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A Membrane-Based Method for Removal of Toxic Ammonia from Mammalian-Cell Culture

DANIEL J. BROSE* AND PAUL VAN EIKEREN

Bend Research, Inc., 64550 Research Rd., Bend, OR 97701

ABSTRACT

Many mammalian cells grown in culture excrete ammonia, which, when it accumulates, limits cell growth and reduces product synthesis. Common tactics for minimizing the effects of ammonia accumulation are uneconomical, requiring large quantities of media and incurring high capital costs. Solution-diffusion membranes were investigated for ammonia removal, and a supported-gas membrane was identified that could be used to remove ammonia rapidly to well below inhibitory levels. Medium treated using this membrane was reused to culture baby-hamster kidney cells, resulting in a cell growth rate that was essentially the same as that for cells grown in fresh medium.

Index Entries: Mammalian-cell culture; ammonia removal; gas membrane.

INTRODUCTION

Ammonia is a toxic waste byproduct of cell metabolism. As it accumulates in the cell-growth medium, it inhibits growth and product synthesis in many types of mammalian cells. For example, ammonia has been shown to inhibit cell growth of chlorioallantoic tissue (*1*), mouse ascitic tumor cells (*2*), 3T3 cultured mouse fibroblast cells (*3*), baby-hamster kidney (BHK) cells (*4*), myeloma cells (*5*), a strain of L mouse cells (*6*), human fibroblast cells used for interferon synthesis (*7,8*), hybridoma cells (*9*), and Madin-Darby canine kidney cells (*10*). The concentration of ammonia that inhibits cells can be very low. For example,

*Author to whom all correspondence and reprint requests should be addressed.

during culturing of mouse–mouse hybridoma cells for antibody production, an ammonia concentration of 2–3 m*M* results in significant inhibition of cell growth (*11*) and antibody production.

The usual tactic for minimizing the effects of accumulated ammonia in mammalian-cell culture is to replace the "spent" medium with fresh medium. Many medium formulations contain expensive animal sera, and replacing large quantities can be expensive. Additionally, replacing spent medium results in high capital costs, because fresh medium must be prepared and sterilized in large quantities, and additional equipment is needed to prepare and store the extra culture medium.

Two other strategies for dealing with the problem of ammonia accumulation have been reported: (1) choosing a cell line that is more resistant to ammonia (*12*) and (2) controlling the amount of glutamine fed to cells in batch culture (*13*). (Glutamine is a primary source of toxic ammonia.) However, few cell lines are resistant to ammonia, and even with tight control over the feeding of glutamine, the accumulation of ammonia is reduced by only 25–30%, and the cell's capability to use other essential amino acids is compromised.

It is conceivable that ammonia could also be stripped from solution by sparging with oxygen or carbon dioxide. However, calculations indicate that the volume of gas needed to effectively remove ammonia is very large, making the cost prohibitive. Furthermore, such large volumes of gases added to mammalian-cell reactors could cause undesirable turbulence, which may lead to cell death.

This paper describes a novel process (*14*) of removing ammonia continuously from cell-culture medium, allowing longer use of the medium. The process is based on the selective transport of ammonia through a membrane; the other cell-culture components are rejected by the membrane and remain in the culture medium. There are two principal benefits of a continuous process that removes ammonia as it is generated by the cells: (1) medium use is significantly reduced, and (2) product titers are greater. Currently, medium is replaced in the culture to reduce ammonia concentrations to subinhibitory levels. However, this added medium also dilutes product concentrations, requiring extensive downstream processing to recover and purify the product.

BASIS FOR MEMBRANE-BASED AMMONIA REMOVAL

Membranes for separations can be categorized into two groups: (1) filtration membranes, and (2) solution-diffusion membranes (*15*). Filtration membranes contain pores. When a solution is forced through a filtration membrane by convective flow, solute molecules smaller than the pores pass easily; solute molecules larger than the pores are retained. Conse-

