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Graduated in
Chemical Engineering and Polymer Science

Implementation of membrane processes for improvement of the detection of food contaminants

Dissertation for obtaining the Master degree in Membrane Engineering

Erasmus Mundus Master in Membrane Engineering

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Abstract

Food borne pathogen is a topmost important concern of present era due to deteriorating the food quality, contributing significant loss in the health aspect, global nutritional demand and economy. A rapid, fast and effective analytic system of pathogen identification and enumeration is the vital most important tool towards minimization of both the grasp and effect of pathogen contamination whereas the performance and accuracy of almost all of presently developed respective sensory tools are localized on the molecular recognition and catalytic processes of the sensor that work for the detection and enumeration. The presence of other biological molecules except the targets imparts significant unwanted ligand interaction which deteriorates its true binding efficiency of the specific ligand with exact orientation and frequency; and thus requires the pre-treatments of the sample to minimize such interference. Keeping this on mind, in this work, a membrane process has developed for the purification of the targets (the cells) by removal of the other unwanted interfering species from the ***Listeria innocua*** loaded food (milk) sample followed by concentration of the sample. Both the type of bacteria and host food were selected due to high demand. The selective concentration of the cells and volume reduction of target cells from the milk sample was accomplished by diafiltration (DF) and tangential flow filtration (TFF) with microfiltration (MF) membrane. The effect of cell loading on the cell purification and concentration by filtration performance of milk was analyzed. Comparative analysis between TFF and DF was done on this aspect and a combined protocol has developed. The analysis of operational parameters (e.g. TMP, CFV, permeate flux behaviour, system configuration, etc.) on the chemical composition of permeate and retentate was studied. A mass-balance based model was developed and used to predict the trend of concentrations of the species along the progress of DF process. The organic load removal from the system was found to be positively correlated with the TMP and CFV involved while the cell recovery was found efficient in an optimum TMP range. The system was found efficient with fastness, effective cell purifying and concentrating capability, high recovery of membrane and easiness to operate with more than 99.7% of viable cell isolation for further use in bio-sensing tool.

Keywords: Membrane processes, milk, food contaminant, membrane rejection, cell isolation.

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Abbreviations

EPS	Extracellular poly - saccharides
TFF	TFF
DF	DF
CFV	Cross Flow velocity
MF	Microfiltration
CVD	Constant volume DF
MWCO	Molecular weight cut off
$q_{water\ (in)}$	Inlet water flow rate [m^3/s]
$q_{water\ (permeate)}$	Permeate water flow rate [m^3/s]
t	Time [second]
$C_{(i)(retentate)}$	Concentration of "i" - th species in the retentate [gm/L]
$C_{(i)(permeate)}$	Concentration of "i" - th species in the permeate [gm/L]
R	Retention coefficient [-]
$C^o_{(i)(retentate)}$	Initial (t=0) concentration of "i" - th species in the retentate [gm/L]
$C^o_{(i)(permeate)}$	Initial (t=0) concentration of "i" - th species in the permeate [gm/L]
P(t)	Total permeation at any time [m^3]
N(t)	Number of diavolumes [-]
V_o	Initial volume of the retentate
T_i	Transmission of the "i" - th species
J(t)	Permeation flux [$m^3m^{-2}.s^{-1}$]
R_t	Total filtration Resistance [m^{-1}]
TMP	Trans membrane pressure [bar]
$P_{entrance}$	Pressure at the entrance of the module [bar]
P_{exit}	Pressure at the exit of the module [bar]
P_p	Pressure at the permeate side from the module [bar]
R_{cp}	Filtration Resistance by concentration polarization [m^{-1}]
R_{rev}	Filtration Resistance by reversible fouling [m^{-1}]
R_{irrev}	Filtration Resistance by irreversible fouling [m^{-1}]
R_m	Intrinsic membrane resistance [m^{-1}]
LRV	Log reduction value [-]
C_p	Cell concentration in permeate [CFU/ml]
C_r	Cell concentration in retentate [CFU/ml]
CFU	Colony forming unit
M	Mass of species [gm]
$Mass_{(i)(permeate)}$	Mass of species "i" in the permeate [gm]
$Mass_{(i)(retentate\ )}$	Mass of species "i" in the retentate [gm]
$Mass_{(i)(membrane)}$	Accumulated mass of species "i" in the membrane [gm]

1 Introduction

1.1 Background and motivation

The quality, content and functionality improvement of foods and supplementary diets are one of the most important strand classifying the modern era. Though there are significantly increased awareness and rule-legalization in this aspect, illness brought by the consumption of pathogen contaminated foods still imparting a significant impact on both the economic and public health. They are responsible for foodborne illness, termed and well known as illness triggered by entering of pathogens into a host body via ingestion of contaminated foods and developed by the resulted food poisoning, a major public health concern having significant and broadly reported negative public health impact [1]. Among the victims, dominant fraction in the world suffer from food-borne diseases caused only due to food spoilage by microorganisms [2]. As an estimation, only in the United States, about 46 million foodborne infections occur each year, along with reported 250,000 hospitalizations and 3,000 deaths. Also, each year, about 31 major known pathogens are acquired in the United States causing an estimated 9.4 million incidences of foodborne illness [3], [4] only in US alone. Alarming incidences of recent worldwide food-borne disease outbreaks indicates a poor food safety status in food processing sector as a major threat to human health [5]–[18]. Among the reported food borne pathogens, bacteria are the most frequently and broadly reported food poisoning source which mostly contaminate the foods due to improper food handling [19]. Once infections by these microbes residing in food brought to the living beings, it directly correlate the complex food web chain that links the consumer with animal, plant, and microbial populations around the world due to the complex and integrated nature of food web, and consequently makes the steps for effective food quality maintenance to be the topmost level of importance. Considering significant threats by foodborne pathogens to global food safety [20], and being the most problematic issues in the food industry [21] causing increased raised demand for control actions; use of higher degree of antimicrobial agents are generally done [22] to maintain the nutritive value and extended shelf-life of food and beverage. But, it bears the potentiality to contribute negatively, and in many cases causing the failure against the global demand for natural, chemical preservative-free, minimally processed, and healthy products which are well balanced and preserve proper microbial safety. In addition, now a days, strict rules and health concerns of consumers have left the food processors with less flexibility to choose preservative substances and methods [2]. Considering all these growing concern over public health, people awareness, and alarming reports of outbreaks, strict steps by decision makers, increasing stringent food safety regulatory

codes for the food processing industries and involved responsible; the proper and effective tool for the controlling of food contamination is a vital concern for global socio-economic strand.

Towards this context, in food processing plant, at both the food and on surfaces in contact of food during processing steps, an effective and precise detection and enumeration method of pathogens are the vital concern and point of consideration towards ensuring the proper safety of foods throughout the entire food supply chain. Globally, both authorized government authorities and food companies constantly carry out microbiological analysis of processing foods as a routine work to identify any contamination as well as to get the state of contamination level for taking effective prevention action to oppose possible respective further emerging risks. Here, it's the time efficiency of proper pathogen detection and enumeration method only which can allow a responsible to develop and implement the on site proper action taking plan and preventive actions. In this context, whereas, numerous global and local critical management strategies are developed and generally followed by the food processing sectors, including Hazard Analysis and Critical Control Points (HACCP) system [23], [24] as an example; and have reported to greatly contribute for improving the food safety status, but in order to gain the best effective advantages of such strategy, a highly effective, fast and better methods of analysis are yet required to be developed and employed.

Milk is a fluid secreted by, and unique to, female mammals for the nutrition of their young. Its composition and properties vary widely between species, reflecting the specific nutritional and physiological requirements of the young of that species [25]. Throughout the world, milk is the most common product which is highly consumed regardless of the source. Among these, cow's milk is the most reputed and popular to the people all over the world for rich nutritional contexts, and is a greatest industrial and economic platform. However, though, because of the excellent sensory properties, being enriched with all the nutrients which are required for the body for rapid growth, assisting to prevent and reduces the risks of many nutritional deficiency diseases and so on for which milk ranks very high among the foods which are considered as the most suitable food for human from birth to senility [26], [27]; but it could possesses significant negative health impact if not well processed to kill the pathogens possibly been present there by diverse mode of contamination. It's because, milk is an excellent medium for microbial growth [28] and when stored at ambient temperature, bacteria and other pathogens can grow and proliferate in milk very fast by different manners [29]. These make milk an idol place for the growth and survival place as well as waterfront of broad ranges of microorganisms including severe pathogens [30]. Contamination in milk by microbes depends on the stages and conditions of breeding process, collection and transportation, feeding, processing as well as the distribution process. Infectious microbes which are found in cow's

milk includes ***Bacillus cereus, Brucella, Campylobacter jejuni, Coxiella burnetii, E. coli O157:H7, Listeria monocytogenes*** and others of the same group***, Mycobacterium avium*** subspecies ***paratuberculosis, Mycobacterium tuberculosis, Salmonella, Staphylococcus aureus, Yersinia enterocolitica*** etc [31]. It's also reported that, almost all the diseases producing pathogens can be found in cow's milk, and about 99% of all the human cases of ***L. monocytogenes*** infections are reported to be caused by the consumption of ***L. monocytogenes*** contaminated foods, especially ready to eat foods like milk [3]. In this context, pasteurization of milk is the most widely process that now a days used to destroy pathogenic bacteria and to inactivate some enzymes, extending the shelf-life and to make the milk edible for the consumers. But afterwards, these require a very strong practical demand for the post analysis, testing and possible further purification for most of the cases. The reason behind is highly controversial and is based on the efficiency of the pasteurization process to kill the pathogens as there exist a lot of supportive reasons for suggesting and leading the post analysis of milk and dairy product for pathogenic assay after this step. For instance, (1) outbreaks of disease in humans by contaminated milk have been traced for the consumption of both unpasteurized milk (by survey on dairy producers, farm employees, and their families, neighbors, and raw milk advocates as well as population consuming several types of cheeses manufactured from unpasteurized milk) and pasteurized milk, (2) entrance of the pathogen loaded milk having any level of degree of contamination bears high capacity on them for hosting and undergoing multiplication in the new nutrient rich medium as well developing persistence biofilms on the surfaces of contact, and subsequent contamination in the entire production chain, (3) pasteurization may not destroy all foodborne pathogens in milk as been reported by many researchers, and (4) many cases of inadequate or faulty pasteurization are incapable for incomplete destruction of all the pathogens being carried in the processing food stream [32]. However, under such scenario of food pathogen destruction, pathogens such as ***Listeria monocytogenes*** are of higher concern as they have very strong capability of surviving and flourishing in the pasteurized milk, their growth after pasteurization is more facilitated by non-competitive growth approach as the bacterial load by other species in pasteurized milk is highly lowered, and thus brings the recontamination of dairy products which in some cases is more faster than initial state.

Considering all these, proper detection and enumerations of pathogens is a vital strand towards minimization and prevention of foodborne disease outbreaks; with consequent high need for effective, rapid, precise, reproducible, easy to operate and accurate detection methods of pathogens in the food matrix among international – national regulators, food producers and/or processors, and researchers. However, the pathogenic contamination food at a minimum level during processing can bring a significant damage on the production as well as possible complete

destructing the production line up, shut downing the processing unit because of the high multiplication capacity of the bacteria within the food matrix which is rich in nutrient with consequent development of the stable inherent biofilms on equipment surfaces and thus grasping the entire production system. This can lead high socio-economic impact, high loss of profit, increase local demand and thus can hamper the smooth production and distribution system; requiring fast pathogen detection and quantification approaches to minimize the respective losses and unwanted process shut downing. Towards this goal, one of the major handicaps is the high time requirement by the traditional detection methods for bacterial quantification in the complex food matrix, which in most of the cases may require more than seven days to obtain a just final result of a species existence. This time limitation harnesses the effective food protection policies and techniques of industry, researchers and regulatory agencies to make the preventive action to be effective because of the fast deterioration of the food products during the prolonged session of experimentation of pathogen detection. In addition, during the prolonged analysis session of the food sample, fast bacterial growth in the sample causes their qualitative and quantitative characteristics to be altered before making the test results to be prepared and justified, and consequently proper identification of presence and quantification of the pathogens and taking further respective efforts to control foodborne disease outbreaks gets limited, requires time and lots of effort. To overcome this, presently many real time and fast bio sensing elements are reported to be developed, applied in the industrial facilities in some extent and many are in research and development stage and in plan to come in the upcoming days. All these works by specific interaction between ligands and active groups of the sensor and bacteria surface. But most of these real-time affinity detecting biosensors are not able to differentiate between the specific bindings of analytes to the immobilized bio-recognition elements and consequently causes the non-specific deposition of non-targeted molecules or other entities (other bacteria, particles, etc.) on the sensor surface with consequent disturbance of the sensor sensitivity, destroys the accurate detection and quantification. This critical problem, mostly, in case of affinity biosensors [33] constructed for the detection of pathogens in complex biological media [34] is a great concern for the present days as it harnessing the implementation growth of these newly developed bio sensors which works very fast and in much reliable way once after exposed to a high and optimized concentrated pathogen rich sample, and can have high potential towards opposing impacts of food contamination. In parallel, stipulating the food business operators to have the full responsibility of food safety to the market by some recent laws (e.g. The EU General Food Law, Regulation 178/2002) makes this situations to be highly concerning for the industrial area [35]. All these are leading towards the necessities for the development of simple and convenient process tools that may help in rapid screening and fast quantification of the pathogen in food sample that allows specific

access of the cells for the bio-sensors to maintain integrity of the food processing system [36] and further proper preventive action planning.

Among diverse food borne pathogenic diseases brought by different pathogens, within few sessions after the first reported case of human listeriosis which is the major impact on human by ***Listeria sp.*** in 1929 by Nyfeldt [37], increased detections was reported during 1980s. Afterword increased number of listeriosis incidence by the raising concise awareness towards the ***Listeria sp.*** in several countries was reported, and the thorough analysis turned it into a globally recognized foodborne disease [38]. Today, this species is the main causative agent of listeriosis, and is a globally well-known foodborne pathogen having its proven and well reported significance for disease outbreaks and severe impact including dominant fatality. It bears the extreme capability to survive and grow over a wide range of environmental conditions including wide temperature (1 to 45^{o}C) and pH range (4.1 to 9.6), can endure by competing with other pathogen in the same host, have capability to grow under refrigeration temperatures, and capable of surviving in freezing and very high salt concentrations [39] etc., all of which offer this pathogen tremendous and distinct capability over other pathogens to overcome the conventional food preservation and safety barriers, and thus possess a high alarming potential risk to human health. Due the natural capability of this bacteria to survive and well adopt with very broad range of condition and environment, many researchers, scientists and industrialist drawn and pointed special attention towards this species, well mentioning as "**a bacteria that knows how to survive**" [40]. In addition, while foodborne illnesses caused by most of the pathogens bears the functionality which is influenced by the geographical impact (e.g. in case of ***Campylobacter sp.*** and ***Salmonella sp.***) as well as surroundings which is just in contact with the cell [41], [42]; but the pathogens in the group of ***Listeria sp.*** has the capability of easy adaptation with the surrounding environment once after transmitted, undergoes growth and full functionality, and can survives in wide range of environmental conditions and medium and cause listeriosis [43], the severe disease associated with widely reported high hospitalization and fatality rates all over the world [44]–[51], once after getting exposed to the human as the host. This group of bacterium is reported to usually infect some determined inhabitants, especially high risk patients such as the elderly, immune suppressed patients and pregnant women; but, it can also affect people who do not have these risk factors. ***L. monocytogenes*** is widespread in nature being part of the faecal flora of many mammals which makes it a common foodborne source, and is acquired by humans primarily through consumption of contaminated food [52]. The dairy process and product are highly liable for the contamination with ***Listeria sp.*** as the conditions at the dairy production facilities, environment (animals and farms), and storage temperatures are within the range of the high adapting and optimum conditions for growth of this pathogenic species [53], [54]. Thus, in case of ***Listeria sp.***, implementation of

rigorous controls after the proper detection and enumeration is most essential to prevent food contamination [55] before the grasping of the entire facility by this species via the processes discussed earlier and to prevent the entire process shutting down. All these forced us for the selecting the ***Listeria sp.*** bacterial species and milk as the host food matrix for our analysis to get the more demand efficient and realistic analysis approach.

The fast, effective and precise tool for detection and easy quantification of such pathogenic bacteria in the food are the main key towards prevention and control of problems related to health and safety in the milk processing and manufacturing environments. Effective destruction of such microbial species is very crucial in food processing since a single trace of pathogenic contamination bears the high potentiality for putting the entire manufacture process in a big risk by the fast growing nature of the bacteria in the milk matrix. Consequently, the manufacturer requires to precisely detect the instant bacterial contamination, undergo the fast enumeration process and then plan for the action to inactivate all microorganisms present in the food, and to take the necessary corrective actions to prevent or retard the new growth of microbial populations, and destruction of the existing growth of present fractions [56]. In addition, the local and global rules and legislations in this aspect are highly tough, where failure to detect an infection at proper time may have terrible ultimate consequences in the local and broad view. Against the pre-mentioned high need for obtaining analytical results in the shortest time possible times, traditional and standard bacterial detection methods may take up to 7 or 8 days to yield only an answer [57] whereas, the effective quantification is the another more tough task yet to do and even in most of the cases, the proper quantification is impossible because of significant alteration in the analytes during this big experimental sessions. These are forcing the respective fellows to draw more and high concentration towards the development of rapid detection and quantification approaches for the pathogen analysis. Most of the presently developed biosensor for the detection of pathogen (e.g. micro fluidic based pathogen sensor [58], Amperometric [59]-[60], piezoelectric [61], impedimetric [62], fluorescent labeling based pathogen sensor for food [63], optical biosensors for foodborne pathogen [64] and so on) detection and counting, both the laboratory based and industrial, functions by real-time detection affinity towards the legends/markers which are not able to completely differentiate between the specific binding of analytes to the immobilized bio-recognition elements and the non-specific response due to the deposition of non-targeted molecules or other entities (protein, fat, particles, organic molecules etc.) on the sensor surface. This non-specific sensor response poses the main governing and critical problem; most particularly for affinity biosensors in case of detecting pathogens in complex biological media [33], especially for widely reported cases of milk [65]. Also, various methods to detect pathogenic bacteria are developed, and reported to work well by antibody probes that specifically bind to target proteins on the pathogen [66] and

amplifies specific target nucleic acids (DNA, rRNA, mRNA) in bacteria using PCR (polymerase chain reaction) or NASBA (nucleic acid sequence based amplification) [67] or by any other means, which is further processed for the quantifications. But the functionally and accuracy is depended on the purity of the bacteriological samples, because of the sensory inhibition by interference of other species (lipids, fats, nucleic acids, polysaccharides) present, making these techniques highly efficient for the laboratory scale with simplified food sample but diminishing the functionality while bringing in contact with the real food sample at any processing location, e.g. at industry, because of the complex nature of the food consisting broad range of components having affinity for the same antibody probes used in the sensing element and thus deteriorating the result and hence the functionality. But it's very important to get the enumeration at shortest possible time and near the functional unit, because of the continuously changing bacterial number in the nutrient rich host by cell growth and multiplication; and thus a proper quantification deviates away as the analyzing time increases while using such processes. All these puts the facility for fast detection and enumeration of the foodborne pathogen questionable, makes of high challenge and is a vital most concerning issue for ensuring food and environmental safety [68]. The situation can be solved with the presently developed biosensor only when the pathogens residing in the analytes can be purified from other contaminants and purified pathogen sample is concentrated to a significant level for detection.

So, against the demand of natural, nutritionally better, free from chemical preservatives and microbiologically safe with extended shelf-life dairy products by the consumers [69], the most important steps to improve the manufacturing system is to develop a process that assist the fast and reliable quantification of viable cells residing in the food sample by offering the production of purified and concentrated pathogens from the test sample . Such an approach will also allow the manufacturer to minimize the exposure time of milk to high temperature (to minimize the bacterial growth) where this exposure at high temperature bears the potentiality to lower the nutritional quality of foods as many nutrients are heat labile, and thus may deteriorate the nutritional quality of food. In addition, since the recent decades, energy optimization and heat recovery is the key focal point for all the industrial processes and the lowering of energy consumption by a better food processing technologies integrated with lowered heating requirement (offered by better control over pathogen by fast reporting) will offer additional opportunities to reduce energy consumption, and can improve sustainability of food production scenario in dairy industries [70]. Such approach is incorporated with the Enterprise Risk and Enterprise Risk Management (ERM) which have attracted a great covenant of attention in this sector, especially in recent years [71]. Also, from the broad studies, research report and case studies; it's widely known about the possible negative impact to human beings by generally used chemical synthetic preservatives as antimicrobials to inhibit the growth of food-borne

pathogens [72], and thus requires to minimize such use which can be only done if the growth of pathogen in processing can brought to the minimum which is governed by the fast pathogen detection and enumeration process in dairy samples. In addition all the presently used techniques for microbial food safety and quality maintaining are related to a significant compromise of the nutritional, functional and sensory characteristics of foods [73], and any alternative innovative process for food preservation and assistance to do so is the key to oppose such alternation of the desired property of the food and are of increasing interest of present time. In addition to all these, it's nothing overflow of hypothesis to say that, because of significant unavailability of economic, easy to adopt, efficient method in this context; there are still constant high needs for enhancement of technological approaches to be used for the efficient detection of pathogenic substances in complex food matrix (e.g. milk), for effective control of quality, optimization of production and processing in food plants; and development of any approach for fast pathogen detection and enumeration in food sample, which can be hybridizable by incorporation with others process will surely contribute towards the efficiency improvement over the processes developed earlier.

