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Studies on the separation of proteins and lactose from casein whey by cross-flow ultrafiltration

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ABSTRACT

A novel strategy has been developed for separation of individual whey protein fractions and lactose from casein whey by a cascade of different molecular weight cut-off (MWCO) cross-flow ultrafiltration (UF) membranes. Centrifugation (166.67 r.p.s., 277 K, 1800 s) followed by microfiltration (MWCO: 0.45×10^{-6} m) were employed for separation of fat molecules and suspended solids from casein whey. Immunoglobulins, such as IgG, IgA, and IgM were separated as retentate of 100 kg mol^{-1} UF membrane; bovin serum albumin, lactoperoxidase, and lactoferrin were separated as retentate of 50 kg mol^{-1} UF membrane; lactose were separated by 5 kg mol^{-1} UF membrane as permeate, and major proteins like β-lactoglobulin (molecular weight 18.3 kg mol^{-1}) and α-lactalbumin (molecular weight 14.2 kg mol^{-1}) were separated by proper control of pH. At pH 5.4 the most dominant whey protein, β-lactoglobulin (isoelectric point 5.2–5.4), formed dimer which was found to have immense effect on the separation characteristics. Hydrodynamic studies were conducted under different trans-membrane pressures (TMPs), 0.686–2.942 bar using four-stages of discontinuous diafiltration (DD) with constant volume concentration factor (VCF 2). In all cases highly purified proteins were obtained at the 3rd stage of DD process under an optimum TMP of 2.06 bar.

Keywords: Ultrafiltration; Whey protein; Lactose; Permeate flux; Trans-membrane pressure; Discontinuous diafiltration stage

1. Introduction

An outstanding development in the field of biotechnology, particularly towards isolation and purification of biopharmaceuticals from crude extract are responsible for proliferations of the downstream process technology [1]. Since protein is directly involved in biological functions, a great deal of emphasis has been placed on developing new tools for protein purification. The fundamental challenges of protein purification are high purity and pilot scale production [2].

Ultrafiltration (UF) is primarily a size-exclusion-based pressure-driven membrane separation strategy, accepted and well practiced processing operation in the dairy industry for whey treatment. Depending on molecular weight of target molecules, UF membranes of specific molecular weight cut-off (MWCO) have been chosen for the separation process. During UF, high molecular weight components, such as protein and suspended solids are rejected, while low

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molecular weight components like mono-saccharides, di-saccharides, salts, amino acids, organic, and inorganic acids pass through the membrane freely. Being a pressure-driven membrane separation process, pressure is applied over the UF membrane, which will facilitate permeation of low molecular weight solute through membrane. One of the major problems of UF technique is the declination of permeate flux with time, which results mostly from deposition of rejected solute over membrane, known as concentration polarization, which often leads to membrane fouling. In fact, fouling plays an important role in the separation of whey proteins by UF. Compared to conventional processes, the use of diafiltration (DF) offers several advantages, including high product purity, elimination of filter aids, and wetting agents. In the batch mode the different stages of DF are carried out in consecutive manner. After the pre-concentration of the batch, the DF liquid is added into the batch tank, and UF continues until the desired concentration of low molecular weight solute molecule in the permeate is reached [3,4].

Dairy industry, a major economical source of tropical and subtropical countries, is generating a large volume of waste liquid effluent, namely whey, having high biological oxygen demand, and chemical oxygen demand. Due to stricter environmental legislations, whey is considered to be a major challenge to the environmental scientists owing to high pollution load [5–7]. Whey is a heterogeneous mixture of different types of proteins, such as immunoglobulins, α-lactalbumin, β-lactoglobulin, bovine serum albumin, and lactoferrin, carbohydrates, such as lactose, fat, etc. those have their unique physico-chemical, functional, nutraceuticals, and medicinal values. The concentrations, characteristics, and pharmaceutical importance of individual whey proteins are described in Table 1.

Therefore, purification of different biomolecules from casein whey will be useful both in dairy, food and pharmaceutical industries, and a large economic boom could be expected. Moreover, the process is also attractive for the implementation of the concept of zero-effluent discharge with respect to dairy effluents. UF was first used in dairy industry for the production of soft cheese in 1969 and this technology rapidly gained popularity in dairy industry [24]. In 1979, about 3% of available world's whey production employed the UF technique, which reached 8–10% in some major cheese producing countries around the world [25]. According to Maubois the first-generation membranes were cellulose acetate symmetric membrane, and used in soft cheese, cottage cheese, feta cheese, and cheddar cheese production [26]. During that period UF technique was used as a sterilization process [27]. The goal of research works was to increase the volumetric permeate flux by changing the volume concentration ratio [28,29]. Maubois and Brule reported that the area of the installed membrane was increased from 300 m^2 in 1971 to 70,000 m^2 in 1981 [25] Maubois and Brule described that during that period the main types of membranes were flat sheet, tubular shape, hollow fiber, and spiral shape [25,26]. A statistical report published by Horton [31] is demonstrated that by December 1982, there were close to 100 plants worldwide that used UF or RO to process their milk, prior to cheese making in some fashion or other, 15 of which were said to be located in North America [31]. It was reported that the automation of UF unit, made long operation times of 20 h per day possible, and the payback period only 8–9 months. As of 1983, over 95% of the Danish feta cheese production of 77,000 ton per year is produced on Pasilac/DDS UF plants [30]. Due to microbial degradation of cellulose acetate membranes, researches were turned to develop polymeric membrane and second-generation membrane [32]. Upto 1990, researches paid more attention to separate protein molecules as a retentate and lactose as permeate. Presently, rationalism and scholarly rebellion of biotechnology with new visions, separation of individual protein molecules from whey, also the valorization of whey took great attention. Besides the polymeric membrane, ion exchange membrane chromatography, gel permeation chromatography, ceramic chromatography, and composite membrane chromatography have also been practiced [33]. The detailed works carried out in this direction have been represented in Table 2.

In the present investigation, without going for mere treatment of whey, the isolation of valuable biomolecules from whey has been attempted. Although works have already been published in the field of whey protein separation with high purity by chromatography, and also membrane-based combinations, and chromatographic separation techniques, but due to high cost, and limited scope of scale-up from bench-scale to commercial production capacity, chromatographic separations are not acceptable. The novelty of this present work is the separation of fractions of individual biomolecules using series of different MWCO (100–5 kg mol^{-1}) cross-flow UF membranes with possible high throughputs. Centrifugation (166.67 r.p.s., 277 K, 1,800 s) followed by microfiltration (MF) (0.45×10^{-6} m) were employed for separation of fat molecules and suspended solids from casein whey. Different fractions of immunoglobulin, such as IgG, IgA, and IgM (molecular weight 150–900 kg mol^{-1}) were separated as retentate of 100 kg mol^{-1} UF membrane; bovin serum albumin (molecular

Table 1
Characteristics and pharmaceutical importance of major proteins present in whey

Protein	Concentration ($kg\,m^{-3}$)	Characteristics	Pharmaceutical importance	Reference
β-lactoglobulin	3.5 ± 0.02	Molecular weight: 18.3 kDa, isoelectric point: 5.2–5.4	Potential sources of essential amino acids. Used in power drinks and confectionary products. Good gelling agent and better foam stabilizer. Regulate phosphorus metabolism in mammary gland. Transporter for vitamin D, cholesterol and retinol. Transfer of passive immunity to the newborn and the enhancement of pregrastic esterase activity	[8–11]
α-lactalbumin	1.4 ± 0.01	Molecular weight: 14.2 kDa, isoelectric point: 4.2	Used in infant formula and as a neutraceutical because of its high tryptophan content. Enhanced whippability in meringue-like formulations. Strong affinity for glycosylated receptors on the surface of oocytes and spermatozoids	[10–13]
Bovin serum albumin	0.4 ± 0.01	Molecular weight: 66 kDa, isoelectric point: 4.9–5.1	Potential of fatty acid binding, anti-mutagenic function, and cancer prevention. Good gelling agent. Used as a standard protein	[10,11,14,15]
Lactoperoxidase	0.06 ± 0.01	Molecular weight: 78 kDa, isoelectric point: 9.6	Catalyzes the oxidation of thiocynate by hydrogen peroxide and generates intermediate products with antimicrobial properties. Used to preserve raw milk quality	[15–18]
Lactoferrin	0.05 ± 0.02	Molecular weight: 78 kDa, isoelectric point: 8	Broad spectrum antimicrobial (antiviral, antibacterial) activity, promotion of iron transfer, and absorption, cancer prevention, cell proliferation, differentiation, and antiparasitic activity. Used as a natural bioactive ingredient in infant formulae, dietary supplement tablets, skin care, and oral health care products	[9,19–21]
IgG	0.2 ± 0.02	Molecular weight: 150 kDa, isoelectric point: 6.5–9.5	Antipathogenic agent	[22,23]
IgA	0.3 ± 0.01	Molecular weight: 320 kDa, isoelectric point: 4.5–6.5	Antipathogenic agent	[22,23]
IgM	0.1 ± 0.01	Molecular weight: 900 kDa, isoelectric point: 4.5–6.5	Antipathogenic agent	[22,23]

weight 66 $kg\,mol^{-1}$), lactoperoxidase (molecular weight 78 $kg\,mol^{-1}$), and lactoferrin (molecular weight 78 $kg\,mol^{-1}$) were separated as retentate of 50 $kg\,mol^{-1}$ UF membrane; and lactose was separated (in the permeate) by 5 $kg\,mol^{-1}$ UF membrane. Separation of close molecular weight protein fractionations, like α-lactalbumin (molecular weight 14.2 $kg\,mol^{-1}$) and β-lactoglobulin (molecular weight 18.3 $kg\,mol^{-1}$) could not be achieved by usual sieving mechanism characteristics of conventional UF technique. In this case, some exclusive property of β-lactoglobulin such as dimerization at a particular pH 5.4 has been exploited to obtain a reasonable separation between α-lactalbumin and β-lactoglobulin. Hydrodynamic studies were also conducted for four-stages DD process, at different trans-membrane pressures (TMPs), ranging from 0.686 to 2.942 bar at constant VCF in order to achieve high permeate flux, lactose, and protein recoveries.

