REVERSE MICELLAR EXTRACTION OF LACTOFERRIN FROM WHEY

Thesis

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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March, 2020

DECLARATION

I hereby *declare* that the Research Thesis entitled **"Reverse micellar extraction of Lactoferrin from whey"** Which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy** in **the Department of Chemical Engineering** is a *bonafide report of the research work carried out by me*. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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CERTIFICATE

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ACKNOWLEDGEMENT

First and foremost I want to thank my research supervisors Dr. I. Regupathi and Dr. Prasanna Belur D. for giving me this opportunity and their continuous guidance and support for the completion of my Ph.D. Their immense knowledge and dedication towards responsibilities have always inspired me.

Besides my supervisors, I would like to thank my RPAC members Dr. Jaganathan T. K. (Dept. of Chemical Eng.) and Dr. Sunil B.M. (Dept. of Civil Eng.) for their valuable and insightful comments.

I extend my gratitude to the Director of NITK; Head of the Department Dr. Hari Mahalingam, and former Heads of the Chemical Engineering Department, Dr. Vidya Shetty and Dr. Raj Mohan B. for providing me with the necessary facilities and support during my research work.

I would like to thank the technical and non-technical staff of our department Mr. Sadashiva, Mrs. Tritila Shetty, Mr Suresh, Mr. Ramesh, Ms. Vijetha, Mrs. Bhavya, Mrs. Sandhya Mrs. Shashikala, Mr. Sukesha, and Mr. Harish for their kind co-operation.

I am also thankful to Dr. K. B. Koteshwara (Professor and Head, Dept. of Pharmaceutics, College of Pharmaceutical Sciences Manipal University) and Mr. Shivanand (Technical Assistant, College of Pharmaceutical Sciences Manipal University) for availing the research facility in their institute.

I owe my thanks to all the research scholars in the department for giving me a friendly environment in the department — my labmates and friends Mr. Basavaraj, Mr. Lister, Dr. Vaisali, Mrs. Rashmi, Dr. Charanya, Dr. Kunal, Dr. Anusha, Ms. Shwetha, for their encouragement and valuable inputs in my study. My life in campus and hostel would not have been so joyful without Mrs. Vrushali, Ms. Sushma, Ms. Suvidha and other hostel friends. My special thanks goes to Mr. Shoaib Haidar, who has always inspired and supported me to pursue my dreams.

Finally, but by no means least, I would like to bow my head and million thanks to my Parents Mr. Sahebrao Pawar and Mrs. Sunanda Pawar for their constant support in my journey of Ph.D. My lovely Sister and Brother-in-law and my supportive Brother and Sister-in-law for their constant support. I cannot forget my cute, adorable niece baby Gandharvikaa; all my cousins who have always cherished me. I would like to say big thanks to my maternal and paternal grandparents, uncle-aunties for always been there to support me.

Swapnali S Pawar

ABSTRACT

Whey, the by-product of dairy industry, contains variety of nutritional valued and commercially important biomolecules. Lactoferrin (LF) is one among the whey protein, present in very less concentration in whey and other biological sources like, saliva, tears, synovial fluid. LF has wide applications in medicinal field as anticancer agent, antiviral activity, maintaining iron level in the body. It is a major component of infant formula and also used in various oral care products and cosmetics. All the mentioned applications require the LF in reasonable amount with high purity. The market demand of LF increased to 262,000 kg in 2017 with the cost ranging from US$500 to US$1,000 per kilogram depending on the purity. The existing technologies/methods used for extraction of LF with required purity are failed to meet the market demand. As an alternative, the reverse micellar extraction (RME), a bulk extraction process, has been developed in the present study for the selective extraction of LF from whey.

The reverse micellar system (RMS) formed by CTAB/n-heptanol was identified for the selective extraction of LF by considering different types of surfactants and solvents. The selected RMS was studied to identify the suitable process condition for the solubilisation of LF in the reverse micellar phase and their effect on the extraction efficiency by conducting the experiments systematically. Initially, the influence of surfactant type, types of salt, phase components and their concentration, pH, co-solvents and phase volume ratio on the forward and back extraction of LF was studied using commercially available LF. The optimised process conditions were then extended to selectively extract the LF from acidic whey. Maximum forward extraction of 98.7% was achieved for LF in CTAB/n-heptanol reverse micellar system at CTAB concentration of 50mM, salt concentration 1.1M and maintaining aqueous phase pH 10.3. Whereas, 94.2% LF was back extracted without any impurities at stripping phase pH 6, 1.5M KCl and 7% n-butanol as a co-solvent. The recycling capacity of the spent reverse micellar phase was studied at optimized extraction condition and the reverse micelle phase may be recycled at least three times without much loss in their extraction capacity. The feasibility of continuous operations of the RME process has also been demonstrated in Rotating Disc Contactors by studying the effect of

variables like rotor speed, dispersed RM phase and aqueous phase velocities on the extraction characteristics.

Keywords: CTAB/n-heptanol, recycling study, rotating disc contactors.

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ABBREVATIONS AND SYMBOLS

AAS	Atomic Absorption Spectrophotometer
AOT	Aerosol OT
α-LA	α-Lactalbumin
ATPE	Aqueous Two Phase Extraction
ATPS	Aqueous Two Phase System
β-LG	β-Lactoglobulin
BDBAC	Benzyl Dodecyl Bis(hydroxyethyl) Ammonium Chloride
BOD	Biological Oxygen Demand
BSA	Bovine Serum Albumin
CAGR	Compound Annual Growth Rate
CBB	Coomassie Brilliant Blue
CMC	Critical Micellar Concentration
CPB	Cetyl Piridium Bromide
COD	Chemical Oxygen Demand
CPE	Cloud Point Extraction
CTAB	Cetyltrimethyl Ammonium Bromide
DDAB	Dimethyl Dioctadecyl Ammonium Bromide
DODMAC	Dimethyl Dioctadecylmethyl Ammonium Chloride
DOLPA	Dioleyl Phosphoric Acid
DTAB	Dodecyl Trimethyl Ammonium Bromide
FCR	Folin-Ciocalteu Reagent
GA	Glucosylammonium
GuHCl	Guanine Hydrochloride
GMP	Glycomacropeptide
HLB	Hydrophilic-Lipophilic Balance
HPLC	High Performance Liquid Chromatography
Ig	Immunoglobulins
LA	Lactosylammonium

LLE	Liquid Liquid Extraction
LF	Lactoferrin
LPO	Lactoperoxidase
PEG	Polyethylene Glycol
PRDC	Perforated Rotating Disc Contactor
RDC	Rotating Disc Contactors
RL	Rhamnolipid
RM	Reverse Micelle/s
RME	Reverse Micellar Extraction
RMS	Reverse Micellar System/s
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SMB	Simulated Moving Bed
TFA	Trifluoroacetic acid
TOMAC	Tri-Octyl Methyl Ammonium Chloride
TRPO	Trialkyl Phosphine Oxide
TX	Triton X

Units

cm	centimeter
g/l	gram/litre
KD	Kilo Dalton
M	molar
m	meter
ml	millilitres
min	minute
mm	millimetre
mg/ml	milligram/millilitre
ml/min	millilitres / minute
mM	milli mol
mPa.s	milipascal-second
ng	nanogram

ppm	parts per millions
rpm	rotation per minute
µl	microliter
µm	micro meter
nm	nanometer

Symols

^{0} C	degree celcius
%	percentage
W_0	Water Content
α	Alfa
β	Beta
Φ	holdup
η	Extraction efficiency
$K_d a$	Mass transfer coefficient

CHAPTER 1

INTRODUCTION

Proteins are one among the essential biomolecules of food, as a source of energy and exogenous amino acids necessary for growth. Diversified complex biological sources from plants, animals and microbes are considered a rich source of proteins. Animal source originated proteins like myoglobin, myosin, collagen, elastin, and haemoglobin have significant health effects on a living organism and some of the plant proteins like cruciferin, soy protein, zein, carmin also plays a vital role in metabolism (Emily Greco et al. 2017). However, the proteins like cytochrome c, cytochrome P 450, insulin and haemoglobin are considered as minor proteins which are required in smaller quantity to regulate the metabolism in a living organism. Cytochrome c is majorly involved in the electron transport chain in the kidney (Mahapatra et al. 2017), cytochrome P450 family regulates cancer drug metabolism (Blackburn et al. 2015), insulin maintains blood sugar level (Altaf et al. 2015), haemoglobin is responsible for maintaining the iron level in the body (Wu et al. 2018). Even though these proteins are available in many biological sources, the lower concentrations in the sources may not be sufficient to impart the required health effect. The functional activities of these proteins are improved by enriching their concentration in the food and pharmaceutical formulation. Hence, some of these proteins are separated and purified from complex biological sources and added with a variety of functional food products and pharmaceutical products to impart the specific function. In this regard, an intensified effort has been made in recent years to develop new separation and purification processes which can be easily scaled-up with biocompatibility and higher selectivity for the biomolecules like proteins, enzymes, nucleic acids, antioxidants, flavonoids etc., from various complex biological sources including fermentation broth, extract from different

animal and plant sources and effluents from multiple industries (Kilikian et al. 2000).

1.1 Milk and whey

Milk proteins are the rich source of essential amino acids that have high biological values. The dynamic composition and its positive impact on health have recognised milk as a powerhouse of nutrients (Marshall 2004). Milk is constitute of lipids (33g/l), saturated fatty acids (19g/l), oleic acid and palmitic acid (8g/l), linoleic acid (1-2g/l), lauric acid (0.8g/l), myristic acid (3g/l), proteins (32g/l), lactose (53g/l), calcium, (1.1g/l), magnesium (100mg/l), zinc (4mg/l), selenium (37µg/l), vitamin A (280µg/l) and E (0.6mg/l), folate (50µg/l), riboflavin (1.83mg/l) and vitamin B12 (4.4µg/l).These milk components have crucial biological functions. Fats, lipids, myristic and palmitic acids present in milk helps to increase the level of high and low-density lipoprotein that works as antioxidants in the body. Minerals like calcium help to increase the bone density whereas, zinc regulates the gene expression. Vitamin E functions as an antioxidant. The cell differentiation is well regulated by vitamin A present in milk. Vitamin B12 has a major role as a regulator in folate metabolism which controls cell division, DNA synthesis, amino acid metabolism (Haug et al. 2007). Whey proteins are rich in branched-chain amino acids namely, leucine, isoleucine and valine and lysine whereas caseins are contained histidine, methionine and phenylalanine in a higher amount. The milk proteins, are majorly categorised into two classes. The first category is soluble proteins which are well known as “whey proteins” and constitutes about 20% of total protein fractions, and another category is insoluble proteins or “caseins” that represents 80% of total protein fractions (Pereira 2014). The amino acids profile is different for both types of protein fractions which divides them into two classes even though both are equally important in nutrition point of view.

1.2 Whey proteins

Whey is a thin liquid, obtained as a by-product during cheese making. Based on the processing conditions, the whey produced in dairy industries are divided into

two categories namely “sweet” and “acid” whey. Sweet whey bares higher fat content whereas acid whey is rich in ash and lactic acid (Carter and Drake 2018). Whey was considered as waste with little or no commercial value by cheese producers. Hence it was either discarded or used as an animal feed. Till the 20th century, the dairy industries have practised the straightforward method of whey disposal, i.e. direct discharge to the ocean, or onto fields. However, environmental issues were raised day by day due to these disposal methods of whey which contains high solid content (~50-70%), high biological oxygen demand (BOD) (>35000 ppm) and chemical oxygen demand (COD) (>60000 ppm). The increased consumption of dairy products like paneer and cheese resulted in the raised production of whey. Worldwide around 180 million metric tons of whey is produced yearly. Parallel in the early of the 21st century, the invention of new nutritional and related technical applications for whey or whey components has come ahead, and it changed the general opinion radically. Now, whey is counted as a co-product of the dairy industry (Carter and Drake 2018).

Whey proteins have extreme nutritional values as compared to other dietary protein sources like meat, egg protein and soy proteins and so forth. The branched-chain amino acids present in whey proteins have crucial biological functions in muscle strengthening, metabolic regulation, glucose homeostasis and lipid metabolism. Also, sulphur containing amino acids present in whey proteins are involved in one-carbon metabolism and protein folding (Smithers 2015).The composition of whey includes; β-lactoglobulin (β-LG: 3-4g/l), α-lactalbumin (α-LA:1.5g/l), bovine serum albumin (BSA: 0.3-0.6g/l), lactoferrin (LF: 0.03-0.1g/l), immunoglobulins (Ig: 0.6-0.9g/l), lactoperoxidase (LPO: 0.0-0.03g/l), glycomacropeptides (GMP: 1-1.2g/l) as whey proteins and lactose, minerals as non-proteinaceous whey component (Du et al. 2013). The minor whey proteins, LF and LPO, are known as critical antimicrobial agents. At the same time, LF along with β-LG and α-LA resist tumour formation (Pereira 2014).

1.2.1 LF

LF is one of the important glycoproteins from medicinal as well as commercial point of view. It is a member of a transferrin protein family. It was first discovered and isolated by Sorensen and Sorensen from bovine milk in 1939 (Baker and Baker 2004). Though milk secreted by mammary gland is the primary source of LF, it also occurs in biological fluids like saliva, tears, synovial and seminal fluids (Steijns and van Hooijdonk 2000). LF is used as a food additive in the meat and poultry industries to inhibit bacterial growth and enhances the shelf life of the meat (Naidu 2002). It is also used in dairy industries to prevent the spoilage and to extend the shelf life of cheese by controlling the growth of *Pseudomonas* (Quintieri et al. 2013). LF inhibits the yeast *Dekkera bruxellesis*, hence used to prevent the deterioration and to improve the quality of the wine (Duran and Kahve 2017). LF has many health benefits and has high biomedical value among the other abundantly present proteins. It is a potential additive for cancer treatment, autoimmune disorders, and antibiotic or antimicrobial therapy where drugs alone have failed to reduce the risks (Adlerova et al. 2008; González-Chávez et al. 2009).

Bioferrin-1000 (tablets), Bioferrin Fish Feed, LF Capsules, whey protein powder obtained from milk are available as a source of the LF in the market. However; there is a global shortage of LF, driven mainly by the demand for infant formula. The market for LF has grown from 45,000 kilograms to 185,000 kg in 2012 and is expected to grow to 262,000 kg in 2017. On the current market, the cost of LF US$500 to US$1,000 per kilogram. The global demand of LF is expected to grow at a compound annual growth rate (CAGR) of 8.4% from 2017 to 2025 accordingly and market size worth $167.9 Million by 2025 according to Grand View Research, US.

1.3 Protein Purification

The valuable minor proteins should be separated and purified from various sources to utilize their complete biological activity when it is used for specific

purposes in the food and pharmaceutical formulations. Some of the purified proteins are used as biochemical agents. The recovery and purity obtained after extraction and purification of the target protein depend upon the source of material used and the steps involved in the purification processes. The separation and purification steps should be adopted such a way that the selected processes should preserve the activity and the unique biological characteristics for their better performance. Generally, the protein recovered from multiple sources have compromised the purity based on the type of source and their percentage recovery (Janson 2012). Cation-anion exchange, chelating, affinity chromatography, gel filtration, ultrafiltration, membrane adsorption etc., methods were exploited to purify target protein. The pre-treatment of raw materials like homogenisation, sonication, chemical/enzymatic treatments, repeated washing, concentrating processes, etc., is the prerequisite for most of the processes. Steps involved in the general protein extraction and purification processes were summarised in figure 1.1.

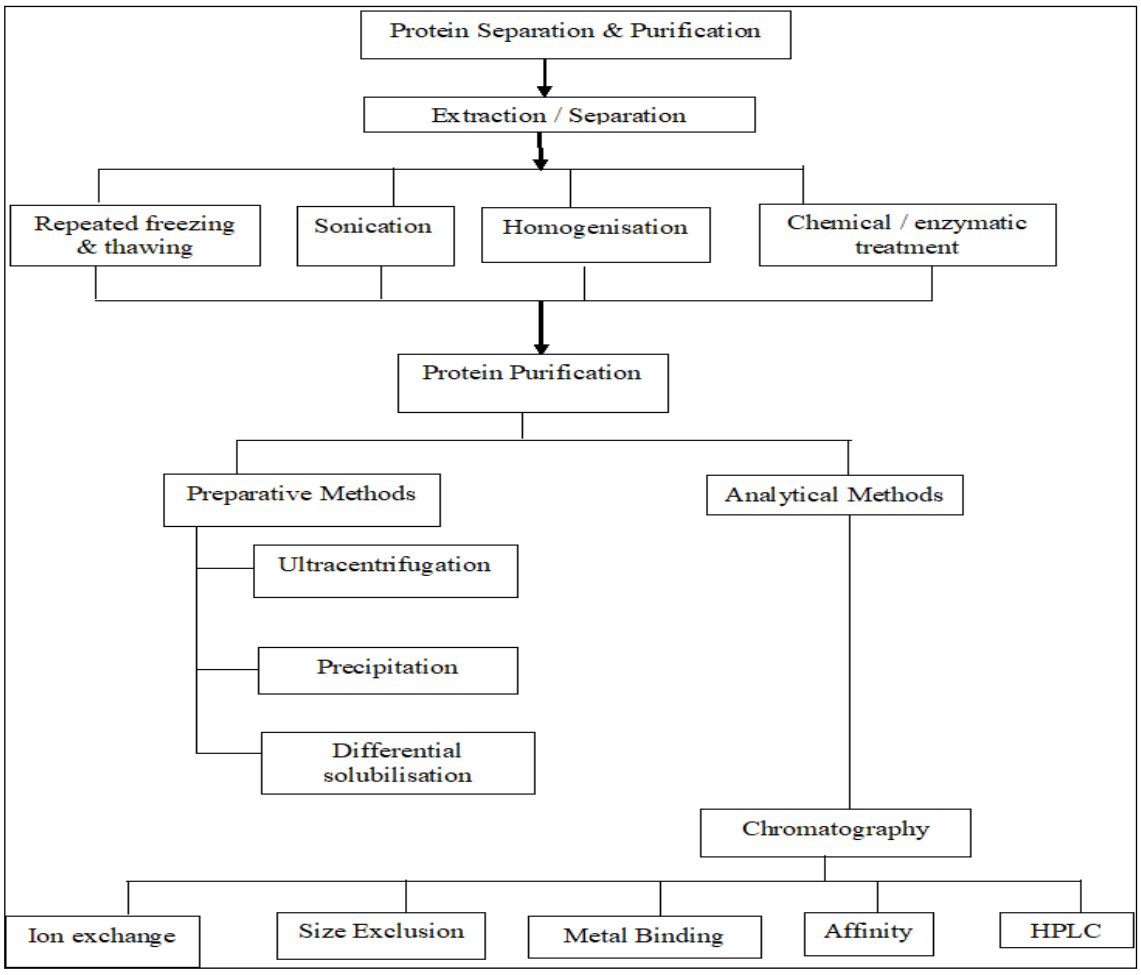


Fig. 1.1: Protein extraction and purification flowchart (Scopes 1994)

To perform pre-treatment, preparative methods like adsorption and precipitation can also be applied. Such pre-treatment methods are time-consuming and increase the number of steps involved in protein separation and purification. Along with this, chances of loss of protein during pre-treatment processes leads to decrease the final yield of the protein (Du et al. 2013). The use of analytical methods for purification is possible only for the small volume of samples. By using chromatography or membrane separation techniques fractions of purified proteins can be collected. As several steps are involved in the protein extraction and purification process, it is laborious as well as time-consuming. Also, the cost of column or membranes used in the purification process is relatively high. The efficiency of column or membranes also found to reduce over the frequent uses, and periodic regeneration of column or replacement of membrane is

required to reactivate the process, even though the similar raw material is used (Janson 2012).

1.4 Liquid-liquid extraction (LLE)

To overcome the problems associated with existing analytical purification methods; methods like LLE may be a better alternative. LLE is a useful method for the isolation of chemical and biological products. It transfers certain components from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other. LLE is extensively used in chemical industries such as aromatic, petrochemical, oils and fats to extract the useful solutes or to remove the impurities (Krishna et al. 2002). LLE can replace the conventional protein separation methods like precipitation and chromatography that are responsible for environmental pollution. Many extraction systems being studied nowadays for the separation and purification of biomolecules are the rewrapped packages of old principles. The non-conventional LLE employs the biocompatible phase forming components like PEG, salts, surfactants, carbohydrates and alcohols that have a mild effect on the bio components, unlike the conventional organic solvents. The characteristic features of these conventional LLE include multiphase properties, ease in solubilising the normally hydrophobic and insoluble species, non-requirement of the use of volatile organic compounds for phase formation and their reliance on the structuring properties of liquid water for forming the multiphase and also their higher solubilising power for a wide variety of biological solutes. The solubilising power and anisotropy make them suitable for extraction of fragile bio-components while the non-requirement of volatile organic compounds for forming phases makes them eco-friendly techniques (Raghavarao et al. 2003). Based on the type of phase formation and their characteristics the conventional extraction processes are classified as cloud point extraction, aqueous two-phase extraction, micellar/ reverse micellar extraction and extractions using thermo-separating polymers.

Aqueous two-phase extraction (ATPE) is an LLE consisting of two immiscible aqueous phases. Water is the main component in the biphasic system formed by the combination of either two polymers, polymer and salt or salt and alcohol and it provides the favourable environment to separate biomolecule. Molecular weight and concentration of phase forming components are the significant factors which affect the phase separation in the aqueous two-phase system (ATPS). Surface properties of components of ATPS influence the partitioning of a solute between two phases. Several proteins, enzymes, monoclonal antibodies, nucleic acids, viruses, cells and organelles have been purified using ATPS system (Iqbal et al. 2016). Even though the ATPE was widely applied for several biomolecules purification; the poor understanding of their phase formation and solute portioning mechanism limits its applications. The separation efficiency and yields of biomolecules are enhanced by coupling the ATPE with affinity ligands or chromatography.

Cloud point extraction (CPE) is the separation of solute in the aqueous micellar solution which is formed by mixing the surfactants (mostly a non-ionic surfactant) with water and turn to be a two-phase system beyond the critical temperature. The system comprises a surfactant-rich phase and a dilute aqueous that does not or contain less micelle above its cloud point by alteration of conditions such as temperature or salt concentration (Raghavarao et al. 2003). Unlike traditional organic LLE, cloud point extraction requires a small amount of environment benign surfactants. CPE has been thoroughly studied for the separation of polychlorinated phenol (Fernández E et al. 1998). It has also applied successfully for the organic pollutants (Xie et al. 2010), vitamins (Casero et al. 1999) and casein proteins (Lopes et al. 2007). However, the elevated critical micellar temperature or cloud point (at which the two-phase formation begin) is the main bottleneck of the process. Thermoseparating polymer systems are yet another modified LLE similar to CPE systems. The use of thermoseparating polymers permits phase separation as the temperature of the thermoseparating polymer solution is increased above the cloud point of

polymers. Once the temperature rises beyond the polymer cloud point, phase separation occurs, and solute gets partitioned in the top water-rich phase. The significance of the method is recycling of polymer phase and less salt concentration during separation. Hence further purification can be done quickly (Ferreira et al. 2008). The purification of various biomolecules like BSA (Berggren et al. 1995), BSA and lysozyme (Persson et al. 1999), apolipoprotein (Persson et al. 2000), amylase (Li et al. 2002), IgG (Ferreira et al. 2008), DNA (Kepka et al. 2004), amylolytic enzyme (Li and Peeples 2004), ciprofloxacin (Chen et al. 2014), has been reported. The thermoseparating polymer provides a mild environment for separation of biomolecules like proteins and enzyme, but a solubilising mechanism is not well understood (Leong et al. 2016).

1.5 Reverse Micellar Extraction (RME)

It is a microemulsion of two immiscible liquids that provide polar core in a solvent phase to solubilise target solute without loss of structure and function. RME is a biphasic process that involves partitioning of a target solute from aqueous feed to an organic phase and then operating the following transfer to a second aqueous (stripping) phase (Kilikian et al. 2000). Reverse micelle (RM) can host the proteins in an aqueous environment, effectively shielding them from an immiscible one. The inverse micellar solvent contains small droplets of water, stabilised within an organic solvent by a surfactant. The protein can move from an original aqueous phase into these small encapsulated water droplets and remain intact into an aqueous core of RM (Anjana et al. 2010). Over the other LLE extraction methods, RME offers several desirable features. Firstly, the partitioning of solute can be regulated by controlling the size and shape of the RM. The second important feature is, partitioning and selectivity of the solute, especially for proteins, can be achieved in RME due to the hydrophobic nature of the protein since RM provide a hydrophobic and hydrophilic environment to the solute simultaneously. Further, convenient recovery of solute from RM phase is possible by exploring the dissembling nature of RM that also make the possibility of reusing the solvent phase which contains the surfactants. In

contrast, efficient recycling of polymer is not developed in case of ATPE (Mathew and Juang 2007). The reverse micellar system (RMS) poses several unique characteristics like thermodynamic stability, low interfacial tension, spontaneous formation of RM, large surface area, the ability to solubilise polar substances. These features make the selective extraction of solute possible (Krishna et al. 2002). RME is based on charge-charge interaction, hydrophobicity, and size of the protein relative to the droplet. The RMS can be easily scaled up, and the principle allows for continuous separation processes. The enlisted features make the RME as the suitable replacement to the other methods like precipitation, ultrafiltration and chromatography.

RM has the number of dynamic properties that help to maximise the solubilisation of solute into it, which may be manipulated by changing the operating conditions. pH, water content (W_0) and ionic strength of the feed phase are critical process parameters that are responsible for the partitioning of the solute to RM phase. Optimum W_0 in the RMs depends upon the size of the solute, and sometimes concentration of the solute apart from the phase forming surfactant and solvents (Krishna et al. 2002). pH of the feed phase is another critical parameter that influences the biomolecule solubilisation in RM phase. The variation in pH tends to modify charge on the solute surface. This changed surface charge of solute and surfactant charge are responsible for improved solute solubilisation in RM (Krishna et al. 2002; Pires and Cabral 1996). The ionic strength of the feed phase has equally influenced the partitioning of the solute. It mediates the electrostatic interaction between solute and surfactant molecule that is responsible for the uptake of the molecule to RM (Kilikian et al. 2000; Krishna et al. 2002). The parameters related to solvent phases like types and concentration of surfactants and the presence of co-solvent also have an impact up to some extent on the partitioning of solute (Pires and Cabral 1996).

1.6 Continuous operation

Continuous extraction using RMS is not only helping to improve production with better extraction efficiency but also helps to reduce the labour cost and

time. In addition, it facilitates the less maintenance and easy automation (Kalaivani and Regupathi 2016). More than 25 kinds of liquid-liquid contactors are used at the industrial level. However, in recent years, the development of new extractants with superior selectivity and their efficient extraction characteristics enhanced the application of agitated columns for the treatment of biomolecular systems. Extractors like mixer/settler, agitated column extractor, centrifugal, and membrane extractors can be used for the continuous liquid-liquid extraction of different biomolecules. Few examples of biomolecule extraction are reported in the literature. The extraction of an α -amylase in aqueous solution by TOMAC reversed micellar phase using two mixer-settler units (Dekker et al. 1986), the extraction of a pure recombinant cutinase by AOT reversed micelles with a perforated rotating disc contactor (Carneiro-da-Cunha et al. 1994a) and the recovery of intracellular proteins from *Candida utilis* in a spray column (Han et al. 1994). Though each extractor has several advantages selection of extraction method limits the use of extractors in continuous operations (Krishna et al. 2002).

Among the extractors, rotating disc contactors (RDC) is widely used in industries due to its ease of operation, less power consumption, high efficiency per unit height, high throughput, low cost and provide increased yields over the others. RDC possess better operational flexibility than conventional sieve plate, packed and spray columns. The rotating element of RDC provides a larger interfacial area and better contact between two phases. The performance of an RDC depends highly on the hydrodynamic conditions, which are determined by the structural and flow parameters and also the physical properties of the contacting phases (Moris et al. 1997). The specific modification has been made on RDC structure to improve the performance by considering the nature of the phases and characteristics of the biomolecules, which includes the asymmetric RDC perforated disc contactor, and open turbine RDC and so on (Kalaichelvi and Murugesan 1998; Moris et al. 1997). The RDC was successfully demonstrated as such or modified for the continuous extraction of different bio-

molecules, namely intracellular proteins (Han et al. 1994), lysozyme (Lye et al. 1996; Nishii et al. 1999; Soltanali et al. 2009) , r-cutinase (Carneiro-da-Cunha et al. 1994a), α-LA and β-LG (Kalaivani and Regupathi 2016) using the conventional LLE systems like ATPS and RME.

1.7 Organisation of Thesis

The thesis is organised as five chapters. 'Chapter 1' presents the generic introduction of the thesis which discusses the necessity and importance of protein purification by considering the merits and demerits of general separation and purification approaches employed. It also gives a brief account of the importance of milk and whey proteins and their purification. The implementation of the conventional LLE for the protein purification also discussed.

'Chapter 2' presents the detailed literature review on the characteristics of the whey proteins, specifically the LF and their purification strategies. The RM formation, factors influencing the RM formation and various bio-molecules purified using RMS along with the influence of various parameters on the forward extraction/ solubilisation of biomolecules into the RM and backward extraction of the same to the fresh stripping solution were discussed. Specifically, the application of RME for the whey proteins also reviewed. Based on the literature review, research gaps and the scope of the present research work were identified. Accordingly, the objectives for the present work were defined and presented.

'Chapter 3' explains the Materials used and Methods adopted in the present work. It explains the experimental methodologies, analytical methods and instruments used to achieve the stated objectives.

The results obtained in different experiments as mentioned in chapter 3 were consolidated, analysed and discussed in detail and presented in the 'Chapter 4' as 'Results and Discussion'. The result and discussion was consolidated under four major sub-sections, which includes the (i) screening and selection of the

suitable RMS for extraction of LF (ii) the use of selected RMS for the extraction and purification of the LF from pure protein solution (iii) implementation of selected RMS for the selective extraction of LF from the synthetic mixture of whey proteins and extend the optimized condition for real whey (iv) implementation of RME for continuous extraction of LF in the RDC.

The important results are summarised, and the significant conclusions drawn in the research work is consolidated in the 'Chapter 5' as 'Summary and Conclusion'. The possible direction for future research work also presented in this chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 Whey and whey proteins

Whey is the watery and thin liquid, obtained as a by-product during cheese making. The significant nutritional properties of whey proteins have grabbed attention and now a day's whey is considered as co-product of dairy industries (Carter and Drake 2018). Whey composition depends upon the cheese making process. The whey produced from the addition of rennet to milk during cheese production is called "sweet whey". Whereas, "acidic whey" is produced by acidifying the milk. Based on the cheese making process the physical and chemical properties of whey varies (Alsaed et al. 2013). Other than water, lactose (-70-72% of the total solids), whey proteins (-8-10%), minerals (-12-15%) and vitamins are the major constituents of both sweet and acid whey. Hence, whey is no longer considered a waste product but the treasure house of nutritionally rich whey components (Panesar et al. 2007). The nutritional values of whey proteins have grabbed attention and hence extracted and purified to make it available on a commercial level. The valuable whey proteins are hence concentrated and available as a commercial product namely, whey protein concentrate or whey protein isolate. The typical steps followed to obtain whey protein concentrate or whey protein isolate from whole milk or whey is shown in figure 2.1, which includes the separation processes like, ultrafiltration, evaporation etc., (Fig.2.1).

The major whey proteins are α-LA (1.5g/l), β-LG (3-4g/l), BSA (0.3-0.6g/l) and Ig (0.6-0.9g/l) whereas proteins like LPO (0.001-0.003g/l) and LF (0.003-0.1g/l) are also present in comparatively less concentration (Du et al. 2013). Even though whey proteins altogether are abundant in nutritional values, the individual proteins in the purified form have unique biological functions.

Therefore, extraction and purification of an individual protein are focused by various researchers since the last few decades (Smithers 2015). Proteins like α-LA, β-LG, BSA and Ig are the major constituents of the whey proteins, and hence, the purification of such proteins is possible with fewer steps with lesser efforts. Whereas, proteins like LF and LPO are the minor proteins but have equal biological importance. Hence, extraction and purification of these proteins and make it available in the market on a large scale is a challenging task (Du et al. 2013; Kalaivani and Regupathi 2015).

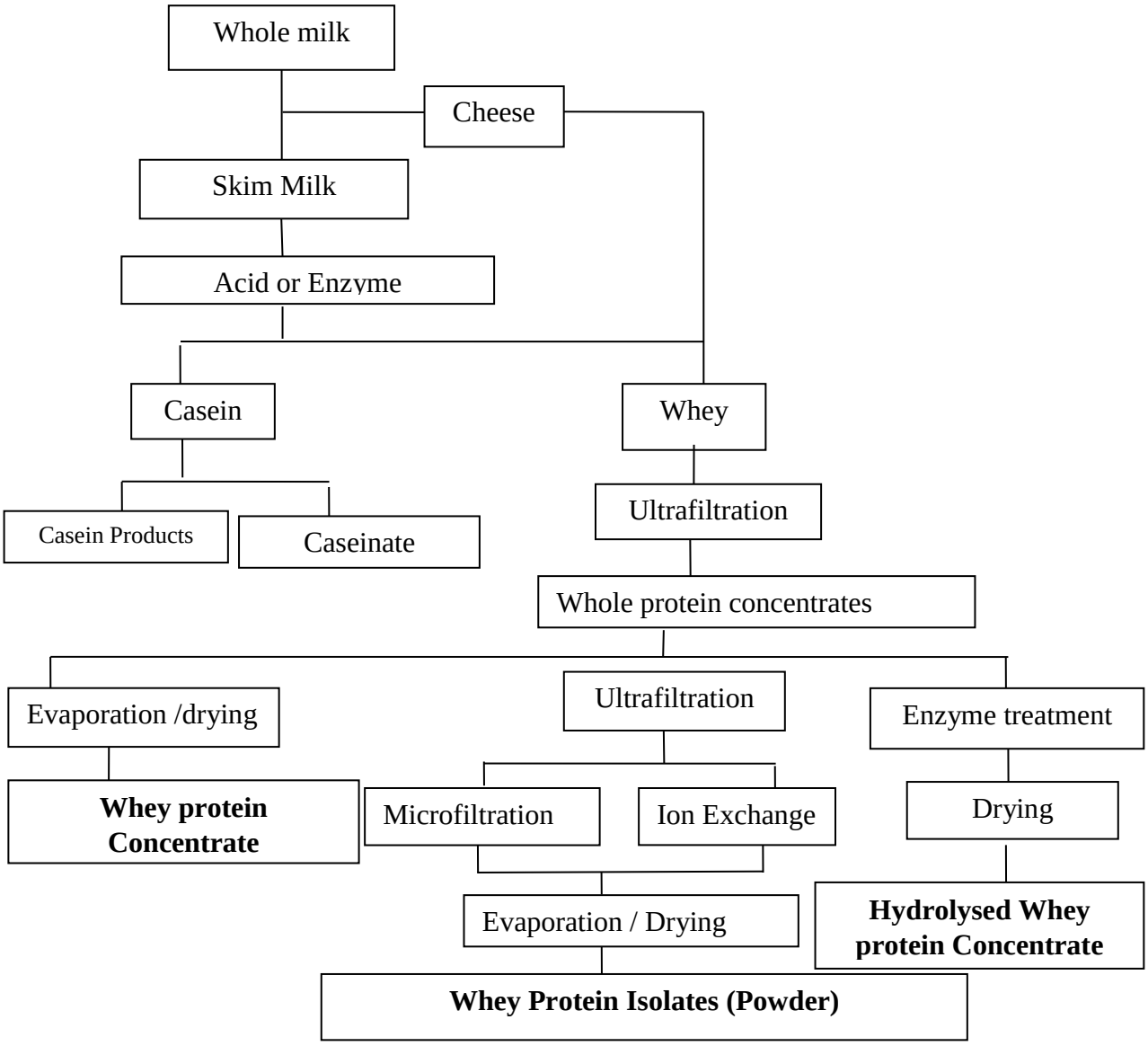


Fig. 2.1: Steps involved in the isolation of whey protein

α-LA is the primary protein in human milk and is therefore extremely important for infant nutrition. Bovine α-LA has high homology with human α-LA. It is a calcium-binding protein that may have a role in calcium transport. It has a high affinity for other metal ions, including Zn^{+2}, Mn^{+2}, Cd^{+2}, Cu^{+2}, and Al^{+3}. It has high cysteine content, which is a building square of glutathione, an amazing cancer prevention agent in the body that assumes an essential job in resistance α-LA is also a rich source of tryptophan. Hence, it also serves as a precursor to the neurotransmitter serotonin and the neurosecretory hormone melatonin. These hormones have a crucial role in regulating neurobehavioral effects such as appetite, sleeping-waking rhythm, pain perception, mood, anxiety and stress control (Permyakov and Berliner 2000). β-LG is a protein of ruminant species and is also present in the milk at higher concentration. *β*-LG also plays an essential role in hydrophobic ligand transport and uptake, enzyme regulation, and the neonatal acquisition of passive immunity. It also functions as a fatty acid or lipid-binding protein (Kontopidis et al. 2004) and retinol carrier and acts as antioxidants (Pereira 2014). GMP contains branched-chain amino acids and but lacks aromatic amino acids like phenylalanine, tryptophan, and tyrosine. GMPs are safe for individuals with phenylketonuria. BSA is a rich source of essential amino acids (Marshall 2004).

Ig is an antibody, or they are known as gamma globulins. It plays a significant role in the immunological activity. Ig is classified into five classes as, IgA, IgD, IgE, IgG, and IgM. Among these, IgG is the primary antibody in adults that constitutes about 75%, and 25% constitutes of the remaining antibodies. IgG also serves as a first-line immune defence to the offspring. IgA is secreted in breast milk transferred to the digestive tract of the newborn infant, providing better immunity (Marshall 2004). LPO is an active antimicrobial agent. Consequently, applications are being found in preserving food, cosmetics, and ophthalmic solutions. Furthermore, LPO is also used in dental and wound treatment. It also acts as anti-tumour and anti-viral agents (Kussendrager and van Hooijdonk 2000). LPO catalyses the peroxidation of thiocyanate and some

halides like iodine and bromium, which generates products that inhibit the growth or kills several bacterial species (Marshall 2004).

2.1.1 LF

LF is a glycoprotein, and a single chain polypeptide of about 80KD containing 1-4 glycan varies according to species. Bovine and human LF consist of 689 and 691 amino acids, respectively. The 3-D structures of bovine (Fig 2.2) and human LF are very similar, but not identical (Moore et al. 1997; Steijns and van Hooijdonk 2000). Each LF comprises two homologous lobes, called the N- and C-lobes, referring to the N-terminal and C-terminal part of the molecule, respectively. LF has a very high isoelectric point. The theoretical pI values for bovine and human LF are 9.4 and 9.5 respectively (Steijns and van Hooijdonk 2000). The gene of LF is located at chromosome no.3 in human and no.9 in the mouse. The size of the gene is around 23kb to 35kb. The LF gene is organised in 17 exons, out of which 15 are identical in mammalian species like cow, pig and mouse (González-Chávez et al. 2009). LF has a very high affinity towards Fe^{3+} ions and shows the property of transferring the same. The iron binding capacity of this milk protein component was discovered in 1960. Along with iron it also has a high affinity for Cu^{2+}, Zn^{2+} and Mn^{2+} (Adlerova et al. 2008).

LF is a dynamic protein and is involved in different biological functions. It is involved in iron metabolism and also helps to maintain the iron level in the body (Adlerova et al. 2008). It protects the infant in the initial day of life during the development of immunity (O'Riordan et al. 2014). LF is helpful to control inflammation caused by microbes. It also acts as an anticancer agent, and inhibits tumour growth and metastasis in organs, like esophagus, tongue, lung, liver, colon and bladder. LF plays a vital role in organ morphogenesis (Ward et al. 2005). The effectiveness of LF for gram-positive and negative and few acid-alcohol microbes has been documented by various researchers (González-Chávez et al. 2009). LF provides protection from viruses which infects humans as well as animals (Steijns and van Hooijdonk 2000). It also stimulates bone growth in vivo (Naot et al. 2005). LF used as a marker for inflammatory

gastrointestinal disorders and colon cancer (Naot et al. 2005). The schematic structural diagram of bovine LF is presented by (Moore et al. 1997) (Fig 2.2)

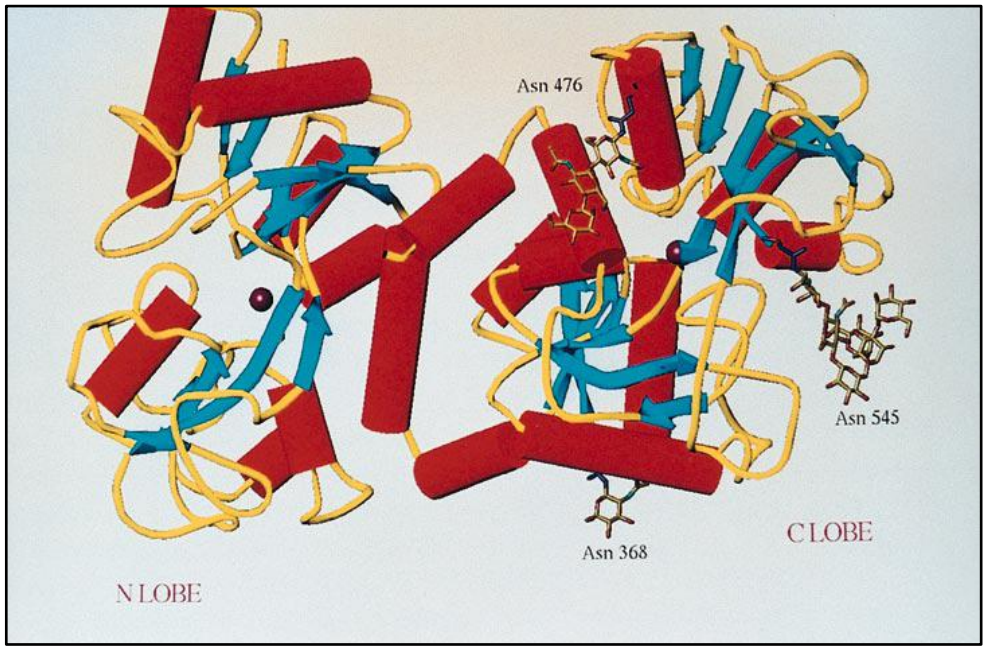


Fig. 2.2: Schematic diagram of bovine LF. Cylindrical shape and arrows indicate α-helices and β- sheets respectively. Purple spheres show Fe molecule. Carbohydrate chains attached to C-lobe are indicated by sticks (Moore et al. 1997)

2.1.2 LF extraction and purification

Various researchers have reported several extractions, and purification methods for the LF solely or with other proteins concentrate since last four decades. In the early years, the chromatography technique was used for the purification of LF (Table 2.1). Al-Mashikhi and Nakai (1987) have used gel filtration techniques for purification of LF and IgG from colostrum milk. Extraction of LF with IgG from bovine colostrum and whey was reported by following cation-anion exchange chromatography (Wu and Xu 2009). A Simulated Moving Bed (SMB) technology was applied by Andersson and Mattiasson (2006) for the extraction of LPO and LF from whey protein concentrate. Carboxymethylcellulose column was used to separate and purify LF and LPO from bovine acidic whey by cation exchange chromatography (Yoshida and Ye-

Xiuyun 1991). The ion exchange membranes were also used to extract LF and LPO (Chiu and Etzel 1997; Ulber et al. 2001). RME of LF using oleic acid sodium salt (Inoue et al. 2005), and cationic surfactant (Anjana et al. 2010) also reported. Fuda et al.(2004) have reported the capture of LF using colloidal gas aphrons. Ndiaye et al. (2010) have used electrodialysis with ultrafiltration for LF extraction and purification. LF and LPO extraction with cation exchange resins was reported by Liang et al. (2011). Lu et al. (2007) have coupled cation exchange chromatography with ultrafiltration to purify LF. Affinity-based separation of LF was reported by Chen et al. (2007). Carvalho et al. (2014) have used a super-macro porous column of polyacrylamide cryogel, loaded with copper ions for LF separation. Partial purification of LF with aqueous two-phase system consisting of PEG 1000, and 4000/ sodium citrate was reported by Costa et al. (2015).

The reported methods were conducted in a batch process for very less volume. The use of expensive column, regeneration at regular time interval and preservation limits the continuous operation of such methods. Moreover, chromatographic methods need the pre-treatment of the raw material. Such requirements of these methods make the separation process as a multistep method. The limitations and drawbacks of methods like chromatography, simulated moving bed, membrane separation, ultrafiltration limits the selective extraction of LF from multiple biological sources like whey. Hence, a need for the development of a simple conventional extraction method that able to extract the LF with high selectivity and easily scaled-up in a continuous operation.