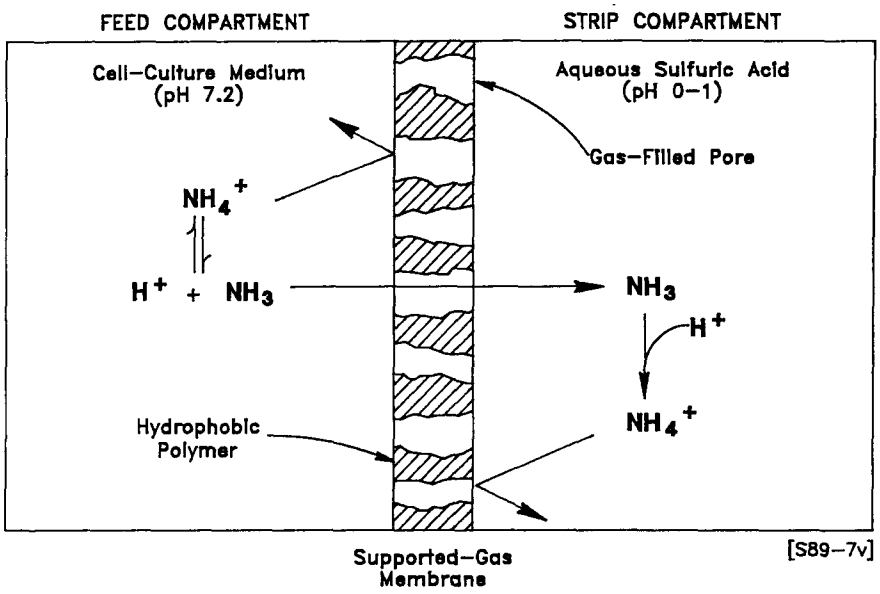

Fig. 1. Ammonia removal by a supported-gas membrane.

quently, the selectivity of this type of membrane is dictated by molecular size.

In most solution-diffusion membranes there is no convective flow through a porous membrane. Instead, solute molecules dissolve in the membrane phase and permeate through the membrane by molecular diffusion. Molecular solutes that exhibit high solubilities in the membrane phase permeate rapidly; molecular solutes that are insoluble in the membrane phase are retained. Thus, selectivity in this type of membrane is closely correlated with solute solubility.

The membranes used in this work are solution-diffusion membranes. Three different types of solution-diffusion membranes were tested, and the supported-gas membrane was identified as the best. A supported-gas membrane consists of a gas layer about 25 μm thick that separates two aqueous solutions. The gas layer is supported and stabilized within the pores of a microporous, hydrophobic polymer film. Since water cannot wet the polymer film, water cannot permeate the pores, and the aqueous solutions on the two sides of the membrane cannot mix. Such supported-gas membranes are specific: only volatile solutes diffuse through the membrane; nonvolatile solutes are completely retained (*16*). Furthermore, supported-gas membranes are reported to be stable for months, even when they are used to separate aqueous solutions that have acid concentrations that differ by 10 or more pH units (*17–20*).

Ammonia removal by a supported-gas membrane is illustrated in Fig. 1. A feed solution containing ammonium ion in equilibrium with dissolved ammonia is on one side of the membrane, and a strip solution containing

aqueous sulfuric acid (H_2SO_4) is on the other side. Ammonia from the feed solution evaporates at the feed-solution/gas interface and is transported by diffusion across the membrane to the gas/strip-solution interface. At this interface, ammonia dissolves in the strip solution and is immediately and irreversibly protonated by the acid in the strip solution, forming ammonium ion. Because the resulting concentration of ammonia in the strip solution is maintained near zero, ammonia is continuously transported from the feed solution to the strip solution.

MATERIALS AND METHODS

Evaluations of Flat-Sheet Membranes

Flat sheets of membranes were evaluated by placing 20 cm^2 of membrane in a two-compartment dialysis test cell. In a typical experiment, 100 mL of 6 m*M* NH_4Cl in 0.10*M* aqueous phosphate buffer at pH 7.2 and room temperature (~25°C) was placed in the feed compartment of the test cell. An equal amount (100 mL) of 0.1*M* H_2SO_4, also at room temperature, was placed in the strip compartment. The solutions were stirred, and the concentration of ammonia in the feed solution was monitored using an ammonia probe (Nurnberg Scientific, Portland, OR) connected to a digital voltmeter. Ammonia concentration was determined by comparing the voltage response of the probe in a sample to that of the probe in standards at pH 7.2.

In each experiment, the ammonia concentration in the feed was monitored as a function of time. The data were correlated using Eq. (1):

$$C = C_0 \times \exp(-PAt / V) \quad (1)$$

where C is the free-ammonia concentration in the feed at any time, C_0 is the initial free-ammonia concentration in the feed, P is the ammonia permeability, A is membrane area, t is time, and V is the feed volume. For a given experiment, the value of P was determined by iteratively adjusting P until the best fit between the experimental and calculated data was obtained.