2. Experimental

2.1. Materials

Raw casein whey was obtained from local sweetmeat industries situated in-and-around Kolkata, India. The pH of the raw casein whey was found to

Table 2
Different methodologies for protein purification from whey

Methodology	Feed stock	Protein of interest	Reference
Ion-exchange resin, electrodialysis or reverse osmosis, and finally vacuum evaporation	Acid casein, sodium caseinate or calcium caseinate	Glycomacropeptide	[34]
Ion-exchange resins, and UF	Milk whey	Glycomacropeptide	[35]
Heat treatment, ethanol precipitation, centrifugation, and ion exchange chromatography	Milk whey	Glycomacropeptide	[36]
De-saltation, lyophilization, and ion-exchange chromatography	Whey of lactic casein	Glycomacropeptide	[37]
UF followed by ethanol precipitation	Proteins of a whey concentrate	Glycomacropeptide	[38]
UF followed by heat treatment, and pH treatment	Whey	Glycomacropeptide	[39]
Different MWCO UF membrane	Sodium caseinate	Glycomacropeptide	[40]
Combination of pepsin treatment, and membrane filtration	Whey protein concentrate	β-lactoglobulin	[41]
Membrane chromatography; membrane	Whey	β-lactoglobulin, α-lactoglobulin, and Bovin serum albumin	[42]
Membrane chromatography; membrane	Whey	β-lactoglobulin, α-lactalbumin, Bovin serum albumin, and IgG	[43]
Ion exchange chromatography	Milk derived products with phenylalanine concentration of 0.5% (w/w)	Glycomacropeptide	[44]
Membrane chromatography	Whey	β-lactoglobulin, α-lactalbumin, and Bovin serum albumin	[45]
Continuous, and discontinuous diafiltration by UF membrane	Whey	β-lactoglobulin and α-lactalbumin	[46]
Anion-exchange chromatography	Non-dialyzable fraction of whey	Glycomacropeptide	[47]
Two step of ion exchange membrane chromatography (anion exchangers of opposite polarity) in series	Whey	Glycomacropeptide	[48]
Anion-exchange chromatography with resin, and Amicon YM100 membrane	Milk whey	IgG and glycomacropeptide	[49]
Exclusion chromatography, and membrane chromatography	Solution of caseinate hydrolyzed by chymosin	Glycomacropeptide	[50]
UF membrane enzymic reactor	Goats' whey	Goat β-lactoglobulin	[51]
Electrochromatography	Whey	β-lactoglobulin, α-lactalbumin, Bovin serum albumin, and IgG	[52]
Ion exchange chromatography	Whey	α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B, Lactoferrin, and Lactoperoxidase	[53]
Affinity—peptide ligand chromatography	Whey protein isolate	α-lactalbumin	[54]
Affinity chromatography	Whey	Glycomacropeptide	[55]
Membrane chromatography	Whey	Lactoferrin	[56]
Liquid-solid circulating fluidized bed ion-exchange extraction	Whey	Whey proteins	[57]
Adjusting pH, and UF	Whey protein concentrate	Glycomacropeptide	[58]
Protein precipitation by trifluoracetic acid followed by chromatography	Sweet whey	Glycomacropeptide	[59]

(*Continued*)

Table 2
(*Continued*)

Methodology	Feed stock	Protein of interest	Reference
Acidification, heating, and UF membrane	Whey protein concentrate of cow, ewe, and goat milk	Caseinomacropeptide	[60]
Affinity ligand chromatography	Whey	β-lactoglobulin	[61]
Two step of ion exchange membrane chromatography	Whey	Whey protein isolate, and glycomacropeptide	[62]
Precipitation with acetic acid/sodium acetate, and trichloroacetic acid (TCA)	Whey	Glycomacropeptide	[63]
Combined UF, and DF process	Acid casein whey	α-lactalbumin	[64]
Size exclusion based gel permeation chromatography, and anion exchange chromatography	Whey	β-lactoglobulin, α-lactalbumin, and Bovin serum albumin	[65]
Combined of UF, and MF with precipitation	Whey	β-lactoglobulin, and α-lactalbumin	[66]
Fractioning with TCA, precipitation with ethanol, and UF membrane	Whey protein isolate	Glycomacropeptide	[67]
Ion exchange chromatography (chitosan as anion exchanger)	Milk whey	Glycomacropeptide	[68]
Membrane chromatography	Whey	β-lactoglobulin	[69]
Ion exchange column chromatography	Lactic acid whey	α-Lactalbumin, and Whey protein isolate	[70]
Gel filtration	Protein fraction	α-Lactalbumin, and β-lactoglobulin	[71]
Ion exchange chromatography	Whey	α-Lactalbumin, Whey protein isolate, Lactoferrin, and Lactoperoxidase	[72]
Two-stage UF process by different MWCO UF membrane	Whey protein isolate	α-Lactalbumin, and β-lactoglobulin	[73]
Enzyme tranglutaminase followed by microfiltration	Whey proteins	Glycomacropeptide	[74]
Ion exchange chromatography, and UF	Milk whey, and whey protein concentrate	Glycomacropeptide	[75]
Adsorption by DEAE-agarose	Protein concentrate of whey	Immunoglobulins	[76]
Gel filtration	Whey	β-lactoglobulin, α-lactalbumin, Bovin serum albumin, and IgG	[77]
Ion exchange column chromatography	Milk	Lactoferrin, and Lactoperoxidase	[78]
Membrane chromatography	Whey	Lactoferrin, Lactoperoxidase, and LFcin	[79]
Different MWCO UF membrane (30 and 50 kg mol^{-1}) in high shear rotating disc membrane module (dead-end membrane module)	Permeate from two-stage UF	β-lactoglobulin and α-lactalbumin	[5]
Membrane chromatography	Casein whey	β-lactoglobulin and α-lactalbumin	[80]
Membrane chromatography	Colostrum, and whey	Lactoferrin	[81]
Membrane chromatography	Whey	Lactoferrin and Lactoperoxidase	[82]
Membrane chromatography	Whey	β-lactoglobulin, α-lactalbumin, and Bovin serum albumin	[83]
Chromatography	Microfiltered whey	β-lactalbumin	[84]
Chromatography	Whey	Whey protein isolate	[85]

(*Continued*)

Table 2
(*Continued*)

Methodology	Feed stock	Protein of interest	Reference
Charged UF membrane	Cheese whey	Glycomacropeptide	[6]
Different MWCO UF membrane (30 and 50 kg mol^{-1}) in high shear rotating disc membrane module (dead-end membrane module)	Casein whey	Individual proteins present in whey	[86]
Ion exchange Mustang Q membrane	Cows whey	β-lactaglobulins A and B, α-lactalbumin, Bovin serum albumin, and immunoglobulins	[87]
UF membrane	Casein whey	Whey protein isolate	[88]
Cation-exchange chromatography	Casein whey	Whey protein isolate-dextran glycates	[89]
Different MWCO UF membrane	Sweet whey	Glycomacropeptide	[90]
Anion-exchange chromatography coupled with a fast protein liquid chromatography (FPLC) system	Whey protein concentrate	β-lactaglobulin, α-lactalbumin, Bovin serum albumin, and immunoglobulins	[91]

vary from 3 to 4, depending upon the quantity of excess acids present in whey resulting from acid caseination. In most of the cases, the sweetmeat industries in India use hydrochloric acid or its equivalent, for casein precipitation.

Highly purified whey proteins i.e. bovin serum albumin, β-lactoglobulin, α-lactalbumin, and trifluoracetic acid (TFA) were procured from Sigma–Aldrich, USA. For the purpose of experimentation, sodium hypo-chloride, hydrochloric acid, and sodium hydroxide (Ranbaxy, Mumbai, India) were used. All other chemicals used during experimentation were procured from Merck (Mumbai, India). The deionized water used in all the experiments was obtained from Arium 611DI ultrapure water system (Sartorius AG, Göttingen, Germany). HPLC grade acetonitrile was purchased from Carbo Erba Reagenti, Rodano, Italy. Different MWCO UF membranes, ranging from 100 to 5 kg mol^{-1} and MF membranes (0.45×10^{-6} m) were purchased from Vivascience AG, Germany.

