow culture of mixed populations of Lactobacilli and Streptococci. J. Appl. Bact. 30:406-409.

15 Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

16 Marshall, K. R., and R. L. Earle. 1975. The effect of agitation on lactic acid by Lactobacillus bulgaricus in a casein whey medium. N.Z. J. Dairy Sci. Technol. 10:123-128.

17 Mathur, B. N., and K. M. Shahani. 1979. Use of total whey constituents for human food. J. Dairy Sci. 62:99-105.

18 Moor, C. V. 1976. Whey protein concentrates: an update. Food Technol. 30:18-42.

19 Ohmiya, K., H. Ohashi, T. Kobayashi, and S. Shimizu. 1977. Hydrolysis of lactose by immobilized microorganisms. Appl. Environ. Microbiol. 33:137-146.

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

21 Pirt, S. J. 1975. Principles of microbe and cell cultivation. Halsted Press, John Wiley & Sons, Inc., New York, New York, p. 29-41.

22 Reddy, C. A., H. E. Henderson, and M. D. Erdman. 1976. Bacterial fermentation of cheese whey for production of a ruminant feed supplement rich in crude protein. Appl. Environ. Microbiol. 32:769-776.

23 Schingoethe, D. J. 1976. Whey utilization in animal feeding: a summary and evaluation. J. Dairy Sci. 59:556-570.

24 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.

25 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.

26 Stieber, R. W., and P. Gerhardt. 1979b. Continuous ammonium-lactate fermentation of deproteinized whey. J. Dairy Sci. In press.

27 Wingerd, W. H. 1970. Lactalbumin as a food ingredient. J. Dairy Sci. 54:1234-1236.

28 Wingerd, W. H., S. Saperstein, and L. Lutwak. 1970. Whey protein concentrate has excellent nutritional properties. Food Technol. 24:758-764.

TABLE 1. Glossary of mathematical symbols

Symbol	Description	Units or Definition
A_m	Area of membrane available for dialysis	cm^2
F_d	Flow rate into and out of dialysate circuit	ml/h
F_f	Flow rate into and out of fermentor circuit	ml/h
K_p	Product inhibition constant	mg/mg
K_s	Substrate limitation constant	mg/ml
P_d	Product concentration in dialysate circuit	mg/ml
P_f^o	Product concentration in fermentor feed	mg/ml
P_f	Product concentration in fermentor circuit	mg/ml
P_{mp}	Permeability of membrane to product	mg/cm^2-h
P_{ms}	Permeability of membrane to substrate	mg/cm^2-h
S_d	Substrate concentration dialysate circuit	mg/ml
S_f^o	Substrate concentration in fermentor feed	mg/ml
S_f	Substrate concentration in fermentor circuit	mg/ml
X_f	Cell-mass concentration in fermentor circuit	mg/ml
Y_{ATP}	Molar growth yield	mg/mmole
α	Substrate/cell ratio	mg/mg
β	Specific maintenance rate	h^{-1}
γ	Product/substrate ratio	mg/mg
μ_m	Maximum specific growth rate of cells	h^{-1}
T_f	Cell-retention time in fermentor circuit	h
R	Ratio of product/substrate membrane permeabilities	$= P_{mp}/P_{ms}$
ϑ	Time factor	$= \mu_m T_f$
Π	Membrane permeability factor	$= P_{ms} A_m / F_f$
ϕ	Flow-rate ratio	$= F_d/F_f$

TABLE 2. Values used for computer-simulated predictions of dialysis continuous fermentations

Figure	μ_m (h^{-1})	K_p (mg/mg)	K_s (mg/ml)	T_f (h)	ϕ	Π	R	P_f^o (mg/ml)	S_f^o (mg/ml)	α (mg/mg)	β (h^{-1})	γ (mg/mg)
1	.25	--	.06	10.0	1.0	1.0	3.0	9.1	150	2.2	0.2	.96
2	.25	0.6	.06	--	--	1.0	3.0	9.1	150	2.2	0.2	.96
3	.25	0.6	.06	--	--	--	3.0	9.1	150	2.2	0.2	.96

TABLE 3. Cell mass produced during prolonged operation of the dialysis continuous fermentation at steady-state conditions

Day	Cell-retention time, T_f (h)	Flow-rate ratio, ϕ	Cell mass, X_f (mg/ml)
57	25.5	2.29	3.04
	24.2	2.26	4.44
	25.7	2.36	4.06
58	28.2	2.69	3.72
	27.4	2.62	3.36
59	26.4	2.46	3.56
	27.4	2.60	3.00
60	27.9	2.66	3.74
	27.5	2.62	2.78
61	27.0	2.53	2.98
	27.0	2.53	2.04
62	26.9	2.53	2.62
	26.3	2.46	2.96
63	27.1	2.55	3.06
	27.7	2.61	2.66
64	27.6	2.61	2.88
	27.6	2.58	2.42
65	27.2	2.53	2.72
	27.4	2.53	2.92
66	27.7	2.58	3.28
	27.8	2.59	3.28
67	28.2	2.61	3.00
	27.9	2.60	3.28
68	27.8	2.58	3.28
	27.6	2.53	3.62
69	27.5	2.54	3.46
	27.2	2.49	3.54
70	27.0	2.47	3.42
	27.5	2.53	3.58
71	27.3	2.52	3.72
mean	27.2	2.54	3.21

TABLE 4. Values used to correlate simulated results with experimental results

Day	μ_m (h^{-1})	K_p (mg/mg)	K_s (mg/ml)	ϕ	Π	R	P_f^o (mg/ml)	S_f^o (mg/ml)	α (mg/mg)	β (h^{-1})	γ (mg/mg)
26-36	.35	2.2	.06	1.0	.5	3.0	9.1	160.3	7.0	1.20	.96
40-49	.35	2.2	.06	2.5	.4	3.0	9.1	160.3	3.0	1.35	.96

TABLE 5. Comparison of dialysis* and nondialysis** results of the continuous fermentation for bacterial cells

DIALYSIS PROCESS				NONDIALYSIS PROCESS			
Cell-retention time (h)	Dry weight (mg/ml)	Cell productivity (mg/ml-h)	Y_{ATP}	Cell-retention time (h)	Dry weight (mg/ml)	Cell productivity (mg/ml-h)	Y_{ATP}
7.6	5.4	.71	5.8	6.4	1.6	.25	5.0
10.4	5.8	.58	4.9	9.3	1.9	.20	4.1
15.2	5.3	.35	4.3	10.2	1.7	.16	3.7
18.6	4.0	.21	3.0	16.4	1.9	.12	3.7
27.5	3.5	.13	2.6	26.7	1.5	.06	2.4

*Flow-rate ratio = 2.5.

**Results of Stieber and Gerhardt (1979b) with feed lactose = 74.6 mg/ml, yeast extract = 2 mg/ml, pH = 5.5, and temperature = 44°C.

TABLE 6. Comparison of culture metabolism at different rates of dialysis and at similar cell-retention times

Flow-rate ratio, ϕ	Cell-retention time, T_f(h)	Substrate/cell ratio, α(mg/mg)	Specific maintenance rate, $\beta(h^{-1})$	Growth metabolism (%)	Maintenance metabolism (%)
0.0 (nondialysis, in reference Stieber and Gerhardt, 1979b)	6.4	11.0	1.00	63.2	36.8
1.0 (dialysis)	7.3	7.0	1.20	44.4	55.6
2.5 (dialysis)	7.6	3.0	1.35	22.6	77.4

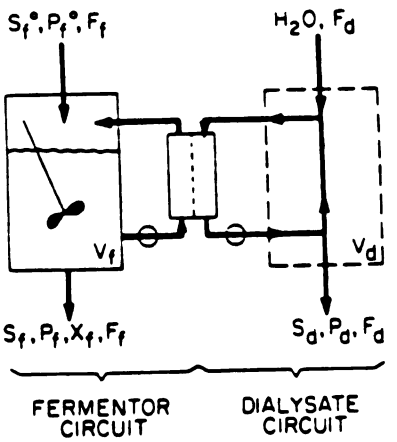


FIG. 1. Schematic of dialysis continuous fermentation system (reprinted with permission from reference Coulman et al., 1977). Symbols are described in Table 1.

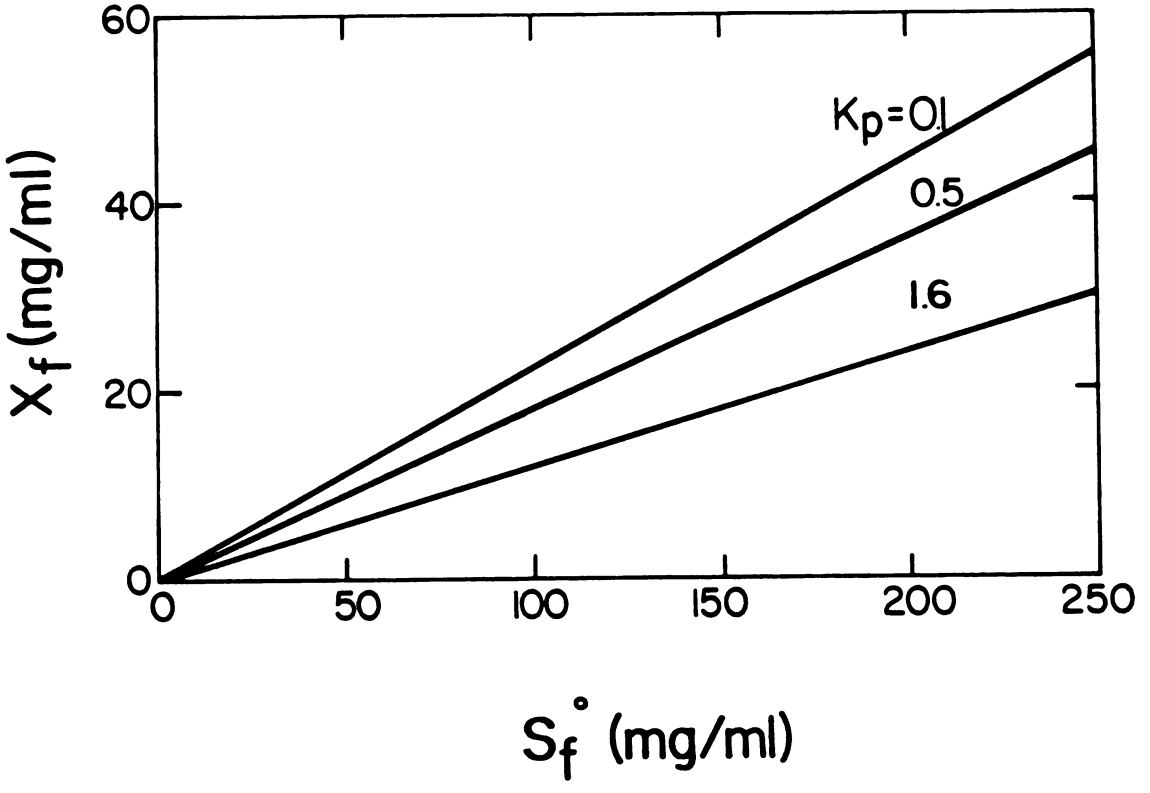


FIG. 2. Simulated effects of changes in the feed substrate concentration (S_f^o) on the cell mass concentration in the fermentor circuit (X_f) at three values of the product-inhibition factor (K_p).

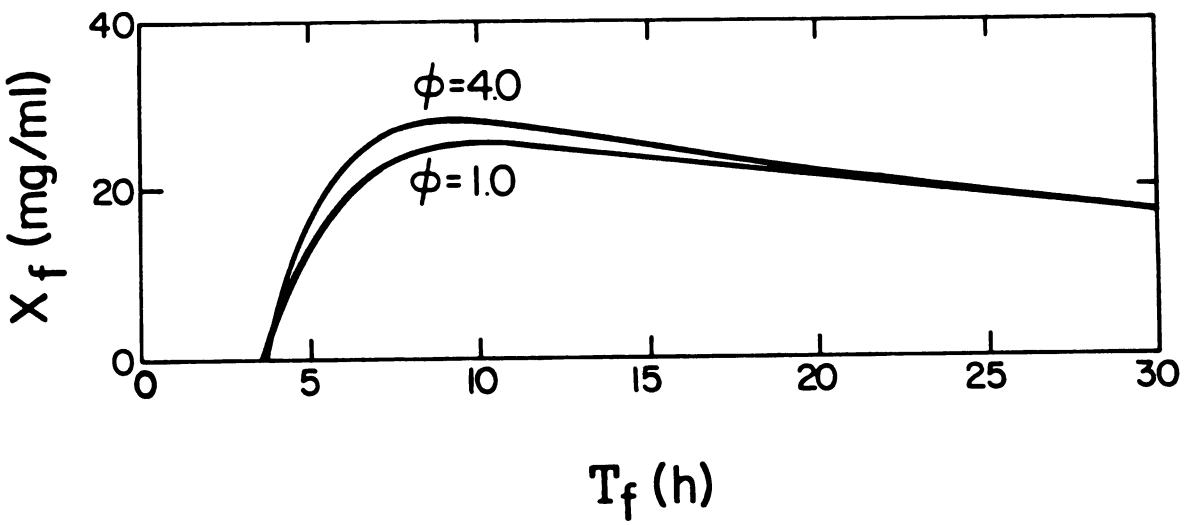


FIG. 3. Simulated effects of changes in the cell-retention time (T_f) on the cell mass concentration in the fermentor circuit (X_f) at two values of the flow-rate ratio (ϕ).

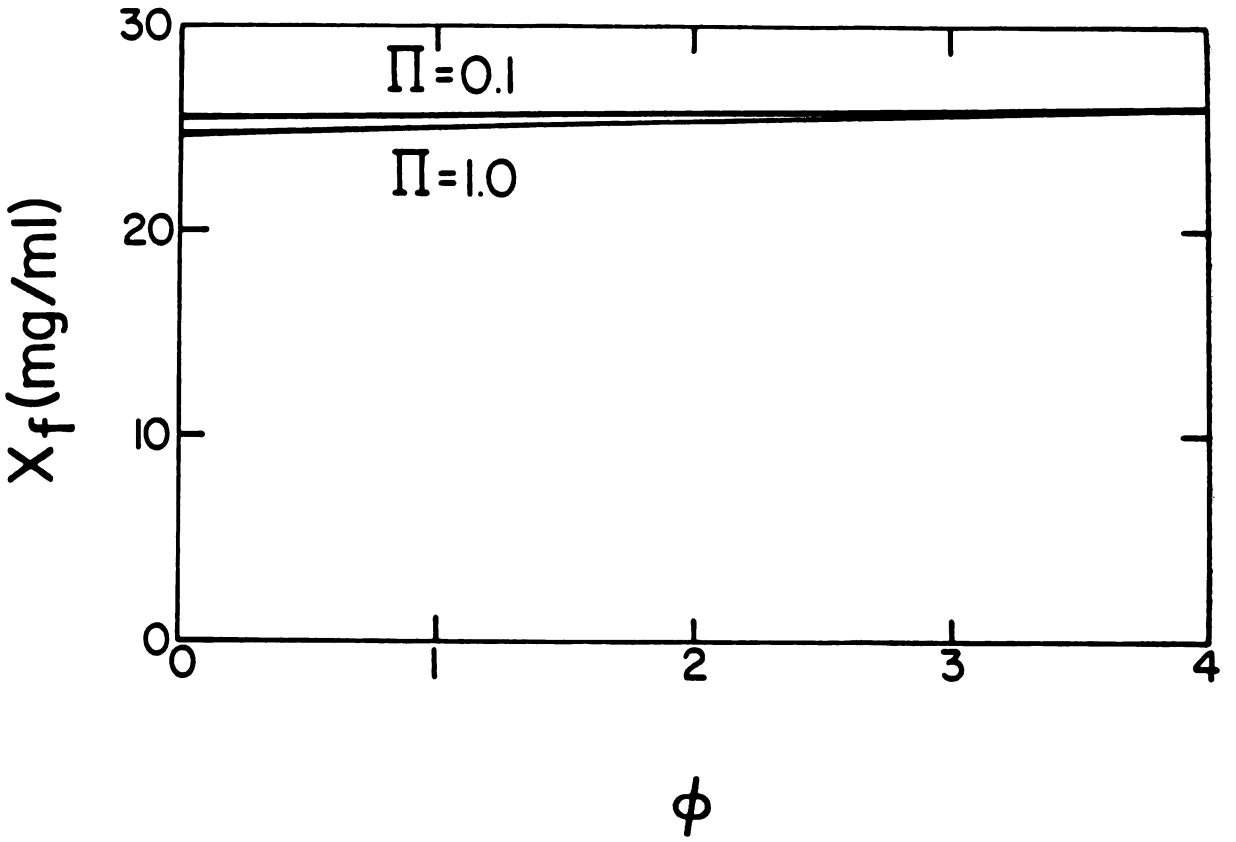


FIG. 4. Simulated effects of changes in the flow-rate ratio (ϕ) on the cell mass concentration in the fermentor circuit (X_f) at two values of the membrane permeability factor (Π).

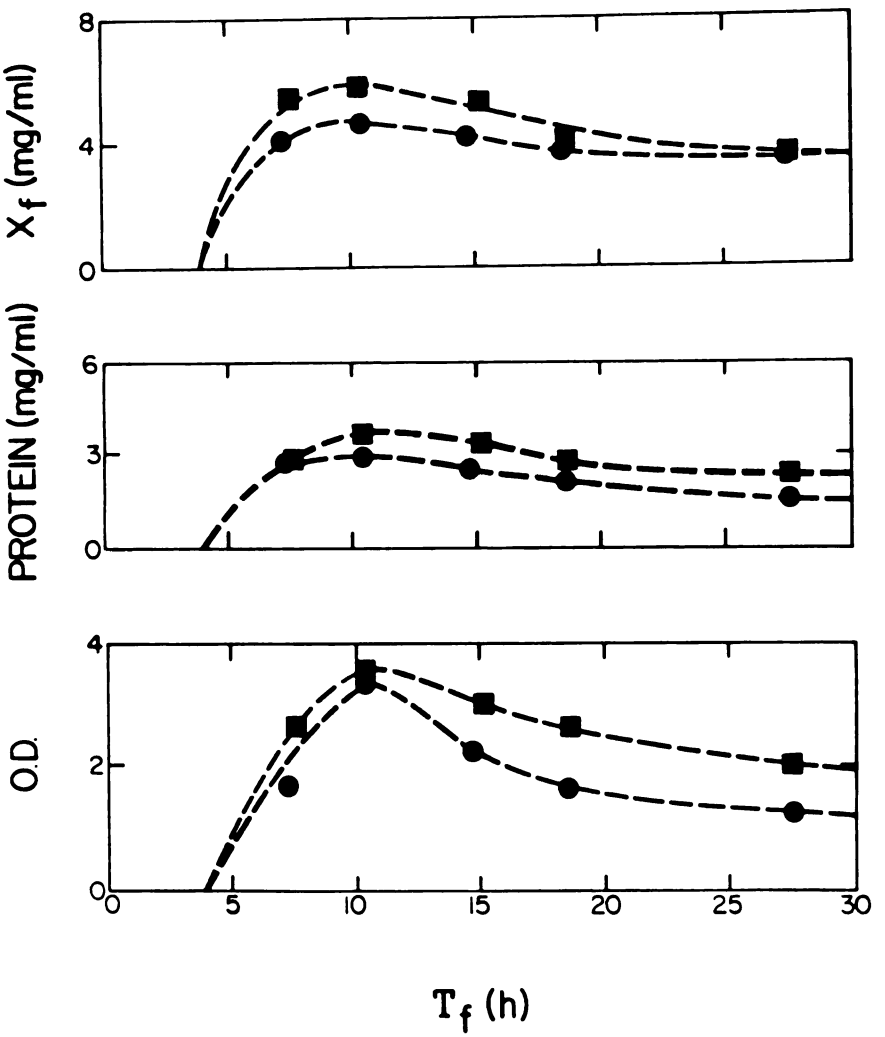


FIG. 5. Experimental effects of changes in the cell-retention time (T_f) and the flow-rate ratio (ϕ) on the cell concentration in the fermentor circuit as determined by dry weight (X_f), protein, and optical density (O.D.). The circle points were obtained at ϕ = 1.0 and during days 26 to 36. The square points were obtained at ϕ = 2.5 and during days 40 to 49. Interpretations of the curves were based on the simulated predictions (Fig. 3).