Table 2.1: Techniques to purify LF and other whey proteins and their limitations

Whey protein	Process	Inference	Limitations	Reference
Ig and LF	Gel filtration technique	99% 83.3% and 92% recovery from colostral, acid and sweet whey was obtained together for Ig and LF	Scale up is difficult	(Al-Mashikhi and Nakai 1987)
LF and LPO	Carboxymethyl cation exchange chromatography	41mg of LPO, 21mg of LF-a and 67mg of LF-b was separated	Ig was also observed in extracts hence not a selective extraction method for LF and LPO	(Yoshida and Ye-Xiuyun 1991)
LF and LPO	Cation exchange membrane	73% LPO and 55% LF was recovered	Scale up is difficult	(Chiu and Etzel 1997)
LF and LPO	Membrane	85% pure LPO and 95% LF obtained from sweet whey	Multistep process	(Ulber et al. 2001)
LF and LPO	Colloidal gas aphrons	Altogether 90% recovery of LF and LPO from sweet whey	Contaminant proteins were also found in the extracted phase hence it is not a selective extraction process	(Fuda et al. 2004)

Whey protein	Process	Inference	Limitations	Reference
LF	Oleic acid sodium salt RMS	Very less extraction (150ng/ml) of LF is obtained from whey	The process is not optimised to improve extraction efficiency	(Inoue et al. 2005)
LF and LPO	Packed bed cation exchange chromatography.	Direct chromatographic capture from raw milk minimises processing time and avoids the fat and casein removal steps	Scale up is difficult	(Fee and Chand 2006)
LF and LPO	Simulated moving bed	48% yield was obtained	Continuous operation is difficult	(Andersson and Mattiasson 2006)
LF	Magnetic affinity separation	Less extraction of LF (62.5%) was observed	Loss of LF has been reported as pre-treatment of acid whey is required	(Chen et al. 2007)
LF	Ultrafiltration coupled with strong cation exchange chromatography	82.46% recovery of LF was obtained from colostral with 94.20% purity	Multistep purification makes the process costly	(Lu et al. 2007)

Whey protein	Process	Inference	Limitations	Reference
LF and IgG	Serial cation-anion exchange chromatography	95% and 96.6% purity of LF and IgG was obtained from bovine colostrum	Difficulty in scale-up of the purification process	(Wu and Xu 2009)
LF	CTAB/Isooctane and CTAB/TX100/Iso octane RMS	100% capture of LF from whey to RM phase was achieved	LF was not further purified by back extraction	(Anjana et al. 2010)
LF	Electrodialysis with an ultrafiltration membrane (EDUF) system	15% extraction of LF was obtained from whey	Limits the selective extraction of LF due to the presence of β-LG	(Ndiaye et al. 2010)
LF and LPO	Cation exchange resins	91% purity of LF was obtained and 67.3% LPO purity was obtained from bovine colostrum	Scale up is difficult	(Liang et al. 2011)
LF	Super-macroporous column of polyacrylamide cryogel, loaded with Cu^{2+}	Purified LF was extracted with the highest yield	Ultrafiltration of whey was required to avoid clogging of the column. The method was developed for very less sample volume (2ml).	(Carvalho et al. 2014)

Whey protein	Process	Inference	Limitations	Reference
LF	Aqueous two-phase extraction with PEG 1000 and 4000/ sodium citrate	1000 fold LF was concentrated in sodium citrate phase	LF was partially purified	(Costa et al. 2015)

2.1.3 LLE of LF

LLE is a well-established unit operation. To overcome the limitations of the chromatographic separation process, LLE may be an alternative process for the selective extraction and purification of LF. It facilitates easy continuous operation on a large scale. However, the conventional organic solvents used in the LLE may denature the fragile protein molecule. The modified non-conventional systems namely ATPE and RME were employed to extract few abundant whey proteins from its pure solutions or complex biological sources. The aqueous biphasic systems including polyethylene glycol 1000 -trisodium citrate (Kalaivani and Regupathi 2015) and polymer - phosphate salts (Zhang et al. 2016) systems were used for the partitioning of α-LA and β-LG from acidic whey. RME of α-LA using ionic surfactants was also reported by (Gomes et al. 2017; Naoe et al. 2004). The possibility of RME of proteins using various RMS was also demonstrated by considering the generic whey protein BSA as a model protein (Pawar et al. 2017b). Specifically, the RM formed by ionic as well as mixed surfactants of ionic and non-ionic surfactants have been implied for the extraction of LPO (Nandini and Rastogi 2010).

2.2 RM

RM, the water-in-oil microemulsions (Fig 2.3), are formed when a small amount of water and surfactant(s) are mixed in an apolar organic solvent above a specific critical concentration. Surfactant forms a monolayer around the water

pool, which separates the water and oil portion and reduces the unwanted solvent-water contact. The term “Microemulsions” was defined by Schulman in 1959 (Schulman et al. 1959). RMs are clear and thermodynamically stable dispersions of two non-soluble liquids with enough amounts of surfactant. In contrast to non-microscopic emulsions, which are thermodynamically unstable, the nano-sized micro-emulsion droplets are formed spontaneously. Although the RMS is heterogeneous on a molecular scale, the phases (aqueous and micellar phase) are in equilibrium and thus thermodynamically stable. The interaction between polar head-groups of the surfactant molecule and the nonpolar tails favours the aggregation of the surfactants at a very specific size and molecular configuration. Microemulsions typically have narrow droplet size distributions. The droplet uniformity of microemulsions is very crucial and has a direct impact on the distribution of resulting particle sizes (Uskoković and Drofenik 2005).

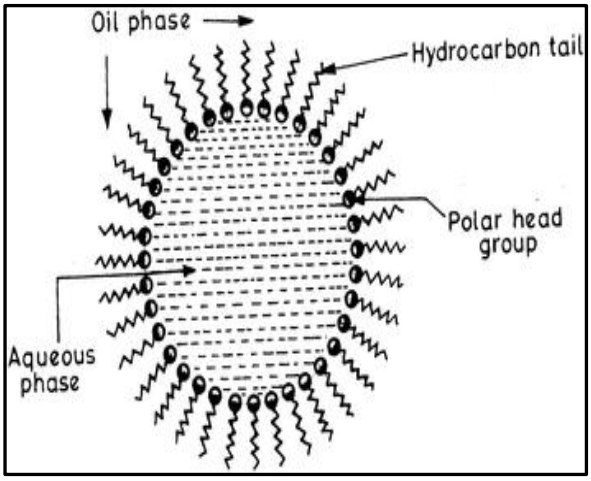


Fig. 2.3: Structure of RM

2.2.1 Surfactants

Surfactants are chemical compounds and can change the properties of the fluid interface. These are widely used in remediation of organic contaminants in oil recovery (Pope and Wade 1995). The surfactant contains a hydrophilic group and hydrophobic moiety (West and Harwell 1992). The whole monomer is commonly known as amphiphile due to its dual nature. The hydrophobic portion of the surfactant monomer is a long hydrocarbon chain, called as the "tail". The

polar "head" group posses anions or cations. The typical surfactant monomer is a tadpole structure (Fig 2.4).

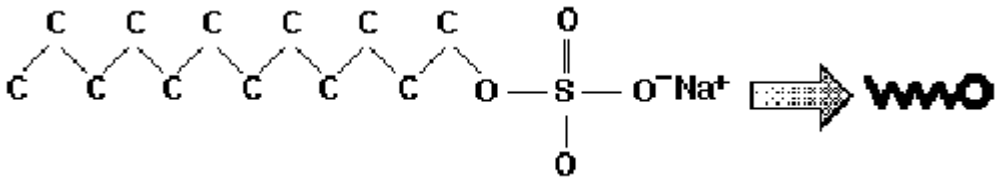
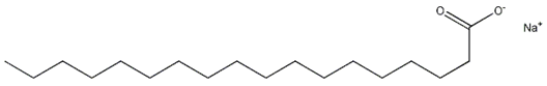
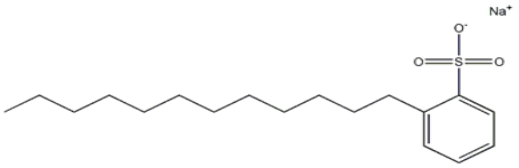


Fig. 2.4: Sodium dodecyl sulphate surfactant monomer

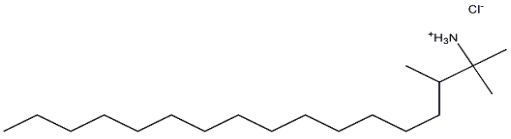
The polar gathering of the surfactant monomer gives high solvency in water. The hydrophobic gathering of the monomer, be that as it may, likes to dwell in a hydrophobic stage, for example, oil. Based on the nature of the polar group, surfactants are mainly categorised as non-ionic, anionic and cationic surfactants. Anionic surfactants (Fig.2.5) have a negatively charged head (example: soaps). Cationic surfactants have a positively charged head (example: alkyl ammonium chlorides). Non-ionic surfactants have a polar, but uncharged, head (example: polyethene ethoxylates) (Salager 2002).



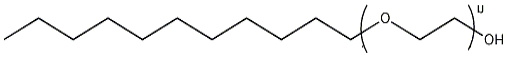
Sodium stearate (soap) (anionic)



Sodium dodecylbenzene sulfonate (anionic)



Trimethylhexadecyl ammonium chloride (cationic surfactant)



Polyethylene ethoxylate (non-ionic)

Fig. 2.5: Types of surfactants

2.3 RME

RM based LLE is a most recognised method for separation and purification of biomolecules. It has the ability to solubilise the solute in water pool of the inverse micelle which is the first step of extraction known as forward extraction. Further, the solute entrapped in water pool surrounded by organic or oil phase can be quickly released by disassembling the RM-structure. The release of solute to the aqueous phase is a crucial step during the RME and is defined as back extraction. The partitioning of solute into the micellar phase can be controlled by changing the shape and size of RM as per the requirement. The key components of RMS, i.e. surfactants and solvents, retain the structural and functional properties of the solute after extraction and purification (Kilikian et al. 2000). Hence, RM based extraction and separation are not only used for biomolecules like protein, enzymes and peptides but it is also applied for the purification of nucleic acids. Along with the proteins and nucleic acids the recovery of peptides, organic acids, antibiotics and steroids with RM is also reported. However, the poor understanding of the solubilisation mechanism restricts the implementation of the process on an industrial scale. The micellar phase components do not lose their functional property during the extraction process, and hence the micellar phase may be reused/recycled number of times over the cycles. The recycling of the recovered micellar phase helps to make the process economical (Krishna et al. 2002).

Since four decades, many researchers have reported the successful extraction of various proteins, enzymes as well as nucleic acids. The literature available briefs the application of micellar extraction process for a number of biomolecules (Table 2.2). The extraction of solutes from the mixture of biomolecule solution containing similar or different proteins and pure solution of the biomolecule was reported. The RME of various biomolecules has been performed using different surfactants individually or a mixture of surfactants. All kind of surfactants, i.e. cationic-anionic and non-ionic surfactants have been explored for the micellar extraction. AOT (anionic surfactant) has been widely used in various

bimolecular extraction like cytochrome c, lysozyme, ribonuclease A, trypsin, α-chymotrypsin, papain, pepsin, horseradish peroxidase, lysozyme, myoglobin and BSA from its synthetic solution as it has the ability to form bigger RM. The anionic surfactants like AOT have also been used to extract IgG from colostral whey, enzymes like inulinase, protease, lipase, chitosanases, nattokinase, laccase, acid phosphatase from different fermentation broths. On the other hand, cationic surfactants are known to form stable reverse micellar structure. Hence, the surfactants like TOMAC, CTAB, DTAB has been used for the extraction of α-amylase, BSA, papain, ovalbumin, kallikrein, amino acids from their synthetic solution whereas, molecules like lipase (fermented rice bran), bromelain (pineapple peel), r-human interferon gamma (fermentation broth), soyhull peroxidase (soybean seeds) also extracted/purified from their respective sources. Very few reports are available for the micellar extraction with non-ionic surfactants like TRPO, tween series, Triton series, e.g. lysozyme, matrine, single-stranded DNA and soy proteins. Few researchers have reported the mixed surfactant based RME (anionic + non-ionic / cationic + non-ionic) for lipase, β-glucosidase, soy proteins etc. in order to get better extraction (Table 2.2).

Table 2.2: RMS employed for various biomolecule extraction

Molecule	Source	RMS	References
The mixture of pure proteins	A mixture of pure proteins	TOMAC/ Rewopal HV5/ isooctane/octanol	(Wolbert et al. 1989)
cytochrome c, lysozyme, ribonuclease A, trypsin, α -chymotrypsin, papain, pepsin, and BSA	Pure protein solution	AOT/isooctane	(Leser and Luisi 1990)
α-chymotrypsin, cytochrome c	Pure proteins solution		(Marcozzi et al. 1991)

Molecule	Source	RMS	References
Horseradish peroxidase	The mixture of pure horseradish peroxidase and alkaline phosphatase		(Paradkar and Dordick 1991)
Pepsin, chymosin	Pure porcine pepsin and bovine chymosin		(Carlson and Nagarajan 1992)
α-amylase	Pure α-amylase	TOMAC/ isooctane	(Hilhorst et al. 1992)
Cytochrome-c	Pure cytochrome-c	AOT/isooctane	(Ichikawa et al. 1992)
Lysozyme and myoglobin	Pure proteins solution		(Nishiki et al. 1993)
Peroxidase	Soybean hull		(Paradkar and Dordick 1993)
Haemoglobin	Pure Haemoglobin	DOLPA/ isooctane AOT/ isooctane	(Ono et al. 1996)
Horseradish peroxidase	Horseradish roots	AOT/ isooctane	(Regalado et al. 1996)
Lysozyme	Standard protein solution	Tween 85 / 2-propanol / hexane	(Vasudevan and Wiencek 1996)
α-amylase	Standard solution	Aliquat 336 and 1% (v/v) n-alcohol / isooctane	(Chang et al. 1997)
Erythromycin Benzyl-penicillin, actidione, oxytetracycline	Synthetic solution	AOT/isooctane	(Fadnavis et al. 1997)
Cytochrome-c, BSA	Pure proteins	AOT/isooctane AOT/Lecithin	(Hong et al. 1997)

Molecule	Source	RMS	References
Inulinase	*K. marxianus* fermentation broth	BDBAC/isooctane/hexanol	(Pessoa Jr and Vitolo 1997)
Lysozyme	Egg white	AOT/isooctane	(Chou and Chiang 1998)
Amino acids	Pure amino acids solution	TOMAC/hexanol/n-heptane	(Barradas et al. 1999)
Cutinase	Fermentation Broth	AOT/isooctane/hexanol	(Carvalho et al. 1999)
Lipase	*C. viscosum* lipase	AOT/Tween 85/isooctane	(Hossain et al. 1999)
Lysozyme, CAB, BSA, β-LG	Pure proteins solution	AOT/isooctane	(Hong et al. 2000)
Acid phosphatase	fermentation broth of *A. niger*		(Soni and Madamwar 2000)
Penicillin acylase	*E. coli* fermentation broth		(Gaikar and Kulkarni 2001)
BSA	Standard protein solution	CTAB/hexanol/petroleum ether	(Zhang et al. 2002)
Lysozyme, ribonuclease-A, cytochrome-c	Pure proteins solution	AOT/isooctane	(Kinugasa et al. 2003)
Lysozyme	Pure lysozyme solution	DODMAC/ isooctane	(Shin et al. 2003a)
		AOT/isooctane DODMAC/isooctane /decanol	(Shin et al. 2003b)
IgG	Colostral whey	AOT/isooctane	(Su and Chiang 2003)

Molecule	Source	RMS	References
Nattokinase	*Bacillus natto.* fermentation broth		(Liu et al. 2004)
Lysozyme and Bovine carbonic anhydrase (CAB)	Pure lysozyme and CAB		(Lee et al. 2005)
Papain	Pure papain solution		(Mathew and Juang 2005)
Protease	*Nocardiopsis sp.* fermentation broth		(Monteiro et al. 2005)
Chitosanases	*B. cereus* fermentation broth		(Chen et al. 2006)
β-galactosidase	Barley extract		(Hemavathi et al. 2008)
Matrine	Pure matrine	TRPO/Cyclohexane	(Dong et al. 2009)
Penicillin-G	Model penicillin-G	AOT/isooctane	(Mohd-Setapar et al. 2009)
Lipase	Fermented rice bran	CTAB/ isooctane	(Nandini and Rastogi 2009)
β-glucosidase	Barley	AOT/Tween 20 AOT/Tween 80 AOT/Triton X-100 AOT/Tween 85	(Hemavathi et al. 2010)
Soy hull peroxidase	Soybean seeds	CTAB/isooctane	(Lakshmi and Raghavarao 2010)
Soybean protein	Soy flour	Triton-X-100/ toluene AOT/toluene	(Zhao et al. 2010)

Molecule	Source	RMS	References
Lipase	*Pseudomonas sp. CSD3* Fermentation Broth	AOT/ isooctane	(Gaikaiwari et al. 2012)
Laccase	*C. versicolor* Fermentation Broth	RL/isooctane/n-hexanol	(Peng et al. 2012)
Kallikrein	Commercial kallikrein	CTAB/n-octane/n-hexanol	(Zhou et al. 2012a)
Chymotrypsin	Red Perch	AOT/isooctane	(Zhou et al. 2012b)
Amoxicillin	Pure amoxicillin solution	AOT/Tween 85	(Chuo et al. 2014)
Tea polysaccharide	Synthetic solution	AOT/Heptane	(Li and Cao 2014)
Enzymes and amino acids	A mixture of pure enzyme and amino acids	AOT/isooctane	(Storm et al. 2014)
Ovalbumin	Synthetic ovalbumin solution	CTAB/ n-Hexane/ 1-hexanol DTAB/ n-Hexane/ 1-hexanol	(Ding et al. 2015)
Lectin	Black turtle bean	AOT/isooctane	(He et al. 2015)
Single-stranded DNA	A synthetic solution of DNA	lauroyl-sn-glycerol-3- phosphocholine (DLPC)/isooctane/1-hexanol	(Maruyama and Ishizu 2016)

Molecule	Source	RMS	References
Bromelain	Pineapple peel	Gemini surfactant/ n-Hexane/ 1-hexanol DTAB/ n-Hexane/ 1-hexanol	(Wan et al. 2016)
Papain	Standard papain solution	CTAB/isooctane	(Prabhu A et al. 2017)
Erythromycin and amoxicillin	Pure antibiotics solution	Sophorolipids	(Chuo et al. 2018)
r-human interferon gamma	*K. lactis GG799* fermentation broth	CTAB/isooctane/n-butanol	(Pandey et al. 2018)

Proteins or any biomolecules can be extracted from an aqueous phase to RM phase. Major interactions that govern the extraction and purification of biomolecules are electrostatic, hydrophobic, van der walls forces and hydrogen bonding (Hilhorst et al. 1992). Among these, electrostatic interactions are most important in biological systems as most of the biomolecules are charged under physiological conditions. The importance of electrostatic interaction lies in a fact that they tend to be highly specific (Gupta et al. 2002). Hence, the interaction between the micelles and solutes may be manipulated for the selective partitioning/ purification of biomolecules by altering certain factors like pH of an aqueous solution, ionic strength, the addition of additives, co-surfactants, etc., along with selecting the appropriate RM phase forming agents like surfactants and organic solvents. The effect of these factors on the extraction efficiency of specific biomolecules needs to be studied for better extraction of any biomolecules and to develop efficient extraction process.

2.3.1 Critical Micellar Concentration (CMC)

The least amount of surfactant required to form RM is defined as CMC. At very less concentration, the surfactant molecules are favourably adsorbed at air-water interface with its hydrophobic tail pointing in the opposite direction to the water surface and reduce the interfacial tension. When the concentration of surfactant in the bulk solution crosses the limiting value, the surfactant molecules agglomerate to form a micelle, which is exhibited by a rapid change in physicochemical properties. The CMC is also known as the concentration at which the micelles first appears in solution. It is determined from the notable changes in the plot of some physicochemical properties of the solution against surfactant concentration (Fig.2.6). The CMC of surfactant can be measured by exploring some of the physicochemical properties like density, viscosity, refractive index, surface tension, turbidity. The knowledge of CMC in RME is a fundamental and essential parameter to determine the minimum surfactant concentration required to form the RM in order to achieve better extraction (Kilikian et al. 2000).

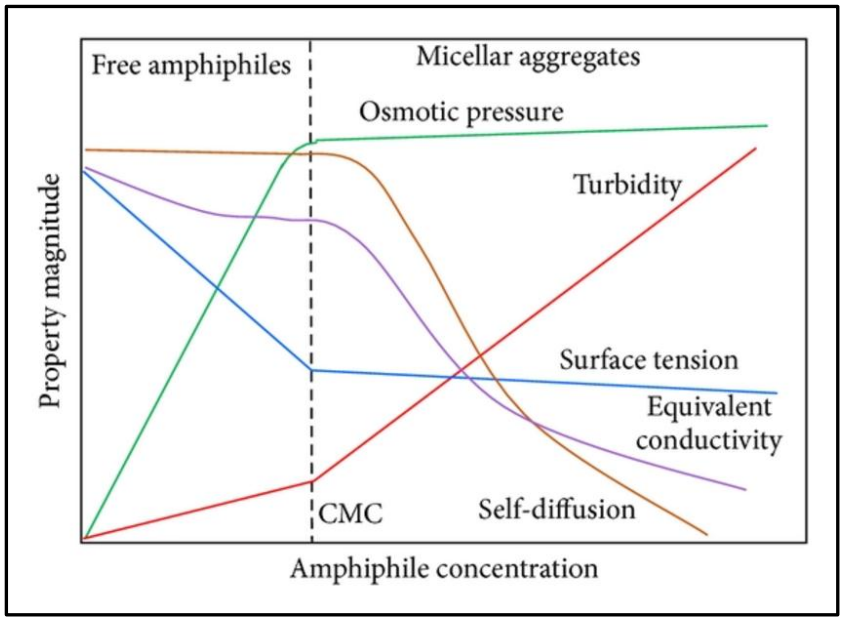


Fig. 2.6: Behaviour of few physiological properties at micelle formation in bulk solution (Salager 2002)

2.3.2 Surfactant type and concentration

Type of surfactants selected for RME plays a crucial role. The protein distribution is mainly dependent on the charge difference between the protein and the surfactant head groups. Also, a surfactant selected may affect protein/enzyme activity (Kilikian et al. 2000). For many biomolecules, RME studies are done with an only ionic surfactant or in combination with a non-ionic surfactant. Hilhorst et al. (1992) used a cationic surfactant (TOMAC) for extraction of α-amylase. Regalado et al. (1996) carried out horseradish peroxidase extraction using anionic (AOT) surfactant. Different cationic surfactants like CTAB, TOMAC used for extraction of glucoamylase for a comparative study to get better extraction efficiency (Forney and Glatz 1995). The use of cationic and ionic surfactants for the extraction of biomolecules showed loss of protein activity in many cases due to their strong charges at the aqueous/organic interface (Krishna et al. 2002). The concentration of surfactant present in organic phase majorly responsible for the better solubilisation of any biomolecules into the RM. Gaikaiwari et al. (2012) have used anionic (SDS & AOT), cationic (CTAB) and non-ionic (TX100 & T80) at a similar concentration, i.e. 25mM for the comparative study of effective lipase solubilisation into RM. It was observed that the use of SDS resulted in denaturation of lipase. Non-ionic and cationic surfactants could not solubilise the lipase in organic phase at the end of forward extraction. Only 33% activity recovery with 4.2 fold purification was obtained with AOT. Whereas, Nandini and Rastogi (2009) have obtained 85.49% enzyme recovery with CTAB in the forward extraction of *A. niger* lipase. The effect of surfactant (CTAB) concentration (0.10–0.25 M) on the forward extraction of lipase showed that the increase in CTAB concentration from 0.10 to 0.20 M increases the extraction efficiency of protein (from 54.37% to 68.49%) and activity recovery of lipase (from 61.50% to 85.49%). Beyond 0.20 M concentration, the activity recovery and extraction efficiency were found to decrease up to 75.69% and 66.71%, respectively. The increase in the activity recovery of lipase with the increase in

surfactant concentration up to 0.20 M may be explained based on the fact that the increase in surfactant concentration increases in some surfactant aggregation and RM, which in turn enhances the extraction. Further increase in surfactant concentration causes micellar clustering, which decreases the interfacial area available to the target biomolecule resulting in a decrease in the extraction capacity of the RM. Also, the inter-micellar collisions occur more frequently because of their large population at a higher surfactant concentration, which results in de-assembling/ deformation of RM and leads to decreased extraction.

The effect of AOT concentration was studied observe its concentration impact on the recovery of nattokinase by Liu et al. (2004). The increased concentration of AOT from 50 to 200 mM, resulted in the gradual increment in the protein recovery. However, the activity recovery has remained the same, which was responsible for minor decrease in purity. Hence, the conclusion was drawn that the increase of protein recovery was due to more transfer of protein impurities.

The concentration of CTAB was varied in the range of 50mM to 250mM to improve solubilisation of soy hull peroxidase. It was observed that solubilisation of the enzyme increased with increase in CTAB concentration. Highest extraction, i.e. was obtained at 150mM, but further increase in CTAB concentration led to a decrease of extraction due to inter-micellar collision (Lakshmi and Raghavarao 2010). A study conducted by Li et al. (2007) for the extraction of BSA using CTAB/isooctane/1-pentanol system have shown that extraction can be increased with increased in CTAB concentration by keeping constant pH value. At pH values of feed phase near to pI of BSA, zero protein is transferred to the micelle phase. Whereas, at pH values more than the isoelectric point, the extraction increased drastically. The obtained enhanced transfer of protein was due to the net negative charge of BSA at pH above its pI, and that increased attraction towards the positively charged polar groups of surfactant plays a vital role in the extraction process. Peng et al. (2012) reported the effect of Rhamnolipid (RL), a kind of bio-surfactant concentration on the solubilisation of laccase in the micellar phase. With the RL concentration

increasing from 2.7 to 3.3mM, the activity recovery, forward extraction efficiency and purification fold to 85.4%, 57.5% and 4.46, respectively. However, the further increasing content of RL resulted in decreases in activity recovery, forward extraction efficiency and purification fold. The effects of surfactant concentration on forward extraction efficiency of single AOT, single Tween 85, and mixed AOT/Tween 85 surfactant systems for amoxicillin were examined by Chuo et al. (2014). The surfactant concentrations were varied between 0 to 200 g/L. The maximum solubilisation of amoxicillin obtained at 50 g/L concentration mixed (AOT/Tween 85) RMS. High amoxicillin extraction was achieved at 50 g/L, i.e. 88%. The efficiency of amoxicillin extraction decreased at higher total AOT/Tween 85 concentration. At concentrations of 100 g/L. However, the optimised AOT concentration for highest extraction would be at least 200 g/L or higher. Whereas, a single Tween 85 surfactant system shows extremely low forward extraction efficiency. Essential to underline, that the expansion of non-ionic surfactant makes the course of action of surfactant particles at the interface more compact and rigid. As the size and properties of the molecule change its behaviour and interaction with amphiphile also found to be changed. Hence, various studies reported suggest optimising surfactant concentration in the organic phase to obtain highest solubilisation of biomolecules.

2.3.3 W_0 and water pool

W_0 plays a significant role in solute solubilisation. The W_0 of the RMs is the ratio of water molecules to surfactant molecules per RM (W_0 = [H_2O] / [Surfactant]) (Krishna et al. 2002). The size of the RM can be calculated with the help of W_0 (Kilikian et al. 2000). W_0 of RM is dependent on the relative solubility of the surfactant in the polar and non-polar solvent. It is expressed as hydrophilic-lipophilic balance (HLB) of the surfactant (Krishna et al. 2002).

The amount of water inside RM is important as biomolecules like protein/ enzyme are entrapped in this water pool. The water pool can be of two types; the water that resides the interior wall of the RM and the free water. Water capture

in RM is different from bulk water. The abnormal behaviour of such water is due to strong interaction between the surfactant polar group and a rupture of the hydrogen-bonded network that exists in bulk water (Krishna et al. 2002). According to Hilhorst et al. (1992) for cationic surfactant used in *a*-amylase extraction, the W_0 of the TOMAC/octane system can be changed by altering TOMAC counterions with ions in the feed phase, and by varying the concentration of alcohol or co-surfactant. For cationic surfactants, the HLB increases as TOMAC<DDAB<BDBAC<CTAB~CPB (Krishna et al. 2002). In the case of AOT/Isooctane RM, it is reported that minimum W_0 required for the solubilisation of a fixed amount of protein. Generally, the diameter of the RM and viscosity of RM phase increases with increasing W_0 due to higher surfactant concentration (Hai and Kong 2008).

2.3.4 Feed phase pH

The ionisation state of the surface-charged groups on the protein molecule is dependent on feed phase pH. The solubilisation of the protein in RMs influenced by charge interactions between the protein and a head group of surfactant. For anionic surfactants solubilisation of solute is favoured at pH values below the isoelectric point (pI) of the solute while it is reverse for cationic surfactants. According to literature; the (pH – pI) value required for optimum solubilisation of proteins with small molecular weight such as cytochrome C, lysozyme, and ribonuclease (MW range 12–14.5 KD) is much lower when compared with the abundant proteins such as α-amylase (MW 48 KD) (Hilhorst et al. 1992) and alkaline protease (MW 33KD) (Pires and Cabral 1996) where (pH – pI) value is around 5. The variation in the size of RM according to solute size in order to hold the solute is the primary reason behind this characteristic. To vary the RM size, higher energy is required, and such high energy can be provided by enhancing the surface charge of the solute. This enhancement of charge density on the biomolecule may be achieved by altering the pH of the feed phase (Krishna et al. 2002).

The impact of aqueous phase pH was examined on lectin extraction from kidney

beans using AOT RMS. The feed phase pH was varied in the range of 3-10. Highest solubilisation and purification factor of lectin in the micellar phase was observed at pH 5.5 due to the increased electrostatic interaction between lectin molecule and polar group of AOT. Beyond pH 6 which near to pI of lectin, maximum solubilisation was observed, however, the purification fold was found to decrease at these conditions. Above and below the pH 5.5 less extraction of lectin was observed due to weak protein surfactant interaction and denaturation of protein (He et al. 2013). Forward extraction of BSA was carried out using the cationic RMS by varying the aqueous phase pH between 6 to 11. The solubilisation of BSA to micellar phase was found to increase with increasing pH; however it was found to decrease beyond pH 11. The precipitation of BSA was noticed below the pH of 6 and above the pH of 11 due to the robust electrostatic interaction between surfactant and BSA molecules (Sun et al. 2011). Solubilisation of yeast lipase with AOT reversed micellar system was studied at various feed phase pH by Yu et al. (2003). It was concluded that pH more than the pI of yeast lipase is favourable for highest, i.e. 85% solubilisation of yeast lipase into RM. As the pH was increased to a near pI value of the protein (i.e., 6), the extraction was found to decrease due to the smaller hydrophobic interaction (Yu et al. 2003). The maximum α-LA solubilisation in the AOT RMS was observed at two different pH 6 and 9.5. But to be very specific complete solubilisation of protein was achieved at pH 6 and appreciable protein solubilisation was also achieved at pH 9.5, i.e. beyond the pI of α-LA (Naoe et al. 2004), which suggest that the electrostatic interaction between solute and surfactant molecules is not the only governing factor responsible for protein transfer to RM phase. The solubilisation of matrine to non-ionic surfactant (TRPO) micellar phase was studied at different pH by (Dong et al. 2009). It was observed that highest extraction of matrine was achieved at pH 9. Beyond pH 9 efficiency was decreased due to the emulsification of the system caused by the increased activity of matrine.

2.3.5 Ionic Strength

The ionic strength has a significant influence on protein solubilisation in RM as it affects the electrostatic interaction. The presence of ions around the head groups of surfactants results in the formation of an electrostatic layer that makes a reduction in electrostatic interaction between solute and surfactant (Kilikian et al. 2000). The same was reported in the study of RME of α-amylase using TOMAC/Isooctane system also. It is concluded that the presence of high ionic strength reduces the electrostatic interaction between the charged solute and surfactant polar groups of the RMs. The increased ionic strength tend to decreases the electrostatic aversion among the charged head gatherings of the surfactants in RM, thereby reduction in the size of RM and can lead to the expulsion of the solubilized proteins (Hilhorst et al. 1992; Krishna et al. 2002). Li et al. (2007) reported that the BSA extraction was reduced at very less salt concentration (0.05 M) of KCl or NaCl. Whereas, the increasing salt concentration (0.05 to 0.1 M) ions forms an electrostatic shield around the wall of micelles polar core, and resulted in the decreased the electrostatic attraction between the charged protein and the inner core of micelles. However, extreme ion concentration may result in more screening effect and the smaller micelles size, which is not favourable for the extraction of any protein or other biomolecules. Though many extraction studies were performed with inorganic salts like KCl or NaCl to improve extraction efficiency, few reports utilized the buffers to maximize the extraction of a target molecule. Naoe et al. (1999) have used phosphate buffer with sugar esters as non-ionic surfactant to improve forward extraction efficiency of cytochrome C. But, effective extraction efficiency could not obtain with increase in buffer concentration due to the sharp reduction in the electrostatic interaction between polar head group of surfactant and protein which ultimately responsible for lesser micellar size and repulsive interaction between polar head groups. Only 42% extraction of cytochrome C has observed at very less buff concentration, i.e. 0.1 M, whereas, the extraction of cytochrome C was not observed beyond the buffer concentration of 0.3 M.

The comparative study of the effect of ionic strength on the extraction efficiency of lysozyme was reported, and less extraction efficiency of lysozyme has observed with $CaCl_2$ as compared to NaCl and KCl due to the presence of Ca ions (Shin et al. 2003a). Similarly, during the purification of peroxidase from horseradish root the use of $CaCl_2$ resulted in reduced solubilisation of the enzyme into the aqueous core of RM in comparison with NaCl and KCl due to divalent nature of Ca ion that creates hindrance with protein interaction (Regalado et al. 1996). Apart from NaCl and KCl; $CaCl_2$ is also used for the extraction of penicillin G. It was observed that the extraction efficiency of penicillin G was improved with the use of $CaCl_2$ as compared to KCl. The molecules having small molecular size can be solubilised in RM using divalent salts like $CaCl_2$ as they form small RM (Mohd-Setapar et al. 2009).

The addition of 1M KBr gave high activity recovery (76%) and purification (4.1 fold) of soy hull peroxidase in the presence of cationic (CTAB) surfactant, when the concentration was varied from 0.5 to 2.5 M. The decreased recovery and purity was observed by increasing the salt concentration beyond 1M due to the strong ionic strength of the aqueous phase, which resulted in loss of enzyme activity also (Lakshmi and Raghavarao 2010). Dilute chaotrophs (urea and GuHCl) were used instead of high salts for the effective back extraction of kallikrein. Kallikrein captured in RM was released effectively in the presence of dilute urea and GuHCl during back extraction. Nearly 100% activity recovery of kallikrein from the commercial product was obtained by adding 0.60 M urea, and for kallikrein from the crude material, the recovery was increased significantly by adding 0.80 M urea and 0.08 M GuHCl in the stripping solution (Zhou et al. 2012a) .

2.3.6 Temperature

The physicochemical properties of RMs are affected by temperature variation. Increasing the temperature may help to improve the protein solubilisation in the RM as well as back extraction (Kilikian et al. 2000). Luisi et al. (1979) reported the effect of temperature on the transfer of α-chymotrypsin in chloroform-

methyltrioctylammonium chloride system. A higher transfer was observed with an increase in temperature from 25ºC to 40ºC; 50%. Marcozzi et al. (1991) observed the increase in α -chymotrypsin recovery when back extraction was performed at 38ºC. The extraction of cutinase was tested between 4 and 40^0 C. The temperature has the strong impact on the extraction. The absolute solubilisation was achieved at 40^0 C. The driving force responsible for the extraction of cutinase is the net hydrophobic interaction of cutinase with the apolar part of the anionic surfactant. The hydrophobic forces majorly depend on the temperature and are least essential at lower temperatures, which decreased the amount of cutinase extracted (100 to 35.6%) as the temperature is reduced from to 4^0 C (Carneiro-da-Cunha et al. 1994b). Forney and Glatz (1995) performed the back extraction of glucoamylase at 35ºC. The enzymatic activity was enhanced from 40% (at room temperature) to 90%. The reduced W_0 at 35ºC resulted in the size-exclusion effect. The effect of temperature on inulinase extraction was also studied by Pessoa Jr and Vitolo (1997). The researchers have reported that W_0 value was increased with increasing temperature, but at temperatures higher than 37ºC inactivate the inulinase and severely affect the yield of active enzyme. Chou and Chiang (1998) have also reported the effect of temperature on hen egg lysozyme extraction with bis-(2-ethylhexyl) sodium sulphosuccinate. According to the study, it was observed that the recovery rate of lysozyme has increased as the temperature of the system was reduced to 10^0 C. The highest specific activity of lysozyme was obtained at the same temperature. Temperature effect is studied by Li et al. (2007) for the extraction of BSA using CTAB/isooctane/1-pentanol system concludes that extraction of protein was found to be low at low temperatures such as 15ºC due to slower movement of BSA and RM at lower temperature; thus the collision between BSA and RM decreased and resulted in less extraction. The forward extraction efficiency of lipase was studied at temperature range 15ºC to 40ºC. The increased extraction of lipase was observed with increasing temperature and highest lipase extraction, i.e. 80% was obtained at 25ºC (Nandini and Rastogi 2009).

2.3.7 Phase volume ratio

Pessoa Jr and Vitolo (1997) conducted the phase volume ratio study for ratios higher than 1 to observe the loading capacity of RM and extraction efficiency. The inulinase solubilisation in BDBAC/ isooctane-hexanol micellar solution was found to decrease with higher volume ratio. Similar results were obtained by Aires- Barros and Cabral (1991) in their study on the purification of lipase in AOT/isooctane micellar solutions. An enhanced volume proportion (Vaq/Vorg) was failed to increase the extraction yield since the micelles were not sufficient to envelop all the inulinase present in the feed broth. Yu et al. (2003) reported an increase in the extraction yield of commercial yeast lipase with an increase in phase volume ratio. The extraction yield was observed to enhance at high phase volume ratio. Around 80% lipase was recovered at Vaq/Vrm proportion 1:4. When the proportion was 1:1, the recovery of lipase was decreased to 50%. Increasing the reverse micellar phase volume tend an increase in the number of RM, and consequently, increase in lipase extraction. Shin et al. (2003a) studied the effect of the phase volume ratio for the extraction of lysozyme at various concentrations with the DODMAC RMS. For a 0.1 g/l lysozyme solution, the highest concentration of lysozyme obtained in the organic phase was about 0.5 ± 0.02 g/l at Vaq/Vorg of 5. For volume ratios higher than 5, a protein-surfactant complex was formed at the aqueous-organic interface, and the extraction efficiency decreased significantly. For a 0.2 g/l lysozyme solution, the highest concentration of lysozyme was found to be about 0.8 ± 0.02 g/l at a volume ratio of 4 and then decreased due to the formation of a precipitate at the interface. For a 0.6 g/l lysozyme solution, the lysozyme concentration of near 1.0 ±0.02 g/l was obtained in the organic phase at Vaq/Vorg = 2, which corresponds to the solubility found in a DODMAC reverse micellar phase. Liu et al. (2004) showed that Vaq/Vorg had a significant effect on the nattokinase transfer to micellar phase from *Bacillus natto*. In the reverse micellar (AOT/ isooctane) extraction, authors obtained maximum nattokinase activity recovery (95%) and maximum total protein (20%) when Vaq/Vrm proportion was 1:3.

Nandini and Rastogi (2009) have reported the impact of phase volume ratio on the back recovery of lipase. According to their study highest back extraction efficiency and activity of lipase has been obtained at a volume ratio of 1.5. The increasing volume ratio has resulted in a lesser back extraction efficiency and activity. During RME of soy protein using AOT/Toluene system, Zhao et al. (2010) found that volume ratio (Vorg/Vaq) should be low for forward extraction and high for back extraction to achieve required concentration. The forward extraction efficiency was found to be less since the increasing volume of organic ratio leads to a decrease in the concentration of surfactant in a system. The effect of the volume ratio of the aqueous to organic (RM containing soy hull peroxidase) phase during back extraction of peroxidase activity recovery indicated that the decrease in a volume ratio from 1.0 to 0.8 resulted in an increase in activity recovery and a purification factor of soy hull peroxidase from 45 to 89% and 3.98 to 4.72, respectively. A further decrease in volume ratio resulted in a decrease in the recovery and purification fold, which could have been due to the decrease in the extraction volume of the stripping solution (Lakshmi and Raghavarao 2010).

Similarly, Gaikaiwari et al. (2012) have reported the phase volume effect on lipase purification for forward as well as back extraction using AOT/isooctane. The increased volume of isooctane tends to enhance the total protein content from 1:1 to 1:5 proportion. At the same time, enzyme recovery was increased only up to a proportion of 1:3 (80.67%) and remained unchanged even after the increased isooctane volume. While in the back extraction of lipase, a range of 1:1 to 1:8 was used to study the effect of phase volume ratio. Total recovery of lipase was unchanged for phase volume 1:1 to 1:6, whereas, at 1:7 and 1:8 ratio enzyme recovery and fold purification were observed to be decreased since phase formation was not observed.

2.3.8 Effect of co-solvent or additives

Various researchers have made attempt to interpret the role of alcohols in RM. Kahlweit et al. (1991) described that the alcohols act as a weak amphiphile

when added to a biphasic system. Hence they are considered as "co-solvents" that partition the aqueous region from the amphiphilic film. Strey and Jonströmer (1992) studied the effect of medium-chain alcohols on the RM in ternary systems; and inferred that the long chain length provides a full elastic module to the mixed film. From spin label experiments on flexible films, Di Meglio et al. (1985) concluded that short-chain alcohols decrease the rigidity of the interfacial film. Penders and Strey (1995) studied the effect of *n*-octanol (C8E0) on the phase behaviour of ternary microemulsion system H_2O–*n*-octane–C8E5 (pentaethylene glycol mono-n-octyl ether). They expected alcohols to bring about two powerful effects: first is variation in the sufficient hydrophilicity of the amphiphilic mixture C8E5+C8E0. The second effect to increase in efficiency (or solubilisation capacity) of the amphiphilic mixture, while at the same time deformation of the three-phase system. The enhanced extraction could be the result of decreased interfacial tension between the aqueous and organic phase (Kahlweit et al. 1988). Nandini and Rastogi (2009) have reported 82.72% enzyme recovery with CTAB/isooctane in the forward extraction of *A. niger* lipase using in the presence of n-hexane and n-butanol as co-solvent at pH 7.0 with 4.094-fold purification. Hilhorst et al. (1992) reported that addition of the co-surfactant Rewopal HV5 (in a 1:4 ratio) and 0.1% octanol to TOMAC reversed micelles during the extraction α-amylase resulted in the enlargement of the micelles, as judged by an increase in W_0 of the organic phase, and the broadening of the transfer profile.

2.4 Back extraction

Back extraction of solute to stripping phase is dependent on specific parameters like the effect of pH in the stripping phase, salts type and their concentration in stripping phase and volume ratio of organic phase to stripping phase. According to Li et al. (2007) at a higher KCl concentration (0.4 M) precipitation of BSA, CTAB, and 1-pentanol molecules occurs which leads to decrease the extraction of protein to stripping aqueous phase from micelles. Hence concentration of salts in back extraction is a crucial parameter, which needs to be studied for

better extraction. pH of the stripping phase also affects the protein back extraction. In the study for BSA extraction, it was shown that pH of stripping solution if adjusted near to the protein pI, back extraction of the protein could be increased. As the electrostatic attraction between the protein molecules and micelles decreases, the protein easily gets transferred from the organic phase to the stripping phase. Along with these parameters, the significant effect of the volume ratio of the organic phase to stripping phase on back extraction and recovering capacity of the stripping phase was also reported. Lee et al. (2004) found that back extraction was relied on the species and concentration of the alcohol and carboxylic acid added to the RMs. Also, co-surfactants suppress the formation of RM clusters and enhance back extraction of protein to aqueous stripping phase.

Different methods can improve the back extraction process. Three possible strategies were stated for improvement of back extraction. The first strategy was related to the stripping phase pH, and salt concentration. The second strategy was in relation to the organic phase, i.e., the components and surfactant amount or addition of different alcohols. Whereas, the third deals with the system temperature and pressure. In these methods, the first one is superior to remaining two as because it can retain RM phase to a larger extent and keep maintain high proteins activity in the aqueous phase, and also crucial in the recycling of the micellar system (Liu et al. 2008).

2.4.1 Effect of co-solvent on back extraction

The addition of co-solvents or additives is essential to obtain optimum back extraction. Alcohol molecules are known to be capable of penetrating the RM and may able to destabilise them. The smaller alcohols are assumed to have better destabilising ability due to high penetration power compared to long chain alcohols. Various alcohols with crucial contributing factors have been applied for successful back extraction of proteins (Mathew and Juang 2007). Amoxicillin recovery from the RM was observed to be increased with the addition of 5% v/v hexanol during the back extraction (Chuo et al. 2014). The

addition of 10–15% isopropanol to the aqueous phase increased the rate of protein release from the RM dramatically and allowed for the nearly complete back transfer of porcine pepsin and 70% back transfer of bovine chymosin (Carlson and Nagarajan 1992). Yu et al. (2003) carried out back extraction of the lipase for various ethanol concentrations (0 to 6%) by volume, with constant ionic concentration and pH. The recovery was approximately 40% when ethanol was 1%. Further, it increases to 68% at 3% ethanol concentration. Later, recovery was constant even at higher ethanol concentration. However, the absence of co-solvent has resulted in zero recoveries at optimised pH and KCl concentration.