2.2. Equipment

In this study, different MWCO cross-flow UF membrane module (100–5 kg mol^{-1}) series were used. Fig. 1 shows the schematic diagram of the experimental setup for membrane processing. All the cross-flow modules were Vivaflow200 (S/N03VF20028) (VivaScience, Germany). The membrane material was poly-ether sulfone (PES), which exhibits no hydrophilic interactions, and is usually preferred for their low-fouling characteristics, broad pH range, and durability. The dimensions of the membrane were overall L/H/W of 126/138/38 mm; channel (W/H) of 10 mm/0.4 mm; the active membrane area was 2×10^{-2} m^2; hold-up volume (module) 5.3×10^{-6} m^3; minimum recirculation volume of less than 20×10^{-6} m^3, and a non-recoverable hold-up of less than 2×10^{-6} m^3. The module could be operated up to a maximum pressure of 4 bar and maximum temperature of 333 K, with a pump flow rate in the range of 3.33×10^{-6}–6.66×10^{-6} $m^3 s^{-1}$.

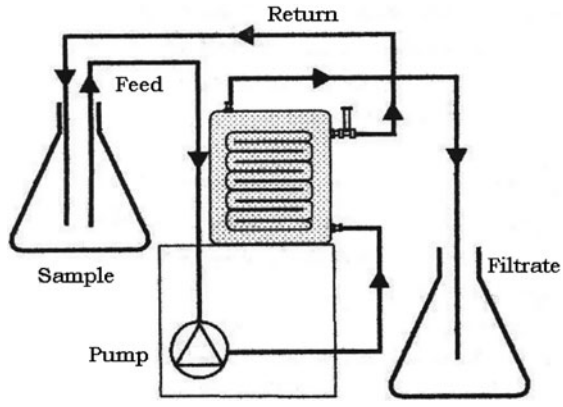

Fig. 1. Schematic diagram of membrane module and experimental set-up.

Hot air oven (Bhattacharya & Co., Kolkata, India), MF unit along with PES membrane of 47×10^{-3} m diameter, and 0.45×10^{-6} m pore size (Sartorius AG, Göttingen, Germany), refrigerated centrifuge (Model: C-24, Remi Instruments Ltd., Mumbai, India), digital pH meter, magnetic stirrer, and digital weighing machine (Sartorius AG, Göttingen, Germany) were used in the experiment.

2.3. Analytical instruments

Lactose was estimated by high-performance liquid chromatography (HPLC) (Perkin–Elmer, Series 200). HPLC system was equipped with RI detector and Spheri

5 amino column (5 μm, 4.6×10^{-3} m × 220×10^{-3} m) [92]. Individual protein concentrations were measured using Water HPLC system (Waters Corporation, Milford, USA) consisting of a Waters 1,525 Binary HPLC pump. Reverse-phase chromatography was performed on silica-based wide pore C_{18} column (15×10^{-6} m, 3.9×10^{-3} m × 300×10^{-3} m) [80].

2.4. Methodology

2.4.1. Feed pretreatment

In order to reduce the extent of membrane fouling, the raw casein whey was first centrifuged at 166.67 r.p.s. at 277 K for about 1,800 s to remove traces of casein particles (colloidal suspension) and fat molecules. The treated whey was then subjected to MF with PES membrane (47×10^{-3} m diameter, pore size 0.45×10^{-6} m), and the filtrate was considered for protein separation by UF membrane.

2.4.2. UF by cross-flow membrane module

The prepared feed was fed to 100 kg mol^{-1} UF membrane to separate IgG, IgA, and IgM as retentate whose molecular weight ranges from 150.0 to 900.0 kg mol^{-1}. The collected permeate was then fed to 50 kg mol^{-1} UF membrane to separate bovin serum albumin, lactoperoxidase, and lactoferrin as retentate whose molecular weights are in the range of 60–78 kg mol^{-1}. The collected permeate which contained only β-lactoglobulins (molecular weight 18.3 kg mol^{-1}), α-lactalbumin (molecular weight 14.2 kg mol^{-1}), and lactose (molecular weight 360) was introduced to 5 kg mol^{-1} UF membrane to separate lactose as permeate. The retentate was divided into two equal parts. In one half, 1(N) NaOH was added to make its pH of 5.3–5.55, and in the other half 1(N) HCl was added to make the pH of 2.8–2.9. These two halves were then subjected to 30 kg mol^{-1} UF membrane separately. Schematic diagram of the proposed work is described in Fig. 2. Permeate volumes were collected at constant time intervals and the permeate flux was calculated according to the following correlation.

$$J = \frac{V}{A \times t} \tag{1}$$

where J = Permeate flux (L m^{-2} h^{-1}), V = Permeate volume (L), A = Effective membrane area (m^2), and t = Time (h).

% Yield of individual protein fractions have been calculated based on the following relation: Eq. (2).

% Purity of individual protein fractions (dry basis) have been calculated from: Eq. (3).

All the UF experiments were conducted in four-stage DD mode with constant VCF of 2 in order to obtain higher purity of target proteins. Different TMPs, ranging from 0.686 to 2.942 bar were used during experimentation to assess the effect of TMP on protein, and lactose permeation.

$$\%\text{yield} = \frac{\text{Amount of target component in the product} \times 100}{\text{Total amount of corresponding component present in the feed}} \tag{2}$$

$$\%\text{purity (dry basis)} = \frac{\text{Amount of target component in the product} \times 100}{\text{Total amount of all the components present in the same product}} \tag{3}$$

2.5. Membrane compaction and water run

Prior to experiments, all the membranes were subjected to compaction for about an hour with ultra-pure de-ionized water at a pressure of 3.432 bar, higher than the highest operating pressure to prevent any possibility of change of membrane hydraulic resistance during UF. Once the water flux becomes steady with no further decrease, it was concluded that full compaction of the membrane has taken place. After compaction, membrane hydraulic resistance (R_m) was determined based on Darcy's law.

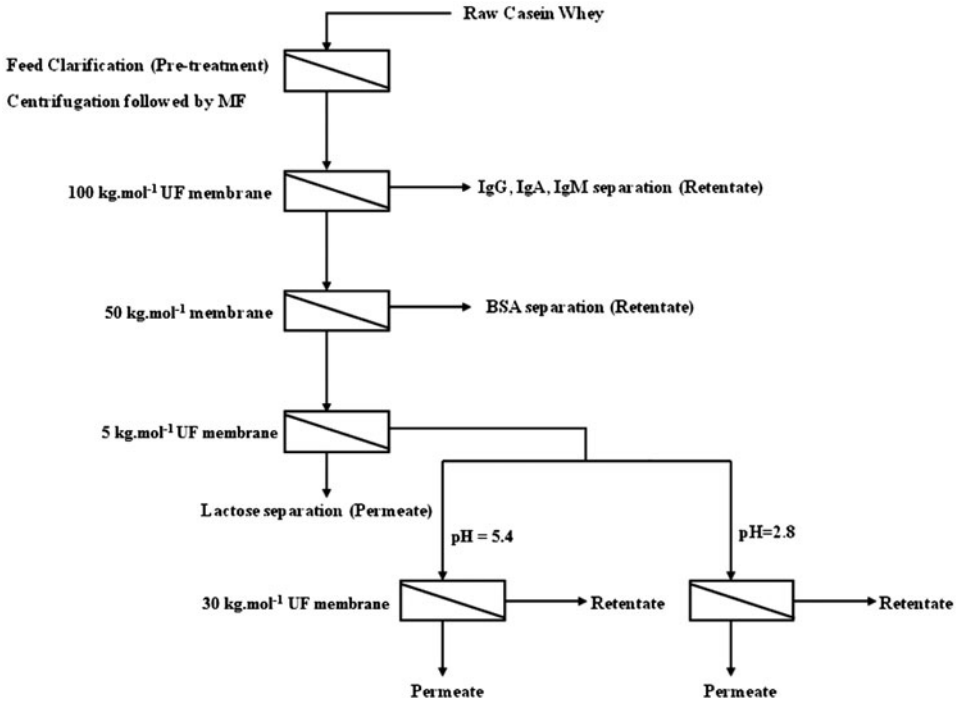

Fig. 2. Schematic diagram of the proposed process.

$$J = \frac{\Delta P}{\mu \times R_m} \tag{4}$$

where ΔP = TMP (bar), μ = dynamic viscosity of water (Ns. m^{-1}), and R_m = membrane hydraulic resistance (m^{-1}).

The slope of water flux versus TMP as obtained from four water runs taken at different TMPs (0.686, 1.373, 2.06, and 2.942 bar) were used to find the numerical value of R_m. In all cases, good correlation coefficient for the least-square fitted straight line of J vs. ΔP was achieved.

