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Ammonia Removal from Mammalian Cell Culture Medium by Ion-Exchange Membranes*

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ABSTRACT

Metabolites such as ammonia and lactic acid formed during mammalian cell culture can frequently be toxic to the cells themselves beyond a threshold concentration of the metabolites. Cell culture conducted in the presence of such accumulated metabolites is therefore limited in productivity. This work demonstrates with laboratory data that a nonporous ion-exchange membrane of the perfluorinated sulfonic acid type can be used to contact the culture medium, and ammonia removed selectivity from the medium without disturbing the process. The technique of pervaporation showed particular promise in this regard. The pervaporation used with inert gas sweep on the permeate side was found superior to that used with vacuum application.

INTRODUCTION

Ammonia, lactic acid, and carbon dioxide are some of the by-products of mammalian cell metabolism. The first two of these, if allowed to accumulate, can lead to inhibition of cell metabolism and cell growth (1–10). For example, ammonia concentration as low as 2 to 3 mmol/L can result

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in significant inhibition of cell growth and antibody production (11). Lactic acid accumulation in the cell culture medium can lead to lowering of pH to suboptimal levels, which impairs cell growth. The existing strategies to deal with the problem include 1) replacing the spent medium with fresh medium when the levels of by-products reach toxic or inhibitive levels, 2) modifying the culture medium so that smaller amounts of waste products are formed because of altered cell metabolism, 3) controlling additions of glutamine (for ammonia) or glucose (for lactic acid), 4) selecting a cell line that is accustomed to higher ammonia or lactic acid concentration and yet has the desired productivity, and 5) stripping ammonia from the culture medium by sparging with a mixture of oxygen and carbon dioxide. Of these options, the first one can be practiced only for small batches as most media formulations require expensive animal sera. Moreover, preparation and sterilization of a medium on a large scale requires additional equipment. Modification of a cell culture medium, or controlled addition of glutamine or glucose, cannot be regarded as desirable solutions because a reduction in waste products is achieved at the expense of the cells' capacity to use some essential amino acids. The fourth strategy also has limited application because only a few cell lines are resistant to ammonia. The last strategy, sparging, requires large volumes of sterile gases. Sparging of gases at high flow rates can lead to practical problems such as foaming and the creation of zones of high shear rates in which cells may be in danger of dying.

An attractive means of increasing the productivity of a cell culture is by using membranes for selective removal of ammonia or other undesirable by-products while the cell culture is in progress. This notion of a process for selective transport of ammonia through a membrane was recently described (12). In that work, a microporous polypropylene hollow fiber bundle ($\sim$1800 cm^2 area and 400 μm inner fiber diameter) was used to separate the culture medium on the lumen side from a dilute aqueous solution (0.4 M) of sulfuric acid on the shell side. Sulfuric acid in this method acts as a chemical sink, which binds ammonia to make ammonium sulfate. The membrane surface area to the volume of the cell culture medium circulated through the lumen was 5.3 cm^{-1}. Ammonia concentration in the medium was reduced from 14 to 0.5 mM in about 5 hours.

Some drawbacks of this method may be noted. Hollow fiber membranes have small inner diameters, so the circulation of the medium through the lumen requires high pressures. Furthermore, for small rates of flow that are typical in hollow fibers, a steep velocity gradient in the lumen causes high shear rates that may be detrimental to the survival of the cells. The cells may also block the surface pores and considerably reduce ammonia transport rates in tests of long duration.

Perfluorosulfonic acid polymer (PFSA) membranes have been used for selective transport of ionic species, amino acids, etc. (13, 14). These membranes are nonporous, and highly hydrophilic in nature. Because of the presence of cation-exchange sulfonic acid groups, these membranes are expected to facilitate transport of ammonia. In addition, because of the nonporous nature of these membranes, transport of whole cells cannot occur across the membrane. These desirable features of PFSA polymeric films motivated us to use this membrane in seeking a solution to the problem of ammonia removal from mammalian cell culture media. We employed three techniques to demonstrate selective ammonia transfer through these membranes: 1) diffusive transport from a source liquid to a sink liquid with and without a chemical sink, 2) pervaporation using vacuum, and 3) pervaporation using an inert sweep gas on the sink side.