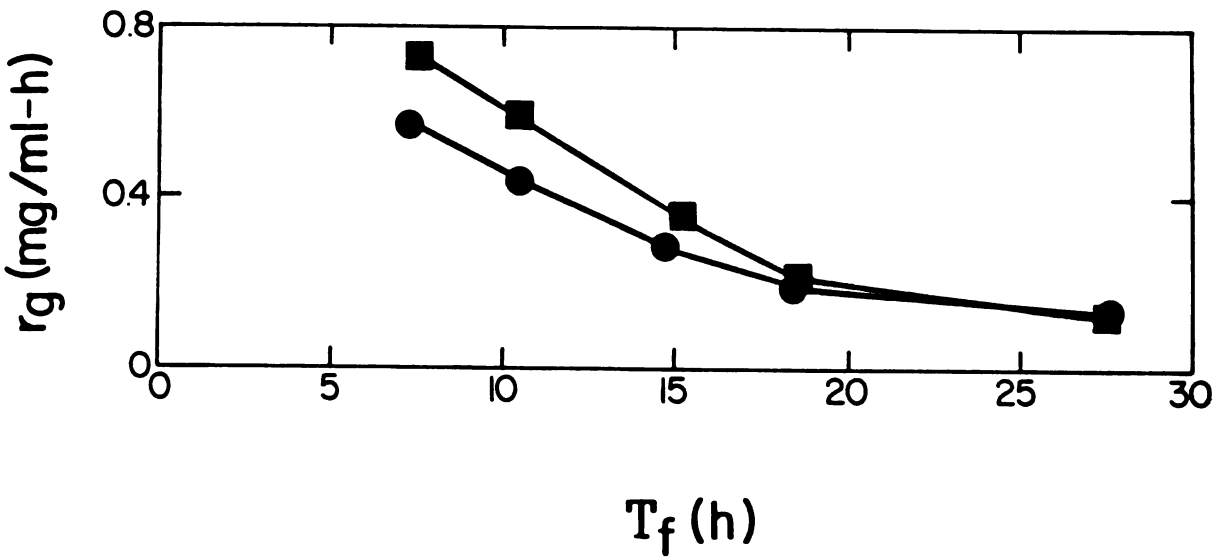


FIG. 6. Experimental effects of changes in the cell-retention time (T_f) and the flow-rate ratio (ϕ) on the cell mass productivity (r_g) of the dialysis continuous process. The circle points were obtained at ϕ = 1.0 and during days 26 to 36. The square points were obtained at ϕ = 2.5 and during days 40 to 49.

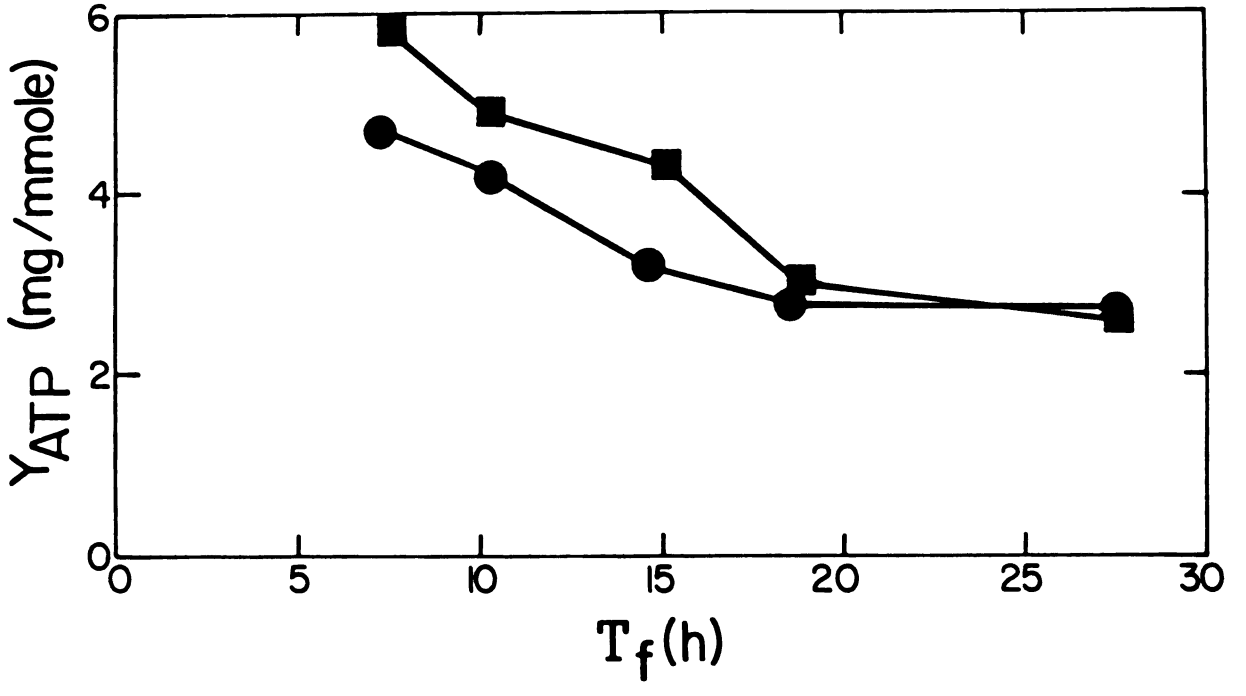


FIG. 7. Experimental effects of changes in the cell-retention time (T_f) and the flow-rate ratio (ϕ) on the molar growth yield (Y_{ATP}). The circle points were obtained at ϕ = 1.0 and during days 26 to 36. The square points were obtained at ϕ = 2.5 and during days 40 to 49.

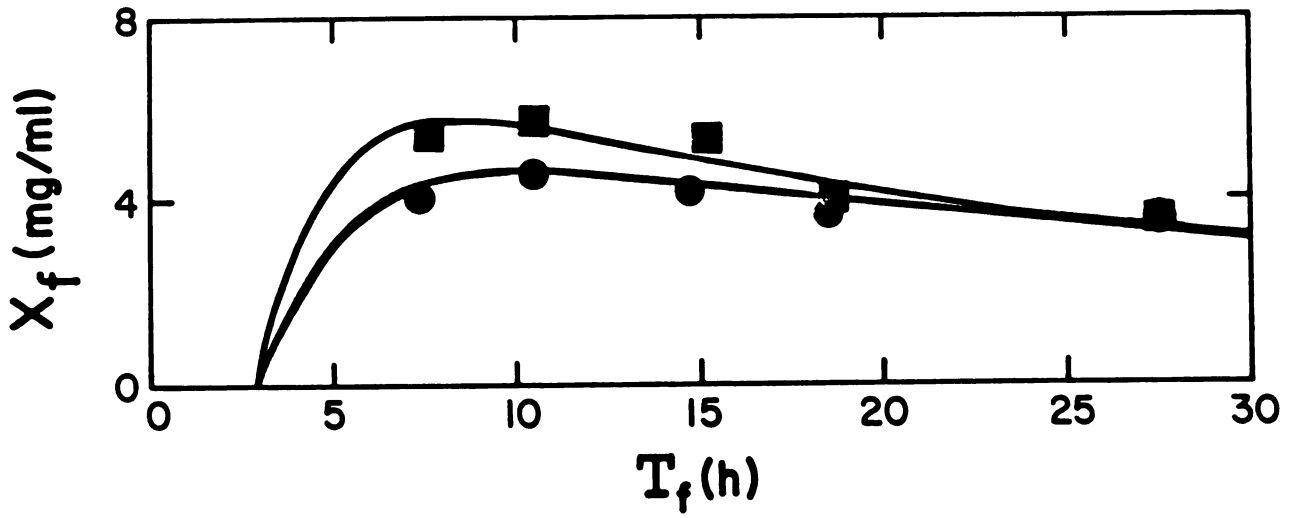


FIG. 8. Simulated effects of changes in the cell-retention time (T_f) and the flow-rate ratio (ϕ) on the cell mass concentration in the fermentor circuit (X_f). The curves were plotted by use of the mathematical model and the values in Table 4. The points were replotted from Fig. 4 to demonstrate the close fit between the experimental results and the simulated results.

ARTICLE V

CONTINUOUS PROCESS FOR AMMONIUM-LACTATE FERMENTATION OF DEPROTEINIZED WHEY

By

R. W. Stieber and Philipp Gerhardt

Accepted by the Journal of Dairy Science.

Continuous Process for Ammonium-Lactate Fermentation of Deproteinized Whey[1]

R. W. STIEBER and PHILIPP GERHARDT

Department of Microbiology and Public Health,
Michigan State University,
East Lansing, Michigan 48824

ABSTRACT

A mathematical model was developed to describe a process for the continuous fermentation of lactose to lactic acid, with neutralization to a constant pH by ammonia. The equations for the steady state were used to computer-simulate the effect of cell-retention time (T_f) on substrate, product and cell-mass concentrations in a single-stage system. The theoretical predictions then were validated by laboratory experiments in which reconstituted and supplemented deproteinized whey was continuously fermented by an adapted culture of Lactobacillus bulgaricus at pH 5.5 and 44 C, without sterilization or asepsis. Lactate concentration was maximum (58.7 mg/ml) at T_f = 27 h, and cell mass was maximum (1.9 mg/ml) at T_f = 9.3 h. The single-stage continuous fermentation of deproteinized whey as compared to that of whole whey, previously described, resulted in similarly high extents of product accumulation and of substrate utilization at similarly high conversion rates. The fermentation of deproteinized whey also was simulated for operation with two stages in series at various retention-time ratios, the results of which predicted that the combined

---

[1]Journal article number 8854 from the Michigan Agricultural Experiment Station.

retention time could be reduced to 17 h with results as good as that with the single-stage operation.

INTRODUCTION

The whey residues of cheese and casein manufacture continue to be a disposal problem for the dairy industry. These residues can be processed by ultrafiltration to obtain concentrates of milk protein (Coton, 1976) of high quality and considerable market potential (Wingerd, 1970; Wingerd et al., 1970; Schingoethe, 1976; DeBoer et al., 1977; Morr, 1976; Delaney, 1976; Mathur and Shahani, 1979). The remaining residue of deproteinized whey retains all of the lactose, however, and a substantial disposal problem still remains.

A potential solution to the problem of economically using whole or deproteinized whey lies with conversion into nitrogen-enriched feedstuff for ruminant animals, accomplished by the fermentation of the lactose into lactic acid and its neutralization by ammonia (Gerhardt and Reddy, 1978; Juengst, 1979). The fermentation can be managed as a batch process (Marshall and Earle, 1975; Reddy et al., 1976), a continuous process (Keller and Gerhardt, 1975; Cox and MacBean, 1977), or a dialysis continuous process (Coulman et al., 1977; Stieber et al., 1977; Stieber and Gerhardt, 1979b). The continuous processes can be managed easily, nonaseptically and indefinitely to produce a uniform product at a useful concentration.

The present study was undertaken to evaluate the use of deproteinized whey instead of whole whey as substrate for the continuous fermentative conversion of the lactose to ammonium lactate. Furthermore, cell-mass was studied for use as a product in itself because of its high nutritional quality (Erdman, et al., 1977). The investigative

procedure was first to develop a mathematical model and use it to simulate a single-stage process for the fermentation. Laboratory experiments then were conducted to test the theoretical predictions. The simulated and experimental results were correlated to evaluate the process, establish values for bacterial metabolic constants, and demonstrate the validity of the model. The validated model finally was used to predict the even greater usefulness of a two-stage process for the fermentation.

THEORY

Design of Fermentation System

Figure 1 shows a schematic of a completely continuous fermentation system designed for the ammonium-lactate fermentation of whey. The symbols correspond to those used previously (Stieber and Gerhardt, 1979b) and are listed in Table 1. The feed into the fermentor, which contains the substrate (lactose) in relatively high concentration (S_f^o), is maintained at a relatively low rate of flow (F_f) consistent with a minimal concentration of unused substrate in the fermentor effluent (S_f). The substrate is converted essentially only to a single product (lactic acid) in the fermentor effluent (P_f). A small concentration of product preexists in the feed (P_f^o). The liquid volume (V_f) and the cell population (X_f) in the fermentor are maintained at constant levels. The addition of ammonia solution is not shown because it does not enter into the theoretical considerations.

For purposes of mathematical modeling, the assumptions were made that: a high rate of mixing insures homogeneity throughout the system; the bacterial metabolic values (μ_m, K_s, K_p, γ, α, and β as identified in Table 1) remain unchanged for a useful period of time;

and the rate of ammonia solution addition is negligible relative to that of substrate feed.

Generalized Mathematical Model

Material-balance equations for the fermentation system were formulated as follows: input + production = output + accumulation. Equations for substrate, product and cell mass in the fermentor are respectively as follows:

$$F_f S_f^o + V_f r_s = F_f S_f + V_f \frac{dS_f}{dt} \tag{1}$$

$$F_f P_f^o + V_f r_p = F_f P_f + V_f \frac{dP_f}{dt} \tag{2}$$

$$F_f X_f^o + V_f r_g = F_f X_f + V_f \frac{dX_f}{dt} \tag{3}$$

Rate equations for substrate utilization, product formation and cell growth were the same as those previously found to be valid (Stieber and Gerhardt, 1979b) and are respectively as follows:

$$-r_s = \alpha r_g + \beta X_f \tag{4}$$

$$r_p = -\gamma r_s \tag{5}$$

$$r_g = \mu_m \left(\frac{S_f}{K_s + S_f + K_p P_f} \right) X_f \tag{6}$$

The rate equations were combined with the material-balance equations, and the variables were defined in dimensionless parameters (Table 2), to obtain a generalized model for the continuous fermentation. The resulting equations for substrate, product and cells are respectively as follows:

$$\frac{d\bar{S}_f}{dt} = \left[-\left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) + \frac{\beta T_f}{\alpha}\right]\bar{X}_f - \bar{S}_f + 1\right]/T_f \qquad (7)$$

$$\frac{d\bar{P}_f}{dt} = \left[\left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) + \frac{\beta T_f}{\alpha}\right]\bar{X}_f - \bar{P}_f + \bar{P}_f^o\right]/T_f \qquad (8)$$

$$\frac{d\bar{X}_f}{dt} = \left[\left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) - 1\right]\bar{X}_f + \bar{X}_f^o\right]/T_f \qquad (9)$$

where $T_f = V_f/F_f$ and is an operational parameter.

Generalized Steady-State Solution

The equations of the generalized model were rearranged and the time derivatives were set at zero to obtain a generalized solution for the steady state. The resulting equations for substrate, product and cell mass are respectively as follows:

$$\bar{S}_f = (\bar{K}_p\bar{P}_f^o + \bar{K}_p + \bar{K}_s)X/(\theta - X + \bar{K}_pX) \qquad (10)$$

$$\bar{P}_f = \left[(1 + \bar{P}_f^o)(\theta - X) - \bar{K}_sX\right]/(\theta - X + \bar{K}_pX) \qquad (11)$$

$$\bar{X}_f = (1 - \bar{S}_f + \bar{X}_f^o)/(1 + \beta\theta/(\alpha\mu_m)) \qquad (12)$$

where $X = (\bar{X}_f - \bar{X}_f^o)/\bar{X}_f = \theta\bar{S}_f/(\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f)$.

Computer Simulated Predictions

The steady-state solution was used to simulate the continuous fermentation of deproteinized whey into ammonium-lactate and cell mass, by programming on a digital computer. Because the simulations were performed before actual experimental fermentations were

conducted, approximations of various conditions were used at first. The resulting simulations for substrate utilization, product formation and cell-mass accumulation were similar to those described previously (Keller and Gerhardt, 1975; Coulman et al., 1977; Stieber and Gerhardt, 1979b) and predicted the conditions used for the following experimental tests.

EXPERIMENTAL MATERIALS AND METHODS

Inoculum

The inoculum, obtained from the fermentor effluent (day 71) of a previous dialysis continuous fermentation (Stieber and Gerhardt, 1979b), was grown at 44 C for 12 to 24 h in a sterile medium of 10% (wt/vol) nonfat milk powder and 90% tap water, stored at 4 C, and transferred biweekly to fresh medium. The original culture, Lactobacillus bulgaricus 2217 (Chris Hanson's Laboratory, Milwaukee, WI), had been selected (Reddy et al., 1976) on the basis of its high rate of acid production in the pH range of 5.0 to 6.0.

Substrate

Dried deproteinized cheese whey (prepared by ultrafiltration; Stauffer Chemical Company, Rochester, MN), used as the fermentation substrate, was rehydrated to contain 62 mg of lactose per ml and was supplemented with 2 mg of yeast extract per ml. The reconstituted whey was made up in 7.5-liter batches without sterilization, stored at 4 C, and held for as long as 10 h in a stirred, heated (78 C) reservoir during use.

Continuous Fermentation System

The fermentation was conducted continuously be metering the whey from a feed reservoir into a fermentor and removing overflow

into a product reservoir, thus maintaining a constant volume in the fermentor. The fermentor contents were mixed thoroughly by stirring and baffling. Nitrogen was continuously purged into the fermentor (1500 ml per min) to force the overflow into the reservoir. Concentrated NH_4OH solution (58%) was automatically fed into the fermentor to maintain a constant pH.

The fermentation was conducted in a 5-liter modular fermentor with automatic temperature control (Microferm model MF 105, New Brunswick Scientific Co., New Brunswick, NJ) and automatic pH control (model pH-40, New Brunswick Scientific). Contents of the fermentor (2.0 to 3.6 liters) were maintained at a temperature of 44 C ± 0.5 C and at pH 5.5 ± 0.05. A plunger-type reciprocating pump (type "P", Bran and Lubbe, Inc., Evanston, IL) was used to meter the flow of whey into the fermentor. The flow rate of ammonia solution and the flow rate of the fermentor effluent were determined at least thrice daily. The system was operated continuously for 17 days.

Analytical Procedures

Samples were taken at least every 8 h from the fermentor effluent. Steady-state data were determined from samples taken at five times the cell-retention time or 60 h after changing a parameter. Lactose in the samples was determined by the colorimetric method of Morris (1948). Lactic acid was determined by use of a gas chromatograph (series 1420, Varian Associates, Palo Alto, CA) equipped with an integrator (model CDS 111, Varian Associates), a thermoconductivity detector, and a stainless steel column (6 ft x 1/8 in O.D.) packed with 10% SP-1000/1% H_3PO_4 on 100/120

Chromosorb WAW (Supelco, Inc., Bellefonte, PA). The samples were prepared by the procedure of Holdeman and Moore (1975).

Dry weight measurements were made by centrifuging cells from 10 ml samples, washing the cells twice with distilled water, and drying the cells at 105 C for at least 18 h.

EXPERIMENTAL RESULTS

Conversion Efficiency and Product Accumulation

Figure 2 shows the experimental results of variation in the cell-retention time (T_f) affecting the residual lactose (S_f) and the accumulation of lactate (P_f) in the fermentor with one-stage operation. The lactose concentration decreased and the ammonium-lactate concentration increased with increased retention time.

Cell Concentration

Figure 3 shows the results of changes in the retention time affecting the cell-mass concentration in the fermentor. With increasing retention time where growth metabolism predominated, the cell concentration at first increased rapidly to a maximum. But beyond a critical retention time where maintenance metabolism superseded growth metabolism, the cell concentration decreased gradually.

Molar Growth Yield

The results of variation in the retention time affecting the molar growth yield (Y_{ATP}, the grams dry weight of cells produced per calculated mole of ATP generated from catabolism) are shown in Figure 4. The molar growth yield decreased with increased retention time; i.e., as the retention time increased, the bacteria increasingly used the energy obtained from catabolism for maintenance and other purposes not associated with growth.

Lactate and Cell Productivities and Lactose Conversion

Figure 5 shows that the lactate productivity and the cell productivity decreased with increased retention time, whereas the percentage of lactose converted to product increased with increased retention time. High lactate and cell productivity, high substrate conversion, and high product concentration are all desirable. Consequently, trade-offs in the regulation of the retention time would be required in designing an operational process.

Product Quality

Samples were regularly analyzed by gas chromatography not only to determine the concentration of lactic acid but also to monitor the possible presence of atypical metabolic products. The results indicated that the fermentation remained homofermentative, i.e., only negligible amounts of products other than lactic acid were formed.

Correlation of Experimental Results With Simulated Predictions

The experimental results were correlated with the simulated predictions to evaluate the metabolic constants of the mathematical model, demonstrate the validity of the model, and evaluate the nature of bacterial metabolism in the process.

The values used for the metabolic constants in the model are listed in Table 3. The values for μ_m, K_s, K_p, α and β were obtained by successive fitting of simulated curves with experimental results. Because the feed reservoir was maintained at 78 C (causing evaporation) and because material balances showed S_f^o to be 74.6 mg of lactose per ml, this value was used instead of 62 mg/ml (which was

measured before the substrate was heated). The value for γ was evaluated from the experimental results.

Figure 2 shows the computer-simulated curves obtained by using the values in Table 3 in the mathematical model to describe the relationships between the retention time (T_f) and the conversion of lactose (S_f) and accumulation of lactate (P_f). Figure 3 shows the relationship between the T_f and the cell mass (X_f). The curves in both figures fitted with the superimposed points of experimental results and thereby demonstrated the validity of the model. With the same values for μ_m, K_s, and K_p, moreover, simulated predictions correlated with experimental results also in a dialysis continuous process (Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979b), additionally validating the model.

The values for K_s and K_p (Table 3) showed that the fermentation was not affected by substrate limitation but was greatly limited by increasing concentrations of product. The relative importance of growth and maintenance metabolism in the fermentation were calculated from knowledge of the values for α and β (Keller and Gerhardt, 1975). At a retention time of 6.4 h, where it was experimentally shown that the cell productivity was greatest (Fig. 5), 63.2 percent of the substrate utilization was calculated to be due to growth metabolism and 36.8 percent to maintenance metabolism. However, at a retention time of 26.7 h, where the lactate concentration was highest (Fig. 2), 29.1 percent of the energy of catabolism was calculated to be due to growth and 70.9 percent to maintenance.

