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Comparative Study of Enantioseparation of Racemic Tryptophan by Ultrafiltration Using BSA-Immobilized and BSA-Interpenetrating Network Polysulfone Membranes

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Effectiveness of Bovine Serum Albumin (BSA) as chiral recognizing protein in enantiomers separation by ultrafiltration technique was studied by immobilizing BSA on the membrane and incorporating BSA as semi-interpenetrating network in the membrane matrix. Separation of racemic tryptophan solution was performed in closed loop cross flow ultrafiltration using BSA immobilized polysulfone membrane and polysulfone membrane having BSA semi-IPN network. The volumetric flux (J_v), the solute flux (J_s), the separation factor (α), and the enantiomeric excess (%ee) of two types of membranes at different trans-membrane pressures and permeation times were determined. BSA semi-IPN membrane exhibits higher volumetric as well as solute fluxes compared to BSA immobilized membrane. Separation factor (α) to the order of 1.89 was achieved with BSA immobilized membrane after 8 h of ultrafiltration and in the same duration BSA-IPN membrane exhibited separation factor (α) to the order of 1.62. BSA immobilized membrane exhibits higher enantiomeric excess (30.8%) compared to BSA semi-IPN membrane (23.8%) after 8 hrs. BSA molecules available on membrane as immobilized or as semi-IPN under go complexion with tryptophan enantiomers differently. BSA immobilized membrane performed better separation and enantiomeric purity; however, the solute flux of the membrane decreases.

Keywords bovine serum albumin; enantiomeric excess (ee); separation factor; solute flux; volumetric flux

INTRODUCTION

There is growing interest in the pharmacological and toxicological properties of the chiral drugs due to increased appreciation of the potential significance of the differential biological properties of their enantiomers (1–3). Basically enantiomeric pure chiral compounds are obtained either by asymmetric synthesis or by separation of racemic mixture. Despite the large number of elaborate enantioselective syntheses for the preparation of single enantiomers, the separation and purification of racemic mixtures to obtain pure enantiomers is inevitable (4). Enantioselective separations have been realized in all possible separation techniques including gas chromatography, high pressure liquid chromatography, thin layer chromatography, electrophoresis, supercritical fluid chromatography, liquid–liquid extractions, etc (5–7). However, these techniques are batch processes and can separate a small amount per run and scaling up of process is expensive. Scientific and economic considerations to prepare optically pure chiral compounds emphasize the need to develop a suitable method for optical resolution, which could be operated continuously and commercialized easily.

In the last two decades membrane separation processes have emerged advantageous over conventional separation processes due to being modular, economical, eco-friendly, simple in operation and easy to scale up. Considering these factors membrane based separation is highly attractive for enantiomeric separation. Liquid membranes as well as solid polymer membranes have been studied for enantiomeric separation. Though liquid membranes showed high enantioselectivity, but they have poor stability (8). Solid polymer membranes being superior in stability therefore are considered to be more suitable for practical uses.

Enantioselective permeation through a solid polymer membrane had been first demonstrated using poly-L-glutamates with amphiphilic n-nonylphenoxy-oligoethyleneglycol side chains (9). C. Thoelen et al. demonstrated the chiral separation of tryptophan with polymer membranes based on poly (γ-methyl-L-glutamate) (10). Aoki et al. had performed comprehensive investigations on various chiral membranes for optical resolution (11). The polymer membranes reported so far are fragile in nature; therefore such membranes have been investigated in diffusion mode of permeation (12–21). The driving force for transport in diffusion cell is the concentration gradient that results in very low permeability. Pressure driven membrane processes such as reverse osmosis, nanofiltration, ultrafiltration etc. are suitable to achieve high permeability. The

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TABLE 1
Experimental results from the literature on the use of BSA as chiral selector in solution system as well as immobilized or grafted on the membrane for enantiomeric separation in membrane processes