In the experimental investigation discussed here, we tested five PFSA-based membranes for ammonia removal efficacy from aqueous media: 1) M1, a composite membrane consisting of a nonporous thin film (about 2 μm) of PFSA polymer on a backing, made by spraying a PFSA solution on a 1.5 mil thick porous PTFE Tetratex backing; 2) M2, a perfluorosulfonic acid polymer membrane film (about 25 μm thick); 3) M3, a composite membrane consisting of a thin nonporous film of PFSA, about 2 μm thick, on a backing, and made by spraying a solution of PFSA on a 3-mil thick porous PTFE Tetratex film thermo-bonded to a polypropylene support; 4) M4, a thin nonporous PFSA film of 2 μm thickness deposited on a commercially available porous polypropylene film; and 5) M5, a 178 μm thick nonporous Nafion PFSA membrane, commercially available from DuPont Company.

MEMBRANE PREPARATION

M2, the thin PFSA polymer membrane (about 25 μm thick), was prepared in the following manner. A rectangular glass frame was mounted on a flat gas plate. A commercially available 5% solution of PFSA of 1100 equivalent mass was poured into the space surrounded by the frame, and spread uniformly. The solvent was allowed to evaporate. The glass plate together with the frame and the membrane film were heated at 100°C for 1 hour. On cooling, the membrane was removed from the glass plate by dipping the glass plate into a batch of ice-cold water.

The composite membranes M1, M3, and M4 were prepared by spraying the PFSA solution on a water-soaked porous matrix of polytetrafluoroethylene or polypropylene. The dried composite membranes were cured by heating at 120°C for 2 hours. The desirable properties of these membranes,

as applied to ammonia removal, are

1. The PFSA polymer forms a nonporous membrane and hence does not permit transport of microorganisms through it. Thus a sterile sink is not needed.
2. Because of the nonporous nature of PFSA membranes, membrane pore blocking due to cell deposition is not a concern.
3. PFSA polymer, and other ion-exchange polymers, swell considerably in water and polar solvents, and therefore permit diffusion of polar solutes.
4. Good transport rates are achieved. Thinner membranes provide correspondingly higher species fluxes.

EXPERIMENTAL

Removal of Ammonia from Aqueous Media by Using a Liquid Sink

The membrane apparatus, the permeation cell of Fig. 1A used for these experiments, was made of a 48.5 mm i.d. 316 stainless steel tube. The cell had two compartments, the source compartment and the sink compartment, each with 190 mL capacity. The compartments were separated by the membrane. The membrane was held in position by means of an O-ring placed between flanges. Each compartment was provided with identical stirrers. The entire cell was placed in a constant temperature water bath. The source and the sink solutions were first placed in the same constant temperature bath and brought to the experimental temperature. Then the solutions were transferred to the respective compartments and stirring at 3 r/s was started. The ammonia concentration in the sink

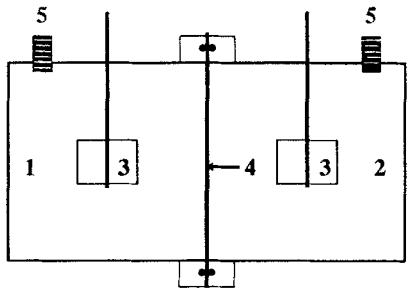

FIG. 1A The membrane apparatus. 1: Source compartment. 2: Sink compartment. 3: Stirrer. 4: Membrane. 5: Sample port.