Mathew and Juang (2005) have reported the impact of alcohol on the back-extraction of papain. Isopropanol, hexanol, propanol and ethanol were used to improve back extraction efficiency of papain with TOMAC RMS. The short-chain alcohols, such as ethanol and *n*-propanol, give slightly less back extraction (70% at 10%, v/v, alcohol) as compared to the branched-chain isopropanol and long-chain hexanol (90% at 10%, v/v, alcohol). Lakshmi and Raghavarao (2010) conducted back extraction of soy hull peroxidise with the addition of isopropanol (5 ~ 25%) and obtained the activity recovery of 90% with purification fold of 4.72. The effect of isopropanol concentration in stripping aqueous phase on total activity recovery, protein recovery and a purification factor of nattokinase were studied by Liu et al. (2004). The presence of more isopropanol tends to enhance protein recovery and total activity to 80.2 and 33.55%, respectively. Purification factor had no significant impact when the isopropanol concentration was more than 5%. Addition of isopropanol probably led to the increase the attractive interaction between RM and the arrangement of AOT molecules in isooctane by rupturing the RM exclusion of nattokinase. Addition of 10% v/v ethanol during backward extraction was found to enhance the recovery of laccase (Peng et al. 2012). Whereas, the addition of isopropanol has no significant effect on the back extraction of tannase (Gaikaiwari et al. 2012). Influence of alcohol on the backward extraction of amoxicillin from

mixed AOT/Tween 85 system was investigated by Chuo et al. (2014). 5% v/v hexanol was added into an aqueous solution with 10 g/L KCl at different pH. The extraction of amoxicillin was increased with pH (3.5 to 5.5). These two significant factors are essential to optimise the process conditions in order to achieve the highest selective extraction of target solute.

2.5 RM extraction of whey proteins

Various factors involved in micellar extraction indicates the necessity to study the physicochemical properties of biomolecules and mechanism involved in extraction and purification of target solute for achieving the required selectivity and extraction efficiency. Many of the micellar extraction had been carried out for the extracellular products or with synthetic solutions of respective molecules. In the case of whey proteins, countable studies with micellar extraction have been reported (Table 2.3). Majorly, the standard protein BSA has been partitioned in different RMS. The suitability of all kind of surfactants i.e. cationic (Li et al. 2007; Xiao et al. 2013; Zhang et al. 1999; Qiang et al. 1998) anionic (Hebbar and Raghavarao 2007; Naoe et al. 2004), non-ionic (Hebbar and Raghavarao 2007) and Gemini (Dong et al. 2013), for the micellar extraction of BSA has been reported. However, few other whey proteins have not extensively studied for the conventional extraction and purification. In case of LF, Anjana et al. (2010) have studied the feasibility of forward extraction of LF from whey using the RMS but failed to analyse the effect of process variables, and hence less extraction efficiency has been reported. RME of α-LA was reported with the anionic RMS by Naoe et al. (2004). Absolute solubilisation of α-LA was obtained in forward extraction whereas, 90% protein transfer was achieved in back transfer to the stripping phase. Qualitative analysis by CD spectrum proved the suitability of micellar extraction process for the α-LA, as the native state of the protein was retained. The comparative study of the different anionic RMS was carried out by Gomes et al. (2017). AOT/ isooctane and AOT/hexane RMS were modified with the addition of alcohols namely isopropanol and butanol as main components of the system. Extraction

of a micellar system with butanol was found to be higher than with propanol (Gomes et al. 2017). RM assisted and RME were compared for the extraction of LPO from whey by Nandini and Rastogi (2010). RM assisted extraction was found to give better extraction efficiency and purification factor as compared to RME.

Table 2.3: RME of whey proteins

Milk protein	Source	RMS	References
BSA	Synthetic BSA solution	CTAB/hexanol/octane	(Qiang et al. 1998)
		CTAB/cibacron blue/hexane-hexanol	(Zhang et al. 1999)
		CTAB/isooctane/1-pentanol	(Li et al. 2007)
		AOT/toluene Triton-X-100/toluene	(Hebbar and Raghavarao 2007)
		CTAC/n-octanol/isooctane	(Sun et al. 2011)
		Gemini surfactant/n-hexane/hexanol	(Dong et al. 2013)
		CTAB/n-hexane/hexanol DTAB/n-hexane/hexanol	(Xiao et al. 2013)
		glucosylammonium (GA) and lactosylammonium (LA) surfactants/n-octane/ hexanol	(Chen et al. 2017)
α-LA	Pure α-LA solution	AOT/isooctane	(Naoe et al. 2004)
		AOT/isooctane/iso-propanol AOT/isooctane/ butanol AOT/hexane/iso-propanol	(Gomes et al. 2017)

Milk protein	Source	RMS	References
		AOT/hexane/butanol	
LPO	Whey	AOT/isooctane CTAB/isooctane/hexanol/butanol	(Nandini and Rastogi 2010)
LF	Whey	CTAB/isooctane CTAB /Triton X100/ isooctane	(Anjana et al. 2010)

Even though successful forward, as well as back extraction, was reported for various biomolecules, few studies have been reported where RME has been coupled with other purification techniques like ion exchange chromatography, precipitation (Table 2.4). The coupling of such purification techniques has made the purification process lengthy and tedious. Such lengthy processes are not feasible to implement for the naturally available crude sources or industrial waste where the quantity of target solute is minimal, hence the additional pre-treatment of the raw source is required.

Table 2.4: RME coupled with various purification techniques

Biomolecule	Source	Extraction technique	Reference
Wheat germ protein	Wheat germ	Ultrasound-assisted AOT/isooctane RME	(Zhu et al. 2009)
Bromelain	Pineapple fruit	CTAB/isooctane/hexanol RME coupled with high-speed counter-current chromatography	(Yin et al. 2011)

Biomolecule	Source	Extraction technique	Reference
Lipase	Fermented rice bran	CTAB/span 80/isooctane RME coupled with liquid emulsion membrane separation	(Bhavya et al. 2012)
Bromelain	Pineapple fruit	CTAB/ isooctane / hexanol/butanol RME coupled with ultrafiltration	(Hebbar et al. 2012)
P-B and pentatricopeptide and repeat-containing protein	Momordica charantia	high-speed counter-current chromatography coupled with AOT+CTAB/isooctane RMS	(Li et al. 2012)
Bromelain	Pineapple core	CTAB/isooctane/hexanol RME coupled with precipitation	(Chaurasiya and Umesh Hebbar 2013)
Lipase	Fermented rice bran	RME coupled with liquid emulsion membrane	(Bhowal et al. 2014)
α-glucosidase	Mouse intestine	Countercurrent chromatography coupled with AOT/isooctane RMS	(He et al. 2016)

2.6 Continuous extraction

RME of commercially valuable molecules in a batch process or lab scale is extensively reported in the literature. However, continuous extractions with RM have been reported for very few molecules. Continuous RME has been demonstrated in conventional and modified contactors. Conventional liquid-liquid column extractors like spray column (Lye et al. 1996), PRDC (Carneiro-da-Cunha et al. 1994a) and packed columns (Nishii et al. 1999) have been used for RME. Protein partitioning kinetics of lysozyme was measured using the

spray column. The operations were performed in semi-batch mode and effect of aqueous phase pH, ionic strength and dispersed phase flow rate on lysozyme extraction kinetics were studied (Lye et al. 1996). Recovery of intracellular protein from *Candida utilis* by RM using simple spray column was reported. Effect of column length, flow rate and some circulations were assessed to examine the extraction of proteins (Han et al. 1994).

Nishii et al. (1999) carried out the extraction of lysozyme using packed columns. The extraction efficiency and overall mass transfer coefficient were examined for various flow rates and different packing material of the column. It was concluded that extraction in a packed column is three times larger than the spray column due to larger dispersed phase hold up. Also, little harm to protein was observed due to moderate mixing in a packed column. Lysozyme extraction from egg white using grasser contactors was reported by (Jarudilokkul et al. 2000). Mass transfer during forward and back extraction and the effect of rotor speed and phase volume ratio were studied to monitor the lysozyme extraction by RM.

2.6.1 RDC

The hydrodynamic and mass transfer characteristics of the RDC with conventional and no-conventional systems are reported in the literature. Kumar and Hartland (1995) proposed a correlation for the determination of holdup in RDC. Data were collected for both with and without mass transfer conditions. The authors have considered dispersed phase hold up to be a function of power dissipation per unit mass, continuous and dispersed phase velocities, physical properties, compartment height and gravitation constant. It was concluded that along with the mentioned parameters diameter of the rotor also plays a significant role in a dispersed phase hold up. Moris et al. (1997) studied the hydrodynamic behaviour of RDC (72mm I-diameter, 1.1m operating height and 22 mixing compartments) using the two-phase water/kerosene system. The entire hold up was found to increase with rotor speed, total throughput and increase in organic/aqueous phase flow ratio. The local hold up was measured at

different column heights and was observed as the maximum at the centre of the column and minimum at the upper and lower parts of the column.

Kalaichelvi and Murugesan (1998) developed a new correlation for the direct estimation of dispersed phase holdup using the conventional system from known operating system variables. Cylindrical glass RDC (0.0762 and 0.1m diameter, 0.9m height) was used for experiments. It was found that initially the holdup increased gradually with dispersed phase flow rates and with further increase in continuous and disperse phase flow rate and rotor speed, the holdup increased sharply. It was observed that the variation in dispersed phase hold up is due to the effect of more fundamental variables like column geometry, phase flow rates, rotor speed and physical properties of the system used. Correlations involving Froude number, phase flow rates, Morton number and geometric factors were proposed for both no solute transfer as well as mass transfer conditions. The separate correlation for no agitation condition was also proposed. Two regions of operations were noted including region one where the disperse phase holdup is nearly independent of rotor speed, covers the operation at low rotor speeds. Region 2 covers higher rotor speed, and higher dependency of dispersed phase holdup on rotor speed was observed.

Carneiro-da-Cunha et al. (1994a) studied the extraction of r-cutinase from fermentation broth to anionic RMS (AOT/isooctane) using perforated RDC (160mm height and 32mm internal-diameter). Studies were carried out at constant flow rates for the different time period. It was observed that yield was increased with an increase in the period. Highest efficiency of 78% was achieved at 70min. Soltanali et al. (2009) carried out the RME of protein in RDC with and without stators. It was noted that the dispersed phase holdup increased with an increase in rotor speed. In the absence of stator rings, holdup increased considerably. The outcome might be due to the size of discs since discs were comparatively bigger and closer to the column walls in the without stator ring configuration that caused an obstacle to the phase flow and diminished the velocity of the droplets.

Even though RDC has been successfully used for the RME, not many detailed studies have been carried out on the mass transfer characteristics, extraction efficiency and hydrodynamics of RDC using RM. Holdup information is essential for the design of RDC as it is needed for the determination of interfacial area mass transfer. Although correlations are available for holdup determination in RDC for RMS, their application for the RMS is limited.

2.7 Research Gap Identified

The conventional separation and purification methods are extensively reported for proteins, enzymes, nucleic acids, antibiotics and many more. A number of reports are available for the extraction and purification of biomolecules including proteins using methods like chromatography, salt precipitation and differential solubilisation etc. Even though the reported techniques are used for a long time, the complete recovery of the product with higher purity is not achieved in most of the cases. The non-conventional methods yield a minimum volume of products, i.e. only fractions of the purified molecule and require more time to accomplish the process. Also, the materials and inputs required for the purification method, i.e. column packing material, the higher concentration of salts, cost of the column etc., has not only made the extraction and purification process uneconomical but also resulted in the lesser yield. Hence these methods are not implemented widely in the industrial scale.

However, the LLE is widely used for a variety of biomolecules from different sources. The modified methods of LLE, i.e. ATPE, CPE, RME etc., are used for a wide range of proteins as well as enzymes. The highest purity and yield of target product has been achieved by such non-conventional LLE methods. The significant advantages of these non-conventional methods include (i) easy availability of phase forming components (polymers, surfactants and organic solvents etc.,) at very low cost, (ii) biocompatibility of the components and phases of the systems, (iii) preservation of native structure and function of the final product. However, the exact mechanism of solute solubilisation and selective partitioning into the phases is not clear for many biphasic systems. The

poor understanding of the processing mechanism has failed to attract the industries for its broad application in the field of the downstream processing. Consequently, the modified LLE methods specifically ATPE, CPE, RME etc., are not used at an industrial level to obtain the bulk purified product by continuous operation.

2.8 Scope and objectives of the work

LF, a glycoprotein, has several biological and commercial applications. There is a huge gap globally between the availability and demand of purified LF. Even though a considerable amount of LF is present in the whey (relatively cheap industrial waste), countable reports are available for the extraction of LF from whey due to its complex nature. Extraction of biomolecules from biological sources by analytical methods like chromatography needs pre-treatment step to concentrate the sample. The cost of the purified product depends upon its purity, its application, and a number of unit operations involved in the extraction and purification process. Hence, there is a need for the development of cost-effective methods, which exhibits higher selectivity with the minimum number of steps for separating the valuable biomolecules from complex biological sources like whey.

Liquid-liquid extraction with biomolecule compatible solvents had the potential to extract the component like LF with higher purity and gives a new way to scale up the process in an economical way. Reverse micellar based LLE system is an exciting option to selectively extract a specific protein from whey or other biological sources since the separation is based on electrostatic interaction and size differences of the target molecules. The modified LLE reduces the process steps and able to purify the LF up to the pharmaceutical grade by employing very few process steps. The cost-effectiveness of the RME of LF may be achieved by reusing the micellar phase and scale-up the process in a continuous operation. The continuously agitated contactors like RDC may be explored to maximise the purification fold and yield. Hence the present research is focused

to identify a suitable RMS for the extraction of LF and improve the extraction efficiency by understanding the effect of various system and operating variables on the extraction and purification process.

The following objectives are framed based on the identified research gap and scope of the present work:

1. Identification of the suitable RMS for the partitioning of LF by considering various surfactants (Ionic and Non-ionic) and using commercially available pure LF.

2. Study of the effect of process and system variables on the extraction of LF from the aqueous solution of pure protein, a solution of standard whey proteins (synthetic whey) and real whey.

3. Study of the reformability/reusability of micelles from the processed RM phase.

4. Implementation of a continuous RM based extraction process in a RDC for the extraction of LF from real whey.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, Proteins and Reagents

Bis-(2-ethylhexyl) sulfosuccinate sodium salt (AOT), Triton X 100 (TX 100) and cetyltrimethylammonium bromide (CTAB) of 99% purity were procured from Sigma Aldrich, India and used in the experiments without further purifications. Organic solvents namely isooctane, hydrochloric acid, n-heptanol, n-decanol, toluene, glacial acetic acid, methanol, n-butanol, n-hexanol, isopropanol were obtained from Loba Chemie Pvt. Ltd India. Acetonitrile, trifluoroacetic acid (TFA) and isopropanol of HPLC grade, Folin-Ciocalteu reagent (FCR), phenol and sulphuric acid (98% purity) were procured from Merck, India. Whey proteins α-LA, β-LG, LF of more than 85% purity and LPO (>150U/mg) were obtained from Sigma-Aldrich. BSA was having purity more than 98% was procured from Hi-media, India. Inorganic salts like potassium chloride (KCl), sodium chloride (NaCl), sodium carbonate (Na_2CO_3), copper sulphate ($CuSO_4.5H_2O$), sodium potassium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$), sodium hydroxide (NaOH) and glucose ($C_6H_{12}O_6$) were purchased from Spectrum Chemicals, India. Mini-PROTEAN® SDS PAGE System (Biorad, India) was used to perform SDS PAGE. The SDS-PAGE kit was procured from Hi-media, India. Protein molecular marker with a range of 5-110 kDa was purchased from Sigma Aldrich. Coomassie brilliant blue R-250 was obtained from Hi-media, India. Millipore grade deionised water was used throughout the experiment.

3.1.2 Instruments

Digital refractometer (RX-5000α, ATAGO CO., Ltd, Japan, with ± 0.00004 accuracies and ± 0.00002 precision), Digital microviscometer (Lovis 2000 M, Anton Paar) with an accuracy of ± 0.005 mPa.s, Digital density meter (DDM 2911, Rudolph, USA with accuracy of ± 0.00005 gm/cm^3 and ± 0.00001 gm/cm^3 precision); Karl-Fischer titrator (Metrohm 899 coulometer), Qudix Scatteroscope, Korea; flame photometer (Elico - CL 378, India); Atomic Absorption Spectrophotometer (AAS) (GBC 932 plus, Australia), Electronic weighing balance (Shimadzu, Japan) with a precision of ± 0.001mg; High performance Liquid Chromatography (HPLC) (Shimadzu, LC-20AD, Japan) with C-18 column (Shim-pack Solar, Shimadzu, Japan, column with size 250 X 4.6 mm l.D. and Capcell Pak C18 MG II, Shiseido, Japan, column with size 4.6ml.D.X 250mm); UV/Vis spectrophotometer (UV3000$^+$, Lab India); refrigerated cooling centrifuge (Kubota 6390, Japan) and magnetic stirrers are some of the essential instruments used in the study.

3.1.3 Synthetic and acidic whey

3.1.3.1 Synthetic LF solution

In order to understand the mechanism of LF transfer to the micellar solution and further to obtain the optimised condition for extraction; it is important to understand the behaviour of LF in RM phase in the absence of other components present in whey. Hence, micellar extraction studies were initially carried out with pure LF solution (feed phase) to select the suitable RMS. A synthetic solution of LF was prepared at a concentration of 0.1mg/ml to mimic the concentration of LF in the whey as reported in the literature (Du et al. 2013). The selected RMS was further improved for the better solubilisation of LF in the organic phase by studying the effect of different variables.

3.1.3.2 Synthetic whey proteins solution

The optimised micellar extraction conditions may be extended to the LF extraction from the acidic whey. However, some non-proteinaceous components

are present in whey which may interfere during the LF extraction. Hence, to understand the effect of such component and other proteins present in the whey on the LF extraction, the optimised conditions obtained with a synthetic solution of LF were used to extract LF from a mixture of whey proteins. This experiment also deduces the role of other whey proteins, which tend to solubilize in RM phase, during the selective partitioning. The synthetic solution of whey proteins (α-LA-1.5mg/ml, β-LG-3mg/ml, LF-0.1mg/ml, BSA- 0.3mg/ml and LPO-0.03mg/ml) was prepared according to the protein concentrations reported in the literature (Du et al. 2013). The results obtained with pure LF solution and synthetic whey solution was scrutinised, and further, the suitable optimised conditions were applied for the RME of LF from real acidic whey, which was prepared in the laboratory.

3.1.3.3 Acidic whey

Whey was prepared by acidification (pH 4.2) of the pasteurised milk using curdling method. Casein was precipitated during acidification, and it was removed by centrifugation at 15,000g for 40 min at 4ºC (Kubota 6930, Japan). The solid free supernatant obtained is the straw-coloured whey and was stored at 4ºC for future use.

Lowry's Assay was used to measure the total protein concentration in whey. The physical and chemical properties like Chemical Oxygen Demand, Biological Oxygen Demand and Total Solid Content (Federation and Association 2005) of whey were determined. The phenol-sulfuric acid assay was performed to measure carbohydrates present in the whey. Metal content in the whey was analysed using Atomic Absorption Spectrophotometer (GBC 932 plus, Australia) for magnesium, zinc, copper and iron. Whereas, the sodium (Na^+) and potassium (K^+) content were measured by a flame photometer (Elico –CL 378, India).

A phenol-sulphuric acid method was used for carbohydrate estimation in whey (Dubois et al. 1956). Glucose (2ml) of different concentrations was hydrolysed

by the addition of 1ml of 5% aqueous solution of phenol in a test tube. Next, to this, 5 mL of concentrated sulphuric acid is added quickly to the reaction solution and allowed to incubate for 10mins at room temperature. Further, tubes were vortexed for the 30s and placed in boiling water bath for 20min to develop a colour complex. The absorbance was recorded at 490nm on a spectrophotometer. Reference solutions are prepared similarly as described above, except that the aliquot of carbohydrate is replaced by double deionised water (Appendix I).

3.2 Method

3.2.1 Screening of RMS

Based on physicochemical properties of surfactants and organic solvents (Table 3.1), six different RMS were selected. Triton X 100/ Isooctane, AOT/ Isooctane, AOT/ n-decanol, CTAB/ Toluene, CTAB/ Isooctane, CTAB/ n-heptanol were chosen for the LF partitioning study. CMC of different RMS was obtained by estimating the physical properties like refractive index (Digital Refractometer, RX-500, ATAGO Co. Ltd. Japan) (Appendix II). W_0 of all selected micellar system at CMC was measured using Karl Fischer Titrator (899 coulometers, Metrohm, Switzerland). The RM size was calculated using Eq. (3.1) (Hebbar and Raghavarao 2007)

$$R_m = 0.175W_0 \qquad (3.1)$$

Table 3.1: Physical and chemical characteristics of phase forming components

System components	Topological surface Area (A°)	Hydro-carbon chain	Rotatable Bond count
TX100	29.5	1	6
AOT	54	2	18
CTAB	0	1	15

Isooctane	18.5	1	2
n-decanol	20.2	1	8
n-Heptanol	20.2	1	5
Toluene	0	0	0

(Data adopted from *pubchem.ncbi.nlm.nih.gov*)

All the six RMSs (Triton X 100/ Isooctane, AOT/ Isooctane, AOT/ n-decanol, CTAB/ Toluene, CTAB/ Isooctane, CTAB/ n-heptanol) are considered for the extraction of LF. Initially, an equal amount of organic phase (containing surfactant concentration above CMC) were mixed thoroughly with an aqueous phase (containing LF concentration 0.1mg/ml) using magnetic stirrer at 800 rpm at room temperature for 20 min. The pure commercial LF was considered to prepare the aqueous phase. Further, the phases were separated by centrifugation at 5000g and 20 min (Remi C-24 plus, India). Protein concentration after LF extraction in each phase was measured using Folin-Lowry's assay described by Lowry et al. (1951). A calibration graph was developed using the BSA (Appendix III), which was utilised to determine the LF concentration. According to the assay, 1ml of the sample was mixed with 5ml of alkaline copper sulphate reagent and allowed to hydrolyse at 100°C for 10 min in a boiling water bath. On cooling the solution, 0.5 ml of 0.5N Folin reagent is added and mixed it correctly.

Further samples were incubated for 45min and absorbance was recorded at 660nm using spectrophotometer (UV3000^{+}, Labindia). The forward extraction efficiency was calculated by using Eq. (3.2). The RMS gave maximum solubility of pure LF was considered for further study.

$$\text{Forward extraction Efficiency (\%)} = \left(\frac{The\ concentration\ of\ LF\ in\ org.phase(mg/ml)}{Initial\ Concentration\ of\ LF\ in\ the\ feed\ phase\ (mg/ml)}\right) \times 100 \qquad (3.2)$$

3.2.2 RME of LF

The extraction characteristics of LF in the RM phase of the selected system (CTAB/ n-heptanol) was initially analysed by considering the aqueous solution of LF prepared by dissolving the commercially available pure LF. The effect of process and system variables of the extraction process on the forward extraction efficiency and backward extraction efficiency of LF were analysed. The knowledge obtained from the initial partitioning studies was extended to study the partition characteristics of the LF from the synthetic whey protein solution, which mimic the protein mixture concentration of whey. The optimum condition obtained during the process was further applied for the real whey prepared in the laboratory.

3.2.2.1 Forward extraction of LF

The forward extraction of LF from the aqueous solution of pure LF was performed. The n-heptanol with CTAB was considered as the organic phase to form the RM phase. 20 ml of the RMS was prepared with a phase volume ratio of 1:1 (organic: aqueous phase) for all the experiments. Forward extraction was carried out by mixing the phases using magnetic stirrer for 20 min at 800 rpm at room temperature. Then the mixture was subjected to centrifugation at 5000 g and 20 min (Remi C-24 plus) for phase separation. The organic phase was carefully separated and used further for back extraction.

The effect of surfactant concentration was studied by varying the CTAB concentration in the organic phase between 10 to 100 mM. The effect of pH on the extraction efficiency was studied by adjusting the aqueous phase pH between 2-11 using the HCl and NaOH. Influence of ionic strength on protein solubilisation to micellar phase was studied by varying the two salts (KCl and NaCl) concentration from 0.1 to 1.3 M. Further; the LF concentration was also varied for a range of 0.04-0.4 mg/ml to study the effect of protein loading in the system. The effect of co-solvent addition on forward extraction was studied by

incorporating the n-butanol as co-solvent at 7 and 15% (V/V). The organic phase to aqueous phase volume ratio on the extraction efficiency was also analysed and reported. In every experiment, the reverse micellar organic phase and the aqueous phases were subjected to the total protein analysis to determine the forward extraction efficiency. The forward extraction efficiency at different operating condition was calculated using Eq.(3.2). Similar experiments are performed to study the forward extraction efficiency for the synthetic whey protein solution and real whey as the aqueous phase. The concentration of LF in organic and aqueous phases were determined through the HPLC analysis. Along with the extraction efficiency, the yield of LF in the organic phase during forward extraction was also calculated (Eq. 3.3).

$$Forward\ Extraction\ Yield\ (\%) = \left[\frac{([LF\ conc.in\ org\ phase]\ mg/ml)\times(Vol\ \ of\ org\ phase\ (ml))}{([LF\ conc.in\ the\ feed\ phase]\ mg/ml)\times(Vol\ of\ feed\ phase\ (ml))}\right]\times 100 \qquad (3.3)$$

3.2.2.2 Back extraction of LF

Back extraction was carried out to extract the LF entrapped in the reverse micellar phase. The organic reverse micellar phase (5ml) obtained from all the three forward extraction processes were subjected to back extraction. 5ml of the organic phase was mixed with an equal volume of fresh stripping phase using the magnetic stirrer for 60 min at 800 rpm and then centrifuged at 5000g for 30 min. The back-extraction efficiency was improved by studying few parameters like pH, ionic strength and addition of additives. The stripping phase pH was varied to study the effect of pH on the back extraction efficiency. Similar to forward extraction, the ionic strength on protein solubilisation to stripping phase was studied by varying the KCl concentration in the range of 0.3 to 1.7 M. The destabilisation of the micelles was further achieved by adding the alcohols (7 to 15% volume) like n-Propanol, n-butanol, n-hexanol and n-decanol as co-solvents. The effect of the phase volume ratio and the contact time (mixing time) were also studied. The separated aqueous and organic phases at each experiment were further subjected to the protein analysis. Folin-Lowry's assay

was used for pure LF solubilisation studies, whereas HPLC analysis was performed to determine the LF concentration for synthetic whey and real whey systems. The backward extraction efficiency (Eq.3.4) and yield (Eq. 3.5) were calculated.

Back Extraction Efficiency (%) =

$$\left[\frac{\text{LF concentration in stripping phase(mg/ml)}}{\text{LF concentration in organic phase(mg/ml)}}\right] \times 100 \quad (3.4)$$

$$Back\ extraction\ Yield\ (\%) = \left[\frac{([LF\ conc.in\ stripping\ phase]mg/ml) \times (Vol\ of\ stripping\ phase\ (ml))}{([LF\ conc.in\ org\ phase]mg/ml) \times (Vol\ of\ org\ phase\ (ml))}\right] \times 100 \quad (3.5)$$

The purity of LF (Eq. 3.6) after back extraction was calculated. The overall efficiency was calculated using Eq. (3.7).

Purity of back extracted LF (%) =

$$\left[\frac{\text{LF conc. in } t\square e \text{ stripping phase (mg/ml)}}{\text{Total protein conc. in } t\square e \text{ stripping phase (mg/ml)}}\right] \times 100 \quad (3.6)$$

$$Overall\ \ Efficiency\ \% = \left[\frac{Conc.of\ Back\ Extracted\ LF\ (mg/ml)}{Initial\ LF\ conc.in\ Whey\ (mg/ml)}\right] \times 100 \quad (3.7)$$

Minitab (18.0) computer program was used for the statistical analysis of the data. All the systems were conducted in triplicate, and the means were reported. Data were tested with analysis of variance (ANOVA) and Tukey's test to compare the means. The significance of means was measured at $P < 0.05$.

3.2.2.3 HPLC Analysis

HPLC (Shimadzu, LC-20AD, Japan) was performed using C18 (Shim-pack Solar, Shimadzu, Japan) column with size 250 X 4.6 mm l.D. Water and acetonitrile with 0.1% trifluoroacetic acid were used as mobile phase. Binary gradient mode was chosen with 0.5ml/min flow rate at column temperature 25º C. The binary gradient mode was maintained at 10% solvent B for 0.01 to 2min, and 90% solvent B was maintained till 15min and till 17min solvent B concentration was 0%. The runtime was 20min. Absorbance was measured at 254 nm using a UV detector. The column was prior equilibrated with mobile phases for 30min for sample injection. Calibration graph for pure LF was obtained with different concentrations (Appendix IV) and used to calculate LF concentration in extracted samples. The samples of standard LF, synthetic whey and acidic whey were analysed for the LF concentration using the chromatogram obtained during the analysis. The LF extracted in the organic phase during forward extraction and in stripping phase during back extraction was also analysed.

3.2.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

In order to assess the purity of extracted LF during forward and back extraction, the SDS-PAGE analysis was performed using 12% resolving gel and 4% stacking gel. Whey, commercially available pure LF, the micellar phase containing LF (obtained during forward extraction) and stripping phase containing LF (obtained during back extraction) were loaded in gel and compared with wide range protein marker. The 10µl volume of each sample was loaded. Electrophoresis was conducted at 75 V, for 3 hrs. Further, gel was stained using staining solution prepared by dissolving 0.05% (W/V) Coomassie brilliant blue (CBB) R-250 in a mixture of glacial acetic acid, methanol and distilled water in the proportion 1:4:5 for 90 min and destained with the same solution without CBB R-250 blue for overnight (Laemmli 1970).

3.2.3 RM characterisation

The Karl-Fischer titrator (Metrohm 899 coulometer) was used to determine the amount of water present in the RM after forward extraction since the W_0 has a significant effect over the size of the RM. The W_0 was represented as a molar ratio of water to surfactant in the reverse micellar phase. The W_0 of the RM was observed to be varying with different process variables. Hence the W_0 and the size of the RM were measured at all the variable combinations at which maximum LF extraction was noticed. The size of the RM is found to vary based on the $W_{0,}$ and the amount of protein entrapped within it. The size of the RM was determined using Qudix Scatteroscope, Korea at each variable combination (Appendix V). The W_0 and the size of the RM at different conditions like empty RM, in the presence of LF in the RM, RM at modified process conditions and the addition of additives were measured and reported.

3.2.4 Continuous extraction of LF from whey

The RDC consist of a cylindrical column provided with the central rotating shaft carrying equally spaced discs, which are positioned at the centre of each compartment made up of stator rings, was fabricated and used in the present study (Table 3.3). The RDC is made up of glass, and its shaft along with rotating disc and stator ring are made of copper. The dimensions of contactors as given in table (3.3) and schematic representation of RDC is as shown in figure 3.1. The light and heavy phases are introduced at the bottom and the top of the column, respectively, and allowed to flow counter-currently. The agitation provided by the discs improves the performance of the contactor by increasing the interfacial area for the mass transfer through the breaking of the dispersed phase droplets. The performance of a RDC depends highly on the hydrodynamic conditions, which are determined by the structural and flow parameters and also the physical properties of the contacting phases (Moris et al. 1997).

Table 3.2: Specifications of RDC

Specification	
The diameter of the column, D_c	2.54 cm
The height of the column, H	51 cm
Rotor diameter D_r	1.524 cm
Stator ring diameter, D_S	1.178 cm
Compartment height, Z_c	2.54 cm
Number of compartments	5nos
Volume of column	320 ml

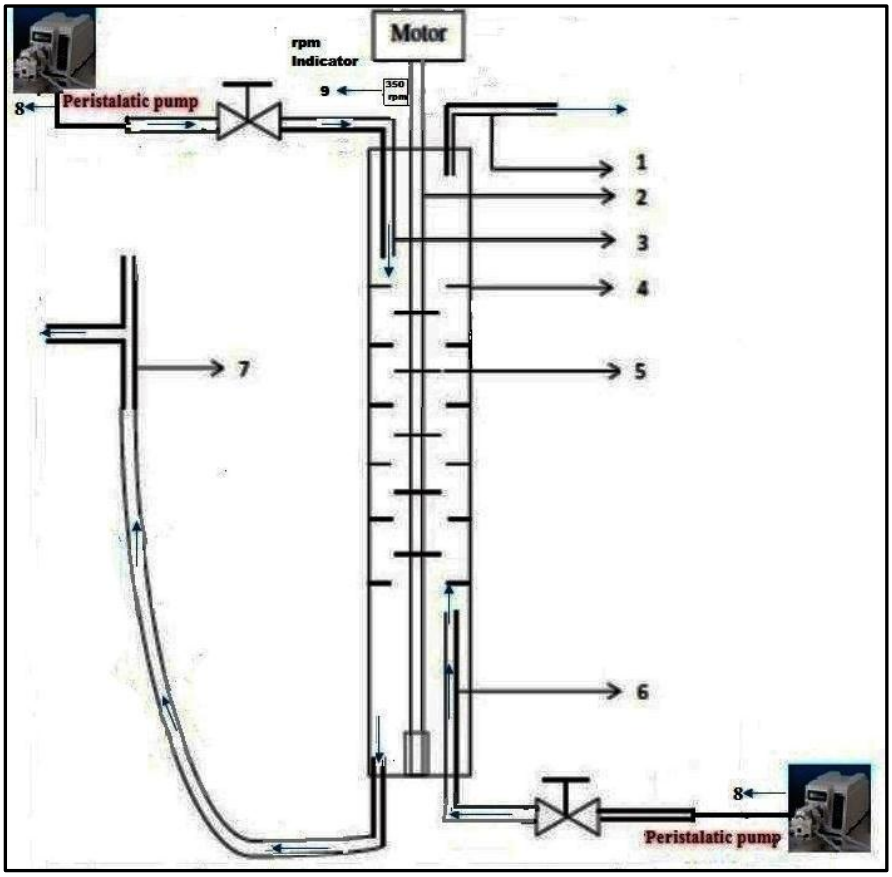


Fig. 3.1: Schematic diagram of RDC: (1) light phase outlet; (2) shaft; (3) heavy phase inlet; (4) stator disc; (5) rotor disc; (6) light phase inlet; (7) heavy phase outlet with adjustable limb (8) peristaltic pump; (9) rpm indicator

The continuous phase (aqueous/heavy phase) was pumped through the inlet present at the top of the column by using a peristaltic pump, and the dispersed phase (organic/light phase) was pumped into the column through the inlet provided at the bottom of the column. Continuous phase outlet was fitted with an adjustable limb which controls the position of the interface above the top stator ring of the column. The outlet of the dispersed phase was collected from the top of the column, whereas the continuous phase will leave from the bottom of the column. The speed of rotation could be adjusted by regulating the DC voltage to the motor and speed was measured by an electronic digital rotation

speed meter.

The RME was performed in the RDC at various operating condition by varying the speed of the rotor, flow rates of the aqueous and organic phases. The aqueous and organic phase flowrate was varied between 4.2 to 7.8 ml/min. The effect of rotor speed was studied between 150 to 450 rpm. At different combination of operating variables, the holdup, mass transfer coefficient and forward extraction efficiency were obtained. The desired flowrates of aqueous and organic phases were admitted into the column, and the rotor speed is fixed at the required level. The column was allowed to reach the steady state, which may be noticed by observing the fixed location of the interface. Once the column reached the steady state, the samples from the outlet of organic and aqueous phases were collected for the protein analysis to estimate the extraction efficiency and volumetric mass transfer coefficient. The equation 3.8, 3.9, and 3.10 were used to calculate extraction efficiency, recovery and volumetric mass transfer coefficient, respectively.

$$Extraction\ efficiency\ \% = \left(\frac{C_{ci}-C_{co}}{C_{ci}}\right) \times 100 \quad (3.8)$$

$$Recovery\ \% = \left(\frac{F_d \times C_{do}}{F_c \times C_{ci}}\right) \times 100 \quad (3.9)$$

$$K_{da} = \frac{l}{d} * ln\left[\frac{C_{di}-(K*C_{ci})}{C_{do}-(K*C_{ci})}\right] \quad (3.10)$$

Where,

Fd- Disperse phase flow rate (ml/min)

Fc- Continuous phase flow rate (ml/min)

C_{ci} – Initial protein concentration in continuous phase (mg/ml)

C_{co} – Concentration of protein in raffinate (mg/ml)

K_{da}- mass transfer coefficient (l/min)

l/d- dispersion volume (ml)

C_{di} – Initial protein concentration in disperse phase inlet (mg/ml)

C_{do} – Concentration of protein in disperse phase outlet (mg/ml)

Further, the holdup of the dispersed phase in the column was measured by volume displacement method by closing all the inlet and outlet of the column simultaneously. As soon as the flow rates are ceased, the interface starts to move towards the downward direction due to the disengagement and accumulation of dispersed phase above the interface. Then the known volume of continuous phase was added to the column to bring back the interface to the original position. The volume of continuous phase added will be used to calculate the holdup of the column (Eq. 3.11).

$$\text{Dispersed phase holdup, } \phi = \frac{\text{The } \textbf{volume} \text{ of the dispersed phase}}{\text{Total contacting volume}} \qquad (3.11)$$

CHAPTER 4

RESULTS AND DISCUSSION

The process development for RME of LF from complex source, whey, was attempted by selecting appropriate RMS. A systematic, methodical approach was adopted to extract LF with the highest purity from the whey. Initially, the screening and selection of suitable RMS were performed by considering the critical process variables like surfactant concentration, feed phase pH and salt concentration using commercially available LF. The physical and chemical properties of phase forming components are responsible for the RM formation to some extent. Hence, considering these properties of surfactants and organic solvents, six different RMS were formed, and their protein solubilising capacity was observed and analysed. The knowledge obtained during the screening is used to choose suitable RMS.

The selected micellar system is used to extract LF from its synthetic solution. The recovery and purity of the target solute can be enhanced by tuning the key variables of the process. The RMS is optimised to transfer LF from aqueous to micellar phase (forward extraction) and further micellar to fresh stripping phase (back extraction) by manipulating the process variables like feed phase pH, surfactant concentration, phase volume ratio, an addition of co-solvent, and protein concentration in feed phase. The RM characterisation is also performed since the W_0 and RM size is majorly influence the partitioning of protein during RME. The qualitative and quantitative analysis of the extracted protein is performed to analyse the efficiency of the RME process.

The behaviour and partitioning of solute could be different when other proteins and components are present in the source. Hence, the obtained optimised process conditions were further extended to extract LF from the synthetic solution prepared by mixing the commercially available whey proteins equivalent to their concentration in the acid whey (i.e., α-LA-1.5mg/ml, β-LG-3mg/ml, LF-0.1mg/ml, BSA- 0.3mg/ml and LPO- 0.03mg/ml). The extraction efficiency may be affected due to the presence of other whey proteins in the solution, which is present comparatively at higher concentration in the actual whey. The influence of other proteins on the LF extraction efficiency was studied, and the process variables are tuned further to improve the selective extraction. However, few non-proteinaceous components present in whey may interfere during the LF extraction. Hence, process conditions obtained for the selective solubilisation of LF from the synthetic whey proteins solution also examined with the acid whey. The reusability of the spent RM phase is not only improving the profitability and sustainability of the process by reducing the processing cost and also reduce the impact on the environment. Hence, the recycling of the micellar phase is one of the crucial parameters to make process cost-effective, is also studied with the optimised conditions.

The increased commercial demand of the LF is one of the major concerns in the current scenario. Hence, the RME of LF has been implemented in a continuous extractor, RDC. The optimized process conditions are considered to analyse the effect of the operating condition of RDC on the extraction efficiency. The extraction efficiency, recovery and mass transfer characteristics of the RDC were analysed at the various rotational speed, continuous and dispersed phase flow rates. The obtained results from all experiments including batch and continuous operation are discussed in detail with reasoning based on the available literature.

4.1 Screening of RMS for LF Solubilisation

Several micellar systems are employed for the selective extraction and purification of various proteins till date. The RMS should be selected such a

way that it should have better compatibility, higher selectivity and capacity with the biomolecule considered. It is also essential to check the characteristics of the RMS to achieve maximum extraction of the target solute. Initially, the physical and chemical properties of the components which form RMS namely, type of surfactants and organic solvents, along with the characteristics of the sources including the type and concentration of all the molecules and the specific bio-molecules present in it should be considered to select the suitable RMS. The physicochemical properties of micellar components majorly influence the extraction efficiency of the target solute. The number of possible RMS suitable for the RME of the target molecule may be formed, and their selective solubilisation characteristics may be studied by comparing the extraction capacity at different operating conditions. Various process variables like feed phase pH, salt concentration, the addition of co-solvent, phase volume ratio, W_0, size of RM and temperature are responsible for the solubilisation of solute to RM.

A number of RMSs formed by different surfactants belonging to three different categories including cationic, anionic and non-ionic surfactants with different solvents were explored to obtain better solubilisation of LF from the synthetic aqueous solution. The characteristics of the surfactants and solvents are analysed to select the RMSs for the present study (Table 3.1). Further, the suitable RMS was screened by studying the effect of essential variables, i.e. feed phase pH, salt concentration, W_0 and size of RM on the solubilisation of LF into the RM phase, i.e., the extraction efficiency. The effect of these variables in the individual RMSs is obtained and analysed to screen a specific system for the selective extraction of LF from the original crude. Specifically, the screening of micellar system will help to select an appropriate RMS for the better solubilisation of LF into RM phase, i.e. forward extraction.

4.1.1 Formation of RMS for LF solubilisation

The physical and chemical properties of the surfactants and organic solvents play an important role in RM formation. Topological surface area, rotatable

bond count and a number of hydrocarbon chain present in surfactant and solvents molecular structure are mainly responsible for the RM formation and also for the amount of water entrapped in the micelle core (W_0) (Mukerjee et al. 1977). The physical and chemical properties of all the selected surfactants and solvents are listed in Table (3.1). From the preliminary experiments on the formation of RM at different combinations using the solvents and surfactants listed in the Table 3.1 and six different RMS were able to form the stable RM (Table 4.1) with the combination of non-ionic surfactant (TX100/ Isooctane), anionic surfactant (AOT/Isooctane, AOT/ n-decanol) and cationic surfactant (CTAB/ Isooctane, CTAB/ Toluene, CTAB/n-Heptanol). The CMC, W_0 and size of the micelles were measured for all the six systems and reported (Table 4.1) at different condition. Specifically, the size and W_0 of the empty micelle and micelle after the solubilisation of LF in the RM at the critical micelle concentration was measured and compared for selecting the best system for the LF solubilisation.

Table 4.1: Physical Characteristics of RMSs

RMS	**CMC (mM)**	**W_0**		**RM size**	
		Empty RM at CMC (mol ratio in ppm)	**With LF at respective surfactant conc. (mol ratio in ppm)**	**Empty RM at CMC (nm)**	**With LF at respective surfactant conc. (nm)**
AOT/Isooctane	1	5.66	-	0.99	-
AOT/ n-decanol	0.8	6.48	-	1.13	-
TX100/ Isooctane	0.3	4.35	9.88	0.76	1.72
CTAB/ Isooctane	2	7.87	13.4	1.37	2.34
CTAB/ Toluene	2	8.95	-	1.56	-

CTAB/n-Heptanol	1	9.64	15.91	1.68	2.78

Even though, the six systems formed the stable RM, the three RMS formed with a cationic surfactant (CTAB/ Toluene) and anionic surfactant (AOT/Isooctane, AOT/n-decanol) were failed to solubilize the LF in the RM phase. However, the other three RMS (CTAB/ Isooctane, TX100/Isooctane and CTAB/n-Heptanol) formed with cationic surfactant able to solubilize the considerable amount of LF.

The formation of RM is best understood for the systems formed with surfactant and solvents, which are possessing topological surface charge, taking in the consideration of the sum of average total surface area of solvent and surfactant, presence of hydrocarbon chain around central molecule and rotatable carbon atoms in solute and surfactants (Mukerjee et al. 1977; Ray and Moulik 1994). Taking all physical and chemical properties in account, a relation between CMC value and W_0 of all RMS has been found and these parameters are proportional to the sum of averages of rotatable carbon atom and average charge on participants in group, which is lower (28) for TX100/Isooctane system at a CMC value of 0.3mM and corresponding less W_0 (4.35). In the case of AOT/Isooctane, the total rotatable bond count and average charge on the system found to be increased to 46.25, as result CMC and W_0 found to be increased to 1mM and 5.66, respectively. A similar result has been observed in AOT /n-decanol system with a little fall in CMC (0.8mM), which was supported by the stated theory and conclusion drawn in the literature.