Evaluation of Hollow-Fiber Membrane Module

Hollow-fiber supported-gas membrane modules were tested to determine the ammonia permeability. The test loop is shown schematically in Fig. 2. The test loop employs a module containing 2.0 ft^2 of microporous polypropylene membrane (Celgard X-20, 400-μm id, purchased from Celanese Separations Products). During typical operation of the test loop, 350 mL of cell-culture medium (Dubelco's Modified Eagles Medium [DME] with 10% (vol) calf serum, obtained from Sigma Chemical Co., St. Louis, MO) with 14m*M* ammonium chloride at pH 7.2 and 37°C was circulated

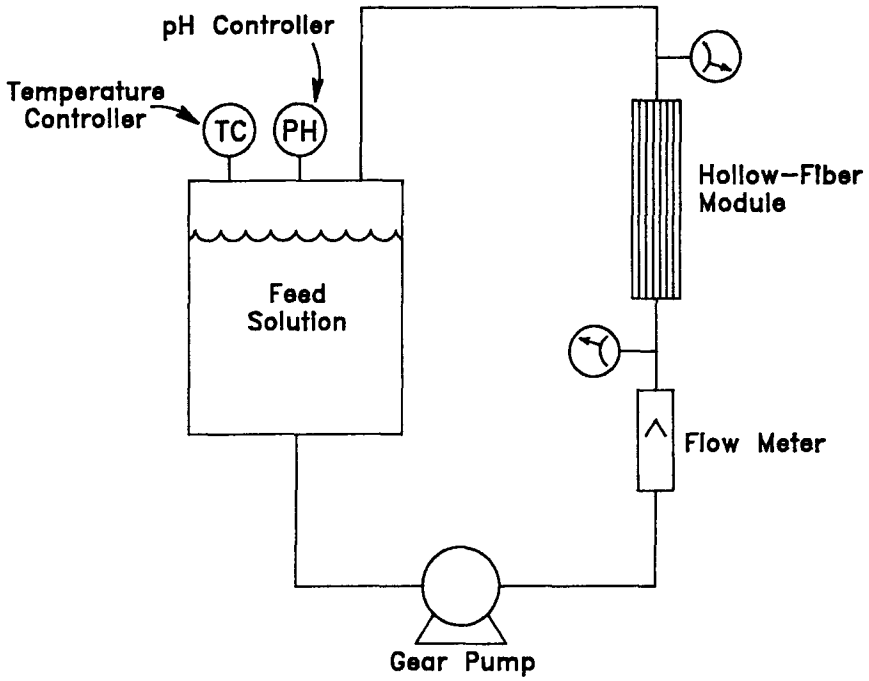

Fig. 2. Test loop for supported-gas hollow fibers, modules.

through the hollow-fiber module at a flow rate of 19 L/h. The shell of the hollow-fiber module was filled with 20 mL of 0.5*M* H_2SO_4; this solution was immobile during the course of the run.

The ammonia concentration in the feed solution was monitored using the ammonia probe as described previously. These data were used to calculate ammonia permeability via the following equation (*19*):

$$C = C_0 \times \exp\left([\pi d^2 v N t / 4V] \times [1 - \exp(4PL/vd)]\right) \quad (2)$$

where d is the fiber's id, v is the linear velocity through a fiber lumen, N is the number of fibers, and L is the fiber's length. (The remaining variables are defined above.) Specifically, ammonia permeability was calculated by iteratively evaluating the expression until the best fit between the experimental and calculated concentration data was obtained.

Assessment of Membrane Stability

The resistance of the supported-gas membrane to permeation of cell-culture medium (i.e., the membrane stability) was tested as follows: Cell-culture medium was circulated through the fiber lumens, and an equal volume of deionized water was circulated through the shell of the hollow-fiber module. The specific conductivity of the deionized water was monitored to determine if the cell-culture medium was penetrating the supported-gas membrane and mixing with the deionized water.

The measure of resistance of supported-gas membranes to permeation of aqueous solutions under pressure—i.e., the "water-entry pressure" —was measured as follows: The lumens of Celgard X-20 fibers were filled with deionized water, DME solution, DME solution with 20% (vol) calf serum, or with deionized water that had varying concentrations of a surfactant (Pluronic F-8, obtained from BASF Corporation, Parsippany, NJ). In each test the pressure of the solution inside the fiber was increased by 5-psi increments until the solution penetrated the membrane and bubbled out of the fiber's pores.