2.6. Membrane cleaning

After each experiment, the membrane was cleaned thoroughly for 1,200 s by deionized water at a pressure of 3.432 bar, higher than the highest operating pressure. After water cleaning, membrane was again cleaned by using a cleaning solution (1×10^{-3} m^3 of 0.5 mM sodium hypo-chloride in 0.1 N sodium hydroxide) for 1,800 s at operating pressure 3.432 bar, subsequently it was thoroughly washed by deionized water for 1,200 s. In each case, the water flux was found to regain by more than 98% of its original value, suggesting the cause of flux decline to be either osmotic pressure limited or due to reversible fouling layer.

2.7. Estimation of protein fraction

Individual protein concentrations were determined using reversed phase high performance liquid chromatography with a silica-based wide pore C_{18} column in the Water Gradient HPLC. In this case, detection was done at UV-280 nm, and the fixed-flow rate at 1.66 $m^3 s^{-1}$. The mobile phase was a binary system of water with 0.1% of TFA (buffer A), and acetonitrile with 0.1% of TFA (buffer B). The gradient condition consisted of a two-step linear binary gradient: buffer A/bufferB = 70/30–35/65 (vol.%), gradient time of 900 s. The column oven temperature was maintained at 298 K [80].

2.8. Estimation of lactose concentration

Lactose was estimated by HPLC (Perkin–Elmer, Series 200). Acetonitrile 75% (v/v) was used as mobile phase at a flow rate of 1.67×10^{-8} $m^3 s^{-1}$ for carbohydrate analysis. Column oven temperature of HPLC was maintained at 298 K [92].

All the experiments were performed in triplicates and the average values were considered.

3. Results and discussions

Whey is a natural mixture of different biomolecules (immunoglobulins, bovin serum albumin, β-lactoglobulin, α-lactalbumin, lactoferrin, lactoperoxidase, and lactose), those that have their unique pharmaceutical and medicinal importance. Therefore, separation of a particular protein fraction requires a very fine-tuned high resolution technique. Different MWCO PES made UF membranes ranging from 100 to 5 kg mol^{-1} in series were selected for this operation to provide a desired retention, and transmission of whey proteins depending on its permeability. Separation characteristics of different whey proteins were investigated under different TMPs (0.686–2.942 bar). All the UF experiments were conducted in four-stages of DD mode at constant volume concentration factor (VCF 2) in order to maintain higher purity of the target protein.

After membrane compaction, series of water runs were taken to evaluate membrane hydraulic resistance R_m, which were found to be $(5.2 \times 10^{14} \pm 0.04 \times 10^{14})\ m^{-1}$ for 5 kg mol^{-1} membrane, $(8.264 \times 10^{12} \pm 0.1456 \times 10^{12})\ m^{-1}$ for 30 kg mol^{-1} membrane, $(3.259 \times 10^{11} \pm 0.02 \times 10^{11})\ m^{-1}$ for 50 kg mol^{-1}, and $(5.05 \times 10^{10} \pm 0.05 \times 10^{10})\ m^{-1}$ for 100 kg mol^{-1} membrane. The higher value of membrane hydraulic resistance for 5 kg mol^{-1} membrane compared to 100 kg mol^{-1} membrane is due to reduced pore size and compact nature of the membrane. Centrifugation (200 r.p.s., 277 K, 1,800 s) followed by MF was employed for separation of fat molecules and suspended solids from casein whey. Reduction of suspended solids was accomplished, from 645 kg m^{-3} in the raw casein whey to 0.5 kg m^{-3} in the pretreated casein whey, which accounts for almost 99% reduction of total solids.

3.1. Hydrodynamic study and protein recovery for 100 kg mol^{-1} membrane

In Fig. 3, steady-state permeate flux for different DD stages, have been plotted against different TMPs (0.686–2.942 bar) for 100 kg mol^{-1} UF membrane. It is observed that permeate flux is decreased with increase of DD stage because high concentration of solute molecules (immunoglobulins) in the feed side, creates the concentration polarization on the membrane surface. With time, concentration polarization layer becomes more compact, and thicker, which attributes to reduced flux till the third stage of DD. It is observed that the steady-state permeate flux in third and fourth stages of DD are almost same (no significant drop in permeate flux), which might be due to the saturation of concentration polarization layer on membrane surface that may lead to the "limiting flux" under a fixed-operating condition. On the other hand, it is observed that permeate flux increases with increase of TMPs (0.686–2.06 bar), and that is similar for TMP 2.06 bar with 2.942 bar. As in the third stage of DD, the concentration polarization layer becomes fully developed, and its hydrodynamic behavior is elucidated in detail for third stage of DD to understand the effect of TMPs. In Fig. 3 (inset), time history of permeate flux is plotted against filtration time for third stage of DD process. In this case, permeate flux is found to increase with the increase in TMPs (0.686–2.06 bar), and at TMP 2.06 bar it becomes almost similar with next one. This may attribute to fully developed concentration polarization layer on membrane surface at high TMP. As the pressure difference across the membrane surface increases, the convective flux increases because of the higher driving force. Higher flux results in more transport of solute molecules towards the membrane surfaces due to higher rejection. This will result in more deposition, and hence higher polarized layer resistance. At higher TMP, flux is generally found to attain a constant value, known as "limiting flux," which is a result of compensation of two effects, higher driving force and high polarized layer resistance. It is further observed from Fig. 3 (inset), that rate of flux decline is higher with low TMP, and permeate flux declines rapidly, then gradually, and eventually it becomes asymptotic with time axis, which is a typical time-history flux profile of any pressure driven separation technique. The possible cause of higher flux and low rate of flux declination at high TMP is that at high TMP on the membrane surface, the feed adjacent to the membrane surface flows at a high velocity (because of cross flow module), creating more turbulence, and sweeps away the deposited solutes from the membrane surface resulting in a reduction of concentration polarization, accompanied by the increase of rate of permeation. Percentages of yields and purities of immunoglobulins, obtained at different TMPs (bar), and stages of DD are depicted in Table 3.

From Table 3, it is observed that % yields of immunoglobulins decreases, though not significantly, with increasing DD stages, and its values remain practically unchanged at the third, and fourth stages of DD process. It may be justified by the fact that due to continuous washing effect during DD stages, and wide pore size distribution on membrane surface, some solutes (immunoglobulins) might pass

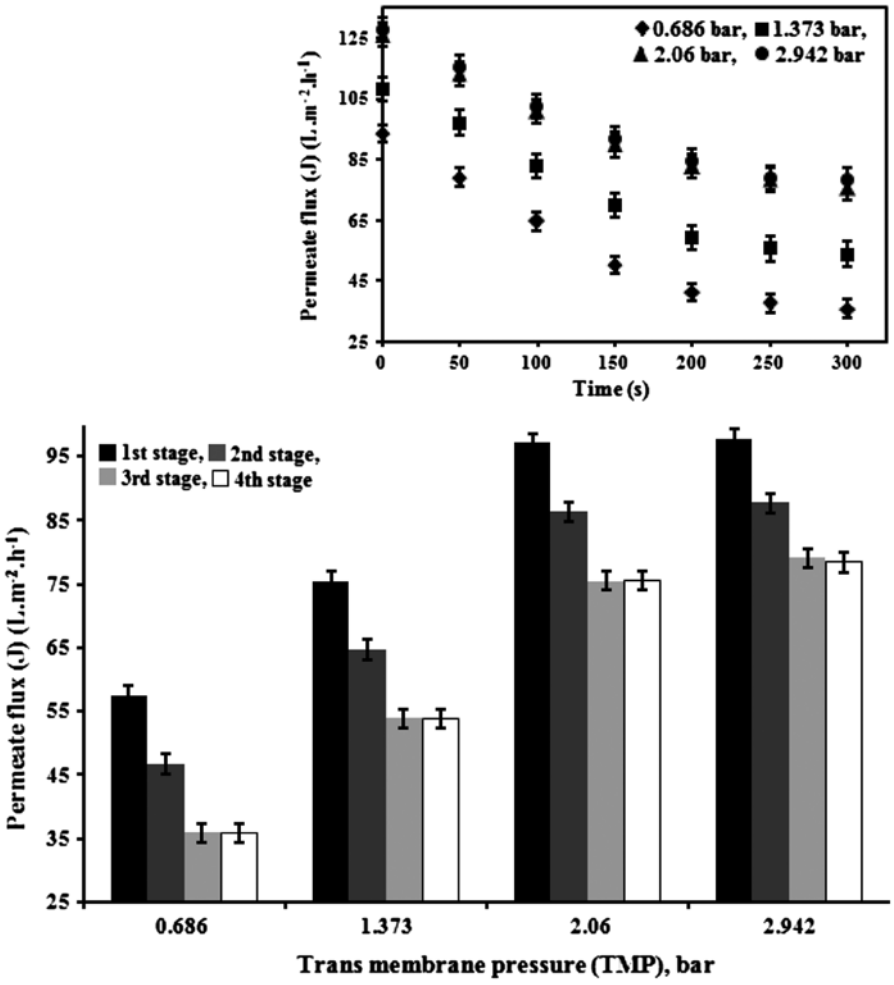

Fig. 3. Steady state permeate flux for 100 kg mol^{-1} UF membrane at different TMPs (0.686–2.942 bar) and stages of DD. (Inset: time history of permeate flux by 100 kg mol^{-1} UF membrane at different TMPs (0.686–2.942 bar) for third stage of DD.).