Whereas, the systems (CTAB/Isooctane-CMC 2mM, CTAB/ n-heptanol- CMC 1mM and CTAB/Toluene- CMC 2mM) in which the solvent and surfactant both are charged, or any one of them shows zero topological surface charges, the availability of side chain hydrocarbon in the components along with the sum of the average of total surface charge of solvent and surfactant plays a major role (Tadros 2005). The side chain hydrocarbons interact with each other in different

symmetry and are responsible for steric repulsion between surfactant and solvent molecules leading to the formation of equilibrium for retention of water within it. As in these cases, the CMC value is high if the system does not have any net charge then, the RM formation is solely performed by the presence of side chain and the steric repulsion between surfactant and solvent molecule (Mukerjee et al. 1977; Ray and Moulik 1994; Tadros 2005). The surfactant required for the formation of the RM is found to be comparatively high due to a week or zero topological surface charges of the components. Accordingly, the size of the RM and corresponding W_0 also increases for the RM formed by CTAB, which enhances the solubilisation of hydrophilic bioactive components in the water pool of the RM. Further, the surfactants CTAB and TX100 contain only one lipophilic chain, therefore not able to form RM in organic media without addition of a second surface active agent, called as co-surfactant. In the case of CTAB/n-heptanol, n-heptanol is used as an organic phase which also acts as co-solvent and aids the formation of micelles. The initial analysis of the surfactants and solvents properties and the CMC, size and W_0 of the micelles suggests that the RM formed by CTAB surfactant may be suitable for the solubilisation of LF in the RM phase. However, the effect of surfactant concentration and aqueous pH on the LF solubilisation may provide much more insight about the suitability of the RMS.

4.1.2 Screening of RMS for the solubilisation of LF

Six different RMS, namely, AOT/Isooctane, AOT/n-decanol, CTAB/Toluene, TX100/Isooctane, CTAB/Isooctane and CTAB/n-heptanol were found to be favourable for the solubilisation of LF to RM by considering the wide combination of different surfactants and solvents. All the systems were subjected to RME of LF, and the forward extraction / solubilisation characteristics of LF was examined by carrying out the RME for 10 min with varying pH and ionic concentration. The RM characteristics like W_0 and size of the micelles and the physical properties of the reverse micellar phase (organic phases) were obtained at different conditions and analysed to identify a specific

system, which is capable of extracting/ solubilising the LF in the RM phase at higher concentration.

4.1.2.1 Effect of surfactant concentration

The effect of surfactant concentration in the organic phase on the LF solubilisation (%) was analysed by varying the surfactant concentration in the range of 10-100mM for all the six RMS. It was observed that the AOT/Isooctane, AOT/n-decanol and CTAB/Toluene RMS failed to solubilise the LF into RM phase even at a higher concentration when compared to its CMC due to the poor electrostatic interaction between surfactant and solute molecule (He et al. 2015). A small amount of LF 2%, 3% and 5% were get transferred to RM for TX 100/ Isooctane (40mM TX100 conc.), CTAB/Isooctane (80mM CTAB conc.) and CTAB/n-heptanol (50mM CTAB conc.), respectively (Table 4.2). The surfactants CTAB and TX 100 require the addition of a second surface active agent, called as co-surfactant for the stable micelle formation. In the case of CTAB/n-heptanol, n-heptanol is used as an organic phase which also acts as co-solvent and aids the spontaneous formation of a greater number of RM to capture more amount of LF. However, the lesser extraction efficiency was found in case of TX100/Isooctane and CTAB/Isooctane due to the longer chain alkane (isooctane) which is more hydrophilic than lipophilic used as organic phase (Street 1994). Further, the concentration of surfactant requirement in the RM for the better extraction of LF is very high in the RMS formed by CTAB when compare to their CMC, which indicates that the extraction mainly depends on the ionic interaction between the surfactant and LF. Hence the subsequent experiments are planned to deduce the effect of pH and ionic strength by varying the electrolyte concentration in the system for the better solubilisation of LF, since the interaction may be improved due to the modification of surface charge of LF.

Table 4.2: Effect of surfactant concentration on the solubilisation of LF in RM

RMS	CMC (mM)	Surfactant Conc. (mM)	LF solubilisation %
AOT/Isooctane	1	10-100	ND
AOT/n-decanol	0.8	10-100	ND
CTAB/Toluene	2	10-100	ND
TX 100/ Isooctane	0.3	40	2
CTAB/Isooctane	2	80	3
CTAB/n-heptanol	1	50	5

***ND: Not Detected**

4.1.2.2 Effect of aqueous phase pH

LF has the isoelectric point (pI) of 9.4. Below pI, LF carries a positive charge, and above the pI, it carries the negative charge (Steijns and van Hooijdonk 2000). To improve extraction efficiency, the pH of the aqueous phase was varied from 2-10 for all RMS. According to Li et al. (2007), better interaction between a positively charged head group of CTAB and negative charge on protein resulted in 5% -CTAB/Isooctane (pH-9.6) and 45% in case of CTAB/n-Heptanol (pH-10) as a result of the capture of LF into RM. However, only 3% of LF transfer has been observed in TX100/Isooctane (pH-7) (Fig.4.1) due to the absence of an electrostatic charge of surfactant. In the case of AOT/Isooctane and AOT/n-decanol, a white precipitate was observed at the interphase when a concentration of surfactants and pH of the aqueous phase was varied. The white precipitate may be the aggregated proteins that occurred when [LF]aq/[S] was high due to low hydrophobicity in the system due to lower surfactant

concentration used in the formation of RM (Lower CMC also). The AOT promotes the protein aggregation rather than protein solubilisation into organic phase (Mohd-Setapar et al. 2009). The extraction was found lesser in the CTAB/ Toluene system due to the organogel formation in the presence of excess water in the organic phase of the CTAB/ Toluene system (Vaidya et al. 2001).

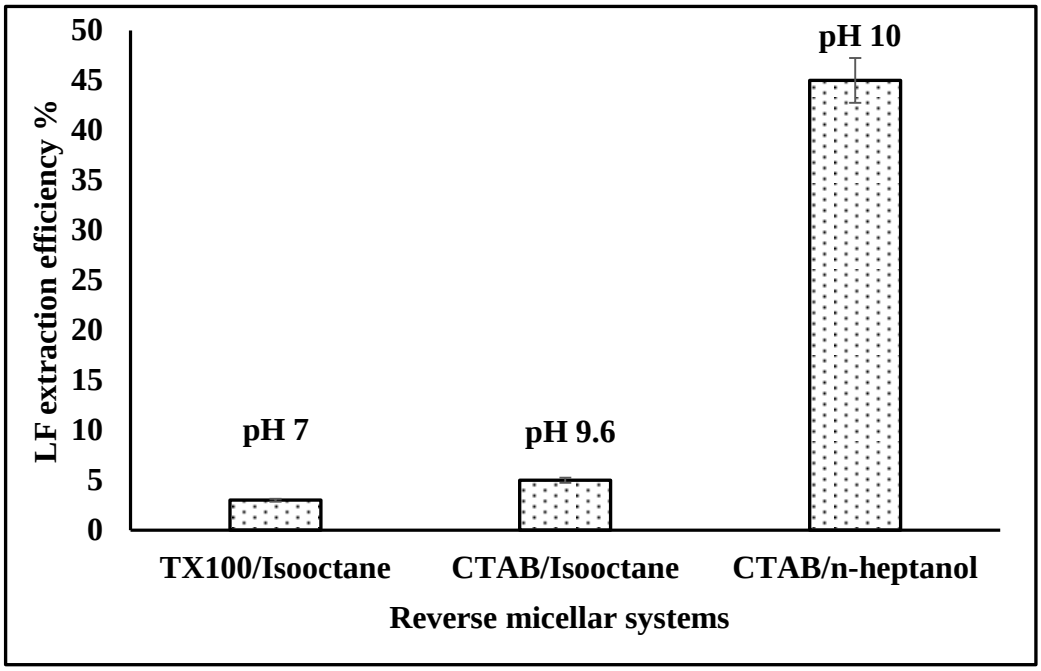


Fig. 4.1: Effect of pH on LF extraction efficiency on TX100/Isooctane, CTAB/Isooctane and CTAB/n-heptanol

4.1.2.3 Effect of Ionic strength

Salt concentration (KCl and NaCl- 0.1to1.3M) was varied in the aqueous phase to achieve better extraction efficiency in the three systems (TX100/Isooctane, CTAB/Isooctane and CTAB/n-heptanol) which gave the significant LF solubilisation. Addition of electrolytes to the aqueous phase of TX100/ Isooctane RMS could not help to improve LF extraction efficiency. 0.8M NaCl concentration in CTAB/ Isooctane found to increase protein transfer efficiency to 7% whereas 0.9M of KCl and 1M of NaCl concentration in case of CTAB/n-heptanol (Table

4.3) resulted in increased protein transfer efficiency 75% and 80% respectively. Addition of ions to the aqueous phase helps to stabilise the micellar structure and also enhances the electrostatic interaction between a polar head group of surfactant molecule and solute and results in better protein partitioning (Lakshmi and Raghavarao 2010).

Table 4.3: Effect of Salt concentration on LF solubilisation

RMS	NaCl		KCl	
	Conc. (M)	LF extraction Efficiency %	Conc. (M)	LF extraction Efficiency %
TX100/ Isooctane	0.1 to 1.3	-	0.1 to 1.3	-
CTAB/Isooctane	0.8	7	-	-
CTAB/n-heptanol	1	80	0.9	75

4.1.3 RM Characterisation

The W_0 and size of the micelles convey the extraction capacity of the RM. The W_0 and size of the micelles not only depends on the surfactant and solvent, it also depends on the characteristics of the biomolecule and the size/ molar mass of the biomolecule. W_0 of micellar systems after extraction of the LF has been measured (Table 4.1) and a notable increase in W_0 has been observed after LF transfer to the organic phase in all RMS. Comparatively, W_0 of non-ionic RMS has observed to be lower than ionic reverse micellar with isooctane and n-heptanol. Further, it was noticed that the size of the RM formed by the cationic surfactant is bigger than the RM formed by other surfactants. The negative surface charge of the LF at higher pH (> pI) and the positive charge of the cationic surfactants promote the interaction and improves the LF solubility in the micelles, and consequently, the size of the micelles is found to increase with the higher amount of LF. The larger number of micelles with higher size in the

CTAB/ n-heptanol than the CTAB/Isooctane improves the extraction. Due to the lack of presence of strong interaction forces; partitioning of LF to organic phase is low which also resulted in less W_0 during LF Extraction in case of TX100/Isooctane (Nagarajan 2002).

Among the six different RMS (Triton X 100/ Isooctane, AOT/ Isooctane, AOT/ n-decanol, CTAB/ Toluene, CTAB/ Isooctane, CTAB/ n-heptanol) selected based on the physiochemical properties of the reverse micellar systems, the three systems formed with non-ionic surfactant and cationic surfactant namely, TX100/Isooctane, CTAB/Isooctane and CTAB/n-heptanol were found to solubilize the LF. CTAB/n-heptanol RMS was found to be a favourable system at a pH of 7 and adding 1M of NaCl or 0.9M of KCl as electrolytes for LF solubilisation. CTAB/n-heptanol system was further used for the subsequent sections of the present work to selectively extract the LF.

4.2 RME of LF from the aqueous Solution

The screening of the various RMS suggests that the CTAB/n-heptanol system is the better RMS for the extraction of LF. The extraction efficiency may be improved by studying the effect of different process variables like feed phase pH, surfactant concentration, additive salt concentration and effect of co-solvent at a constant concentration of LF in the aqueous solution. These variables are studied in detail for the forward extraction of LF to the RM phase as well as back extraction of LF from the RM phase using the fresh striping phase. The optimum process condition at which the highest extraction of LF was identified by varying one of the process variables at a time by maintaining the other variables at a constant value. The yield percentage of LF was further improved by studying the loading capacity of the micellar phase at different LF concentration in aqueous feed phase and the volume ratio (volume of organic phase/ volume of aqueous phase). The change in micelle characteristics like the size of the RM and the W_0 of the micellar phase is also measured at different process condition to understand the effect of variables on the micellar characteristics.

4.2.1 Forward Extraction

The solubilisation of LF to the reverse micellar phase from the aqueous LF solution (forward extraction) is studied in detail. The extraction/ solubilisation efficiency may be improved by increasing the interaction between the surfactant and solute molecules in the RMS. As the electrostatic and hydrophobic forces are the primary forces for the RME, the net forces available for the better LF solubilisation was obtained by studying the effect of different variables which includes the surfactant concentration, aqueous phase pH, addition of additives like electrolyte salt and co-solvents etc., The results obtained from the study of each variable are presented and discussed in detail.

4.2.1.1 Surfactant concentration

The CTAB concentration in n-heptanol was varied from 10 mM to 100 mM, which is above the critical micellar concentration of CTAB/n-heptanol system (1 mM), to study the effect of surfactant concentration in the micellar system on the forward extraction of LF. The LF transfer from aqueous to organic phase was found to increase till the surfactant concentration of 70 mM. As much as 85% LF was transferred to the micellar phase (Fig.4.2) at 70 mM CTAB concentration. A gradual increase in the W_0 was also observed till the CTAB concentration of 50 mM, and further, it remained constant despite increasing surfactant concentration. The increased LF transfer to micellar phase with increasing CTAB concentration was due to the increase in a number of RM and a corresponding increase in W_0 (Hebbar et al. 2008; Krishna et al. 2002). Also, the higher surfactant concentration and increased RM in number are ultimately responsible for the increased protein solubilisation to RM (Krishna et al. 2002). Further, the RM size was not varied much by increasing the surfactant concentration which indicates that the enhanced extraction of LF was not because of the change in the size of the RM but due to the increase in the number of micelles and interfacial area between the micelles and aqueous phase at higher surfactant concentration. However, the transfer of LF to micellar phase was observed to decrease beyond 70 mM concentration of CTAB due to the

inter-micellar collision, micellar clustering and collapse of micellar structure (Nandini and Rastogi 2009).

Further, the enhanced surfactant concentration results in decreased protein uptake from aqueous phase due to rupture of RM (Chuo et al. 2014) and leads to gradual percolation and interfacial deformation along with change in micellar shape as well as clustering (Krishna et al. 2002).

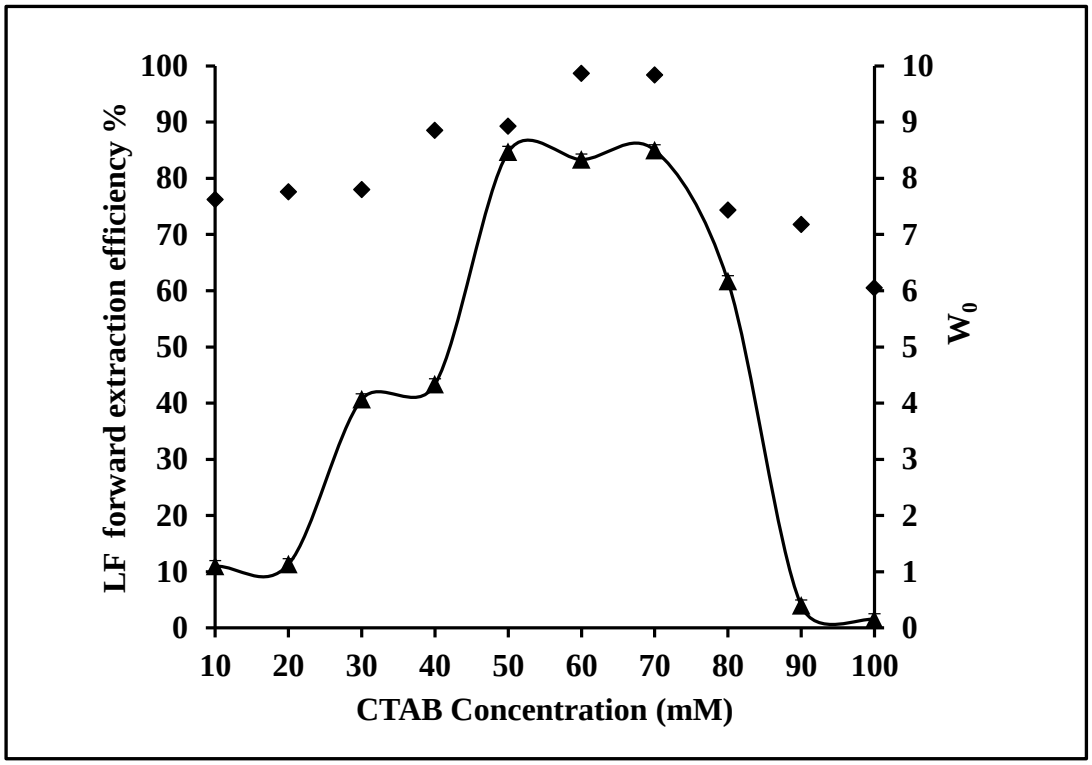


Fig. 4.2: Effect of CTAB concentrations on LF solubilisation (▲) into CTAB/n-heptanol RM and W_0 (♦) at an initial aqueous phase pH of 7.5

4.2.1.2 Effect of aqueous phase pH

Aqueous phase pH plays a crucial role in protein transfer to reverse micellar phase as it determines the ionisation state of surface-charged groups present on protein. The transfer of protein to RM is regulated by electrostatic interaction between proteins and the surfactant head groups (Ono et al. 1996). The net protein surface charge can be manipulated and modify the interaction between

the LF and RM by varying the solution pH. However, the protein transfer to RM phase occurs above the isoelectric point (pI) of the protein in case of cationic surfactants whereas it may happen below the pI for anionic surfactant (Krishna et al. 2002). The CTAB is a cationic surfactant and hence the LF, which has the pI of 9.4 and 9.5 for bovine and human LF, respectively (Steijns and van Hooijdonk 2000), is expected to interact with the RM around the pI of the protein. The protein charge may be varied by increasing the pH of the aqueous solution higher than the pI of LF. The aqueous phase pH was varied between 2 to 11 to study the effect of pH on the LF extraction (Fig. 4.3). The extraction efficiency was very low in acidic pH, but it was found to increase at basic pH. Maximum of 96.66% LF entrapment into the RM was observed at a pH 10, which is slightly above the pI of the LF (9.4). Above the pI of LF, the net charge of the LF changes to negative. Thus, negatively charged LF was found to interact with the positively charged head group of CTAB and facilitates the capturing of LF into RM (Li et al. 2007).

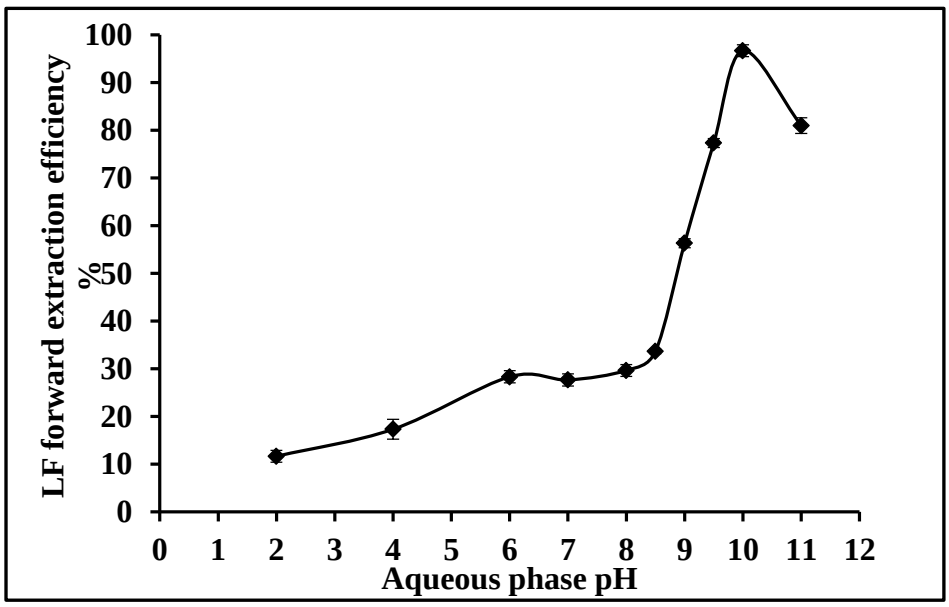


Fig. 4.3: Effects of aqueous phase pH (♦) on the forward efficiency of LF from a solution containing the LF concentration of 0.1 mg/ml.

Further, the size of the RM and protein molecule also has a significant effect on the extraction efficiency. Size of RM can also be increased by increasing the

number of the charged group on proteins through the manipulation of aqueous phase pH. According to Krishna et al. (2002), proteins with a less molecular weight (MW range 12KD-14.5KD) may require lesser pH-pI (i.e. less than 2) compared to larger molecular weight protein (MW range 33KD-48KD), which is around 5, for optimum protein transfer. With some surfactant concentration the size of the RM with LF was found to be much higher than the corresponding RM without LF (Table 4.4). The higher pH-pI and molecular size of LF (78-80KD) are favoured for the higher solubilization of LF to RM (Steijns and van Hooijdonk 2000). Hence the larger RM were formed at the pH of 10. A similar effect was explained for the extraction of bromelain, whose pI is 9.5 (Hebbar et al. 2008).

Table 4.4: W_0 and the size of RM at different process conditions

System Parameters	Without LF		With LF		LF Extraction Efficiency %
	W_0 (Molar ratio in ppm)	RM size	W_0 (Molar ratio in ppm)	RM size	
40mM CTAB/n-heptanol + water	8.532	3.51µm	8.852	--	43.33
50mM CTAB/n-heptanol + water	8.826	6.15µm	9.854	6.79µm	84.66
60mM CTAB/n-heptanol+ water	8.854	6.79µm	9.867	--	83.33
80mM CTAB/n-heptanol+ water	9.867	7.67µm	7.431	--	61.66

System Parameters	Without LF		With LF		LF Extraction Efficiency %
	W_0 (Molar ratio in ppm)	RM size	W_0 (Molar ratio in ppm)	RM size	
50mMCTAB/n-heptanol+ water+ (0.9M) KCl	7.085	5.60µm	9.197	134µm	97
50mMCTAB/n-heptanol+ water+ (0.9M) KCl+ n-Butanol (7%)	8.234	5.08µm	8.248	236µm	46
50mM CTAB/n-heptanol+ water+ (0.9M) KCl+ n-Butanol (15%)	7.931	3.73µm	7.947	4.89µm	13

4.2.1.3 Effect of ionic strength

The LF extraction efficiency may be further improved by modifying the ionic strength present between the molecules in RMS. The literature suggests the addition of salts may vary the protein solubilisation in reverse micellar phase, since the size and W_0 of the micelle may differ with respect to the modified electrostatic effect (Hebbar et al. 2008; Ono et al. 1996; Wan et al. 2016). The water forming salts like NaCl and KCl are generally considered to improve the forward extraction (Wan et al. 2016). The effect of salts was studied by adding the KCl and NaCl at different concentration (0.1 to 1.3M). The extraction efficiency was found to increase gradually at lower concentrations of both the

salts. However, the efficiency was found to decline beyond the salt concentration of 1 M for both the salts (Fig. 4.4).

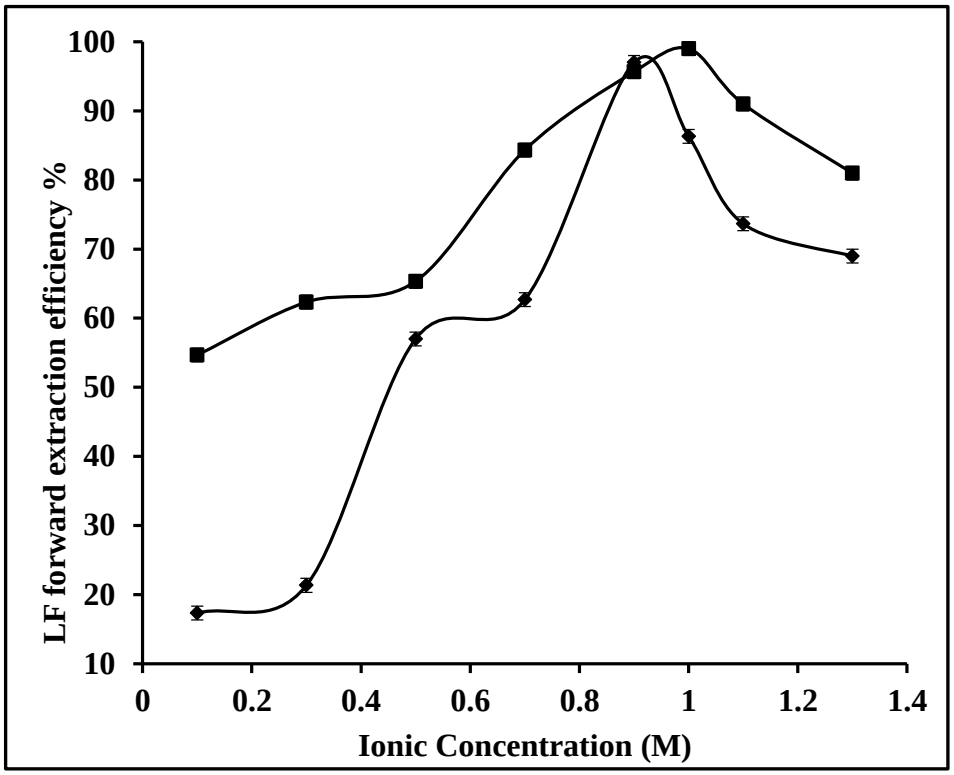


Fig. 4.4: Effect of inorganic salts KCl (♦) and NaCl (■) on the forward extraction of LF at an initial aqueous phase pH of 10 ±0.1

At lower salt concentration the repulsive force between the surfactant head groups decreased and hence the size of the micelle with a protein found to increase (Hebbar et al. 2008; Tonova and Lazarova 2008). The stable emulsion of water was also observed due to less interfacial tension at lower salt concentration. Consequently, the W_0 of the RM and extraction efficiency was found to increase. As the concentration of salt increases, the stability of the RM tends to increase with the reduction in size. The electrostatic interaction between CTAB head group and LF also decreased due to the interaction between the cationic surfactant head with the chloride ions. Thus the attractive interaction between the LF and CTAB was shielded due to the decreased Debye length and reduction in the thickness of the electric double layer (Tonova and Lazarova 2008). Hence, forward extraction efficiency was found to decrease with

increased ionic strength due to the smaller RM size (Chuo et al. 2014; Lakshmi and Raghavarao 2010). The presence of different cations, sodium and potassium, pronounce almost similar effect on the LF solubilisation. The maximum capture of protein to RM was observed at KCl concentration 0.9M (97%) and 1M (99%) of NaCl concentration (Fig.4.4).

4.2.1.4 Effect of LF concentration

The LF extraction capacity of the RMS was studied by varying the concentration of LF in the aqueous solution between 0.04 and 0.4 mg/ml at the optimum surfactant concentration and pH of the system since the LF concentration in the whey was 0.03-0.1mg/ml (Du et al. 2013). A negligible amount of protein was solubilised into the organic micellar phase until the LF concentration of 0.06 mg/ml in aqueous phase due to the lesser W_0 of the micellar system. The lower protein concentration is not sufficient to decrease the interaction between the surfactant heads and hence not able to stretch the micelles. Accordingly, W_0 and size of the RM was found to increase with increasing LF concentration from 0.08mg/ml (3.53µm) to 0.1mg/ml (6.79µm) but RM size and corresponding W_0 was found to be decreased from 6.79µm to 341.1nm as LF concentration was increased to 0.4 mg/ml (Fig. 4.5).

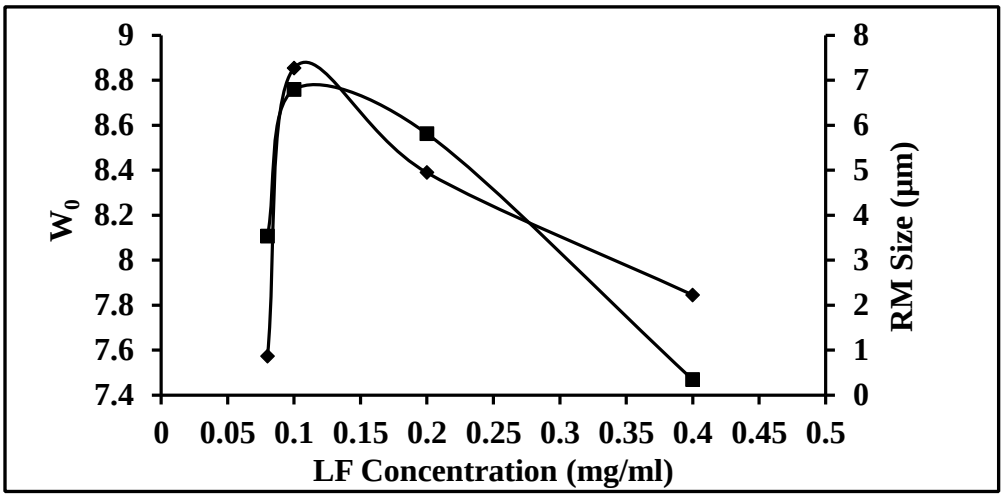


Fig. 4.5: Effect of various initial concentration of LF on RM size (■) and W_0 (♦).

Relatively higher concentration of surfactant in the organic phase also degrades the LF present in lower concentration. A significant quantity of LF solubility in the reverse micellar phase was noticed at 0.08 mg/ml, and the maximum solubility was observed at an LF concentration of 0.1mg/ml (Fig.4.5). However, the solubility was not improved beyond the concentration of 0.1 mg/ml, since all the positively charged head groups of CTAB may be engaged by negatively charged proteins at this concentration. Further, the additional LF solubility was restricted due to almost constant W_0 of the system with increasing concentration (Fig.4.5). Similar observations were reported by Mohd-Setapar et al. (2009) in penicillin extraction with anionic surfactant AOT with the reasoning that optimum surfactant could be different for increased penicillin concentration in feed phase which ultimately results in low extraction efficiency with same surfactant concentration.

4.2.1.5 Effect of phase volume ratio *(V_{org}/ V_{aq})*

For the effective extraction of the protein, the LF has to be extracted/ concentrated in a smaller volume of RM phase. This phenomenon may be examined by calculating the volume ratio (organic/aqueous phase). Generally, this ratio should be lower for forward extraction and higher for back extraction in an effective extraction system (Krishna et al. 2002). Effect of a phase volume ratio was studied by varying the ratio from 0.2 to 1.6 by maintaining the other variables at a constant value which provided maximum extraction efficiency. As the volume ratio increased, the extraction efficiency was found to increase until the volume ratio of 1. The amount of CTAB increases in the total system till the ratio of 1 and hence the extraction efficiency was found to increase (Fig. 4.6). However, the extraction efficiency was found to decrease beyond the ratio of 1 due to the change in organic phase volume which resulted in the change of CTAB concentration in the total mixture (Zhao et al. 2010).

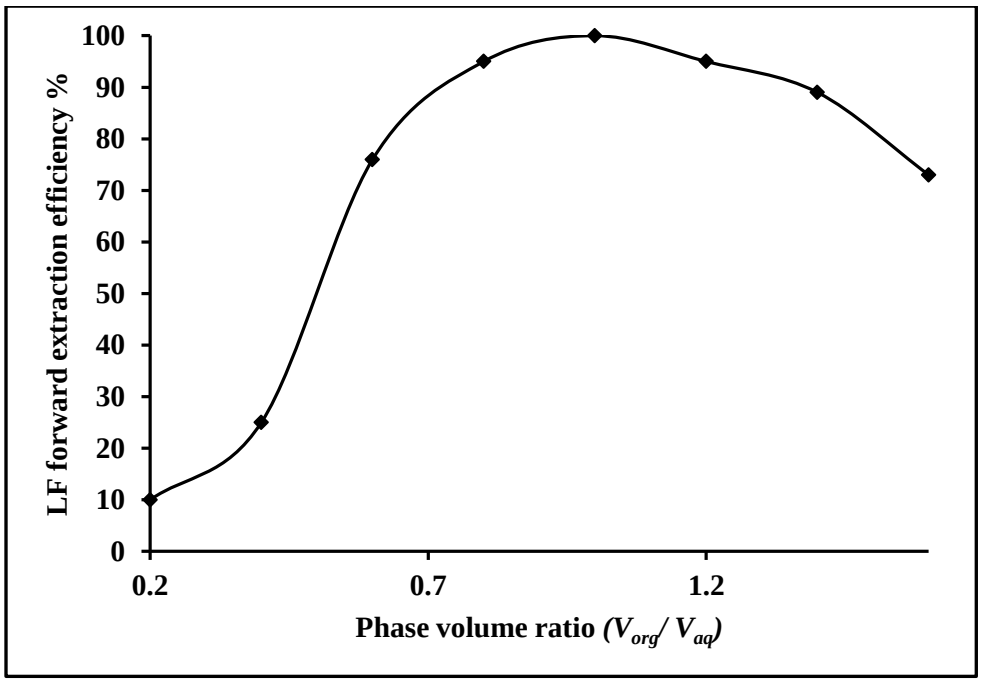


Fig. 4.6: Effect of phase volume ratio (♦) on forward extraction of LF

4.2.1.6 Effect of co-solvent addition

The cationic surfactants tend to form smaller micelle size than any other surfactant type, and the size of RM can be modified with the addition of alcohol as co-surfactant/co-solvent (Mathew and Juang 2007) through the interfacial resistance reduction and fusion/fission of the inverse micelles (Mukhopadhyay et al. 1990). The medium chain length alcohols tend to increase the size of the RM through the reduction of surface tension due to the adsorption of alcohol at the emulsion interface. The presence of alcohol changes the micellar properties due to the micelle-micelle and micelles-protein interactions. The n-butanol was considered as a co-solvent during the forward extraction of LF. The effect of alcohol was analysed in two different LF concentration (0.2 and 0.4 mg/ml) solutions by adding 7 and 15 % (V/V) n-butanol with CTAB/n-heptanol and (0.9M) KCl system. The addition of n-butanol resulted in increased protein extraction efficiency (Table 4.5). Improved extraction may be the result of increased hydrophilic interaction between organic and feed phase (Krishna et al. 2002). The RM size increases (5.81µm to 27.2µm for 0.2 mg/ml and 807µm for

0.4mg/ml) due to the increased hydrophilic force and W_0, since the alcohol adsorbed at the interface reduces the hydrophobic interaction between the hydrophobic tails of the surfactant and electrostatic repulsion between the charged head group (Mathew and Juang 2007). However, the higher concentration of alcohol reduces the stability of the RM and lead to denaturation of the protein.

Table 4.5: Effect of co-solvent on LF extraction efficiency

n-Butanol	LF concentration (mg/ml)	Forward extraction efficiency %
7%	0.2	80
	0.4	50
15%	0.2	80
	0.4	62.5

4.2.1.7 RM characterisation

The size and number of RM and the resulted W_0 in RM phase are mainly responsible for the solubilisation of LF in the RM phase, apart from the other interactions between the molecules due to hydrophobic and ionic interactions. The experiments were conducted initially to measure the size of RM and corresponding W_0 in the system by changing the surfactant and LF concentration. The expansion and contraction of the RM size and extraction efficiency of LF are based on the net ionic strength and hydrophobic forces caused due to the addition of alcohol as co-surfactant and electrolyte salts to modify the ionic strength. The characterisation of RM was performed at the conditions, which show a favourable LF solubilisation into RM phase (Table 4.4).

From the Table (4.4), it was observed that the size of the RM found to increase from 3.51 µm to 7.67 µm as the concentration of CTAB increases from 40 mM to 80 mM without the addition of LF. Accordingly, the W_0 of the RM has been found to increase from 8.532 to 9.867 (Table 4.4). The size of the RM and W_0 of the RM system found to increase further with the addition of LF at different concentration (Fig.4.2). The maximum extraction efficiency of LF and W_0 were observed at 50 mM CTAB, even though the size of the RM was increased beyond this concentration. Size of RM was found to increase from 6.15 µm to 6.79 µm with a corresponding increase in W_0 of RM from 8.826 to 9.854 when LF was entrapped in RM without altering other process conditions at 50 mM CTAB. The increasing surfactant concentration in the organic phase leads to the formation of bigger RM, and such larger RM may be useful for enhanced solubilisation of larger biomolecules in the RM phase. The size of the RM with LF was found to be higher than the corresponding RM without LF (Table 4.4). Increase in RM size with increasing W_0 may be due to the increased number of hydrogen bond at each acceptor due to high water-surfactant molecule ratio. Increased water-surfactant ratio allows more access of hydrogen bonding site to water that helps to solubilise LF into RM and resulting in increased RM size compared to empty RM (Jeffrey and Saenger 1991).

The size of the RM was found to increase drastically from 5.60 µm to 134 µm during the addition of KCl at the concentration of 0.9 M with an increase in W_0 from 7.1 to 9.2. As Cl^- ions tend to attract towards positively charged CTAB head group, the attraction of Cl^- ions to the surfactant head group results in increased thickness of the electrical double layer and increase in size of RM (Fathi et al. 2012). Further RM size and W_0 were measured with the addition of co-solvent. When 7% n-butanol (V/V) was added at LF concentration of 0.1 mg/ml RM size was found to be 236 µm (Table 4.4). RM size was found to decrease up to 4.89 µm for 0.1 mg/ml of LF when 15% n-butanol (V/V) was added. Addition of n-butanol resulted in a decrease in the electrical percolation threshold (φt) which resulted in a reduction of the thickness of the electrical

double layer and ultimately reduce the size of RMs (Mathew and Juang 2007). Hence, the addition of co-solvent in the forward extraction of LF is not recommended.

Further, RM size and W_0 analysis were carried out for a micellar solution containing various LF concentrations (Table 4.4). Concentration of LF was increased from 0.08 mg/ml to 0.4 mg/ml. Size of RM was found to increase from 3.53 µm to 6.79 µm for LF concentration of 0.08 mg/ml to 0.1 mg/ml, respectively. However, further increase in LF concentration of 0.2 mg/ml to 0.4 mg/ml, size of RM was found to decrease up to 341.1nm even though the CTAB concentration of 50 mM was maintained. Perhaps this could be due to weak intermolecular interaction between protein and surfactant molecules as a result of unbalanced charges on protein and surfactant molecules. At low LF concentration, the micellar size was observed to increase due to optimal intermolecular interaction between protein and surfactant molecule. As the protein concentration tends to increase, intramolecular forces in protein molecule mask the protein-surfactant interactive forces which result in charge unbalance and ultimately the reduction of micelle size (Jeffrey and Saenger 1991). Characterisation of RM not only helped to understand the effect of LF concentration on RM size and W_0 but it also confirmed the degree of solubilisation of LF into RM due to the variation observed in size and W_0.

4.2.2 Back Extraction

The back extraction of LF from the RM phase to the aqueous stripping phase is carried out by destabilizing the RM. The destabilization generally occurred with the variation of pH, an addition of additives to modify the ionic strength of the system, counter surfactant and alcohols as co-solvent to the stripping phase based on the RM characteristics and the entrapped biomolecules in it. Apart from these system variables few other operating variables like the modification of the equilibrium characteristics through the change of volume ratio and contact time of the phases in the process also have the significant effect in the destabilization of the micelles to release the entrapped bioactive components.

The back extraction may help to obtain the purified products and paved the way to recycle the micelles for the economic operations. Hence, some of the mentioned parameters which are essential for the back extraction of the LF from the organic micellar phase are optimized to obtain better back extraction efficiency.

4.2.2.1 Effect of stripping phase pH

The back extraction of LF from reverse micellar phase to fresh stripping phase was carried out by altering the pH of fresh aqueous phase (Mohd-Setapar et al. 2009) and electrostatic interactions using a different concentration of electrolytic salt, KCl. The back extraction can be achieved through the electrostatic repulsion between the RM and LF. The effect of the pH value of the stripping aqueous solution was studied between the pH values of 2 to 10 (Fig.4.7). Maximum of 91% LF was released from RM at pH value of 6. In general, the proteins gain the net surface charge based on the isoelectric pH of the protein (pI). Above the pI, the net surface charge would be negative and vice versa (Krishna et al. 2002; Steijns and van Hooijdonk 2000). The pI of the LF was reported as 9.5 (Steijns and van Hooijdonk 2000), and hence the LF attained the positive charge at the pH of 6 and released from the RM due to the electrostatic repulsion between a positively charged head group of surfactant and protein (Pires and Cabral 1996).

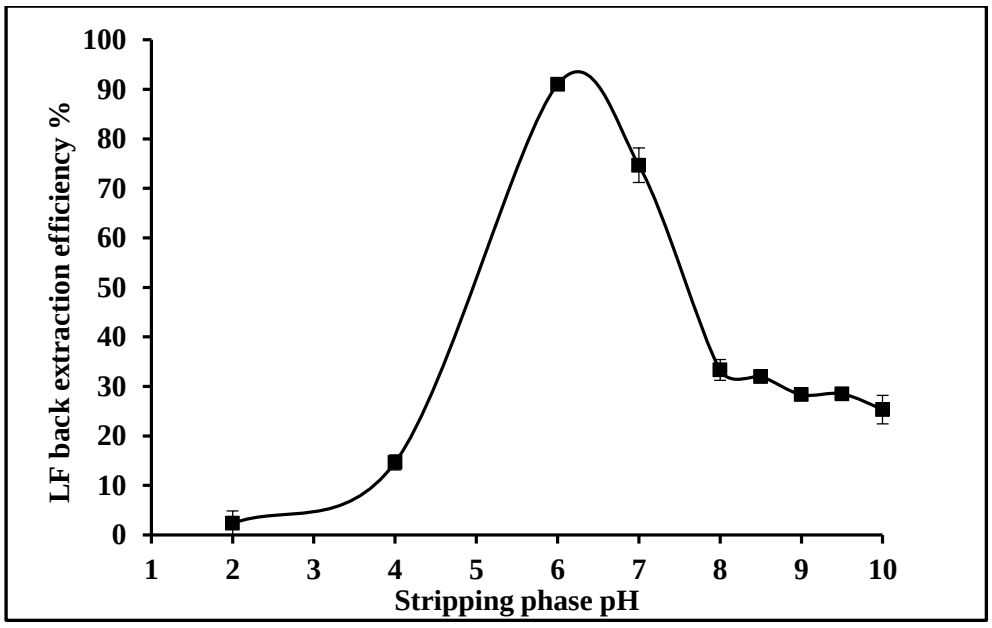


Fig. 4.7: Effects of stripping phase pH on the back extraction (■) efficiency of LF from a solution containing the LF concentration of 0.1 mg/ml.

4.2.2.2 Effect of ionic strength

The ionic strength developed due to pH variation may not be sufficient to overcome the micelle-micelle, and micelle-protein interaction exists in the reverse micellar phase due to the strong electrostatic interaction. Further, the protein encapsulated micelles interacted between them and leads to the formation of micelle aggregates and cluster, resulting in decreased back extraction of LF. The ionic strength of the fresh aqueous phase solution reduces this effect to a certain extent as a result of the Debye screening effect. The electrostatic interaction was further reduced by adding the electrolyte salt KCl with the aqueous phase. KCl was considered since the larger K^+ ions are capable of causing higher solubilisation as compared to other ions with smaller sizes such as Na^+ (Zhao et al. 2010). Further, K^+ cations are chaotropes in nature and help to destabilise the hydrophobic aggregates and increase the back extraction of proteins (Gaikaiwari et al. 2012). The effect of electrolyte salt KCl on the back extraction of LF was studied by adding the KCl solution at different concentrations of 0.3 to 1.7M (Fig. 4.8).

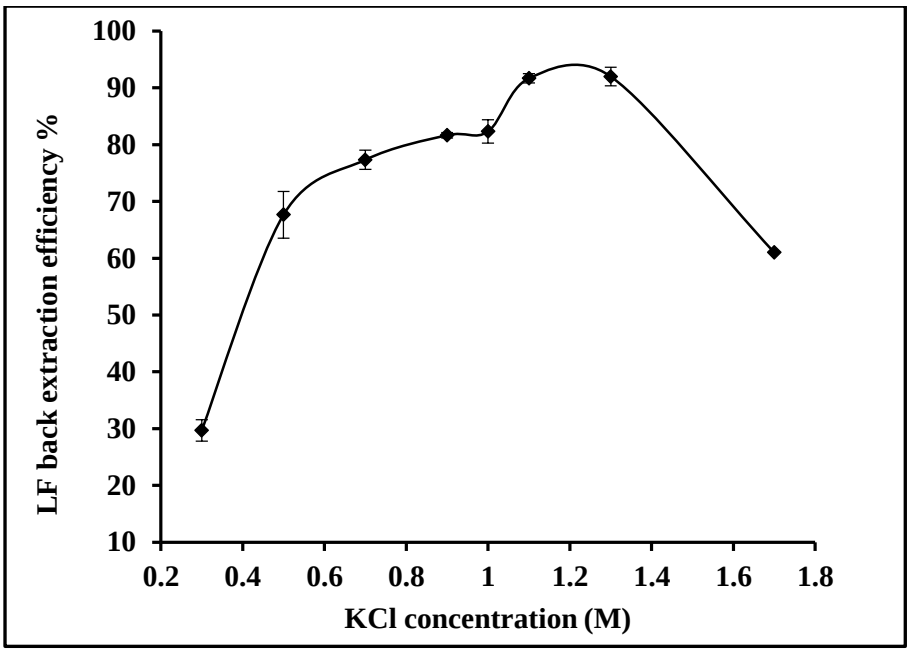


Fig. 4.8: Effect KCl (♦) on the back extraction efficiency at a stripping phase pH of 6.

The maximum back extraction of LF (92%) was obtained at 1.3 M to 1.5 M of KCl. Addition of KCl reduces the size of RM due to the stronger interaction between the cationic surfactant head with the chloride ions and thus squeezing out the solutes contained in the RM. However, the K^+ cations represented as a chaotrophic agent which destabilises the RM by disrupting the hydrogen bonding network between the molecules, resulting in increased extraction of LF into the aqueous phase (Pires and Cabral 1996). The addition of higher concentration of KCl (>1.7M) reduces the LF release from RM since the increased ionic strength resulted in cloudy phase formation (Li et al. 2007) due to the denaturation and precipitation of proteins. Hence the LF back extraction was favourable between the KCl concentrations of 1.3 M to 1.5 M.