Tests for Growing Mammalian Cells

Anchorage-dependent BHK cells were grown in microcarrier cultures by adding 3.2×10^7 cells and 0.3 g of Cytodex I microcarrier to 100 mL of medium (DME with 10% [vol] calf serum) in five shaker flasks. One flask contained no ammonia, and the other flasks were spiked with ammonia at concentrations of 4m*M*, 7m*M*, 10m*M*, and 13mM. The flasks were agitated at 45–55 rpm in an incubator containing 10% CO_2 at 37°C, and samples were taken periodically from each flask over 140 h. Citric acid was used to dissolve the microcarriers in each sample, and cell nuclei were counted under a microscope.

The growth rates of BHK cells in membrane-treated medium were determined as follows: Medium (DME with 10% [vol] calf spiked with 14m*M* ammonia and treated using the membrane-based process until the ammonia concentration was reduced to less than 0.5m*M*. BHK cells were grown in the membrane-treated medium as described above; samples were removed at regular intervals and analyzed for cell concentration.

RESULTS AND DISCUSSION

Evaluation of Flat-Sheet Membranes

Three different types of solution-diffusion membranes were tested for ammonia permeability: supported-gas membranes, supported-liquid membranes, and a dense polymeric membrane. The results, shown in Table 1, indicate that the Celgard 2400 supported-gas membrane has the highest permeability for ammonia. This is consistent with ammonia's high vapor pressure and its known high diffusivity in supported-gas membranes. Additionally, Table 1 shows that ammonia permeability in the supported-gas membrane increases as the ammonia concentration and temperature increase.

Evaluation of Hollow-Fiber Membrane Module

Supported-gas membranes in hollow-fiber modular form were tested for ammonia permeability. Figure 3 shows typical ammonia removal data

Table 1
Membrane Evaluation Results

Membrane	Conditions			Ammonia permeability, cm/h
	NH_3, m*M*	pH	Temperature, °C	
Celgard 2400[a] supported-gas membrane	200	7.2	37	7.0
Celgard 2400 supported-gas membrane	20	7.2	23	4.6
Trioctylphosphine oxide-loaded supported-liquid membrane[b]	5	7.2	23	0.8
Diethylhexyl phosphoric acid-loaded supported-liquid membrane[c]	5	7.2	23	3.5
Dimethylsiloxane polymeric membrane[d]	10	7.2	23	<0.1

[a]Celanese Separations Products, Charlotte, NC.
[b]Trioctylphosphine oxide, Kodak Lab and Research Products, Rochester, NY, loaded into Celgard 2400
[c]Diethylhexyl phosphoric acid, Daihachi Chemical Industry Co., Osaka, Japan, loaded into Celgard 2400
[d]Dimethylsiloxane, UOP Fluid Systems, San Diego CA

when ammonia-spiked cell-culture medium was circulated through the hollow-fiber module. Using the data in Fig. 3 and Eq. (2), we calculated an ammonia permeability for the hollow-fiber module of 16 cm/h. The curve drawn through the data in Fig. 3 is the predicted concentration-vs.-time curve obtained using an ammonia permeability of 16 cm/h.

An analysis of the data in Fig. 3 leads to three important conclusions about permeation of ammonia through the hollow-fiber membranes in the module:

1. The permeability of ammonia through hollow-fiber membranes is higher than that through flat-sheet membranes. (We obtained an ammonia permeability of 7.0 cm/h using flat-sheet supported-gas membranes.)
2. The process lowers the ammonia concentration in the feed solution to well below 1m*M*. (This is consistent with the high driving forces for ammonia transport that result from irreversible conversion of ammonia to ammonium ion on the strip side of the membrane.) This indicates that the supported-gas membrane can effectively extend the life of cell-culture medium, in which the concentration of ammonia must not exceed 2m*M* for mammalian cells to grow well.
3. The results in Fig. 3 are correlated well using Eq. (2) and a single value of ammonia permeability. This indicates that the ammo-