Table 3
Yield (w/w) (%) and purity (%) of immunoglobulins under different TMPs (bar) and stages of DD

	Trans-membrane pressure (TMP), (bar)							
	0.686				2.942			
IgG, IgA, IgM (100 kg mol^{-1} retentate)	Stage 1	Stage 2	Stage 3	Stage 4	Stage 1	Stage 2	Stage 3	Stage 4
Yield (w/w) (%)	85	78	70	70	68	64	60.5	60.5
Purity (%)	35	51	65	65	52	65	76	76

through the porous channel of membrane. After the third stage of DD process, things would become much more stable, resulting the yield of immunoglobulins remain practically constant during the third, and fourth stage of DD process. In contrast to the trend observe for % yields, % purities of immunoglobulins are increased with increasing DD stages. During DD process, due to the washing effect, lower molecular weight of solutes passed through the membrane pores, lead to high purity of rejected proteins. It is further observed that % yields of immunoglobulins decrease, not significantly, with

increase of TMPs, up to 2.06 bar, has the similar value with 2.942 bar. Due to high driving force on the membrane surface at TMP 2.06 bar, and asymmetric distribution of membrane pore size, some high molecular weight solute might permeate, leading to decrease in the % yields of proteins in retentate. On the other hand, % purities of immunoglobulins are increased up to TMP 2.06 bar, and it is remained unchanged at 2.942 bar. The protein permeation has indirect responses depending on permeate flux, which is strongly influenced by TMPs. Increase of TMP results in higher convective flux, as well as more transport of solute molecules towards the membrane surface. Effects of TMPs on both yields and purities show negligible change after TMP 2.06 bar, because of stabilization of higher solute permeation (due to higher solvent flux) and high rejection of solutes on membrane surface.

3.2. Hydrodynamic study and protein recovery for 50 kg mol^{-1} membrane

In Fig. 4, % reduction of initial flux for each DD stage has been plotted with different TMPs for 50 kg mol^{-1} membrane. It is observed that % reduction of permeate flux decreases with increases of TMPs, and in the case of TMP 2.06–2.942 bar it becomes similar. This may be justified by the fact that high TMP provides more turbulence on membrane surface (because of cross flow module), which is the cause of high permeation and reduces the concentration polarization. For TMP 2.06–2.942 bar concentration polarization layer becomes compact, attributed to similar reduction value of permeate flux. It is also observed that % reduction of permeate flux for the third stage of DD is high compared with the second stage of DD, which attributes that increase of

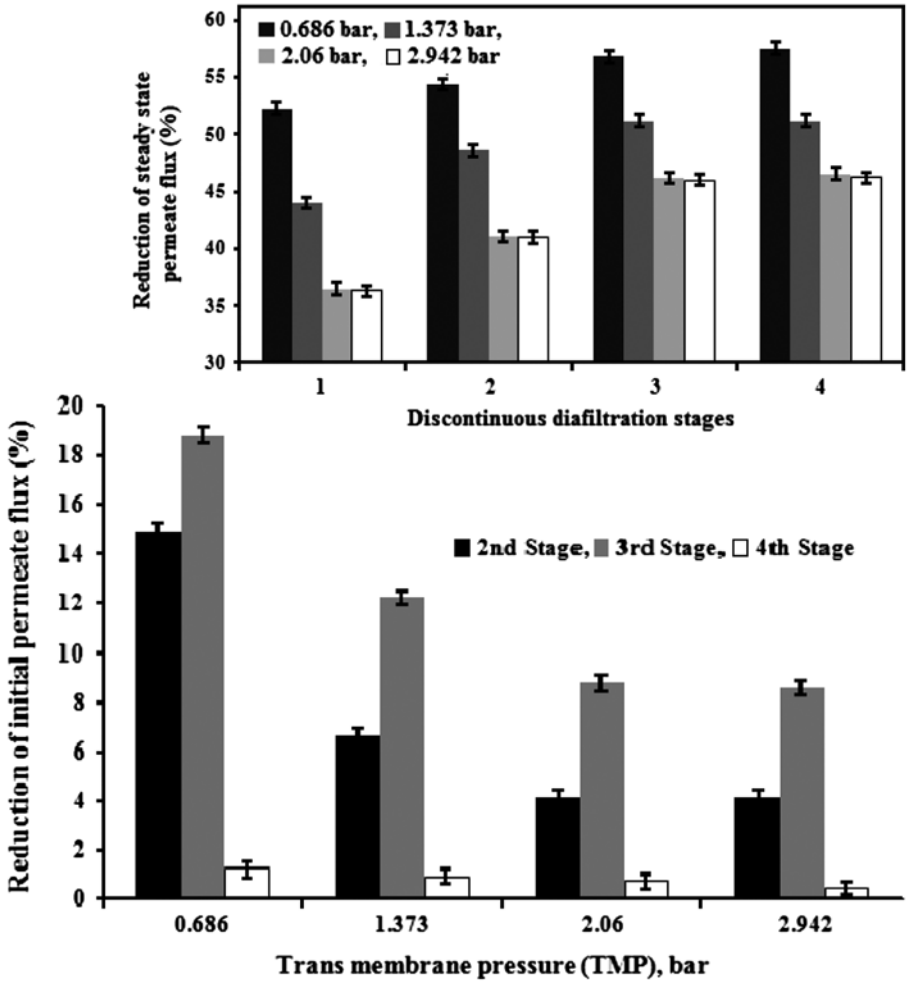

Fig. 4. Reduction of initial permeate flux (%) for different stages of DD and TMPs (0.686–2.942 bar). (Inset: reduction of steady state permeate flux from initial flux (%) at different stages of DD and TMPs (0.686–2.942 bar)).

thickness of concentration polarization layer on membrane surface with time progress. Lower values of % reduction of permeate flux for the fourth stage of DD process at different TMPs (0.686–2.942 bar) signifies the saturation of concentration polarization layer on membrane surface. Without exception, similar trend of time history permeate flux (like 100 kg mol^{-1} membrane) was observed for all cases (data not shown). Moreover, in Fig. 4 (inset), % reduction of steady-state permeate flux from initial flux for each TMP has been plotted for different DD processes, and it is observed that with increase of DD process % reduction of steady-state permeate flux increases, whereas with increase of TMP % reduction of steady-state permeate flux decreases. This may be justified by the fact that with increasing DD stages, the concentration polarization layer becomes more depleted with low molecular weight solutes, because of enhanced permeation through the membrane due to the "washing effect," which may result in more compaction of the concentration polarization layer. This will result in decrease in flux with increasing DD stage. Further, increasing TMPs in a particular DD stage results in decreasing % reduction of steady-state permeate flux from initial flux, which may be explained based on higher driving forces across the membrane surface. In Fig. 5, % yields and purities of bovin serum albumin in retentate of 50 kDa membrane have been plotted with different DD stages for TMP 2.942 bar. The figure reveals that the % yields of bovine serum albumin at retentate side decreases, not significantly, with increase of stages of DD (first stage–third stage), whereas the % purities of bovin serum albumin increase with increase of DD stages. In the same figure (inset of Fig. 5), % yields and purities of bovin serum albumin at retentate side of 50 kDa

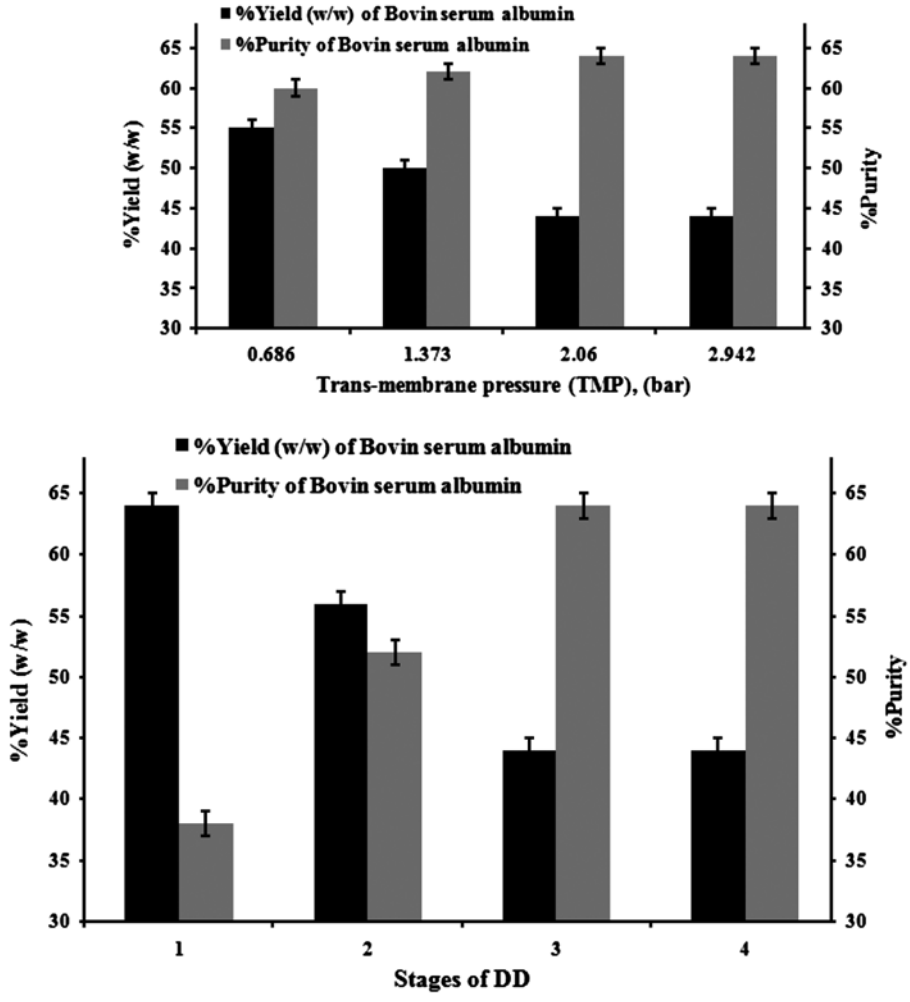

Fig. 5. Yield (w/w) (%) and purity (%) of bovin serum albumin at different stages of DD for TMP 2.942 bar. (Inset: yield (w/w) (%) and purity (%) of bovin serum albumin for different TMPs (0.686–2.942 bar) at third stage of DD).