4.2.2.3 Effect of co-solvent

Further to reduce the interaction between micelle-micelle and micelle-bio-molecules, different alcohols were studied, since alcohols have amphiphilic property as a co-surfactant. To enhance the back extraction efficiency, many researchers have studied the effect of the addition of alcohol as co-solvent

(Mathew and Juang 2007), since the back extraction was controlled by the interface resistance than the diffusional resistance in the reverse micellar phase and an aqueous phase. Hemavathi et al. (2010) reported that the addition of alcohol could reduce the interactions by reducing the micellar interface resistance. Alcohol is well known to break and destabilise the RM through the coalescence of reduced interface resistance inverse micelles in the solution (Mukhopadhyay et al. 1990). The experiments were conducted to understand the effect of alcohol chain length and their concentration during back extraction. Four different alcohols, namely n-propanol, n-butanol n-hexanol and n-decanol were used in the range 3% to 15% V/V (Fig. 4.9). Maximum back extraction (98%) was obtained with n-propanol and n-butanol. Even though higher chain length alcohols are reported as suitable alcohol for back extraction of solutes than the lower chain length alcohol (Mathew and Juang 2007), the LF was successfully back extracted with n-propanol. Back extraction efficiency with higher alcohols (n-hexanol and n-decanol) was found to be lesser since the long chain alcohols cannot penetrate into the RM and smaller alcohols can easily penetrate an interfacial layer of RM and disrupt the hydrophobic surfactant-protein interaction (Hong and Kuboi 1999). Further, the higher chain length alcohols at higher concentration lead to protein degradation (Ono et al. 1996).

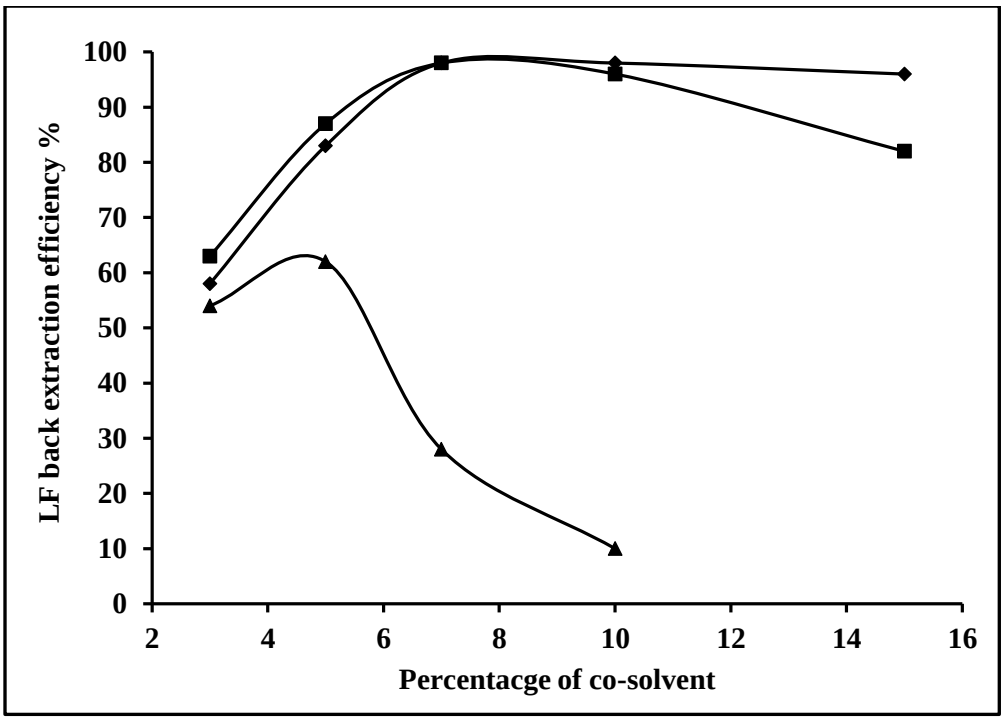


Fig. 4.9: Effect of co-solvent addition (▲-n-hexanol, ■- n-butanol and ♦-n-propanol) during back extraction of LF from CTAB/n-Heptanol RM phase using aqueous stripping solution at a pH of 6.

4.2.2.4 Effect of phase volume ratio and phase contact time

Other than the system variables, the effect of operating variables like phase contact time and a volume ratio of the phases were also studied for the back extraction of LF. The phase volume ratio (V_{aq}/V_{org}) was varied from 0.2 to 1. It was observed that the back extraction increased with increasing phase volume ratio and maximum back extraction was obtained at the ratio of 1 (Fig. 4.10), which indicates that the stripping phase has limited recovery capacity (Li et al. 2007).

Similarly, the phase contact duration was also found to have a significant influence on the recovery of protein as the protein transferred from organic phase to aqueous phase across the interphase by overcoming the interfacial resistance for mass transfer (Chuo et al. 2014). Effect of phase mixing duration

on back extraction was studied for a range of 15 to 120 minutes at a magnetic stirrer speed of 800 rpm. It was observed that less time of phase mixing results in no protein extraction to stripping phase as the external forces fail to destabilise the micellar structure to squeeze out protein. When the mixing time was increased from 30 to 90 min, the recovery of LF increased from 0% to 93%. The optimum mixing time was about 60 minutes (Fig.4.11).

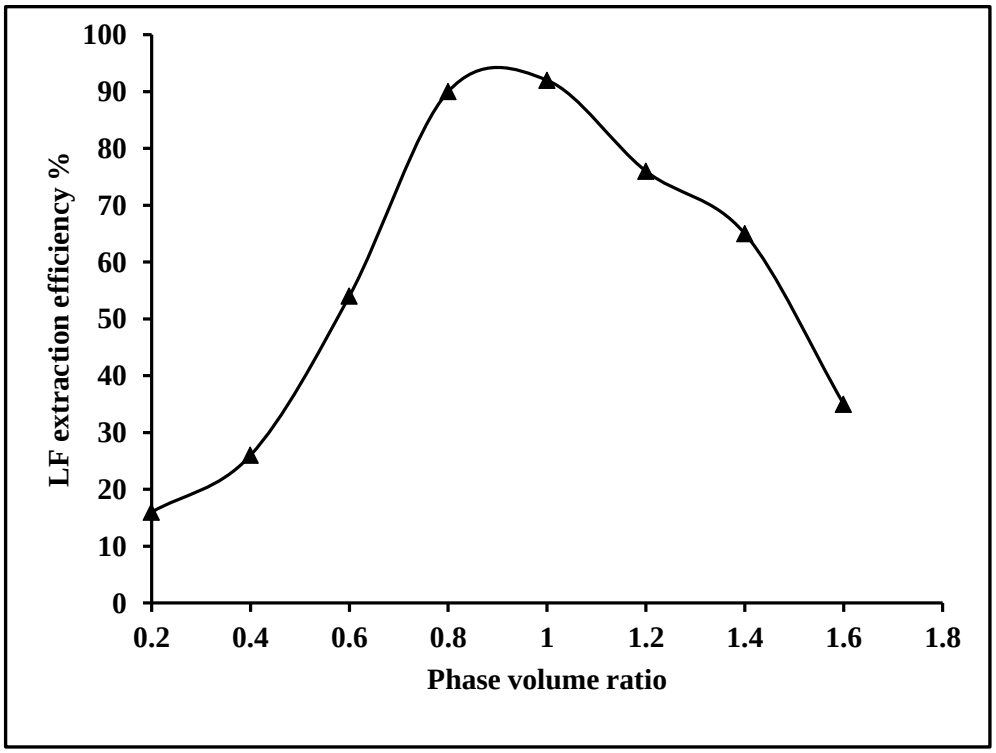


Fig. 4.10: Effect of phase volume ratio (▲) on back extraction of LF

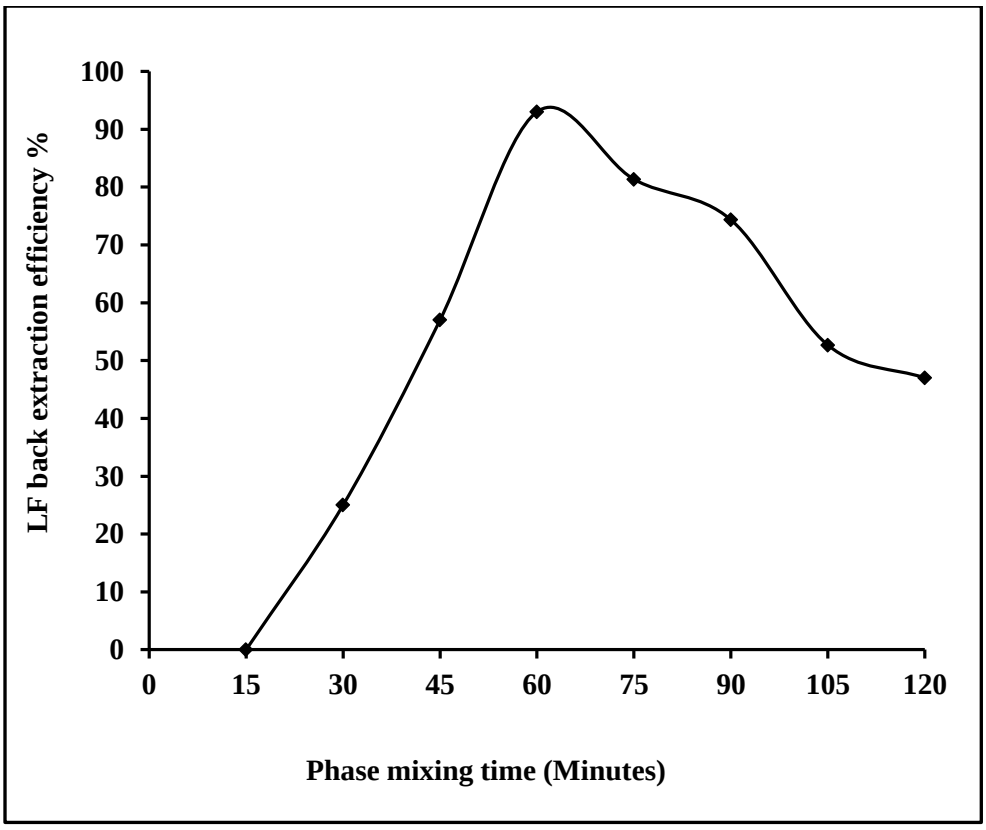


Fig. 4.11: Effect of phase mixing time (♦) on back extraction of LF

4.2.3 Qualitative analysis of extracted protein

The presence of LF in the stripping phase after back extraction was confirmed by performing the RP-HPLC analysis. After studying each back-extraction parameter, HPLC analysis was done to confirm the back extraction. The chromatograms of the sample collected from the back-extraction experiment, which gave the highest extraction efficiency were compared with commercially available LF (Fig. 4.12). With appropriate chromatographic conditions, the peak for the elution of pure LF was observed at 5.3 minutes (a) and back extracted LF at 5.25 minutes (b). In the case of back extracted LF, the slight shift was observed in peak elution due to the presence of different ions in the sample solution. However, it is assumed that there are no significant structural changes due to the RME as the observed peak pattern is similar to the pure LF peak.

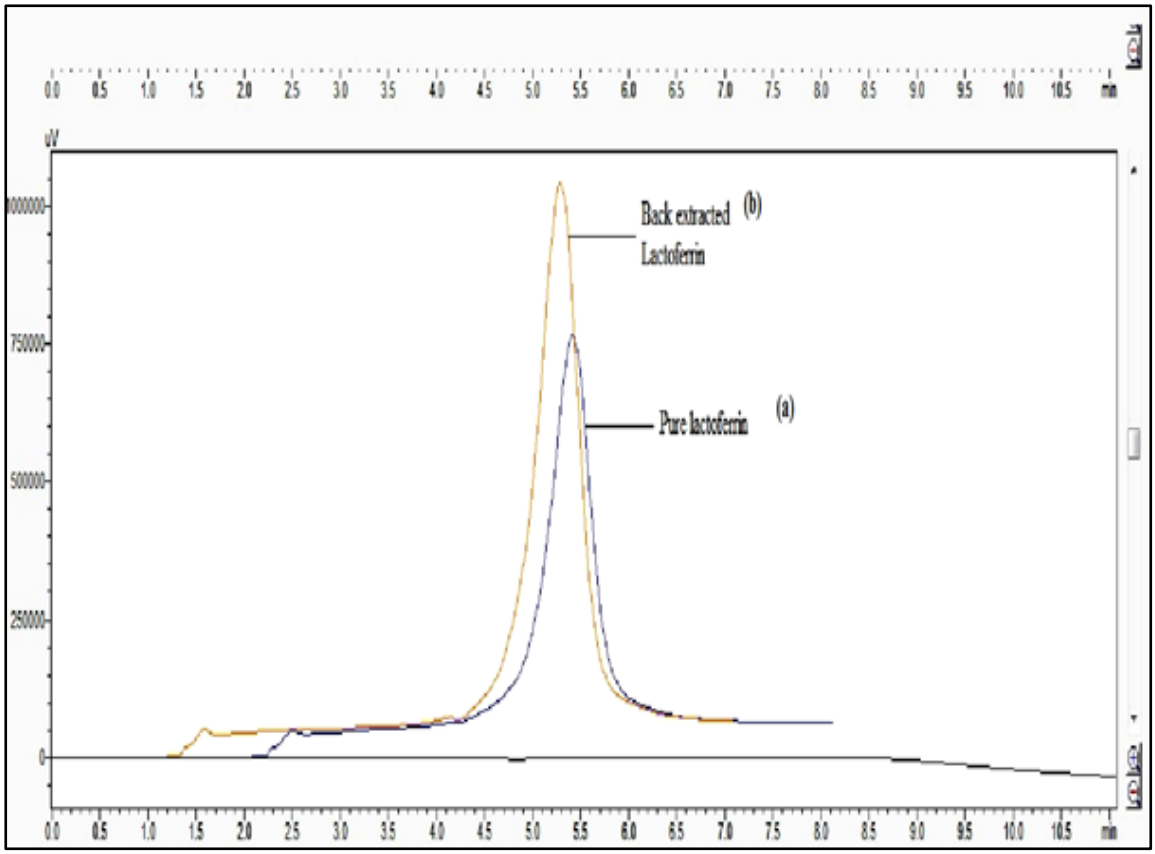


Fig. 4.12: HPLC chromatogram of commercial LF (a) and back extracted LF (b)

The CTAB/ n-heptanol RMS is studied for forward and back extraction of LF from its synthetic solution by analysing the important process variables like aqueous phase pH, ionic strength, phase volume ratio and co-solvent and almost all the LF might be solubilised during the forward extraction in the RMS having 50mM CTAB concentration in the n-heptanol with the addition of 0.9M KCl at pH 10. A notable increase in RM size has been observed, i.e. 5.60µm to 134µm at the optimised forward extraction conditions. 98% of LF was back extracted at pH 6 with the addition of small amount of co-solvent (7% n-propanol or n-butanol) and electrolyte (1.3M KCl). LF was found to be stable after back extraction to the fresh stripping phase, which was confirmed by the HPLC analysis. The selected RM system proved its suitability for LF extraction from a synthetic solution. The selected RMS (CTAB/ n-heptanol) with the identified

process condition was further considered for the selective extraction of LF from the synthetic whey proteins solution and whey.

4.3 RME of LF from synthetic whey proteins solution and bovine acidic whey

The optimised conditions obtained with pure LF solution are further extended for the selective extraction of LF from the synthetic solution prepared by mixing the commercially available whey proteins equivalent to their concentration in the whey and bovine acidic whey prepared in the laboratory. The extraction behaviour LF in the micellar phase due the presence of other components (impurities) like metals, other whey proteins and the influence of these impurities on the extraction efficiency and the selectivity is described in this section. The effect of operating conditions like feed phase pH, salt concentration, phase volume ratio, the addition of co-solvents and loading capacity of the micellar phase were studied. The forward and back extraction experiments were performed by considering the knowledge and conditions obtained during the solubilisation of LF to the micellar phase from section 4.2.

The synthetic whey was prepared by mixing the whey proteins according to protein concentration present in whey to mimic the real whey. Bovine acidic whey was prepared by the acidification of the commercially available pasteurized toned milk (Nandini, Karnataka). The results were compared and analysed with those obtained during LF extraction from aqueous LF solution. Further, the recycling capacity of micellar phase is studied to check the reusability of the micellar phase components obtained after back extraction of the LF, since the micellar phase components are able to reform the RM structure.

4.3.1 Whey characterisation

Whey is a complex mixture of proteins, carbohydrates and traces of minerals. The physicochemical properties of whey were analysed (Table 4.6) and compared with the literature. The composition of the components in the whey is

essential to improve the selective extraction of LF by understanding their effects during the extraction process (Fee and Chand 2006). Even though the whey properties are reported by (Du et al. 2013; Smithers 2015), the analysis of the whey prepared in the present work is essential since the properties and composition of whey are dependent on the preparation methodology and the source of milk. Table 4.6 listed out the values obtained in the present study for each parameter. It was observed that the protein and carbohydrates concentrations were within the concentration range reported in the literature. However, the lower concentration of metal ions (sodium, potassium, calcium, magnesium) was observed when compared to the literature (Table 4.6).

Table 4.6: Whey characterisation

Parameters	**Present work**	**Literature Value**	**Reference**
BOD	21000 ppm	>35,000 ppm	(Smithers 2015)
COD	41680 ppm	˜80,000 ppm	
Total Protein	6.473 mg/ml	6.8 mg/ml	(Du et al. 2013)
Total Solids	3.0658 %	6.5 %	
Total Carbohydrate	43.5 mg/ml	47 mg/ml	
Density	1.01553 gm/cm^3	1.0654 gm/cm^3	
Minerals			
Sodium	97.5 ppm	500 ppm	
Potassium	257.3 ppm	1500 ppm	
Calcium	189.1 ppm	600 ppm	
Magnesium	72 ppm	100 ppm	
Zinc	2.26 ppm	1.5 ppm	
Iron	0.529 ppm	0.6 ppm	

4.3.2 Forward Extraction

The variation in the concentration of metals and other impurities in the whey may significantly influence the selective extraction of LF. Hence, the optimum conditions reported in the literature for the partitioning of LF from the synthetic solution of LF (Pawar et al. 2017a) may not be the optimum process condition for the whey. The forward extraction was carried out at the optimised condition (50mM CTAB in n-heptanol at pH 10 with the addition of 1M NaCl) for synthetic whey which was prepared by mixing the pure whey proteins (α-LA, β-LG, LF, BSA and LPO) and real whey prepared in the laboratory. However, relatively less transfer of LF (88% - synthetic whey and 84.66% - real whey) to RM was observed at this condition when compared with the 100% extraction of LF from synthetic solution. Hence the experiments are performed to improve the extraction efficiency by studying the effect of essential process variables like aqueous phase pH, ionic strength and phase volume ratio, and the addition of additives during the forward and back extraction of LF from the complex sources by understanding the interferences caused by the other proteins and impurities.

4.3.2.1 Effect of aqueous phase pH

Many useful proteins are present in the whey, and each protein differs in their physicochemical properties, structure and functions. The variation in these properties can be exploited to purify a specific protein selectively from the complex protein mixture like whey. The change in surface charges in response to the changed pH (Krishna et al. 2002) is one of the critical characteristics of the selective extraction of the target protein. The changes in aqueous phase pH are found to change the charge density on protein or any solute (Hossain et al. 1999). Specifically, the electrostatic interaction between the solutes and the charged surfactant head group is the major driving forces during the RME of any biomolecule, which may be different for each protein present in the solution.

The forward extraction of LF from the synthetic solution of pure LF to micellar phase was studied at different pH and observed that pH 10 was the suitable pH for the highest solubilisation of LF. In the whey, the proteins like α-LA, β-LG and BSA are present in high concentration compared to LF. Hence, there is a possibility of interference of such proteins during the entrapment of LF into RM (Fee and Chand 2006). Also, the weak molecular interaction between surfactant head group (positively charged) and LF (negatively charged) lead to the lesser extraction of LF at pH 10 (Li et al. 2007). Hence, the pH of the aqueous phase was slightly adjusted within the range of 10 to 11 to improve the extraction efficiency by increasing the interaction between the molecules. The maximum solubilisation of 96.33 and 97.46% of LF into the micellar phase has been observed at pH 10.3 for synthetic and real whey, respectively (Fig. 4.13).

The increase in pH of aqueous phase resulted in partial precipitation of major proteins like α-LA, β-LG and BSA. The precipitation is the result of high protein-surfactant ratio. The increased ratio results in the decrease of the hydrophobicity of the system and tends to aggregate proteins instead of solubilising into micellar phase as the surfactant concentration is less. Similar precipitation of solute was observed during micellar extraction of Penicillin-G extraction by Mohd-Setapar et al. (2009). Consequently, loss of total protein (6.8 to 2.13 mg/ml) in the aqueous phase was observed. Also, the increased pH led to conformational changes of α-LA (Dhanapati et al. 1997) and irreversible transformation of β-LG (Tanford et al. 1959). Furthermore, an increase in pH found to reduce the LF extraction efficiency by up to 62% for synthetic as well as real whey (Fig.4.13).

As the pH of the solution gradually increases, accordingly the concentration of basic ions (OH^-) in the aqueous solution increases. When both phases are mixed to solubilise LF into RM phase, basic ions tend to interact with a positive head group of CTAB; but at the same time, LF bears negative charge above its isoelectric point (9.4) (Steijns and van Hooijdonk 2000). This intermolecular interaction between LF and the polar head group of CTAB tended to increases

the basic ion concentration in the aqueous phase and resulted in the repulsion of the LF molecule. Thus, the solubilisation of LF in RM beyond the equilibrium, the electrostatic interaction was limited (Pires and Cabral 1996). Similarly, W_0 (8.1) was found to be high at pH 10.3, and subsequent fall (4.3) was observed as the extraction efficiency decreases at pH 11.

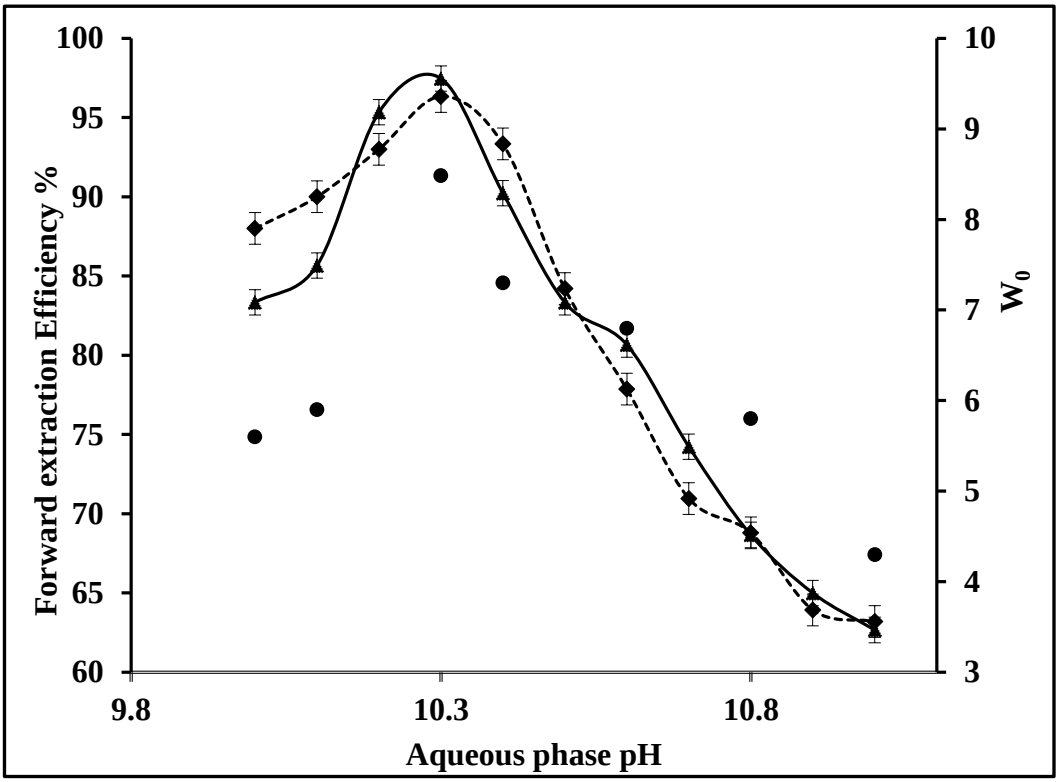


Fig. 4.13: Effect of aqueous phase pH on the forward extraction of LF. (♦, synthetic whey; ▲, real whey; ●, W_0 (real whey)).

4.3.2.2 Effect of Ionic strength

The electrostatic repulsion between the surfactant head groups in the RM may be modified by varying the ionic strength through dissolving the electrolyte salts. The absence of ions in the system results in accumulation of surfactant and/or the surfactant and protein complex which leads to precipitate the protein with surfactant molecule and hinders the phase separation (Mohd-Setapar et al. 2009). The addition of electrolyte salts at an optimum concentration may improve the solubilisation of solute into the organic phase.

Further, the addition of electrolyte helps to reduce the interfacial tension of the solution and thereby improved the formation of RM through the inverse emulsion. The addition of 1 M NaCl improved the solubility of LF (99%) to the RM phase during the extraction of LF from the aqueous solution of pure LF (Pawar et al. 2017a), which reveals that the ionic strength has a significant effect over the solubilization of the LF to the RM phase. Hence, the effect of ionic strength on the LF solubilization from the synthetic whey mixture and real whey to micellar phase was studied (Fig.4.14).

The maximum amount of LF solubilisation to RM phase from synthetic whey and real whey was observed as 98.04% and 98.7% at the NaCl concentration of 1.1M. Whereas, a very sharp drop in LF transfer to RM, i.e. 79.66% to 25.66% in synthetic whey and 80.66% to 34.56% in case of real whey were observed between 1.3 and 1.5 M of NaCl (Fig.4.14). When electrolytic strength is increased ions tend to form an electrostatic layer and surrounds the micelles polar core that tend to reduce electrostatic attraction between the charged protein and the charged inner core of micelles. In other words, the interfacial tension is being lowered when salt was added and helps to improve solute solubilisation to micellar phase. Along with this, the smaller ions (Cl^-) produce less screening effect and allow more protein to entrap in RM. The anions pronounce more effect on the extraction than the cations. During LF extraction, anions (Cl^-) were also transferred into the reversed micelles by electrostatic interaction (Li et al. 2007). The decrease of LF transfer efficiency with the increase of salt concentration is the result of reduced Debye length due to the increased electrostatic interaction between ion and surfactant and reduced interaction between solute and surfactant (Tonova and Lazarova 2008).

The increased salt concentration in the system also alters the RM size and resulted in the exclusion of LF from RM and affects extraction efficiency (Nandini and Rastogi 2009). The direct effect of W_0 on RM size and extraction efficiency has been reported at different operating conditions (Pawar et al. 2017a). Therefore, the size of micelles was measured at optimised parameters

for the forward extraction of LF from a synthetic solution of whey as well as real whey. RM size was around 138 µm for both synthetic and real whey. However, the size of RM was remarkably less for the empty RM in both cases (7.83µm). Accordingly, the W_0 of RM was also found to be less in both micellar solutions i.e.7.6. As the W_0 of RM is increased, the water-CTAB ratio is increased and allows access of more hydrogen bonding site on surfactant as well as water to solubilise protein in the RM (Jeffrey and Saenger 1991).

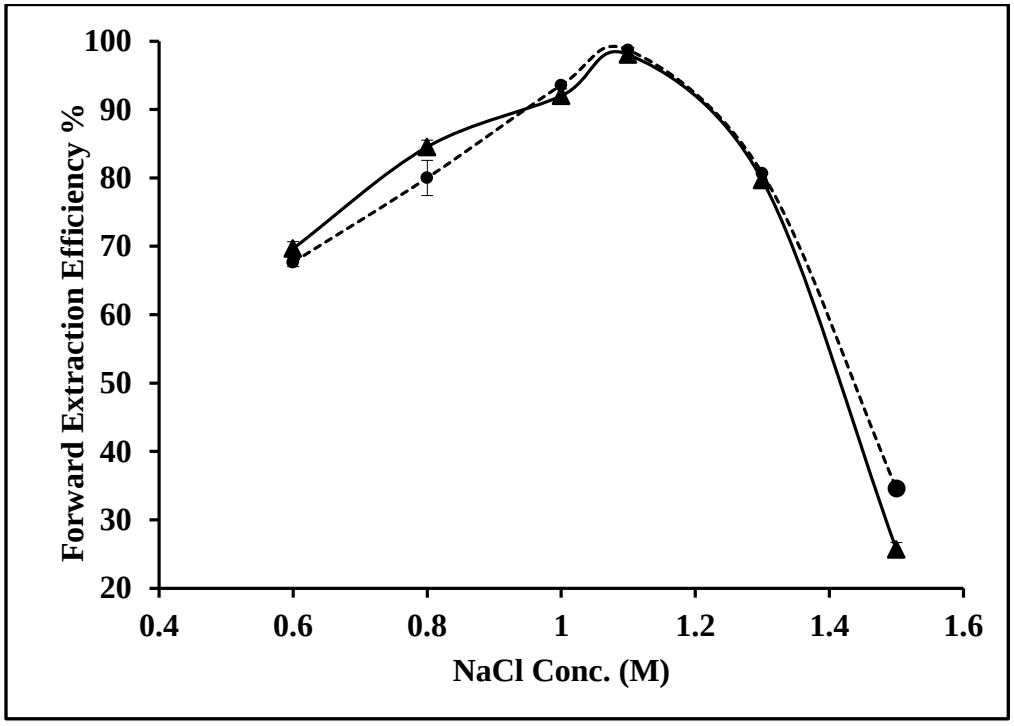


Fig. 4.14 Effect of salt concentration on the forward extraction of LF (▲, synthetic whey; ●, real whey)

4.3.2.3 Effect of phase volume ratio on forward extraction (Vorg/Vaq)

The phase volume ratio (V_{org}/V_{aq}) was studied to improve the yield of LF in the RM phase by understanding the maximum solubility of LF in the RM. The experiments were conducted between the phase volume ratios of 0.25 to 2.0 and found that the extraction efficiency of LF increases from 138.47% with increasing phase volume ratio to 256.31% till the phase volume ratio of 0.3.

Further, the extraction efficiency was found to gradually decline with increasing volume ratio and reduced to 49.84% at the ratio of 2 (Fig.4.15).

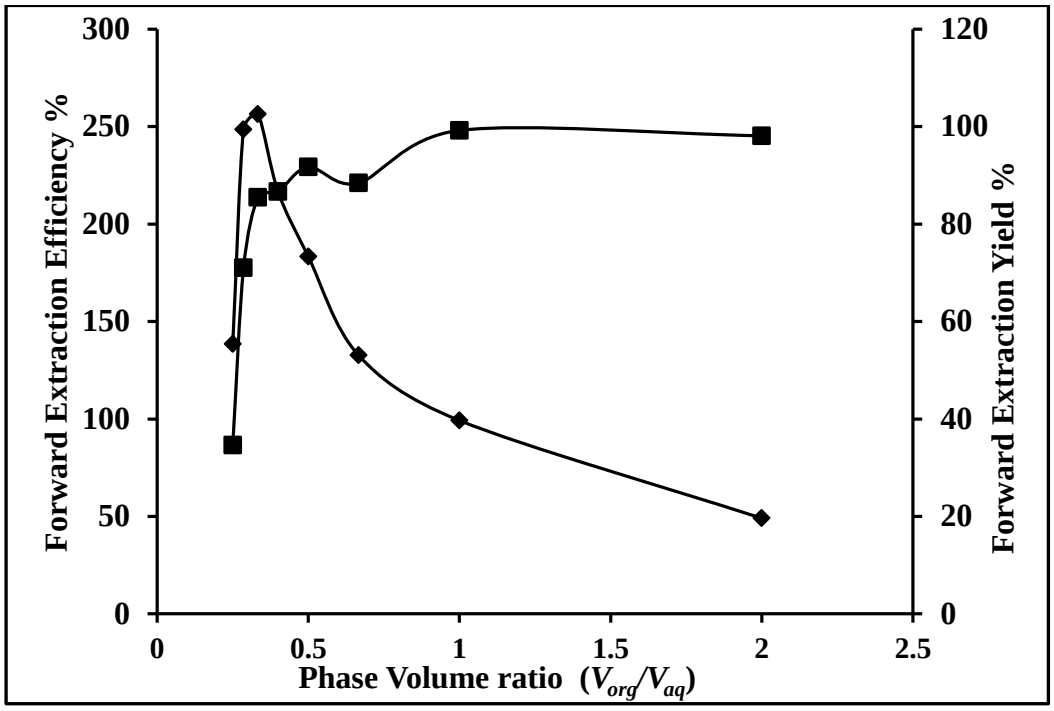


Fig. 4.15: Effect of phase volume ratio (V_{org}/V_{aq}) on forward extraction efficiency (♦) and yield (■) of LF

The highest yield of LF in the RM phase was observed at the volume ratio 1. Even though the maximum LF concentration was achieved at the volume ratio of 0.3, the relatively lesser yield was observed due to the lower volume of RM phase. At lower phase volume ratio, the volume of the organic phase is not sufficient to accommodate all the LF from the aqueous phase.

Further the RM also less stable due to the saturated concentration of LF in the RM phase and consequently resulted in the leakage of the aqueous phase from the organic phase (Bhavya et al. 2012). However, the LF concentration in the RM phase decreases with increasing volume ratio due to the decrease in overall surfactant concentration in the system (Li et al. 2007) and hence the RM was less stable with reduced interactive force with LF. Accordingly lesser extraction

efficiency was observed at higher volume ratio. The yield is not decreased at higher volume ratio since sufficient RM are available to accommodate all the LF with a lower concentration (Fig.4.15).

4.3.2.4 Effect of concentration of LF in whey

The maximum capacity of the RM may be realised by studying the yield with increasing LF concentration in the crude. The maximum quantity of LF may be selectively partitioned to the RM phase at the optimum volume ratio with maximum possible equilibrium concentration of LF in the organic phase, which was the function of the LF concentration in the feed phase. Hence, the experiments are planned to increase the LF yield in the RM phase by increasing the LF concentration in the crude during the forward extraction. The effect of increasing LF concentration from 0.2 to 0.7mg/ml was studied during the forward extraction by maintaining the other process conditions as constant. Highest LF recovery (76.5%) was obtained when LF concentration was maintained at 0.2 mg/ml in the whey. The drastic fall in the recovery of LF was observed as the concentration of LF was increased beyond 0.2 mg/ml. W_0 was found to be constant (8.4) for all the RM formed irrespective of LF concentrations. Hence, the size of the micelles is not changing with the increased load of LF concentration. The decreased solubility of LF to organic phase was due to the insufficient number of CTAB molecules and a lesser number of RMs in the organic phase (Fig. 4.16). It was believed that the increased concentration of target protein in the feed phase might demand a higher concentration of surfactant to increase the extraction efficiency by providing ample amount of RM (Mohd-Setapar et al. 2009).

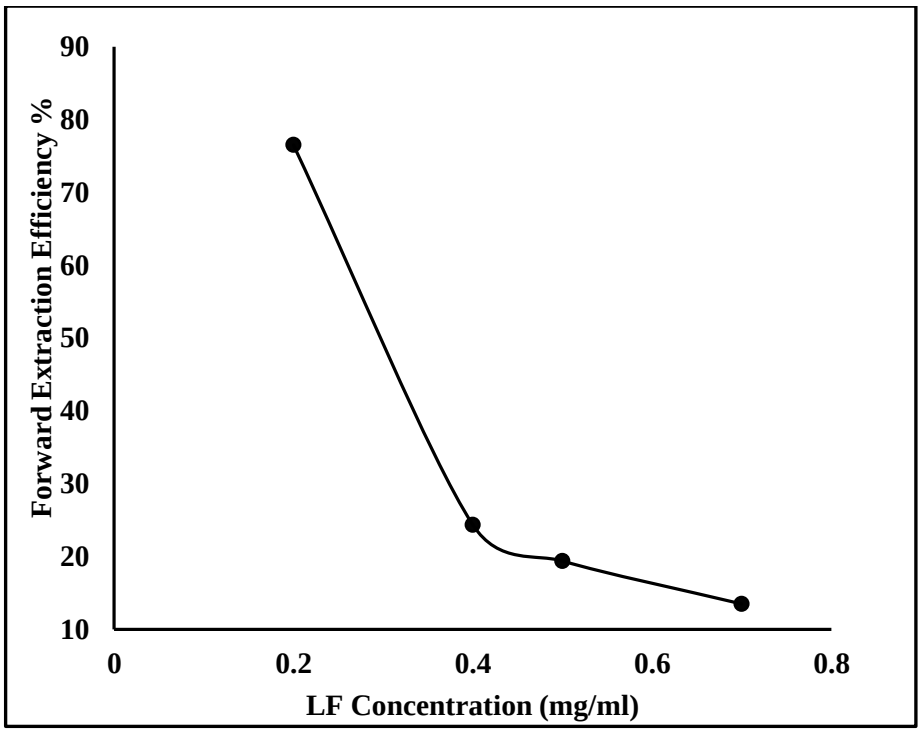


Fig. 4.16: Effect LF concentration on forward extraction (●)

4.3.3 Back Extraction

4.3.3.1 Effect of salt concentration

Selectively extracted LF to the RM phase has to be back extracted to a fresh aqueous stripping phase to recover the LF from the RM phase. The process condition should be maintained in such a way that the interactive forces between the RM and LF should be destroyed and LF should be released from the RM. The repulsive force between protein and a polar head group of surfactant is mainly responsible for releasing the protein from RM (Krishna et al. 2002; Nandini and Rastogi 2009). Such repulsive force may be created by modifying the ionic strength of the system through the addition of appropriate concentration of ions and change the pH of the aqueous stripping phase. The back extraction of LF from the RM phase of forward extraction with pure synthetic solution was studied in previous section and reported that the pH 6 with the addition of small amount of co-solvent (7% n-propanol or n-butanol) and electrolyte (1.3 to 1.5 M KCl) is the optimum condition to back extract the

LF. The chaotrophic ions like KCl were found to be more useful to rupture RM (Gaikaiwari et al. 2012). The back-extraction efficiency was studied in the present study by extending the findings. 93.42% and 94.2% of LF was back extracted from the micellar phase (obtained from forward extraction process) to the stripping phase at 1.5M KCl concentration with synthetic and real whey, respectively (Fig 4.17).

The increased KCl concentration (>1.7M) in stripping phase resulted in precipitation of protein at the interface that ultimately reduces the LF extraction efficiency for both synthetic as well as real whey to 54.5% and 44.9%, respectively (Fig. 4.17). Along with potassium ion, the co-solvent n-butanol also acts as a chaotropic agent that helps to weaken the hydrogen bonding network between a water molecule and also reduces the stability of the native state of proteins by weakening the hydrophobic effect (Salvi et al. 2005). The effect of n-butanol as co-solvent at different concentration was studied but failed to increase the extraction efficiency significantly other than 7%, which was reported earlier for the pure LF extraction studies.

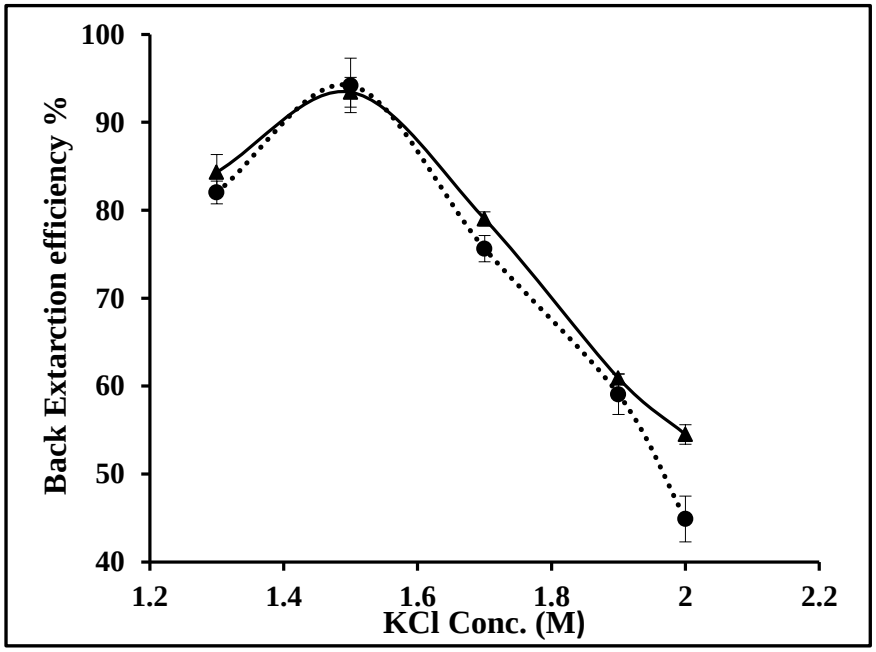


Fig. 4.17: Effect of salt concentration on the back extraction of LF. (▲, synthetic whey; ●, real whey)

4.3.3.2 Effect of phase volume ratio

The effect of volume ratio (Vorg/Vaq) on the back-extraction efficiency and yield of LF was studied for real whey to obtain the actual quantity of stripping phase added to the system for the better back extraction. It was observed that the back extraction efficiency was increased from 9.4% to 97.77% as the volume ratio increases from 0.25 to 1 and it was found to decrease to 67.27% as volume ratio increased to 2. However, the yield was found to increase from 37.98% to 97.77% to the volume ratio of 1 and gradually decreased after that to 33.63% by increasing the volume ratio to 2 (Fig.4.18). The higher volume of stripping phase at lower volume ratio tends to increase the ionic strength and rupture the RM. The hydrogen bonding of the protein with water molecules got weaken and the proteins released from the RM. The yield and extraction efficiency found to decrease at the higher volume ratio two due to the relatively lesser volume of stripping phase which may not be able to accommodate all the LF released from the RM phase. However, the less extraction efficiency was noticed at extreme volume ratios of 0.25 and 2, due to the denaturation and precipitation of LF (Salvi et al. 2005). Analysis of variance (ANOVA) for the studied variables also shows that each variable has a significant effect on extraction efficiency of LF for synthetic as well as crude whey since means end the column with different letters differs significantly ($P < 0.05$) (Appendix VI).

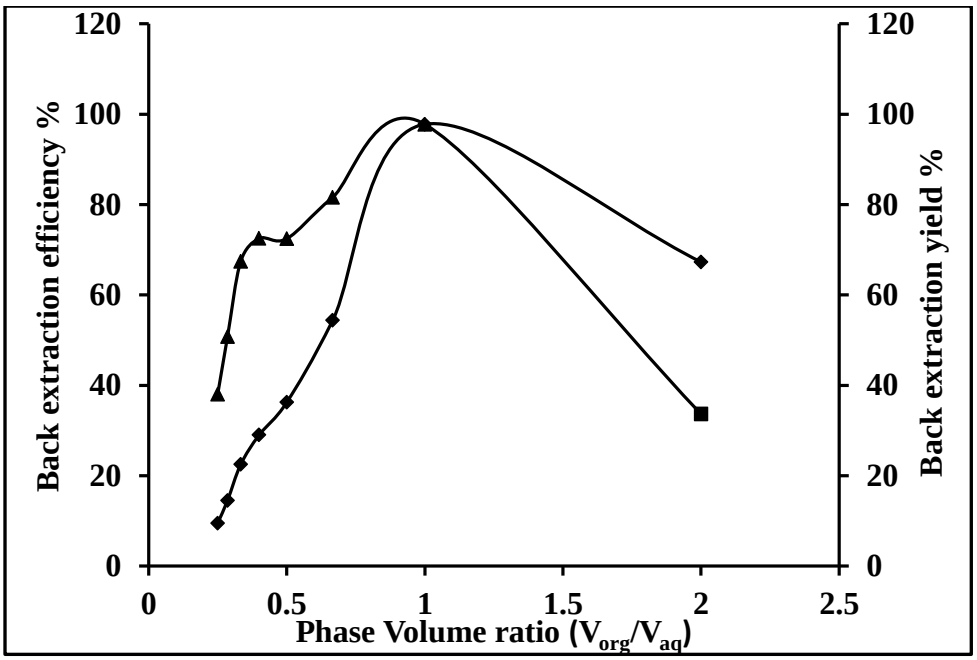


Fig. 4.18: Effect of phase volume ratio (V_{org}/V_{aq}) on back extraction (extraction efficiency (♦) and yield (■) of LF

4.3.3.3 Purity analysis of extracted LF

The RP-HPLC was utilised to obtain the chromatogram of LF, and other proteins profile at different stages of the extraction and the concentrations were quantified. The area of the chromatogram peak corresponding to the LF for different samples were compared with the standard graph developed at different concentration of LF. The LF peak elution was obtained at 9.038 min (Fig.4.19.a) for the standard LF. The synthetic whey prepared by dissolving the pure proteins like α-LA, β-LG, BSA and LPO with LF corresponding to the whey composition was subjected for the HPLC, and the obtained chromatogram is presented as (Fig. 4.19.a). With similar chromatographic conditions, forward as well as back extracted LF sample was analysed, and peaks were observed at 9.017min (Fig.4.19.b) and 8.851 (Fig.4.19.c) min, respectively. In the case of RM phase sample obtained from the forward extraction, the additional peaks are obtained other than the proteins peaks (Fig.4.19.b). Those peaks are belonging to the solvent and surfactant, which was confirmed by comparing with the chromatogram obtained for the empty RM without LF. Interestingly, the LF

alone selectively get extracted from the whey as well as the synthetic solution of whey proteins by leaving all the other proteins and impurities during the forward extraction, which was confirmed by the chromatogram with a single peak for LF at 9.017min along with the peak of solvent and surfactant. However, a slight shift in the LF peak elution time was observed for the RM phase and stripping phase when compared with the standard LF elution time due to the interference of organic components and ions in the samples.