Ref. no.	Separation system	Feed compound	Feed concentration	Permeability	Separation factor
23	Solution system	Tryptophan	2×10^{-4} M	6.3×10^{-4} M/m$^2 \cdot$ s at 1.5 bar	91% purity with 89% recovery
22	Solution system and immobilized	Phenylalanine and Leucine	0.05 mM and 0.0024 mM	0.8–1.1 m^3 m^{-2} d^{-1}	Less than 1 for Leucine
25	Solution system	Tryptophan	2×10^{-6} M to 1×10^{-4} M	Not given	D/D + L = 80%
26	Solution system	Tryptophan	10^{-4} M	Not given	L-D/L + D = < 0.4
27	Solution system	Tryptophan	0.3 mM−2 mM	Not given	$<2-11$
28	Solution system	Ibuprofen	Ratio BSA/Ibup. 3.4 mol	18.5 L/m^2.h	%ee 23 or less
29	BSA immobilized by grafting	Tryptophan	0.02 mM	10–80 ml/h	Not given
30	BSA immobilized	Tryptophan	1 mM	1.5 ml/min	Separation factor 12
31	Solution system	Tryptophan	0.4 mM	Not given	Purification factor 0.6–1
38	Solution system	Tryptophan	3×10^{-3} % w/v	21 l/m^2-h	%ee 33 at pH 9.2 & BSA 0.07% w/v
This study	BSA immobilized BSA-IPN membrane	Tryptophan	0.1% w/w	J_v (IPN) 4.71/m^2-h & (Immb) 2.81/m^2-h	%ee (Immb) 30.8 & (IPN) 23.8 $\alpha = 1.89$ (Immb) & $\alpha = 1.62$ (IPN)

ultrafiltration process offers very high permeability at low pressure therefore considered to be more practical for optical resolution using chiral selective legends such as bovine serum albumin (BSA) (22–30), deoxyribonucleic acid (DNA) (31–34) etc., immobilizing on the membranes or adding in the feed solution. Earlier reports (22–25) have clearly demonstrated feasibility of ultrafiltration for enantiomers separation using BSA as chiral selector in solution system. The use of BSA (26–30) and DNA (31–35) as immobilized or grafted on ultrafiltration membranes have been documented. Cai Lian et al. have reported modification of ceramic membranes by BSA chiral separation (36). Chiral separation of tryptophan by single-pass affinity ultrafiltration using hollow fiber membrane module and BSA as chiral selector in feed solution has also been reported (37). Experimental results indicated in the literature on the use of BSA as chiral selector in solution system as well immobilized or grafted on the membrane for enantiomeric separation in membrane processes have been collected, compared and as well as included in the manuscript as Table 1.

These studies have given a clear understanding of the preferential complexion of one of the enantiomers normally based on feed characteristics; however, a clear understanding of the effect of the process parameters such as pressure and permeation time on volumetric flux, solute flux, separation factor, enantiopurity, solutes profile in permeate etc. have yet to be achieved. Further studies reported have been conducted in dead end mode of ultrafiltration whereas for all large-scale separations cross-flow ultrafiltration is advisable.

The study aims to determine the effectiveness of BSA as chiral selector in cross flow ultrafiltration membrane process for enantiomers separation by immobilizing BSA on ultrafiltration membrane and by introducing interpenetrating cross linked network of BSA in membrane matrix.

EXPERIMENTAL

Materials

Polysulfone (Udel P-3500; Solvay advanced Polymers, USA), BSA MW 69,000 Dalton (fatty acids free, 98% pure)

(S.D Fine Chemicals Ltd. Mumbai (India)), Racemic, d and l tryptophan, and glutaraldehyde (25% solution) were procured from Sigma Chemicals, USA). All other chemicals and reagents (AR grade) were used in the study.