membrane have been plotted with different TMPs (0.686–2.942 bar) for the third stage of DD process. The figure depicts that with increase of TMPs (0.686–2.06 bar), the % yields of bovine serum albumin decreases, and the purities increase (64% at the third stage and TMP 2.06 bar). The reasons of these observations may be explained by above-mentioned justifications.

3.3. Hydrodynamic study and protein recovery for 5 kg mol^{-1} membrane

Lactose was separated out by 5 kg mol^{-1} UF membrane at different TMPs (0.686–2.942 bar) and four-stages DD at constant VCF 2. It is observed that permeate flux increases with increase of TMPs (0.686–2.06 bar), whereas it decreases with increase in the DD stages (up to the third stage). Further increase in TMPs and DD stages, there has no effect in permeate flux. In Fig. 6 steady-state permeate flux for the third stage of DD process, and TMP 2.06 bar have been plotted for three different MWCO UF membrane, i.e. 100, 50, and 5 kg mol^{-1}. The permeate flux has been found to decrease with reduction of MWCO of the membrane (100–5 kg mol^{-1}) because of more compact nature of membrane. As the MWCO of the membrane decreases, the average pore size of the membrane becomes smaller resulting in higher membrane hydraulic resistance, and less permeability through the membrane. For a fixed MWCO membrane, increase of TMPs, results in higher permeate flux because of more driving force across the membranes. But after TMP 2.06 bar the concentration polarization layer will approach the steady-state because of the balance between the higher driving force, and higher deposition of the solute brought about by enhanced convective flow of the solvent towards the membrane. This will lead to asymptotic approach towards the

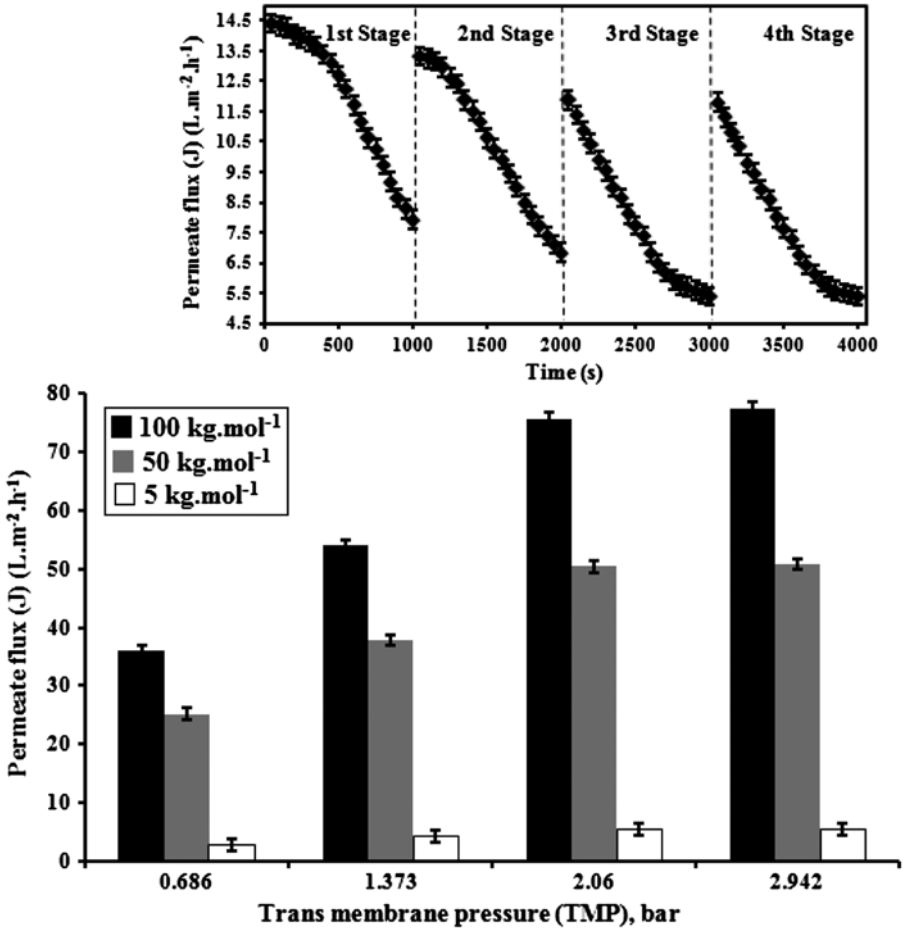

Fig. 6. Steady-state permeate flux for different MWCO UF membrane at constant TMP 2.06 bar and third stage of DD. (Inset: time history of permeate flux by 5 kg mol^{-1} UF membrane at constant TMP 2.06 bar).

"limiting flux" phenomenon. In Fig. 6 (inset) time history of permeate flux through the DD steps for 50 kg mol^{-1} UF membrane at TMP 2.06 bar is depicted. In this case permeate flux is found to decrease with time, which is a typical time history permeate flux profile for any pressure driving process. Permeate flux reduces sharply at the third, and fourth stages of DD process compared to initial two stages. This may be justified by the fact that with time progress, the concentration polarization layer becomes thicker, which attributes to lower permeation, as well as high resistance. Moreover, it is observed that time history flux profile for all TMPs was similar with all cases (data not shown). In Fig. 7, recovery of lactose by 5 kg mol^{-1} UF membrane under different TMPs (0.686–2.06 bar) at constant DD stage is elucidated. From this