The SDS-PAGE analysis also performed to confirm the selective extraction of LF from the crude mixture. The image consisting of protein marker (Lane 5), whey (Lane 4), Pure LF (Lane 3), organic phase of forward extracted LF (Lane 2) and stripping phase of back extracted LF (Lane 1) was obtained (Fig.4.20). The RM phase of forward extraction was loaded in Lane (2), but due to hindrance caused by the solvent of the organic phase, the protein band is not visible in the respective lane (2). The figure (5) indicates that the appearance of a single band of stripping phase (Lane1) corresponds to protein band in lane (3, 4 and 5) and absence of other protein bands which were visible in lane 4 confirms the presence of LF alone in the stripping phase. The obtained result is in concurrence with the RP-HPLC results and confirms the selective extraction of LF.

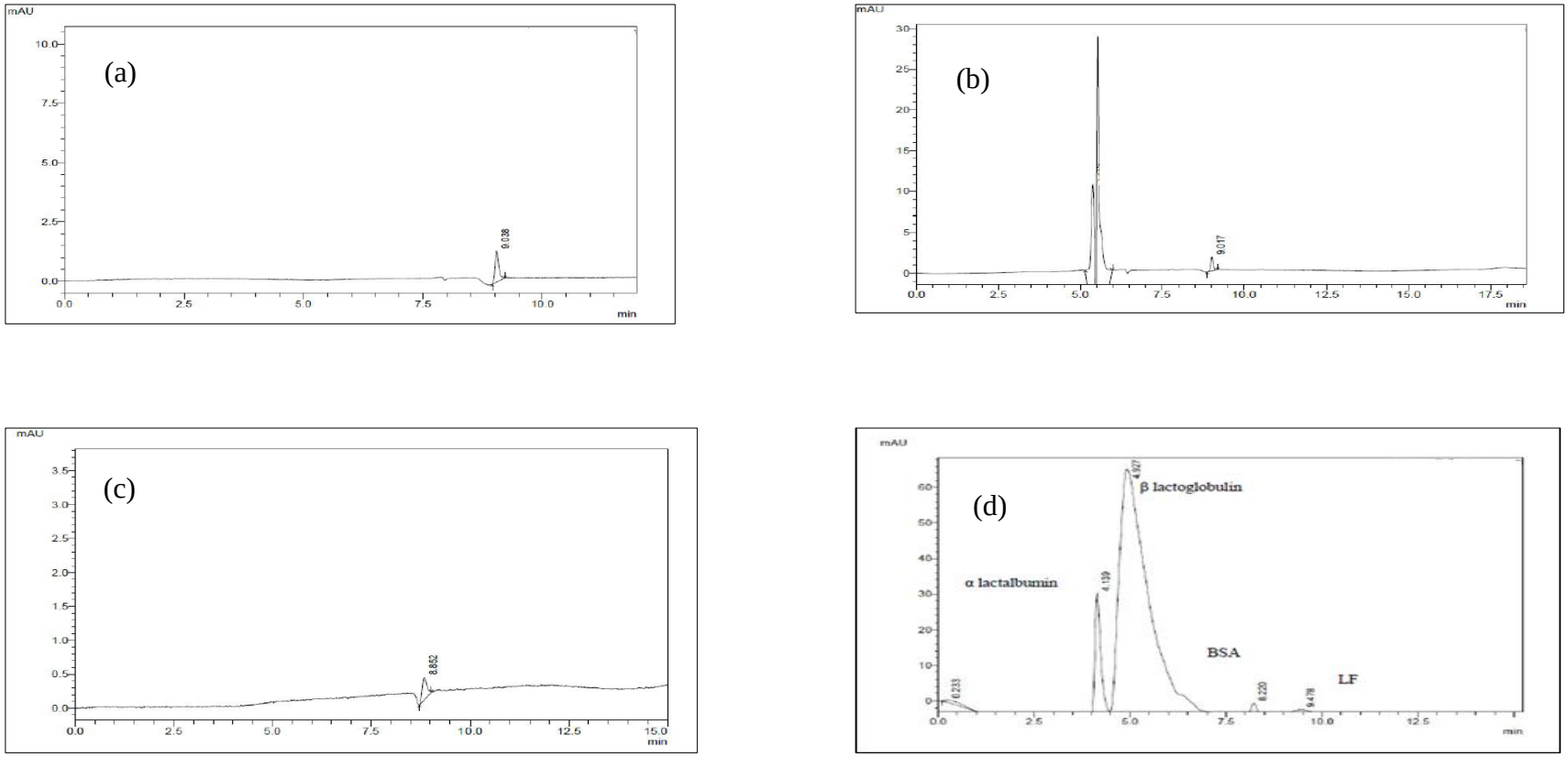


Fig. 4.19: HPLC chromatogram of (a) pure LF; (b) forward extracted LF; (c) back extracted LF; (d) whey proteins present in the synthetic whey.

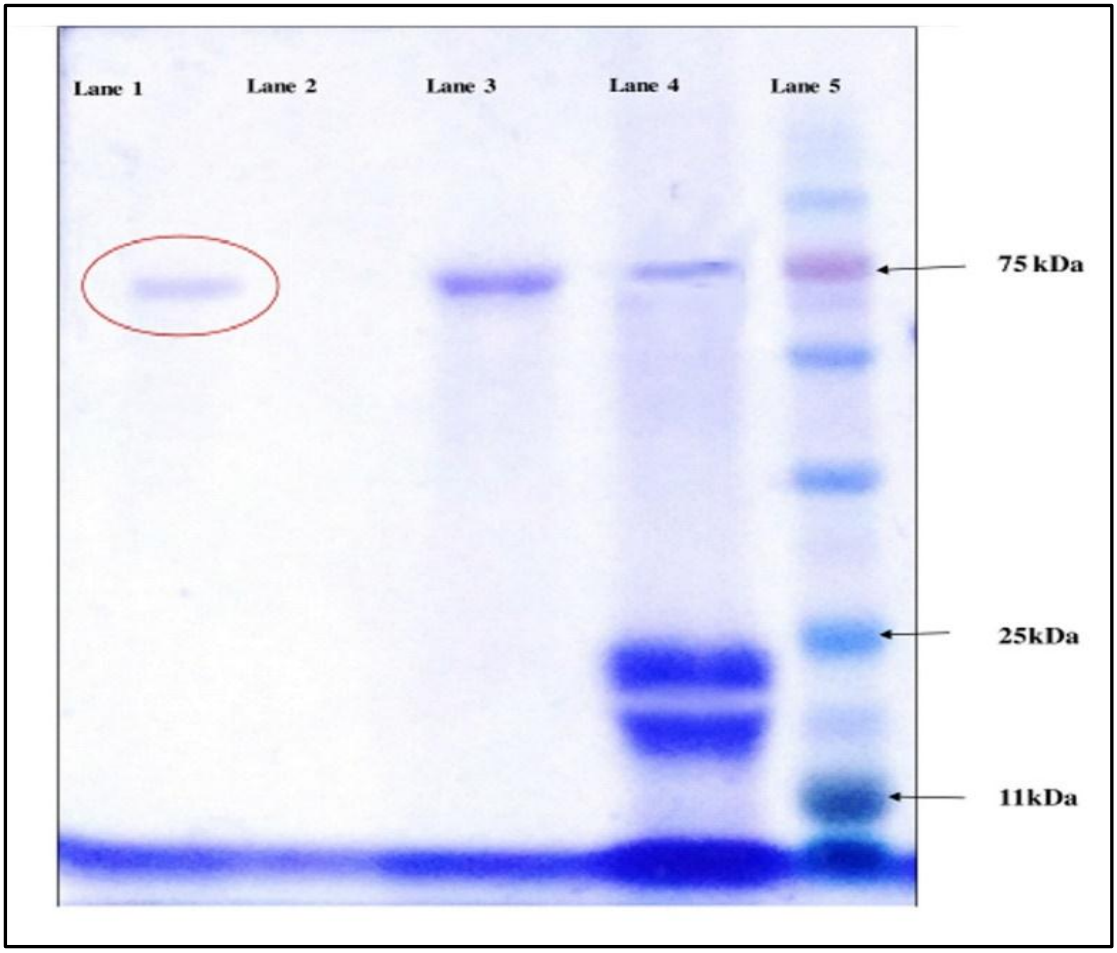


Fig. 4.20: SDS PAGE profile of back extracted LF (lane 1), forward extracted LF (lane 2) and pure LF (lane 3), whey (Lane 4) and protein Marker (Lane 5) at optimised condition

4.3.4 Recycling of RM phase for LF extraction

The studies were conducted to recycle the micellar phase obtained after the stripping operation for the extraction of LF from the fresh whey in order to make the process cost-effective, and sustainable with reduced spent organic phase disposal. The RM phase was reused for some cycles, and their extraction capacity and efficiency were analysed (Fig.4.21). The highest forward extraction efficiency of LF of 97.5% obtained at the first cycle found to decrease to 53% in the fourth cycle gradually. Similarly, the back-extraction efficiency of LF also remarkably decreased from 95.43% to 46.22% from first to the fourth cycle during recycling (Fig.4.21). The decreased extraction efficiency over the number of cycles could be due to loss of CTAB during extraction of LF(Nandini and Rastogi 2009).

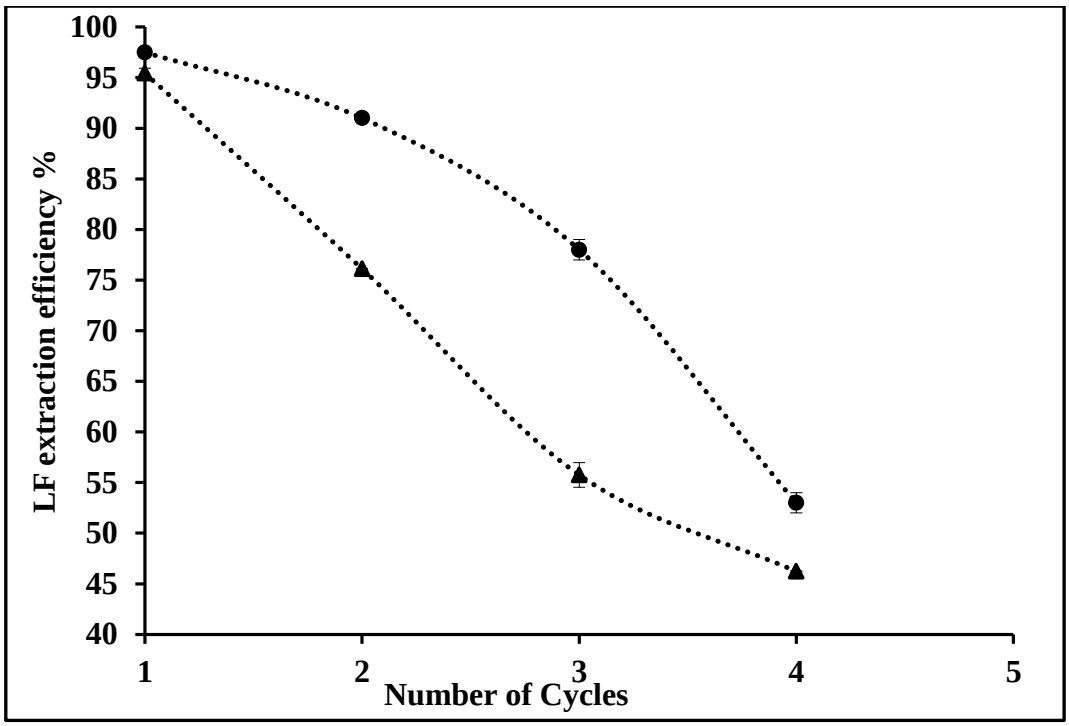


Fig. 4.21: Recycling of organic phase for LF (●) forward and (▲) back extraction

The decrease in extraction efficiency after each cycle was analysed by studying the RM size and W_0 of the RM phases. The RM with LF after the forward extraction was found to be stable till the fourth cycle with a similar RM size of 128μm. The number of RM in the organic phase may decrease after each cycle due to the loss of CTAB even though significant increase in W_0 was observed between the empty RM (7.432) to RM which contains LF, the W_0 of the RM phase found to be similar (9.018) after each cycle due to the simultaneous reduction in number of surfactant molecules and resultant number of RM with their W_0. The difference in W_0 and size of empty and LF containing RM was the result of increased molecular interaction between chloride ions, protein molecule and a positively charged head group of surfactant present in organic phase that is responsible for increasing the thickness of electric double layer for RM containing LF (Fathi et al. 2012). Further, the RM size and W_0 may differ if the ionic strength of the system varies due to the presence of Na^+ and K^+ ions. The ions K^+ from the stripping phase and Na^+ from the fresh whey may accumulate into the RM phase due to the recycling operation. Na^+ and K^+ concentrations were measured once the organic phase was obtained after back extraction at each cycle.

The concentration of Na^+ was found to be almost constant (4ppm) till the fourth cycle, however a slight increase in K^+ ion from 0.5 ppm for the first cycle to 3.5 ppm after the 4^{th} cycle was observed. The instability of the RM after the fourth cycle may be due to the combined effect of loss of surfactant and increased concentration of K^+ ion. A detailed study is required to improve the extraction efficiency by makeup the organic phase with the addition of additional solvent and surfactant. The requirement of the additional amount of organic phase should be accessed by analysing the RM characteristics including the size and W_0.

The 98.7% of LF was solubilised into RM at the salt aqueous phase pH 10.3 with the addition of 1.1 M NaCl from model whey and back-extracted into the stripping phase containing 1.5M KCl and 7% n-butanol as a co-solvent at pH 6. At the same operating condition, 94.2% of LF was recovered from bovine acidic whey without the interference of other whey proteins. The purity of the back extracted LF was confirmed with SDS PAGE as well as RP-HPLC analysis. The recycling of RM phase shows the feasibility of the process in industrial scale with the addition of an additional quantity of surfactant to retain the maximum extraction efficiency.

4.4 Continuous Extraction of LF

A number of extractors/contactors are utilized to perform the LLE process for the extraction of the variety of solutes including organic and biomolecules using the conventional organic-aqueous systems. However, the in-depth studies on the hydrodynamic and mass transfer characteristics of these extractors using modified LLE systems like RMS and ATPS are in scarcity in the literature. The distinguishing physical and chemical characteristics of the modified systems from the conventional systems made the difficulty to implement these processes in the continuous contactors. Further, very less mixing intensity is good enough to extract the solutes from one of the phases to another phase, since the phases are formed within the contactor or frequent reassembling of the dispersed phase droplets due to their lesser interfacial tension and density difference between the phases. Specifically, the interphase mass transfer resistance is very less, and hence it is not a controlling factor during the solute transport across the interphase. The lower interfacial tension and

density difference also provide the additional difficulties like (i) stable emulsion formation at higher mixing intensity (ii) phase inversion at higher flow rates (iii) attainment of flooding at lower velocity of the phases (iv) lower rate of phase separation due to the similar physical characteristics of the phases. By considering the characteristics of the RMS and the issues/ difficulties associated with the continuous operation, the 'RDC', is selected for the implementation of RME in the continuous process.

The RDC was implemented for the forward extraction of LF from the whey, i.e., to transfer the maximum amount of target solute, LF to the micellar phase. The mixing of the phases by the rotating disc and the counter current flow of phases helps to improve the mass transfer. The turbulence generated due to the movements of the rotor disc placed at the centre of the column helps to enhance the uniform dispersion of the micelle into the continuous phase (aqueous phase) (Treybal 1980). The extent of mixing, which may be controlled by the velocities of the phases (flow rates) and coalescence and reassembling of the micelles due to the instantaneous phase separation within the contactor and the speed of the rotating discs regulates the rate of LF transfer and yield. Effective separation/ extraction of LF in RDC depend upon the column geometry and operating conditions (phase flow rate and rotor speed) which influence the dispersion of the micellar phase.

The CTAB/n-heptanol system was used for the continuous extraction of LF in the continuous extractor, RDC. The optimised conditions obtained for the forward extraction of LF from whey was considered to study the effect of different operating variables for the extraction efficiency and other mass transfer characteristics. The continuous phase was the whey, and the dispersed phase was the organic solvent and surfactant mixture (CTAB/n-heptanol). The continuous phase was fed through the top inlet and the dispersed phase fed through the bottom inlet by using peristaltic pumps. Countercurrent flow is maintained, and the samples after the extraction were collected from the top and bottom outlet points for the analysis of LF content. The holdup of RM phase (Φ), extraction efficiency (η) and recovery of LF (R) along with dispersed

phase volumetric mass transfer coefficient ($K_d a$) at different operating conditions were computed and analysed.

4.4.1 Effect of disperse phase flow rate:

The effect of dispersed phase flow rate (4.2, 6.6 and 7.8ml/min) on the extraction efficiency and recovery of LF and RM phase holdup and volumetric mass transfer coefficient was studied at different continuous phase flow rates (4.2, 6.6 and 7.8ml/min) and rotor speed (150, 350 and 450 rpm). The change in dispersed RM phase holdup with the disperse phase flow rates at three different rotor speeds (150,350 and 450 RPM) is shown in figure 4.22.

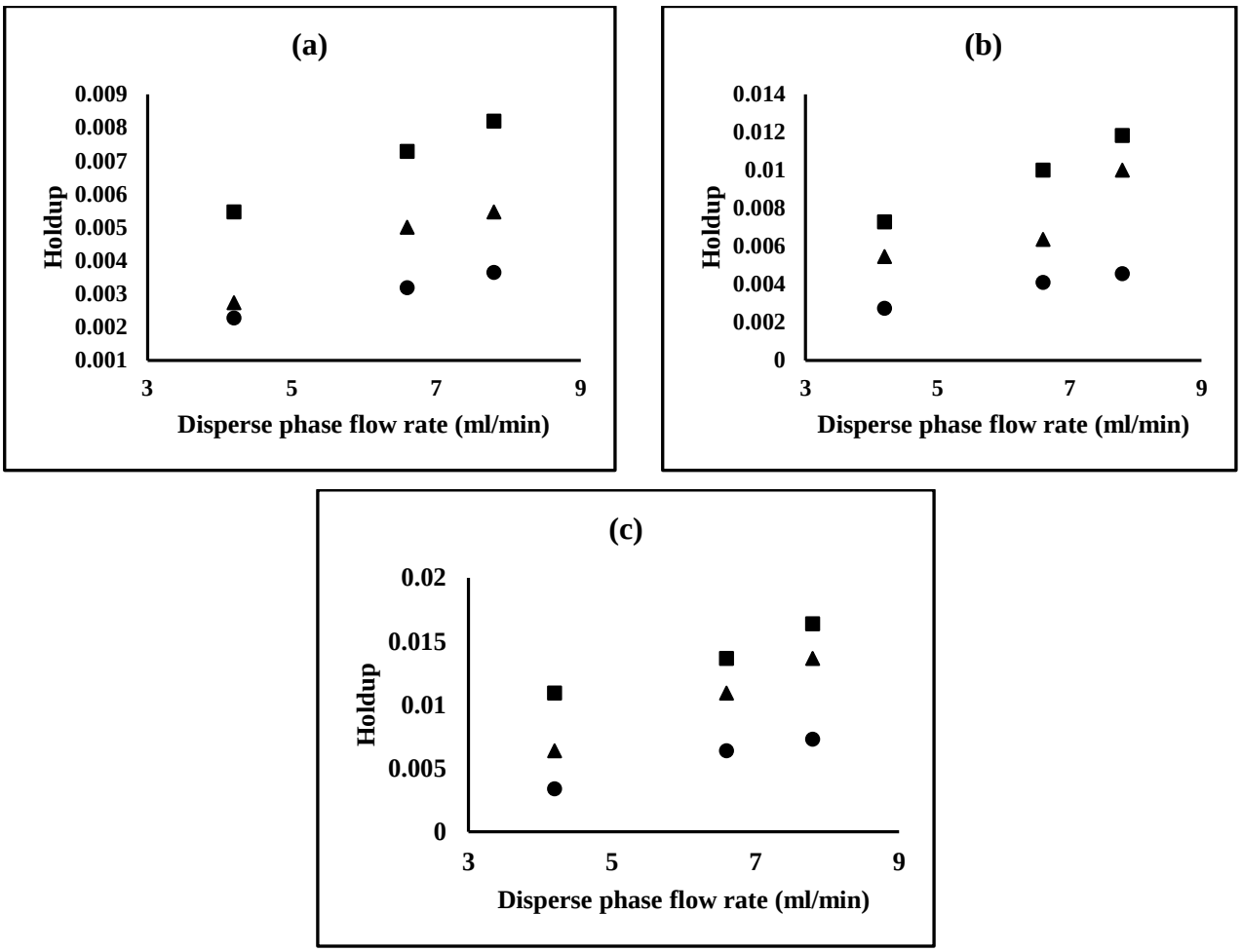


Fig. 4.22: Variation of dispersed phase holdup with disperse phase flow rate (● 4.2ml/min; ▲ 6.6ml/min; ■ 7.8ml/min) at different rotor speed (a) 150rpm (b) 350rpm (c) 450rpm

The holdup of the RM phase found to increase with increasing the RM phase flow rate, and rotor speed since more the number of RMs formed due to the increase in surfactant and solvent quantity with the velocity of the dispersed phase. These RMs subsequently reassembled a number of times within the contactor due to the increased kinetic and buoyancy force offered by the increased velocity and rotor speed that act on the droplets (Barhate et al. 2004). The increased rotor speed also enhances the holdup by splitting the RMs into smaller RMs and retaining them for a longer duration since the raising velocity of the smaller micelles decreased. The stator rings of the contactor interfere with the rising RM droplets and reduce the velocity of the RMs which increases the holdup further.

The increase in a holdup with increasing dispersed phase flow rate also indicates the increase in the interfacial area available for mass transfer. The larger number of smaller RMs with the higher interfacial area and longer residence time at an increased dispersed phase flow rate and rotor speed resulted to the increasing trend of extraction efficiency (Fig. 4.23), recovery (Fig.4.24) and the mass transfer coefficient (Fig.4.25). When the number of RM within the RDC increases with respect to the particular flow rate, it leads to enhanced interaction with hydrophilic LF with RM. The solute transfer in the RMS inherently a spontaneous process, i.e., the solutes may be entrapped inside the RMs during the formation of micelles, and hence the solute (LF) transport may not be similar to the conventional mechanism, which was controlled by the films at the interphase and surface area of the dispersed droplets. However, the increased trend of RM phase holdup was observed due to the reassembling of the micelles when they experience the higher kinetic energy imparted by the rotating discs and the flow rates of the phases. The similar trend of increasing extraction efficiency and recovery was reported by Kalaivani and Regupathi (2016) for α-LA in the RDC for ATPS.

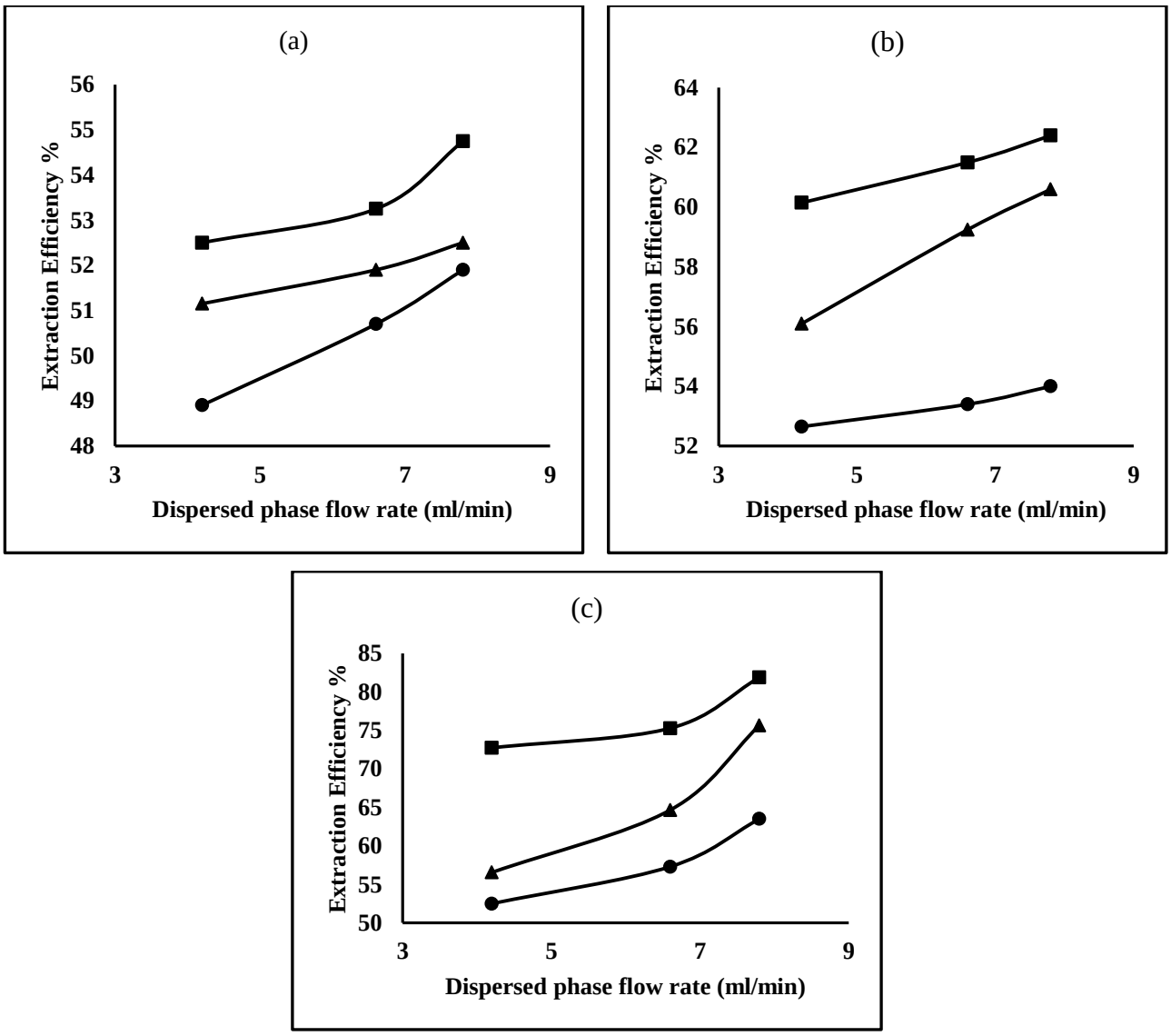


Fig. 4.23: : Effect of disperse phase flow rate and rotor speed (● 150rpm; ▲ 350rpm; ■ 450rpm) on extraction efficiency of LF at continuous phase flow rate (a) 4.2ml/min (b) 6.6ml/min (c) 7.8ml/min

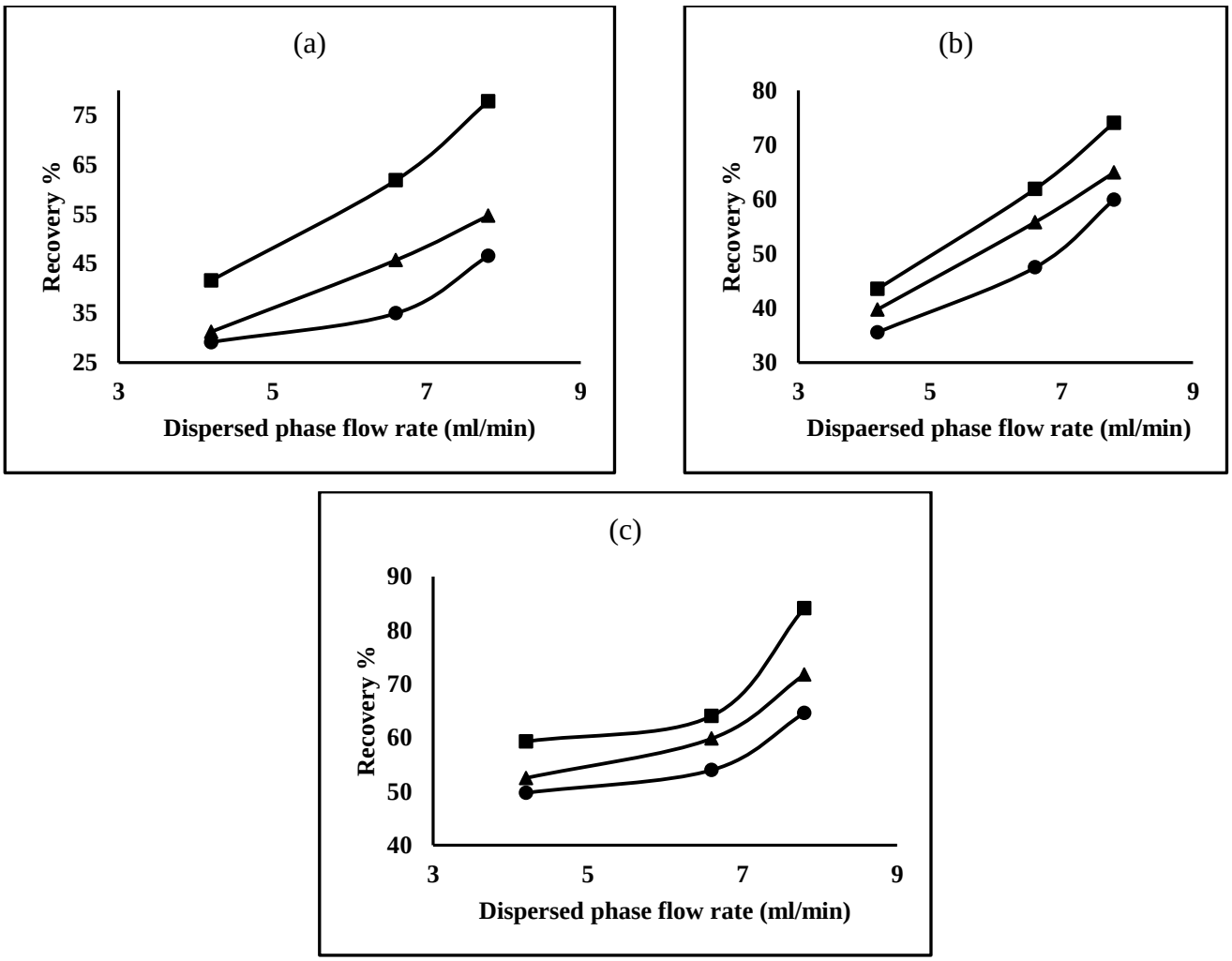


Fig. 4.24: Effect of disperse phase flow rate and rotor speed (● 150rpm; ▲ 350rpm; ■ 450rpm) on a recovery of LF at continuous phase flow rate (a) 4.2ml/min (b) 6.6ml/min (c) 7.8ml/min

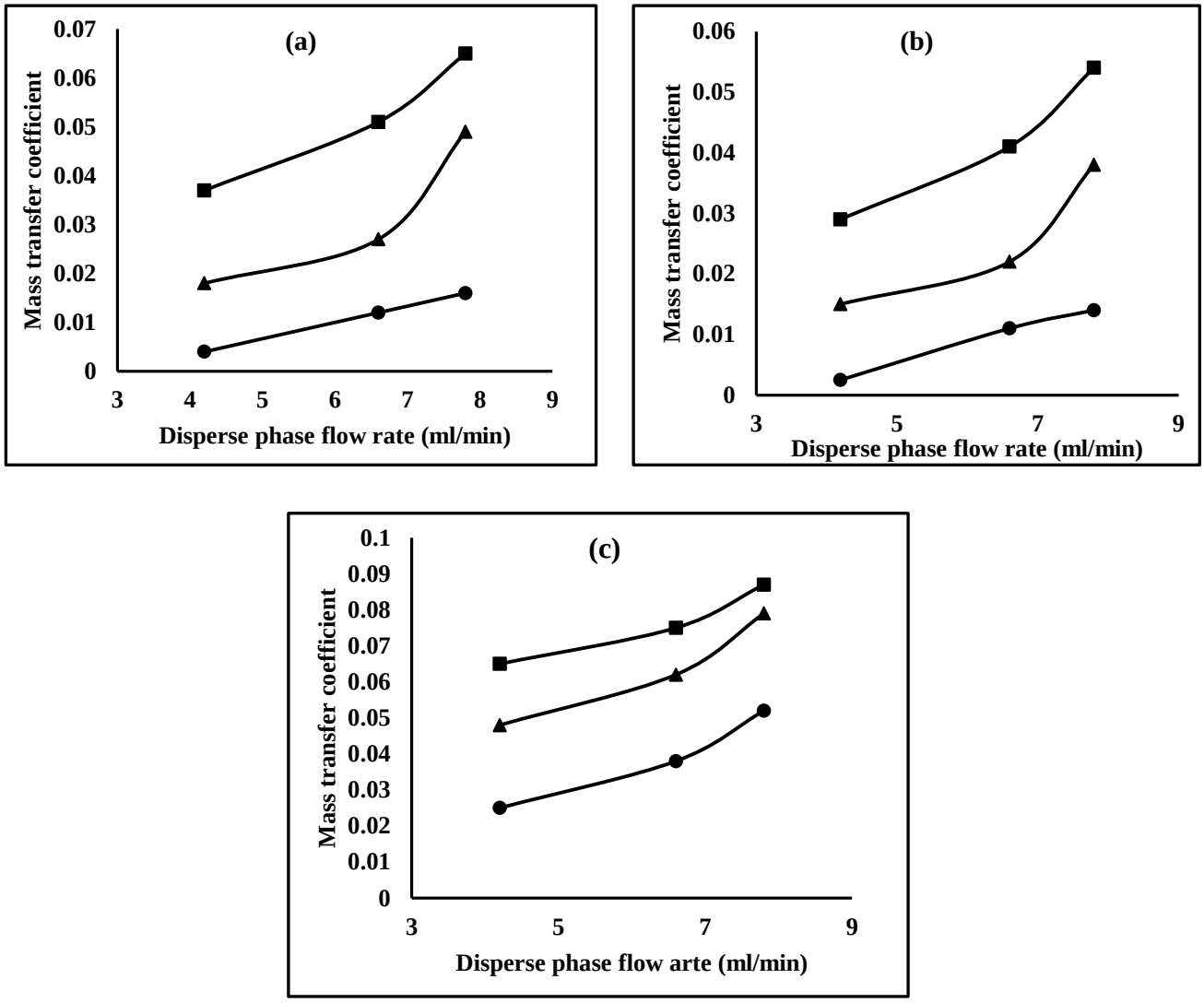


Fig. 4.25: Effect of disperse phase flow rate and rotor speed (● 150rpm; ▲ 350rpm; ■ 450rpm) on mass transfer coefficient of LF at continuous phase flow rate (a) 4.2ml/min (b) 6.6ml/min (c) 7.8ml/min

The dispersed RM phase flowrate increases the volumetric mass transfer coefficient due to the increased turbulence caused by the dispersed phase velocity and rotor speed, which increases the reassembling rate of the RMs. The surface renewal and the internal mixing rate also enhanced while the reassembling rate increases for the RMs. During the reassembling process, the interactive regions of the surfactant molecules exposed to the LF and the LF may be easily entrapped within the micelles. The increased effect of dispersed phase on the mass transfer coefficient was noticed at higher flowrate and agitation.

4.4.2 Effect of continuous phase flow rate:

The effect of continuous phase flow rate (4.2, 6.6, 7.8ml/min) on the performance of RDC was studied and analysed at different flow rates of RM phase and rotor speed (150, 350, 450 rpm). With the increase in continuous phase velocity the dispersed phase holdup was observed to increase due to the drag force applied on the dispersed RM phase (Fig.4.26) (Igarashi et al. 2004) by the continuous phase velocity. The effect is more influential when the extraction was performed at higher disperse phase flow rate for all rotor speeds. However, the continuous flow rate increases the holdup almost linearly due to the exertion of combined forces such as buoyancy, drag and friction of droplets on the uprising RM droplets and retain the droplets for longer duration in the contactor. At steady state operation, the number of smaller RM formed at higher RM phase flow rate and rotor speed interacts with each other and leads to the destabilization of the RMs. The subsequent reassembling and formation of the new RMs with smaller size increases the extraction efficiency (Fig.4.27) and recovery (Fig.4.28) of LF with increasing mass transfer coefficient (Fig. 4.29), but it is relatively lesser than the extent observed at the increased dispersed phase flow rate. Even though, the kinetic energy offered by the continuous aqueous phase flowrate retained the droplets, relatively less mass transfer coefficient and recovery could be observed due to the less mean residence time of the continuous phase in the column (Cavalcanti et al. 2008). In the present study, highest recover of LF, i.e., 84.10% was obtained at dispersed and continuous phase flow rate 7.8 ml/min at a rotor speed of 450 rpm. As the continuous phase flow rate increases, the interaction between the RM formed in the dispersed phase and LF in continuous phase also increases. The increased interaction between LF and RM resulted in enhanced entrapment of LF to the RM in the dispersed phase and resulted in increased extraction efficiency and recovery.

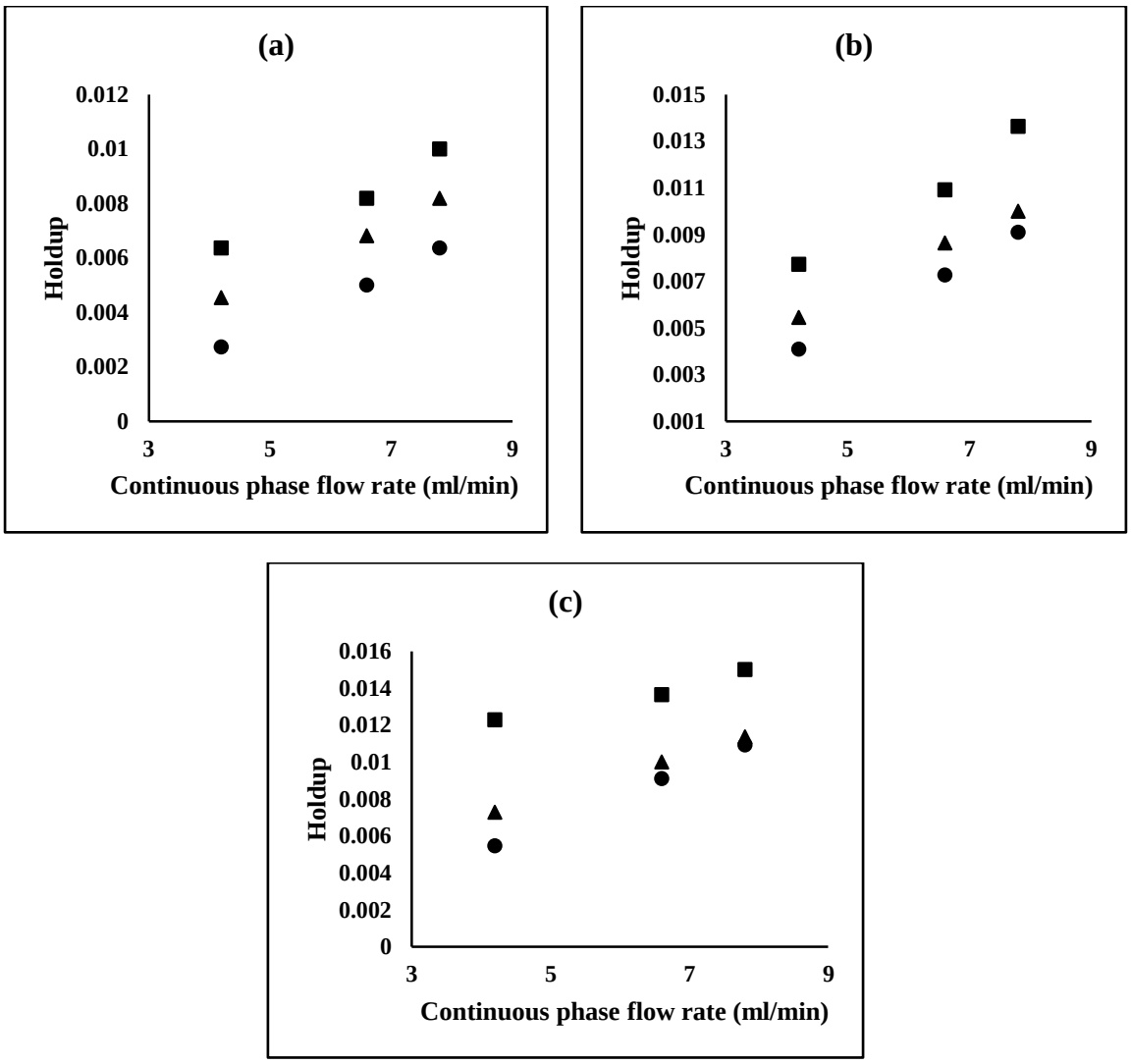


Fig. 4.26: Variation of dispersed phase holdup with continuous phase flow rate (● 4.2ml/min; ▲ 6.6ml/min; ■ 7.8ml/min) at different rotor speed (a) 150rpm (b) 350rpm (c) 450rpm

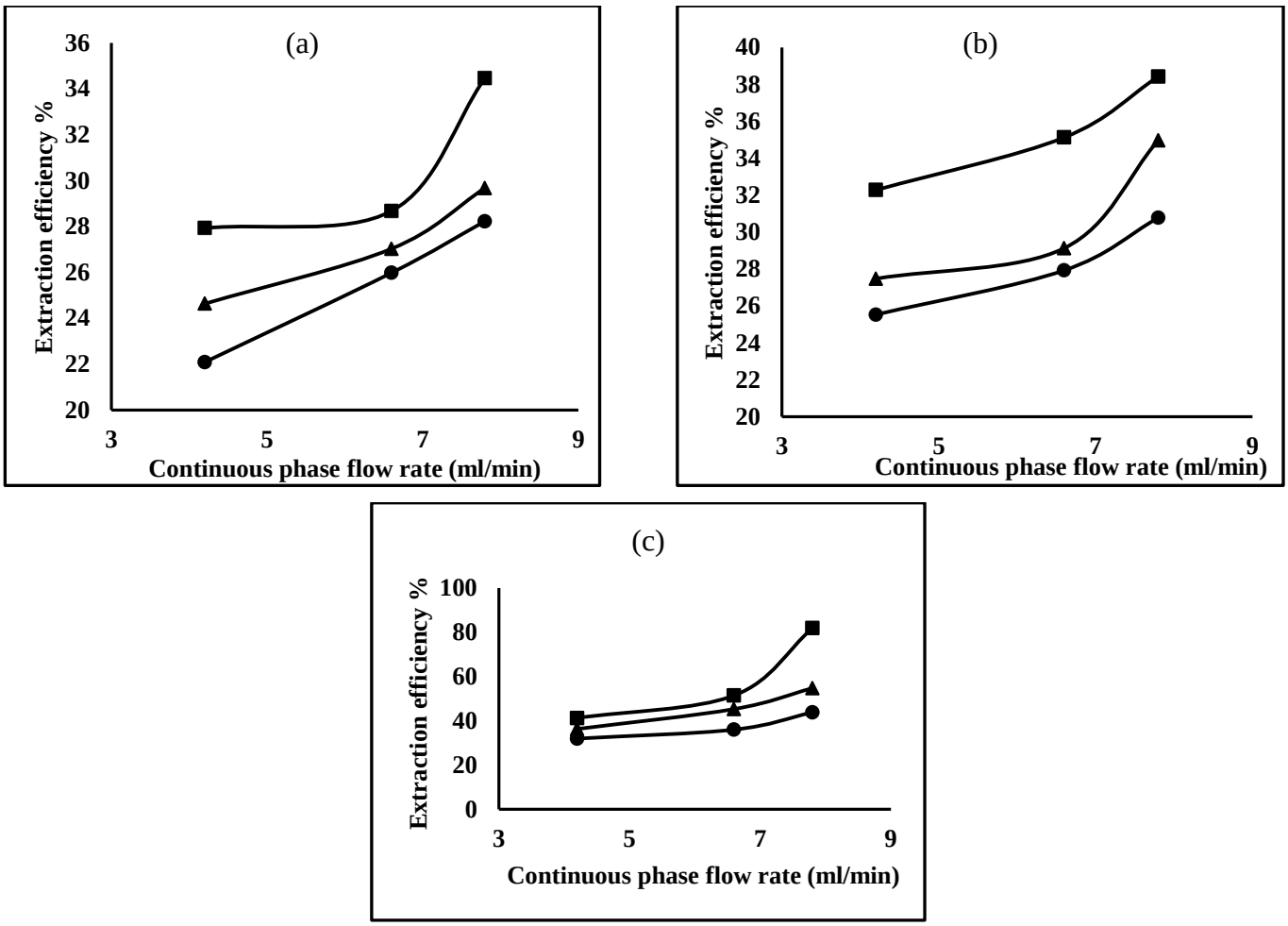


Fig. 4.27: : Effect of continuous phase flow rate and rotor speed (● 150rpm; ▲ 350rpm; ■ 450rpm) on extraction efficiency of LF at disperse phase flow rate (a) 4.2ml/min (b) 6.6ml/min (c) 7.8ml/min