Preparation of Membranes

1. Preparation of asymmetric membrane: Asymmetric membranes were prepared by diffusion induced phase separation (DIPS) technique. In this process, a thin layer of the polymer dissolved in an appropriate solvent is cast on a suitable support and phase inversion is induced by immersing the polymer solution film in a non-solvent bath. Homogeneous polysulfone solution in dimethylformamide (15% w/w) was prepared by stirring continuously overnight. The polysulfone solution was cast on the polyester non-woven fabric (support), controlling the thickness and immersed into the water (non-solvent) gelation bath.
2. BSA semi-interpenetrating network (BSA semi-IPN) membrane: BSA semi-interpenetrating network (BSA-IPN) membrane consists of cross-linked network of BSA chains in polysulfone matrix. Interpenetrating network membrane was prepared by in-situ cross linking of BSA molecules by glutaraldehyde in polysulfone solution. First, BSA solution in dimethylsulfoxide (DMSO) (5 mg/ml) was prepared then 2 ml glutaraldehyde (25%) solution was added drop wise to fully dissolved BSA under continuous mixing. The reaction mixture was then added to 15% polysulfone solution in dimethylacetamide and thoroughly mixed for 4 h using mechanical stirrer. The solution was evacuated to remove air bubbles. The membrane was prepared on non-woven polyester fabric support by wet phase inversion method similar to polysulfone membrane. The membrane was washed with de-ionized water till uncross-linked BSA was completely removed. The evidence of BSA in the membrane was recorded by EDX mode of SEM of membrane samples.
3. Immobilization of BSA on polysulfone membrane: BSA was immobilized on membrane by ultrafiltration technique by permeating BSA solution (2 mg/ml) in phosphate buffer (at pH 7) through membrane for 4 h at 344.7 kPa pressure. The membrane was removed from filtration unit and washed with deionized water to remove excess amount of BSA from the membrane surface. BSA adsorbed on the membrane surface and in the pores was cross linked using glutaraldehyde by passing glutaraldehyde (5% solution) through the membranes for 4 h at 344.7 kPa pressure, and finally membranes were cured at 60°C for 10 min. The amount of BSA immobilized on the membrane was estimated by determining the concentration of BSA in solution by UV-Vis spectrophotometer at 280 nm for feed and permeate.

METHODS

Characterizations

Pore Size Analysis

Pore size analysis of membranes was done by Capillary Flow Porometer (Porous Materials Inc, USA, Model 1500 AEX), considering the pores as a capillary. First, the membrane samples were soaked in a wetting liquid, Porewick having low surface tension (γ) 16 dynes/cm^2 and contact angle is zero i.e., it wet the membrane samples fully and spontaneously fills all the pores in the sample. Then the gas was blown through the membrane and the pressure of the gas was gradually increased. The basis of the porometry can be presented as

$$D = 4\gamma \, \mathrm{Cos}\,\theta/p \tag{1}$$

Where D is the diameter of a pore, θ is the contact angle, and p is differential pressure. For wetting liquid Porewick $\theta = 0°$, the equation becomes

$$D = 4\gamma/p \tag{2}$$

From this equation diameter of pores in the membrane is calculated. The data of Porometric analysis of membranes is shown in Table 2.

Enantiomers Analysis

Concentrations of tryptophan enantiomers in permeate of ultrafiltration were estimated on HPLC (Waters) fitted with Photodiode array detector at 278 nm, using Crownpack column (CR+, Diacel chemical co.). Concentration of racemic tryptophan in feed and permeate was determined by UV-Vis spectrophotometer at 280 nm.

TABLE 2
Porometry results of the membranes

Membrane identity	Bubble point pressure (psi)	Bubble point pore dia. (μ)	Maximum pore size distribution	Dia. (μ) at (mpsd)
Polysulfone	65.62	0.1012	5824.55	0.0843
PS-BSA Immobilized	101.367	0.0655	3267.56	0.0670
PS-BSA semi-IPN	89.356	0.0943	5907.18	0.0346

Pressure Driven Filtration

The ultrafiltration experiments were conducted in cross flow and closed loop mode on filtration module having four cells connected in series. Each cell has a circular shape membrane having an effective membrane area of $0.00195\,m^2$. The concentration of feed and permeate was recorded at specified intervals so the observed value is not cumulative instead it is value at that time. Flux values were recorded at various pressures from 68.9 to 344.7 kPa and times ranging from 2 to 12 h were determined at constant temperature of 25°C after achieving steady state when decline in flux is nominal which was achieved after two hours of permeation.

The performance of the membrane separation process is exhibited in terms of Membrane Flux (Permeability) and Separation (Selectivity).

Membrane Flux: Membrane flux is a measure of productivity of the membrane process, and is described as the volumetric flux (J_V) and solute flux (J_s) as well.

Volumetric flux (J_V): Volumetric flux (J_V) is the volume of liquid in liter permeated through per unit area of membrane per hour. On the basis of the Hagen-Poiseuillie equation, volumetric flux (J_V) is expressed by the following equation:

$$J_v = n\pi r^4 \Delta p / 8\,\mu l \tag{3}$$

Here, n is number of pores, r is radius of membrane pores, Δp is trans-membrane pressure, μ is viscosity of the fluid, and l is the membrane thickness. Volumetric flux (J_V) is in direct relation to trans-membrane pressure (Δp), it is therefore essential to determine volumetric flux (JV) of membrane at various pressures.