However, even though many highly sensitive detection tools have been developed and more yet to come in the near future, sample preparation methods, such as target (cell or nucleic acid) concentration/purification are the upmost important and first steps for the detection of pathogenic bacteria in test samples when any bio-sensing tools are applied for the detection and enumeration process. The scenario is more crucial when low concentrations of pathogenic bacterial cells are present in relatively large volume of samples which is rich in other interfering constituents (e.g. as in milk), and thus a cell purification and enrichment method becomes vital most important step for detection for such cases [74]. Though some nucleic acid based testing protocol can be a vital counterpart for the cell detection method, but in such approach requirement of various sample preparations steps such as, using chaotropic salt technologies, SPRI (Solid Phase Reversible Immobilization) polyethylene glycerol (PEG) based technologies, and CST (Charge Switch Technology) through pH control and so on makes the total process highly labor intensive and time consuming [75]. Considering all these, a cell purification and concentration steps by proper filtration with a suitable filtration technology can be good approach to keep the detection process to be simpler, faster, and easy to adopt and in coherence with presently developed sensing tools to reduce the energy, effort and chemicals requirement for total detection process. In this concern, Membranes are developed and designed with broad range of pore size distribution and functionality which can be effectively used for this purpose once after proper selection and methodology is developed. However, the application of membrane process for the pathogen isolation is pretty historic, can be considered to be started with the MF process, the oldest membrane process which was developed with most priority

towards bacteriological assay for rapidly determination of the safety for drinking water supplies in 1945 [76]. Such a well-defined pathogen-isolation system can be considered to have applicability over diverse food matrices and food type. It should be able to offer fast isolation, should well adopt with the reference control by undergoing minimized cell loss or deterioration, must should have high sensitivity and specificity for the isolation of the target cells by most effective separation of the other constituents from the food matrix, have to be easy to operate and to interpret the results, should offer the feasibility to count or detect the lost/damaged/adsorbed/newly developed cells, as well as effective for the further qualitative and quantitative enumeration and cost efficiency while treating the harvested cells with the bio-sensor. In this context, a properly selected MF process can be of high efficiency for cell harvesting because of the high flux at considerably lowered operational TMP than that for other membrane process and can make the process faster. The RO, and other low pore sized membrane system can be used for higher retention of the bacteria from the host solution (or the dissolved molecules), but is of no facility for the isolation of the cells from the complex food matrix consists of proteins, lipid etc (as in milk, blood etc) because of their bigger sizes. In addition, the MF process offers the lowered stress exerted on the processing fluid by offering lowered operational shear and TMP which works for minimized damage of the cell during the filtration process than that for UF and NF systems, thus assists in maintaining the cell intigrity. This higher cell integrity can be considered as very important criteria in this context, when the enumeration of the isolated cells is the main target. Moreover the higher pore sizes of MF membrane allows higher broad sized particle removal from food matrix, and reduced the need for multiple staged filtration and consequent losses of the target cell at different inter stages involved. But, though the microfiltration process is assisted by the low pressure operation, comparative high permeation flux, and by highly response of flux on cross flow velocity, available in convenient flat or cylindrical geometries of membrane module, but the process efficiency harnesses by the deposition and intrusion of macromolecules, colloids and particles onto and into the microporous membrane [77] and thus need to optimize for the specific applications.

Against the high need of purified cell suspension and concentration of these purified cells to be high enough for easy detection by biosensors, the conventional culturing and growth method offers the workability for cell detection in 5-7 days after the enrichment of the cells by growth. But, due to the prolonged session and increment of the cell concentration by cell growth, the cell quantification by using this cell enriched suspension is far than accurate and requires complex calculations to get an estimation. In this context, bacterial cells are very broadly discussed and reported to be isolatable from host sample matrix by using simple size exclusion techniques [78], most preferably by filtration processes like membrane filtration. Microfiltration of cell

suspension is most widely reported to be used for such aspect for cell concentration without any lengthy culturing and enrichment steps [79]–[81], [82]–[86]. Most conventionally it's done by passing the host sample through a flat-sheet MF membrane by a syringe pump, having an attached MF membrane piece holder. But, in any of such cases, requirement of the subsequent steps for recovering the microbial cells from the surface of the used membrane is troublesome, and bears the chances of losing the bacteria and wasting of time before using the captured bacteria for quantification in a suitable microfluidic biosensor platform. In addition, presence of the steps like removal of the membrane from the filtration system, recovery of the attached bacteria as well as dealing with very small volume of the bacteria involved after filtration; are added obstacles to make the process simpler and faster. Importantly, such approach can't be used without any pretreatment or dilution for the real biological fluid, because of high degree of fouling development within shortest possible filtration time, which is imparted from the nature of complex food matrix (e.g. milk). The resulted high degree of deposition on membrane is brought by wide range of constituents having broad range of physico-chemical property (e.g. fats, oils, particles, vitamins and proteins etc) being present on the test sample. Also, while processing with bacteria loaded biological fluid, e.g. milk, presence of membrane pores having sizes higher than the minimum dimension of the viable cells causes the cells to be permeated under application of drag force of the permeation stream once after attachment with the membrane surface. Also, EPS from bacteria can be released due to the action of high shear stress imparted by such filtration [87] which may cause increment of filtration resistance [88], can interact with the deposited proteins on the membrane to make the cake to be more severe for filtration [89], possible enhanced cell attachment on the membrane at the concentration stage of the dead end filtration because of increased cell concentration [90], increased biofouling on the membrane because of the high cell concentration at the membrane at the early stage [91] and is considered as one of the main obstacles of MF membrane [92] and causes the membrane to be less usable for the recovery and reuse. In addition, while filtration of the cell suspension with the intended purpose of cell isolation, especially when dead end filtration approach is followed, because of such fouling, increased differential pressure faced by a cell after once get immobilized into the membrane can cause it to deform elastically [93], penetrated into the membrane matrix, and bears capability to further capture the internal structures of the membrane by growth [93] and release of associated products. Considering all these, a TFF system can be an excellent alternative for the efforts to isolate cells by membrane filtration. This mode of widely used and broadly accepted in the biotechnology industry and laboratory analysis [93]–[96] which offers the lowered and controllable deposition by cross flow velocity and thus lowers the associated problems. TFF system possesses tremendous facility preventing the sedimentation of large molecules on the membrane surface and thus reduces fouling, and minimizes the aforementioned related impacts [96]. Consequently it offers the possibility of

processing of large quantity of pathogen loaded sample [97] with comparatively lower filtration performance alteration. In addition, because of the minimized shear force experienced by the bacteria as well as less interaction with membrane, TFF systems are reported to be highly in efficient terms of performance (filtration rate) and cell viability [98] which are of topmost importance when the system is employed for harvesting of viable cells to be used for quantification. However, when the TFF is bought in the mode of constant volume DF (DF), the gradual lowering of the bulk concentration of the species further assist more decline of deposition on membrane and thus maintain the smoother operation. Also, such process bears additional advantage of higher cell integrity while processing of bacteria rich sample and when viable pure cells with structural integrity are main concerned target. More details of these systems are discussed in the later section of the report.

Considering all these backdrop and demands, as in deliberately, applicability of a membrane technology has demonstrated in this work for assisting the current technologies of food borne pathogen detection by overcoming sensory inhibition of non-target species, for improving the pathogen sensor workability and working range; and is of high effectiveness for making the traditional viable cell enumeration methods be of better efficiency by incorporating effective pre-filtration, isolation and concentration of the target cells. In addition, it will improve and positively contribute in the field of new biosensor innovation and engineering and assist faster, reliable and more precise assays to improve the present scenario of the food contamination prevention, control, making the dairy production processes smoother and of better inherent safety as well as will contribute for the development of the global heath and food safety. The work is aimed for development of a microfiltration based DF-TFF integrated process for ease harvesting and concentration of the viable cell present in a complex food matrix of milk. Based on the aforementioned discussion of the high and proven need of such process for the dairy sectors, and being ***Listeria. sp.*** as the most alarming dairy pathogen which requires very fast detection and enumeration for effective control among the pathogens this field, we selected milk and ***Listeria sp.*** as our host complex food – pathogen system for the evaluation of this testing process. Among the ***Listeria species,*** the non pathogenic ***Listeria innocua*** was selected because of its exceptional similarity with the pathogenic species of ***Listeria*** group, the ***Listeria monocytogenes.*** Along the experimental session, individual efficiency of TFF and DF process were tested for this same aspect and was further combined to make a hybrid cell purification and concentration technique for opposing the drawbacks of individual steps, to develop a faster and effective isolation method of these bacterial cell for sensing and quantification in the complex sample matrix of contaminated real milk. All the stages of filtration and processing were analyzed for the respective efficiency, characterized for effective combination and integrity of the entire developed approach. The removal of the organic load from milk sample was achieved

at significant level and developed method was found efficient for concentrated viable cell isolation. A well fitted mathematic modeling for predicting the trend of organic load elimination from the testing milk sample were developed and implemented. The optimum condition for the isolation of bacterial cell from the sample without deteriorating their functionality and integrity was derived and tested.

1.2 Objectives

Based on the aforementioned backdrops and present high demand of biosensor implementation in rapid, precise, easy and reliable pathogen detection and enumeration techniques for carrying out simultaneous improvement of the quality of food, awareness and better following up of legalizations in the fast growing sector of food industries; it's of high concern to make the cell harvesting and concentration process to be faster, precise, reproducible, simple and hygienic. But, these requirements can be fulfilled only when a good, highly efficient, precise and reproducible method of bacteria isolation and concentration from the alalysing complex food matrix is developed. Keeping on mind about the possible potential broad applicability of membrane in this aspect, the goals of this project are:

- Selection of a membrane process for performing selective concentration of a high demanding bacteria (***Listeria sp.***) residing at the complex food matrix (the milk).
- Determination of a process flow design of experiment which allows minimized pre-treatment and keeps the process simple, isolated, hygienic and smooth to operate.
- Determination of the influence of operating condition on the organic load removal performance and to derive optimum.
- Development of a suitable model for the system to predict and define the removal profile of organic species from the analyzing sample along the progress of cell isolation process.
- Analysis of the cell retention and concentration efficiency by the process.
- Deriving an optimum process condition for the technique to adapt in real scale.
- Identification and analysis of parameters, situations and their influences on any observed bacterial leakage during filtration process. Determination of conditions and their impacts to make the isolated cells to be ready for direct quantification (by biosensor) with minimized cell loss resulted deviations, and additional requirement of correction factor incorporation.

2 Theory

2.1 Modelling of DF system

In case of DF, continuous removal of a species (solute) from the retentate occurs along time which is comparable with a continuous stirred tank reactor (CSTR), but been incorporated with a membrane at outlet stream [99] imparting the selective exit of respective species through the exit stream. This implies that, the equations governing the elimination of a species from DF system would be rather similar with those used for CSTR, but the difference will be introducing a rejection defining parameter in case of DF based on system performance characteristics. **Figure 1** shows a schematic drawing of a continuous stirred tank reactor (CSTR) and of a DF system with the flow characteristic parameters. This rejection defining parameter (rejection coefficient $\boldsymbol{R_i}$) on a feed solution consists of broad range of molecular weight species possesses different values for different solute species based on the size, shape, hydrodynamics, physico-chemical characteristics and different interactions. For instance, some species of very small size (e.g. Salt) can have a negligible rejection coefficient ($R \approx 0$), while the others may have values higher than zero, magnitude of which is the function of aforementioned properties of the species.

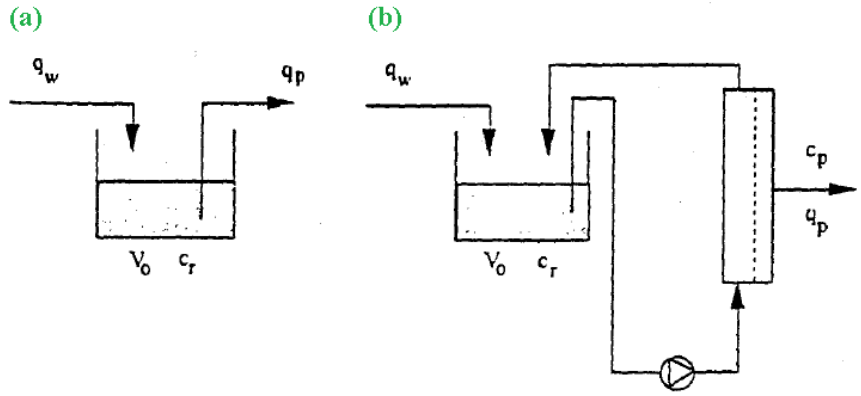

Figure 1: Schematic diagram of (a) a CSTR and (b) a DF system [99].

In both the cases, the permeation nature of a species generally possesses expression in the form of generalized exponential decline which in case of ideal DF is a function of time along the progress of DF, keeping other rate influencing parameters (e.g. $\boldsymbol{R_i}$) of expression to be constant. But in our case, continuous variation of permeation flux along the progress of DF was observed which causes the variation of such permeation influencing parameter (imposed to the permeation defining equations) values along progress of DF, been arisen from the feed (retentate) side. Consequently an empirical equation [**Equation 1**], similar to that of the ideal exponential interpretation, having demonstrated high correlation ($R^2 > 0.99$) with the observed permeation data of the species (i) was selected to define the permeations of respective species.

Defining the permeation natures by the selection of such empirical equation imparts simplicity on the development model with offering easy assessing scope of continuous variation of the rate influencing parameters (e.g. $\boldsymbol{R_i}$).

Such equation can be written as:

$$\boldsymbol{C_{(i)(permeate)}(t,N) = C^{o}_{(i)(permeate)} \times exp\left[-\frac{P(N,t)}{V_o} \times m_i\right]} \qquad \textbf{Equation 1}$$

Where,

$$\mathrm{P(N,t) = q_{w(t)} \times t} \qquad \textbf{Equation 2}$$

and $\boldsymbol{m_i}$ is the dimensionless rate constant representing the exponential accumulation rate of the permeating species (i) in the permeate stream. Higher values of $\boldsymbol{m_i}$ stands for faster and easier permeation of respective species (i) through the membrane during DF process. Here, $\boldsymbol{C^{o}_{(i)(permeate)}}$ is the predicted instantaneous concentration of the respective species at permeate just at the beginning (N, t = 0) of the DF.

The membrane rejection coefficient of a species (i) can be expressed as,

$$\boldsymbol{C_{p\,(i)(permeate)}(N,t) = (1 - R_i(N,t))\, C_{(i)\,(retenate)}(N,t)} \qquad \textbf{Equation 3}$$

Here, $\boldsymbol{R_i(N,t)}$ represents the membrane retention coefficient of the respective species at that instance. The reason for selection of an empirical equation for defining the permeation rate of a species and further development of the modeling equations based on that equation is the observed time depended variation of flux, which in term can influence different performance factors, e.g. continuous influence on "$\boldsymbol{R_i}$" and "$\boldsymbol{C_i}$" values along the progress of DF. Approaching and developing formulations in this demonstrated way offers the simplicity of the model as well as eliminates the possible complexity which can be imposed while incorporation of time depended such "$\boldsymbol{R_i}$" and "$\boldsymbol{C_i}$" values along the filtration time, and interprets the system better. The time depended transmission values of a species (i) along the DF ($\mathrm{T_i(N,t)}$) were determined by the **Equation 4**.

$$\mathrm{T_i(N,t) = 1 - R_i(N,t) = \frac{C_{(i)(permeate)}(N,t)}{C_{(i)(retenate)}(N,t)}} \qquad \textbf{Equation 4}$$

The mass balances for solvent (and replacement buffer) of the system can be written as:

$$\boldsymbol{q_{water\,(in)}(N,t) = q_{water\,(permeate)}(N,t)} \qquad \textbf{Equation 5}$$

The mass balances for solute (i) of the system can be written as:

$$\text{Mass}^{\circ}_{\text{(i)(permeate)}}(\text{N,t}) + \text{Mass}^{\circ}_{\text{(i)(retentate)}}(\text{N,t}) + \text{Mass}^{\circ}_{\text{(i)(membrane)}}(\text{N,t}) = 0$$

or,

$$\boldsymbol{q_{water\ (permeate)}(N,t) \times C_{(i)(permeate)}(N,t) =}$$

$$-\boldsymbol{V_o}\frac{\boldsymbol{dC_{(i)(retenate)}(N,t)}}{\boldsymbol{dt}} + \text{amount of "i" in membrane}(\boldsymbol{N,t}) \qquad \textbf{Equation 6}$$

Here, the Mass° represents the rate of change of the respective mass.

Cumulative amount of species "i" permeated is:

$$\boldsymbol{M_{(i)\ (permeate)} = \int_0^t \int_0^N C_{(i)\ (permeate)}(N,t) \times P(N,t) dN dt} \qquad \textbf{Equation 7}$$

And consequently, amount of a permeating species accumulated on the membrane is:

$$\boldsymbol{M_{(i)\ adsorbed\ in\ membrane} = [C^{o}_{(i)(retenate)} \times V_o] - \int_0^t \int_0^N C_{(i)\ (permeate)}(N,t) \times P(N,t) dN dt}$$

Equation 8

The residual concentration of the species (i) in the retentate at any instance is:

$$\boldsymbol{C_{(i)\ (retenate)}} = \frac{\boldsymbol{M_{(initial\ in\ the\ feed\ tank)} - M_{(in\ the\ permeate)} - M_{(i)\ adsorbed\ in\ membrane}}}{\boldsymbol{V_o}}$$

$$= \frac{\boldsymbol{C_0 V_o - \int_0^t \int_0^N C_{(i)\ (permeate)}(N,t) \times P(N,t) dN dt - M_{(i)\ adsorbed\ in\ membrane}}}{\boldsymbol{V_o}}$$

Equation 9

The boundary conditions for the intigration are:

$$at\ , \boldsymbol{t = 0}\ , \boldsymbol{q = 0}\ and\ \boldsymbol{N = 0};\qquad => \qquad \boldsymbol{C_{(i)(permeate)} = C^{o}_{(i)(permeate)}}$$

$$at\ , \boldsymbol{t = t, q = q}\ and\ \boldsymbol{N = N};\qquad => \qquad \boldsymbol{C_{(i)(permeate)} = C^{o}_{(i)(permeate)}}$$

These equations were solved numerically altogether with the experimental data's by Matlab [100] and Microsoft Excel [101] to quantify the respective parameters and comparison.

2.2 Flux, permeability and resistances calculation

In cases of DF and concentration experiments, complete recycling of the retentate stream to feed tank was done and the permeate flux (J) [$m^3m^{-2}s^{-1}$] was determined from timed collection log of the membrane permeations from a digital balance and by using **Equation 10**.

$$J(t) = \frac{1}{A}\frac{dP(t)}{dt}$$ **Equation 10**

Here, **A** is the total active membrane area in m^2, **P(t)** is the cumulative permeate volume in m^3 obtained after filtration time, ***t*** (in second). The duplication of the experiments showed reproducibility with a maximum of ± 5 % error.

The hydraulic permeability of the membrane was tested by sterile distilled water permeation and calculated from the slope of Darcy's law [**Equation 11**].

$$J = \frac{TMP}{\mu R_t}$$ **Equation 11**

TMP was calculated by using **Equation 12**; where, $P_{entrance}$, P_{exit} and P_p are the pressures at the entrance of module, at the exit of module and at the permeate side respectively.

$$TMP = \frac{P_{entrance} + P_{exit}}{2} - P_p$$ **Equation 12**

The permeability (**P**) of the membrane is the slope of the plot obtained from **Equation 11** and represented by **Equation 13.**

$$P = \frac{1}{\mu R_t}$$ **Equation 13**

Among the many models describing the increment of membrane resistance (i.e. decline in permeate flux by fouling and deposition on the membrane surface during filtration), the resistance-in-series model [102], [103] is used to subdivide the net resistance into all the individual resistances as given by **Equation 14.**

$$R_{total} = R_m + R_{cp} + R_{rev} + R_{irrev}$$ **Equation 14**

The intrinsic resistance of membrane [R_m] is mainly characterized by pore shape, size, tortuosity, membrane material, membrane thickness, and so on which can be controlled only by the proper controlling of these parameters during the manufacturing stage of respective membrane. All other resistances are depended on the operation mode and system involved. The resistance on the membrane by adsorption [R_{rev}] stands for permeation resistance imparted by thermodynamically unavoidable adsorption of the constituents from the feed material which is thus recoverable by proper water wash (or rinsing). Generally, the contribution on the total resistance by this resistance is small [104] comparing to the relative magnitude of the other resistance contributors. Filtration resistance by concentration polarization [R_{cp}] is imparted by the accumulation of the material adjacent to the membrane surface which is recoverable by "*rinsing*" the membrane surface with pure water just after completing the use of membrane

[105]. Irreversible resistance on the membrane [R_{irrev}] is imparted by the irreversible accumulation of the solutes in the membrane matrix and membrane surface.

