NON-CONVENTIONAL EXTRACTION OF BOVINE LACTOPEROXIDASE FROM WHEY

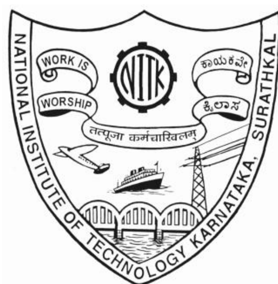
Thesis

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

SHWETHA KARANTH



DEPARTMENT OF CHEMICAL ENGINEERING

NATIONAL INSTITUTE OF TECHNOLOGY KARNATAKA,

SURATHKAL, MANGALORE -575025

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DECLARATION

I, Shwetha Karanth hereby *declare* that the Research Thesis entitled "**NON-CONVENTIONAL EXTRACTION OF BOVINE LACTOPEROXIDASE 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 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.

Shwetha Karanth
(165047CH16F05)
Department of Chemical Engineering

Place: NITK, Surathkal
Date:

C E R T I F I C A T E

This is to *certify* that the Research Thesis entitled "**NON-CONVENTIONAL EXTRACTION OF BOVINE LACTOPEROXIDASE FROM WHEY"** submitted by Shwetha Karanth (Register Number: 165047CH16F05) as the record of the research work carried out by her, is *accepted as the Research Thesis submission* in partial fulfilment of the requirements for the award of degree of Doctor of Philosophy.

Research Guide

Dr. I. Regupathi

Associate Professor

Dept. of Chemical Engineering

NITK, Surathkal

Chairman – DRPC

Dr.P.E. Jagadeeshbabu

Head of the Department

Dept. of Chemical Engineering

NITK, Surathkal

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ABSTRACT

Food spoilage is a global industrial issue, and consumers are vary of the ill-effects of chemical preservatives. Bovine Lactoperoxidase (LP- EC 1.11.1.7), a natural antimicrobial, is used as bio preservative. Industries are pursuing new purification methods as conventional techniques like chromatography and membrane separation suffer drawbacks. The present work investigates the use of non-conventional liquid-liquid extraction (LLE) techniques to extract and purify LP. The stability of LP was explored in various phase forming components of LLE, viz. polymers, surfactants, salts sugars, polyols, and alcohols. The surfactant systems showed compatibility, and the Reverse Micellar Extraction (RME) was studied to extract the LP from aqueous solution using systems formed by ionic and non-ionic surfactant mixtures to reduce the denaturation of LP. Tween series surfactants with Aerosol-OT (bis-(2-ethylhexyl) sulfosuccinate) showed better extraction than Triton and Span series. Complete extraction of LP occurred with the RM formed by 90mM Aerosol-OT/8 mM Tween 80 in isooctane and a maximum of 95.5% back extraction efficiency with 66% active LP recovered using pH of 10.5, 1M KCl, and 60 mM cetyltrimethylammonium bromide system. Further, selective RME of LP was extended to whey. A maximum of 86% LP was extracted from acid whey at pH 9.5 with the addition of 0.2M KCl, using 115 AOTmM / Tween 80 23mM surfactant blends in the organic phase. Active LP of 80% with 112% extraction efficiency was achieved with a stripping phase of 1.5M KCl at pH 10.5 and 60 mM CTAB in the organic phase.Further, Rhamnolipid-based RME was studied to avoid the adverse effect of synthetic surfactants. A novel back extraction strategy using pH-specific protonation – deprotonation of the Rhamnolipid headgroups was used during back extraction. The optimized extraction conditions resulted in 96.65% LP extraction and 85.71% active LP recovery with 8.4 fold purification. The recovered LP from acid whey studies was qualitatively analyzed using RP-HPLC. The antimicrobial activity of the extracted LP showed a good reduction in colony-forming units of *S. aureus* and specifically exhibited a bacteriostatic effect.

Keywords: Whey protein, bioseparation, surfactant, reverse micelle extraction, purification

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ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
AOT	Sodium bis(2-ethyl hexyl) sulfosuccinate
B.E.	Back extraction
BCA	Bicinchoninic Acid
BS	Biosurfactant
BSA	Bovine Serum Albumin
CD	Circular Dichroism
CMC	Critical micelle concentration
CTAB	Cetyltrimethylammonium bromide
DOLPA	Dioleyl phosphoric acid
F.E.	Forward extraction
HLB	hydrophilic-lipophilic balance
IgG	Immunoglobulin G
23KCl	Potassium Chloride
LP	Lactopeorxidase
LPS	Lactopeorxidase system
mg	milligrams
mL	millilitre
NaCl	Sodium Chloride
NaDEHP	Sodium bis(2-ethylhexyl)phosphoric acid
PEG	Polyethylene oxide or polyoxyethylene
PEO/ POE	Polyethylene oxide or polyoxyethylene
PF	Purification Fold
PPG	Polypropylene glycols
ppm	Parts per million
PVP	Polyvinylpyrrolidone
RLs	Rhamnolipids
RMs	Reverse micelles
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
RPM	Rotation Per Minute
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulphate
TFA	Tri-FluoroAceticacid
TOMAC	Trioctylmethylammonium chloride
TSA	Tryptose soya Agar
TX	Triton X
UV	Ultraviolet