Similar calculations, when correlated with the Y_{ATP} values (Figure 4) and extrapolated to 100 percent, resulted in a mean

Y_{ATP}^{max} of 8.0; this value, which describes the maximal cell mass produced per mole of ATP generated from catabolism, agrees with values reported by Forrest and Walker (1971). The product/substrate ratio (γ) of the fermentation was .96 mg/mg, i.e., 96 percent of the lactose utilized was converted to lactate and 4 percent was incorporated into bacterial cells. On a substrate efficiency basis, the fermentation thus would be better for production of lactate than of bacterial cells.

Simulated Two-Stage Operation

Keller and Gerhardt (1975) improved the conversion efficiency for the continuous fermentation of whole whey by the employment of two equal fermentors in series. In this system the effluent of the first fermentor, containing residual lactose (S_f), lactate (P_f) and cell mass (X_f), flows directly into the second fermentor. With the values for the metabolic constants in Table 3 and the steady-state solution, the two-stage operation was computer simulated for the fermentation of deproteinized whey. The effluent concentrations (S_f, P_f, and X_f) from the first fermentor were used as the feed concentrations (S_f^o, P_f^o, and X_f^o) into the second fermentor. Also, it was assumed there was no lag in growth of the cells entering the second fermentor.

Figure 6A shows the simulated effects of changes in the ratio of the retention time of the second fermentor (T_{f2}) to that of the first fermentor (T_{f1}) on the total retention time ($T_{f1} + T_{f2}$) required to achieve 60 mg of lactate per ml in the effluent of the

second fermentor. The shortest time required was 17 h at T_{f2}/T_{f1} = 1.0 (8.5 h for each stage), which compared to about 26 h for the single-stage operation (T_{f2}/T_{f1} = 0).

Figure 6B shows the simulated effects of changes in T_{f2}/T_{f1} on the accumulation of cell mass in the effluent from the second fermentor. The cell mass was greatest in a two-stage operation with a total retention time of 17 h (8.5 h for each stage) and lowest in the single-stage operation. Thus, two stages provided the optimal conditions for increased cell mass, resulting in an increased rate of converting lactose to lactate.

DISCUSSION

A continuous process for the ammonium-lactate fermentation was previously studied in our laboratory using unsupplemented whole whey (Keller and Gerhardt, 1975). A comparison of the prior results with whole whey and the present results with deproteinized whey, each operated as a single-stage fermentation and at comparable retention times, is shown in Table 4. Use of the two substrates resulted in a similar extent of lactate accumulation and rate of lactose utilization. The percentage of lactose conversion was greater with whole whey than with deproteinized whey; however, the latter was more concentrated in the feed, making the comparison difficult. The concentration of 59 mg of lactate per ml in the effluent from the fermentor probably was near the maximum which a continuous culture without dialysis can tolerate, thus limiting the extent of substrate conversion. Liquid deproteinized whey, before concentration or drying, contains about 46 mg of lactose per ml, which should be capable of complete conversion to product by the continuous process. The rate of lactose utilization with whole

whey was similar to that with deproteinized whey, even though the latter was supplemented with yeast extract. Moreover, the inoculum for the latter fermentation was obtained from a culture with a 163 day history of continuous ammonium-lactate fermentation and thus was well adapted for the fermentation. With these differences taken into account, whole whey probably is a somewhat better substrate for the fermentation but either is practicable. With whole or deproteinized whey, continuous operation of the fermentation is of simple operation, can be managed for prolonged periods of time (perhaps indefinitely), does not require sterilization or asepsis, and produces a uniform product at useful concentration. The ammonium-lactate fermentation is one of the few processes where continuous operation appears more practicable than the conventional batch operation.

Mathematical modeling and computer simulation provide powerful but sometimes unappreciated tools for predicting the results to be expected in a continuous fermentation process. Laboratory experiments need be conducted only to validate the predictions by using a relatively limited number of changes at preselected critical points. The experimental results in turn are used to establish constants and perhaps to indicate additional terms in the mathematical equations. The model can then be further used to evaluate the results. By this process of successive theoretical prediction and experimental validation, the model becomes increasingly accurate and useful to predict how the fermentation should be conducted for accomplishing practical objectives.

The rate equation for substrate utilization in the present mathematical model is the same as in a prior one (Keller and Gerhardt,

1975). However, the prior model requires knowledge of the relative values of α and β (i.e., the ratio of α to β) to simulate substrate utilization and product formation, whereas the present model does not. The absolute values of these constants are required to simulate cell mass concentration with either model. In both, the relative values for the constant were the same (11.0).

The present mathematical model for the fermentation is simple, has a sound biological basis (Stieber and Gerhardt, 1979b), and has values for constants which are realistic. The model was particularly useful for evaluating the metabolic constants of the culture, which were helpful in simulation studies of a dialysis continuous process (Stieber and Gerhardt, 1979b) and also in predictions of the usefulness of a two-stage dialysis or cell-recycle process for the fermentation (Stieber and Gerhardt, unpublished). The validity of the model was demonstrated by the correlation of simulated predictions with experimental results in both the prior studies of continuous fermentation with dialysis (Stieber and Gerhardt, 1979b) and the present studies without dialysis. Consequently, the simulated predictions of the two-stage process (Fig. 6) should be realized experimentally. Furthermore, the model should be useful for on-line control and optimization of such a process for industrial use.

ACKNOWLEDGMENT

This work was supported by grant ENG 76-17260 from the National Science Foundation.

REFERENCES

1 Coton, S. G. 1976. Recovery of dairy waste. In G. G. Birch, K. J. Parker, and J. T. Worgan (ed.), Food from Waste, Applied Science Publishers, Barking, England, p. 221.

2 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.

3 Cox, G. C., and R. D. MacBean. 1977. Lactic acid production by Lactobacillus bulgaricus in supplemented whey ultrafiltrate. Australian J. Dairy Technol. 32:19.

4 DeBoer, R., J. N. DeWit, and J. Hiddink. 1977. Processing of whey by means of membranes and some applications of whey protein concentrate. J. Soc. Dairy Technol. 30:112.

5 Delaney, R. A. M. 1976. Composition, properties and uses of whey protein concentrates. J. Soc. Dairy Technol. 29:91.

6 Erdman, M. D., W. G. Bergen, and C. A. Reddy. 1977. Amino acid profiles and presumptive nutritional assessment of single-cell protein from certain lactobacilli. Appl. Environ. Microbiol. 33:901.

7 Forrest, W. W., and D. J. Walker. 1971. The generation and utilization of energy during growth. Adv. Microbiol. Physiol. 5:213.

8 Gerhardt, P., and C. A. Reddy. 1978. Conversion of agroindustrial wastes into ruminant feedstuff by ammoniated organic acid fermentation: a brief review and preview. Dev. Ind. Microbiol. 19:71.

9 Holdeman, L. V., and W. E. C. Moore. 1975. Anaerobe Laboratory Manual. VPI Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, p. 113.

10 Juengst, F. W., Jr. 1979. Use of total whey constituents - animal feed. J. Dairy Sci. 62:106.

11 Keller, A. K., and P. Gerhardt. 1975. Continuous lactic acid fermentation of whey to produce a ruminant feed supplement high in crude protein. Biotechnol. Bioeng. 17:977.

12 Marshall, K. R., and R. L. Earle. 1975. The effect of agitation on the production of lactic acid by Lactobacillus bulgaricus in a casein whey medium. New Zealand J. Dairy Sci. Technol. 10:123.

13 Mathur, B. N., and K. M. Shahani. 1979. Use of total whey constituents for human food. J. Dairy Sci. 62:99.

14 Morr, C. V. 1976. Whey protein concentrates: an update. Food Technol. 30:18.

15 Morris, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science. 107:254.

16 Reddy, C. A., H. E. Henderson, and M. D. Erdman. 1976. Bacterial fermentation of cheese whey for production of a ruminant feed supplement rich in crude protein. Appl. Environ. Microbiol. 32:769.

17 Schingoethe, D. J. 1976. Whey utilization in animal feeding: a summary and evaluation. J. Dairy Sci. 59:556.

18 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.

19 Stieber, R. W., and P. Gerhardt. 1979. Improved models of dialysis and nondialysis continuous processes for ammonium-lactate fermentation. Proc. 2nd Internat. Conf. Computer Appl. Ferm. Technol. In press.

20 Stieber, R. W., and P. Gerhardt. 1979. Dialysis continuous process for ammonium-lactate fermentation: improved mathematical model and use of deproteinized whey. Appl. Environ. Microbiol. 34:487.

21 Wingerd, W. H. 1970. Lactalbumin as a food ingredient. J. Dairy Sci. 54:1234.

22 Wingerd, W. H., S. Saperstein, and L. Lutwak. 1970. Whey protein concentrate has excellent nutritional properties. Food Technol. 24:758.

TABLE 1. Glossary of Mathematical Symbols

Symbol	Description	Units
F_f	Flow rate into and out of fermentor	ml/h
K_p	Product inhibition constant	mg/mg
K_s	Substrate limitation constant	mg/ml
P_f^o	Product concentration in feed	mg/ml
P_f	Product concentration in fermentor	mg/ml
r_g	Rate of cell growth	mg/ml-h
r_p	Rate of product formation	mg/ml-h
$-r_s$	Rate of substrate utilization	mg/ml-h
S_f^o	Substrate concentration in feed	mg/ml
S_f	Substrate concentration in fermentor	mg/ml
t	Time	h
V_f	Volume of liquid in fermentor	ml
X_f^o	Cell-mass concentration in feed	mg/ml
X_f	Cell-mass concentration in fermentor	mg/ml
Y_{ATP}	Molar growth yield	mg/mmole
α	Substrate/cell ratio	mg/mg
β	Specific maintenance rate	h^{-1}
γ	Product/substrate ratio	mg/mg
E	Efficiency of lactose conversion	%
μ_m	Maximum specific growth rate of cells	h^{-1}
T_f	Retention time of cells in fermentor	h

TABLE 2. Glossary of Dimensionless Parameters

Type	Symbol and Definition	Description
Material parameters	$\bar{P}_f^o = P_f^o/(\gamma S_f^o)$	Product factor in feed
	$\bar{P}_f = P_f/(\gamma S_f^o)$	Product factor in fermentor
	$\bar{S}_f = S_f/S_f^o$	Substrate factor in fermentor
	$\bar{X}_f^o = \alpha X_f^o/S_f^o$	Cell factor in feed
	$\bar{X}_f = \alpha X_f/S_f^o$	Cell factor in fermentor
Kinetic parameters	$\bar{K}_p = \gamma K_p$	Product-inhibition factor
	$\bar{K}_s = K_s/S_f^o$	Substrate-limitation factor
	$\theta = \mu_m T_f$	Time factor

TABLE 3. Values Used to Correlate Simulated Predictions with Experimental Results

Symbol[a]	Value
K_p	2.3 mg/mg
K_s	0.07 mg/ml
P_f^o	2.8 mg/ml
S_f^o	74.6 mg/ml
X_f^o	0 mg/ml
α	11.0 mg/mg
β	1.0 h^{-1}
γ	0.96 mg/mg
μ_m	0.35 h^{-1}

[a]Symbols are described in Tables 1 and 2.

TABLE 4. Comparison of Continuous Ammonium-Lactate Fermentation of Wheys at Two Retention-Time Levels with Single-Stage Operation

Substrate	Cell retention time, T_f (h)	Lactose in feed, S_f^o (mg/ml)	Lactose in fermentor, S_f (mg/ml)	Lactate in fermentor, P_f (mg/ml)	Rate of lactose utilization, r_s (mg/ml-h)	Efficiency of lactose conversion, E (%)
Whole whey without supplementation (Keller and Gerhardt, 1975)	15.0	48	7.0	52	2.73	85.4
	31.0	48	6.5	53	1.34	86.5
Deproteinized whey with 2 mg/ml of yeast extract	16.4	74.6	28.7	46.6	2.80	61.6
	26.7	78.9	21.2	58.7	2.16	73.1

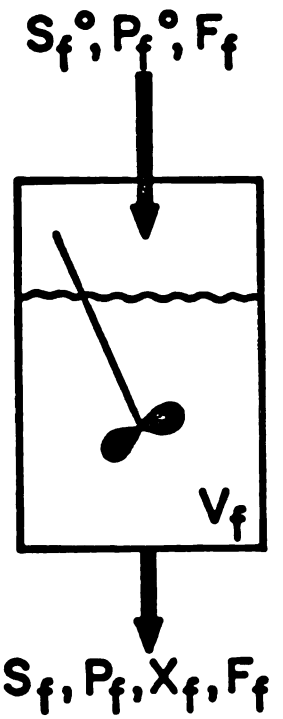


Fig. 1. Schematic of one-stage continuous fermentation system. Symbols are described in Table 1.

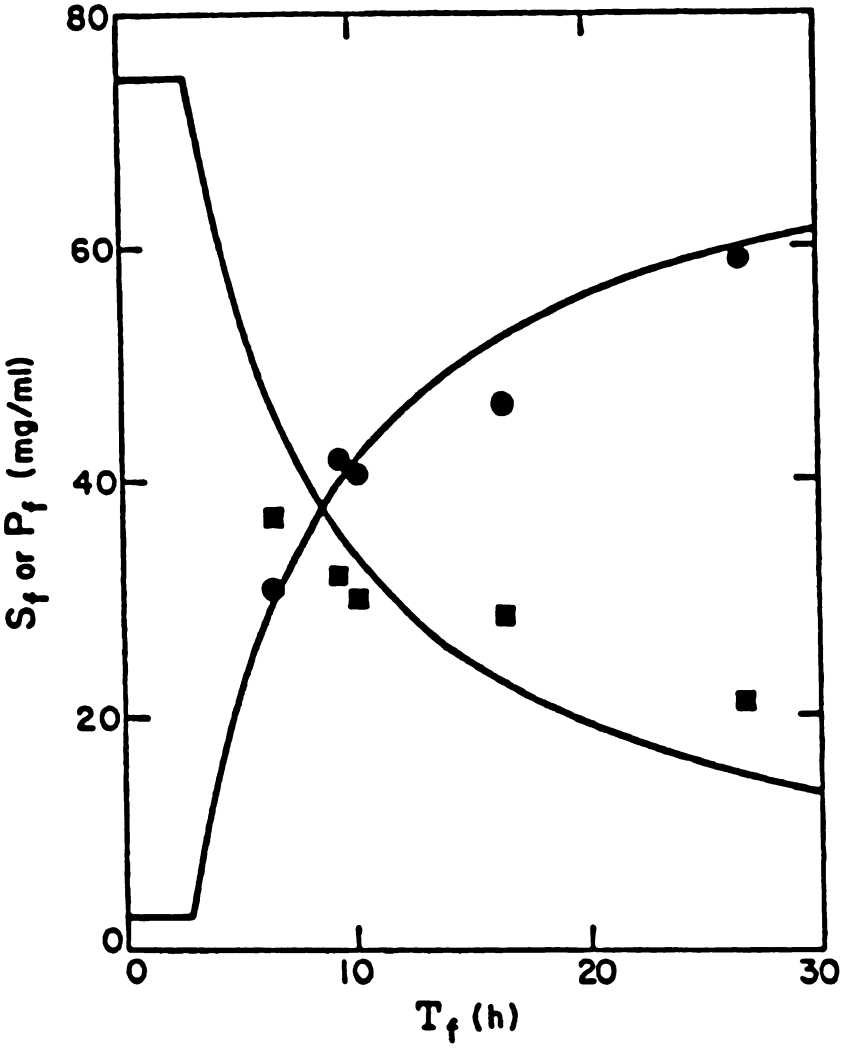


Fig. 2. Computer-simulated effects of cell retention time (T_f) on residual lactose (S_f) and accumulated lactate (P_f) in the fermentor with one-stage operation. The curves were plotted by use of the steady-state solution and the values in Table 3. The points (squares for S_f, circles for P_f) are experimental data and demonstrate the fit between the experimental results and the computer simulations.

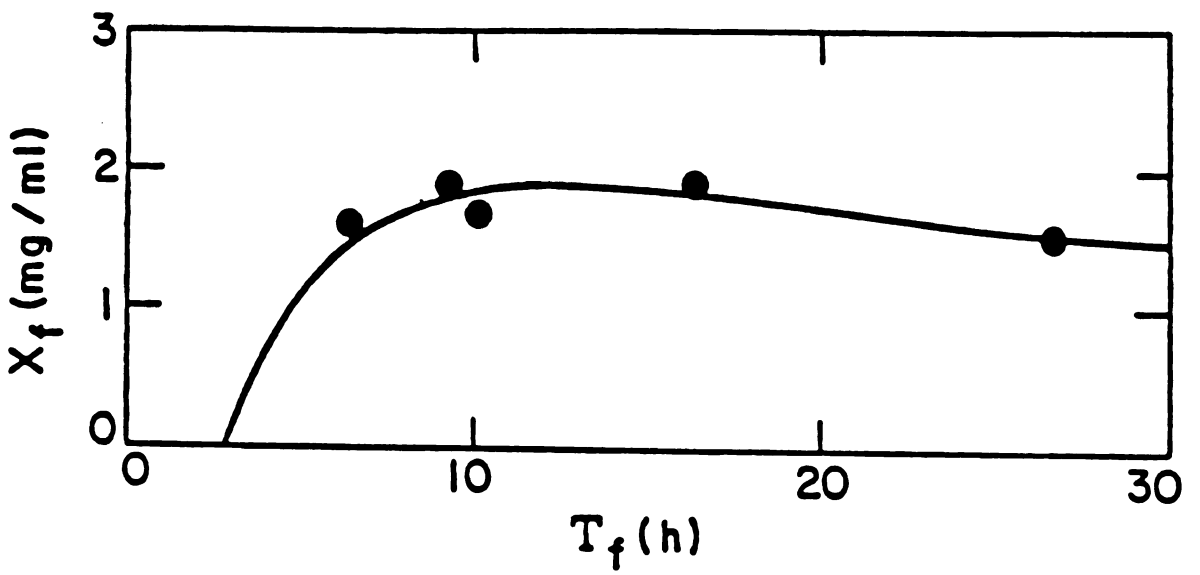


Fig. 3. Computer-simulated effects of cell-retention time (T_f) on cell-mass concentration (X_f) in the fermentor. The curve was plotted by use of the steady-state solution and the values in Table 3. The points are experimental data and demonstrate the fit between the experimental results and the computer simulation.

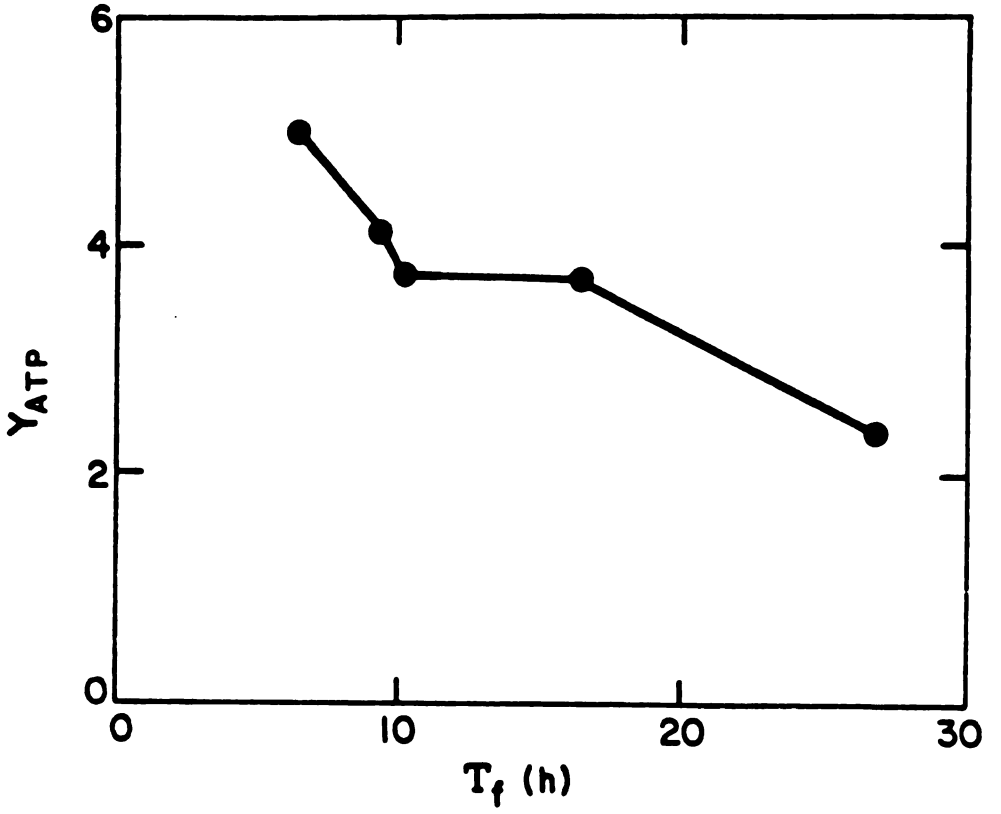


Fig. 4. Experimental effect of changes in cell retention time (T_f) on the molar growth yield (Y_{ATP}).