Solute flux (J_s): Solute flux (J_s) is the amount of dissolved solute in grams or moles permeated through per unit area of membrane per hour and is represented as:

$$\text{Solute flux } (J_s) = Q/A \cdot t \tag{4}$$

where Q is the amount of dissolved solute permeated, A is the effective membrane area, and t is the time of permeation.

Enantioseparation: In an enantiomeric separation process, the membrane selectivity for one of the paired enantiomers is responsible for optical purity which is defined in terms of the separation factor (α) and % enantiomeric enrichment or excess (%ee).

Separation factor (α): Separation factor (α) is defined as:-

$$\frac{\alpha = J_{S,d}/J_{S,l}}{C^{df}/C^{lf}} \tag{5}$$

Where $J_{s,d}$ and $J_{s,l}$ refer to the fluxes of d-tryptophan and l-tryptophan respectively and C^{df} and C^{lf} are concentrations of enantiomers d-tryptophan and l-tryptophan in the feed. Since feed was the solution of the racemic tryptophan, the separation factor (α) may be defined as

$$\alpha = J_{s,d}/J_{s,l} \tag{6}$$

Enantiomeric enrichment or excess (%ee): The enantiomeric excess is a measure of optical purity of a chiral compound and expressed as % enantiomeric excess or % ee. It is indicative of the percentage of one enantiomer in %excess of the other in the sample.

$$\%ee = (C^{lP} - C^{dP}/C^{lf} + C^{df}) \times 100 \tag{7}$$

Here, C^{lP} and C^{dP} are concentrations of l-tryptophan and d-tryptophan in permeate respectively.

RESULTS AND DISCUSSIONS

Volumetric Flux (J_v)

The variation in volumetric flux (J_V) of racemic tryptophan solution through membrane and pure water flux (PWP) of polysulfone membrane at different trans-membrane pressures and times is depicted in Figs. 1 and 2 respectively. The volumetric Flux (J_V) and PWP of membranes vary on increase in trans-membrane pressure (Δp) in accordance with the Hagen-Poiseuillie equation; however, the increase rate is not followed systematically. Initially the increase rate is higher and decreases gradually.

The BSA-IPN polysulfone membrane exhibited higher flux compared to BSA immobilized membrane, because

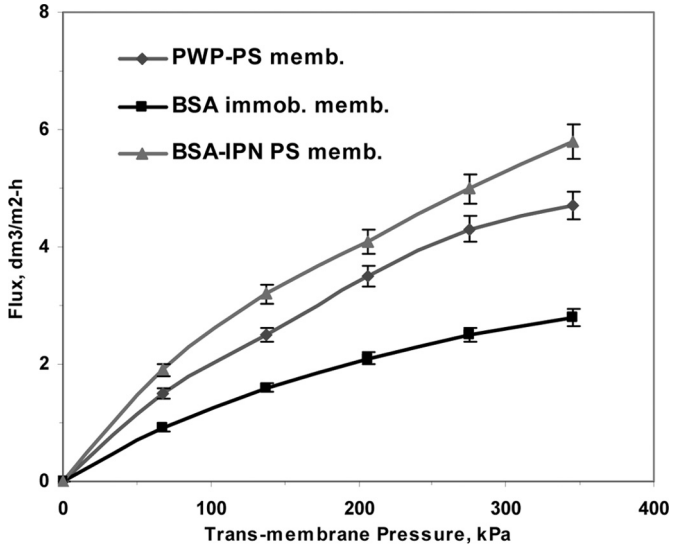

FIG. 1. Variation in volumetric flux of membranes and pure water flux (PWP) of polysulfone membrane at different trans-membrane pressures (feed 0.1% racemic tryptophan).

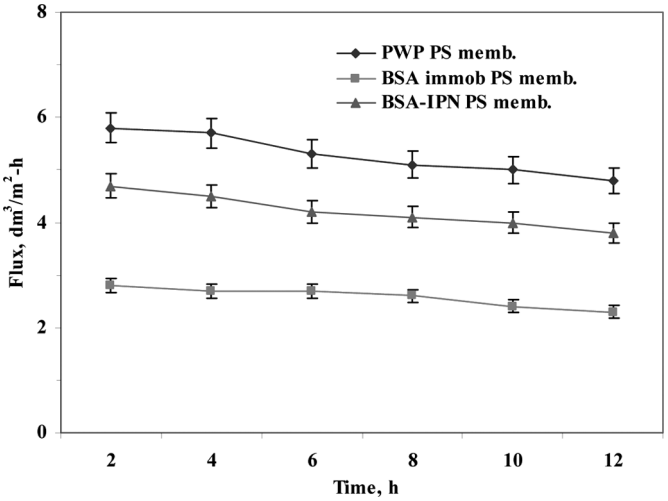

FIG. 2. Time dependence variation of volumetric flux of membranes, and pure water flux (PWP) of polysulfone membrane (feed 0.1% racemic tryptophan, at 344.7 kPa).