The intrinsic and the fouling layer resistance were calculated using **Equation 14**, **Equation 15, Equation 16** and **Equation 17**; where, J_0 is the distilled water flux obtained for the un-used membrane [106] and the measurement of $R_{fouling} (= R_{cp} + R_{rev} + R_{irrev})$ was based on flux obtained during filtration of milk.

$$R_m = \frac{TMP}{\mu J_o} \qquad \textbf{Equation 15} \qquad \text{and} \qquad R_{fouling} = \frac{TMP}{\mu J_o} - R_m \qquad \textbf{Equation 16}$$

During the concentration of the retentate sample by TFF, the concentration factor of the sample was calculated as:

$$concentration\ factor = \frac{(V_{initial} - V_{final})}{V_{initial}} \qquad \textbf{Equation 17}$$

2.3 Quantification of the Bacterial cell concentrations and recovery

Concentration of the cells was expressed in the form of CFU/ml. Log reduction value (LRV) was used for demonstrating the relative permeation of the bacteria from the retentate sample. The LRV was defined as the ratio of number of CFU of the bacteria in the retentate at any time to that in permeate at that instance **Equation 18**.

$$\text{LRV} = \frac{C_r \times volume\ of\ retentate}{C_p \times volume\ of\ permeate} \qquad \textbf{Equation 18}$$

Where, C_r and C_p stands for the bacterial cell concentration [CFU/ml] in the retentate and the permeate side respectively.

Cell concentration factor and the recovery efficiency were calculated according to the following equations:

$$concentration\ factor = \log\left[\frac{cell\ concentration\ \left(\frac{CFU}{ml}\right)\ in\ the\ concentrate}{cell\ concentration\ \left(\frac{CFU}{ml}\right)\ in\ the\ initial\ original\ sample}\right] \qquad \textbf{Equation 19}$$

Cell recovery by the individual process [%] =

$$\log\left[\frac{cell\ concentration\ \left(\frac{CFU}{ml}\right)\ in\ the\ concentrate \times volume\ of\ the\ concentrated\ sample\ (ml)}{cell\ concentration\ \left(\frac{CFU}{ml}\right)\ in\ the\ initial\ original\ sample \times volume\ of\ the\ original\ sample\ (ml)}\right] \qquad \textbf{Equation 20}$$

The total recovery [%] of bacteria by the process was defined as the percentage of total bacteria gained after the dual step of filtration (DF and concentration), and was calculated by using **Equation 21.**

Total cell recovery [%] =

$$\frac{\left[C_{cell\ (retentate)} \times V_{concentrate}\right]_{after\ TFF}}{\left\{\begin{array}{l}\left[C_{cell\ (retentate)} \times V_{concentrate}\right]_{after\ TFF} + \left[C_{cell\ (retentate)} \times V_{retentate}\right]_{after\ DF} \\ + \left[C_{cell\ (permeate)} \times V_{permeate}\right]_{after\ TFF} + \left[C_{cell\ (permeate)} \times V_{permeate}\right]_{after\ DF}\end{array}\right\}}$$

Equation 21

3 Materials and methods

3.1 Materials

3.1.1 Milk and contaminated milk

Commercially available semi skimmed milk, "leite Agros meio gordo", was used to test this filtration performance (as received in the final dispensable form) without any modification. The constituents of this milk and their respective proportions are tabulated in **Table 1.** For the preparation of the contaminated milk, the same milk was contaminated with the selected bacteria up to a specific level of contamination.

Table 1: Constituents and their proportions in the used semi skimmed milk.

Constituent(s)	Amount(s) [gm per 100 ml]
Lipids	1.60
Sugars	5.10
Proteins	3.40
Salt	0.10
Vitamins and minerals:	
Vitamin B12	0.3710E-6
Potassium	0.16
Calcium	0.12
Phosphorous	0.093
Iodine	20×10E-6
Riboflavin (vitamin B_2)	0.17×10E-3

3.1.2 Bacteria

Listeria innocua, the non-pathogenic species among the six species belonging to the group ***Listeria sp.***, was selected as the model bacteria for this experiment. It is a rod shaped gram positive bacteria having longest dimension of 1-2 µm and the smallest dimension of 0.45-0.55 µm. The species bears the high similarity with the ***Listeria monocytogenes*** except the capability of the pathogenic property on the host and most widely used as the model bacteria for the laboratory scale testing of ***Listeria sp***.

3.1.3 Bacteria enriched (contaminated) Milk

The culture of ***L. innocua*** was prepared in BHI (agar), grown for 18 hours at 30 ºC, and then this culture was used for loading of bacteria in milk. A 199 ml of milk (specified in **section 3.1.1**) was inoculated with 1 ml of diluted 10^{-2} (~10^{7} CFU/ml) ***L. innocua*** culture. So, the resultant milk solutions had ***L. innocua*** concentration of ≈ 10^{5} CFU/ml. The bacteria loaded milk sample was preserved in the cold storage (near 4^{o}C of temperature) and was used for the further experimentation within shortest possible time in order to minimize the effect of bacterial growth (before using in the experiment) on the filtration performance. This section was done with appreciated collaboration of Dr. Susana Rodrigues and Dr. Wilson Antunez from the Laboratório de Bromatologia e Defesa Biológica, Portugal.

3.1.4 Reagents and chemicals

The Analysis of total protein was done by Folin-Ciocalteu's reagent (Merck). All other chemicals used in this experiment were of analytical grade, received from Sigma, Merck and Pierce and were used without any form of additional purification.

3.1.5 Membrane

The microfiltration membrane used for the experiment was made of regenerated cellulose having average pore size of 0.45 µm [Model: HY18606 from Sartorius (Germany)].

3.2 Methods

3.2.1 Experimental methods

3.2.1.1 Cross flow filtration and DF

The experimental setup for the DF experiment is schematically shown in **Figure 2**. The suitable sized piece of membrane was placed on the membrane support made of mesoporous steel within the module (locally designed, made of stainless steel; and equipped with the feed/ retentate channel, permeation compartment, feed inlet channel and retentate outlet channel, permeate collection channels and rubber sealing to oppose the leakage) and screwed well for making the system to be air tight. All the used tubing and valves were made of sterilizable silicon rubber and Teflon respectively. The flow circuit of the system [**Figure 2**] was equipped with series combination of pressure sensors and pumps to complete the closed circuit. Flow to and from feed reservoir through this closed retentate circuit was induced by using feed pump. The permeate channels were extended to the permeate collection vessel mounted on the high precision balance equipped with electronic data logging. Prior to each experiment, the membrane was compacted by permeation of the sterile water in tangential flow mode with

stepwise increment of the TMP up to a level above the analyzing TMP. Each of these steps was continued until a stable permeation flux was established under respective TMP. Afterward, the clean water permeability of the membrane was determined prior to start of each experiment. For the DF experiment, a fixed amount of the sample was taken in the feed reservoir and constant volume was maintained by addition of buffer at rates equal to that of permeates by using buffer feeding pump and tubing. All the experiments were done in the laminar flow chamber under sterile condition and at room temperature. After completion of DF step, transformation to the concentration step was done by immediate turning off the buffer addition pump and closing of the buffer addition valve.

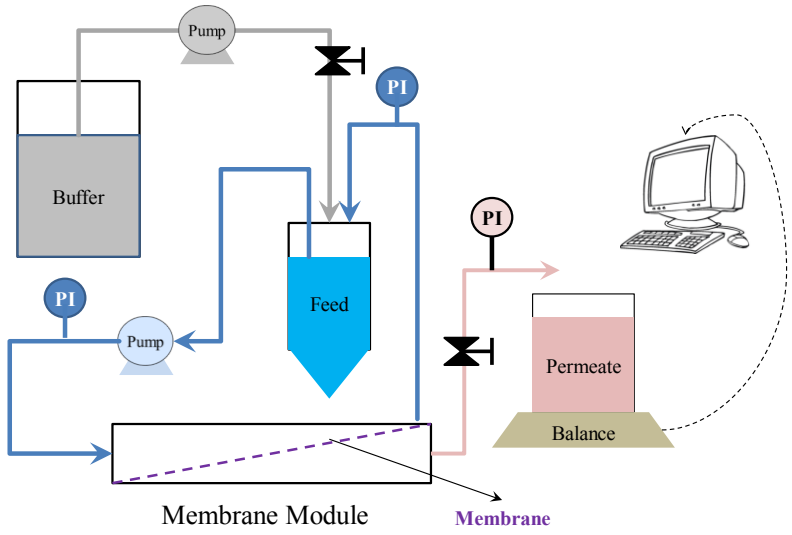

Figure 2: Experimental setup for the DF experiments **(** – **,** – **,** – represents the buffer flow line, milk circulation line and permeate flow channel respectively; and "PI" stands for the pressure indicator (sensor)**)** in the respective positions.

For the experimentation in concentration in TFF mode, the experimental setup was of same configuration and equipments except the elimination of the buffer addition section **[**Figure 3].

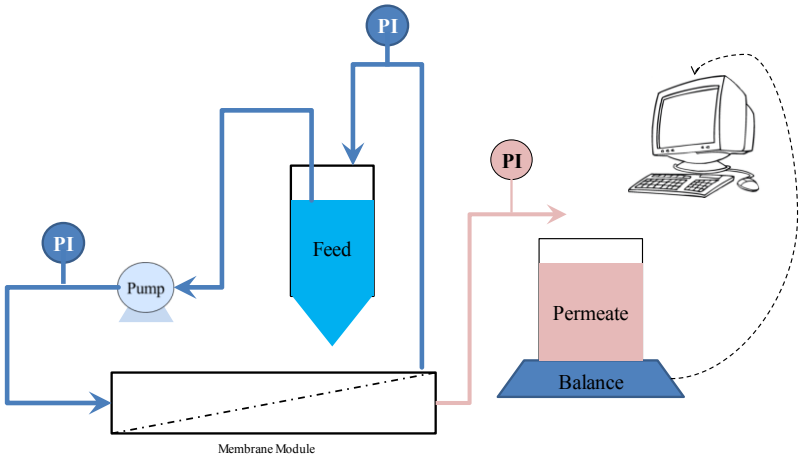

Figure 3: Experimental setup for the TFF experiments **(**– and – represents the milk circulation line and permeate flow channel respectively; and **PI** stands for the pressure indicator (sensor)**)**.

3.2.1.2 Sterilization of the equipments and auxiliaries

The entire filtration system, components, cleaning solution and rinsing distilled waters were sterilized by autoclaving at 121^{o}C under 20 bar of pressure for 20 minutes prior using for the filtration experiments. For the sterilization of the membrane module and pump heads, 70% ethanol was used and later rinsed with sterile distilled water to remove the ethanol prior starting of experimentation. The surfaces of laminar flow chamber was washed with 70% ethanol prior using and further sterilized with UV radiation altogether with all the equipments and auxiliaries. For the sampling and quantifications of the parameters; sterile pipette, sterile pipette filler, sterile micro syringe and needles were used, and proper care was taken for handling of samples, preservations and during assay in order to minimize any external contamination. All personal protective cares based on the lab rules were taken while experimentation with contaminated milk samples. After use, discarding of the waste from the biologically contaminated samples was done only after autoclaving to kill bacteria and subsequent to additional rinsing with 70% ethanol.

3.2.1.3 Cleaning of membrane

After using, the membrane was washed in different stages in order to accomplish maximum possible recovery of the membrane. Simultaneously, different fouling was quantified from the cleaning stages. At first, rinsing (at very low flow rate) of the retentate circuit was done with sterile distilled water for 0.50-1.0 minutes to remove the accumulation in the tubing with measurement of water permeability. Then, the filtration of the sterile water through the

membrane was done at low TMP (≈ 0.02 bar) for 10-15 minutes unless the turbidity of the washing solution turns to undetectable and the sterile distilled water permeability of the membrane was measured; and from that, reversible fouling was quantified. Afterwards, the membrane was rinsed with 0.20 N NaOH solution of 40-50^{o}C in non-permeation state (rinsing the membrane surface) at low TMP (≈ 0.05 bar) for 20-30 minutes depending on the existence of the detectable suspended particle in the washing solution. During this session, the cleaning solution was changed multiple times based on until how long the elimination of the substances from the membrane surface was detectable. After obtaining stable condition with no more observed removal of substances to the washing agent, the system was turned on the filtration mode at low TMP (≈ 0.07 bar); and was continued for 5-10 minutes. After that, the system was operated in filtration mode with distilled water (of room temperature with continuous changing of water) until the water pH in the both side of the TFF system comes to normal water pH, and the water permeability was measured again. This measurement was used for the determination of the irreversible fouling by comparing with initial membrane permeability's. This process was continued unless maximum possible recovery of the membrane permeability was achieved.

3.2.2 Analytical Methods

3.2.2.1 Membrane characterization

The characterization results altogether with the reference methods are incorporated in the result and discussion section.

3.2.2.2 Determination of Protein

For the determination of total protein in samples, spectrophotometric Lowery method [107] was used with some modification as been developed and reported by [108]. To a brief, the UV absorbance intensity at 750 nm of wavelength showed by **copper - protein - Folin Ciocalteu reagent** complex was measured by a spectrophotometer for different standard protein solutions. These data's were used for the preparation of calibration curve and was further used for protein quantification of unknown samples. The milk solution, as received, was diluted with phosphate buffer for the preparation of standard solutions. The linear region of the calibration plot was considered for the assay and all the testing samples were diluted to that range by dilution with buffer prior to total protein quantification (in order to get the correlation factor, $R^2 \geq 0.99$).

3.2.2.3 Determination of Sugar

The sugar concentration in the retentate and the permeate streams at different stages of DF was determined by high pressure liquid chromatography with a Varian Metacarb 87-H

column. The standard solution of lactose, glucose and galactose (the main sugar constituents of the milk) were prepared by dissolving the respective quantities in distilled water and peak areas obtained for with different standard sugar solution concentrations at corresponding elution time (using 0.10 N H_2SO_4 as mobile phase) were used for the construction of calibration curve which were further used for the quantification of sugars in the test samples (prepared by filtration through 0.45 µm filter). The peak areas at the respective elution times were compared with the calibration curve to get the respective concentrations.

3.2.2.4 Determination of Fat

The fat assay was done after extraction of the fatty component from lyophilized milk samples by hexane. The sample-hexane mixer was centrifuged to physically separate the fat rich extract phase followed by complete drying for fat quantification as previously developed in the lab and reported by [109]. At least, three replications were done and the average value was used for the total lipid content estimation.

3.2.2.5 Biological assay and quantification

Quantification of the ***Listeria innocua*** in milk sample was done by plating with selective Palcam culture as developed and reported by [110]. It is a selective differential medium for the isolation and detection of ***Listeria innocua***, ***Listeria monocytogenes*** and other ***Listeria sp***. from foods and clinical specimens which is widely used for the isolation and enumeration of this species and recommended by APHA, AFNOR and the International Dairy Federation for the detection of ***Listeria sp.*** in milk and milk products [111]–[114]. Further details of the procedure are reported elsewhere [114]. This section was done with kind collaboration of Dr. Susana Rodrigues and Dr. Wilson Antunez from the Laboratório de Bromatologia e Defesa Biológica, Portugal.

3.2.2.6 Determination of buffer capacity

The buffering capacity of the milk can be considered as the indicator of the elimination/replacement of the buffering components of the milk, as it is contributed by the presence of acid-base compounds in the milk matrix, depends on several compositional factors including small constituents (e.g. inorganic phosphate, citrate, organic acids) and milk proteins (caseins and whey proteins) [115] residing in the its milk matrix. The total buffer capacity is induced by the individual buffering capacity of all the components present in milk, any natural or induced impacts on the milk, and consequently variations in the composition of milk significantly alter the buffering capacity [115]–[120]. Consequently, the buffer capacity alteration resulted by elimination of these diverse constituents can be a vital strand to demonstrate the overall organic load elimination (by a process like membrane separation

technology), even when the overall removal performance it not well detectable at very low level of individual concentrations. It may also serve by fast indication of overall removal rate of the components from milk apart from analysis of the individual components when precise estimations about removal of these are not required. In this context, the buffer capacity of milk sample at different stages of DF and TFF was considered, assessed and compared for effectiveness of the process performance. Experimentally, a 2.0 ml of testing sample was taken in a plastic container and well precise 0.1 ml of 1M HNO_3 was continuously added until the pH gets reached to a stable value. After each addition of 0.1 ml 1M HNO_3, the sample was immediately well vortexed for 30 seconds to homogenize the mixture and to complete the physico-chemical changes, and the final pH was recorded. The same protocol was followed for the fresh milk sample (before using for the filtration experiment), for pure replacement buffer used in the DF and relative comparison was considered for the performance analysis. All the experiments were done at room temperature ($25 \pm 2^{o}C$).

3.2.2.7 Determination of viscosity

Viscosity of the samples was determined by Brookfield Viscometer [model: DV-II] equipped with required sized spindle and specified amount of sample as mentioned in the protocol offered the manufacturer. Further details about the testing process can be found elsewhere [121], [122].

4 Results and discussion

4.1 Dependence of flux on TMP and CFV

In order to assess the influence of CFV and TMP on the permeation flux of milk, a fixed amount of milk were filtered in TFF filtration mode with complete recirculation of permeate in to the feed reservoir for opposing the effect of any feed composition alteration by the losses of organic loads due to membrane permeation. During filtration in this mode, the continuous increment of the CFV with parallel increment of TMP was done and the permeate fluxes were recorded. The main target was to investigate the possibility of minimizing solute accumulation in the vicinity of membrane by the sweeping action of increased CFV; which instead may cause elevated deposition on the membrane at increased TMP by the increased drag force towards membrane surface by high permeation stream velocity (because of higher flux at high TMP). This approach was selected due to high requirement of lowered processing time during cell harvesting as the growth of bacteria can introduce higher deviation on the number of harvested cells from that of initial. Also, it offers lowered retention time of the bacteria in the system and thus minimizes the net amount of the EPS release, and corresponding additional interaction, deposition and fouling.

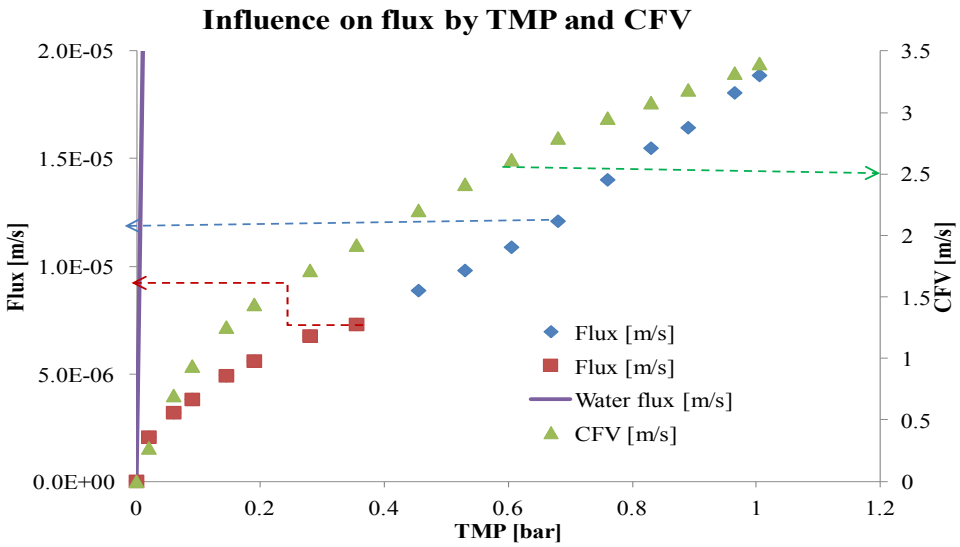

Figure 4: Effect of the applied TMP and CFV on the permeation flux during filtration of milk.

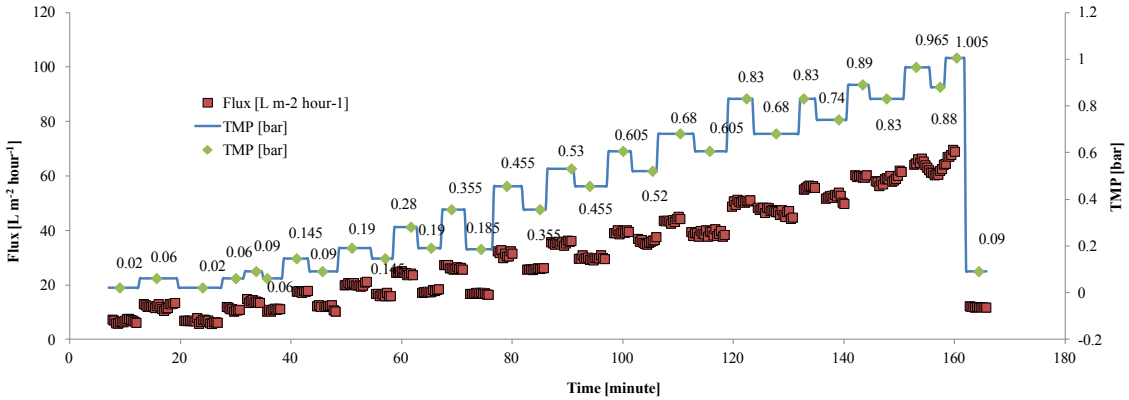

Figure 5: Observed variation of the permeation flux by the stepwise change in the TMP and CFV.