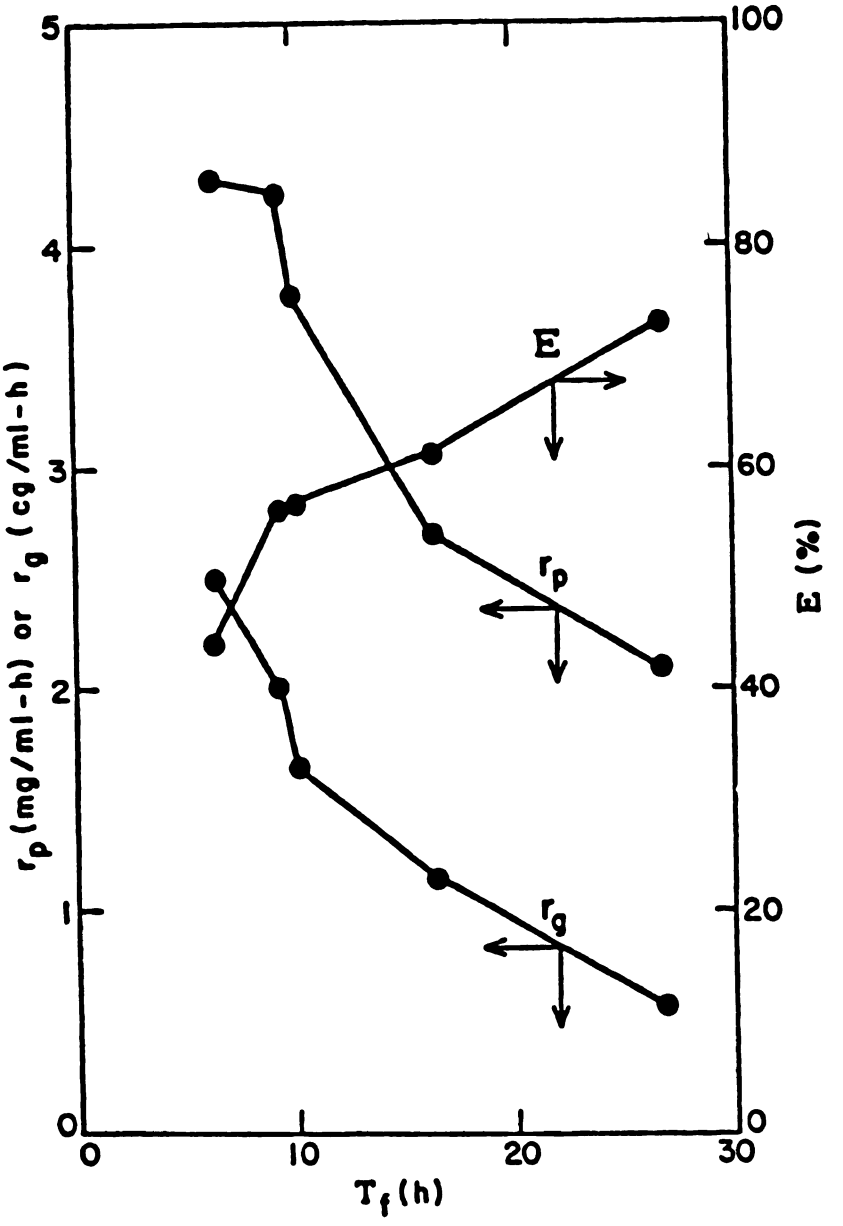


Fig. 5. Experimental effect of changes in cell retention time (T_f) on efficiency of lactose conversion $E = (1 - \bar{S}_f) \times 100$, rate of lactate formation $r_p = (P_f - P_f^o)/T_f$, and rate of cell growth ($r_g = X_f/T_f$).

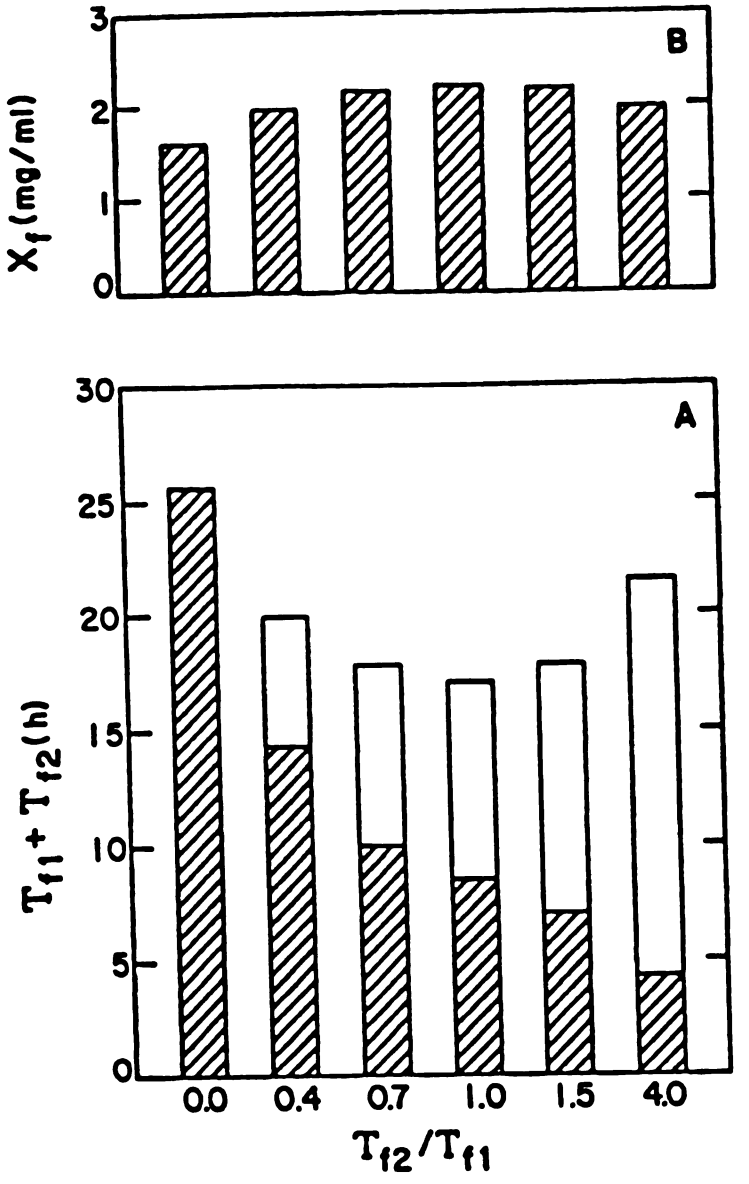


Fig. 6. Computer-simulated two-stage continuous operation for the fermentation. A - The effects of changes in the ratio of retention time of the second fermentor to that of the first fermentor (T_{f2}/T_{f1}) on the total retention time ($T_{f1} + T_{f2}$) required to achieve 60 mg of lactate per ml in the effluent of the second fermentor; the hatched portion of the bars represents T_{f1} and the remaining portion represents T_{f2}. B - The effects of changes in T_{f2}/T_{f1} on the accumulation of cell mass in the effluent of the second fermentor (X_f).

ARTICLE VI

DIALYSIS CONTINUOUS PROCESS FOR AMMONIUM-LACTATE FERMENTATION: SIMULATED PREFERMENTOR AND CELL-RECYCLING SYSTEMS

By

R. W. Stieber and Philipp Gerhardt

Dialysis Continuous Process for Ammonium-Lactate Fermentation: Simulated Prefermentor and Cell-Recycling Systems

R. W. STIEBER and PHILIPP GERHARDT

Department of Microbiology and Public Health,
Michigan State University,
East Lansing, Michigan 48824

SUMMARY

A generalized mathematical model, previously developed and experimentally validated, was modified and used to computer-simulate the outcome of two systems for increasing the rate and extent of substrate conversion of a dialysis continuous process for the ammonium-lactate fermentation of deproteinized whey. The addition of a nondialysis continuous prefermentor would not improve the process. However, the addition of cell recycling (feedback) would greatly improve the process. With 100% recycling the cell-retention time would be reduced ninefold, and the extent of converting substrate to product would be increased 25% and accomplished almost entirely by maintenance metabolism.

INTRODUCTION

A dialysis continuous process can be used effectively for the fermentive production of ammonium lactate and Lactobacillus cells from whole or deproteinized whey (Stieber et al., 1977; Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979c). The products have commercial potential, including use as a nitrogenous feed supplement for ruminants (Juengst, 1979; Gerhardt and Reddy, 1978; Reddy et al., 1976; Keller and Gerhardt, 1975; Coulman et al., 1977). In the process, a high concentration of the whey

(e.g., 240 mg of lactose per ml) is continuously fed into a fermentor, the contents of which are dialyzed through a membrane against a continuous stream of water. Relative to nondialysis continuous or batch processes for the fermentation, the dialysis continuous process enables the use of more concentrated substrate, has a higher rate of substrate utilization and additionally produces a dialysate effluent of cell-free ammonium lactate. With deproteinized whey, however, the effluent from the fermentor contains a considerable residuum of lactose which lessens the value of the process.

Increasing the concentration of cell mass in the fermentor may be a way to increase both the extent and the rate of substrate conversion. Thus, the objective of this study was to test by computer simulation the feasibility of improving the dialysis continuous process with deproteinized whey either by the recycling of cells or by the addition of a nondialysis prefermentor that is optimized for cell-mass accumulation.

Recycling (feedback) of cells in a continuous fermentation process is used to retain cells in a fermentor and thereby to increase the biomass concentration in the fermentor, allowing increased throughput and decreased retention time (Fencl, 1966; Schroeder, 1971; Pirt, 1975). Importantly for the present study, recycling also can be used to reduce substrate concentration in the fermentor effluent (Grieves et al., 1964). Wall growth, a form of cell retention, was modeled (Topiwala and Hamer, 1971) and a prediction of increased biomass productivity was experimentally validated (Wilkinson and Hamer, 1974). Using filtration to retain cells in a fermentor, Pirt and Kurowski (1970) obtained a fourfold increase in biomass productivity.

Topiwala and Khosrovi (1978) theoretically studied product accumulation and its effect on process water recycle in a biomass production process. Weissman and Benemann (1979) demonstrated that recycling can change the outcome of species competition in a mixed continuous culture of Spirulina gertleri and a Chlorella sp. Other studies on recycling with heterogenous populations have been concerned with an activated-sludge process (Ramanathan and Gaudy, 1969; Ramanathan and Gaudy, 1971; Gaudy and Srinivasaraghaven, 1971).

The use of a nondialysis continuous prefermentor from which the effluent goes into a dialysis fermentor is analogous to the "multi-stream" two-stage process modeled by Pirt (1975). He also reviewed the results obtained with other such processes and concluded that they extend the applicability of continuous culture. In particular, such processes may have use for producing secondary metabolites and for obtaining steady-state conditions that sometimes are unobtainable with single-stage systems.

In the present study, a generalized mathematical model of the dialysis continuous process for the fermentation, previously developed and experimentally validated (Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979c), was modified to incorporate a second feed stream for cells, substrate and product into the fermentor. The modified model was used to computer-predict the outcome of adding cell-recycling or a prefermentor to the dialysis continuous process with deproteinized whey as substrate.

MATHEMATICAL MODEL

Design of Fermentation System

Figure 1 shows a schematic of a dialysis continuous process for the ammonium-lactate fermentation of whey with two feed flows into the fermentor circuit. The symbols correspond to those used previously (Stieber and Gerhardt, 1979a) and are listed in Table 1. A usual feed flow (F_f) contains concentrated substrate (S_f^o), a small concentration of product (P_f^o) and no cells. A second cell-feed flow (F_{cf}) contains cells (X_{cf}) obtained either from recycling, (i.e., the return from the fermentor-circuit effluent) or from the effluent of a prefermentor (i.e., a single-stage nondialysis continuous fermentor with feed whey of normal concentration), and may or may not contain residual lactose (S_{cf}) and accumulated ammonium-lactate (P_{cf}). Further rationale for the design of the fermentation system and the assumptions for purposes of mathematical modeling were detailed by Coulman et al. (1977), but an additional assumption for the present process is that there is no lag-growth phase for the cells entering the fermentor circuit.

Generalized Model

From Figure 1, a set of material-balance equations was developed for substrate, product and cell mass in the fermentor circuit and for substrate and product in the dialysate circuit (not shown). Rate-relationship equations for substrate utilization, product formation and cell growth were the same as those found to be valid previously (Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979b; Stieber and Gerhardt, 1979c). The rate equations were combined with the

material-balance equations and the variables were defined in dimensionless parameters (Table II) to obtain a generalized model for the fermentation.

The resulting equations for the fermentor circuit are as follows:

$$\frac{d\bar{S}_f}{dt} = \left[-(1 + \Pi)\bar{S}_f - \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) + \beta T_f/\alpha\right]\bar{X}_f + \Pi\bar{S}_d + \Delta\bar{S}_{cf} + 1 - \Delta\right]/T_f \quad (1)$$

$$\frac{d\bar{P}_f}{dt} = \left[-(1 + R\Pi)\bar{P}_f + \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) + \beta T_f/\alpha\right]\bar{X}_f + R\Pi\bar{P}_d + \Delta\bar{P}_{cf} + (1 - \Delta)\bar{P}_f^o\right]/T_f \quad (2)$$

$$\frac{d\bar{X}_f}{dt} = \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) - 1 + \Delta X\right]\bar{X}_f/T_f \quad (3)$$

where $T_f = V_f/F_f$ and is an operational parameter.

For the dialysate circuit, the corresponding equations are:

$$\frac{d\bar{S}_d}{dt} = \left[-(\phi + \Pi)\bar{S}_d + \Pi\bar{S}_f\right]F_f/V_d \quad (4)$$

$$\frac{d\bar{P}_d}{dt} = \left[-(\phi + R\Pi)\bar{P}_d + R\Pi\bar{P}_f\right]F_f/V_d \quad (5)$$

Generalized Steady-State Solution

The equations of the generalized model were rearranged and combined, and the time derivatives were set at zero, to obtain a generalized solution for the steady state.

Generalized equations for substrate, product and cells in the fermentor circuit are as follows:

$$\bar{S}_f = \frac{(\bar{K}_p - \bar{K}_p\Delta X)\left[1 + \bar{P}_f^o + \Delta(\bar{P}_{cf} + \bar{S}_{cf} - \bar{P}_f^o - 1)\right] - (\bar{K}_s - \bar{K}_s\Delta X)(\pi_p R\Pi - 1 - R\Pi)}{(1 + \Pi - \pi_s\Pi)(\bar{K}_p - \bar{K}_p\Delta X) - (\theta - 1 + \Delta X)(\pi_p R\Pi - 1 - R\Pi)} \quad (6)$$

$$\bar{P}_f = \frac{\left[\Delta(1 + \bar{P}_f^o - \bar{S}_{cf} - \bar{P}_{cf}) - 1 - \bar{P}_f^o\right](\theta - 1 + \Delta X) + (\bar{K}_s - \bar{K}_s\Delta X)(1 + \Pi - \pi_s\Pi)}{(\theta - 1 + \Delta X)(\pi_p R\Pi - 1 - R\Pi) - (\bar{K}_p - \bar{K}_p\Delta X)(1 + \Pi - \pi_s\Pi)} \quad (7)$$

$$\bar{X}_f = \frac{1 - \bar{S}_f + \Pi(\bar{S}_d - \bar{S}_f) + \Delta(\bar{S}_{cf} - 1)}{1 + \beta T_f/\alpha - \Delta X} \quad (8)$$

where $\pi_s = \Pi/(\phi + \Pi)$ and $\pi_p = R\Pi/(\phi + R\Pi)$.

For the dialysate circuit, the corresponding generalized equations are the same as those used previously (Stieber and Gerhardt, 1979a):

$$\bar{S}_d = \Pi\bar{S}_f/(\phi + \Pi) \quad (9)$$

$$\bar{P}_d = R\Pi\bar{P}_f/(\phi + R\Pi) \quad (10)$$

Equations 6 to 10 comprise a generalized steady-state solution for substrate, product and cells in the fermentor and dialysate circuits of the system.

COMPUTER SIMULATIONS

Dialysis Continuous Process With Recycling of Cells

The generalized steady-state solution was used to simulate the dialysis continuous process with recycling of cells for the fermentation of deproteinized whey lactose to ammonium lactate and cells. The feed-cell ratio (X) and the feed-flow ratio (Δ) are dimensionless operating parameters not contained in the previous dialysis model (Stieber and Gerhardt, 1979a) and are defined in Table II with the

other operating parameters. The use of X enables the simulation of substrate utilization and product formation without knowledge of cell concentration. However, the model cannot predict cell-concentration data unless values are given for the substrate/cell ratio (α) and the specific maintenance rate (β). Operationally, Δ should be kept small because the smaller that it is, the less that the feed-substrate concentration (S_f^o) is diluted. The product of Δ and X represents the fraction of cells from the fermentor-circuit effluent which is recycled. Thus, Δ can easily be maintained small if the cells from the fermentor-circuit effluent are concentrated.

The simulations were made by arbitrarily setting the feed-flow ratio (Δ) at .2 and the substrate (S_{cf}) and product (P_{cf}) concentrations in the cell-feed flow (F_{cf}) at 0, and by varying the feed-cell ratio (X) and the time factor (θ). All other parameters and conditions (Table III) were the same as those used before (see Table 5 in reference Stieber and Gerhardt, 1979a; and Table 4 in reference Stieber and Gerhardt, 1979c), which closely simulated the experimental results.

Figure 2 shows the simulated effects of changes in the time factor (θ) on the substrate factor in the fermentor circuit ($\bar{S}_f$) at various fractions of cell-recycle (ΔX). $\bar{S}_f$ decreased with increased θ. More importantly, $\bar{S}_f$ can be reduced completely and at a much smaller θ with total recycling of cells ($\Delta X = 1.0$) than with none ($\Delta X = 0$). The latter prediction (at $\Delta X = 0$) corresponded with experimental results of 75% conversion of lactose to products at a retention time of 27.5 h (Stieber and Gerhardt, 1979a). When given dimensions, the simulated outcome with total recycling of cells

predicted practically complete conversion of lactose (an increase of 25%) at a retention time of only 2.9 h. Furthermore, calculations of growth-associated metabolism ($\alpha\mu$) and nongrowth-associated (maintenance) metabolism (β) showed that, with total recycling of cells, the conversion would be accomplished solely by the latter.

The effect of changes in the time factor (θ) on the cell factor in the fermentor circuit ($\overline{X}_f$) at various fractions of cell-recycling (ΔX) is shown in Figure 3. The highest value of $\overline{X}_f$ occurred with total recycling of cells at $\theta = 1.0$ and corresponded to 10 mg dry weight of cells per ml for the ammonium-lactate fermentation.

Dialysis Continuous Process With Prefermentor

A two-stage dialysis continuous process with deproteinized whey was simulated by use of the experimental results from a nondialysis continuous process at parameters where the cell mass concentration was maximum (S_{cf}, P_{cf} and X_{cf} in Figures 1 and 2 of reference Stieber and Gerhardt, 1979b). The first (nondialysis) stage is optimized for cell-mass accumulation and the effluent from it flows into the second (dialysis) stage, which is also fed with concentrated substrate. The process is simplified in that there is no concentration or separation step between the prefermentor and the dialysis fermentor. With this system, the only way to manipulate the amount of cells entering the dialysis fermentor (X_{cf}) is to change the feed-flow ratio (Δ).

Unlike the simulation of cell recycling, a specific value for the feed-cell ratio (X) was required initially to simulate the effects of changes in the feed-flow ratio (Δ) and the time factor (θ) on the dialysis continuous process with a prefermentor. Whereas X does not vary with θ for the simulation of cell recycling, X does vary with θ

for simulating the dialysis process with a prefermentor because X_{cf} is constant and $\overline{X}_f$ varies with θ (Fig. 3). Thus, to begin the prefermentor simulation, the results from cell-recycling simulation were used to choose the proper value for X (with X_{cf} = 1.85 mg/ml) at each value of Δ.

It was thought that the feed of cells (X_{cf}) into the dialysis fermentor would increase the cell mass in the fermentor circuit (X_f) and favorably affect the rate of lactose utilization (mg/ml-h) and the extent of lactose conversion. Previous work (Stieber and Gerhardt, 1979a) had established the parameters that would accomplish adequate product concentrations. Consequently, the present simulations compared the dialysis process without cell input to the dialysis process with increasing input of cells from a prefermentor with respect to the rate of substrate utilization ($-r_s$), the extent of lactose conversion (E), and the cell-mass accumulation (X_f). The simulations were made by varying the time factor (θ) over a range of feed-flow ratios (Δ), with initial feed-cell ratios (X) determined as described above. All other parameters and conditions (Table III) were the same as those used previously (Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979c), which closely simulated the experimental results.

The simulated results of changes in the feed-flow ratio (Δ) affecting the rate of substrate utilization ($-r_s$) in the dialysis fermentor are shown in Figure 4. At all cell-retention times (T_f), the maximum rate of substrate utilization occurred with no input of cells from a prefermentor.