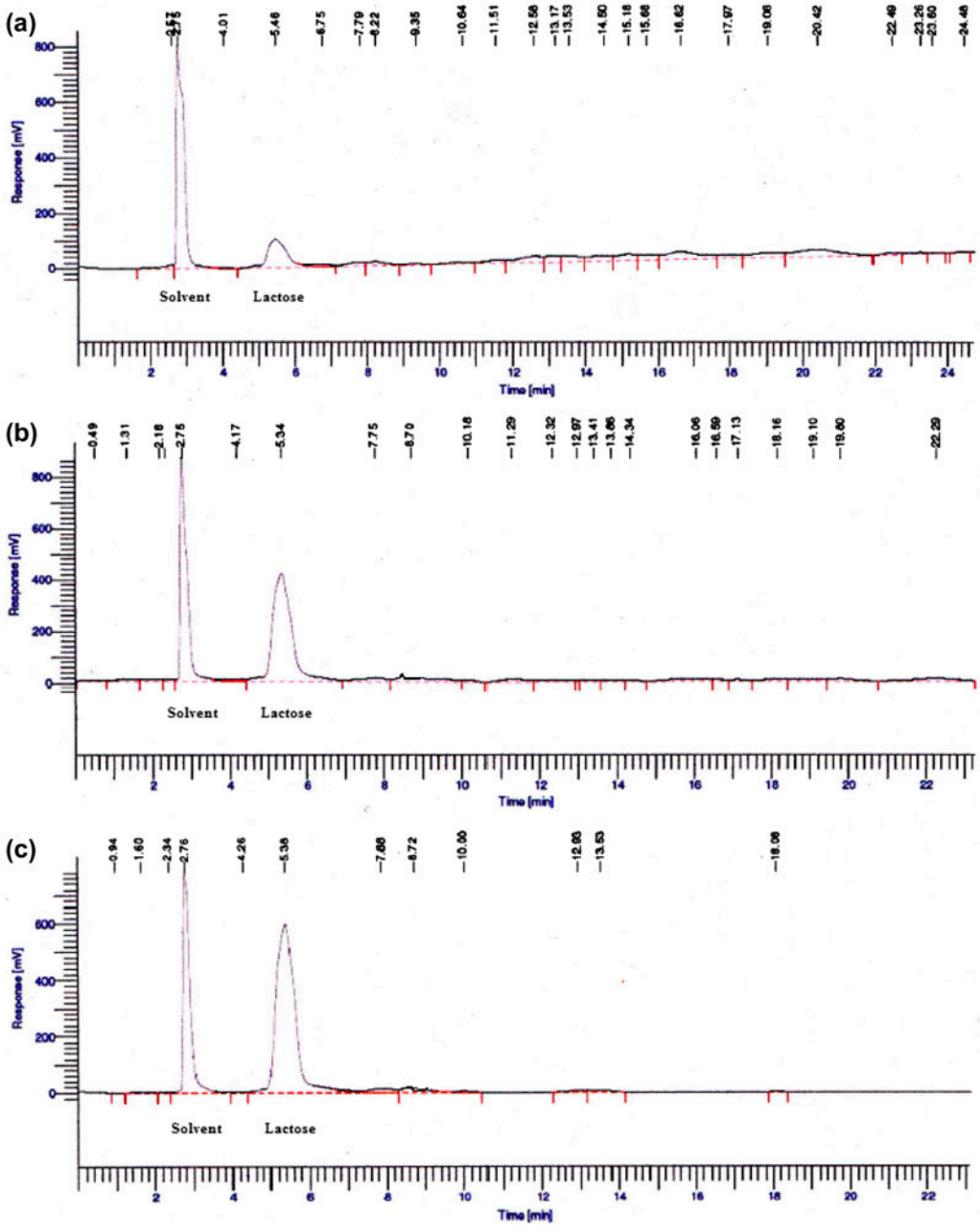

Fig. 7. HPLC chromatogram of the permeate side of 5 kg mol^{-1} UF membrane for different TMP (2.06 bar) and constant DD stages. (a) TMP 0.686 bar; (b) TMP 1.373 bar; and (c) TMP 2.06 bar.

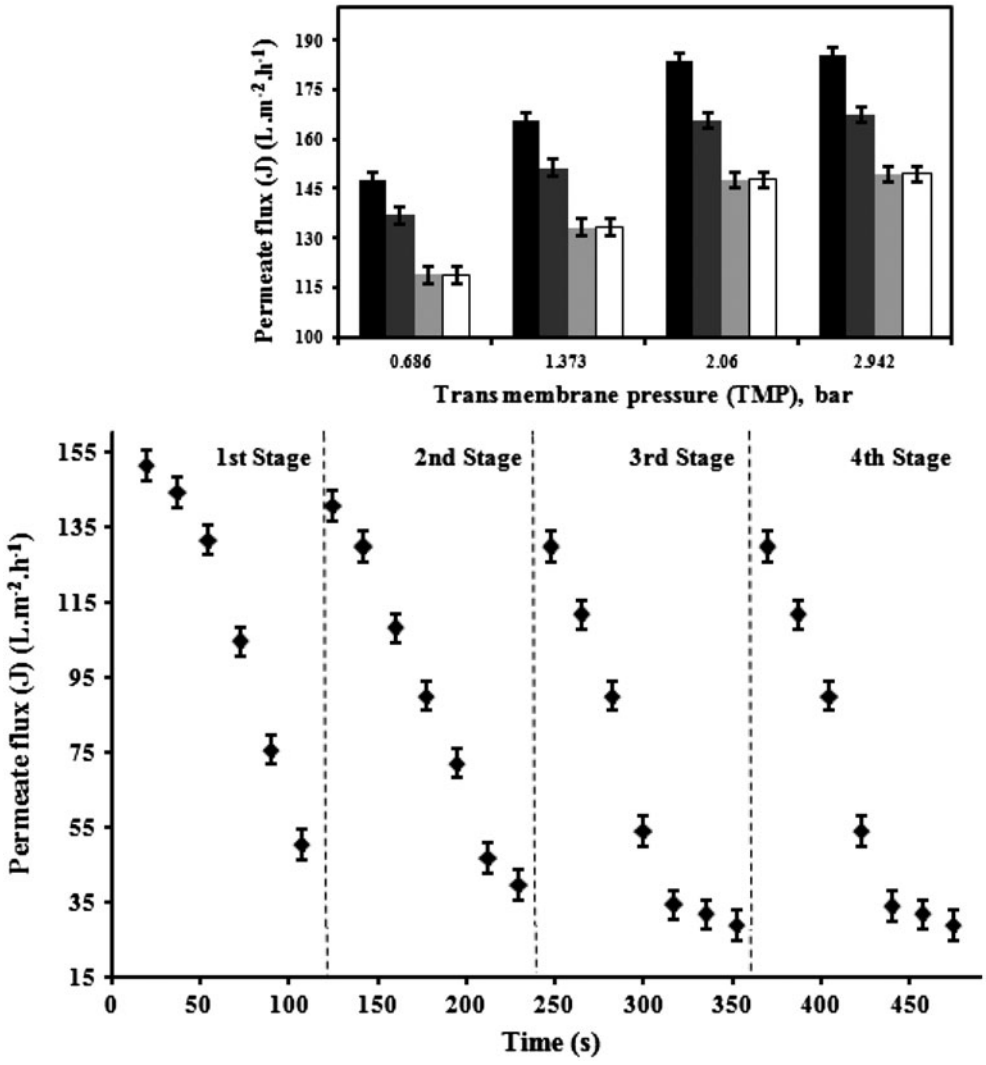

Fig. 8. Time history of permeate flux by 30 kg mol^{-1} UF membrane at constant TMP 2.06 bar, and pH 5.4. (Inset: steady state permeate flux for 30 kg mol^{-1} UF membrane at different TMPs (0.686–2.942 bar) and stages of DD for pH 2.8).

figure it is observed that concentration of lactose increases in permeate side with increase of TMP. Moreover, it is observed that with increase of DD stages of up to the third stage, purity of lactose in permeate side increased. This may be vindicated by the fact that dual effect, i.e. continuous washing during DD process, and high driving force, developed by TMP, provides positive influence on lower molecular weight lactose permeation. At the third stage of DD process, and TMP 2.06 bar 99% pure lactose was recovered with an overall yield of nearly 95%. HPLC analysis shows that at the third stage of DD process under TMP 2.06 bar, β-lactoglobulin and α-lactalbumin are more concentrated with respect to feed solution (figure not shown).

3.4. Hydrodynamic study and protein recovery for 30 kg mol^{-1} membrane

The most challenging step of the proposed separation technique is the separation of similar molecular weight β-lactoglobulin (molecular weight 18.3 kg mol^{-1}), and α-lactalbumin (molecular weight 14.2 kg mol^{-1}) by 30 kg mol^{-1} UF membrane. It is reported that at 293 K, bovine β-lactoglobulin forms a dimer at pH 5.2–5.5, while the monomeric native state is stable at pH values below 3, and at low ionic strength in absence of salts [93–97]. To study the overall performance at two different pHs, one above, and the other below, the isoelectric point of β-lactoglobulin (isoelectric point 5.2–5.4), experiments were carried out at

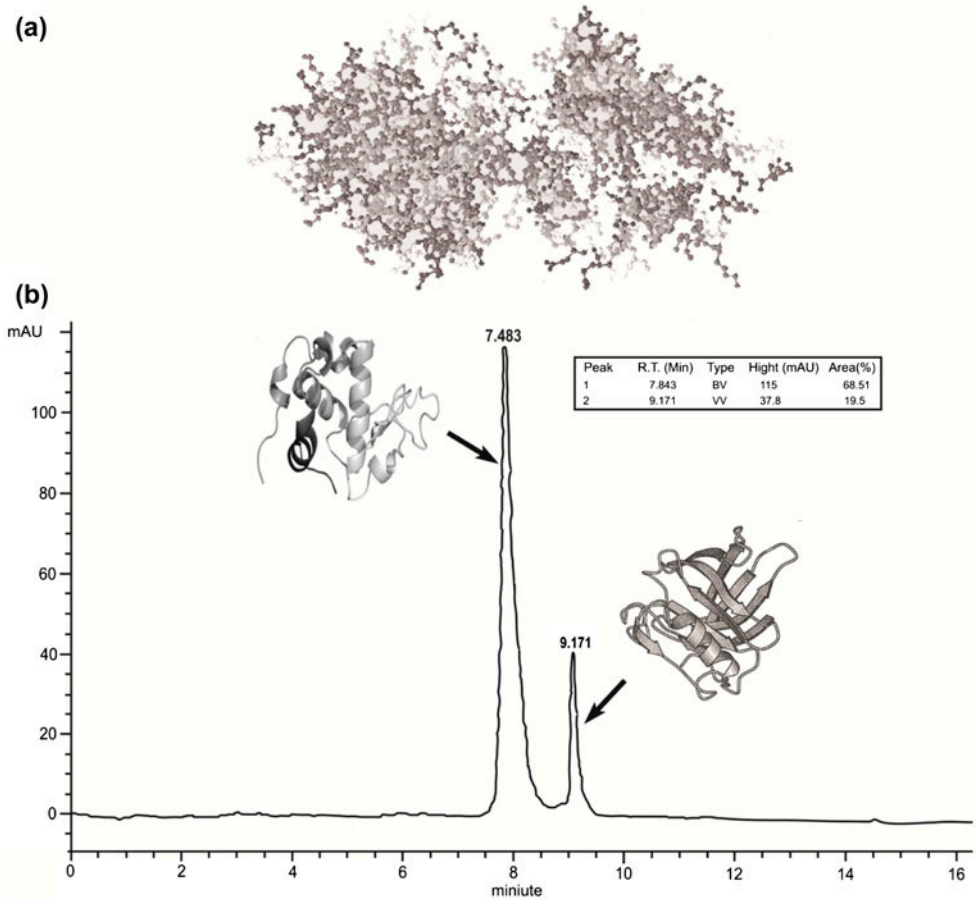

Fig. 9. (A) Dimer structure of β-lactoglobulin at pH 5.4 (figure obtained from Adams et al. [94]); (B) HPLC chromatogram of 30 kg mol^{-1} UF membrane permeate at TMP 2.06 bar, third stage of DD, and pH 5.4.