Figure 4 demonstrated the effect of TMP and cross flow velocity on the permeation flux, most preferably of the milk serum from the milk matrix during filtration. From this plot, it evident about the steady increment of flux with elevation of TMP under associated CFV. From the comparison between the pure water flux (and permeability) with that for filtration of milk [**Figure 4**], it's clear about the possible immediate accumulation of organic loads (proteins, fats etc) on the membrane vicinity just after starting of the filtration process. Since, even from the lowest TMP conditions, the observed flux is much lower than that of pure water flux, the permeation can be considered to be carried out above the critical flux region along the entire experimental TMP range and the steady increment of the flux with TMP is the indication of the positive impact of CFV on the permeation flux for this system, and can make the filtration process faster. The Reynolds number in the entire experimental framework was ranging within the $0 < Re < 2300$ and thus it's evident about the laminar nature of the flow in all the experimental conditions. In this aspect, since the positive response of the permeation flux on CFV is high in case of laminar flow of such system, increment of CFV was done to the maximum possible value (under a specific TMP) to minimize the degree of accumulation near membrane. This increased CFV found to work well by enhanced sweeping out of the components from the membrane vicinity, allowing carrying out of filtration experiment faster via high permeation flux.

As shown in **Figure 5**, establishment of stable flux at all the TMP-CFV pairs can be consequence of no additional time depended accumulation of organics in the vicinity of the membrane to impose flux decline during the session of each step. It can be the consequence of high permeations of the concerning species of the milk samples (proteins, sugars and some fats) due to their smaller dimension than that of the average membrane pore size. Considering this fact and as the DF causes the steady dilution of the sample along time from the very beginning [123], this duration of each step was considered enough for the testing purpose. Also, as the

fluxes between two consecutive TFF step under identical TMP remains same while having a TFF step of elevated TMP between them, it indicates the minimized (or no) additional deposit on the membrane during the filtration at elevated TMP (incorporated with elevated CFV as mentioned in previous section); demonstrating the effective sweeping out of the solute from membrane vicinity by the respective CFV's [124]. Here, a similar method reported in [125], where the author's followed a similar approach for the detection of irreversible fouling in the membrane with stepwise increment and decrement of the TMP, is used for analyzing influence of CFV on deposition at elevated TMP by permeation flux. Thus instead of gradual lowering of the flux towards a limiting value with gradual TMP increment, which is the general case of TFF above critical flux (under constant CFV for all the TMP's), observed steady increment of the permeation flux with TMP increment resulted from increased sweeping effect of elevated CFV is found effective for the making the system faster with lowered additional fouling and resistances incorporation.

However, as here observed degree of irreversibility of fluxes (counted by the ratio of the fluxes between two subsequent steps of same applied TMP and CFV) is negligible; so, the net effect can be considered as continuous maintaining of the resistance imposed by boundary layer thickness to a comparatively stable value via opposing the high TMP associated possible accumulation of the organic loads by balanced sweeping action of the associated increased CFV. So, an immediate deposit on the membrane surface just after the starting of the filtration process and further relatively constant accumulation (by concentration polarization, deposition) in the vicinity of the membrane along the experimental TMP (and CFV) range can be considered.

During filtration of milk, a complex biological system having broad particle size distribution (PSD) (offered by proteins, fats, sugars and others constituents), the increment of the CFV in parallel of TMP increment serves by opposing TMP influenced additional accumulation of these particles near the membrane. Also, though the whole milk is not compressible, but there exist compressive nature in the milk constituents [126]; e.g. in case of casein micelle and fat, and can be of significant role in case of nature of the deposition (such as high degree of compaction within the deposit matrix) at elevated TMP and contribute for higher fouling. For instance, the high water content of the casein micelle refers them to have a colloidal microgel like structure which is deformable under the application of stress [127] and can undergo high compaction under stress imposed by filtration flux stream with causing high resistance to permeate flux. The same effect can be considered for the microfiltration of deformable fat globules and all of these are requires to overcome for maintaining a steady flux. Also, the degree of compaction in the concentration polarization and deposit layer are positively influenced by TMP, and consequently requires suppressing by sweeping action of high

tangential flow velocity. In addition, during filtration of milk loaded with bacteria, possible higher amount of deposition and higher degree of compaction in deposition adjacent to the membrane surface (while filtration at increased TMP but without increasing CFV) can cause the bacteria to be entrapped within the regions covered by the deposit, and thus able to increase the concentration polarization of the bacterial cells. Under such case, these partially immobilized bacteria can move forward towards the membrane pore by the actions of: mass growth of bacteria due to multiplication, forwarding of rod shape bacteria towards the membrane pore by the drag imposed by the permeation flow stream; and can possess possible high contributions for the bacterial leakage from retentate. So, considering the fast permeation rate and lowered possibility of the cell loss; aforementioned elevation of CFV in parallel of TMP increment was selected for cell harvesting experiments.

4.2 Performance of concentration by TFF

The main target of this section was to investigate the organic load removal performance during concentration of milk sample in TFF mode. In this context, a fixed amount of milk was concentrated and the removal performance of the organic components under different operating conditions was evaluated. Experiments were done at 3 different TMP with pre-mentioned parallel increment of the CFV and, degree of organic load removal and fouling characteristics were taken in consideration for performance assessment.

4.2.1 Removal of organic load by TFF

For analysis of organic load removal performance by concentration in TFF mode, removal of total protein and fat after volume reduction of the sample by filtration were considered [**Figure 6**]. Since, the sugar molecules are easily permeable due to very small size comparing to molecular weight cut off of the used membrane, imposing easy permeation, it was not considered for the assessment.

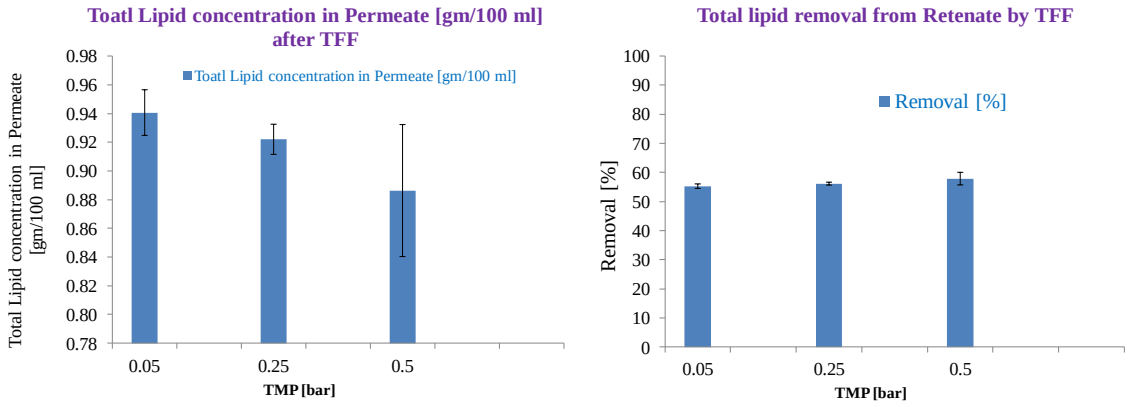

Figure 6: Removal of total lipid during concentration by TFF at different experimental TMP.

The removal of the total protein after the end of concentration experiment was of 30.4%, 33.57% and 43.70 % for the operating TMP of 0.05, 0.25 and 0.50 bar respectively. In parallel, only 50-60% of total fat was found to eliminate from the retentate [**Figure 6**]. However, this residual degree of organic load (which are well known for binding staining molecules and interfering with sample detection while using biosensor [128]) is not satisfactory for the smooth workability of the biosensor [129], [130].

4.2.2 Flow, flux and hydrodynamics

Concentration of the retentate was done to the maximum possible limit offered by the experimental setup and variation of flux along the progress of concentration factor, fouling behavior and recovery of the membrane were analyzed [**Table 2**]. The flow condition was maintained as discussed in **section 4.1.**

Table 2: Membrane permeability and recovery after concentration in TFF mode.

TMP	**CFV**	**Membrane status**	**Permeability**	**Recovery of the permeability**	**Filtration time**	**Concentration factor**
[bar]	[m s^{-1}]		[m s^{-1} bar^{-1}]	[%]	[minute]	[-]
0.05	0.67	before use	0.00210	n.a.	120	3.1
		after water wash	0.00140	67.0%		
		after cleaning	0.00204	97.1%		
0.25	1.65	before use	0.00252	n.a.	38	3.4
		after water wash	0.00214	84.93%		
		after cleaning	0.00248	98.4%		
0.50	2.25	before use	0.00171	n.a.	9.5	3.1
		after water wash	0.00133	77.67%		
		after cleaning	0.00168	97.8%		

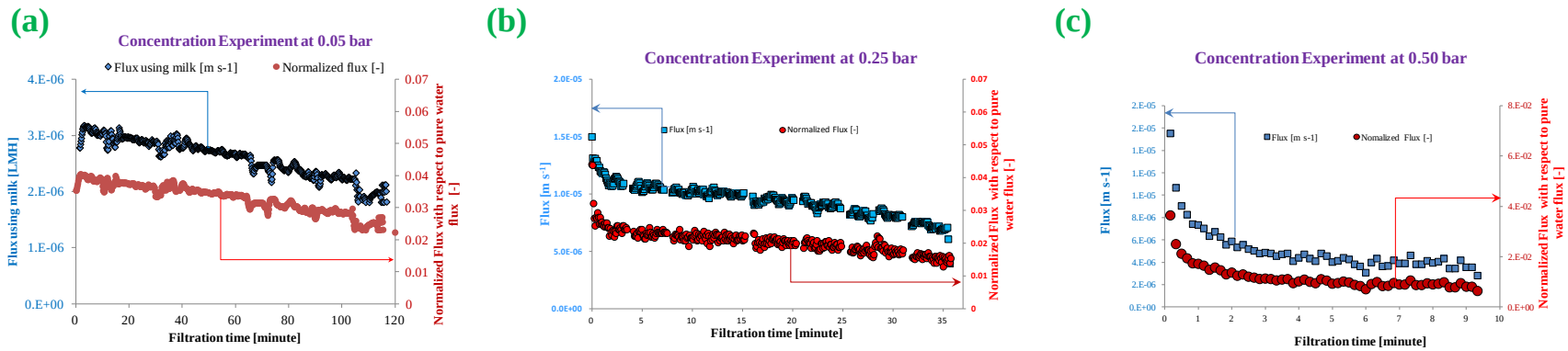

Figure 7: variation of flux and normalized flux (ratio of milk flux to water flux) along the progress of conbcentration during TFF of milk under diffrent TMP: (a) under TMP of 0.05 bar, (b) under TMP of 0.25 bar and (c) under TMP of 0.50 bar.

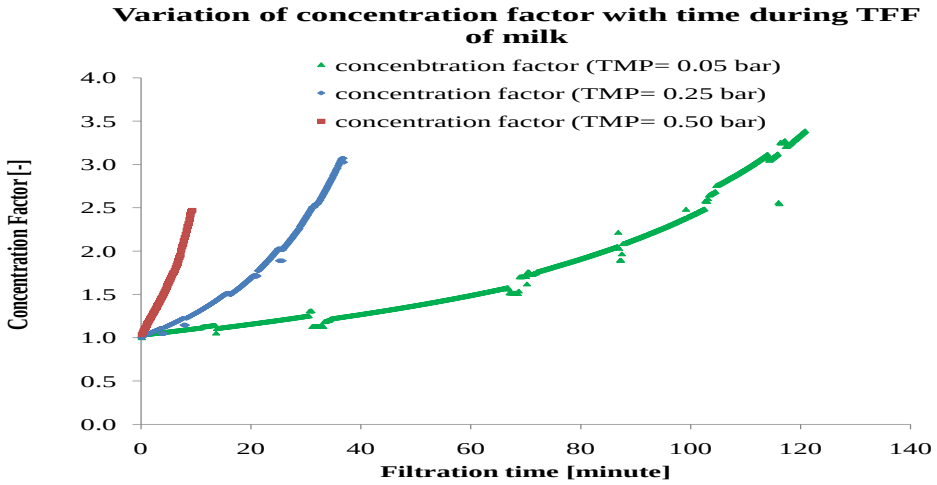

Figure 8: variation of concentration factor along the progress of TFF under different TMP.

The experimental output is demonstrated in the figures [**Figure 7** and **Figure 8**] indicating continuous decline of the membrane flux along the entire session of TFF for all the testing TMP which can be result of continuous deposition of substances on the membrane surface and accumulation in the vicinity of membrane. The lowering rate of flux was found to occur faster in case of filtration at elevated TMP [**Figure 7** and **Figure 8**] indicating quicker fouling of the membrane during concentration at high TMP. The time requirement for obtaining a specific concentration factor was found to lower with the increment of the TMP which was due to increased flux at elevated TMP. Considering the main target of the work, since in case of filtration of bacteria loaded milk, observed increment of the fouling rate can be also further assisted with bacterial cell entrapment in the fouling layer, further possible easy undergoing of cell deformation and permeation from this region through the membrane by the drag force of the permeation stream [131]; based on these observed findings of fouling nature, the TFF experimentation can be considered not efficient for the isolation of cell with maintaining the cell wall integrity. Also, as this effect on cell is higher at elevated flux (result of high TMP) and fouling showed to be faster at elevated TMP, concentration experiments were kept limited within this experimental TMP range. It should be noted that, the fouling nature can be even faster in case of increment of the TMP without increasing the CFV. Based on observed smaller degree of solute removal from the milk under this concentration factor achieved, the concentration in TFF mode can be considered non effective for organic load removal from milk. A possible way of improving the performance can be use of high feed sample volume, but observed steady progress of fouling at elevated TMP and being very low flux at lower TMP, this approach will not only require pre-processing of the milk sample (e.g. dilution prior to concentration and need of additional additives for the possible better flux and elimination of the organic loads by TFF); but also associated with possible higher compensation of both the recoverable membrane permeability, increased effort and time requirement for recovery because of the prolonged fouling, and consequent lowered reusability. Because of the high mass of the organics in the retentate (while concentrating high volume of milk sample), it can result very high degree of concentration polarization and deposits of the organics adjacent to the membrane surface retarding forward flow of the organic matters, development of increased fouling during TMP induced permeations.

In addition to all these, while using bacteria loaded milk, effort for selective enrichment of the bacterial species by this approach can be of significant deficiencies because of gradual increment of the bulk concentration of bacteria in the retentate stream along the progress of TFF governing possible accumulation of the bacteria on the deposited layer on the membrane (resulting bio-fouling), agglomeration of the cells with entrapment of the organics within it. It can result in more lowering of organic load removal from retentate. Also, once the cell get trapped on the deposit, because of higher deforming shear force at high TMP, the EPS can be

released from the bacteria which can further interfere with the other constituents of medium matrix to influence the organic removal and enhance membrane fouling [132]. This higher concentration of the bacteria just near the membrane surface bears high potentiality for them to move through the membrane pore due to their cell wall flexibility, possible alignment of this rod shaped bacteria along the permeation stream at elevated permeation flux, bacterial motility of ***Listeria sp.*** once after entrapment in the pore; and by this manner, bacterial cell may transport from cake layer to the membrane permeate. Since, the diameter of the bacteria (0.45-0.50 µm) is almost similar to that of the average size of the membrane pore diameter (0.45 µm); entrapment of one edge of bacteria at the pore mouth is feasible which can further get permeated through the pore by the action of drag force of permeation stream. Meanwhile, obviously there are some pores in the membrane having higher diameter and in contact of them, the bacteria can be lost by permeation more easily [133] and this effect is more obvious in case of higher cell concentration in the vicinity of the membrane which can result while direct concentration of the cell suspension for prolonged time. All of these cell permeations increases with the increment of the required time for concentration; and as direct concentration of milk shows very fast fouling due to presence of organic loads and causes consequent higher required processing time, the cell loss while direct concentration of contaminated milk (without any purification to remove the organic load prior to concentration) can be higher. Also, higher cell accumulation in the membrane surface requires washing of the membrane to recover the cells [80] which is troublesome and imparts complexity in the process. In addition to high fouling by deposition of organic loads; due to higher possible biofouling, wash ability and reusability of the membrane declines and requires higher need of the more efficient sterilization prior to further use - contributing negatively on the number of possible experiment by per piece of membrane.

All these condition sharply deviated out this filtration mode for using in efficient selective cell purification and concentration. Consequently, the DF step prior to concentration was taken in consideration to overcome these essential limitations of this process. Also, since the DF process works with simultaneous elimination of the solutes and consequent lowering of the bulk concentration of the solute by continuous addition of replacement buffer, the DF offers better experimental performance by different terms including: lowered concentration polarization [134], bringing more efficient desalting (as well as removal of inorganic and tinny molecules) and exchange of the host buffer [135], higher adaptability with the biological samples by means of lowered deposition on the vicinity of membrane [135], lowered fouling and high recoverable usability of membrane [136] with consequent optimized and lowered membrane area requirement [137], small and easy adaptability for laboratory scale experimentation [138], high recovery as a primary step for the targets [139], maintaining the physico-chemical property, integrity and dispersion of the solutes because of addition of buffer

having identical pH and conductivity, as well as well easy accessibility to industrial bio-processing area [140] and so on.

4.3 Performance of DF coupled with a concentration step

4.3.1 Filtration configuration for organic load removal and cell concentration

Membrane filtration is an unique separation system capable of offering simultaneous concentration and fractionation of species present in the retentate stream, and the DF process can be considered as an unique supportive example of this strategy. This aspect can be symbolize by [141] **"Killing two birds with one stone"** – and in our case, here, we employed DF process for the simultaneous removal of organic load from milk and retaining hosting bacterial cells within the system. Among the different type of conventionally used DF process, namely, volume-decreasing DF process [142] for removal of impurity from liquid suspension medium with continuous decline of the net volume by means of buffer addition at a fractional rate of the permeation [143]; constant volume DF process of maintaining the constant retentate volume by addition of buffer at a rate equal to that of permeation [144]; batch DF operated with addition of buffer up to the initial volume of the sample when the feed volumes went down to a specific level [123] and so on; in our experiment, targeting on the minimized bacterial and organic species accumulation in the vicinity of the membrane during experimental session (associated with the potential to cause the high fouling and cell permeations), continuous constant volume DF was selected for the purification of the bacteria from the complex milk matrix. By lowered accumulation in the vicinity of the membrane due to continuous lowering of the bulk concentrations due to permeations and maintaining constant volume of the retentate; this selected approach bears the potentiality of offering less bacteria to be permeated by means of motility, mass growth rate, further forwarding along the permeate and within pores (accessed by convection resulted drag force, motility once after entrapment in the pore and/or by the combined action of multiple of these). In addition, increment of CFV was done in parallel of TMP increment for more lowering of the concentration polarization. By cell purification through this way, we believe to minimize the higher concentration of the bacteria near the membrane during the DF process and consequent lowered entrapment in the cake layer and/or forwarding with the convection stream towards the membrane pore to maintain minimized bacterial leakage. Once the cell purification is done, further mass volumetric concentration of the recovered bacteria was done by the concentration in TFF mode as it was found to be a fast process for elimination of the solvent from this complex medium even in presence of other constituents of the milk matrix [**Section 4.2.1** and **4.2.2**]. It also bears the capacity for some additional residual organic load removal. But, when the TFF is applied alone, it failed to effectively remove the organic load because of high concentration of organic load been present

in retentate. Here in this work, at first, assessment of organic removal and hydrodynamic performance of aforementioned DF process was done for non-contaminated milk. Afterwards same experiments were replicated with the same milk, but after well controlled enrichment with the analyzing model bacteria (***Listeria innocua***) and the effect of cell loading on the filtration performance were studied. Quantification of species accumulated on the membrane surface was done by the mass balance of those respective organic compounds after completion of filtration experiment. These measurements were cross checked with the value derived from model equations [**section 2.1**] describing the time dependent permeations of the individual species. Analysis of protein, fat and sugars were done as distinct quantity as they are present in the major proportion. The removal performance of the other residuals (and trace contributor) components were evaluated indirectly as a whole by the overall buffer capacity estimations, as the magnitude of this parameter is a function of compositional factors including small constituents (inorganic phosphate, citrate, organic acids), milk proteins (caseins and whey proteins) and others [115], [145]; and this parameter possesses the capability to indicate of the respective compositional change.

4.3.2 Removal of organic load by constant volume DF

The DF of contaminated [**Figure 9**] and non-contaminated milk [**Figure 10**] showed the steady lowering of the total protein concentration in the retentate along the progress of DF. The aim was focused to analyze the influence of TMP on the trend, fastness and net amount of protein removal during DF where all the experimental TMP was associated with the respective CFV, applied by the action of the feed pump as mentioned earlier. Also, whether the DF performance is pressure depended or not, how the permeations of the proteins are influencing by the TMP, required DF time and diavolumes for a specific amount of protein removal, influence of cells (when present in the milk matrix) on the rejection properties of total organics and fouling behaviors to get idea of any possible interaction between cells with the other organic constituents of milk and the membrane were studied.