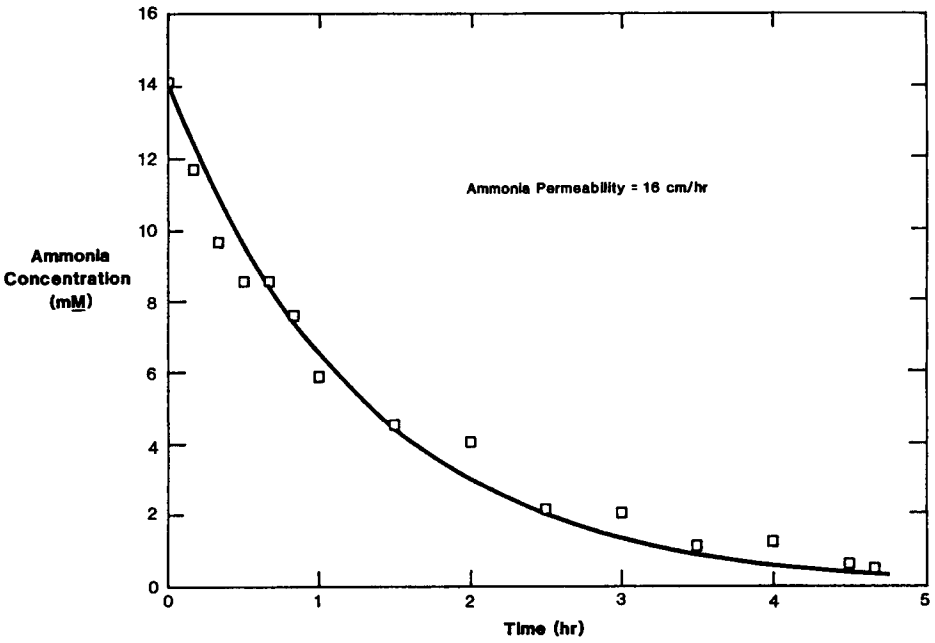

Fig. 3. Ammonia removal by a hollow-fiber membrane module as a function of time; feed consists of 14m*M* ammonium chloride in cell-culture medium at pH 7.2 and 37°C.

nia permeability is constant over the entire range of ammonia concentrations encountered in the feed. Furthermore, this result confirms the validity of the theoretical model, which assumes that ammonia permeability through the membrane is constant and rate-limiting, regardless of ammonia concentration in the feed. Finally, this result allows us to use the theoretical model to design membrane modules at large scale that operate over the entire ammonia-concentration range.

Assessment of Membrane Stability

A crucial feature of the process is that the membrane must act as a highly selective barrier that allows the passage of ammonia, but not sulfuric acid. A supported-gas membrane can fail if the surface tension between the hydrophobic polymer in the pores and the aqueous solution becomes so low that the surface "wets" and water displaces the gas in the pores. A wetted membrane allows the two solutions to mix, and a change in pH and conductivity of the solutions on both sides of the membrane will occur. Experiments were conducted to evaluate the long-term wetting resistance of the membrane on the basis of two criteria: (1) the resistance to permeation of cell-culture medium, and (2) the resistance to permeation of aqueous solutions under pressure.

Table 2
Effect of Surfactant
on Supported-Gas Membrane Water-Entry Pressures

Pluronic F-68 surfactant concentration, wt%	Water-entry pressure of Celgard, psi
	Average[a] of 3 tests
0	185±0
0.01	182±3
0.02	177±3
0.03	170±0
0.04	170±0
0.06	172±3
0.08	172±3
0.20	153±3

[a]Standard deviations are shown.

Cell-culture medium was circulated on the lumen side of Celgard X-20 hollow fibers, and deionized water was circulated on the shell side. The conductivity of the deionized water solution was measured as a function of time during the experiment: The conductivity did not change significantly over a 20-h time-period.

Stability can also be estimated from the hydrostatic pressure required to force a liquid into the pores and displace the air in the pores of the membrane. This unique pressure is termed the "water-entry pressure." Comparing the water-entry pressure of membranes that are in contact with various solutions provides a convenient method of comparing the effect of solution composition on membrane stability. Solutions containing surfactants are especially important to test, because surfactants are used in cell-culture media as antifoaming agents and are known to lower the water-entry pressure of supported-gas membranes.

The water-entry pressure of Celgard X-20 hollow fibers in contact with deionized water was 185 psi; the water-entry pressure of these fibers when in contact with DME solution or DME with 20% (vol) calf serum was also 185 psi. When surfactant was added to water, the water-entry pressure decreased. These data, shown in Table 2, lead to two important conclusions:

1. At a concentration of surfactant typically used in cell-culture media (0.02% [wt]), (*21*) the water-entry pressure of the supported-gas membrane is high—nearly the same as that when there is no surfactant in the water.
2. The supported-gas membrane resists wetting, even in the presence of surfactants at concentrations far in excess of those used in typical formulations of cell-culture media.