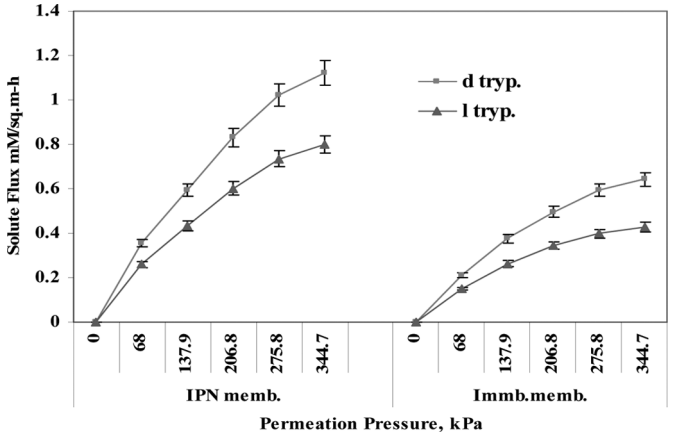

FIG. 3. Solute flux (J_s) of d & l enantiomers of tryptophan through BSA modified membranes at different permeation pressures (feed 0.1% racemic tryptophan, at 344.7 kPa).

during BSA immobilization few molecules of BSA seem to have attached at pore opening of few pores, thus such pores have been blocked by BSA molecules thereby the porosity of the membrane has decreased resulting in less flux. The decrease in porosity of the membrane has been evidenced from porosity analysis of membranes (Table 2). The pore diameter for the membranes at maximum pore size distribution order is Polysulfone > BSA immobilized Polysulfone > BSA-semi IPN. The pore diameter detected at maximum pore size distribution is 6.7×10^{-8} m in BSA immobilized with respect to 8.43×10^{-8} m for polysulfone membrane. This indicates a decrease in the pore size of the membrane as a result of immobilization.

Time dependence study shows the decrease in volumetric flux with time; however, the rate of decrease is also not uniform. The BSA immobilized membrane exhibited marginally lower rate of decrease with respect to BSA semi-IPN membrane in same duration. Time dependence decrease in flux is possibly due to compaction phenomenon and concentration polarization.

Solute Flux (J_s)

The solute flux (J_s) of d and l enantiomers of tryptophan through BSA modified membranes at different permeation pressures are shown in Fig. 3. It is seen that BSA semi-IPN polysulfone membrane exhibited higher fluxes for both enantiomers compared to BSA-immobilized polysulfone membrane. Higher solute fluxes are a consequence of higher volumetric flux of BSA semi-IPN membrane (4.7 dm^3/m^2-h *vs* 2.8 dm^3/m^2-h) for BSA immobilized membrane at 344.7 kPa. The solute flux of both enantiomers increases as permeation pressure increases, however, the increase rate is not uniform. Further the flux of d-tryptophan is higher than that of l-tryptophan through both types of membranes. There is 0.27% rise in d-tryptophan flux and 0.19% rise in l-tryptophan flux for each kPa pressure increase in case of BSA semi-IPN membrane whereas for BSA immobilized membrane the rise in flux of d-tryptophan and l-tryptophan for each kPa pressure rise is 0.15% and 0.10% respectively. A comparatively higher flux of d-tryptophan is indicative of its preferential permeability by both types of membranes. It is due to the fact that BSA undergo complexion with tryptophan enantiomers differently (22).