The extent of substrate conversion (E) increased with increased Δ (Figure 5). Most of the increased conversion, however, occurred

because the feed substrate concentration (S_f^o) was diluted by the input from the prefermentor. The dilution effect of Δ on S_f^o (DS_f^o) represents the amount of feed substrate per total volume entering the fermentor and this also is shown in Figure 5.

Figure 6 shows the simulated effects of changes in the cell-retention time (T_f) on the concentration of cell mass in the fermentor circuit (X_f) at various feed-flow ratios (Δ). Most importantly, X_f decreased with increased Δ. The cell mass was greatest in the dialysis fermentor without cell input ($\Delta = 0$) from the prefermentor.

DISCUSSION

Two systems for increasing the rate and extent of substrate conversion for the continuous ammonium-lactate fermentation of deproteinized whey were modeled and simulated: a two-stage process in which a first nondialysis stage (prefermentor) is optimized for cell mass, the effluent of which goes into a second dialysis stage that is also fed with concentrated substrate; and a single-stage dialysis process with recycling of cells.

The outcomes predicted that the addition of a prefermentor to the dialysis fermentor would only marginally increase the extent of substrate conversion to product and would actually slow the rate of substrate conversion in the dialysis fermentor. The cause of the latter effect is that the effluent of the prefermentor contains lactic acid as well as cells; as more cells from the prefermentor are fed into the dialysis fermentor, more lactate is fed and so further decreases the rate of the fermentation (equations 1, 2 and 3). The addition also would complicate the process. Moreover, the cells from the

prefermentor, upon entering the dialysis fermentor, would be affected by a large increase in substrate concentration and thus a lag-growth phase may result. The use of a prefermentor, furthermore, would lower the concentration of cell mass in the dialysis fermentor. The cause is that with increased feed into the dialysis fermentor from the prefermentor, the feed substrate concentration (S_f^o) is increasingly diluted (Figure 5). In a continuous culture, the cell mass is directly proportional to the feed substrate concentration that can be metabolized by the cells (Stieber and Gerhardt, 1979c). Altogether, the addition of a nondialysis prefermentor to the dialysis fermentor would not improve the process.

On the other hand, the recycling of cells would greatly improve the dialysis continuous process for the ammonium lactate fermentation. of deproteinized whey. The rate equations of the model for substrate utilization, product formation and cell growth have been shown previously to be valid (Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979b; Stieber and Gerhardt, 1979c). The lag-growth phase of the recycled cells upon entering the fermentor should be minimal if the process is managed at steady-state conditions and if a percentage of the cells can be separated from the fermentor-circuit effluent, concentrated and returned quickly to the fermentor circuit without drastic changes. Accordingly, the simulated predictions of increased rates and extents of substrate conversion to product should be realized experimentally.

The recycling of cells also increases the cell mass in the fermentor circuit, which results in greater rates of substrate utilization and product formation. Practically, the recycling of cells would be

accomplished by separation (centrifugation, filtration or sedimentation), and damaged and dead cells may result. Perhaps only 80% cell recycling can be realized, which would still much improve the conversion efficiency and rate of fermentation. On the other hand, the application of cell recycling increases cost and complicates operation. The advantages must compensate the disadvantages if cell recycling is to be useful.

The limiting factor for the recycling of cells is the problem of separating the cells from the effluent and returning the cells to the fermentor without losing cell activity (Gledhill et al., 1973). An alternative system, which may accomplish the same objectives as cell recycling and yet not have the mechanistic problems, may be to use a membrane to retain the cells in the fermentor. In the dialysis continuous processes studied to date (Coulman et al., 1977; Stieber and Gerhardt, 1979a), membranes have been used primarily for the removal of inhibitory products and not for retaining cells. However, the purpose might be accomplished by using a batch fermentor in which the substrate is continuously fed to the fermentor culture by way of membranes and the product is continuously removed from the culture by way of membranes. Essentially 100% cell recycling might be accomplished in this way, amounting essentially to an immobilized cell system.

ACKNOWLEDGEMENT

This work was supported by grants ENG 76-17260 and DAR 79-10236 from the National Science Foundation.

This was assigned journal article no. _____ from the Michigan Agricultural Experiment Station.

REFERENCES

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

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

3. R. W. Stieber and P. Gerhardt, J. Dairy Sci., (1979c) submitted.

4. F. W. Juengst, Jr., J. Dairy Sci., 62, 106 (1979).

5. P. Gerhardt and C. A. Reddy, Dev. Ind. Microbiol., 19, 71 (1978).

6. C. A. Reddy, N. E. Henderson, and M. D. Erdman, Appl. Environ. Microbiol., 32, 769 (1976).

7. A. K. Keller and P. Gerhardt, Biotechnol. Bioeng., 17, 997 (1975).

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

9. Z. Fencl, in Theoretical and Methodological Basis of Continuous Culture of Microorganisms, I. Malek and Z. Fencl, Eds., Academic Press, New York, 1966, p. 67.

10. E. D. Schroeder, Water Research, 5, 29 (1971).

11. S. J. Pirt, Principles of Microbe and Cell Cultivation, John Wiley & Sons, New York, 1975, p. 45.

12. H. H. Topiwala and G. Hamer, Biotechnol. Bioeng., 13, 919 (1971).

13. T. G. Wilkinson and G. Hamer, Biotechnol. Bioeng., 16, 251 (1974).

14. S. J. Pirt and W. M. Kurowski, J. Gen. Microbiol., 63, 357 (1970).

15. H. H. Topiwala and B. Khosrovi, Biotechnol. Bioeng., 20, 73 (1978).

16. J. C. Weissman and J. R. Benemann, Biotechnol. Bioeng., 21, 648 (1979).

17. M. Ramanathan and A. F. Gaudy, Jr., Biotechnol. Bioeng. 11, 207 (1969).

18. M. Ramanathan and A. F. Gaudy, Jr., Biotechnol. Bioeng. 13, 125 (1971).

19. A. F. Gaudy, Jr., and R. Srinivasaraghaven, Biotechnol. Bioeng., 16, 723 (1971).

20. R. B. Grieves, W. O. Pipes, W. F. Milbury, and R. K. Wood, J. Appl. Chem., 14, 478 (1964).

21. R. W. Stieber and P. Gerhardt, J. Dairy Sci., (1979b) in press.

22. W. E. Gledhill, I. D. Hill, and P. H. Hodson, Biotechnol. Bioeng., 15, 963 (1973).

TABLE I

Glossary of Mathematical Symbols

Symbol	Description	Units
A_m	Area of membrane available for dialysis	cm^2
F_d	Flow rate into and out of dialysate circuit	ml/h
F_f^o	Flow rate into fermentor circuit	ml/h
F_f	Flow rate out of fermentor circuit	ml/h
F_{cf}	Flow rate of cell feed into fermentor circuit	ml/h
K_p	Product inhibition constant	mg/mg
K_s	Substrate limitation constant	mg/ml
P_d	Product concentration in dialysate circuit	mg/ml
P_f^o	Product concentration in fermentor feed	mg/ml
P_f	Product concentration in fermentor circuit	mg/ml
P_{cf}	Product concentration in fermentor cell-feed	mg/ml
P_{mp}	Permeability of membrane to product	mg/cm^2-h
P_{ms}	Permeability of membrane to substrate	mg/cm^2-h
r_g	Rate of cell growth	mg/ml-h
r_p	Rate of product formation	mg/ml-h
$-r_s$	Rate of substrate utilization	mg/ml-h
S_d	Substrate concentration in dialysate circuit	mg/ml
S_f^o	Substrate concentration in fermentor feed	mg/ml
S_f	Substrate concentration in fermentor circuit	mg/ml
S_{cf}	Substrate concentration in fermentor cell-feed	mg/ml
t	Time	h
V_d	Volume of liquid in dialysate circuit	ml
V_f	Volume of liquid in fermentor circuit	ml
X_f	Cell-mass concentration in fermentor circuit	mg/ml
X_{cf}	Cell-mass concentration in fermentor feed	mg/ml
α	Substrate/cell ratio	mg/mg
β	Specific maintenance rate	h^{-1}
γ	Product/substrate ratio	mg/mg
E	Extent (efficiency) of lactose conversion	%
μ_m	Maximum specific growth rate of cells	h^{-1}
T_f	Cell-retention time in fermentor circuit	h

TABLE II

Glossary of Dimensionless Parameters

Type	Symbol and definition	Description
Material parameters	$\bar{P}_d = P_d/(\gamma S_f^o)$	Product factor in dialysate circuit
	$\bar{P}_f = P_f/(\gamma S_f^o)$	Product factor in fermentor circuit
	$\bar{P}_f^o = P_f^o/(\gamma S_f^o)$	Product factor in fermentor feed
	$\bar{P}_{cf} = P_{cf}/(\gamma S_f^o)$	Product factor in fermentor cell feed
	$\bar{S}_d = S_d/S_f^o$	Substrate factor in dialysate circuit
	$\bar{S}_f = S_f/S_f^o$	Substrate factor in fermentor circuit
	$\bar{S}_{cf} = S_{cf}/S_f^o$	Substrate factor in fermentor cell feed
	$\bar{X}_f = \alpha X_f/S_f^o$	Cell factor in fermentor circuit
	$\bar{X}_{cf} = \alpha X_{cf}/S_f^o$	Cell factor in fermentor feed
Operational parameters	$R = P_{mp}/P_{ms}$	Ratio of product/substrate membrane permeabilities
	$X = X_{cf}/X_f$	Feed-cell ratio
	$\Delta = F_{cf}/F_f$	Feed-flow ratio
	$\Pi = P_{ms}A_m/F_f$	Membrane permeability factor
	$\phi = F_d/F_f$	Flow-rate ratio
Kinetic parameters	$\bar{K}_s = K_s/S_f^o$	Substrate-limitation factor
	$\bar{K}_p = \gamma K_p$	Product-inhibition factor
	$\theta = \mu_m T_f$	Time factor

TABLE III

Values Used for Computer Simulations of Fermentations

Use	Figures	K_p (mg/mg)	K_s (mg/ml)	P_f^o (mg/ml)	P_{cf} (mg/ml)	R	S_f^o (mg/ml)	S_{cf} (mg/ml)	X_{cf} (mg/ml)	α (mg/mg)	β (h^{-1})	γ (mg/mg)	Δ (ml/ml)	μ_m (h^{-1})	Π
For predictions of dialysis continuous process with cell recycling	2, 3	2.2	.06	9.1	--	3.0	160.3	--	--	7.0	1.2	.96	.2	.35	.5
For predictions of dialysis continuous process with prefermentor	4, 5, 6	2.2	.06	9.1	38.6	3.0	160.3	34.0	1.85	7.0	1.2	.96	--	.35	.5

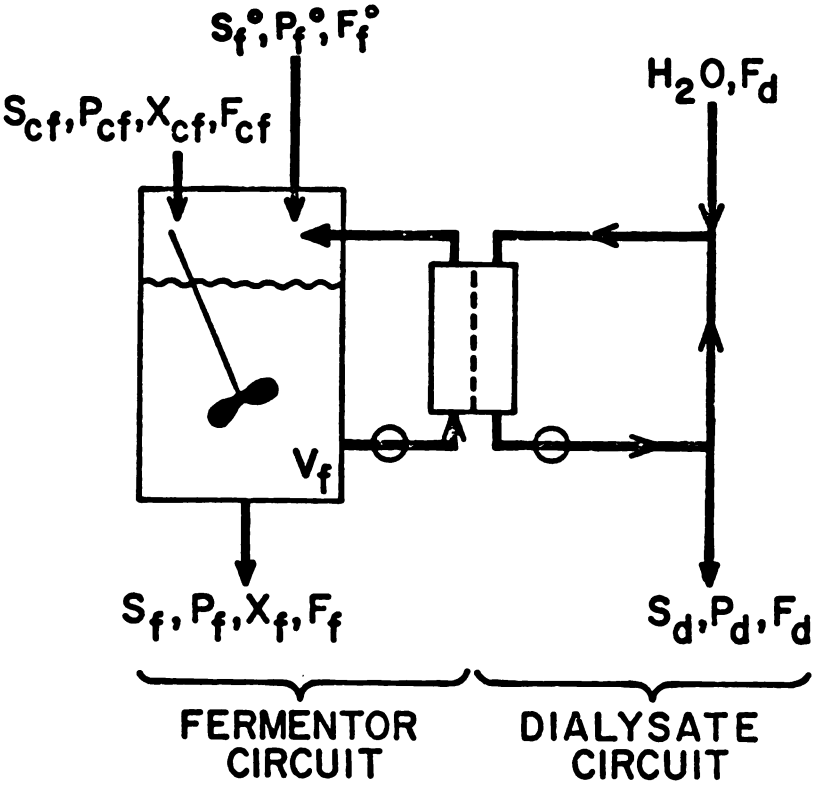


Fig. 1. Schematic of dialysis continuous fermentation system with a cell-feed flow in addition to the usual feed flow. Symbols are described in Table I.

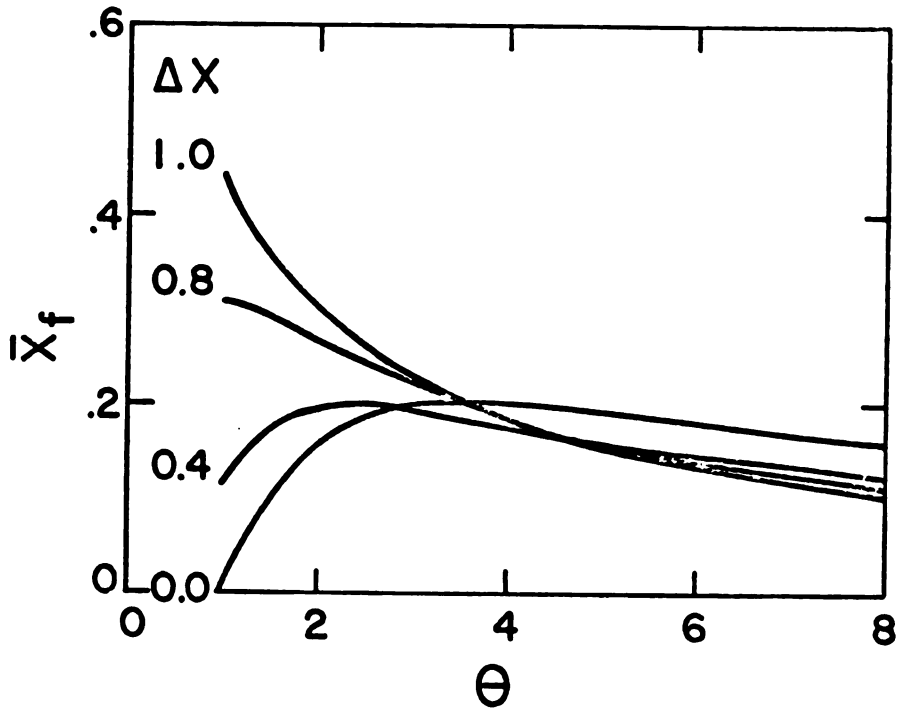


Fig. 3. Simulated effects of changes in the time factor (θ) on the cell factor in the fermentor circuit ($\bar{X}_f$) at four fractions of cell-recycling (ΔX).

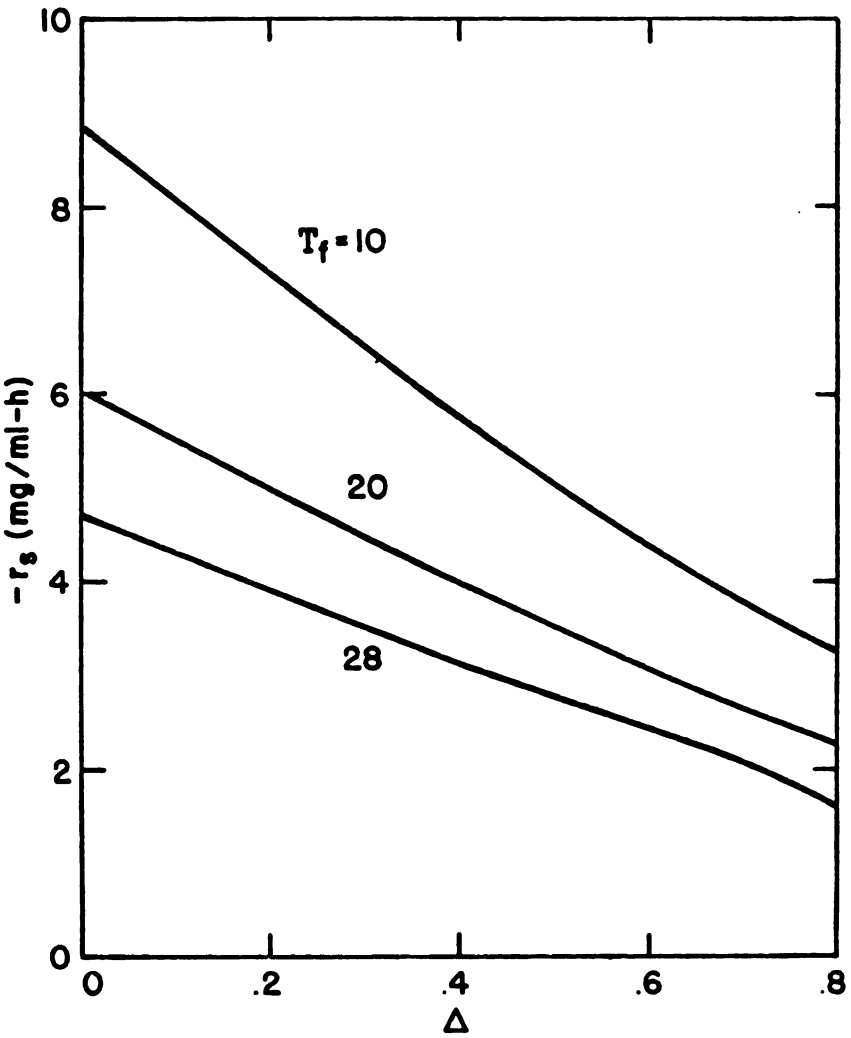


Fig. 4. Simulated effects of changes in the feed-flow ratio (Δ) on the rate of substrate utilization ($-r_s$) at three cell-retention times (T_f) for the dialysis continuous process with a prefermentor. $-r_s = \left[S_f^o F_f (1 - \Delta) + S_{cf} F_{cf} - (S_f F_f + S_d F_d)\right] / (T_f F_f)$.

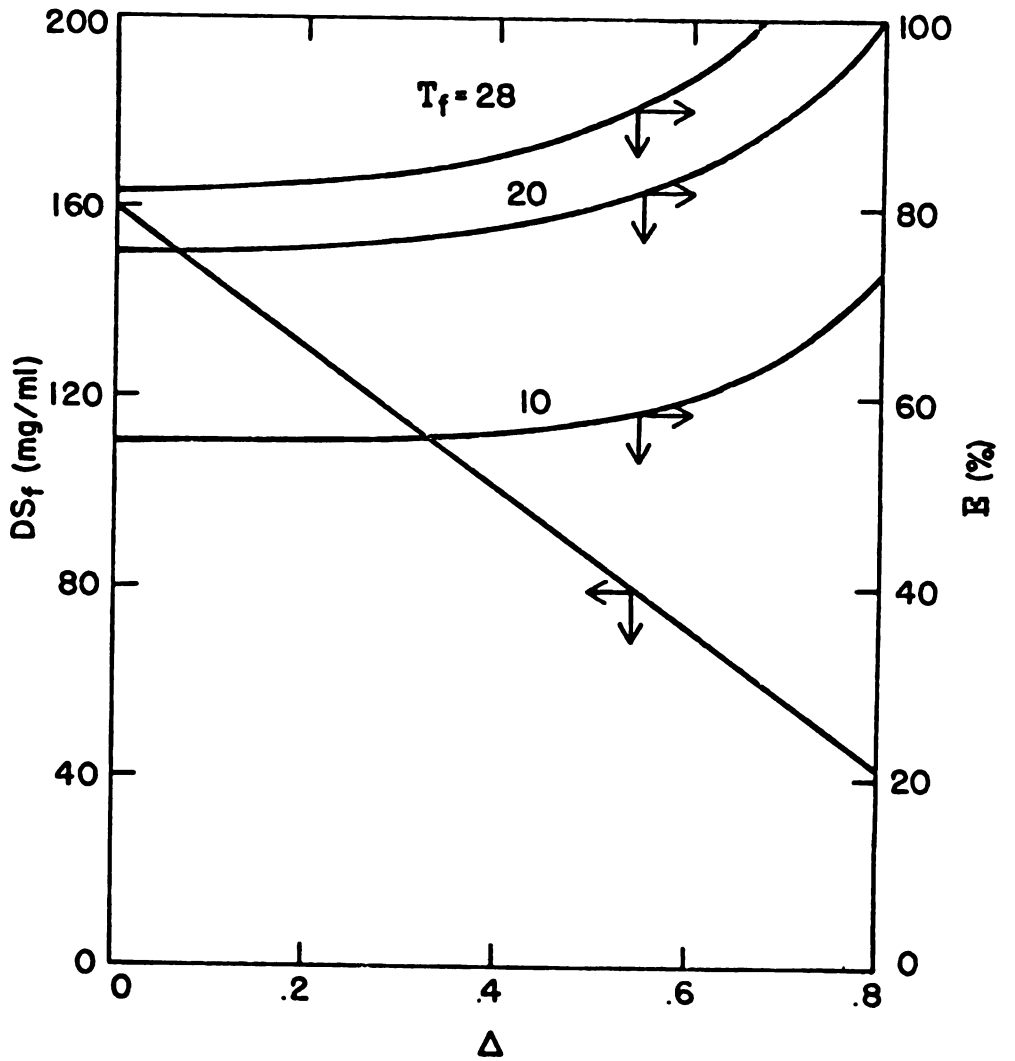


Fig. 5. Simulated effects of changes in the feed-flow ratio (Δ) on the extent of substrate conversion (E) and on diluting the feed substrate concentration (DS_f^o) at three cell-retention times (T_f) for the dialysis continuous process with a prefermentor. $E = \left[1 - (F_f S_f + F_d S_d)/(F_{cf} S_{cf} + (1 - \Delta) S_f^o)\right] \times 100$. $DS_f^o = (1 - \Delta) S_f^o + \Delta S_{cf}$.