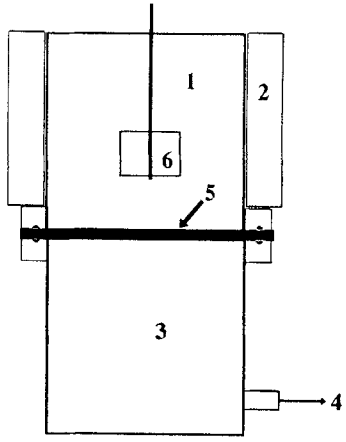

FIG. 1B Pervaporation cell. 1: Source compartment. 2: Jacket. 3: Vacuum compartment. 4: Vacuum connection. 5: Membrane. 6: Stirrer.

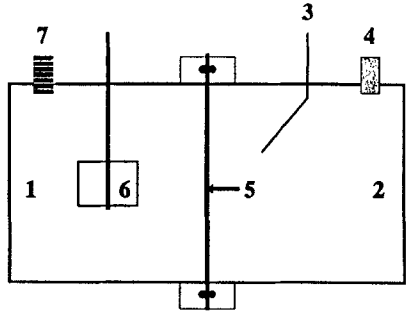

FIG. 1C Gas sweep cell. 1: Source compartment. 2: Gas sweep. 3: Gas inlet. 4: Gas outlet. 5: Membrane. 6: Stirrer. 7: Sample port.

solution was measured at the end of a predetermined period either by HPLC or by an ammonia-selective electrode.

Removal of Ammonia by Pervaporation

The pervaporation cell of Fig. 1B was made of a 52 mm i.d. 316 stainless steel tube equipped with a variable-speed stirrer. The total liquid capacity

of the source side of the cell was 150 mL. The sink side was separated from the source solution by a porous metallic plate which supported the membrane. The membrane was held in position by means of two O-rings placed between flanges. A jacket was provided for the source solution to control its temperature. The source solution containing 15 mM NH_4Cl was charged into the cell and stirred at 3 r/s. Water from a constant temperature water bath was circulated through the jacket to maintain the source solution at a desired temperature. Vacuum was then applied to get an absolute pressure of 2 torr on the sink side. Two receivers placed in Dewar flasks containing liquid nitrogen were used in the vacuum line to condense and freeze the permeate. The ammonia concentration in the permeate was measured after bringing the frozen permeate to ambient temperature. The source solution was maintained at pH 7.2 by using 0.1 M phosphate buffer. This apparatus and its use were described in more detail elsewhere (15).

Removal of Ammonia by Nitrogen Sweep

The gas sweep cell (Fig. 1C) for this experiment was made of a 316 stainless steel tube, 48.5 mm i.d., and was equipped with a variable speed stirrer on the source side. The solution capacity of the source compartment was 100 mL. The sink side was separated from the source side by a membrane held in position by means of O-rings. During the experiment the entire cell was immersed in a constant temperature water bath maintained at 37°C. One hundred milliliters of 15 mM ammonium chloride was charged to the source side and continuously stirred at 3 r/s. Nitrogen gas from a cylinder was introduced into the sink side at a flow rate of 100 mL/s (at 25°C) through a stainless steel coil placed in the same water bath. The temperature of both sides of the membranes was 37°C during the experiment. Ammonia, together with water vapor, was removed continuously by the nitrogen stream. The liquid level in the source compartment was maintained by adding distilled water from time to time.

RESULTS AND DISCUSSION

Evaluation of Membranes

All the membranes (M1 to M5) were first evaluated at 22°C for ammonia transport using an aqueous solution of ammonia (450 mM) as a source solution and deionized water as a sink solution. Figure 2 shows the results. Membranes M2 and M5 are PFSA membranes without any support and with thicknesses of 25 and 178 μm, respectively. Initial rates of ammonia transport across these membranes were 8.95 and 1.24 mol/h·m^2, respectively. The initial fluxes were computed from the slopes of the sink con-