BSA is known to possers several binding sites for tryptophan molecules, termed as the primary binding sites and secondary binding sites. l-tryptophan binds with one of the primary binding sites in a highly stereo-specific manner. l-tryptophan binds to a secondary site also with low complexion constant. The secondary binding site is not a stereo-specific site and both d- and l-tryptophan can bind with it competitively with same complexion constant (23). The binding constant of BSA with l-tryptophan is reported to be $4.4 \times 10^4\,M^{-1}$ and for d-tryptophan is $0.510 \times 10^4\,M^{-1}$ (27). Hence large numbers of l-tryptophan molecules interact with BSA molecules in the membrane results into low concentration of l-tryptophan in the permeate. D-tryptophan interacts with BSA though comparatively less, therefore permeated preferentially. Preferential complexion of l tryptophan with BSA molecules might be the reason of low permeation of l-tryptophan. These observations confirm the interaction of tryptophan molecules with BSA present in the membrane surface.

The time dependence of the solute flux (J_s) of enantiomers of tryptophan through BSA modified membranes is shown in Fig. 4. It is seen that the flux of both enantiomers (d-tryptophan and l-tryptophan) through both types of membranes decreases with time. The decrease is rapid for

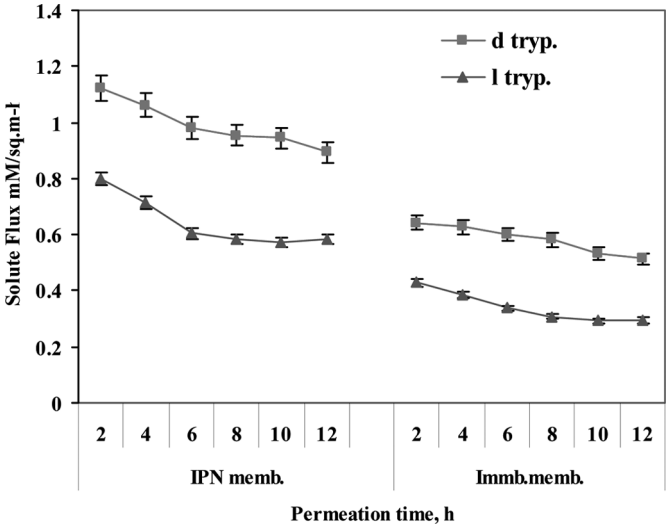

FIG. 4. Variation in solute fluxes of enantiomers of tryptophan through BSA modified membranes with time (feed 0.1% racemic tryptophan, at 344.7 kPa).

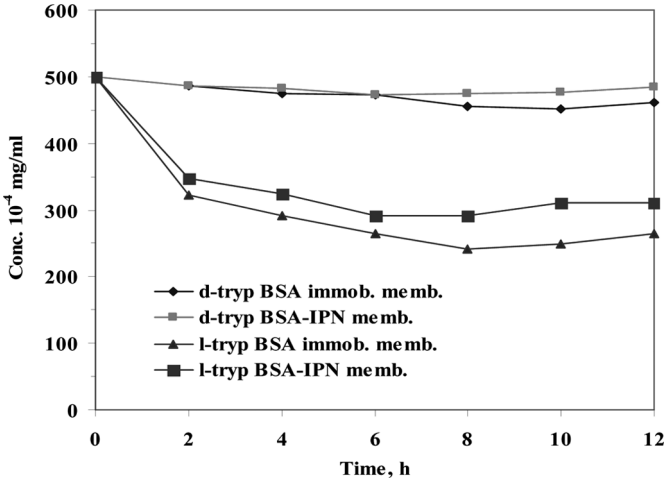

FIG. 5. Variation in concentration of enantiomers of tryptophan in permeate with time (feed 0.1% racemic tryptophan, at 344.7 kPa).

BSA semi-IPN membrane compare to BSA immobilized membrane. Further the decrease in flux of l-tryptophan is more in comparison to d-tryptophan. In case of BSA semi-IPN membrane the flux of l-tryptophan decreases sharply up to 6 hrs (24%) and the flux of d-tryptophan decreases 12% only in the same period. In case of BSA immobilized membrane the decrease in flux of l-tryptophan is 9.3% and of d-tryptophan is 6% in the same period. The higher decrease in the flux of l-tryptophan is the result of a combined effect of decrease in volumetric flux of membranes and interaction of l-tryptophan molecules with the BSA molecules present in the membrane. The rapid decrease in the solute fluxes of enantiomers in case of BSA semi-IPN membrane is due to higher compaction of BSA-IPN membrane as a result of stretching of BSA net work in polysulfone matrix. The differential decrease in fluxes of d and l enantiomers indicates that the mechanism of transport of d- and l-tryptophan is different for two types of membranes. The BSA immobilized membrane transport d-tryptophan more due to preferential interaction with l-tryptophan and BSA semi-IPN membrane transport tryptophan due to preferential adsorption and diffusion through the membrane.