From these experimental plots [**Figure 9** - **Figure 10**], it's evident about the high efficiency of this DF process for fast (high removal within 6 diavolumes, operation at flux higher than that for the concentration in TFF mode), efficient and effective lowering of the total proteins from the medium and effective replacement of the solvent by the replacement buffer. From the nature of the concentration profile in the retentate side, its evident about the time (and diavolumes) depended organic removal from the retentate. Both the transmission and protein removal rate is very fast at the beginning which is found to decline along time. Also, the similar natures of transmission and removal rate from the retentate were found for other organics (discussed later). A possible reason can be the gradual lowering of the respective bulk

concentration in the retentate solution, and consequent lowering of the solute concentration in the vicinity of the membrane surface. This in turn lowers the permeation rate of respective species via lowered exposure and influence of that species to the permeation streams. In parallel, as the transmission is positively correlated with the concentration of the species [**section 2.1**] in the retentate, the transmission followed the similar nature.

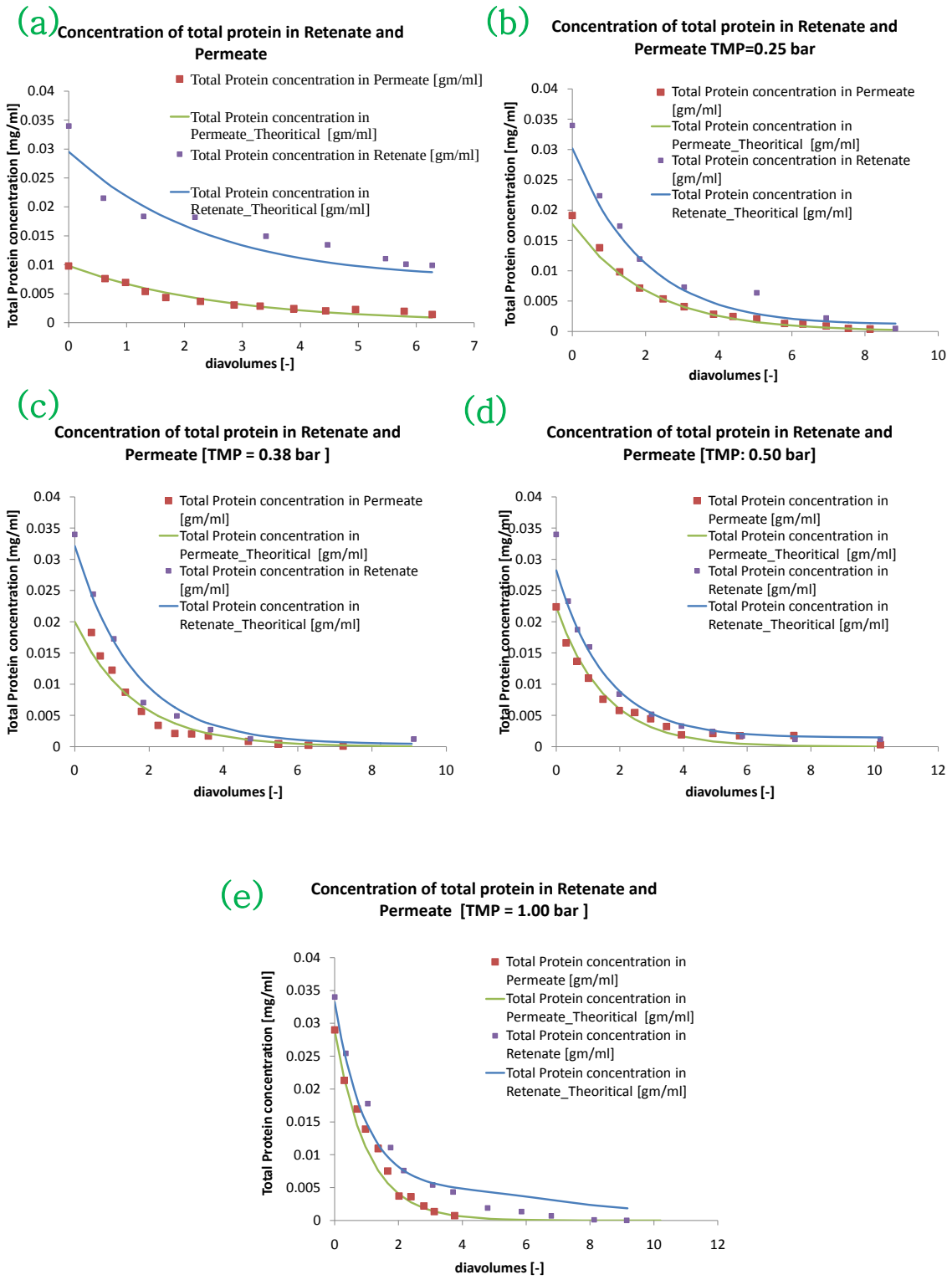

Figure 9: Total Protein concentration variation in the feed and permeate side during DF of contaminated milk.

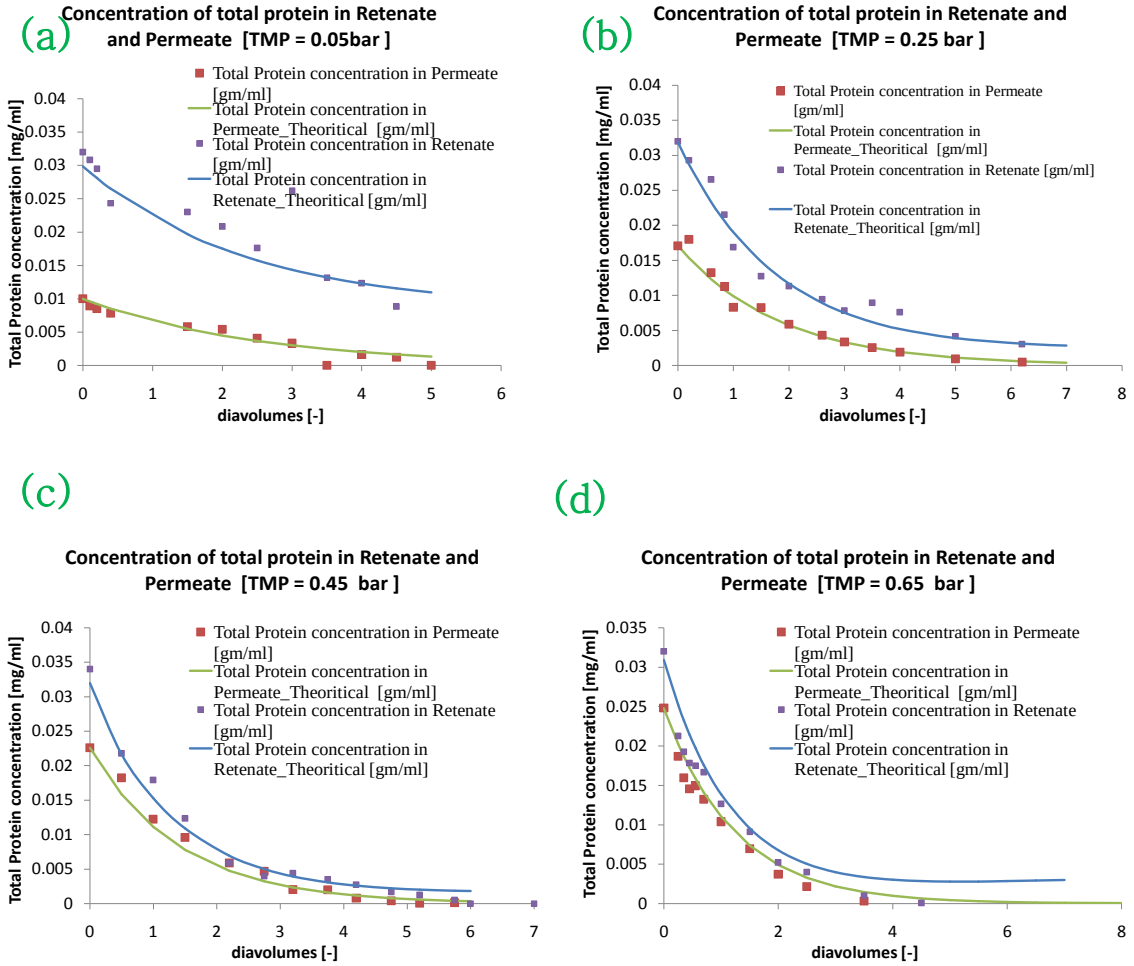

Figure 10: Total Protein concentration variation in the feed and permeate side during DF of non-contaminated milk.

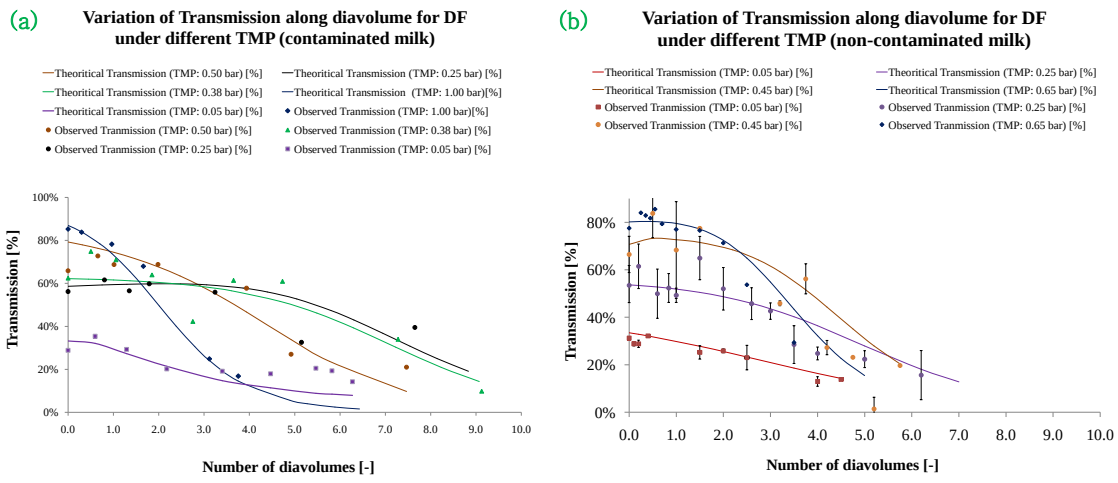

Figure 11: Variation of Transmission along the progress of DF: (a) non-contaminated milk and (b) contaminated milk.

Both the contaminated and non-contaminated milk showed good fitting with the model developed in the **section 2.1.** In addition, the organic charge removal and buffer replacement characteristics of DF process were found identical for both contaminated and non-contaminated milk. This replacement of the solvent by the buffer, as demonstrated in the later sections, is representative of the identical elimination nature of the dissolved components from the sample. The obtained values of model parameters after treating of experimental protein removal data's with the developed model are tabulated in **Table 3 - Table 4**.

Table 3: Summerized result and derived model parameters values of DF with contaminated milk.

TMP [bar]	**CFV** [m/s]	**Protein in membrane** [iii] [gm]	$C^{o}_{(permeate)}$ [i] [gm/ml]	m [ii] [-]	**Reynolds number [-]**	**Flow Type**
0.05	0.3473	0.05473	0.0098	0.38	325.22	Laminar
0.25	1.1692	0.07283	0.0191	0.54	1094.80	Laminar
0.36	1.4008	0.11811	0.0201	0.64	1311.63	Laminar
0.50	1.6293	0.00888	0.0224	0.68	1525.65	Laminar
1.00	2.2895	0.16013	0.0290	0.88	2143.79	Laminar

Table 4: Summerized results and derived model parameters values of DF with non-contaminated milk.

TMP [bar]	**CFV** [m/s]	**Protein in membrane** [iii] [gm]	$C^{o}_{(permeate)}$ [i] [gm/ml]	m [ii] [-]	**Reynolds number [-]**	**Flow Type**
0.05	0.3473	0.04672	0.010	0.40	325.22	Laminar
0.25	1.1692	0.06482	0.018	0.55	1094.80	Laminar
0.50	1.6293	0.09246	0.023	0.70	1463.26	Laminar
0.65	1.8106	0.12434	0.026	0.83	1695.34	Laminar

[i] : the 'm' values were determined by the fitting of the model equations described in theory section.

[ii] : the '$C^{o}_{(permeate)}$ values were determined by the fitting of the model equations described in theory section.

[iii]: the values was determined by the commutative mass balance.

From these, it's evident about the strong dependence of the TMP (associated with respective CFV) on the amount of total protein removal from the feed side at any diavolumes [**Figure 12 (a)**]. Also, the organic load removal was found faster while performing DF at higher TMP a quantified view of which can be observed from the parameter **"m"** values. Higher **"m"** values are representative for the faster permeation of the concentrated analyzing species from the retentate side [**section 2.1**]. This higher removal rate of the organic constituents from the

milk sample at elevated TMP can be considered to be the consequence of observed increased permeation flux of the milk serum through the membrane at elevated TMP (discussed in later sections), and corresponding positive influence of increased flux on the suspended organic compounds permeation due to increased induced drag force by permeate stream. Also, as predicted from the plots and summarized in **Table 4** and **Table 5**, concentration of protein in the permeate obtained just after commencing of the DF process was found to increases with the increment of the TMP, indicating the DF process is working in the pressure depended mode and the efforts for increment of TMP will serve for faster permeation of the organic loads from the feed side. However, within the used range of TMP and CFV in this experiment, this effect was not linear and the influence was found to gradually flatten with the increment of the TMP values [**Figure 12 (b)**]. Also, based on the modeling equations and the simulations done by using the Microsoft excels [101] and Matlab [100] with the experimental data, and from **Table 4 - Table 5,** it's reasonable enough to consider non significant dependency of the bacterial load on the total protein removal performance during DF process (since, both the **"m"** and $C^{o}_{(permeate)}$ values are identical for the contaminated and non contaminated milk under identical operational conditions). Consequently, as there was no observed influence of the cell on the total protein removal, the permeation nature was considered identical for the lipid and sugar too; and the lipid and sugar removal data obtained for the experimentation with non-contaminated milk was considered equivalent with those of the contaminated milk.

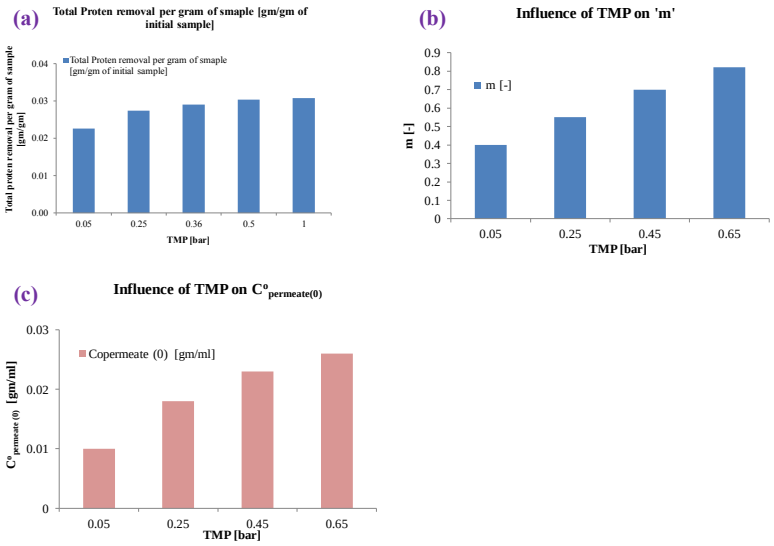

Figure 12: influence of TMP on: (a) the total protein removal at a certain diavolumes (diavolumes = 4.00), (b) influence on 'm' and (c) influence on $\boldsymbol{C^{o}_{permeate}}$.

Also analysis transmission nature, fouling at different stages in the DF process and membrane recovery after washing showed identical variation along diavolumes for both the contaminated and non contaminated milk suggesting non existence of efficient and significant interaction between the cell and milk constituents to influence the rejection properties. It can be considered as an evidence of not enough secretions of extracellular proteins and EPS polysaccharide which can be released by the bacteria to generally cause interaction with these constituents and membrane [132], [146]–[149], [150], [151] during such filtration process. Consequently, the physicochemical interactions between the suspended organic components in the serum can be considered not to be significantly altered between the contaminated and non contaminated milk. Also, the interaction between the cell and milk constituents can be neglected in the same way [152], [153]. The reason for non significant EPS release can be the lower retention time of the cell in the filtration system than that required by the cells for EPS proteins and polysaccharide secretions. In addition, the required time for the effective biofilms formation, a multi cellular layer of adherent bacteria surrounded by a matrix of extracellular polysaccharide [154]**,** is much higher [155] than the duration of this experiment with this bacteria**.** During these short period, processing of the ***Listeria innocua*** rich milk was done before significant secretion of EPS by the cells [131], for which negligible deviations (with respect to that of the non contaminated milk) of the membrane permeability recovery after water wash was also observed. As there was no significant contribution on the filtration performance after cell incorporation, the consideration of same removal characteristics in contaminated and non contaminated milk filtration was considerable for the lipid and sugar. The condition is especially demanded for the determination of the true concentration of sugar as in case of contaminated milk, respective experimental measurements of sugars can be significantly altered during the experimental session by the activity of the bacteria [156]–[160]. Also, using bacteria rich sample can irreversibly contaminate and deteriorate the HPLC columns generally used for the sugar assay, and can also contribute the error in estimation by the consumption of sugar during prolonged analytical session of sugar assay. Even the cells are killed prior the estimations, the dead cell can also contribute for significant inconsistency.

However, some positive deviations (higher than the theoretical estimation) in the observed net transmission values at higher diavolumes can be due to rapid flux increment while filtrations at elevated TMP, which may cause the comparative higher protein concentration in permeate and consequent higher transmission value. Here, it should be noted that, the flux increment is faster in case of high TMP operation [**Figure 19** to **Figure 20**]. Observed diavolumes (and hence time) depended rejection coefficient can be because of continuous lowering of the extent concentration polarization, varying accumulation of the constituents in

the vicinity of the membrane altogether with the impact of increasing flux as discussed earlier, and accordingly, the transmission lowered along the progress of DF. The overall transmission value at any diavolumes was found higher for the DF at elevated TMP which can be because of the higher drag force induced permeations by observed elevated flux at high TMP. The observed positive dependence of the rate defining parameter "m" on TMP [**Figure 12 (c)**] can be a supportive strand in this aspect. From **Table 3** and **Table 4**, for both the contaminated and non-contaminated milk, ultimate protein accumulation on membrane was found to increase with increment of TMP, which may be possibly due to higher penetration of the proteins in the membrane matrix because of observed faster permeation rates of proteins at elevated TMP, and consequent possible entrapment of some proteins in very narrow pores as well as possible deterioration of the conformation (denaturation and gelation) [161]–[164], [127] of the molecule by high induced shear force resulted by the high flux and thus gaining more adsorption capacity than that for the initial.

In case of total lipid, the cumulative removal and rate at which the lipid is removed from the retentate was found to increase with the increment of the TMP [**Figure 14**]. But the removal rate and extent of removal was found lower than that for protein. Total fat removal was found maximum at 0.65 bar of TMP when about 60 % of the total lipid initially present was found to permeate which is considerably small comparing to the more than 92 % of protein removal under all the experimental TMP. Also, the "**m**" and $\mathbf{C^{o}_{(lipid)}}$ values were found lower in case of lipid permeations but it showed the similar dependence on TMP as was in case of protein [**Figure 13**]. This lower removal of fat may be due to presence of the significant fraction fat in the globular form having diameter higher than that of the average pore size of the membrane [165]. Even though, there may be some fat globule permeated under increased drag force resulted (imposed by permeation stream) possible deformation [166]–[169] once after getting in contact with free membrane pore. Also, the rated membrane pore size (0.45 µm) is the mean of the pore size distribution estimated by conventional methods and there are always some pores having diameter higher than this reported average [170], [171], [171], [172]. Consequently, there is chances for enhanced lipid removal from the milk by more increasing the TMP but, the higher observed leakage of the cells through the membrane after this TMP (as discussed in the later section) limited such action to take. Moreover, in our experiment, the flow is laminar and so, there is no turbulence enhanced fragmentation of the large fat globules to be cut into smaller fragments which can assist lipid permeations. But, the residual fat in retentate is low enough to be separated by additional centrifugation of the concentrated sample. In this context, an alternative option can be multi staged membrane pore size based fractionation methods (step wise permeation of the analyzing milk sample in a series of membrane filtration

with respective size based recovery of species in individual filtration steps), but is troublesome as well as critical during implementation for the pathogenic species rich samples because of exposure. Also, the losses of species in each step can add the higher deviation and dominant error for the final quantification of initial number of bacteria which was present in the sample: the main target goal. Consequently, the developed and demonstrated approach of fat removal can be considered effective.