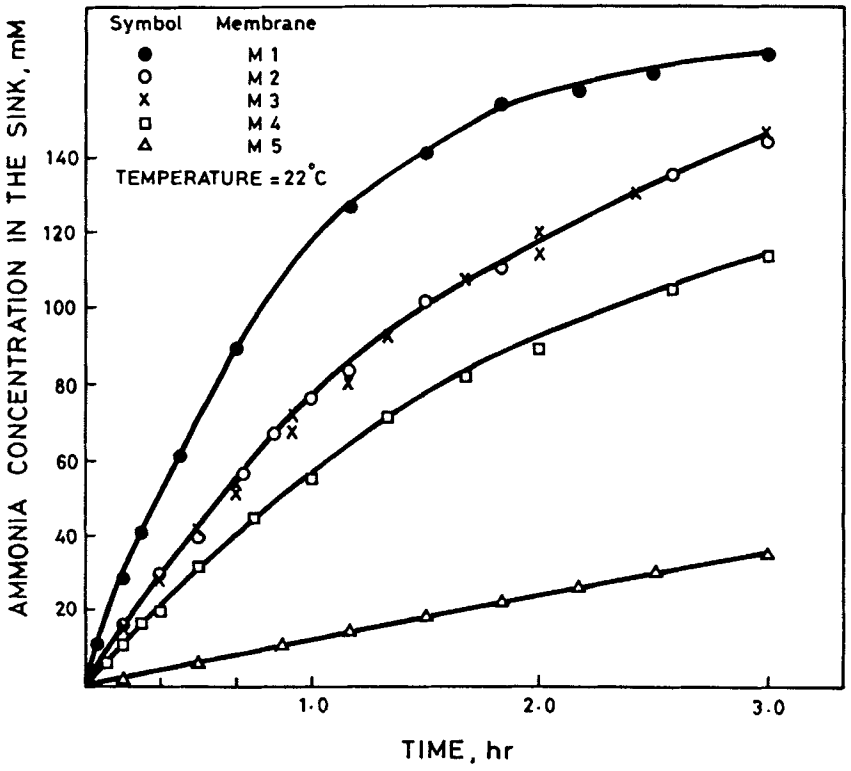

FIG. 2 Effect of membrane type on permeation of ammonia.

centration versus time at zero time, as discussed for this type of permeation experiments by Sikdar (13). As expected, the transport rate was inversely proportional to the membrane thickness. A comparison among the transport rates for the membranes indicates the superiority of the composite membrane M1 over the other membranes.

Membrane M1 exhibited an initial ammonia flux (17.9 mol/h·m^2) twice that given by Membrane M2 (8.95 mol/h·m^2). The porous backing of M1 offered significant transport resistance, otherwise for this 2 μm PFSA film thickness we would have expected an initial flux several times greater than a factor of 2. In a commercial application, the latitude one has in choosing a backing material, thin yet strong, with high porosity, will critically determine superior performance. Ammonia transport characteristics of Membranes M2 and M3 were identical, which merely indicates that the resistance offered by the 1.5 mil Tetratex backing was comparable to the

3 mil thick Tetratex film thermobonded to a polypropylene support. No technical specifications about these support materials, however, were available. Both M2 and M3 exhibited about 35% higher initial ammonia flux than did M4, which is a 2 μm PFSA film on a commercial porous polypropylene support. Membranes M1, M3, and M4 were all composite membranes (with 2 μm thick PFSA film) differing only in the support material. The comparative fluxes for these membranes indicated the effectiveness of the backing material.

No detailed permeation study was planned for all membranes. The screening data discussed here enabled us to select one membrane, the composite M1, for testing the efficacy of the concept of removing ammonia through a PFSA-type membrane for cell culture applications.

Effect of NH_3 Concentration

The effect of ammonia concentrations on ammonia permeation through M1 was investigated by varying the ammonia concentration of the source solution (15, 50, and 450 mM) at 22°C. Figure 3 shows the results. The initial flux was proportional to the ammonia concentration, as expected from Fick's law of diffusion. In all these cases the pH of the ammonia solution was above 11.5. At such pH values, more than 95% of ammonia exists as NH_3 in the solution. Thus it is safe to conclude at this stage that the observed transport of ammonia through the membrane was due to the diffusion of free NH_3 through the solvated hydrophilic layer of PFSA.