The time dependence study of the concentrations of tryptophan enantiomers in permeate and retentate are given in Figs. 5 and 6 respectively indicated that the complexion of BSA with tryptophan enantiomers is reversible and time dependent. The flux of l-tryptophan through BSA modified membranes decreases rapidly up to 6 hours, and thereafter the decrease is nominal that indicates the binding sites for l-tryptophan all have been occupied within 6 hrs and thereafter only few sites are available for binding and bind molecules get detached form BSA site thus flux of l-tryptophan normalizes.

Separation Factor (α)

The variation of separation factor (α) with permeation time is displayed in Fig. 7 Higher separation factor ($\alpha = 1.89$) has been observed with the BSA immobilized membrane after 8 hours and thereafter the separation factor declines as the permeation time increases. BSA semi-IPN membrane exhibits low separation factor ($\alpha = 1.62$) in similar permeation time. Higher separation factor for BSA immobilized membrane might be due to the fact that more BSA molecules are present on the surface of the membrane for complexion because immobilization is a surface phenomenon whereas in case of IPN

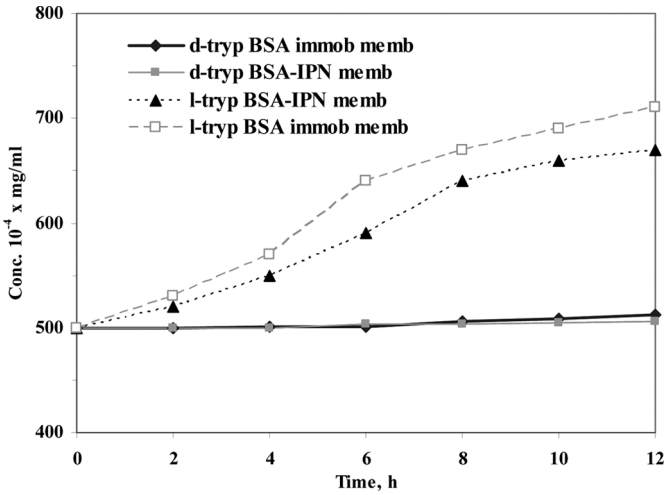

FIG. 6. Variation in concentration of enantiomers of tryptophan in retentate with time (feed 0.1% racemic tryptophan, at 344.7 kPa).

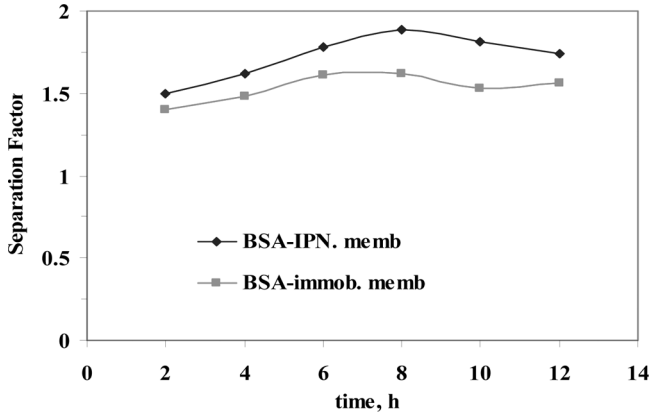

FIG. 7. Separation factors of membranes with time, (feed 0.1% racemic tryptophan, at 344.7 kPa).

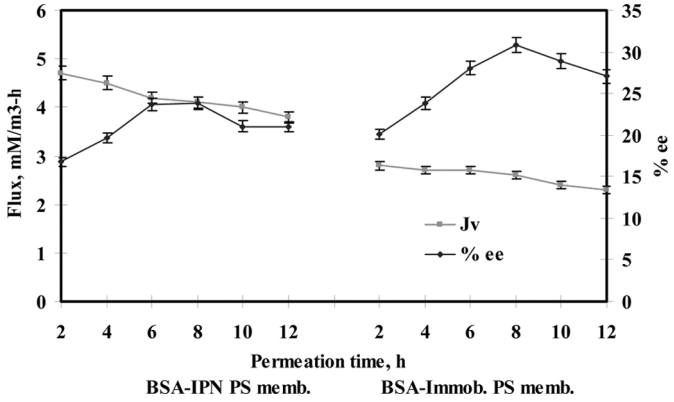

FIG. 9. % ee vs. volumetric flux of membranes at different permeation time.