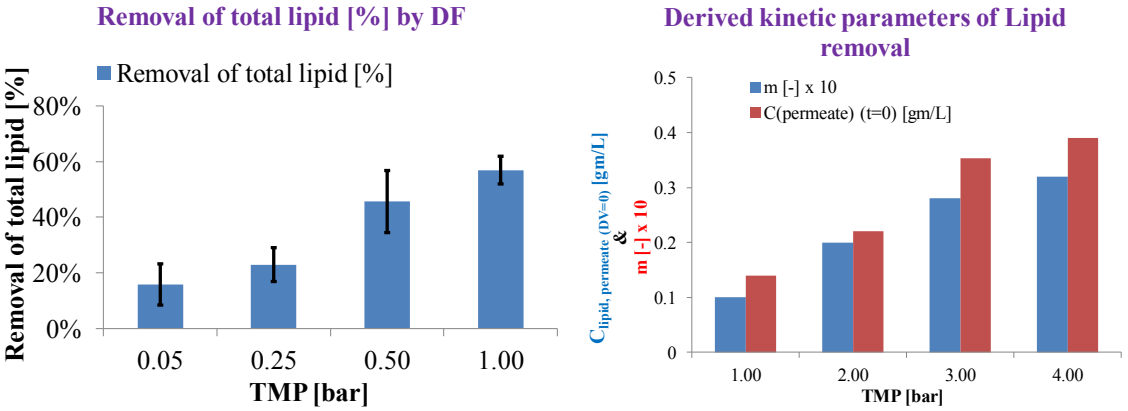

Figure 13: Dependence of "m" and C^{o}_{lipid} on the TMP in removal of total lipid by DF.

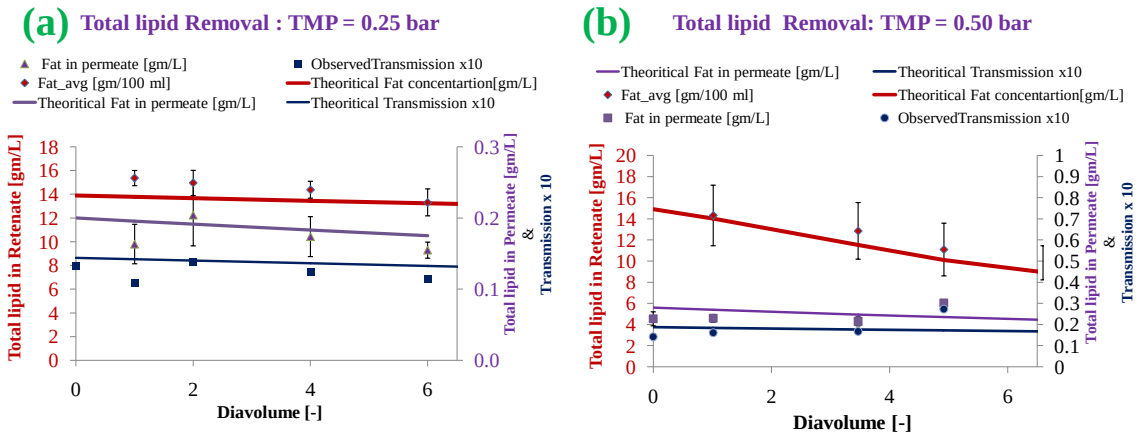

Figure 14: Removal of total lipid from the retentate side for during DF of non-contaminated milk under different TMP.

Removal of sugar [**Table 5**] was fastest among the observed organic load removal rates (**"m"** values ranging from 0.80 to 0.98) and was most effectively removed. It may be because of the very low size of the sugar molecules than the MWCO of the used membrane. Also, some affinity of the sugar molecule towards the other constituents of used milk (during presence in the serum), and homogeneous presence of all the residual sugar in the serum can assist this easy and fast permeation. Also, as these molecules (sugars) have lower affinity for the used membrane and broadness of membrane pore size does not affect the permeation of sugars due to their very low molecular weight [173]-[174], their permeation is more preferential than retention and accumulation on the membrane. However, the removal rate gradually lowers along the progress of DF which is due to the gradual lowering of the sugar (and also for other constituents analyzed) concentration in the vicinity of membrane along the progress of DF as discussed earlier.

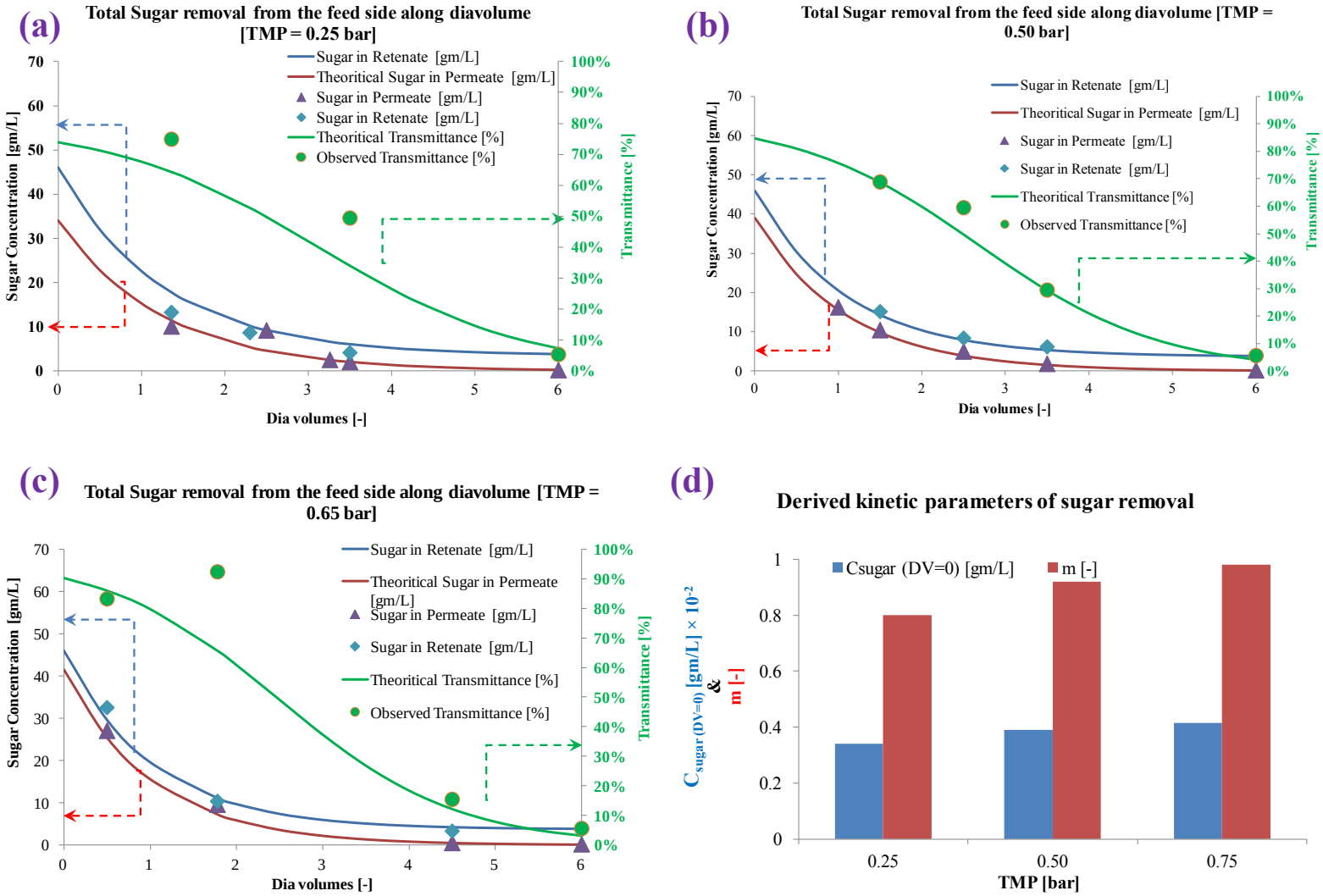

Figure 15: Removal of sugar from the Retentate side at different TMP.

Table 5: Removal of total sugars from the retentate by DF of non-contaminated milk.

TMP [bar]	$C^{0\ i}_{(sugar)(permeate)}$ [gm/L]	m [ii] [-]	Removal [%]	Sugar in membrane *(in deposited layer)* [iii] [gm]
0.25	34.00	0.80	91.80%	0.0047
0.50	35.00	0.92	92.20%	0.0074
0.65	36.00	0.98	92.24%	0.0076

[i] : the 'm' values were determined by the fitting of the model equations described in theory section.

[ii] : the '$C^{0\ i}_{(sugar)(permeate)}$' values were determined by the fitting of the model equations described in theory section.

[iii]: the values was determined by the commutative mass balance.

Regarding to the accumulation of the organic substances on the membrane, a sharp lowering of the accumulated mass of protein and sugar in the membrane surface along the progress of DF was observed. The accumulation of fatty component on the membrane was found almost stable along the progress of DF (discussed and showed in **section 4.6**). The accumulation of a species on the membrane was calculated by the respective mass balance. The difference between protein losses from the retentate and accumulation in permeate, which is corresponding to the protein accumulation in the membrane, was found to increase with increment of the applied TMP. The elevated protein accumulation on the membrane at high TMP [**Table 3** and **Table 4**] can be because of the propagation of the accumulated proteins within the membrane matrix (pores) and compaction of the adsorbed layer due to observed high permeation rate (discussed in **section 4.6**) related convective drag force. But, in all the cases, the protein accumulation on the membrane surface was trace comparing to that of the net initial amount present in the milk sample (concentration = 34 grams of protein /Litre of milk sample). However, protein accumulation profile of **Figure 21** showed the steady lowering of accumulated protein in the membrane along time which can be because of time and bulk concentration dependence of adsorption rate of the species on the membrane. At the beginning of the filtration, because of the high concentration of the protein in the retentate side, the protein adsorption is faster which lowers sharply because of the steady lowering of bulk protein concentration at a rate depending on the permeation rate of the protein and the hydrodynamics involved. This resulted lowering of bulk concentration may cause continuous desorption of proteins initially adsorbed on the membrane surface until the end of the filtration process. Though the adsorption of the proteins on used slightly negatively charged regenerated cellulose membrane is pH depended (as the membrane bears higher hydrophilicity and zeta potential at lowered pH), but, since, the pH was maintained to the initial level by means of buffer (of same pH as of milk serum (6.7±0.3)), there was no pH influenced variation on the protein adsorption during the entire experimental session. Also, considering the protein adsorption is faster and of high extent at lowered pH [175], this stable operating pH may be a contributor for observed low

protein adsorption. In addition, as in all the cases, the DF time was lower than that required for adsorption equilibrium establishment (generally 4-10 hours [176], [177]) for different proteins present [175], the adsorbed protein fraction can be lower. All the experiments are done under same bulk area of membrane and reported comparative results here are also based on bulk membrane area. The true area revealed from the BET adsorption can be used for the further quantification of relative blocking of the true surface area. In this case, as the proteins are much bigger than the N_2 molecules, consideration of a fractional lowering of the area is required to get the more realistic pattern [175]. More details on the variation of the accumulated protein in the membrane along DF progress are discussed in later section.

The observed very low sugar accumulation in deposit on the membrane [**Table 5**] (it is reasonable to consider the no sugar adsorption on the membrane due to from the nature of the polymer used in the membrane) can be the consequence of continuous washing up of the sugar from the deposit by the permeate streams. Also, because of the lowering of the sugar concentration in the bulk, continuous back diffusion can also contribute for the lowered sugar adsorption in the membrane. The comparatively higher TMP independence of the ultimate amount of adsorbed sugar in the membrane (comparing to that of proteins and fats) can be because of very fast permeations (**"m"** values ranging from 0.80 to 0.98) which allows lower residual sugars left at the course of DF.

The accumulation of the lipids in the membrane after DF experiments was higher which can be because of the physical deposition of larger fat globules on the membrane assisted by drag force of permeation stream. Also, as lipids are most retaining species in our experiments with very low permeation rates ("m" values found to range from 0.03 to 0.07 along the experimental conditions); their contribution on the deposition can be comparatively higher. But this deposition is mostly reversible in nature as higher degree of recovery in hydraulic membrane permeability was achieved by water wash after use of membrane. Also, since the lowering of the fat concentration in the bulk (at retentate side) in all the DF experiment was minimum, the back transport to the bulk by concentration gradient is also very low (further supported by higher size of the fat globules) and may contribute for this accumulation at higher proportion on the membrane.

4.4 Variation of buffering capacity

The comparative plot of alteration of buffer capacity of the milk after and prior to DF [**Figure 16**] can be used to illustrate the efficiency of used DF process for overall removal of the organic and inorganic loads from the milk matrix. The buffer capacity showed by the milk after

DF was almost similar with that of the pure replacement buffer used and significantly lower than that of the milk before DF [**Figure 16**]. As milk possesses its own buffer capacity based on the minerals, vitamins, salts, acid-base and other compounds; effective removal of them by replacement buffer will alter the original buffer capacity of the milk. These constituents are the main concerns which interact with the sensory properties of the bacterial sensor by their physico-chemical characteristics, harness the sensitivity and performance of the bio-sensor, and thus require confirming about their removal prior to use of bio-sensor. A summarized view of the buffering contributor is showed in [**Table 6**]. After the removal of the proteins been confirmed by the protein assay, this buffer capacity alteration can be used to identify the bulk removal of the residual phosphate, carbonate, citrate, lactate and other salts. The milk, when is richer in fat, lactose, protein (especially caseins) and minerals such as calcium, magnesium and inorganic phosphate; is reported to be of higher buffer capacity because of the inherency of acidification properties [178]. The effect is also well established for different other biological fluids [179]. During buffer capacity estimations, the molecular changes induced by the addition of the acid in milk includes acidification, protonation of acid groups including de-mineralization of casein and decrease of solubility, hydration and zeta potential influence of caseins, interactions with all other constituents are most described in the deferent research results [180]–[189], and is representative of the components which are present in the matrix as well as their respective proportions. The fast decline of buffer capacity can be considered for very fast elimination of inorganic phosphate [190], imparting considerable buffering capacity on milk, at the earlier stages of the DF because of their very low molecular weight. Though the addition of the acidic components causes the casein aggregation and precipitation at the lowered pH (4.6) assisted by other respective components present; but during this acidification of the retentate samples, there was no observed precipitation in the diafiltered samples which can be because of the effective respective removals during DF. It is representative of minerals elimination during the process which was able to assist this precipitation of the residuals.

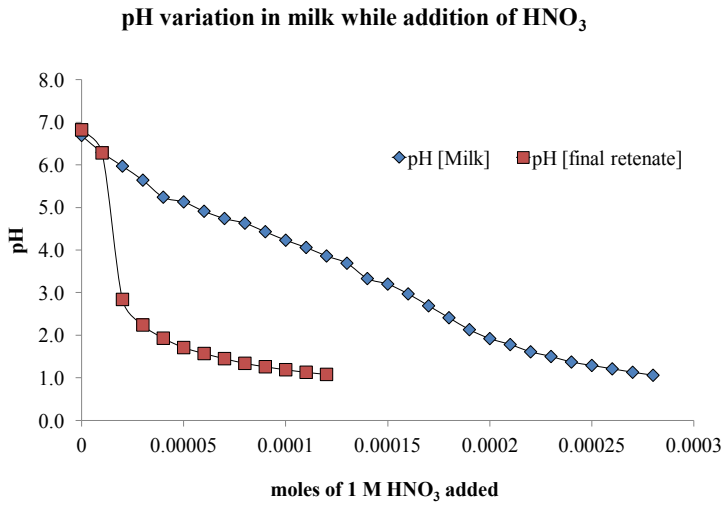

Figure 16: Buffer capacity of the unused milk and the final retentate [final retentate after DF at 0.25 bar, the entire remaining DF showed similar conditions].

Table 6: Contribution of milk constituents to its overall buffering capacity (reported by different Authors).

[191]		[118]		[192]	
Proteins	35.00%	Caseins	36.00%	Caseins	32.70%
Salts (phosphate, carbonate, citrate, lactate)	65.00%	Whey proteins	5.40%	Rennet whey	46.40%
		Salts	58.60%	Colloidal calcium phosphate	20.90%

4.5 Change of viscosity during DF

There was sharp variation of the retentate viscosity along the progress of DF [**Figure 17**]. The viscosity of water (0.91 ± 0.07 Centipoise) is much smaller than that for the used semi skimmed milk (1.90 ± 0.2 Centipoise) for which continuous replacement of the milk constituents and serum with exchange buffer leads the retentate sample towards lower viscosity state during DF.

The viscosity is correlated with the Reynolds number and permeation flux, the resulted lowered viscosity causes increment of Reynolds number [193] and can contribute for increment of the permeation flux [194]; thus bears high significance on the process performance. Viscosity of the milk is contributed by the composition and interaction between constituents of milk

matrix, but because of the continuous elimination of these milk constituents from the retentate side, the viscosity of the circulating sample in the retentate loop gradually declines. This decline of viscosity of the retentate sample contributes for the increment of the permeation flux and bears the capability of concentration polarization lowering during filtration [195]–[197]. Also, it can significantly contribute on the permeation natures of the species and contributes for lower possibilities of colloids and other suspended particles to foul high flux MF membranes. In addition, it can affect the retention of desirable and undesirable solutes [198]. So, this alteration of the viscosity of the processing retentate fluid during DF with MF membrane can be of significant impact on the process performance characteristic.

However, the observed variation of the viscosity with the progress of DF (in term of diavolumes) [**Figure 17**] is a sharp demonstration of efficient elimination of the target components from the milk matrix since the viscosity of milk is mainly constituted by the fat, total proteins and other compounds been present there altogether with their physico chemical interactions [199]. Elimination of these constituents shifts the system to a lower viscosity state. In addition, while processing, viscosity of milk also influences the shear stress involved because of the increment of CFV [200], [201]. With the increment of the shear rate, the deformable (e.g. fat globules) constituents been present there may deform to get well aligned along the stress, and also denaturation of proteins can occur. In addition, the viscosity is also influenced by the bacterial activity on the milk (in presence). The adulteration of the milk associated with the bacterial interactions may result in the adsorption of oxygen and nitrogen, and cause a decline in the dissolved carbon dioxide level. Solubility and equilibrium of the salts can also get changed in some extent. Fat globule can agglomerate in some amount and can get phase separated, leaving some cream layer at the surface based on the extent of adulteration. The denaturation of proteins and the influence on the fat is rather slow process making the impact to be significant with the passing of time. But, no observed significant variation between the initial viscosities among samples of used milk confirms absence of such bacterial interference to occur. To maintain this, in all the experimentations, milk were used within the shortest possible times after controlled incubation of bacteria, consequently had not expected and observed any significant change in the viscosity by bacterial enrichment of the milk. Also, continual depletion of the dissolved gases, which is function of the living cell and their growth rates, was rather reasonable to neglect because of the slower nature of these influencing process comparing to the fast sessions of experiments.

In case of the full milk, the major contributors of the viscosity are fats and viscosity value is mainly governed by total fat concentration. Consequently it's always higher for the whole bovine milk (3.85 to 4.85 % of fat) than that for the semi skimmed and skimmed milk.

The viscosity of the skimmed milk is almost similar to that of the skim after the elimination of the compressible fat globules. In our case, the semi skimmed milk bears the viscosity governed by the fat contents, the proteins and other inorganic constituents altogether with their configurations and interactions. The contribution on viscosity by the other major components, sugars, existing at the concentration of 4.6 %, is almost zero [202]. So, the net change in the viscosity that we observe here is by the alteration of the fat and protein constituents and to some extent, by the modified interaction due to compositional change of vitamins, minerals and other interfering constituents based on their own retention time in the retentate. The trend of the viscosity variation with progress of DF is well consisted with some other experimental evidence reported by [202], and the observed nature of the viscosity decline with the dilution and elimination of the constituents from the milk is unswerving with that for the three different milk studied by the same authors. The observed relative exponential dependency of viscosity on diavolumes [**Figure 17**] can be because of the exponential elimination of the proteins and fats from the retentate along the progress of diavolumes. However, after the end of DF, the effective removal of the species was associated with final viscosity of the retentate having magnitude very close to the pure buffer [**Figure 18**].

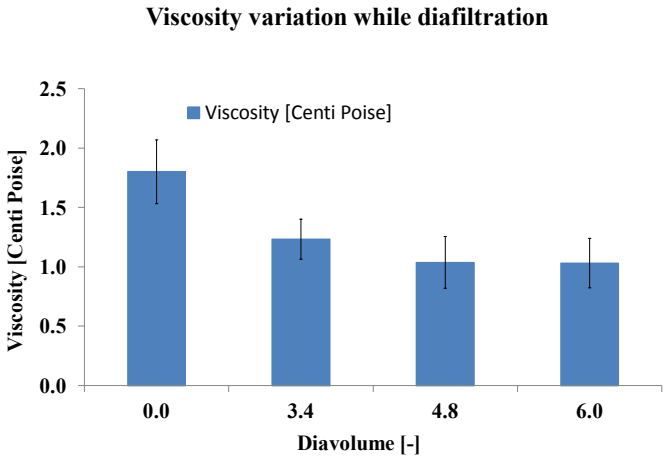

Figure 17: Viscosity at different stages during the progress of DF process.