At pH 7.2, typical of mammalian cell culture media, however, a substantial portion of the ammonia exists in the protonated form, NH_4^+, in equilibrium with NH_3. In order to ascertain the exact diffusing species for experiments run at pH 7.2, NH_3 species concentration in the source solution was varied by varying the pH of the solution (7.2, 7.65, and 8.10) using 0.1 M sodium phosphate buffer and 15 mM concentration of NH_4HCO_3. On the sink side, the same buffer solution was employed without NH_4HCO_3. Figure 4 plots the ammonia removal rate against free ammonia concentration in the solution. The proportionality of the ammonia flux to the free NH_3 concentration again confirms the observation that ammonia was the diffusive species.

Effect of the Sink Environment

For these experiments a simulated cell culture medium was employed as the source solution. First a solution of MEM Eagle in 0.1 M sodium phosphate buffer was prepared (16). The simulated culture medium was prepared by mixing MEM Eagle solution (90% by volume) with calf serum (10%). The pH of the medium was adjusted to 7.2 at 37°C. Ammonium

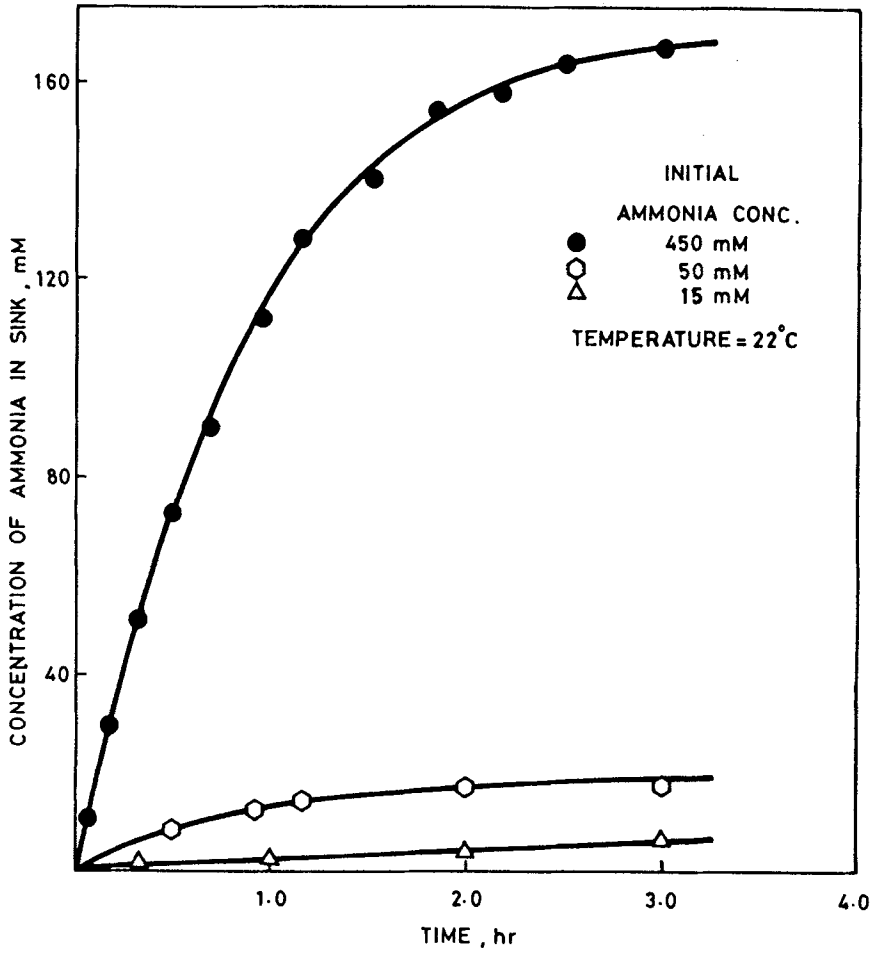

FIG. 3 Effect of ammonia concentration on permeation of ammonia.