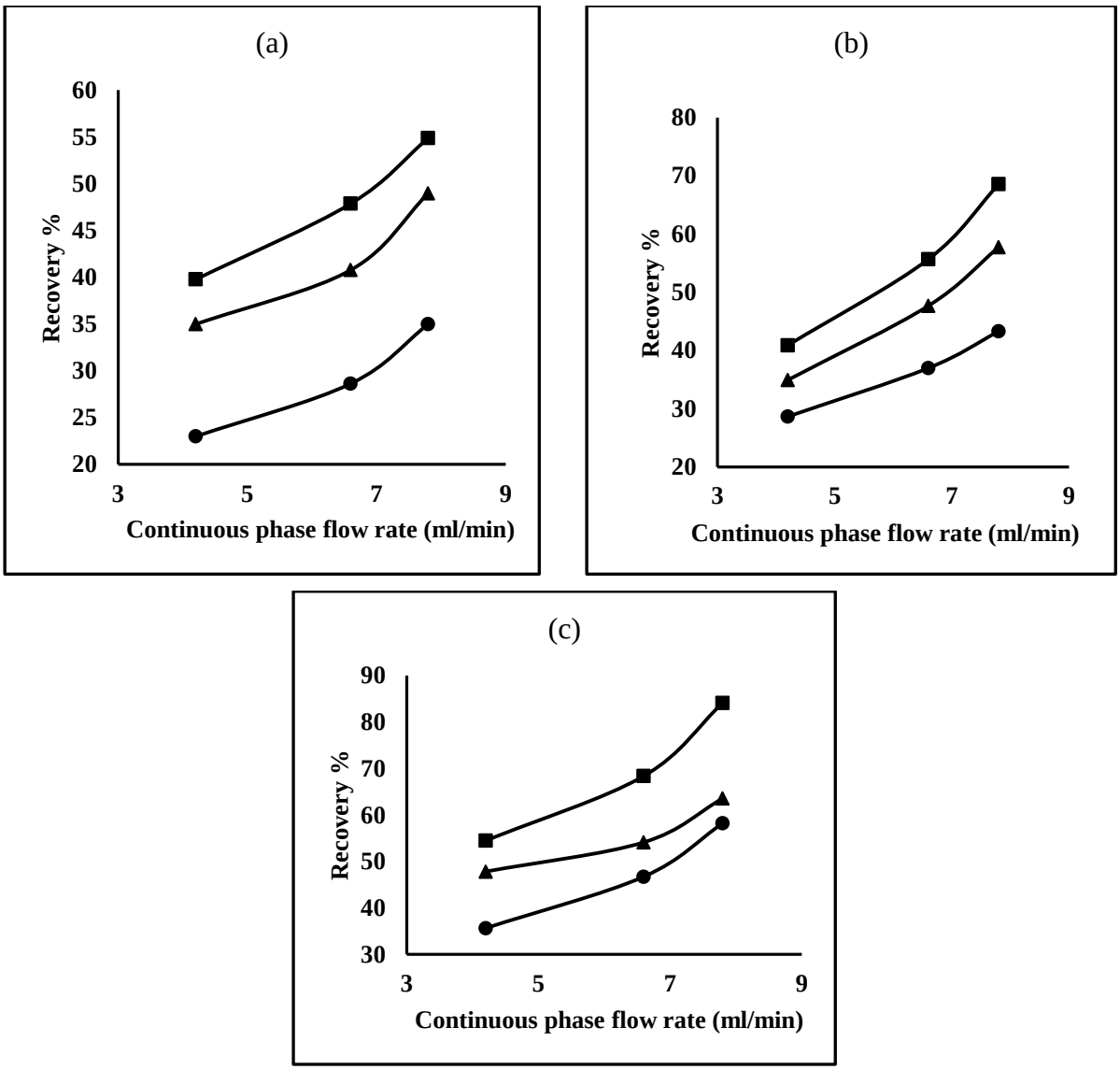


Fig. 4.28: Effect of continuous phase flow rate and rotor speed (● 150rpm; ▲ 350rpm; ■ 450rpm) on a recovery of LF at disperse phase flow rate (a) 4.2ml/min (b) 6.6ml/min (c) 7.8ml/min

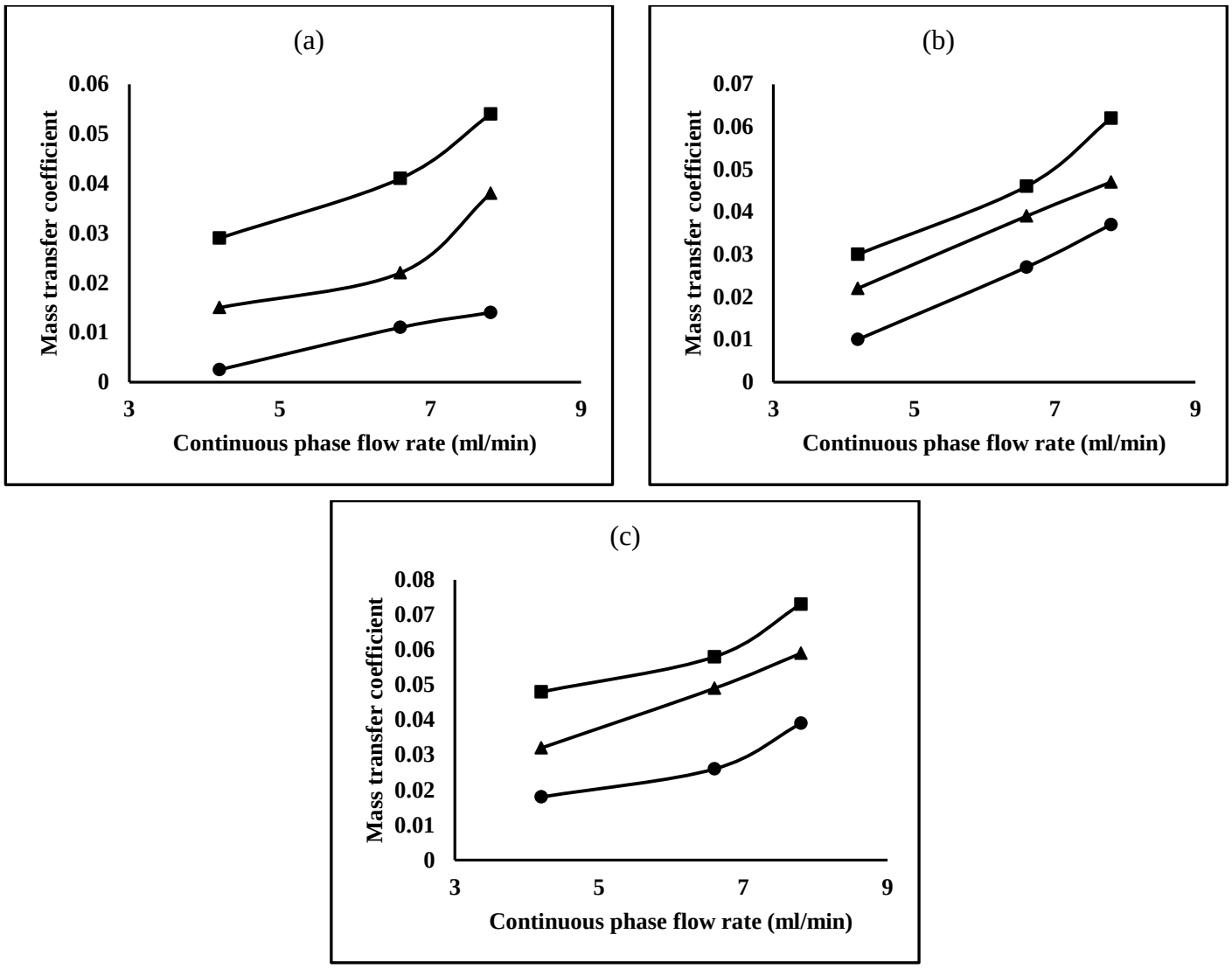


Fig. 4.29 : Effect of continuous phase flow rate and rotor speed (● 150rpm; ▲ 350rpm; ■ 450rpm) on mass transfer coefficient of LF at disperse phase flow rate (a) 4.2ml/min (b) 6.6ml/min (c) 7.8ml/min

4.4.3 Effect of rotor speed:

Rotor speed plays a vital role in the simultaneous destabilization and formation of RMs by reassembling the surfactant molecules. The effect of rotor speed on the extraction parameters and column performance was studied at different flow rates of both the phases. The holdup was found to increase with increasing the rotor speed at different dispersed RM phase (Fig.4.22) and continuous phase (Fig.4.26) flow rates. The dispersed RMs are scattered almost uniformly within the continuous phase present in each of the compartment made by the stator rings due to the shearing action

applied by the centrally placed rotor disc. The dispersion caused due to the turbulence created by the rotor disc is directly proportional to the rotor speed. As the turbulence of the phases increased due to the imparted kinetic energy by the rotor discs, more the dispersed RMs were retained, and they get split into smaller micelles by the simultaneous destabilization and reformation of larger micelles. Further, the smaller RM droplets spend more time within the mixing zone and thus resulted in increased holdup (Fig. 4.22 and 4.26).

As the rate of simultaneous destabilisation and reformation of RMs increases with increasing rotor speed, the mass transfer coefficient also found to increases at larger extent with higher dispersed RM phase flowrate (Fig. 4.25) than the aqueous continuous phase (Fig. 4.29). The increased mass transfer is the result of higher turbulence created by the movement of the rotor disc in the mixing zone. Also, the increasing rotor speed tends to form a greater number of smaller secondary RM by breaking down larger RM which comes in contact with moving disc. This tends to decrease the terminal velocity of the droplets and increases the interfacial area which ultimately results in higher mass transfer (Kalaivani and Regupathi 2016; Sarubbo et al. 2005). The smaller droplets further experience lesser terminal velocity and reside for more time in the column. The increased retention time in the column and enhanced rate of surface renewal of RMs due to the reassembling the surfactant molecules helps to transfer the LF from the bulk continuous phase to micellar droplets at the higher rate. As the RMs reside for more time in the column and effectively interacted with LF molecule for a longer duration, the enhanced extraction efficiency and recovery were noticed with increasing rotor speed. The interfacial turbulence caused by the rotor is responsible for the shape, size and water content of the RMS, which are further related to the number of LF molecules entrapped within the RMs.

From the developed method for LF reverse micellar extraction approximately 30-40% final cost of the purified LF can be reduced than the current market price.

CHAPTER 5

SUMMARY AND CONCLUSION

The major observations and results obtained during the development of selective extraction of LF from whey using the RMS are consolidated and presented.

- Six different RMSs namely, TX 100/ Isooctane, AOT/ Isooctane, AOT/ n-decanol, CTAB/ Toluene, CTAB/ Isooctane, CTAB/ n-heptanol were selected based on some of the chemical and physical properties of the surfactants and solvents to identify the suitable RMS for the solubilisation of LF.
- The Physiochemical properties including CMC, W_0, size of the RMs, etc., were also measured for all the possible systems which are capable to solubilize the LF.
- The RMs formed by the surfactant/ solvent combination of CTAB/n-heptanol with the addition of 1M of NaCl or 0.9M of KCl as electrolytes at pH of 7 was found to be a favourable system for the maximum solubilisation of LF.
- Process variables like aqueous phase pH, ionic strength, phase volume ratio and co-solvent was studied to obtain highest forward and back extraction of LF from its synthetic solution in the CTAB/ n-heptanol RMS. The improved solubility of LF was observed at 50mM CTAB in n-heptanol and addition of 0.9M KCl in the aqueous solution at a pH of 10 during the forward extraction.
- 98% of LF was back extracted to the fresh stripping phase by destabilizing the RMs at pH 6 with the addition of small amount of co-solvent (7% n-propanol or n-butanol) and electrolyte (1.3M KCl).
- A notable increase in RM size has been observed, i.e. 5.60µm to 134µm at the optimised forward extraction conditions.

- The stability of LF after back extraction was analysed by HPLC and observed similar retention time for standard LF and the back extracted LF.
- The selected CTAB/ n-heptanol was extended to selectively extract the LF from model whey as well as bovine whey and 98.7% LF was solubilised into RM at the aqueous phase pH of 10.3 with the addition of 1.1 M NaCl. Further, the maximum LF was back-extracted into the stripping phase containing 1.5M KCl and 7% n-butanol as a co-solvent at pH 6. Maximum of 94.2% LF was recovered from bovine acidic whey without the interference of other whey proteins.
- The purity of the back extracted LF was confirmed with SDS PAGE as well as RP-HPLC analysis.
- The recycling of RM phase shows the feasibility of recycling the spent RM phase at least 3 cycles with minimal loss of extraction efficiency. The recycling of RM phase also shows the feasibility of the process in industrial scale.
- Continuous RME was attempted in RDC, and the effect of phase velocities and rotor speed on the extraction efficiency and recovery of LF was studied.
- The increase in the disperse phase velocity resulted in the increased dispersed phase holdup, LF recovery and mass transfer coefficient.
- The significant effect of rotor speed on the extraction of LF was observed. At lower rotor speed and phase flow rate, moderate RM phase holdup and mass transfer coefficients were observed. Whereas, the favourable extraction and recovery of LF are achieved at higher rotor speed as well as phase flow rate.
- Maximum recovery (84.10%) of LF was obtained at dispersed and continuous phase flow rate at 7.8ml/min at rotor speed 450rpm.

The following significant conclusions were made based on the selective RME and purification of the LF from the whey.

- The high value, minor protein LF was successfully purified without any other impurities from the complex waste source whey by employing the RMS formed by CTAB/n-heptanol.

- A systematic selection and development of RMS through a sequence of experiments starting from the solubilisation of LF from the simple LF solution, synthetic whey and acidic whey was successful in arriving at an optimal RMS.
- The optimized CTAB/n-heptanol system was successfully implemented in the continuous extractor RDC for the selective extraction of LF with a recovery of 84.10 % from acidic whey.
- The developed RME method gives the advantage to avoid the pre-treatment steps involved in the separation and purification methods and reduces the steps involved in the purification of LF from complex biological sources.
- The present study contributes towards the development of efficient and alternate RME and purification of value-added products in its native state selectively from the complex biological sources or effluents.

Scope for future work

Commercial implementation of the process needs further research in the following area

- In-depth study on the recycling, the spent RMs with the addition of makeup surfactant.
- Integration of forward and back extraction in the in continuous operation at a larger scale by considering the scale-up parameters.
- Cost analysis and industrial scale feasibility studies by integrating the process in the existing milk processing units.

Studies on biological characteristics of the extracted LF to confirm its natural state.

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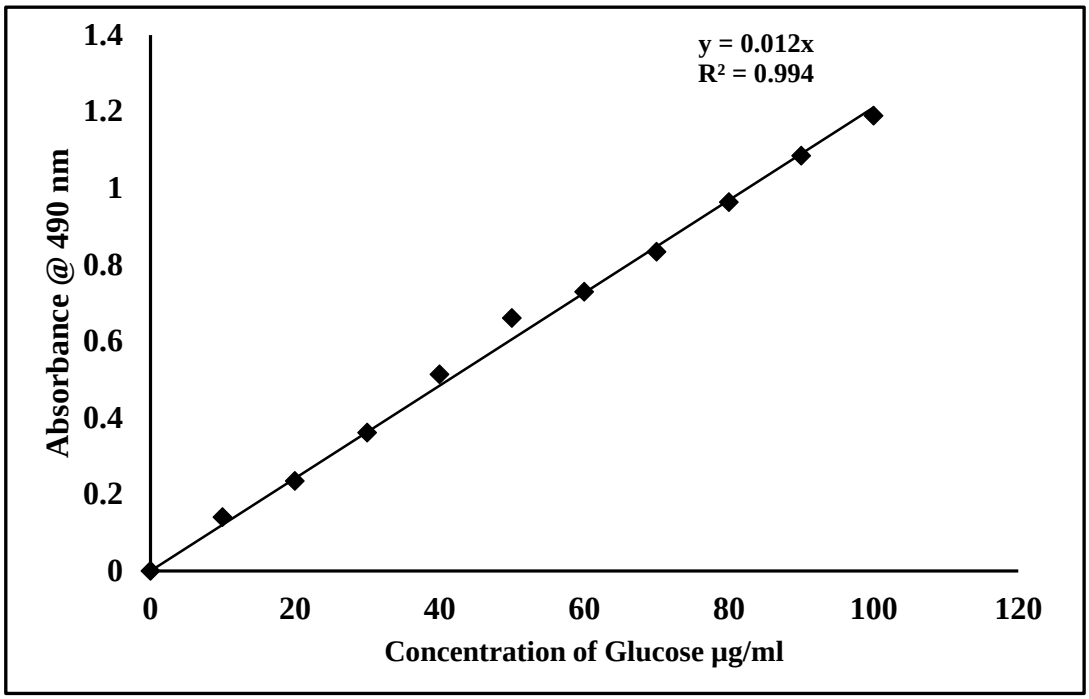
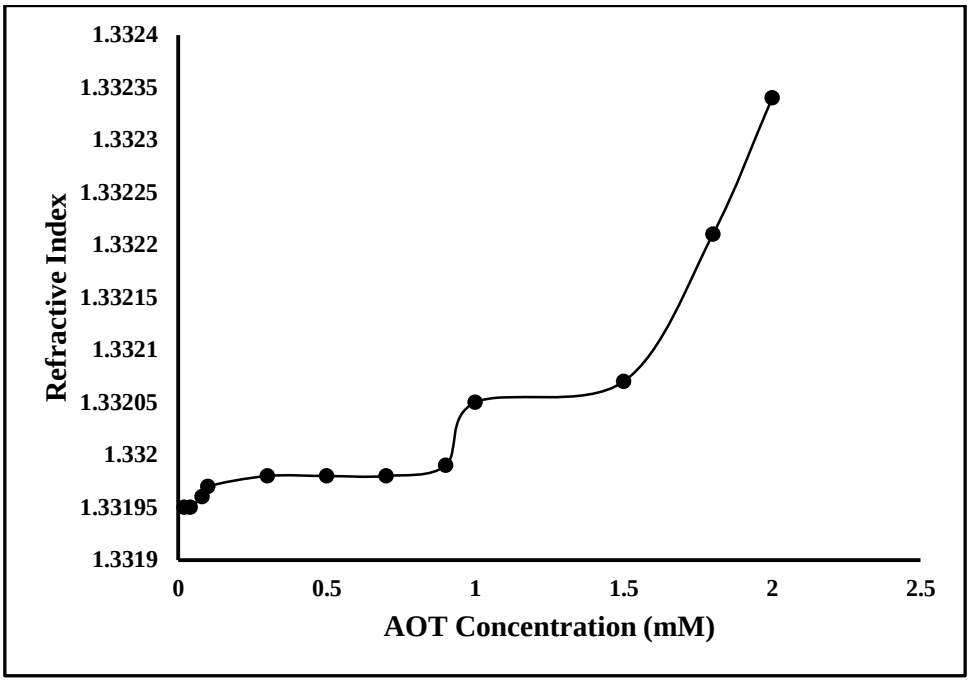
Appendix I

Glucose calibration graph by phenol-sulphuric assay

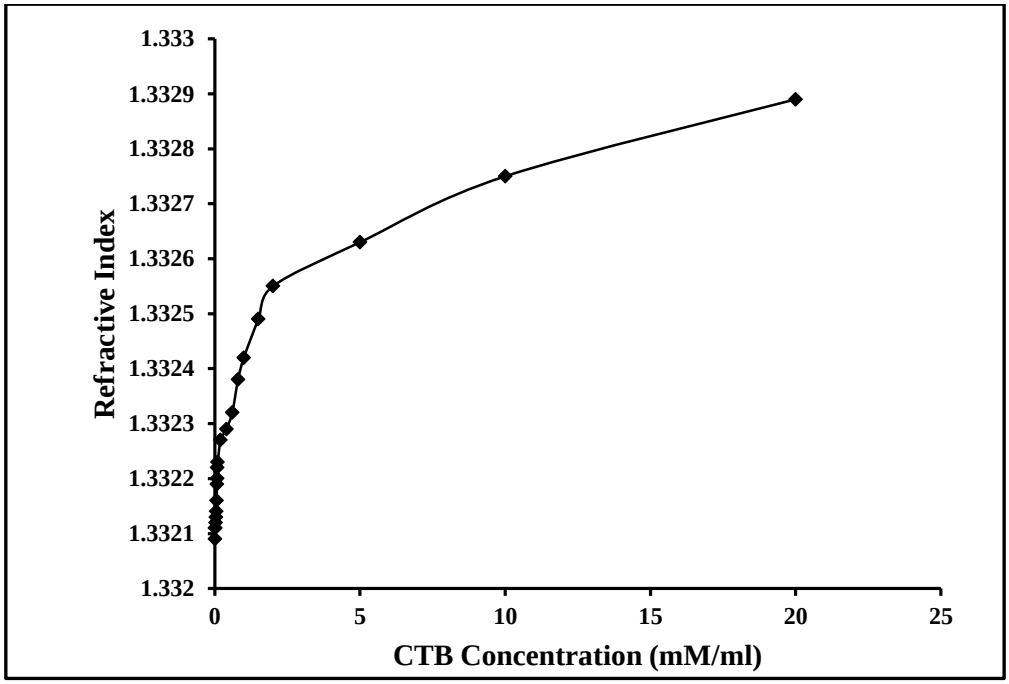


Appendix II

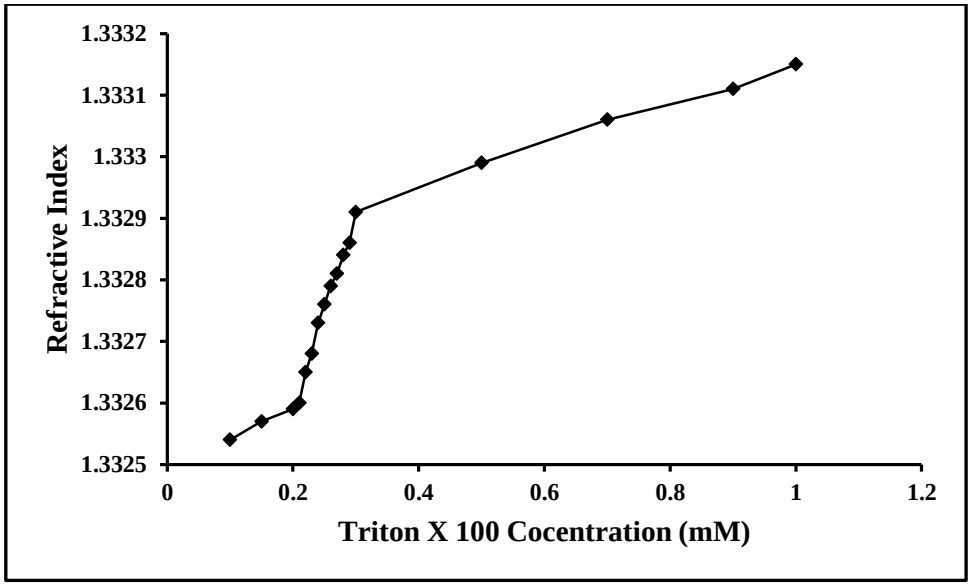
II-1 CMC of AOT/Isooctane RMS (1mM)



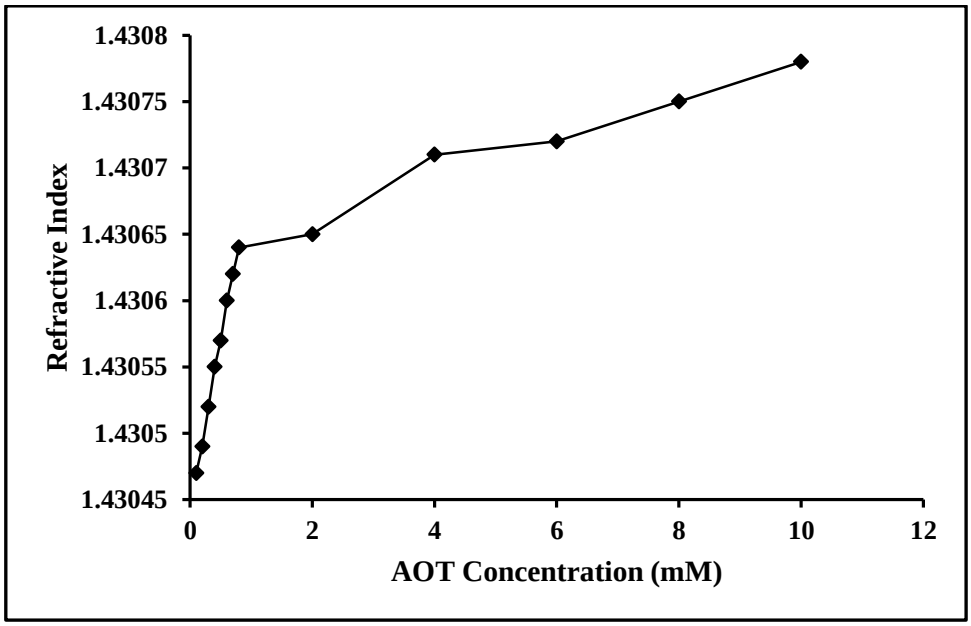
II-2 CMC of CTAB/Isooctane RMS (2mM)



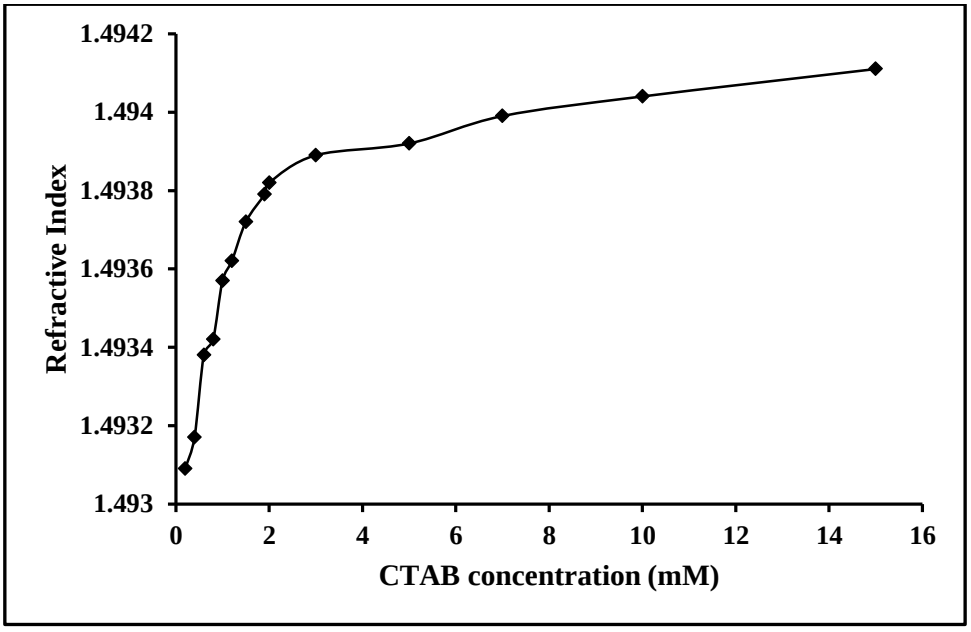
II-3 CMC of TX 100/Isooctane RMS (0.3mM)



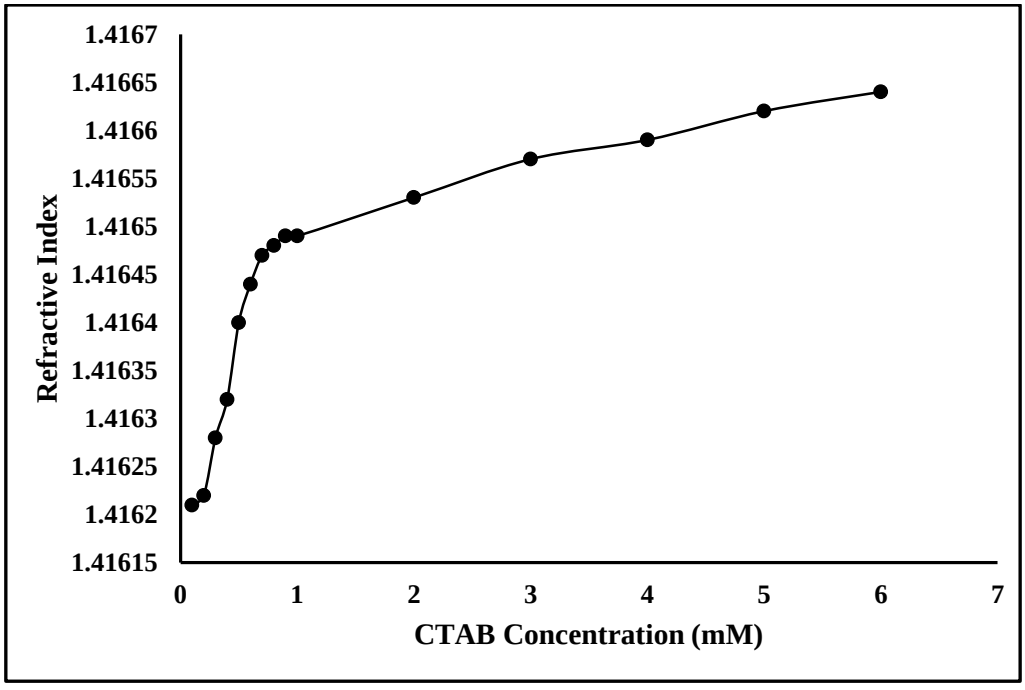
II-4 CMC of AOT/n-decanol RMS (0.8mM)



II-5 CMC of CTAB/Toluene RMS (2mM)

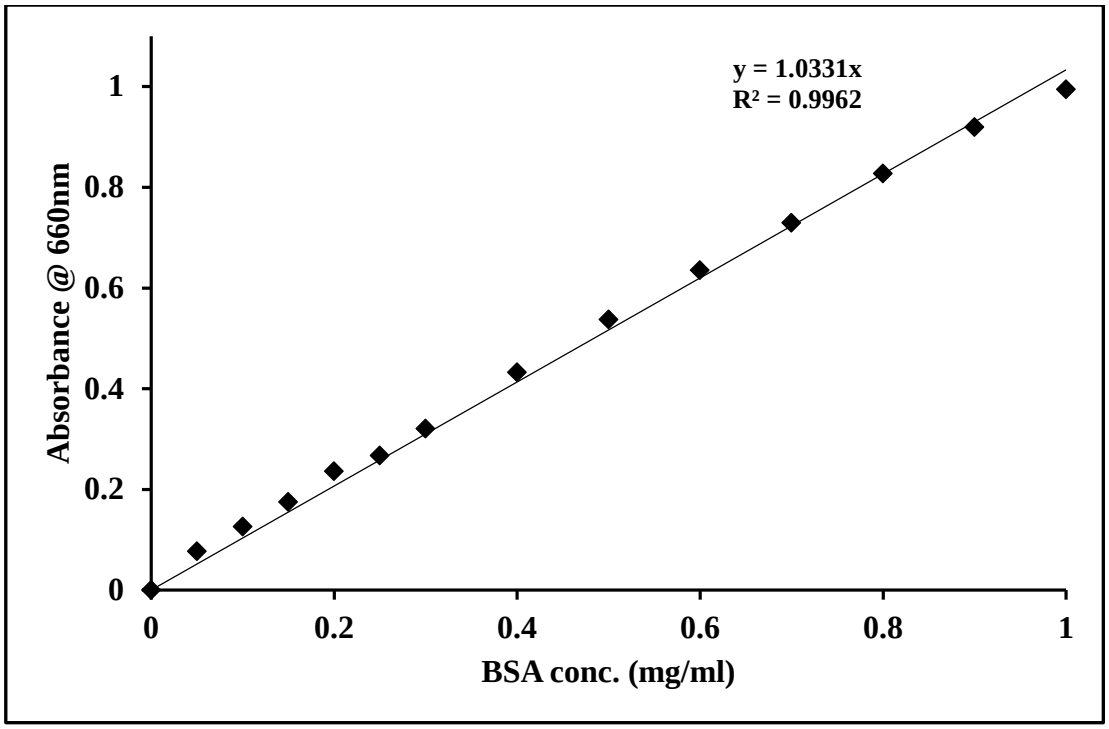


II-6 CMC of CTAB/n-heptanol RMS (1mM)



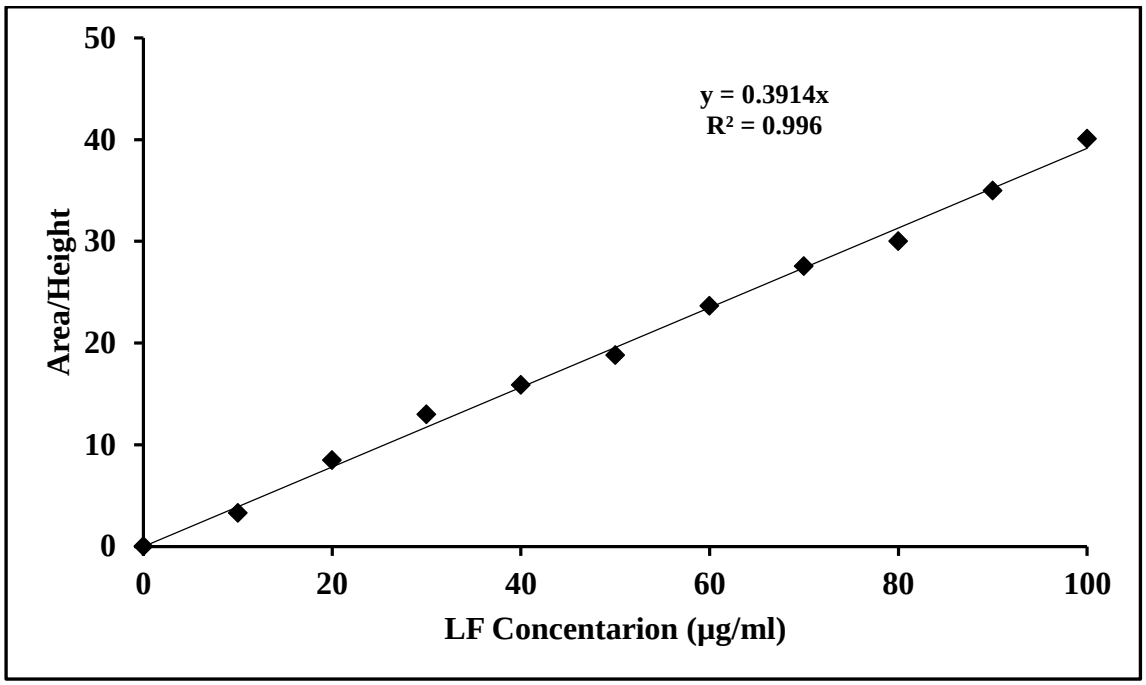
Appendix III

BSA calibration graph by Lowry's assay



Appendix IV

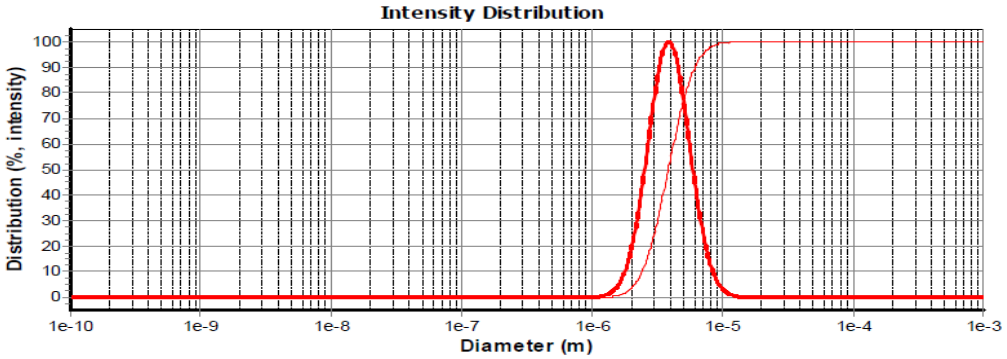
LF Calibration graph by HPLC



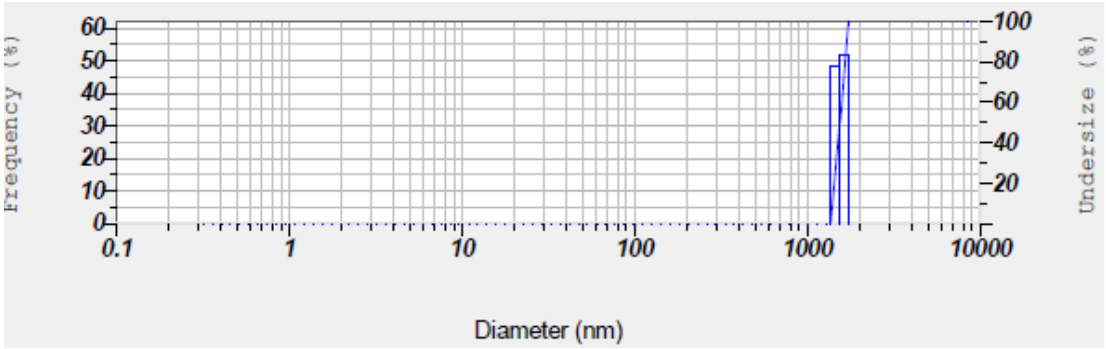
Appendix V

RM size analysis

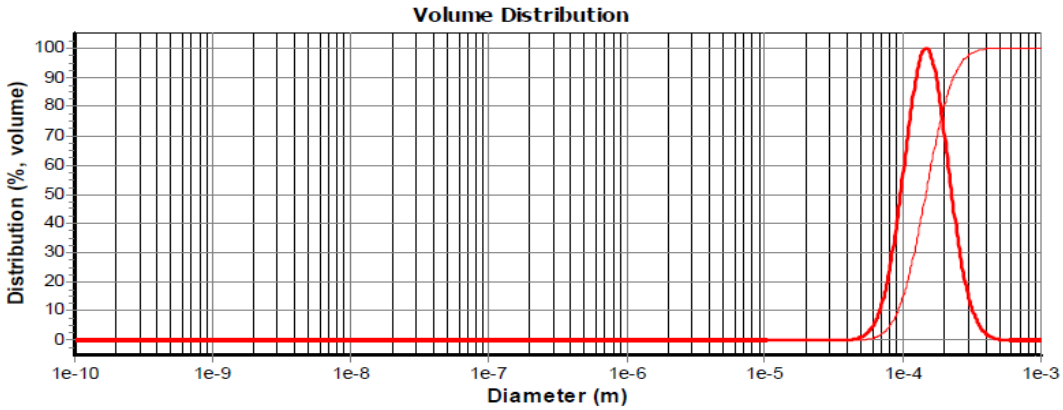
V-1: 50mM CTAB +n-heptanol



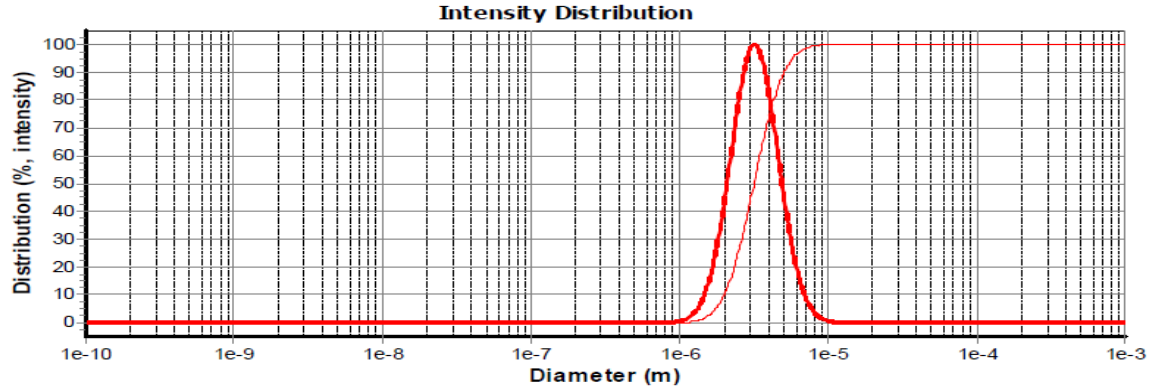
V-2: 50mM CTAB/n-heptanol+ KCl +LF



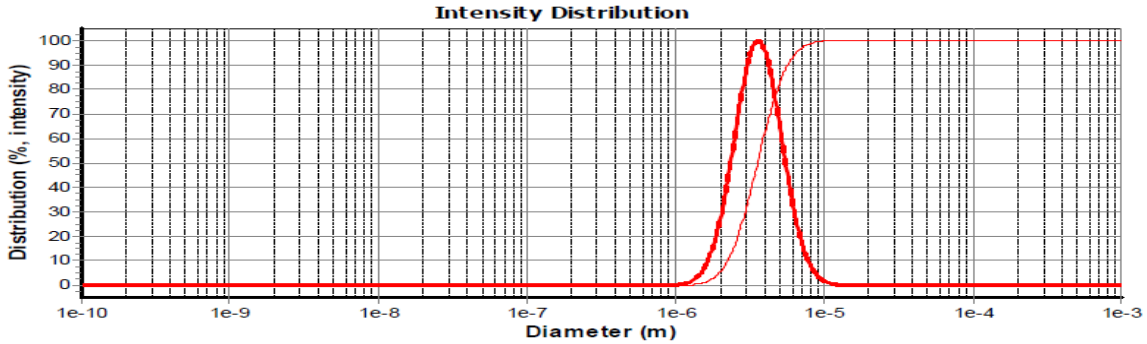
V-3: CTAB/n-heptanol +KCl +n-butanol (15%)



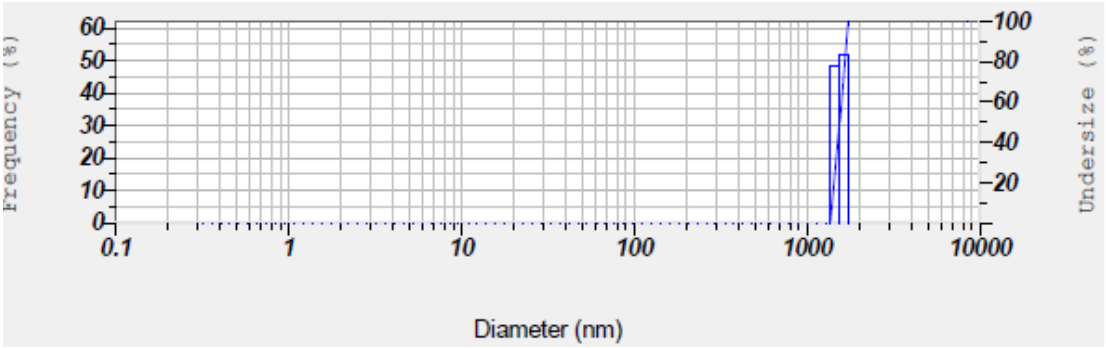
V-4: CTAB/n-heptanol +KCl+n-butanol (7%)



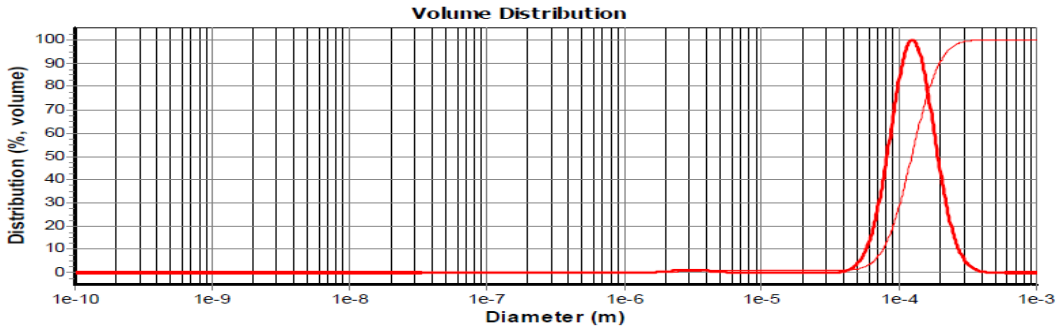
V-5:50mMCTAB/n-heptanol+ water+ (0.9M) KCl



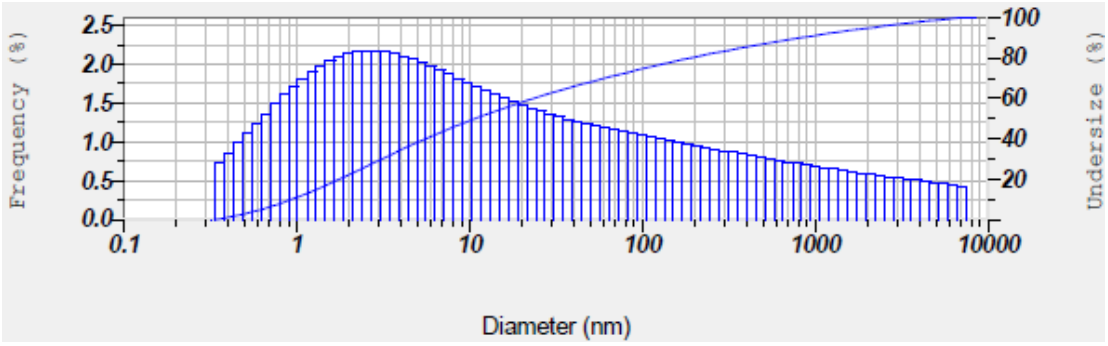
V-6: 50mMCTAB/n-heptanol+ water+ (1.3M) KCl + whey