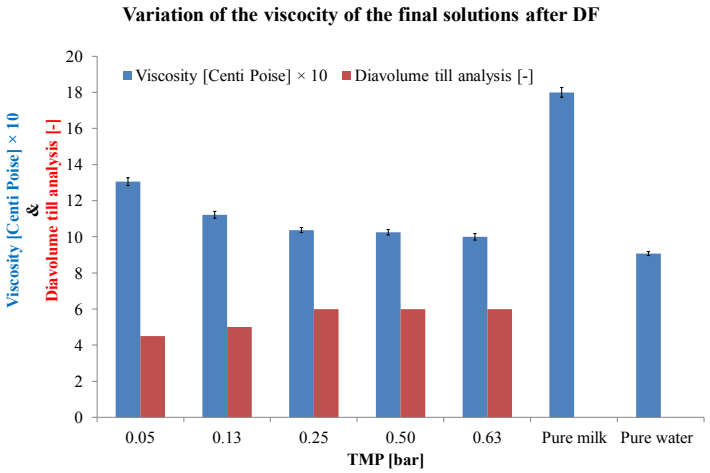

Figure 18: final viscosity after DF at different applied TMP.

4.6 Flow, flux and hydrodynamics

The variation of permeation flux along the progress DF and TFF of milk under different TMP is showed in **Figure 19** - **Figure 20.** In all the experimental cases, the flow was laminar (Reynolds number < 2300), TMP was almost constant and all other respective experimental conditions were kept identical for both the contaminated and non contaminated milk.

Observed increased permeation flux while the DF at elevated TMP was the consequence of the higher driving force for solvent permeation and lowered deposition related permeation resistance at increased CFV. The immediate normalized flux (ratio of flux obtained with milk to that for distilled water) obtained just after the commencing of DF was identical with that for respective TMP-CFV pairs as obtained in **section 4.1.** But, afterwards the flux was found to gradually increase with time, rate of which was found higher for the DF at elevated TMP. For DF at high TMP, the flux increased sharply along progress of DF until achieving a plateau followed by lowering of the permeation flux ([**Figure 20**] (for non contaminated milk) and [**Figure 19**] (for contaminated milk)**).** During the TFF stage, the gradual lowering of the permeation was observed extent of which was higher at high TMP operation.

Gradual lowering of retentate viscosities with the increment of the diavolumes [**Figure 21**] can be vital reason for this continuous increment of the flux along the progress of diavolumes as the flux is inversely related with the viscosity. This lowering of viscosity is consequence of continuous elimination of the organic loads from the milk matrix along the progress of DF [203]. In addition, as shown in the **Figure 21 (c)**, the sharp lowering of accumulated protein in the membrane can also contribute for the continuous increment of the permeation flux. The possible reason for this lowering of accumulated mass of proteins on the membrane may be

fragmentation of the deposited casein micelles (accumulated on the membrane surface at the earlier stage of DF) caused by the elimination of the calcium ion (Ca^{2+}) from their core and consequent conversion to very small and easily permeable sub micelle, and thus lowering the filtration resistance contributed by accumulated protein on the membrane. As this effect is not immediate and proceeds along time (and diavolumes) by continuous elimination of the core calcium's from the micelle by developed osmotic pressure [204] due to the elimination of calcium from the bulk retentate stream, the effect of protein dissociation on filtration flux is time depended. In addition, in our case, used MF has a mean pore size of 0.45 µm which may undergo these mechanisms very fast due to exerted high drag force of permeation stream on adsorbed and deposited casein. This mechanisms can be further supported by the reported experiment of [205] and [206]. The accumulated protein in the membrane is represented with respective flux at different diavolumes in **Figure 21**. The more details on the mechanism of casein micelles dissociation (mean diameter of 50-300 nm) to sub-micelles (having mean diameter of 2-3 nm) can be found elsewhere [207], [208]**.** It can be the governing process for the lowered casein dissociation as there was no significant temperature change during the experiment (25-29 ^{o}C) for causing such fragmentation. This dissociation of the adsorbed micelle and further permeation in the form of sub-micelle may also contribute for the observed lowered amount protein accumulation on the membrane after DF. Though, together with the casein proteins, whey proteins can also contributes for the gel-like deposit adjacent to the membrane surface, but the very small sizes of whey protein (comparing to the average membrane pore size) allows their easy permeations from there along times. The observed lowering of the permeation flux after reaching the maximum [**Figure 14**] may be contributed by the irreversible fouling by the adsorption of accumulated fat, and proteins on the membrane [**Figure 21**]. Also, the increased flux at such stage (of high diavolumes) may assist the penetration of the deformable fat globules [209], [210] on the membrane pores by the higher drag force resulted from high permeation rate, block the pores and contribute for this observed lowering of flux. The observed trend was similar for the DF with contaminated and non contaminated milk. However, the deposition of the fat on the membrane undergone minimum lowering along the progress of DF, thus offered negligible contribution for the increment of flux. Accumulation of sugar on the membrane was found to be very small which can be because of very small size of the molecule and respective easy washing out from the membrane surface by the permeation streams.

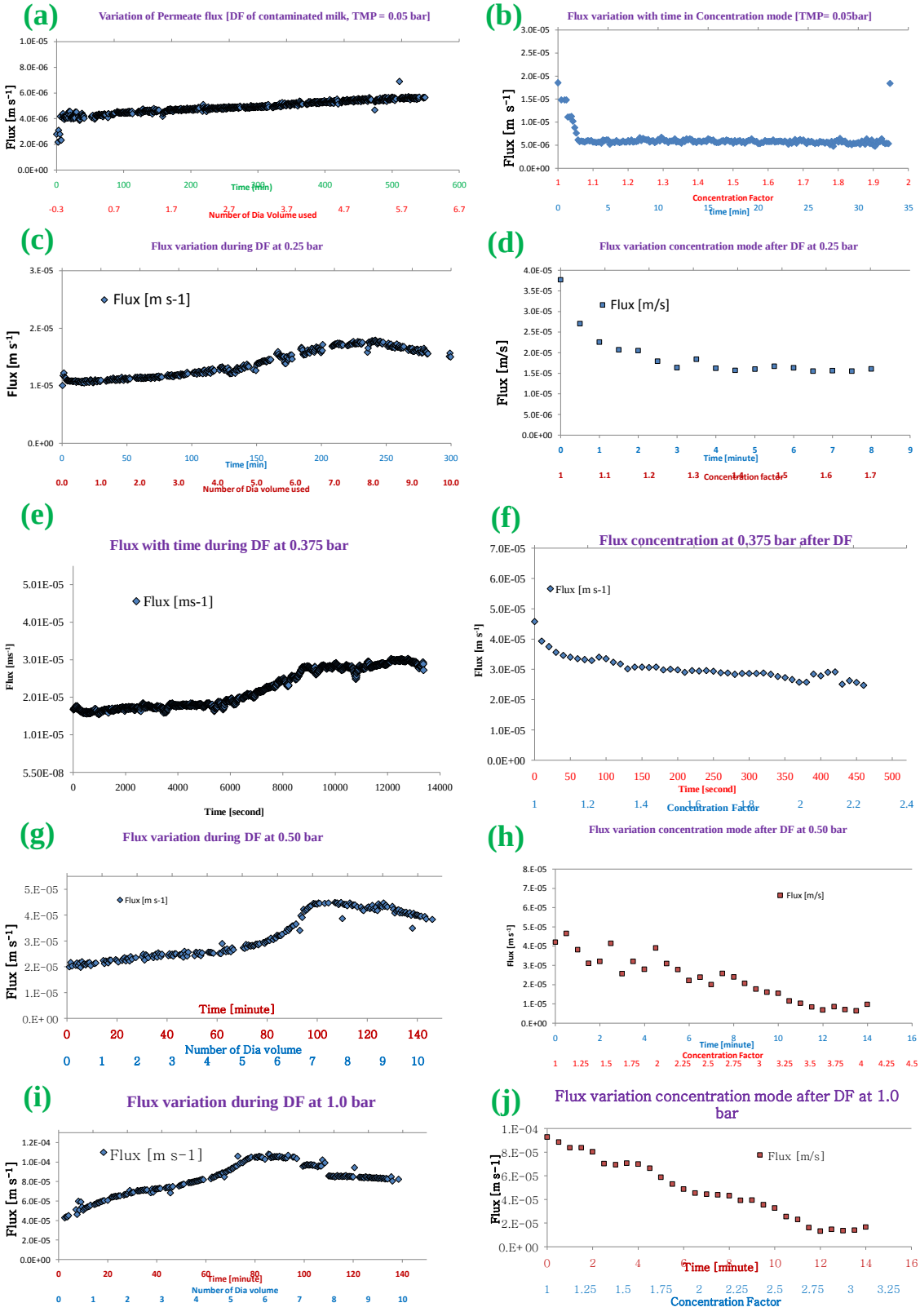

Figure 19: behaviour of the flux with progress of DF of contaminated milk: (a) DF at TMP of 0.05 bar, (b) TFF after DF at 0.05 bar; (c) DF at TMP of 0.25 bar, (b) TFF after DF at 0.25 bar,

(e) DF at TMP of 0.375 bar, (f) TFF after DF at 0.375 bar; (g) DF at TMP of 0.50 bar, (h) TFF after DF at 0.50 bar, (i) DF at TMP of 1.00 bar and (j) TFF after DF at TMP of 1.00 bar.

The observed trend of flux variation with diavolume for non-contaminated milk is:

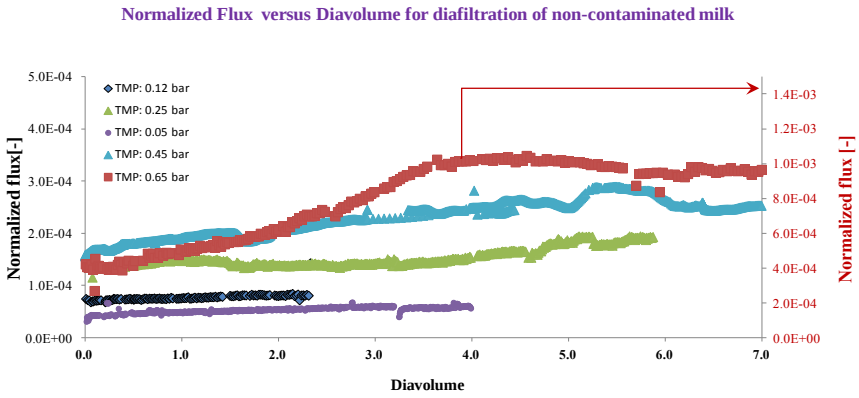

Figure 20: Normalized Flux versus Dia volume while DF (non contaminated milk)

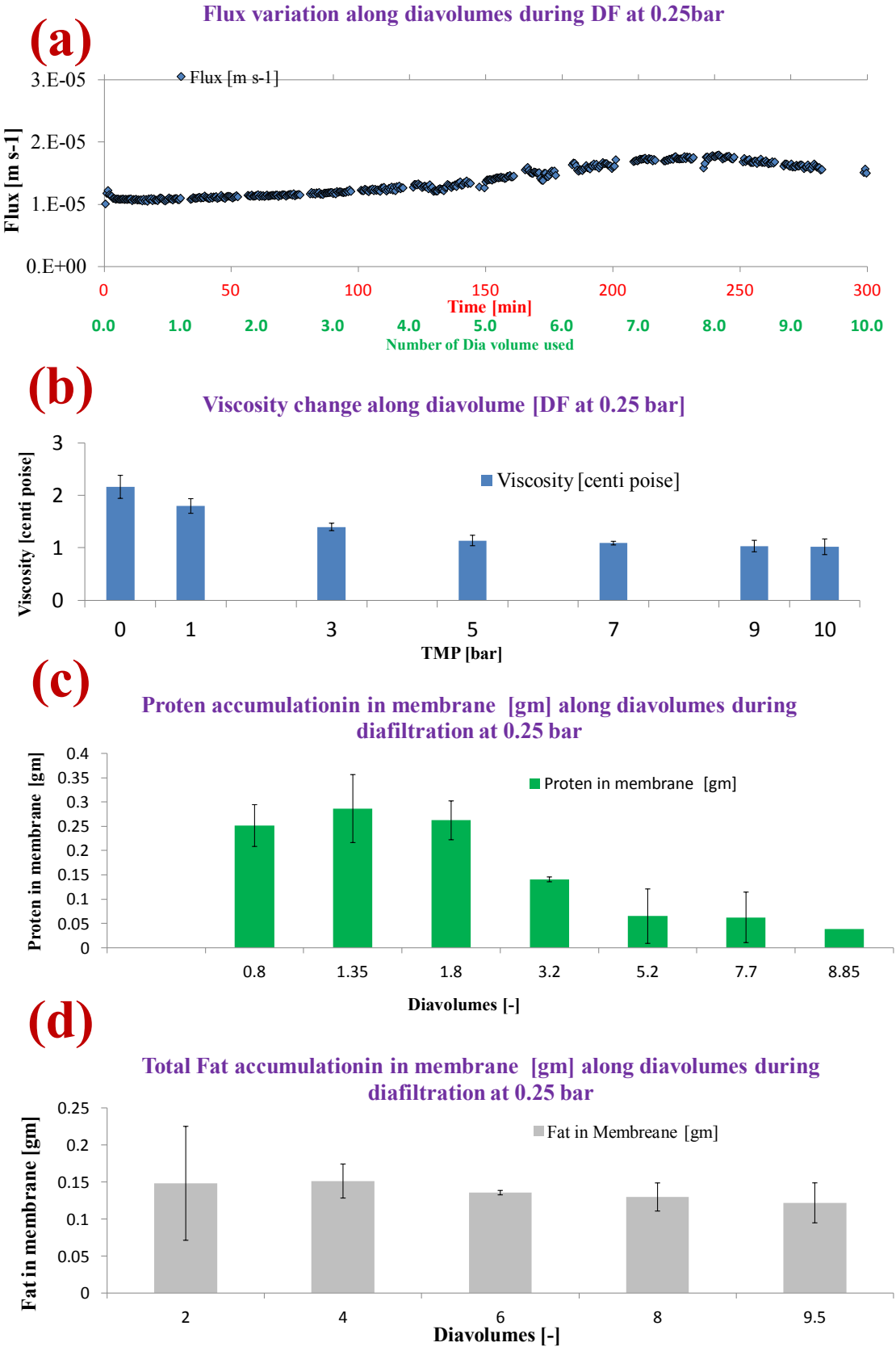

Figure 21: The variation of: (a) permeate flux , (b) viscosity in the retentate side, (c) protein and (d) fat accumualtion in the membrane along diavolumes for DF at 0.25 bar of TMP.

After using the membranes, it was possible to recover the permeability to a significant level (a minimum recovery of 90%) indicating the reusability of the membranes for multiple experimentation and testing purpose. Contribution of different resistances on the total resistance at different stages of DF of contaminated milk under different applied TMP's is tabulated in **Table 7**.

Table 7: Relative contributions of the reversible and irreversible resistances in the total resistance and observed fouling at different operational TMP's.

TMP [bar]	Normalized resistances [%]		Recovery [%]
	$R_{irreversable}/R_{foulant}$	$R_{reversable}/R_{foulant}$	
0.05	4.75%	29.93%	93.2%
0.25	10.27%	12.42%	88.3%
0.38	1.41%	32.33%	97.9%
0.50	6.69%	37.68%	89.3%
1.00	7.35%	24.77%	90.2%

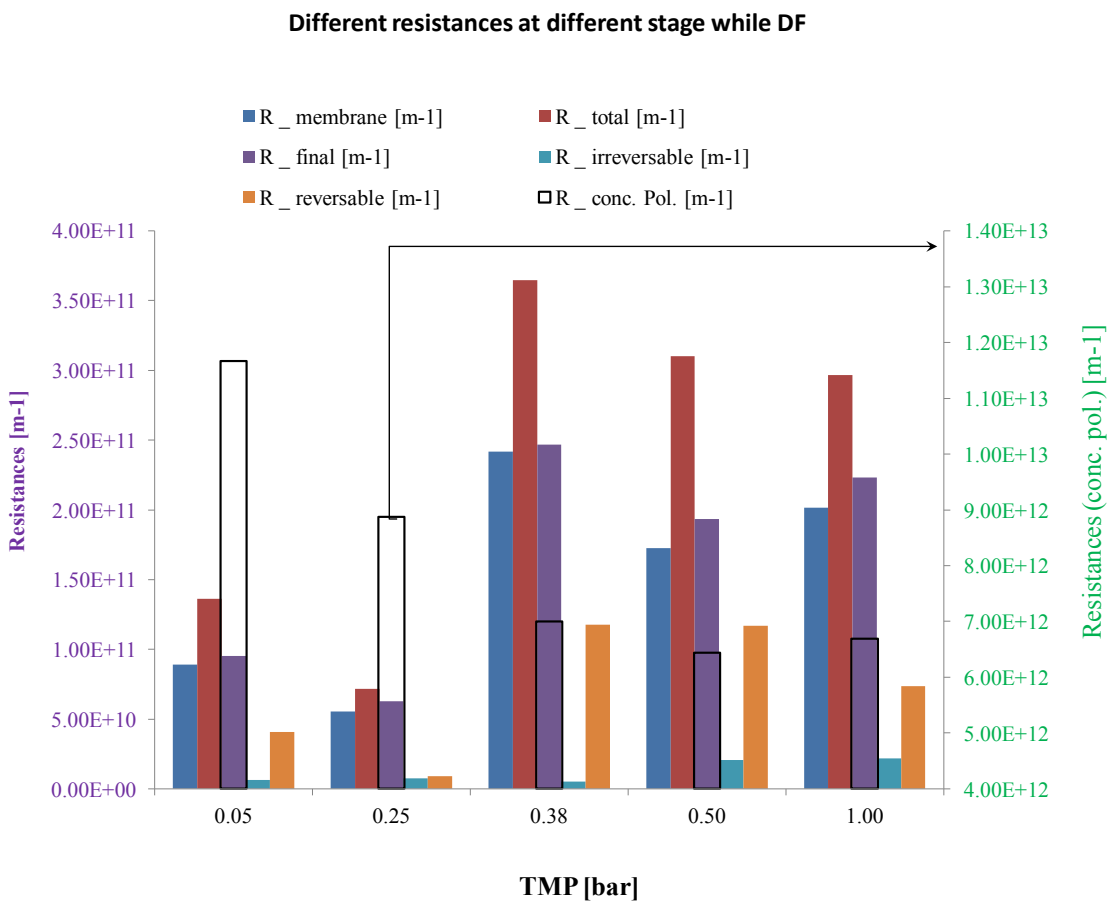

Figure 22: Different resistances at different stage while performing DF-TFF of contaminated milk; R_membrane, R_final, R_total, R_reversable, R_irreversable, R_conc.pol. Are the

contribution on the resistance by membrane intrinsic property, residual fouling after washing, total resistance after DF, fouling by reversable deposition (removable by water wash), fouling by iereversable deposition (removable by chemical wash), and fouling by concentration polarization respectively (the measurements for the Resistance by concentration polarization is represented by the “green” axis while anothers are represented in the “violet” axis).

5 Harvesting of bacteria by the DF process

Though more efficient is the observed organic load removal from milk by DF at elevated TMP, but for retaining the cells using microfiltration membrane, system always requires the optimization based on efficiency of cell harvesting [211]. In this section, the cell harvesting efficiency is checked under different experimental conditions. It was considered that, though the cells are in growth phase, the difference between specific growth rates in the retentate and permeate side was not high enough to consider as different entity. Also, since the time length from loading the milk with cells to time of doing the cell count analysis was kept same in all the cases, no significant contribution by the cell growth in cell recovery during experimentations was considered. In addition, the experiments were done within shortest possible time (lower than lag phase length) after loading of milk with bacteria for opposing cell growth effect during filtration.

5.1 Effect of TMP on cell retention during DF and concentration step

The performance parameter for retaining the bacteria on test sample for both the DF and TFF steps was assessed by the Log reduction value (LRV) [**Equation 22**], a mathematical term (representing "log increase") demonstrating the relative number of alive microbes permeated from the milk sample during filtration. From the definition [**Equation 23**], for a specific diavolumes (in case of purification step) or concentration factor (in case concentration step), the increment of LRV indicates higher retention of the bacteria and thus better cell harvesting efficiency. The experimental outcomes are represented in **Figure 23** and **Figure 24**. Also, in parallel, overall cell recovery is quantified to demonstrate the cell gained for further quantification by biosensor.

As discussed in the methodology section, for isolation of the concentrated cells from contaminated milk samples, selective enrichment of the cells was done by DF step with subsequent volume reduction by concentration step. In both of the stages, the LRV and the losses of cells from the retentate side was found to depend significantly on the applied TMP (and hence on fluxes) [**Figure 23**]. At lowest operated TMP within the experimental range (0.05 bar), the LRV was found to be minimum indicating the maximum loss (and hence permeation) of the bacteria (72.52%) from the testing milk sample [**Figure 23 (b)**]. Further increment of the TMP showed sharp lowering of the bacterial losses through the membrane and can be considered as continued until a TMP value of 0.25 bar, followed by stable cell loss until 0.50

bar of TMP. Afterwards, more increment of TMP showed lowering of cell recovery (but at comparatively slower rate than that for LRV increment rate at low TMP region). The overall trend was found similar in both the cell purification and concentration step; but, in all the experimental TMP conditions, the LRV values were found higher for the concentration step [**Figure 23 (a)**], indicating higher cell recovery in this step (in comparison with cell purification step).