chloride was dissolved in this solution to give 15 mM concentration and used as the source solution. Several different sink solutions were used: 0.025 M aqueous sulfuric acid, 0.1 M sodium phosphate buffer at pH 7.2, and MEM Eagle in 0.1 M sodium phosphate buffer (pH 7.2). In one series of experiments with 0.1 M phosphate buffer at pH 7.2, ammonium chloride was replaced by ammonium bicarbonate in the source solution. The results are provided in Fig. 5. For all sink solutions of pH 7.2, the permeation rates of ammonia were comparable. Permeation was also independent of

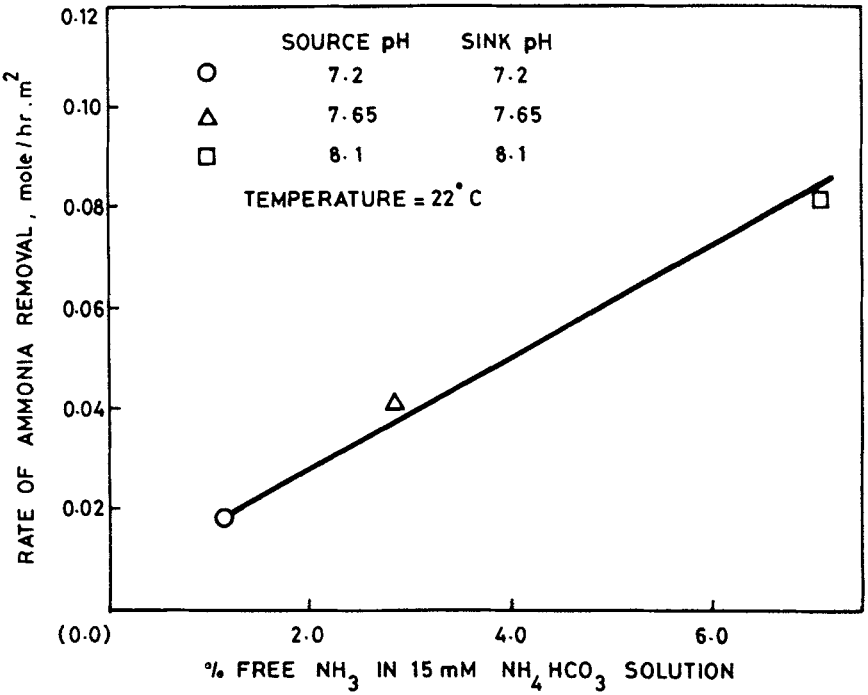

FIG. 4 Effect of free NH_3 concentration on rate of NH_3 removal.

the nature of the ammonia salt. For the experiments with the sulfuric acid sink, we would have expected somewhat higher ammonia fluxes, since in the sink solution free ammonia concentration was always zero because of chemical conversion to ammonium sulfate. From the data presented in Fig. 5, this expected result was not clearly demonstrated.

PFSA membranes were selective for ammonia. To establish this, we measured spectra in the 200 to 800 nm range of the following sink solutions: 1) 0.025 M aqueous sulfuric acid at the end of the experiment, 2) 0.025 M aqueous sulfuric acid before the experiment but spiked with ammonium chloride to make the ammonia concentration equal to that of 1). No difference in the spectra was observed. This fact indicated that the transport of other components did not take place for the duration of the experiment.