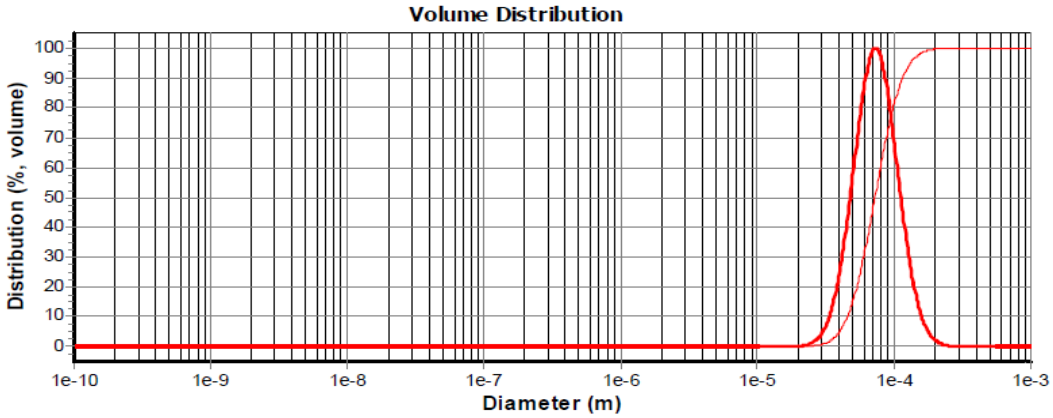
V-7: 50mM CTAB/n-heptanol+ water+ (0.9M) KCl+ n-Butanol (15%) +LF



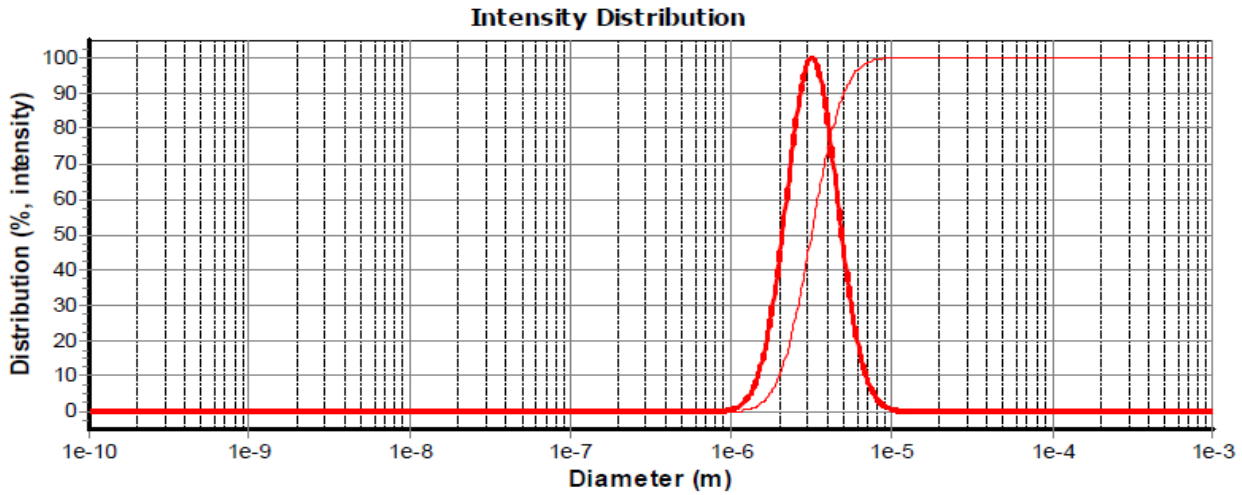
V-8: 50mMCTAB/n-heptanol+ water+ (0.9M) KCl+ n-Butanol (7%) +LF



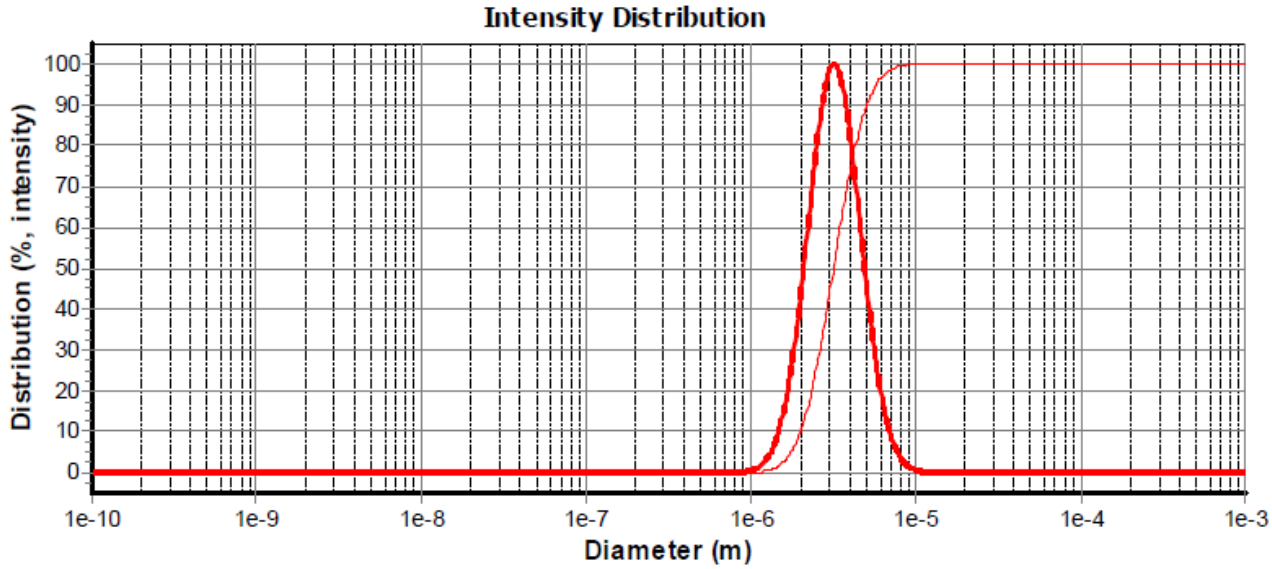
V-9: 40mM CTAB/n-heptanol + water



V-10: 50mM CTAB/n-heptanol + water+ LF



V-11: 60mM CTAB/n-heptanol+ water



Appendix VI

ANOVA Statistical analysis data

One-way ANOVA: synth whey FE effi versus NaCl

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
NaCl	6	0.6, 0.8, 1.0, 1.1, 1.3, 1.5

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
NaCl	5	10184.1	2036.82	537.46	0.000
Error	12	45.5	3.79		
Total	17	10229.6			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.94671	99.56%	99.37%	99.00%

Means

NaCl	N	Mean	StDev	95% CI
0.6	3	69.667	1.528	(67.218, 72.116)
0.8	3	84.53	2.84	(82.08, 86.98)
1.0	3	92.00	2.00	(89.55, 94.45)
1.1	3	98.040	1.298	(95.591, 100.489)
1.3	3	79.667	1.528	(77.218, 82.116)
1.5	3	25.67	2.08	(23.22, 28.12)

Pooled StDev = 1.94671

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

NaCl	N	Mean	Grouping			
1.1	3	98.040	A			
1.0	3	92.00		B		
0.8	3	84.53			C	
1.3	3	79.667			C	
0.6	3	69.667				D
1.5	3	25.67				E

Means that do not share a letter are significantly different.

One-way ANOVA: real whey be eff versus KCl

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
KCl	5	1.3, 1.5, 1.7, 1.9, 2.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
KCl	4	4514.75	1128.69	174.67	0.000
Error	10	64.62	6.46		
Total	14	4579.37			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.54205	98.59%	98.02%	96.83%

Means

KCl	N	Mean	StDev	95% CI
1.3	3	82.033	1.617	(78.763, 85.303)
1.5	3	94.20	1.90	(90.93, 97.47)
1.7	3	75.63	2.87	(72.36, 78.90)
1.9	3	59.07	3.21	(55.80, 62.34)
2.0	3	44.90	2.75	(41.63, 48.17)

Pooled StDev = 2.54205

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

KCl	N	Mean	Grouping		
1.5	3	94.20	A		
1.3	3	82.033		B	
1.7	3	75.63		B	
1.9	3	59.07			C
2.0	3	44.90			D

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
1.5 - 1.3	12.17	2.08	(5.34, 18.99)	5.86	0.001
1.7 - 1.3	-6.40	2.08	(-13.22, 0.42)	-3.08	0.069
1.9 - 1.3	-22.97	2.08	(-29.79, -16.14)	-11.07	0.000
2.0 - 1.3	-37.13	2.08	(-43.96, -30.31)	-17.89	0.000
1.7 - 1.5	-18.57	2.08	(-25.39, -11.74)	-8.95	0.000
1.9 - 1.5	-35.13	2.08	(-41.96, -28.31)	-16.93	0.000
2.0 - 1.5	-49.30	2.08	(-56.12, -42.48)	-23.75	0.000
1.9 - 1.7	-16.57	2.08	(-23.39, -9.74)	-7.98	0.000
2.0 - 1.7	-30.73	2.08	(-37.56, -23.91)	-14.81	0.000
2.0 - 1.9	-14.17	2.08	(-20.99, -7.34)	-6.83	0.000

Individual confidence level = 99.18%

One-way ANOVA: FE eff versus LF conc in feed phase

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
LF conc in feed phase	4	0.2, 0.4, 0.5, 0.7

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
LF conc in feed phase	3	5062.52	1687.51	831.03	0.000
Error	4	8.12	2.03		
Total	7	5070.65			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.425	99.84%	99.72%	99.36%

Means

LF conc in feed phase	N	Mean	StDev	95% CI
0.2	2	76.50	2.83	(73.70, 79.30)
0.4	2	24.375	0.177	(21.577, 27.173)
0.5	2	19.400	0.283	(16.602, 22.198)
0.7	2	13.4950	0.1061	(10.6974, 16.2926)

Pooled StDev = 1.425

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

LF conc in feed phase	N	Mean	Grouping
0.2	2	76.50	A
0.4	2	24.375	B
0.5	2	19.400	B
0.7	2	13.4950	C

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.4 - 0.2	-52.13	1.42	(-57.93, -46.32)	-36.58	0.000
0.5 - 0.2	-57.10	1.42	(-62.90, -51.30)	-40.07	0.000
0.7 - 0.2	-63.00	1.42	(-68.81, -57.20)	-44.21	0.000
0.5 - 0.4	-4.98	1.42	(-10.78, 0.83)	-3.49	0.081
0.7 - 0.4	-10.88	1.42	(-16.68, -5.08)	-7.64	0.005
0.7 - 0.5	-5.90	1.42	(-11.71, -0.10)	-4.14	0.047

Individual confidence level = 98.48%

One-way ANOVA: Synthetic whey be eff versus KCl

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
KCl	5	1.3, 1.5, 1.7, 1.9, 2.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
KCl	4	3180.11	795.027	280.93	0.000
Error	10	28.30	2.830		
Total	14	3208.41			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.68226	99.12%	98.77%	98.02%

Means

KCl	N	Mean	StDev	95% CI
1.3	3	84.33	2.52	(82.17, 86.50)
1.5	3	93.42	2.16	(91.26, 95.59)
1.7	3	79.017	1.062	(76.853, 81.181)
1.9	3	60.903	0.633	(58.739, 63.067)
2.0	3	54.500	1.277	(52.336, 56.664)

Pooled StDev = 1.68226

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

KCl	N	Mean	Grouping			
1.5	3	93.42	A			
1.3	3	84.33		B		
1.7	3	79.017			C	
1.9	3	60.903				D
2.0	3	54.500				E

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
1.5 - 1.3	9.09	1.37	(4.57, 13.61)	6.62	0.000
1.7 - 1.3	-5.32	1.37	(-9.83, -0.80)	-3.87	0.020
1.9 - 1.3	-23.43	1.37	(-27.95, -18.91)	-17.06	0.000
2.0 - 1.3	-29.83	1.37	(-34.35, -25.32)	-21.72	0.000
1.7 - 1.5	-14.41	1.37	(-18.92, -9.89)	-10.49	0.000
1.9 - 1.5	-32.52	1.37	(-37.04, -28.00)	-23.68	0.000
2.0 - 1.5	-38.92	1.37	(-43.44, -34.41)	-28.34	0.000
1.9 - 1.7	-18.11	1.37	(-22.63, -13.60)	-13.19	0.000
2.0 - 1.7	-24.52	1.37	(-29.03, -20.00)	-17.85	0.000
2.0 - 1.9	-6.40	1.37	(-10.92, -1.89)	-4.66	0.006

Individual confidence level = 99.18%

One-way ANOVA: sythetic whey FE efficiency versus pH

* NOTE * Cannot draw the interval plot for the Tukey procedure. Interval plots for comparisons are illegible with more than 45 intervals.

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
pH	11	10.0, 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, 11.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	10	4616.19	461.619	213.74	0.000
Error	22	47.51	2.160		
Total	32	4663.70			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.46959	98.98%	98.52%	97.71%

Means

pH	N	Mean	StDev	95% CI
10.0	3	88.000	1.000	(86.240, 89.760)
10.1	3	90.00	1.73	(88.24, 91.76)
10.2	3	93.000	1.000	(91.240, 94.760)
10.3	3	96.333	1.528	(94.574, 98.093)
10.4	3	93.333	1.155	(91.574, 95.093)
10.5	3	84.20	1.87	(82.44, 85.96)
10.6	3	77.87	1.87	(76.11, 79.63)
10.7	3	70.967	1.457	(69.207, 72.726)
10.8	3	68.80	1.93	(67.04, 70.56)
10.9	3	63.933	1.007	(62.174, 65.693)
11.0	3	63.200	1.114	(61.440, 64.960)

Pooled StDev = 1.46959

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping						
10.3	3	96.333	A						
10.4	3	93.333	A	B					
10.2	3	93.000	A	B					
10.1	3	90.00		B	C				
10.0	3	88.000			C	D			
10.5	3	84.20				D			
10.6	3	77.87					E		
10.7	3	70.967						F	
10.8	3	68.80						F	
10.9	3	63.933							G
11.0	3	63.200							G

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
10.1 - 10.0	2.00	1.20	(-2.29, 6.29)	1.67	0.836
10.2 - 10.0	5.00	1.20	(0.71, 9.29)	4.17	0.014
10.3 - 10.0	8.33	1.20	(4.04, 12.63)	6.94	0.000
10.4 - 10.0	5.33	1.20	(1.04, 9.63)	4.44	0.007
10.5 - 10.0	-3.80	1.20	(-8.09, 0.49)	-3.17	0.114
10.6 - 10.0	-10.13	1.20	(-14.43, -5.84)	-8.45	0.000
10.7 - 10.0	-17.03	1.20	(-21.33, -12.74)	-14.20	0.000
10.8 - 10.0	-19.20	1.20	(-23.49, -14.91)	-16.00	0.000
10.9 - 10.0	-24.07	1.20	(-28.36, -19.77)	-20.06	0.000
11.0 - 10.0	-24.80	1.20	(-29.09, -20.51)	-20.67	0.000
10.2 - 10.1	3.00	1.20	(-1.29, 7.29)	2.50	0.355
10.3 - 10.1	6.33	1.20	(2.04, 10.63)	5.28	0.001
10.4 - 10.1	3.33	1.20	(-0.96, 7.63)	2.78	0.229
10.5 - 10.1	-5.80	1.20	(-10.09, -1.51)	-4.83	0.003
10.6 - 10.1	-12.13	1.20	(-16.43, -7.84)	-10.11	0.000
10.7 - 10.1	-19.03	1.20	(-23.33, -14.74)	-15.86	0.000
10.8 - 10.1	-21.20	1.20	(-25.49, -16.91)	-17.67	0.000
10.9 - 10.1	-26.07	1.20	(-30.36, -21.77)	-21.72	0.000
11.0 - 10.1	-26.80	1.20	(-31.09, -22.51)	-22.33	0.000
10.3 - 10.2	3.33	1.20	(-0.96, 7.63)	2.78	0.229
10.4 - 10.2	0.33	1.20	(-3.96, 4.63)	0.28	1.000
10.5 - 10.2	-8.80	1.20	(-13.09, -4.51)	-7.33	0.000
10.6 - 10.2	-15.13	1.20	(-19.43, -10.84)	-12.61	0.000
10.7 - 10.2	-22.03	1.20	(-26.33, -17.74)	-18.36	0.000
10.8 - 10.2	-24.20	1.20	(-28.49, -19.91)	-20.17	0.000
10.9 - 10.2	-29.07	1.20	(-33.36, -24.77)	-24.22	0.000
11.0 - 10.2	-29.80	1.20	(-34.09, -25.51)	-24.84	0.000
10.4 - 10.3	-3.00	1.20	(-7.29, 1.29)	-2.50	0.355
10.5 - 10.3	-12.13	1.20	(-16.43, -7.84)	-10.11	0.000
10.6 - 10.3	-18.47	1.20	(-22.76, -14.17)	-15.39	0.000
10.7 - 10.3	-25.37	1.20	(-29.66, -21.07)	-21.14	0.000
10.8 - 10.3	-27.53	1.20	(-31.83, -23.24)	-22.95	0.000
10.9 - 10.3	-32.40	1.20	(-36.69, -28.11)	-27.00	0.000
11.0 - 10.3	-33.13	1.20	(-37.43, -28.84)	-27.61	0.000
10.5 - 10.4	-9.13	1.20	(-13.43, -4.84)	-7.61	0.000
10.6 - 10.4	-15.47	1.20	(-19.76, -11.17)	-12.89	0.000
10.7 - 10.4	-22.37	1.20	(-26.66, -18.07)	-18.64	0.000
10.8 - 10.4	-24.53	1.20	(-28.83, -20.24)	-20.45	0.000
10.9 - 10.4	-29.40	1.20	(-33.69, -25.11)	-24.50	0.000
11.0 - 10.4	-30.13	1.20	(-34.43, -25.84)	-25.11	0.000
10.6 - 10.5	-6.33	1.20	(-10.63, -2.04)	-5.28	0.001
10.7 - 10.5	-13.23	1.20	(-17.53, -8.94)	-11.03	0.000
10.8 - 10.5	-15.40	1.20	(-19.69, -11.11)	-12.83	0.000
10.9 - 10.5	-20.27	1.20	(-24.56, -15.97)	-16.89	0.000
11.0 - 10.5	-21.00	1.20	(-25.29, -16.71)	-17.50	0.000
10.7 - 10.6	-6.90	1.20	(-11.19, -2.61)	-5.75	0.000
10.8 - 10.6	-9.07	1.20	(-13.36, -4.77)	-7.56	0.000
10.9 - 10.6	-13.93	1.20	(-18.23, -9.64)	-11.61	0.000
11.0 - 10.6	-14.67	1.20	(-18.96, -10.37)	-12.22	0.000
10.8 - 10.7	-2.17	1.20	(-6.46, 2.13)	-1.81	0.765
10.9 - 10.7	-7.03	1.20	(-11.33, -2.74)	-5.86	0.000
11.0 - 10.7	-7.77	1.20	(-12.06, -3.47)	-6.47	0.000
10.9 - 10.8	-4.87	1.20	(-9.16, -0.57)	-4.06	0.018
11.0 - 10.8	-5.60	1.20	(-9.89, -1.31)	-4.67	0.004
11.0 - 10.9	-0.73	1.20	(-5.03, 3.56)	-0.61	1.000

Individual confidence level = 99.83%

One-way ANOVA: BE eff versus PV ratio

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
PV ratio	8	0.25, 0.28, 0.33, 0.40, 0.50, 0.60, 1.00, 2.00

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
PV ratio	7	12249.7	1749.96	719.38	0.000
Error	8	19.5	2.43		
Total	15	12269.2			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.55968	99.84%	99.70%	99.37%

Means

PV ratio	N	Mean	StDev	95% CI
0.25	2	8.625	1.223	(6.082, 11.168)
0.28	2	14.135	0.530	(11.592, 16.678)
0.33	2	24.17	2.40	(21.63, 26.71)
0.40	2	27.50	2.12	(24.96, 30.04)
0.50	2	36.21	0.00	(33.67, 38.75)
0.60	2	54.705	0.417	(52.162, 57.248)
1.00	2	96.05	2.43	(93.51, 98.59)
2.00	2	66.460	1.146	(63.917, 69.003)

Pooled StDev = 1.55968

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

PV ratio	N	Mean	Grouping
1.00	2	96.05	A
2.00	2	66.460	B
0.60	2	54.705	C
0.50	2	36.21	D
0.40	2	27.50	E
0.33	2	24.17	E
0.28	2	14.135	F
0.25	2	8.625	F

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.28 - 0.25	5.51	1.56	(-0.67, 11.69)	3.53	0.086
0.33 - 0.25	15.55	1.56	(9.37, 21.72)	9.97	0.000
0.40 - 0.25	18.88	1.56	(12.70, 25.05)	12.10	0.000
0.50 - 0.25	27.59	1.56	(21.41, 33.76)	17.69	0.000
0.60 - 0.25	46.08	1.56	(39.90, 52.26)	29.54	0.000
1.00 - 0.25	87.42	1.56	(81.25, 93.60)	56.05	0.000
2.00 - 0.25	57.84	1.56	(51.66, 64.01)	37.08	0.000
0.33 - 0.28	10.04	1.56	(3.86, 16.21)	6.43	0.003
0.40 - 0.28	13.37	1.56	(7.19, 19.54)	8.57	0.000
0.50 - 0.28	22.08	1.56	(15.90, 28.25)	14.15	0.000
0.60 - 0.28	40.57	1.56	(34.39, 46.75)	26.01	0.000
1.00 - 0.28	81.91	1.56	(75.74, 88.09)	52.52	0.000
2.00 - 0.28	52.33	1.56	(46.15, 58.50)	33.55	0.000
0.40 - 0.33	3.33	1.56	(-2.85, 9.51)	2.14	0.466
0.50 - 0.33	12.04	1.56	(5.86, 18.22)	7.72	0.001
0.60 - 0.33	30.53	1.56	(24.36, 36.71)	19.58	0.000
1.00 - 0.33	71.88	1.56	(65.70, 78.06)	46.09	0.000
2.00 - 0.33	42.29	1.56	(36.11, 48.47)	27.11	0.000
0.50 - 0.40	8.71	1.56	(2.53, 14.89)	5.58	0.007
0.60 - 0.40	27.20	1.56	(21.03, 33.38)	17.44	0.000
1.00 - 0.40	68.55	1.56	(62.37, 74.73)	43.95	0.000
2.00 - 0.40	38.96	1.56	(32.78, 45.14)	24.98	0.000
0.60 - 0.50	18.49	1.56	(12.32, 24.67)	11.86	0.000
1.00 - 0.50	59.84	1.56	(53.66, 66.02)	38.37	0.000
2.00 - 0.50	30.25	1.56	(24.07, 36.43)	19.40	0.000
1.00 - 0.60	41.34	1.56	(35.17, 47.52)	26.51	0.000
2.00 - 0.60	11.76	1.56	(5.58, 17.93)	7.54	0.001
2.00 - 1.00	-29.59	1.56	(-35.77, -23.41)	-18.97	0.000

Individual confidence level = 99.58%

One-way ANOVA: synthtic whey ef eff versus phase vol ratio

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
phase vol ratio	8	0.25, 0.28, 0.30, 0.40, 0.50, 0.60, 1.00, 2.00

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
phase vol ratio	7	74527.5	10646.8	2287.08	0.000
Error	8	37.2	4.7		
Total	15	74564.8			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.15759	99.95%	99.91%	99.80%

Means

phase vol ratio	N	Mean	StDev	95% CI
0.25	2	136.74	2.45	(133.22, 140.25)
0.28	2	246.00	2.83	(242.48, 249.52)
0.30	2	258.15	2.61	(254.64, 261.67)
0.40	2	217.265	1.039	(213.747, 220.783)
0.50	2	181.97	1.93	(178.45, 185.48)
0.60	2	141.805	1.138	(138.287, 145.323)
1.00	2	97.32	2.40	(93.80, 100.84)
2.00	2	50.55	2.13	(47.03, 54.06)

Pooled StDev = 2.15759

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

phase vol ratio	N	Mean	Grouping
0.30	2	258.15	A
0.28	2	246.00	B
0.40	2	217.265	C
0.50	2	181.97	D
0.60	2	141.805	E
0.25	2	136.74	E
1.00	2	97.32	F
2.00	2	50.55	G

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.28 - 0.25	109.26	2.16	(100.72, 117.81)	50.64	0.000
0.30 - 0.25	121.42	2.16	(112.88, 129.96)	56.28	0.000
0.40 - 0.25	80.53	2.16	(71.99, 89.07)	37.32	0.000
0.50 - 0.25	45.23	2.16	(36.69, 53.77)	20.96	0.000
0.60 - 0.25	5.07	2.16	(-3.47, 13.61)	2.35	0.370
1.00 - 0.25	-39.42	2.16	(-47.96, -30.87)	-18.27	0.000
2.00 - 0.25	-86.19	2.16	(-94.73, -77.65)	-39.95	0.000
0.30 - 0.28	12.15	2.16	(3.61, 20.70)	5.63	0.007
0.40 - 0.28	-28.74	2.16	(-37.28, -20.19)	-13.32	0.000
0.50 - 0.28	-64.03	2.16	(-72.58, -55.49)	-29.68	0.000
0.60 - 0.28	-104.19	2.16	(-112.74, -95.65)	-48.29	0.000
1.00 - 0.28	-148.68	2.16	(-157.22, -140.14)	-68.91	0.000
2.00 - 0.28	-195.45	2.16	(-204.00, -186.91)	-90.59	0.000
0.40 - 0.30	-40.89	2.16	(-49.43, -32.35)	-18.95	0.000
0.50 - 0.30	-76.19	2.16	(-84.73, -67.65)	-35.31	0.000
0.60 - 0.30	-116.35	2.16	(-124.89, -107.81)	-53.93	0.000
1.00 - 0.30	-160.83	2.16	(-169.38, -152.29)	-74.54	0.000
2.00 - 0.30	-207.61	2.16	(-216.15, -199.07)	-96.22	0.000
0.50 - 0.40	-35.30	2.16	(-43.84, -26.76)	-16.36	0.000
0.60 - 0.40	-75.46	2.16	(-84.00, -66.92)	-34.97	0.000
1.00 - 0.40	-119.94	2.16	(-128.49, -111.40)	-55.59	0.000
2.00 - 0.40	-166.72	2.16	(-175.26, -158.18)	-77.27	0.000
0.60 - 0.50	-40.16	2.16	(-48.70, -31.62)	-18.61	0.000
1.00 - 0.50	-84.65	2.16	(-93.19, -76.10)	-39.23	0.000
2.00 - 0.50	-131.42	2.16	(-139.96, -122.88)	-60.91	0.000
1.00 - 0.60	-44.49	2.16	(-53.03, -35.94)	-20.62	0.000
2.00 - 0.60	-91.26	2.16	(-99.80, -82.72)	-42.30	0.000
2.00 - 1.00	-46.77	2.16	(-55.32, -38.23)	-21.68	0.000

Individual confidence level = 99.58%

One-way ANOVA: FE yield versus Pv ratio

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Pv ratio	8	0.25, 0.28, 0.30, 0.40, 0.50, 0.60, 1.00, 2.00

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Pv ratio	7	6831.60	975.943	243.09	0.000
Error	8	32.12	4.015		
Total	15	6863.72			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.00369	99.53%	99.12%	98.13%

Means

Pv ratio	N	Mean	StDev	95% CI
0.25	2	32.31	3.26	(29.04, 35.57)
0.28	2	69.12	2.64	(65.85, 72.39)
0.30	2	83.78	2.34	(80.51, 87.04)
0.40	2	84.98	2.31	(81.71, 88.25)
0.50	2	90.00	1.41	(86.73, 93.27)
0.60	2	94.535	0.757	(91.268, 97.802)
1.00	2	98.600	0.849	(95.333, 101.867)
2.00	2	98.545	0.643	(95.278, 101.812)

Pooled StDev = 2.00369

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Pv ratio	N	Mean	Grouping				
1.00	2	98.600	A				
2.00	2	98.545	A				
0.60	2	94.535	A	B			
0.50	2	90.00		B	C		
0.40	2	84.98			C		
0.30	2	83.78			C		
0.28	2	69.12				D	
0.25	2	32.31					E

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.28 - 0.25	36.82	2.00	(28.88, 44.75)	18.37	0.000
0.30 - 0.25	51.47	2.00	(43.54, 59.40)	25.69	0.000
0.40 - 0.25	52.67	2.00	(44.74, 60.61)	26.29	0.000
0.50 - 0.25	57.70	2.00	(49.76, 65.63)	28.79	0.000
0.60 - 0.25	62.23	2.00	(54.30, 70.16)	31.06	0.000
1.00 - 0.25	66.29	2.00	(58.36, 74.23)	33.09	0.000
2.00 - 0.25	66.24	2.00	(58.31, 74.17)	33.06	0.000
0.30 - 0.28	14.66	2.00	(6.72, 22.59)	7.31	0.001
0.40 - 0.28	15.86	2.00	(7.93, 23.79)	7.92	0.001
0.50 - 0.28	20.88	2.00	(12.95, 28.81)	10.42	0.000
0.60 - 0.28	25.41	2.00	(17.48, 33.35)	12.68	0.000
1.00 - 0.28	29.48	2.00	(21.55, 37.41)	14.71	0.000
2.00 - 0.28	29.42	2.00	(21.49, 37.36)	14.69	0.000
0.40 - 0.30	1.20	2.00	(-6.73, 9.14)	0.60	0.998
0.50 - 0.30	6.22	2.00	(-1.71, 14.16)	3.11	0.148
0.60 - 0.30	10.76	2.00	(2.83, 18.69)	5.37	0.009
1.00 - 0.30	14.82	2.00	(6.89, 22.76)	7.40	0.001
2.00 - 0.30	14.77	2.00	(6.84, 22.70)	7.37	0.001
0.50 - 0.40	5.02	2.00	(-2.91, 12.95)	2.51	0.310
0.60 - 0.40	9.56	2.00	(1.62, 17.49)	4.77	0.018
1.00 - 0.40	13.62	2.00	(5.69, 21.55)	6.80	0.002
2.00 - 0.40	13.57	2.00	(5.63, 21.50)	6.77	0.002
0.60 - 0.50	4.53	2.00	(-3.40, 12.47)	2.26	0.407
1.00 - 0.50	8.60	2.00	(0.67, 16.53)	4.29	0.033
2.00 - 0.50	8.55	2.00	(0.61, 16.48)	4.26	0.034
1.00 - 0.60	4.06	2.00	(-3.87, 12.00)	2.03	0.518
2.00 - 0.60	4.01	2.00	(-3.92, 11.94)	2.00	0.532
2.00 - 1.00	-0.05	2.00	(-7.99, 7.88)	-0.03	1.000

Individual confidence level = 99.58%

One-way ANOVA: real whey FE eff versus Nacl

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Nacl	6	0.1, 0.6, 0.8, 1.1, 1.3, 1.5

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Nacl	5	7943.12	1588.62	309.64	0.000
Error	12	61.57	5.13		
Total	17	8004.68			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.26507	99.23%	98.91%	98.27%

Means

Nacl	N	Mean	StDev	95% CI
0.1	3	93.567	1.343	(90.717, 96.416)
0.6	3	67.67	3.21	(64.82, 70.52)
0.8	3	80.00	1.95	(77.15, 82.85)
1.1	3	98.700	0.794	(95.851, 101.549)
1.3	3	80.67	2.03	(77.82, 83.52)
1.5	3	34.57	3.18	(31.72, 37.42)

Pooled StDev = 2.26507

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Nacl	N	Mean	Grouping		
1.1	3	98.700	A		
0.1	3	93.567	A		
1.3	3	80.67		B	
0.8	3	80.00		B	
0.6	3	67.67			C
1.5	3	34.57			D

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.6 - 0.1	-25.90	1.85	(-32.11, -19.69)	-14.00	0.000
0.8 - 0.1	-13.57	1.85	(-19.78, -7.35)	-7.34	0.000
1.1 - 0.1	5.13	1.85	(-1.08, 11.35)	2.78	0.130
1.3 - 0.1	-12.90	1.85	(-19.11, -6.69)	-6.98	0.000
1.5 - 0.1	-59.00	1.85	(-65.21, -52.79)	-31.90	0.000
0.8 - 0.6	12.33	1.85	(6.12, 18.55)	6.67	0.000
1.1 - 0.6	31.03	1.85	(24.82, 37.25)	16.78	0.000
1.3 - 0.6	13.00	1.85	(6.79, 19.21)	7.03	0.000
1.5 - 0.6	-33.10	1.85	(-39.31, -26.89)	-17.90	0.000
1.1 - 0.8	18.70	1.85	(12.49, 24.91)	10.11	0.000
1.3 - 0.8	0.67	1.85	(-5.55, 6.88)	0.36	0.999
1.5 - 0.8	-45.43	1.85	(-51.65, -39.22)	-24.57	0.000
1.3 - 1.1	-18.03	1.85	(-24.25, -11.82)	-9.75	0.000
1.5 - 1.1	-64.13	1.85	(-70.35, -57.92)	-34.68	0.000
1.5 - 1.3	-46.10	1.85	(-52.31, -39.89)	-24.93	0.000

Individual confidence level = 99.43%

One-way ANOVA: real whey FE efficiency versus pH

* NOTE * Cannot draw the interval plot for the Tukey procedure. Interval plots for comparisons are illegible with more than 45 intervals.

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
pH	11	10.0, 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, 11.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	10	4148.63	414.863	278.72	0.000
Error	22	32.75	1.488		
Total	32	4181.38			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.22003	99.22%	98.86%	98.24%

Means

pH	N	Mean	StDev	95% CI
10.0	3	83.333	1.155	(81.873, 84.794)
10.1	3	85.667	1.528	(84.206, 87.127)
10.2	3	95.333	1.155	(93.873, 96.794)
10.3	3	97.467	0.503	(96.006, 98.927)
10.4	3	90.233	0.681	(88.773, 91.694)
10.5	3	83.333	1.528	(81.873, 84.794)
10.6	3	80.667	1.528	(79.206, 82.127)
10.7	3	74.233	1.150	(72.773, 75.694)
10.8	3	68.667	1.528	(67.206, 70.127)
10.9	3	65.000	1.000	(63.539, 66.461)
11.0	3	62.667	1.155	(61.206, 64.127)

Pooled StDev = 1.22003

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping						
10.3	3	97.467	A						
10.2	3	95.333	A						
10.4	3	90.233		B					
10.1	3	85.667			C				
10.5	3	83.333			C	D			
10.0	3	83.333			C	D			
10.6	3	80.667				D			
10.7	3	74.233					E		
10.8	3	68.667						F	
10.9	3	65.000							G
11.0	3	62.667							G

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
10.1 - 10.0	2.333	0.996	(-1.231, 5.898)	2.34	0.442
10.2 - 10.0	12.000	0.996	(8.436, 15.564)	12.05	0.000
10.3 - 10.0	14.133	0.996	(10.569, 17.698)	14.19	0.000
10.4 - 10.0	6.900	0.996	(3.336, 10.464)	6.93	0.000
10.5 - 10.0	0.000	0.996	(-3.564, 3.564)	0.00	1.000
10.6 - 10.0	-2.667	0.996	(-6.231, 0.898)	-2.68	0.271
10.7 - 10.0	-9.100	0.996	(-12.664, -5.536)	-9.14	0.000
10.8 - 10.0	-14.667	0.996	(-18.231, -11.102)	-14.72	0.000
10.9 - 10.0	-18.333	0.996	(-21.898, -14.769)	-18.40	0.000
11.0 - 10.0	-20.667	0.996	(-24.231, -17.102)	-20.75	0.000
10.2 - 10.1	9.667	0.996	(6.102, 13.231)	9.70	0.000
10.3 - 10.1	11.800	0.996	(8.236, 15.364)	11.85	0.000
10.4 - 10.1	4.567	0.996	(1.002, 8.131)	4.58	0.005
10.5 - 10.1	-2.333	0.996	(-5.898, 1.231)	-2.34	0.442
10.6 - 10.1	-5.000	0.996	(-8.564, -1.436)	-5.02	0.002
10.7 - 10.1	-11.433	0.996	(-14.998, -7.869)	-11.48	0.000
10.8 - 10.1	-17.000	0.996	(-20.564, -13.436)	-17.07	0.000
10.9 - 10.1	-20.667	0.996	(-24.231, -17.102)	-20.75	0.000
11.0 - 10.1	-23.000	0.996	(-26.564, -19.436)	-23.09	0.000
10.3 - 10.2	2.133	0.996	(-1.431, 5.698)	2.14	0.563
10.4 - 10.2	-5.100	0.996	(-8.664, -1.536)	-5.12	0.002
10.5 - 10.2	-12.000	0.996	(-15.564, -8.436)	-12.05	0.000
10.6 - 10.2	-14.667	0.996	(-18.231, -11.102)	-14.72	0.000
10.7 - 10.2	-21.100	0.996	(-24.664, -17.536)	-21.18	0.000
10.8 - 10.2	-26.667	0.996	(-30.231, -23.102)	-26.77	0.000
10.9 - 10.2	-30.333	0.996	(-33.898, -26.769)	-30.45	0.000
11.0 - 10.2	-32.667	0.996	(-36.231, -29.102)	-32.79	0.000
10.4 - 10.3	-7.233	0.996	(-10.798, -3.669)	-7.26	0.000
10.5 - 10.3	-14.133	0.996	(-17.698, -10.569)	-14.19	0.000
10.6 - 10.3	-16.800	0.996	(-20.364, -13.236)	-16.86	0.000
10.7 - 10.3	-23.233	0.996	(-26.798, -19.669)	-23.32	0.000
10.8 - 10.3	-28.800	0.996	(-32.364, -25.236)	-28.91	0.000
10.9 - 10.3	-32.467	0.996	(-36.031, -28.902)	-32.59	0.000
11.0 - 10.3	-34.800	0.996	(-38.364, -31.236)	-34.93	0.000
10.5 - 10.4	-6.900	0.996	(-10.464, -3.336)	-6.93	0.000
10.6 - 10.4	-9.567	0.996	(-13.131, -6.002)	-9.60	0.000
10.7 - 10.4	-16.000	0.996	(-19.564, -12.436)	-16.06	0.000
10.8 - 10.4	-21.567	0.996	(-25.131, -18.002)	-21.65	0.000
10.9 - 10.4	-25.233	0.996	(-28.798, -21.669)	-25.33	0.000
11.0 - 10.4	-27.567	0.996	(-31.131, -24.002)	-27.67	0.000
10.6 - 10.5	-2.667	0.996	(-6.231, 0.898)	-2.68	0.271
10.7 - 10.5	-9.100	0.996	(-12.664, -5.536)	-9.14	0.000
10.8 - 10.5	-14.667	0.996	(-18.231, -11.102)	-14.72	0.000
10.9 - 10.5	-18.333	0.996	(-21.898, -14.769)	-18.40	0.000
11.0 - 10.5	-20.667	0.996	(-24.231, -17.102)	-20.75	0.000
10.7 - 10.6	-6.433	0.996	(-9.998, -2.869)	-6.46	0.000
10.8 - 10.6	-12.000	0.996	(-15.564, -8.436)	-12.05	0.000
10.9 - 10.6	-15.667	0.996	(-19.231, -12.102)	-15.73	0.000
11.0 - 10.6	-18.000	0.996	(-21.564, -14.436)	-18.07	0.000
10.8 - 10.7	-5.567	0.996	(-9.131, -2.002)	-5.59	0.001
10.9 - 10.7	-9.233	0.996	(-12.798, -5.669)	-9.27	0.000
11.0 - 10.7	-11.567	0.996	(-15.131, -8.002)	-11.61	0.000
10.9 - 10.8	-3.667	0.996	(-7.231, -0.102)	-3.68	0.040
11.0 - 10.8	-6.000	0.996	(-9.564, -2.436)	-6.02	0.000
11.0 - 10.9	-2.333	0.996	(-5.898, 1.231)	-2.34	0.442

Individual confidence level = 99.83%

One-way ANOVA: BE eff versus Recycle

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Recycle	4	1, 2, 3, 4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Recycle	3	2884.39	961.462	1118.56	0.000
Error	4	3.44	0.860		
Total	7	2887.82			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.927119	99.88%	99.79%	99.52%

Means

Recycle	N	Mean	StDev	95% CI
1	2	95.430	0.707	(93.610, 97.250)
2	2	76.1200	0.0141	(74.2998, 77.9402)
3	2	55.75	1.71	(53.93, 57.57)
4	2	46.2200	0.0990	(44.3998, 48.0402)

Pooled StDev = 0.927119

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Recycle	N	Mean	Grouping
1	2	95.430	A
2	2	76.1200	B
3	2	55.75	C
4	2	46.2200	D

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
2 - 1	-19.310	0.927	(-23.086, -15.534)	-20.83	0.000
3 - 1	-39.680	0.927	(-43.456, -35.904)	-42.80	0.000
4 - 1	-49.210	0.927	(-52.986, -45.434)	-53.08	0.000
3 - 2	-20.370	0.927	(-24.146, -16.594)	-21.97	0.000
4 - 2	-29.900	0.927	(-33.676, -26.124)	-32.25	0.000
4 - 3	-9.530	0.927	(-13.306, -5.754)	-10.28	0.002

Individual confidence level = 98.48%

One-way ANOVA: FE eff versus Recycling

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Recycling	4	1, 2, 3, 4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Recycling	3	2320.38	773.458	687.52	0.000
Error	4	4.50	1.125		
Total	7	2324.88			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.06066	99.81%	99.66%	99.23%

Means

Recycling	N	Mean	StDev	95% CI
1	2	97.500	0.707	(95.418, 99.582)
2	2	91.00	0.00	(88.92, 93.08)
3	2	78.00	1.41	(75.92, 80.08)
4	2	53.00	1.41	(50.92, 55.08)

Pooled StDev = 1.06066

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Recycling	N	Mean	Grouping		
1	2	97.500	A		
2	2	91.00		B	
3	2	78.00			C
4	2	53.00			D

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
2 - 1	-6.50	1.06	(-10.82, -2.18)	-6.13	0.012
3 - 1	-19.50	1.06	(-23.82, -15.18)	-18.38	0.000
4 - 1	-44.50	1.06	(-48.82, -40.18)	-41.96	0.000
3 - 2	-13.00	1.06	(-17.32, -8.68)	-12.26	0.001
4 - 2	-38.00	1.06	(-42.32, -33.68)	-35.83	0.000
4 - 3	-25.00	1.06	(-29.32, -20.68)	-23.57	0.000

Individual confidence level = 98.48%

LIST OF PUBLICATIONS BASED ON RESEARCH WORK

RESEARCH PUBLICATIONS

- Pawar, S. S., Iyyaswami, R., and Belur, P. D. (2017). "Reverse micellar extraction of lactoferrin from its synthetic solution using CTAB/n-heptanol system." *J. Food Sci. Technol.*, 54(11), 3630–3639.
- Pawar, S. S., Regupathi, I., and Prasanna, B. D. (2017). "Reverse micellar partitioning of Bovine Serum Albumin with novel system." *Resour. Technol.*, 3(4), 491–494.
- Pawar, S. S., Iyyaswami, R., and Belur, P. D. "Selective Extraction of Lactoferrin from Acidic whey using CTAB/n-heptanol Reverse Micellar System." *J. Food Sci. Technol.*, 56(5),2553-2562.

CONFERENCE PUBLICATION

- Pawar, S. S., Regupathi, I., and Prasanna, B. D. (2017). "Screening of Reverse Micellar System for the Extraction of Bovine Lactoferrin". *International Engineering Symposium (IES), Kumamoto University, Japan, 1st to 3rd March 2017*, P.P. 844-847.

CURRICULUM VITAE

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- **M.Tech in Biotechnology (68.70%), 2008-20010**

Dept. of Biotechnology and Bioinformatics
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- **B.Sc. in Biotechnology (68.77%), 2003-2007**

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Work Experience

Worked as **Lecturer** at **MGM's College of Engineering and Technology, Navi Mumbai,** from **July 2011-December 2013.**

Research Papers Published:

- Pawar, S. S., Iyyaswami, R., and Belur, P. D. (2017). "Reverse micellar extraction of lactoferrin from its synthetic solution using CTAB/n-heptanol system." *J. Food Sci. Technol.*, 54(11), 3630–3639.

- Pawar, S. S., Regupathi, I., and Prasanna, B. D. (2017). "Reverse micellar partitioning of Bovine Serum Albumin with novel system." *Resour. Technol.*, 3(4), 491–494.

International Conference:

- **Presented a research paper entitled, "**Reverse micellar partitioning of Bovine Serum Albumin with novel system**." Technoscape 2016, VIT Vellore, Tamilnadu, October 2016.**
- **Presented a research paper entitled, "**Screening of Reverse Micellar System for the Extraction of Bovine Lactoferrin". ***International Engineering Symposium (IES), Kumamoto University, Japan, 1st to 3rd March 2017***.

Declaration:

I hereby declare that all the information mentioned above is true to the best of my knowledge.

Swapnali S. Pawar