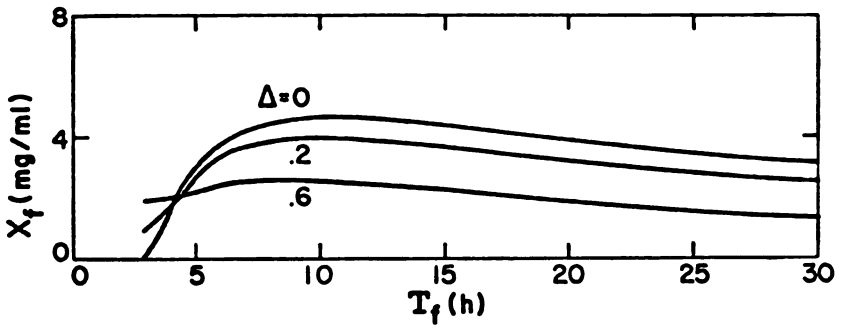


Fig. 6. Simulated effects of changes in the cell-retention time (T_f) on the cell-mass concentration in the fermentor circuit (X_f) at three feed-flow ratios (Δ) for the dialysis continuous process with prefermentor.

ARTICLE VII

DIALYSIS CONTINUOUS PROCESS FOR AMMONIUM-LACTATE FERMENTATION: SIMULATED AND EXPERIMENTAL DIALYSATE-FEED SYSTEMS

By

R. W. Stieber and Philipp Gerhardt

Dialysis Continuous Process for Ammonium-Lactate Fermentation: Simulated and Experimental Dialysate-Feed Systems

R. W. STIEBER and PHILIPP GERHARDT

Department of Microbiology and Public Health,
Michigan State University,
East Lansing, Michigan 48824

SUMMARY

A generalized mathematical model, previously developed and experimentally validated, was modified and used to computer simulate a dialysate-feed system for operating a dialysis continuous process for the ammonium-lactate fermentation of whey. Outcomes predicted that the feeding of substrate into the fermentor via dialysis should greatly improve the production of cell mass and cell-free metabolite. Experiments were conducted to test selected predictions. Dried cheese-whey ultrafiltrate was rehydrated to contain a normal concentration of lactose (62 mg per ml) supplemented with yeast extract, charged into a 5-liter fermentor without sterilization, adjusted in pH (5.5) and temperature (44°C), and inoculated with an adapted culture of Lactobacillus bulgaricus. A series of steady-state conditions was managed without asepsis for 26 days. Results showed that the dialysate-feed system is a useful way to obtain immobilized cells for the purpose of producing a cell-free metabolite at a high rate. The substrate consumed by the cells is converted only via maintenance metabolism and is sterilized by dialysis.

INTRODUCTION

Previous studies of a dialysis continuous process for the ammonium-lactate fermentation of whey (Coulman et al., 1977; Stieber et al., 1977; Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979c) were conducted in a system in which substrate is fed into a fermentor and the fermentor contents are dialyzed against a stream of water to remove an inhibitory product (lactic acid), thus maximizing the rate and extent of substrate (lactose) conversion. Relative to nondialysis continuous or batch processes, this dialysis continuous process enabled the use of more concentrated substrate, had a higher rate of substrate utilization, produced cells at a higher rate and concentration, and additionally produced a dialysate effluent containing a cell-free product.

An alternative way to operate a dialysis continuous process is to dialyze the fermentor contents against a dialysate stream containing substrate, primarily for the purpose of retaining cells in the fermentor and thereby increasing the concentration of cells and throughput of substrate. In this dialysate-feed system, the substrate enters the fermentor by diffusion through membranes and thereby is sterilized. Similarly, a metabolite product leaves the fermentor by dialysis and thus is free of cells.

The dialysate-feed system was studied by use of the ammonium-lactate fermentation of cheese whey by Lactobacillus bulgaricus as a model. The fermentation advantageously can be conducted without sterilization or asepsis because of the restrictive conditions of low pH (5.5), high concentration of undissociated acid, high temperature (44^{o}C), and anaerobiosis. The disposal of whey is a

problem in the dairy industry, and the product of the fermentation has commercial potential, including use as a nitrogen-enriched feed supplement for ruminant animals (Gerhardt and Reddy, 1978; Juengst, 1979). Consequently the fermentation also has important economic implications.

A mathematical model, previously developed and experimentally validated (Stieber and Gerhardt, 1979a; Stieber and Gerhardt, 1979c), was modified and used to computer-simulate the outcomes of operating the dialysate-feed system for the fermentation. The simulations were used to determine the optimum conditions for producing either ammonium-lactate or lactobacillus cells. Laboratory experiments then were conducted to test selected predictions. (This paper was presented in part at the American Chemical Society Annual Meeting, September 10-15, 1978, Miami Beach, Florida.)

THEORY

Design of Dialysate-Feed System

Figure 1 shows a schematic of the dialysate-feed system. The symbols correspond to those used previously (Stieber and Gerhardt, 1979a) and are listed in Table I.

The feed into the dialysate circuit contains the substrate (lactose) in normal concentration (S_d^o) and is maintained at a relatively high rate of flow (F_d). A small concentration of product (lactic acid) preexists in the whey feed (P_d^o). The dialysate circuit consists only of tubing, pump and the dialysate side of the dialyzer, and contains a relatively small volume (V_d) which is continuously circulated. Substrate which is not transferred by the dialyzer to the fermentor circuit remains in the dialysate effluent (S_d).

Product from the whey feed as well as that which is transferred from the fermentor circuit by the dialyzer remains in the dialysate effluent (P_d).

In the fermentor circuit, the liquid volume (V_f) and cell mass (X_f) are maintained at constant levels. Substrate that is transferred from the dialysate circuit by the dialyzer is converted to product (P_f). Unused substrate remains in the fermentor effluent (S_f) along with P_f and X_f.

Further rationale for the design of a dialysis continuous fermentation system and the assumptions for purposes of mathematical modeling are detailed by Coulman et al. (1977).

Generalized Model

From Figure 1 a set of material-balance equations (not shown) was developed for substrate, product, and cell mass in the fermentor circuit and for substrate and product in the dilaysate circuit. Rate-relationship equations for substrate utilization, product formation and cell growth were the same as those used previously (Stieber and Gerhardt, 1979a). The rate equations were combined with the material-balance equations and the variables were defined in dimensionless parameters (Table II) to obtain a generalized model for the system.

The resulting equations for the fermentor circuit are as follows:

$$\frac{d\bar{S}_f}{dt} = \left[-(1 + \phi\Pi)\bar{S}_f - \theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) + \frac{\beta T_f}{\alpha}\bar{X}_f + \phi\Pi\bar{S}_d\right]/T_f \qquad (1)$$

$$\frac{d\bar{P}_f}{dt} = \left[-(1 + \phi R\Pi)\bar{P}_f + \theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) + \frac{\beta T_f}{\alpha}\bar{X}_f + \phi R\Pi\bar{P}_d\right]/T_f \quad (2)$$

$$\frac{d\bar{X}_f}{dt} = \left[\theta\left(\frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f + \bar{K}_p\bar{P}_f}\right) - 1\right]\bar{X}_f/T_f \quad (3)$$

where $T_f = V_f/F_f$.

For the dialysate circuit, the corresponding equations are as follows:

$$d\bar{S}_d = \left[-(1 + \Pi)\bar{S}_d + \Pi\bar{S}_f + 1 + 1/\phi\right]F_d/V_d \quad (4)$$

$$d\bar{P}_d = \left[-(1 + R\Pi)\bar{P}_d + R\Pi\bar{P}_f + \bar{P}_d^o + \bar{P}_d^o/\phi\right]F_d/V_d \quad (5)$$

Generalized Steady-State Solution

The equations of the generalized model were rearranged and combined, and the time derivatives were set at zero, to obtain a generalized solution for the steady state.

The resulting equations for substrate, product and cell mass for the fermentor circuit are as follows:

$$\bar{S}_f = \frac{\bar{K}_s(R\Pi - R\Pi\pi_p + 1/\phi) + \bar{K}_p(\pi_p\bar{P}_d^o + \pi_p\bar{P}_d^o/\phi + \pi_s + \pi_s/\phi)}{(\theta - 1)(R\Pi - R\Pi\pi_p + 1/\phi) - \bar{K}_p(\pi_s\Pi - \Pi - 1/\phi)} \quad (6)$$

$$\bar{P}_f = \frac{(\theta - 1)(\pi_p \bar{P}_d^o + \pi_p \bar{P}_d^o/\phi + \pi_s + \pi_s/\phi) + \bar{K}_s(\pi_s \Pi - \Pi - 1/\phi)}{(\theta - 1)(R\Pi - R\Pi\pi_p + 1/\phi) - \bar{K}_p(\pi_s \Pi - \Pi - 1/\phi)} \quad (7)$$

$$\bar{X}_f = \frac{\phi\Pi(\bar{S}_d - \bar{S}_f) - \bar{S}_f}{1 + \beta\theta/(\mu_m \alpha)} \quad (8)$$

where $\pi_s = \Pi/(\phi + \Pi)$ and $\pi_p = R\Pi/(\phi + R\Pi)$.

For the dialysate circuit, the corresponding equations are as follows:

$$\bar{S}_d = (\Pi\bar{S}_f + 1 + 1/\phi)/(1 + \Pi) \quad (9)$$

$$\bar{P}_d = (R\Pi\bar{P}_f + \bar{P}_d^o + \bar{P}_d^o/\phi)/(1 + R\Pi) \quad (10)$$

Computer-Simulated Predictions

The generalized steady-state solution (equations 6 to 10) was used with a digital computer to simulate operation of the system. Because the fermentation was simulated before laboratory experiments were conducted, approximate values of various parameters were used (Table III). The values for μ_m, K_s, K_p, γ, and R were obtained from previous dialysis fermentations with deproteinized whey (Stieber and Gerhardt, 1979a). The values for α and β were obtained similarly (Stieber and Gerhardt, 1979c). The values for S_d^o and P_d^o were chosen arbitrarily.

The simulation outcomes in Figure 2 show the effects of changes in the cell-retention time (T_f) affecting the residual substrate (S_d) and the accumulation of product (P_d) in the dialysate circuit at a constant flow-rate ratio (ϕ). As is characteristic of conventional

continuous fermentation processes, the substrate decreased and the product increased with increasing retention time. The simulation outcomes were almost identical at other values of ϕ (e.g., ϕ = 1.0 or 20.0).

In Figure 3 are shown the simulated effects of changes in the membrane permeability factor (Π) on the substrate concentration in the fermentor circuit (S_f) and in the dialysate circuit (S_d). A high value of Π (at least 8.0) is required to obtain a concentration of substrate in the fermentor circuit similar to that in the dialysate circuit. The substrate concentration rather than the product concentration was simulated to demonstrate the membrane permeability requirement, because it was thought that the fermenting culture would be limited mostly in obtaining substrate for metabolic purposes.

Figure 4 shows the simulated effects of changes in the cell-retention time (T_f) affecting the cell mass concentration (X_f) at three flow-rate ratios (ϕ). At first, with increasing retention time where growth metabolism (α) predominated, X_f increased to a maximum. But beyond a critical retention time, maintenance metabolism (β) superseded that of growth and then X_f decreased. X_f also increased greatly with increased ϕ. In fact, the results predicted that the cell-mass productivity of the present process (3.5 mg/ml-h at ϕ = 20.0) would be about 20-fold greater than that for an ordinary continuous process for the same fermentation (Stieber and Gerhardt, 1979b) at a retention time of 10 h, where X_f was greatest in both processes.

Design, Solution and Simulation of Dialysate-Feed, Immobilized-Cell System

The system can be modified to maximize the production of the metabolite product by eliminating the fermentor effluent stream (Figure 5), which results essentially in an immobilized-cell system with the metabolite product removed only in the dialysate effluent. Schultz and Gerhardt (1969) termed this a system with a continuous reservoir and a batch fermentor.

A generalized steady-state solution was obtained for this dialysate-feed, immobilized-cell system by setting the fermentor flow rate (F_f) equal to zero and reducing equations 6 to 10. For the fermentor circuit, the resulting equations for substrate, product and cell mass are as follows:

$$\bar{S}_f = 0 \tag{11}$$

$$\bar{P}_f = \frac{\pi_p \bar{P}^o_d + \Pi_s}{R\Pi - R\Pi\pi_p} \tag{12}$$

$$\bar{X}_f = \frac{D\Pi}{(1 + \Pi)\beta/\alpha} \tag{13}$$

where the hydraulic dilution rate (D) equals F_d/V_f.

For the dialysate circuit, the corresponding equations are as follows:

$$\bar{S}_d = 1/(1 + \Pi) \tag{14}$$

$$\bar{P}_d = (R\Pi\bar{P}_f + \bar{P}^o_d)/(1 + R\Pi) \tag{15}$$

This solution importantly indicates that the concentrations of substrate (S_f and S_d) and product (P_f and P_d) in the fermentor and dialysate circuits are independent of the hydraulic dilution rate (D). The accumulation of cell mass (X_f) is linear with respect to D. By use of values for the constants listed in Table III, a graphical representation of the effects of changes in D on S_d, P_d, and X_f is shown in Figure 6. The simulation outcomes predict that a satisfactory low level of S_d (< 5 mg/ml) and a high level of P_d can be obtained no matter what dilution rate is used. No change in the permeability factor (Π) is required with increased D as long as D is increased by decreasing the volume of the fermentor circuit (V_f). However, if D is increased by increasing the flow rate into the dialysate circuit (F_d), there must be a concurrent increase in the permeability (P_{mp} and P_{ms}) or the area (A_m) or the dialyzer membranes to maintain the desired concentrations of S_d and P_d.

Figure 6 also shows that the accumulation of cell mass (X_f) is directly proportional to the dilution rate (D). In other words, given a constant flow rate of feed (F_d) into the system, no matter what the fermentor-circuit volume (V_f) is, X_f remains unchanged. Thus the conversion of substrate to product importantly would be accomplished only by maintenance metabolism of the cells.

The steady-state solution also predicts complete substrate utilization in the fermentor circuit (see equation 11). The cell mass (X_f) would increase in the fermentor circuit until substrate becomes limiting, assuming that there is no accumulation of an inhibitory product in the fermentor circuit.

Design of Dialysate-Feed, Fermentor-Throughput System

The system can be further modified by eliminating the dialysate effluent stream (Figure 7), so that the metabolite product as well as the cells are removed only in the fermentor effluent.

A generalized steady-state solution was obtained for this dialysate-feed, fermentor-throughput system by setting the dialysate effluent flow rate (F_d) sequal to zero, so that the flow rate of the dialysate feed (F_d^o) equals that of the fermentor effluent (F_f). Equations 6 to 10 were then reduced as before.

EXPERIMENTAL MATERIALS AND METHODS

Inoculum

The inoculum culture was obtained from the fermentor effluent (day 71) of a previous dialysis continuous fermentation (Stieber and Gerhardt, 1979a) which originally was started with Lactobacillus bulgaricus 2217 (Chris Hanson's Laboratory, Milwaukee, Wisconsin).

Substrate

Dried deproteinized whey (prepared by ultrafiltration of whole sweet-cheese wheys by Stauffer Chemical Co., Rochester, Minnesota) was rehydrated to contain 62 mg of lactose per ml and was supplemented with 2 mg of yeast extract per ml. The reconstituted whey ultrafiltrate was made up in 7-liter batches without sterilization, stored at 4°C and held in a stirred, heated (64°C) reservoir during use.

Fermentation Systems

The experimental dialysis continuous fermentations were operated at a temperature of 44°C and pH of 5.5 in the fermentor and with essentially the same equipment as used previously (Stieber et al.,

1977; Stieber and Gerhardt, 1979a). However, membrane filters of 0.2 μ_m pore diameter (Versapore 200; Gelman Instrument Company, Ann Arbor, Michigan) were used instead of the smaller pored dialysis membrane used before. Eight membranes, providing 2,300 cm^2 of effective area, were used in the dialyzer. The pressures in the fermentor and dialysate circuits varied throughout the fermentation and were balanced by manually changing the circulation rate (2 to 4 liters per min.) in the fermentor circuit. A series of steady-state conditions was managed without asepsis and without interruption for 26 days.

Analytical Procedures

Samples were taken at 8 h intervals from the dialysate effluent and from a glass "T" inserted in the tubing between the fermentor and dialyzer. Lactose in the samples was determined by the colorimetric method of Morris (1948). Lactic acid was determined by the colorimetric method of Pryce (1969). Cell-mass measurements were made by centrifuging the cells from 10 ml samples, washing the cells twice with distilled water, and drying them at 105°C for at least 18 h.

EXPERIMENTAL RESULTS

Dialysate-Feed, Immobilized-Cell System

The dialysate-feed, immobilized-cell system (Figure 5) was operated at steady-state conditions ($D = 0.92\ h^{-1}$) for 13 days (Table IV). As predicted by the simulations, a low level of residual lactose (S_f) and a high level of lactate (P_f) in the fermentor circuit were maintained throughout the operation. Complete lactose utilization did not occur in the fermentor circuit because lactate accumulated to inhibitory levels. During operation of the system, the pressures in the fermentor and dialysate circuits kept changing,

resulting in variation of the fermentor volume. The variation had little effect on the lactose and lactate concentrations (Table IV), but did affect the cell-mass concentration. Although the fermentor volume (V_f) may change, the total cell mass (X_f) in the fermentor should remain unchanged (Figure 6). The values for X_f listed in Table IV are corrected values that are based on a mean V_f of 2,685 ml.

Examination of the data for cell mass (X_f) shown in Table IV indicated that three different periods occurred during the 13 days of operating the system. For the first few days X_f remained at an average value of 1.02 mg/ml, suggesting that the energy resulting from catabolism was used only for maintenance (nongrowth associated) purposes. Next there was a four day period of increasing X_f during which the extent (E) and the rate ($-r_s$) of substrate utilization also increased, though to a much lesser degree. In the period of the last six days, X_f again remained stable and at a mean value (4.54 mg/ml) which was closely predicted by the model (Figure 6).

At a similar dilution rate (D) and extent (E) and rate ($-r_s$) of substrate utilization, the cell mass (X_f) was 4.5 times greater in period 3 than in period 1. Substrate was not limiting throughout the fermentation except on day 16 where the steady-state condition of the experiment was interrupted with dilute feeding. An explanation for the increased cell mass in period 3 is that the interruption with dilute feeding caused each cell to lower its metabolic rate because of the lower ratio of substrate to cells. After the interruption, with correct feed concentration and with cells adjusted to a lower metabolic rate, the cells increased their metabolism to include that for growth and then the cell mass increased to a new level.

This transition occurred in period 2. Growth metabolism ceased at the start of period 3, and the cells remained at the low metabolic rate. However, E and $-r_s$ remained the same as in period 1 because of the cell-mass increase. Microscopic examination showed that there was no difference in the morphology of the cells during the three periods: the cells appeared homogenous and healthy, and very little cell debris was observed. This explanation postulates that L. bulgaricus can maintain itself at different metabolic rates in a given environment. The metabolic rate depends on the initial start-up conditions (e.g., cell and substrate concentrations, temperature, etc.). An analogous situation was observed with a continuous culture of Bacillus linens growing exponentially (Finn and Wilson, 1954). The culture was interrupted twice and, following each interruption, the system achieved a new steady population of different size. Though the metabolic rate of each B. linens cell did not change, the accumulation of cell mass depended on the initial conditions and not on a limiting concentration of substrate.

From material-balance data (Table IV), the equivalent lactose concentration in the dialysate-feed stream (S_d^o) was calculated by use of a conservation-of-mass equation. The mean calculated value (60.5 mg/ml) agreed well with the mean analytical value (62 mg/ml) for S_d^o, confirming that no significant portion of the substrate was lost to products other than lactic acid.