Ammonia Removal by Pervaporation

Several experiments were carried out with selected membranes (M1, M2, and M3) at 37°C, the temperature at which mammalian cell cultures

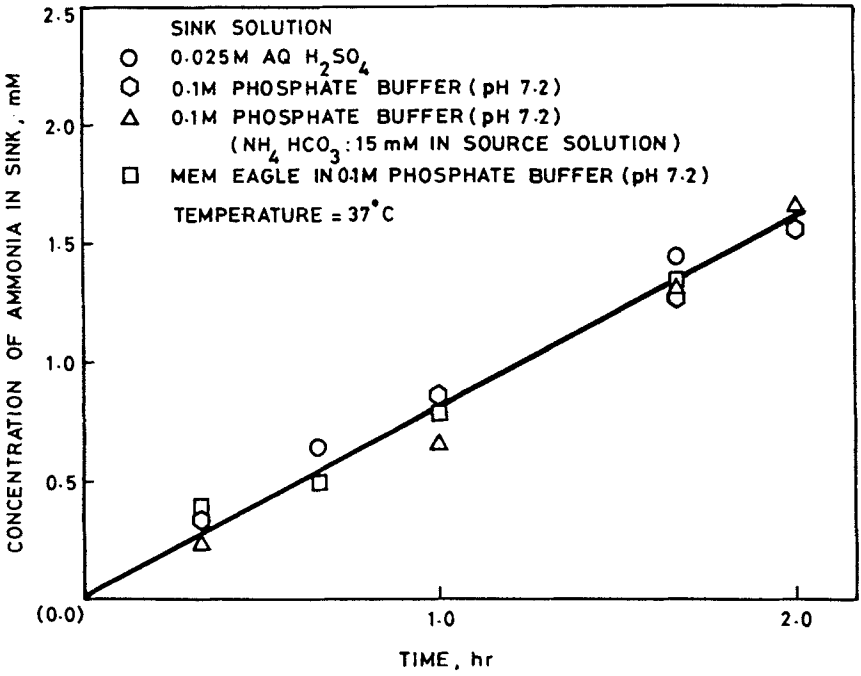

FIG. 5 Effect of sink environment on permeation of ammonia.

are run. The results are presented in Table 1. These results show that the flux for ammonia removal is almost 10–12 times higher than for the corresponding conditions using liquid sinks (data presented in Fig. 4). Also, ammonia fluxes through M1 and M2 membranes were only dependent on the pH and the temperature, and not on the use of MEM Eagle solution.

TABLE 1
Initial Rate of Ammonia Removal by Pervaporation at 37°C

Source solution	Membrane	Rate, mM/(h·m^2)
1. 0.1 M sodium phosphate buffer (NH_4Cl = 15 mM)	M1	1.6
2. Same as 1	M2	1.1
3. Same as 1	M3	0.91
4. 10% calf serum with 90% MEM Eagle in 0.1 M sodium phosphate buffer (NH_4Cl = 15 mM)	M1	1.58
5. Same as 4	M2	1.15

TABLE 2
Initial Rate of Ammonia Removal at 37°C by Nitrogen Sweep

Source solution	Membrane	Rate, mM/(h·m²)
1. 0.1 M sodium phosphate	M1	20.05
2. Same as 1	M2	14.12

Removal of Ammonia by Nitrogen Sweep

Results obtained with this strategy are presented in Table 2. The transport flux of ammonia for this strategy was much higher than even for pervaporation. Though no experiment was performed with a simulated cell culture medium in this case, the results of such experiments would be expected to be comparable to those presented in Table 2.

Among the three strategies, the last strategy gave far higher ammonia fluxes than the other two. In a process version of this strategy, the nitrogen gas can be recycled, if necessary, after its ammonia content is scrubbed with dilute sulfuric acid. The scrubbing will also ensure that recycled nitrogen is saturated with water, thereby eliminating the need to add make-up water to the cell media.

CONCLUSIONS

1. Among the membranes employed, the composite membrane consisting of a 2 μm layer of PFSA on a 1.5 mil Tetratex PTFE film exhibited the highest flux for ammonia under otherwise identical conditions. The PFSA composition was highly selective for ammonia.
2. All experimental strategies gave encouraging results for pervaporation as a promising method for commercial use. The chosen mode of operation will largely be determined by the economic consideration of creating a vacuum as against handling a gas sweep.

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