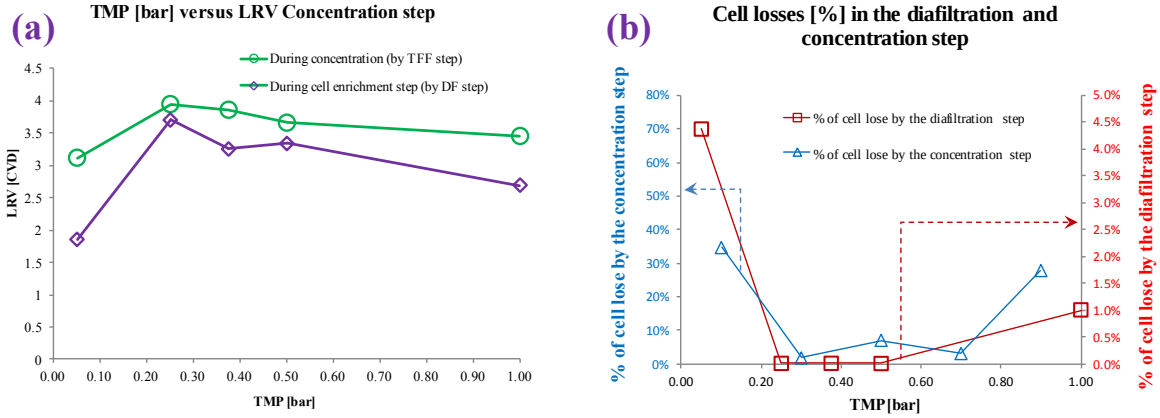

Figure 23: (a) variation of LRV with operational TMP for the DF mode (-◊-) and during concentration mode (-O-)**; (b)** variation of the % of cell looses during the selective cell enrichment session of the bacteria by DF (–Δ–) and during the concentration mode (–□–).

As discussed earlier, there was formation of cake layer on the membrane surface and concentration polarization just after starting of the filtration process. The thicknesses of these layers are function of TMP and shear force exerted by the CFV of flowing stream. From the observed effects of their imparted resistances [**Figure 22**], resistance by concentration polarization is appeared maximum at TMP of 0.05 bar with gradual lowering afterwards. The concentration polarization effect can be considered almost stable for the TMP's above 0.25 bar. It can affect the contact of cell and membrane pore in three ways – 1) the lowered degree of mass accumulation in the vicinity can offer lower resistance for the cell to get contact with the membrane pores, 2) very high concentration polarization at 0.05 bar may work for the higher retention of the cells in this massive accumulated layer which can forward to the membrane by different transport modes of cell itself, and 3) the relative compactness of these layers can contribute in different way on cell-membrane contact. However, as discussed in the section of the flow, flux and hydrodynamics, effort were made to increase the CFV in parallel of the increment of the TMP with main target of lowering of deposit layer thickness and concentration polarization as there was immediate formation of the deposit layer after starting of the filtration. This resulted lowered mass accumulation adjacent to the membrane can offer lower retention time of the cell (by lowering of entrapment in this accumulated layer of fats and proteins) to be present in such region, consequently lowers the frequency of cells comes in contact of the membrane and membrane pores (average pore size of the membrane is 0.45 µm and smallest

dimension of the rod shape cell (diameter) is 0.45-0.50 µm). By this way, during the session of DF step, number of the propagating bacteria may come to the membrane pore mouth, immobilized at the pores and can exist in the membrane matrix. From this stage, just after the entrapment of bacteria with a pore, further cell division will allow it to propagate through membrane filter matrix. These growing cells bears the potentiality of adaptation with the local morphology because of wall flexibility, and it will contribute for the easiness of the permeation too. It can be better explained as bacterial infiltration which can be defined as growth resulted mass flux of the bacteria through the membrane pore [212]. This type of bacterial permeation may be further assisted by growth of the cell mass once after immobilization of the parent cell within the membrane pore and can further propagated because of the motility. Even in absence of the permeation stream, these processes can contribute for the ***Listeria innocua*** permeation [213]. So, though the net like microstructure of the regenerated cellulose membrane bears the capability of offering higher retention time of the cell within membrane matrix and thus supposed to offer high retention of cells, permeation of the bacteria imposed by these mechanisms harnesses the performance. Different degree of influence of these mechanisms in the retention property of membrane can be the main reason of observed different degree of bacterial leakage under different operating conditions. In addition, the used membrane 0.45 µm average pore size rated, and is of fibrous materials (Regenerated Cellulose) offering the pore size distribution (PSD) by the relative orientation, interconnection and alignment of the fibers. There is always some broadness in the PSD which offers some bigger sized pores that may allow the easier permeation of the cells [214].

The main reason of observed cell permeation through the membrane is the higher length to diameter ratio of the used cell. Used rod shape bacterial cell have a smaller dimension (cell diameter: 0.47-0.50 µm) which almost equal to the average pore size of the membrane (average pore diameter: 0.45 µm) and thus bears possibility of permeation through the membrane while coming in contact of the membrane pores with favorable condition. This can contribute for the observed higher permeations at very low and high TMP. In case of low TMP operation, as the deposits may not be attached so strongly with the membrane and may not be compacted enough to effectively block (by deposition of large and deformable compounds, e.g. fat globules) the oversized membrane pores, and thus these oversized pores of membrane may remains more accessible for the bacterial cells. On the other hand, because of the high permeation flux at elevated TMP, Peclet number is also high indicating higher convective flux towards the membrane surface; and thus may cause rod shape cells to be aligned along permeation flow stream and consequently can get exposed to the membrane pore mouth with projection of their sectional area which is identical with the average area of pore mouth. The cross-sectional area

of the cell being identical with the average of the pore of the membrane, and exposure of bacteria in a orientation parallel with the membrane surface can allow easy penetration of the cells into the membrane pores (or may cause the attachment with the pores) [215], [216]. Afterwards, once after getting immobilized in the membrane pore or matrix, these cells can further permeate through the membrane by cell infiltration mechanism [213]. Also, the flagellar motility of the ***Listeria innocua*** [217], [218] can assists such permeation for making it faster. In addition, the continuous cell multiplication of these cells (after getting incorporated with the membrane matrix) can cause the forwarding of newly generated cells along the permeate streams through the membrane, and thus may contribute for higher viable cell count in permeate. However, easy elastic deformability of the cell [219] can also assist for getting permeated with alteration of shape and without damaging the cell wall integrity, once after getting penetrated in the membrane matrix at elevated TMP and under exposure to drag forces of respective high fluxes. Also, among two extreme analyzed TMP's, comparatively smaller cell permeation at maximum TMP can be because of the higher accumulation of organics in the membrane and thus comparatively higher blocking of the pores then that for the lowest TMP used [**Figure 22**].

At lower TMP than this maximum, the Peclet number is lower and consequently, the system will have fewer bacteria to be transported to the membrane surface by the convective stream in a way so as to project their sectional area on membrane pore (by alignment along the permeation flow stream). Also, the cells are more aligned along the cross flow stream which offers cell cells to be projected to the membrane pores with their length (1-2 µm) and thus lowers the cell losses by permeation. Also, the lowered flux causes membrane adjacent mass accumulative layer to be less compacted by the permeation drag force, and will lower the degree of entrapment of the cells in the organic deposited layer. Consequently, the cell permeation into the membrane by cell infiltration and flagellar motility from the deposit will decline than that for the higher TMP operation. Also, permeation rates at these TMP range may not be of enough to expose high drag force for the cells to be deformed and passed through the membrane. Consequently, the cell losses are lowered in the TMP range of 0.25 to 0.50 bar.

At lowest experimental TMP (0.05 bar), the permeation of the cells was found maximum (almost 70% of the cells were found to permeate the membrane). It can be because of low flux and consequent lowered drag force exerted on the solutes towards the membrane (by the permeation stream) at lower TMP. So, the deposited layer may not be compacted enough to create sufficient restriction to the bacterial motility once after the cell reach the vicinity of membrane or get trapped in the mass accumulated layer. This can also enhance the retention time of the cell in the vicinity of the membrane surface. Thus forwarding motion of the bacteria

towards membrane surface through the accumulated gel layer by flagellar motion can be high. Also, the low CFV respective to this TMP may not be high enough to wash out the cells from this region to bring back to the bulk of the retentate stream. Because of higher retention time in the deposit layer, tumbling motility [220]–[223] of the cell, flagellar motion incorporated with drag force of permeation stream [222], [224]–[226] towards the permeation direction, multiplication of these retained cells [227]–[229]; these cells can reach to the membrane surface comparatively easily, get exposed to the oversized membrane pores and can get stick with membrane pores by some part of the cell with further penetration into the pores. All of these cell penetration and immobilization can further result the permeation of the cells (drag force and flagella assisted) and can contribute for higher cell count in permeate by these possible pass through the membrane with permeation stream, cell multiplication and motion of the cell.

There was no observed influence of cells on the permeation nature of the organic species, flux and fouling in comparison with the same experimentation with non contaminated milk which in turn indicates that, the removal mechanisms do not rely upon the interactions between microorganisms within this used range of bacteria concentration [230], [231]. Since the bacteria loaded milk had almost similar cell concentration during all the experiments, the effect of cell concentration on the comparative cell loss analysis was neglected. Also, it has been reported about the non significant influence of the cell concentration on filtration performance of milk under this contamination level of such bacteria [231].

5.2 Cell recovery in DF and TFF

After organic load removal from the cell loaded milk by DF, during the concentration of cell suspension by volume reduction by TFF step, LRV (and the cell losses) from the system demonstrated the almost similar variation with TMP as was obtained for respective DF steps. But the magnitude of the relevant log reduction values was higher (i.e. lower cell loss from retentate) in the concentration step [**Figure 23**]. One reason can be very low processing time during the concentration of the cell suspension than that for the purification step, for which the net cell permeations during the concentration step can be lowered. Also, cells in concentration step may undergo increased agglomeration with progress of cell suspension concentration [131], which can significantly lower the cell permeation through membrane as these agglomerates possesses bigger sizes than an individual cell. In addition, consideration should be taken about the pre-fouled stage of membrane before starting of cell concentration step due to possible deposits of the fat globules [**Figure 21 (d)**], and thus oversized pores can be blocked with deposition and penetration of deformable fat globules. This can also contribute for the lowered cell permeation during the concentration step. Here, the effect of cell interaction with the membrane by any release of EPS can be neglected as predicted in earlier sections.

5.3 Overall cell recovery and optimum condition

The observed overall performance of cell isolation under different TMP condition is showed in [**Figure 24**]. It demonstrates that, though the cell purification is positively correlated with the TMP, but it's the lowered cell recovery which harnesses application of high TMP considering cell recovery as the main target. Considering all these, the process can be considered for efficient extraction of more than 99.7% of cells from the milk matrix, which are further treatable with biosensor for the fast detection and enumeration, by operating the process in TMP ranges of 0.25 to 0.50 bar. Also, though the liner dimension of the used cell is much higher than average membrane pore size, due to the small section area of the cell and deformability, retention performance is required to control with the operational parameter. However, this optimum range is governed by the PSD of the membrane, the nature of the cell, relative degree of the forces faced by the cells and the hydrodynamic condition involved.

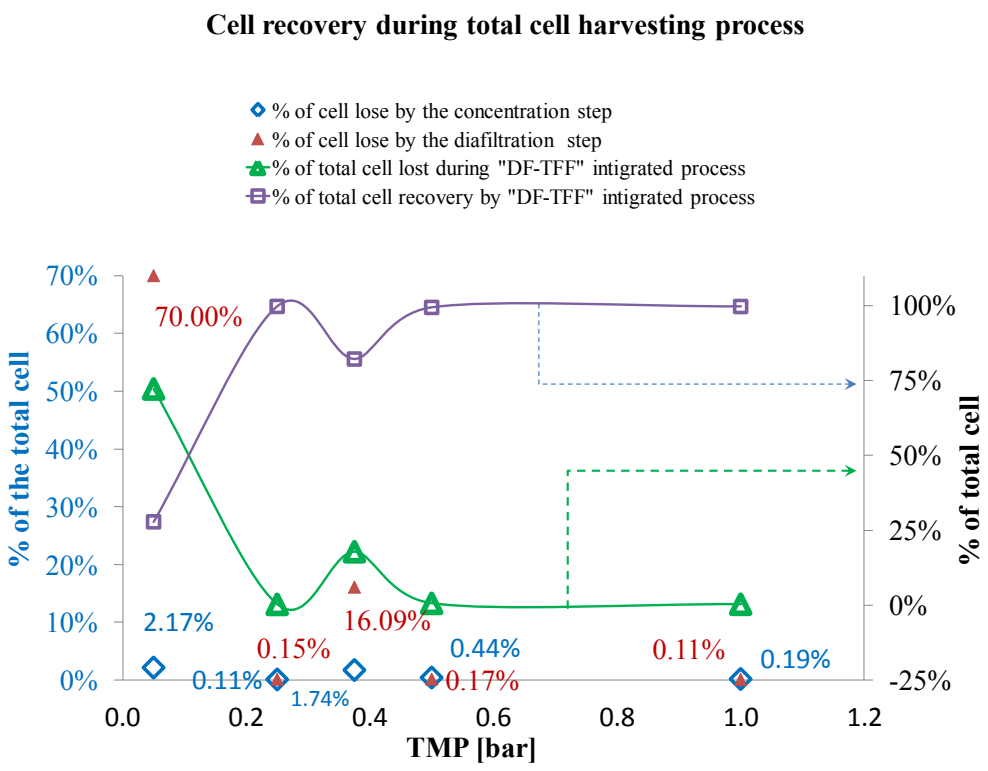

Figure 24: TMP dependence of % of the cell losses in during the purification step (-Δ-), % of the cell lost during concentration step (-◊-), % of overall cell losses during the entire session of cell harvesting (-Δ-) and % of cell recovered during the entire session of the cell harvesting (-□-) under this experimental condition. The % of the total cell representative axis for the (-Δ-) and (-

◊-) are the "blue" colored axis while **"black"** colored axis stands for the others. Here, "% of cells" was calculated based on the counting of the total viable cells.

6 Conclusion

The study demonstrated the high efficiency for the selective enrichment of the ***Listeria innocua,*** a cell highly comparable and used for the performance analysis of the ***Listeria monocytogenes*** which is the most pathogenic form of the ***Listeria*** group having widely reported significant global impact, from the complex food sample (milk) by effective elimination of the high organic loads by microfiltration based DF and TFF steps. Both the DF and concentration steps involved in the cell isolation and concentration were operated in cross flow mode and found to be fast, of minimized disturbance and efficient for the viable cell isolation for further analysis by bio-sensing tools.

The filtration and cell harvesting was faster with increment of TMP, but the cell lose from the system was found to be depended on the operating condition. In the TMP region of 0.25 to 0.50 bar, the viable cell harvesting was found to be maximum. Considering the degree of organic load removal rate and cell recovery, DF and cell concentration at TMP of 0.25 bar was found to be most effective with more than 99.7% cell recovery as well as removal of more than 99% and 91.80% of the total proteins and sugars respectively from the sample. Under this condition, the removal of total fat content was 30-50 %. But the residual fat concentration was low enough for separating by an additional centrifugation, physico-chemical process or an additional membrane filtration step prior to further analysis by biosensor. The overall removal trend of organic load found higher at elevated TMP but the cell recovery was the issue which limits the high TMP operation. The increment of the CFV in parallel of increment of TMP was found effective for improving the permeation flux to make the process faster. The fouling was found recoverable to a desired level by effective washing of the membrane and reusability of the membrane for multiple analyses was determined. Based on the observed data, a broad and through analysis for the possible modes of cell losses during such filtration was made. Considering the fastness of the process and lowering of residual species concentrations, operation in the TMP range of 0.25 to 0.50 bar can be considered to be of soaring enough to allow the sample to be analyzed by a biosensor for the intended purpose. In all the cases of experimentation, the recovery of the membrane was acceptable and enough for effectively carrying out multiple analysis by a since piece of membrane (more than 7 experiment per membrane).

The selected regenerated cellulose acetate polymeric membrane was found efficient for such isolation of the cells with simultaneous eliminations of the organic loads. Here it should be noted that, the works were proceeds with a membrane having some pore size distributions. The

cell rejection performance as whole may not be theoretically comparable with a membrane of very narrow, finely defied and uniformly sized pores; but the demonstrated system is cheaper, easy to adopt and offers high viable cell recovery from the sample (more than 99.7%) in a fast way.

The developed model based on the mass balance of the species involved was found to well define the system and was effective for predicting the trends of the organic load removal from bacteria loaded milk. Also, the model parameter and their influence on the nature of organic load removal are discussed. In addition, the model was found able to quantify rapidness of the organic load removal by this process at elevated TMP (with associated CFV) and showed applicability for the optimization of such process.

Finally, the demonstrated method for fast isolation and concentration of ***Listeria innocua*** cell from the complex milk matrix leads towards the usability of this system for pathogenic species of this group as well as for other similar bacteria. It opens a fast, cheaper and thus better way for completion of such work in the fields of food-borne pathogens, clinical diagnostics and other biological environment monitoring. The easy to use, fastness, lower exposure to the work environment and safety standards of this method acts to make it well considerable for the processing of food pathogens arena. Also, the observed retentions by the demonstrated filtration process (and thus recovery) for this rod shape bacteria is high enough to consider this process for isolating the other cells having higher length to diameter ratio (e.g. spherical cells) and minimum dimension within the length to diameter range of the used ***Listeria innocua*** cells.

7 Recommendations & future work

The experiments open an approach for processing vast variety of contaminated biological fluids to obtain purified and concentrated bacteria which can be further used for the fast bacterial detection and enumeration by biosensors in the field of health, food and biotechnological processes. Also, easy adoptability, cheaper and operability of this method will assist the easy testing of newly developed and emerging biosensors, and in assessing their applicability's for real field. Based on the analysis of the results obtained from this experimental framework, we recommend the following further works and aspects that can be considered for making this method to be more efficient, faster, much well defined and of high applicability towards its further goals.

- Caring out the same experiment in chip based scale systems, e.g. in microarrays and microfluidic bio-chips, to reduce the holdup volume of the system and to obtain higher concentration factor of the cell at the end with simultaneous observation of the effect of high cell concentration on its permeation nature and membrane fouling.
- The work demonstrated the applicability of conventional MF membrane having some broadness in the pore size distribution to be effective for such isolation of cell. Also, the observed efficiency is higher than that for competitive other conventional multistage processes which are incorporated with more exposures of cells with the environments and causes significant losses of cells at different inter-stages. It would be a nice approach to observe the motion of the cell under the action of pressure, flow as well as cell motility and growth associated motions in the vicinity of the membrane to get the more insight of the phenomenon. Analysis can be brought by transparent filtration module after marking the cells with a detection system. We recommend confocal laser scanning microscopy based bacteria location tracing after staining with fluorescent dyes to detect the motions of bacteria in such membrane system and further analysis based on dynamic distribution of bacteria in milk.
- Development of simulation and modeling in the micro scale activity based on force balances that a bacteria faces during its travel from the bulk of the liquid to the membrane pores with competition with other molecules. It can give a good insight and opening for such membrane to be more effectively used in such approaches.
- Comparative analysis of the respective system with a spherical bacteria loaded milk to compare the effect of bacterial cell shape on the rejection performance. Incorporation of the cell wall elasticity and flexibility in the modeling section on the pre-mentioned approach

and to develop the theoretical core foundation contribution on cell permeation through membrane during processing complex biological fluids under such conditions.

- Development of an inherent method for such MF process based cell isolation by considering the interactions between the cells, membrane and other constituents involved to well define all the experimental regions of filtration.
- Using the same technology for isolation and concentration of the cells from different contaminated biological fluid (clinical diagnostics for body fluids and monitoring of other food-borne pathogens) with different pathogens and under different degree of cell loading to construct the deep insight of the process.
- Analysis of the total cells in the retentate and permeate side by other conventional method to check cell death, and analyzing the effect of operating conditions on the cell fate as well as influence on overall quantification. Further analysis can be considered to study the effect of filtration condition on the fatality of the cells and thus making the process more precise.
- Analysis of the performance of the cell recovery after removal of lipid by an additional stage of centrifugation or filtration.
- Analysis of such continuous membrane based filtration systems incorporated with different pore sized membrane to carry out size based fractionation of the sample, comparative analysis of the cell recovery and detection efficiency by treating this isolated bacteria by biosensor.
- Analysis of the bacterial entrapment on the membrane, penetration and bio fouling by analysis of SEM images of membrane taken at different stages of filtration. Establishment of the correlation on the respective aspects discussed and thorough analysis.
- Investigation of the effect of module design and hydrodynamic condition on bacteria permeation by using different type and shaped modules.
- Analysis of the effect of pretreatment (e.g. addition of surfactant) of the contaminated milk on the improvement of flux, viability of the cells involved, interaction with other organic loads and membrane fouling with respective optimization.

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