The dialysate-feed, immobilized-cell system was also operated on fermentation days 4 through 7 at steady state conditions. The cell mass (X_f) remained stable (mean X_f = 2.66 mg/ml) at a dilution

rate (D) of 0.15 h^{-1} for the four days. The residual lactose was 8.7 mg/ml in the fermentor circuit (S_f) and 29.5 mg/ml in the dialysate circuit (S_d). The lactate was 52.5 mg/ml and 36.0 mg/ml in the fermentor and dialysate circuits (P_f and P_d), respectively.

Dialysate-Feed, Fermentor-Throughput System

The dialysate-feed, fermentor-throughput system (Figure 7) was operated on fermentation days 10 and 11 to demonstrate the process. At steady-state conditions and a retention time (T_f) of 7.6 h, the mean fermentor effluent concentrations of cell mass (X_f) and lactate (P_f) were 0.63 mg/ml and 34.3 mg/ml, respectively. The system may have application for a continuous fermentation in which the substrate contains vitamins or other labile molecules that must be continuously sterilized by dialysis. Also, the energy requirements of the system may be less than those which utilize the usual procedures of heating.

DISCUSSION

The experimental results indicated that dialysis provides a novel and useful method for immobilizing microbial cells for the purpose of producing a cell-free metabolite at a high rate without appreciable expenditure of substrate for cell growth. A particular advantage of this particular method is that each cell is not physically immobilized, thus allowing maximum cell surface area for fermentation activity. Further, since the cell population as a whole is immobilized, the process can be operated at a hydraulic dilution rate greater than the maximum specific growth rate of the bacterium. The cell mass should increase in the fermentor circuit until substrate becomes limiting or until an inhibitory concentration of product accumulates. Thus, removal of the inhibitory concentration by dialysis

should result in complete substrate utilization in the fermentor circuit. Other advantages are that dialysis serves also to sterilize the feed and the process produces a cell-free fermentation metabolite.

The limiting factor in the process is the solute exchange capacity of the dialyzer, as indicated by the large differences between S_f and S_d and between P_f and P_d in Table IV. Further, the differences increased as the fermentation progressed.

Improvement in the design of the present dialyzer (Schultz and Gerhardt, 1969) seems attainable (Dawids and Boe, 1977a; Dawids and Boe, 1977b). Membrane permeability also should be improvable; the membrane used in the present study was relatively thick, resulting in decreased performance. Improvement also should be obtainable by using a greater membrane area.

Another potential for improvement of the process is to maintain a constant pressure differential between the fermentor and dialysate circuits. It was unknown why the pressure kept changing and gradually increasing throughout the fermentation, as the chemical potential was always lower in the fermentor circuit. A pressure monitoring and regulating system for the process is being investigated.

The computer simulations of the model were useful for predicing the characteristics of the systems. The simulations also increased that the primary system (Figure 1) could be useful for the continuous production of a high concentration of cells at a high rate. Though the process is not simple, it may have potential for the continuous production of a valuable enzyme or cell, or for producing starter cultures for the dairy industry.

ACKNOWLEDGMENT

This work was supported by grants ENG 76-17260 and DAR 79-10236 from the National Science Foundation.

REFERENCES

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

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

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

4. R. W. Stieber and P. Gerhardt, J. Dairy Sci, submitted (1979c).

5. P. Gerhardt and C. A. Reddy, Dev. Ind. Microbiol., 19, 71 (1978).

6. F. W. Juengst, Jr., J. Dairy Sci., 62, 106 (1979).

7. C. A. Reddy, H. E. Henderson, and M. D. Erdman, Appl. Environ. Microbiol., 32, 769 (1976).

8. A. K. Keller and P. Gerhardt, Biotechnol Bioeng., 17, 997 (1975).

9. R. W. Stieber and P. Gerhardt, J. Dairy Sci., in press (1979b).

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

11. D. L. Morris, Science, 107, 254 (1948).

12. J. D. Pryce, Analyst, 94, 1151 (1969).

13. R. K. Finn and R. E. Wilson, J. Agri. Food Chem., 2, 66 (1954).

14. S. Dawids and C. Boe, Acta. Med. Scand. Suppl., 607, 7, (1977a).

15. S. Dawids and C. Boe, Acta. Med. Scand. Suppl., 607, 17, (1977b).

TABLE I

Glossary of Mathematical Symbols

Symbol	Description	Units
A_m	Area of membrane available for dialysis	cm^2
D	Hydraulic dilution rate	h^{-1}
F_d^o	Flow rate into dialysate circuit	ml/h
F_d	Flow rate out of dialysate circuit	ml/h
F_f	Flow rate out of fermentor circuit	ml/h
K_p	Product inhibition constant	mg/mg
K_s	Substrate limitation constant	mg/ml
P_d^o	Product concentration in dialysate feed	mg/ml
P_d	Product concentration in dialysate circuit	mg/ml
P_{mp}	Permeability of membrane to product	mg/cm^2-h
P_{ms}	Permeability of membrane to substrate	mg/cm^2-h
r_g	Rate of cell growth	mg/ml-h
r_p	Rate of product formation	mg/ml-h
$-r_s$	Rate of substrate utilization	mg/ml-h
S_d^o	Substrate concentration in dialysate feed	mg/ml
S_d	Substrate concentration in dialysate circuit	mg/ml
S_f	Substrate concentration in fermentor circuit	mg/ml
t	Time	h
V_d	Volume of liquid in dialysate circuit	ml
V_f	Volume of liquid in fermentor circuit	ml
X_f	Cell-mass concentration in fermentor circuit	mg/ml
α	Substrate/cell ratio	mg/mg
β	Specific maintenance rate	h^{-1}
γ	Product/substrate ratio	mg/mg
E	Efficiency of lactose conversion	%
μ_m	Maximum specific growth rate of cells	h^{-1}
T_f	Cell-retention time in fermentor circuit	h

TABLE II

Glossary of Dimensionless Parameters

Type	Symbol and Definition	Description
Material parameters	$\bar{P}_d^o = P_f^o/(\gamma S_d^o)$	Product factor in dialysate feed
	$\bar{P}_d = P_d/(\gamma S_d^o)$	Product factor in dialysate circuit
	$\bar{P}_f = P_f/(\gamma S_d^o)$	Product factor in fermentor circuit
	$\bar{S}_d = S_d/S_d^o$	Substrate factor in dialysate circuit
	$\bar{S}_f = S_f/S_d^o$	Substrate factor in fermentor circuit
	$\bar{X}_f = \alpha X_f/S_d^o$	Cell factor in fermentor circuit
Operational parameters	$R = P_{mp}/P_{ms}$	Ratio of product/substrate membrane permeabilities
	$\Pi = P_{ms}A_m/F_d$	Membrane permeability factor
	$\phi = F_d/F_f$	Flow-rate ratio
Kinetic parameters	$\bar{K}_s = K_s/S_d^o$	Substrate-limitation factor
	$\bar{K}_p = \gamma K_p$	Product-inhibition factor
	$\theta = \mu_m T_f$	Time factor

TABLE III

Values Used in Computer Simulations

Symbol[a]	Value
$\bar{K}_p$	2.2
$\bar{K}_s$	0.001
$\bar{P}_d^o$	0.04
R	3.0
S_d^o (mg/ml)	68.0
α (mg/mg)	7.0
β (h^{-1})	1.2
γ (mg/mg)	0.96
μ_m (h^{-1})	0.35

[a]Symbols are described in Tables I and II.

TABLE IV

Data From Operation of the Dialysis Immobilized-Cell System at Steady-State Conditions

Period	Day	$D(h^{-1})$[a]	X_f (mg/ml)[a]	S_f (mg/ml)	S_d (mg/ml)	P_f (mg/ml)	P_d (mg/ml)	E(%)[b]	$-r_s$ (mg/ml-h)[c]	S_d^o (mg/ml)[d]
		.095	1.85	17.6	27.8					
	13	.096	1.11	5.2	12.6	19.5	19.5	57.4	1.64	29.6
		.093	0.91	10.0	23.4	33.5	31.0	56.6	2.83	53.9
		-	0.96	12.5	26.7	36.5	30.8	51.5	-	55.0
	14	.089	0.92	12.4	26.2	41.5	35.0	55.4	2.90	58.7
ONE		.092	1.02	14.9	28.2	43.8	34.5	53.2	2.95	60.2
		.092	1.07	11.8	21.8	47.0	35.0	59.1	2.98	54.3
	15	.091	1.09	13.8	27.3	45.8	35.8	55.0	3.02	60.6
		.091	1.17	11.2	19.8	49.0	41.3	66.2	3.55	58.6
		.094	0.93	6.7	14.0	33.0	28.3	64.8	2.42	39.8
	16	.092	1.03	7.5	18.8	38.3	29.8	59.2	2.50	46.1
	Mean[e]	.091	1.02	12.4	24.8	42.4	35.1	56.7	3.04	57.3
		.092	1.48	13.5	29.7	43.5	32.0	49.8	2.70	59.2
		.090	1.60	13.8	23.1	47.5	35.5	58.8	2.96	56.1
	17	.088	2.58	8.4	22.5	55.3	36.3	60.0	2.98	56.3
		.091	3.73	10.7	22.7	46.0	36.8	60.2	3.13	57.0
		.092	2.86	8.0	19.2	54.0	42.3	67.5	3.67	59.0
	18	.092	3.04	8.8	24.7	59.0	41.8	61.4	3.61	64.0
TWO		.091	3.72	10.4	21.8	54.0	42.5	64.7	3.65	61.8
		.088	3.95	7.0	16.5	58.3	45.6	70.1	3.79	61.8
	19	.091	4.08	7.2	21.0	59.0	46.5	67.7	4.01	65.0
		.092	4.17	6.5	22.3	58.5	41.3	63.5	3.57	61.1
		.086	3.92	5.0	21.3	53.0	46.5	67.4	3.78	65.3
	20	.095	3.94	5.0	19.3	54.5	43.3	67.9	3.86	60.1
	Mean	.091	-	8.7	22.2	53.6	40.9	-	-	60.6
		.093	4.59	8.0	23.9	56.0	39.5	60.8	3.44	60.9
		.094	4.45	5.3	20.8	51.5	38.8	63.6	3.40	57.1
	21	.093	4.18	8.4	27.9	50.5	42.0	58.6	3.69	67.4
		.091	4.43	9.4	25.3	49.0	39.3	59.3	3.34	62.1
		.091	4.45	8.0	23.9	52.5	38.0	59.8	3.22	59.4
	22	.093	4.63	8.0	23.9	49.0	37.5	59.4	3.25	58.9
		.092	4.69	11.3	30.1	49.0	36.3	52.9	3.10	63.9
		.093	4.55	7.1	26.8	53.2	37.6	56.8	3.26	62.1
THREE	23	.089	4.52	7.4	24.5	52.0	36.8	58.3	3.05	58.8
		.095	4.78	4.2	23.6	50.3	34.3	57.4	3.03	55.4
		.094	4.91	5.0	27.2	45.0	32.3	52.3	2.80	57.0
	24	.091	4.77	6.3	25.7	47.0	34.8	55.7	2.94	58.0
		.097	4.42	7.3	32.6	51.0	37.5	51.8	3.38	67.6
		.095	4.37	7.6	32.0	49.0	37.3	52.1	3.31	66.8
	25	.094	4.34	6.2	31.4	54.0	35.8	51.5	3.12	64.7
		.094	4.12	5.8	29.8	53.8	34.8	52.0	3.02	62.1
		.094	4.81	3.8	30.0	56.3	40.5	55.9	3.57	68.0
	Mean	.093	4.54	7.0	27.0	51.2	37.3	56.4	3.23	61.8

[a] Based on the 13 day mean V_f of 2,686 ml.

[b] $E = (1 - S_d/S_d^o) \times 100$.

[c] $-r_s = (S_d^o - S_d)P_d/V_f$, where V_f = 2,686 ml.

[d] $S_d^o = S_d + P_d - P_d^o$, where P_d^o = 2.5 mg/ml.

[e] The mean does not include day 13 (start-up) and the last 2/3 of day 16 (diluted feed).

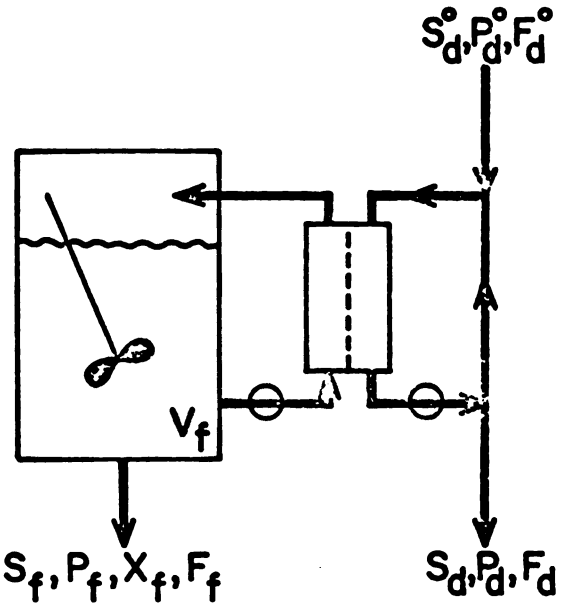


Fig. 1. Schematic of dailysate-feed system. Symbols are described in Table I.

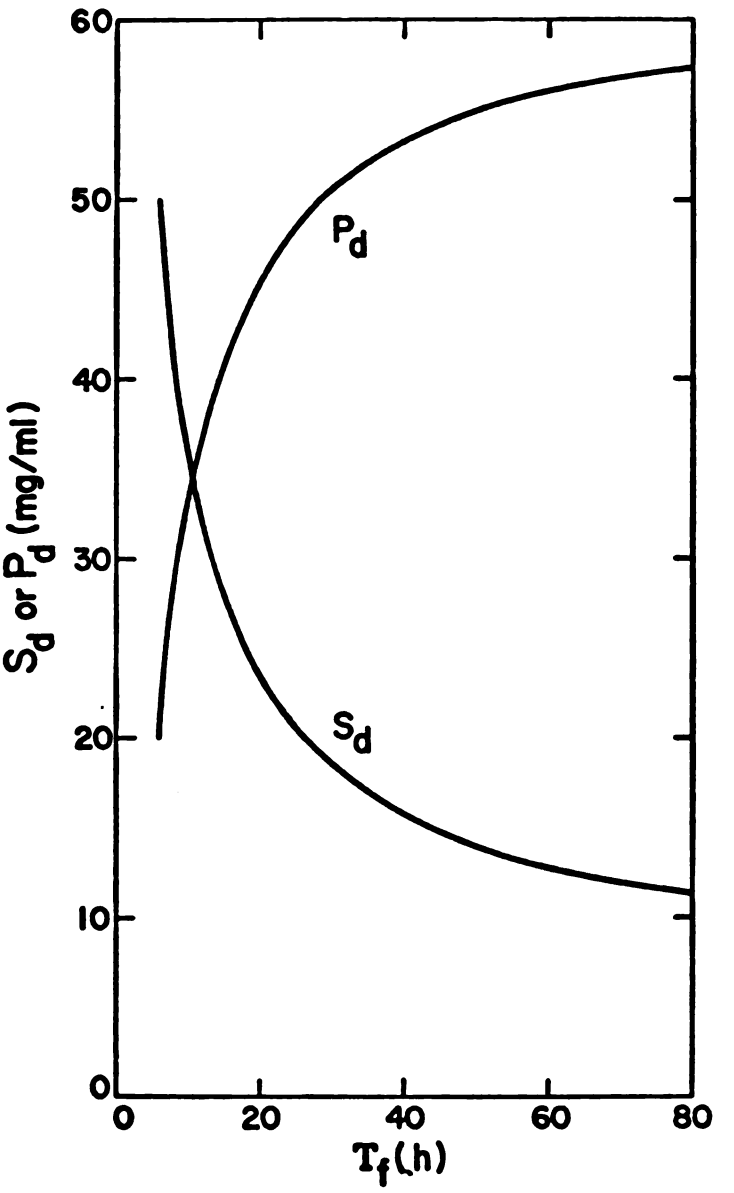


Fig. 2. Simulated effects of changes in cell-retention time (T_f) on residual substrate (S_d) and accumulation of product (P_d) in the dialysate circuit, when ϕ = 10.0 and Π = 10.0.

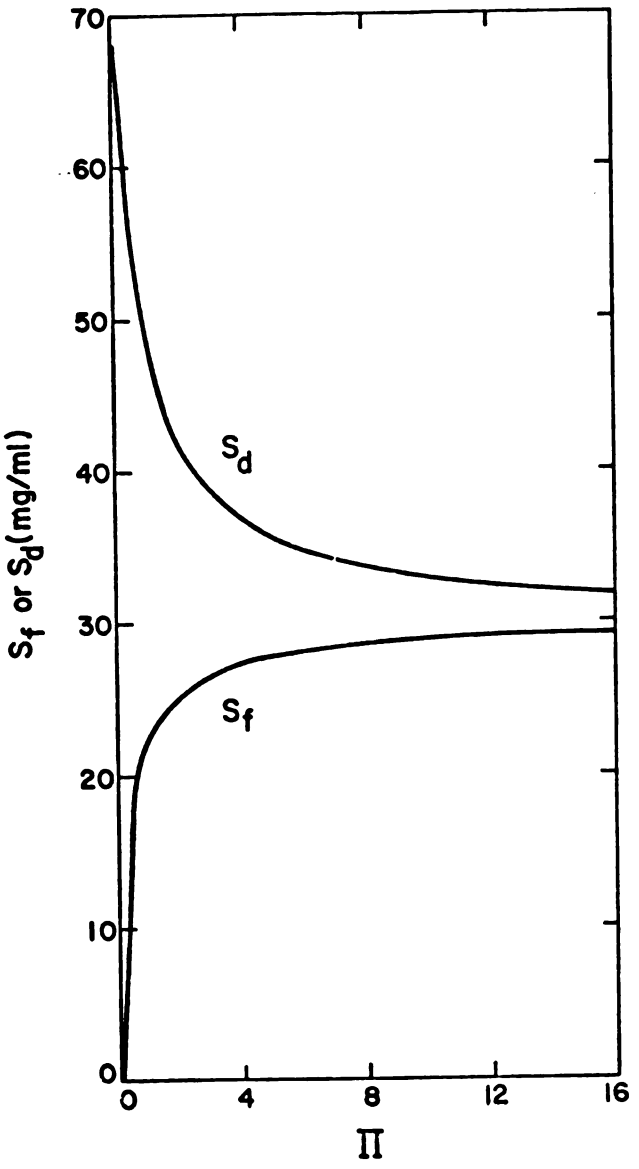


Fig. 3. Simulated effects of changes in the membrane permeability factor (Π) on substrate in the dialysate circuit (S_d) and the fermentor circuit (S_f), when $\phi = 10.0$ and $\theta = 4.0$.

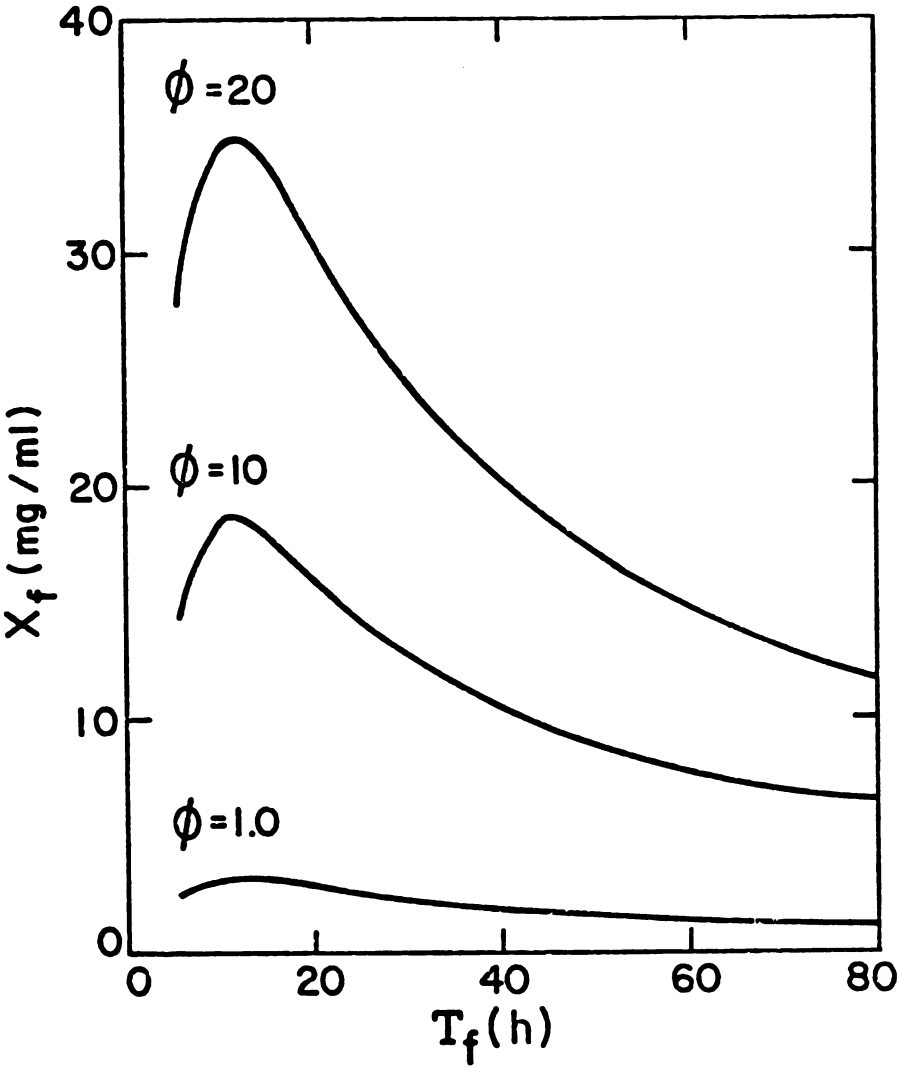


Fig. 4. Simulated effects of changes in cell-retention time (T_f) on cell mass (X_f) in the fermentor circuit at three flow-rate ratios (ϕ), when Π = 10.0.