membrane BSA molecules are distributed throughout the matrix of the membrane. The E-DEX analysis of the BSA immobilized membrane samples indicate higher content of sulphur and nitrogen ($S = 3.21$ & $N_2 = 10.92$ wt. % vs. $S = 2.72$ & $N_2 = 9.55$ wt. % in BSA-IPN membrane) which is an evidence of more BSA molecules in BSA immobilized membrane.

% Enantiomeric Enrichment or Excess (% ee)

The time dependent study of enantiomeric excess (% ee) of d-tryptophan in permeates of membranes is shown in Fig. 8, and % ee in relation to volumetric flux (J_V) with time is given in Fig. 9. It is seen that BSA immobilized membrane exhibits higher enantiomeric excess (30.8%) compared to BSA semi-IPN membrane (23.8%). Further, it is observed that enantiomeric enrichment (% ee) increases steadily up to 8 hrs. After 8 h% ee starts the decline trend for both types of membranes. The time dependency of %ee indicates that BSA tryptophan complexion equilibrium is reached within 8 hrs. After 8 h no more complexion sites on BSA are available for free so the maximum enrichment is achieved. Complexion being reversible hence sites get free due to desorption of tryptophan molecules. The higher %ee of BSA immobilized membrane has resulted due to the presence of more BSA molecules on the surface of the membrane for complexion which is evident from the E-DEX analysis of membrane samples.

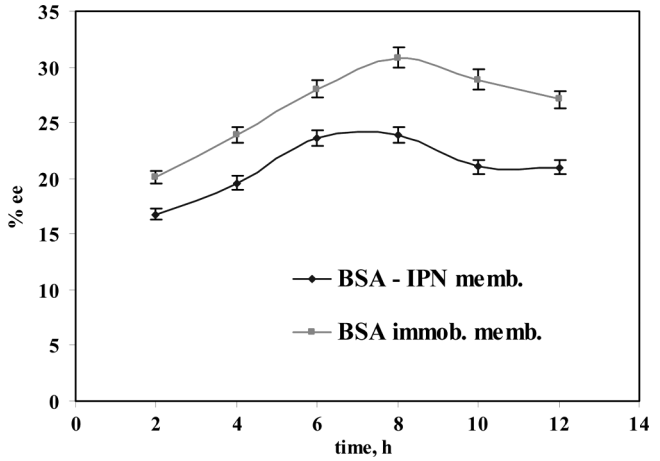

FIG. 8. Enantiomeric excess of membrane with time (feed 0.1% racemic tryptophan, at 344.7 KPa).

CONCLUSION

Enantiomers separation of racemic tryptophan was performed by ultrafiltration membrane process in a closed-loop and cross-flow mode using BSA modified polysulfone membranes. The observations of the study conclude that BSA can be used as chiral selector protein in enantiomeric permeation of α-amino acids by ultrafiltration. Volumetric and solute flux of enantiomers through membranes increases with permeation time and transmembrane pressure. BSA semi-IPN polysulfone membrane exhibit higher volumetric as well as solute fluxes compare to BSA immobilized membrane. A separation factor to the order of 1.89 was achieved with BSA immobilized membrane after 8 hrs. BSA immobilized membrane exhibits higher enantiomeric excess (30.8%) compared to BSA semi-IPN membrane (23.8%) after 8 h. BSA molecules available on membrane as immobilized or as semi-IPN undergo complexion with tryptophan enantiomers differently having preferential complexion with l-tryptophan might be the reason for enantiomers separation.

ABBREVIATIONS

Volumetric flux	(J_V)
Solute flux	(J_s)
Enantiomeric enrichment or excess	(%ee)
Separation factor	(α)

Flux of d-tryptophan	$J_{s,d}$
Flux of l-tryptophan	$J_{s,l}$
Concentrations of enantiomer d-tryptophan in the feed	C^{df}
Concentrations of enantiomer l-tryptophan in the feed	C^{lf}
Concentrations of enantiomer l-tryptophan in permeate	C^{lP}
Concentrations of enantiomer d-tryptophan in permeate	C^{dP}

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