Table 3
Growth Rate for BHK Cells at Various Concentrations of Ammonia

Ammonia concentration, m*M*	BHK growth rate, cells/mL/h	Relative growth rate, %
0	12,000	100
4	8500	71
7	6500	54
10	5800	48
13	4500	38

Tests with Growing Mammalian Cells

A series of experiments was conducted with BHK cells to address two issues: (1) the suitability of using BHK as a model for ammonia-sensitive mammalian cells, and (2) the suitability of reusing membrane-treated medium for growing mammalian cells. Table 3 shows the rate of growth of BHK cells grown in shaker flasks containing 0m*M*, 4mM, 7m*M*, 10m*M*, and 13m*M* ammonia. As expected, the presence of ammonia slowed the growth rate of the BHK cells; the growth rate in medium that contained 13m*M* ammonia was 62% lower than in ammonia-free medium. The sensitivity of BHK cells to ammonia demonstrates that this cell line is a suitable model for evaluating the ammonia-removal strategy.

Cells were grown in cell-culture medium that had been both spiked with 14m*M* ammonia and subsequently stripped of ammonia using the membrane-based process. The BHK cell-growth data obtained from this experiment, as well as cell-growth data from those flasks containing 0m*M*, 7m*M*, and 13m*M* ammonia, are plotted in Fig. 4. This figure shows that the growth rate for BHK cells in the membrane-treated medium is essentially the same as that for cells grown in fresh medium. These results demonstrate that the membrane process removed ammonia without removing desirable nutrients from cell-culture medium.

CONCLUSIONS

We have demonstrated a novel and inexpensive membrane-based method of removing ammonia from mammalian-cell culture. The process relies upon the rapid removal of ammonia from cell-culture medium by transport across a supported-gas membrane. Medium treated using this membrane can be used to grow BHK cells in a shaker-flask culture. These cells multiplied as fast as cells that were grown in fresh medium.

A continuous ammonia-removal process based on supported-gas membranes promises substantial improvements over existing systems. First, because ammonia is removed from the culture medium, the process should improve the productivity of mammalian-cell culture. Second,

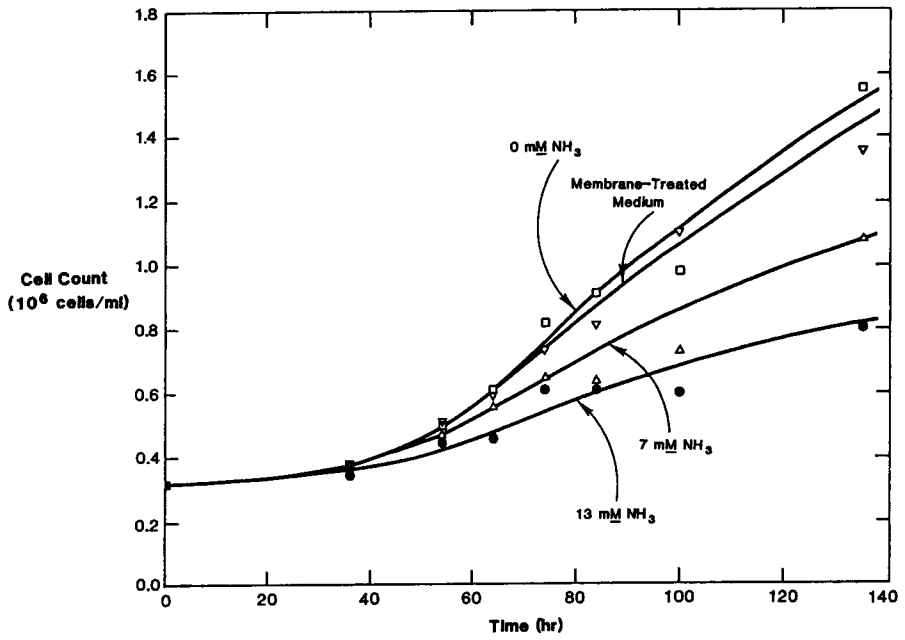

Fig. 4. Cell concentration as a function of time for shaker flasks with various concentrations of ammonia and for a shaker flask with membrane-treated medium.

because there will be less dilution of the culture medium, product concentrations should be higher, and downstream processing costs should be reduced. Third, because the process allows treatment and partial reuse of medium, medium costs should be reduced substantially. When viewed in terms of the cost of media for animal, microbial, plant, and other cell cultures—$737 million in 1987 (22)—the savings to industry in cell-culture medium promises to be substantial.

ACKNOWLEDGMENTS

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