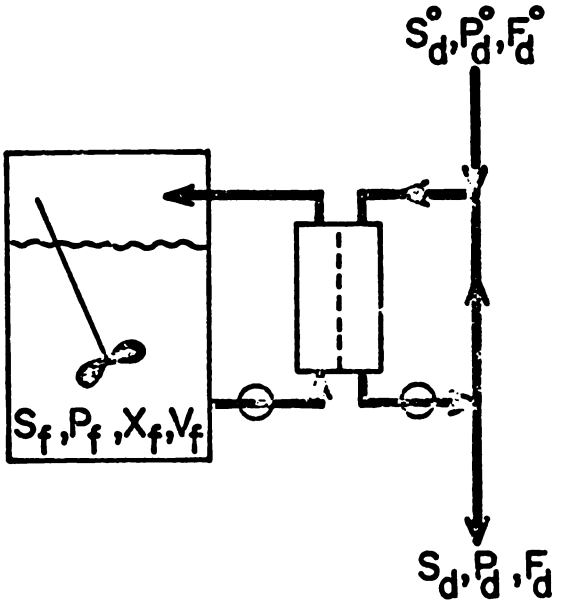


Fig. 5. Schematic of dialysate-feed, immobilized-cell system. Symbols are described in Table I.

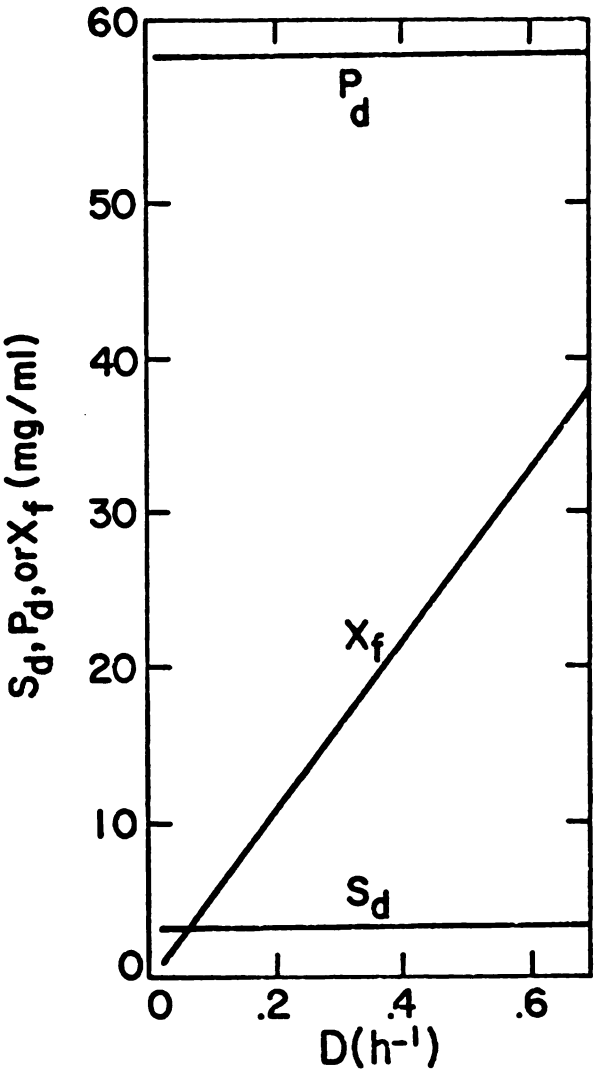


Fig. 6. Simulated effects of changes in hydraulic dilution rate (D) on residual substrate (S_d) and accumulation of product (P_d) in the dialysate circuit and cell mass (X_f) in the fermentor circuit, when $\Pi = 10.0$.

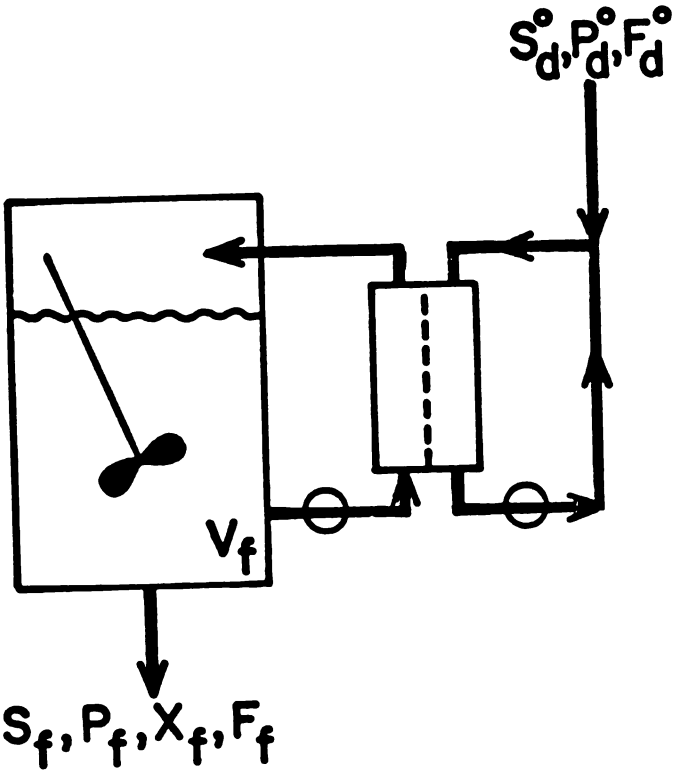


Fig. 7. Schematic of dialysate-feed, fermentor-throughput system. Symbols are described in Table I.

GENERAL DISCUSSION

The simulated and experimental results together with the review in this thesis all indicate that membranes can be used in continuous fermentation processes to enhance microbial growth, substrate utilization, metabolite production and product recovery, whether the product be metabolites or cells.

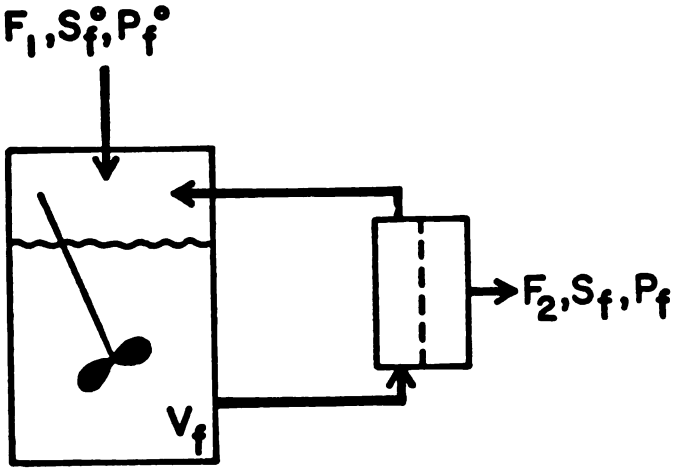
The potential for membrane technology lies principally with its use to retain microbial cells in a fermentor, thereby increasing the cell population and allowing increased throughput of substrate (Article VII). Such a process can produce a cell-free metabolite at a usefully high concentration and rate, and a concentrated cell suspension by continuously or periodically harvesting a volume from the fermentor.

The use of membranes to relieve inhibitory effects by metabolites is obviously limited to fermentations which produce such metabolites. In dialysis systems applied to these fermentations, ways must be found to circumvent the production of large volumes of dilute permeate, an environmental liability. For the ammonium-lactate fermentation this was accomplished by feeding substrate at a high concentration into the fermentor, so that the resulting permeate, though diluted by water, still contained a useful concentration of metabolite (Articles I through IV). For a cycloheximide fermentation, Kominek (1975) used an extractor to eliminate the production of

large volumes of permeate. These continuous methods also eliminate the need for a large reservoir vessel, which often is required in a batch dialysis process (Schultz and Gerhardt, 1969).

For the potential of dialysis in fermentation to be realized, the solute exchange capacity of the present dialyzer must be improved. The best membranes for dialysis appear to be membrane filters (pore diameter of between .05 and 4.0 μm) which separate only cells from solutes. The use of vast areas of membrane not only has physical limitations, but is limited in maintaining turbulence on membrane surfaces and in preventing membrane fouling. The available dialyzers preclude use of mycelial and other organisms that cause rapid fouling. These considerations along with scale-up problems indicate that the potential for use of membranes in fermentation probably lies with refinements of dialysis such as electrodialysis or microfiltration. These processes can increase solute transfer across the membranes.

A schematic of a microfiltration fed-batch process which may have application for many types of microbial cells in fermentation is as follows:



The process is designed for the continuous production of cell-free metabolites and for intermittent harvesting of a concentrated suspension of cells. Operationally, the process is started batchwise. The fermentor is filled with a medium containing a relatively low concentration of substrate and the fermentor is inoculated. As the cells reach an exponential rate of growth, a feed of substrate into the fermentor and an effluent flow from the microfilter are started at the same exponential rate. Because of the relatively low substrate concentration in the medium and in the feed, diffusible metabolites will not accumulate to inhibitory levels. Thus for lactobacilli in the ammonium-lactate fermentation, growth in a confined volume would proceed at the exponential rate until the cell mass becomes unmanageable or the microfilter becomes fouled. At such times, a volume (e.g., 90%) of the concentrated cell culture is harvested from the fermentor, the volume is replaced with the medium, and operation is resumed as before. For an aerobic fermentation, the optimal time for cell harvest may be when the cell mass reaches a level at which oxygen becomes growth limiting.

Computer control is required for this process in order to maximize substrate utilization and cell mass productivity and, thus, to increase the utility of the membranes. As applied to the ammonium-lactate fermentation, the sensors needed for computer control are several. Conductance in the microfilter effluent can be monitored by a conductivity bridge and this information can give the lactate concentration (P_f; Article III). With the exponential flow rates, F_1 and F_2, equal to each other and knowledge of the lactose concentration in the feed (S_f^o) and P_f in the effluent, the computer

can maximize substrate utilization by changing the flow rates. The lactate concentration can be checked by monitoring the rate of ammonia addition (load cell or flowmeter) and by knowing the fermentor volume (V_f; level probe). Similarly, F_1 and F_2 can be determined. Knowing F_1, S_f^o, P_f, and V_f and using the mathematical model (Article III), the computer can instantly calculate the cell mass concentration, and can predict and adjust the time for cell harvest. As the fermentation progresses, the pressure in the conduits may have to be increased automatically (e.g., by increasing the speed of a circulating pump or by closing a valve) to compensate for membrane fouling, in order to maintain the appropriate F_2. A pressure transducer is needed in the conduits to alarm for excessive pressure, which also may indicate harvest time.

This computer control scheme maximizes substrate utilization and cell mass and metabolite production, thereby increasing the potential for use of membranes in fermentation. The computer also is necessary to acquire all the data provided by the sensors, to obtain further data by calculations (e.g., cell mass) and, to make the data readily available.

The microfiltration process is presented to highlight the potential for application of membrane technology in fermentation. Further, it emphasizes that the use of membranes requires complex control systems. Presently, a microfilter needs to be designed specifically for microbial purposes; the remaining equipment is conventional. The capital investment in such a process is great, but the fermentation potential for production and recovery of both cell mass and metabolites appears greater.

SUMMARY-ABSTRACT

Dialysis continuous culture processes were mathematically modeled, computer simulated and experimentally tested, and the literature was reviewed, to assess the potential for application of membrane technology to industrial fermentations. The ammonium-lactate fermentation of whey was used as a model system for the experiments because of its commercial potential. The simulated and experimental results both showed ways that dialysis continuous processes can maximize substrate utilization and produce high cell populations and cell-free metabolites at high rates. The dialysis processes were a considerable improvement over conventional continuous processes.

In one system for dialysis continuous fermentation, whey of high concentration was fed into a fermentor while the fermentor contents were dialyzed through a membrane against water to relieve the inhibitory effect of metabolites. The fermentation was modeled and a solution for the steady state was used to simulate the fermentation on a digital computer. The results showed the effects of various parameters on the process and predicted that it could be operated efficiently. To test the predictions, dried sweet-cheese whey was rehydrated to contain 230 mg of lactose per ml, charged into a 5-liter fermentor, adjusted in pH (5.3) and temperature (44^{o}C), and inoculated with Lactobacillus bulgaricus. The fermentor and dialysate circuits were connected, and a series of steady-state conditions was managed without

sterilization or asepsis for 94 days. As time progressed, the fermentation remained homofermentative and increased in conversion efficiency, although membrane fouling necessitated dialyzer cleaning about every 4 weeks. With a retention time of 19 h, 97% of the substrate was converted into products and cells. Relative to nondialysis continuous or batch processes for the fermentation, the dialysis continuous process enabled the use of more concentrated substrate, was more efficient in the rate of substrate conversion, and additionally produced a second effluent of less concentrated but purer ammonium lactate.

The model was improved by incorporating separate terms for substrate limitation and product inhibition into the equation describing the rate of cell growth. The improved model was validated using the results from previous experiments with whole whey and also in subsequent experiments with deproteinized whey. The latter showed that the cell accumulation, cell productivity, and molar growth yield were greater than those obtained from a conventional continuous process for the fermentation and that the dialysis process could produce a continuous effluent containing 50 mg of cell-free ammonium lactate per ml. The whole whey proved to be a better substrate than the deproteinized whey.

In a second system for dialysis continuous fermentation with a prefermentor, simulations predicted that the addition would not bring improvement. In a third system with 100% recycling (feedback) of cells, simulations predicted that the retention time would be reduced

9-fold and the conversion of substrate (deproteinized whey) to product would be increased by 25%, accomplished almost entirely by maintenance metabolism.

In a fourth system, the use of dialysis for substrate feed as well as product removal also was modeled and simulated. The simulations predicted that the feeding of substrate into the fermentor circuit via the dialysate circuit has potential for production and recovery of both cell mass and cell-free metabolite. The experimental tests indicated that the fourth system would be a useful method for immobilizing microbial cells to produce a cell-free metabolite at a rate greater than the maximum specific growth rate of the microbe, and additionally would serve to filter-sterilize the substrate.

LITERATURE CITED

LITERATURE CITED

1. Abbott, B. J. 1978. Immobilized cells, p. 91-123. In D. Perlman (ed.), Annual reports on fermentation processes, Vol. 2, Academic Press, Inc., New York.

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

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

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

5. 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.

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

7. Algire, G. H. 1957. Diffusion chamber technique for studies of cellular immunity. Ann. N.Y. Acad. Sci. 69 (Article 4):663-667.

8. 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.

9. 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.

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

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

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

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

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

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

16. 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.

17. 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.

18. 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.

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

20. 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.

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

22. 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.

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

24. 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.

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

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

27. 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.

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

29. 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.

30. 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.

31. 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.

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

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

34. 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.

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

36. 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.

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

38. 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.

39. 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.

40. 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.

41. 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 45:373-383.

42. 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.

43. 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.

44. 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.

45. Brooks, J. R., S. H. Sturgis, 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.

46. 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.

47. 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.

48. 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.

49. 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.

50. 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.

51. 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.

52. 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.

53. 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.

54. Dawids, S., and C. Boe. 1977. A new parallel plate dialyzer. I. Development and testing of membrane support. Acta Med. Scand. Suppl. 607:7-16.

55. Dawids, S., and C. Boe. 1977. A new parallel plate dialyzer. II. Description of design and in vitro performance. Acta Med. Scand. Suppl. 607:17-24.

56. Dawids, S., and S. Vojnovic. 1977. A new parallel plate dialyzer. III. Preliminary experience on clinical performance. Acta Med. Scand. Suppl. 607:27-29.

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

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

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

60. 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.

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

62. 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.

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

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

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

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

67. Eipert, E. F., L. Adorini, and J. Couderc. 1978. A miniaturized in vitro diffusion culture system. J. Immunol. Methods 22:283-292.

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

69. 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.

70. Favero, M. S., N. J. Petersen, L. A. Carson, W. W. Bond, and S. H. Hindman. 1975. Gram-negative water bacteria in hemodialysis systems. Health Lab. Sci. 12:321-334.

71. Fensom, A. H., W. M. Kurowski, and S. J. Pirt. 1974. The use of ferricyanide for the production of 3-keto sugars by non-growing suspensions of Agrobacterium tumefaciens. J. Appl. Chem. Biotechnol. 24:457-467.

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

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

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

75. 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.

76. 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.

77. 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.

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

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

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

81. 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.

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

83. Gerhardt, P., and D. M. Gallup. 1965. Process and apparatus for dialysis fermentation. U.S. Patent 3,186,917.

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

85. 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.

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

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

88. 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.

89. 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.

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

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

92. 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.

93. 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.

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

95. 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.

96. Hardt, F. W., J. C. Young, L. S. Clesceri, and D. R. Washington. 1971. Ultrafiltration for the control of recycled solids in a biological system. Environ. Sci. Technol. 5:345-349.

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

98. 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.

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

100. 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.

101. 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.

102. 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.

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

104. 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.

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

106. 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.

107. 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.

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

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

110. 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.

111. 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.

112. 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.

113. Jensen, A., B. Rystad, and S. Melsom. 1974. Heavy metal tolerance of marine phytoplankton. I. The tolerance of three algal species to zinc in costal sea water. J. Exp. Mar. Biol. Ecol. 15:145-157.

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

115. Johnson, K. T., C. G. Hill, Jr., and C. H. Amundson. 1976. Electrodialysis of raw whey and whey fractionated by reverse osmosis and ultrafiltration. J. Food Sci. 41:770-777.

116. 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.

117. 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.

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

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

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

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

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

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

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

125. 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.

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

127. 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.

128. 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.

129. 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.

130. 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.

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

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

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

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

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

136. 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.

137. 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.

138. 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.

139. 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.

140. 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.

141. 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.

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

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

144. 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.

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

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

147. 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.

148. 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.

149. 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.

150. 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.

151. 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.

152. Metchnikoff, E., E. Roux, and T. (Aurelli) Salimbeni. 1896. Toxine et antitoxine cholerique. Ann. Inst. Pasteur 10:257-282.

153. Michaels, A. S. 1968. New separation technique for the CPI. Chem. Eng. Prog. 64:31-43.

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

155. 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 in ex vivo hemodialysis culture. J. Infect. Dis. 135:51-54.

156. 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.

157. 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.

158. 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.

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

160. 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.

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

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

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

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

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

166. Osebold, J. W., P. M. Outteridge, L. D. Pearson, and R. A. DiCapua. 1970. 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.

167. 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.

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

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

170. Petersen, B. H., T. 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.

171. 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.

172. Pirt, S. J., and W. M. Kurowski. 1970. An extension of the theory of the chemostat with feedback of organisms. Its experimental realization with a yeast culture. J. Gen. Microbiol. 63:357-366.

173. 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.

174. 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.

175. Poyton, R. O., and D. Branton. 1970. A multipurpose microperfusion chamber. Exp. Cell Res. 60:109-114.

176. 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.

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

178. 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.

179. 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.

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

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

182. 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.

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

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

185. 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.

186. 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.

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

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

189. 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.

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

191. 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.

192. 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.

193. 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.

194. 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.

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

196. Rose, G. G., and T. Yajima. 1978. Terminal bronchiolar-alveolar (TB-A) units in circumfusion system cultures. In Vitro 14:557-580.

197. 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.

198. 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.

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

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

201. 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.

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

203. 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.

204. 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.

205. 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.

206. 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.

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

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

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

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

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

212. 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.

213. 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.

214. 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.

215. 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.

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

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

218. 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.

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

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

221. 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.

222. 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.

223. 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.

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

225. 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.

226. 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. Immunol. 102:442-450.

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

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

229. 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.

230. Vieth, W. R., and K. Venkatasubramanian. 1974. Part. IV. Process engineering for immobilized enzyme systems. Chem. Technol. 4:434-444.

231. Vilpo, J. A. 1972. Proliferation, survival and malignancy of chloroleukaemia cells grown in diffusion chambers in vivo. Annales Medicinae Experimentalis et Biologiae Fenniae 50:168-174.

232. 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.

233. 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.

234. Wang, D.I.C., A. J. Sinskey, and T. A. Butterworth. 1970. Enzyme processing using ultrafiltration membranes, p. 98-119. In J. E. Flinn (ed.), Membrane science and technology, Plenum Press, New York.

235. 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.

236. 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.

237. 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.

238. 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.

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

240. Young, P. 1974. An introduction to electrodialysis. J. Soc. Dairy Technol. 27:141-150.

241. 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.