two different pHs, i.e. 2.8 and 5.4. It was expected that due to monomer-dimer equilibrium of β-lactoglobulin at pH 5.4, which offers high molecular weight of β-lactoglobulin (molecular weight 36.5 kg mol^{-1}), most of the β-lactoglobulin was rejected by 30 kg mol^{-1} UF membrane, while α-lactalbumin passed as permeate stream. Under this investigation the effect of TMPs (0.686–2.942 bar), DD stages and pH of solution on permeate flux, and rejection have been thoroughly investigated by 30 kg mol^{-1} UF membrane. In Fig. 8 time history of permeate flux is plotted for pH 5.4. Without exception, time history permeate flux showed a typical declining nature for each of the DD stage, and flux declination was more rapid at the third and fourth stages, compared to other, similar to previous observations. In the same figure (Fig. 8, inset) steady-state permeate flux is plotted for different TMPs (0.686–2.942 bar), and stages of DD for pH 2.8. From Fig. 8 it is observed that for two cases (pH 5.4 and 2.8) ultimate concentration polarization is manifested at the third stage of DD process, and also the positive influence of TMPs on permeate flux. The possible cause for reduction of permeate flux with time progress, and high value of permeate flux with increase of TMPs are elucidated in previous section. From these figures it is also observed that reduction of permeate flux is high at pH 5.4 compare with pH 2.8. This could be explained by the fact that at pH 5.4 β-lactoglobulin forms a dimer, which creates the concentration polarization on membrane surface, whereas lower molecular weight α-lactalbumin passes through the porous channel of the membrane. Under this investigation it was also observed that time required for achieving steady-state permeate flux in case of pH 2.8 is higher than the other pH value, pH 5.4 (data not shown). This may be justified by the fact that at pH 2.8, both α-lactalbumin and β-lactoglobulin stay at monomeric forms, and they passes through the porous channel of membrane, which attributes lower concentration polarization. In Fig. 9, a HPLC chromatogram of 30 kg mol^{-1} UF membrane permeate at TMP 2.06 bar, third stage of DD, and pH 5.4 is shown.

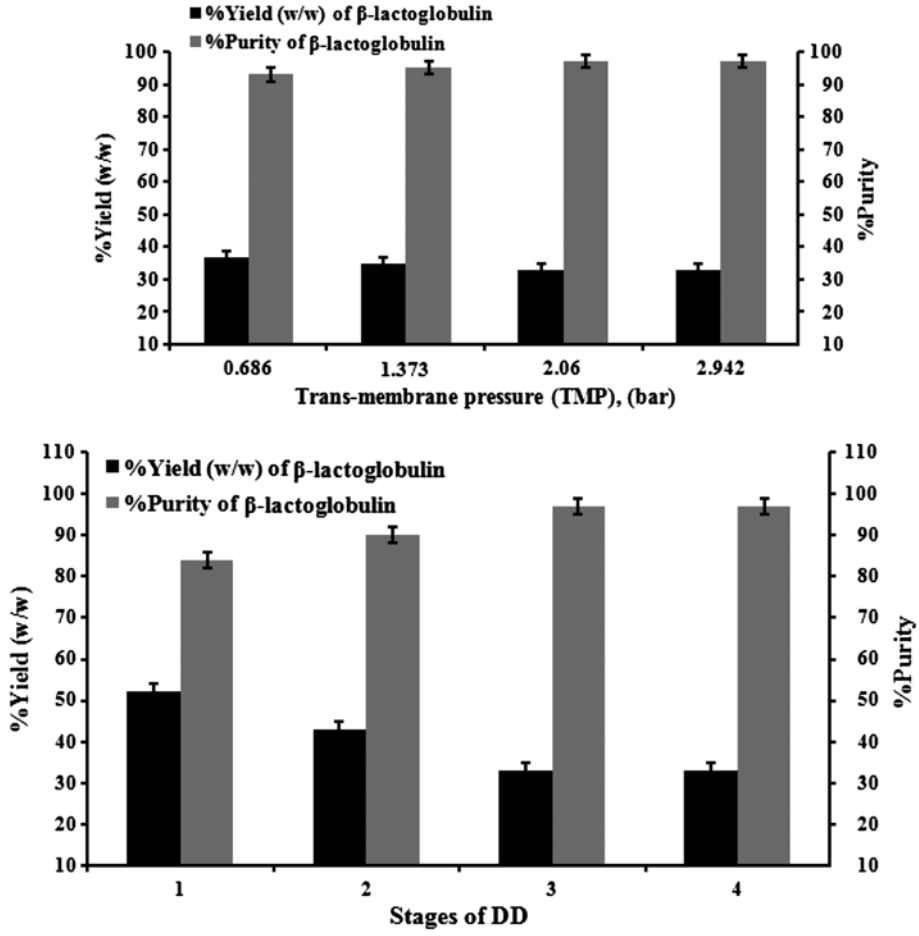

Fig. 10. Yield (w/w) (%) and purity (%) of β-lactoglobulin at different stages of DD for TMP 2.942 bar. (Inset: yield (w/w) (%) and purity (%) of β-lactoglobulin for different TMPs (0.686–2.942 bar) at third stage of DD).

Although there are two peaks in the chromatogram (one is for α-lactalbumin, and another one is for β-lactoglobulin), but compared to pick area, the pick area dedicated for α-lactalbumin is very high compared to other. This may be justified by the fact that at pH 5.4, β-lactoglobulin stays under monomer-dimer equilibrium, which offers low fractional β-lactoglobulin at permeate side. In Fig. 10, % yields and purities of β-lactoglobulin in retentate of 30 kDa membrane have been plotted with different DD stages for TMP 2.942 bar, pH 5.4. From the figure it is observed that the % yields of β-lactoglobulin at retentate side decreases, with increase of DD stages (first stage to third stage). The reduction of % yields of β-lactoglobulin in retentate side may be explained due to monomer-dimer equilibrium of β-lactoglobulin at pH 5.4 as some β-lactoglobulin molecules in monomeric state pass through the porous channel of membrane during the washing at DD process facilitating the reverse reaction of the monomer-dimer equilibrium, as per the Le Chatelier's principle [98]. Contradictorily, % purities of β-lactoglobulin increase with increase of DD stages. In the same figure (inset of Fig. 10) % yields and purities of β-lactoglobulin at retentate side of 30 kDa membrane have been plotted with different TMPs (0.686–2.942 bar) for the third stage of DD process. The figure depicts that with increase of TMPs (0.686–2.06 bar), % yields of β-lactoglobulin decrease, and the purities increase (95% at the third stage, and TMP 2.06 bar). The reasons of these observations have been mentioned earlier.

It is expected that the proposed methodology should be a viable route of waste treatment as well as recovery of different valuable biomolecules from whey in industrial scale.

4. Conclusion

In the present investigation, different fractions of individual whey proteins (immunoglobulins, lactoferrin, lactoperoxidase, bovin serum albumin, α-lactalbumin, and β-lactoglobulin), and lactose have been

purified by cascade of different MWCO cross-flow membrane module (100–5 kg mol^{-1}). Hydrodynamic studies were conducted under different TMPs (0.686–2.942 bar) at constant VCF. It was found that for all cases permeate flux increased with TMP 0.686–2.06 bar (later considered as optimum TMP), and with subsequent increase of TMP up to 2.942 bar, it remained virtually unchanged, possibly due to fully developed concentration polarization layer on membrane surface. High TMPs (2.06–2.942 bar) on the membrane surface provides higher cross-flow velocities, resulting in more turbulence, leading to reduction in concentration polarization, and hence higher flux. Further, it was found that permeate flux decreases with increase in DD stages, and it stabilized at the third, and fourth stages of DD process possibly due to compactness of concentration polarization layer. Major whey proteins β-lactoglobulin (molecular weight 18.3 kg mol^{-1}) and α-lactalbumin (molecular weight 14.2 kg mol^{-1}) are separated by controlling the pH. At pH 5.4 the most dominant whey protein β-lactoglobulin (isoelectric point 5.2) exists in dimer form, which is found to have immense effect on the separation characteristics. The % purities of proteins and lactose were increased sharply with increasing DD stages due to the washing effect. Effects of TMPs have been found positive on % purities of proteins and lactose. The proposed process strain could be used for fractionation of different whey proteins and recovery of lactose from whey with substantially good yield. Further, the proposed process may be extended to possible industrial application with suitable scale-up strategy.

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