CHAPTER ONE

1. INTRODUCTION

1.1 Food Spoilage and antimicrobials

The increasing demand for ready-to-eat, fresh, minimally processed food has led to either canned or frozen food. These days food products cross multiple national borders to reach the consumer food table. Safe food production and preservation are growing issues and challenges with an ever-increasing population. Behavioral and lifestyle changes have intensified the risk of food contamination and food borne diseases. Food from both plant and animal origin consists of water, carbohydrates, lipids, fats, proteins, and minerals. These substances can take part in chemical, enzymatic, or microbial activities during transportation and storage and leading to food spoilage(Sharif et al. 2017). Food contamination and spoilage is a significant problem during the processing and storage in the food industry, and currently, the effort on the reduction of food spoilage is an important objective for the industries. Incidents of both biological and chemical contamination of food are surprisingly increasing during transportation and storage in the past few yearsand eventually cause diseases in consumers(Bondi et al. 2017). Some food contaminants also include natural toxins from vegetables and fruits like aflatoxins, ochratoxins, goitrogens, lectins, trypsin inhibitors, salicylates, patulin. Microbial spoilage of food is caused by fungi and bacteria that grow on food and produce substances that render the food unfit for human consumption. Spoilage is very common in protein-rich food such as fish, meat, poultry items & dairy products. Gram-positive rod Lactic acid bacteria are a common contaminant in various vacuum-packed food. Lactic acid bacteria are identified as *Lactobacillus, pediococcus spp., streptococcus, leuconostoc spp.* Aerobic bacteria attack the meat and poultry initially and provide slime and other conditions favorable for the growth of anaerobic bacteria(Amit et al. 2017).

Many chemicals known as artificial preservatives regarded as safe and approved by FDA kill the microbes and /or stop their growth. Some of the antimicrobials are

benzoates, nitrites, propionate, gaseous sulphur dioxide and sulphite salts, parabens. However, Benzoates are reported to cause asthmatic reactions in aspirin-sensitive individuals. Sorbates and nitrites are known to be mutagenic, genotoxic, carcinogenic (Mamur et al. 2010). They are useful in their undissociated forms as antimicrobials, and the same can form genotoxic compounds in combination (Hartman 1983). Sulfites are reported to cause adverse clinical effects in sensitive individuals, ranging from dermatitis, urticaria, flushing, hypotension, abdominal pain, and diarrhoea to induce anaphylactic and asthmatic reactions (Taylor et al. 1986). These chemical antimicrobials are a common topic in public discussions as they are known to be harmful with serious side effects. Also, several physical and chemical properties of food like pH, temperature, the presence of salts, metal ions, lipids, proteases, and sugars affect the antimicrobial activity of antimicrobials (Juneja, Dwivedi, andYan2012).

As an alternative to chemical preservatives, the food industry has moved slowly towards Biopreservatives because of the growing concerns of health, shelf-life, taste trends &safety issues. In the age of "back to organics", everyone is looking for natural ingredients. Natural antimicrobials are being used synergistically with other techniques to prevent food spoilage (Elsser-Gravesen and Elsser-Gravesen 2013). Biopreservation, hence has a new market,and Bacteriocins, Ovo-antimicrobials, Lacto-antimicrobials, Phyto-antimicrobials, and acid-antimicrobials offer the choice depending on the target organisms (Naidu 2000).

1.2 Lactoperoxidase and Lactoperoxidase system

Nature's complete food, "milk," is the best source of bioactive components. Antimicrobial proteins from milk include lactoferrin, lactoperoxidase (LP), lysozyme, lactoglobulins, lactolipids. These have significant applications in food industries as antimicrobial agents and multi-nutrient. Amongst these milk proteins, Lactoperoxidase (EC 1.11.1.7) is the inherent antimicrobial system in milk. LP is also secreted by salivary and other mucosal glands. Mostly abundant in bovine milk, it has a concentration range from 30-50mg/L(Yingling 2016). The enzyme alone is inactive

against microbes; however, the reaction product catalyzed by LP is harmful toinfect microbes. Generation of antimicrobial reaction product hypothiocyanate from thiocyanate ion is catalyzed by lactoperoxidase in the presence of hydrogen peroxide and is reported as Lactoperoxidase system (LPS). This highly reactive oxidizing agent destroys bacteria, viruses and fungi. Elsewhere (commercially) LPS constitutes Lactoperoxidase, Glucose oxidase, Glucose, Sodium Thiocyanate. Besides, LPS is generally recognized as safe (GRAS) by the American Food and Drug Administration (FDA). The formulation for LPS at a level of 300 mg/L milk or 300ppm is advised with the composition of Lactoperoxidase: 1.25%, Glucose oxidase: 0.75%, Glucose: 30%, Sodium Thiocyanate: 5%, Sucrose: 63%. Range of market applications of LPS including food products**,** infant formula, sports, and functional food, pharmaceuticals, veterinary and feed specialties, personal care products.

LPS is studied extensively and used as an antimicrobial in various areas. Unlike few antimicrobials, LP has a broad range of activity, both bacteriostatic and bactericidal, detrimental to both gram-positive and gram-negative alike(Arqués et al. 2008a; Björck et al. 1975) and extending the activity to few fungi (Jacob et al. 2000; Popper and Knorr 1997) and virus (Shin et al. 2005). Its anti-listerial activities can be exploited in the food industry (Elliot et al. 2004) and the dairy industry in particular (Seyoum et al. 2015; Barrett et al. 1999). Studies have shown that combining LPS with other antimicrobial systems can better affect *Listeria* destruction (Bravo et al. 2014; Montiel et al. 2012; Zapico et al. 1998). LPS is used in dental care products and is assumed to prevent/ inhibit plaque accumulation (Kirstila et al. 1994; Adams et al. 2017). They are available commercially under various trade names Biotene, Zendium, oral balance, Bio-Xtra for oral care. They are used in pharmaceutical topical creams under trade names Flaminal® Hydro and Flaminal®. In tropical countries, the LPS system can successfully prevent milk spoilage before reaching the dairy plant for pasteurization. Many field studies have proven that milk spoilage can be prevented by activating LPS through the addition of Hydrogen peroxide and Thiocyanate(Asaah et al. 2007; Kamau et al. 2010).

1.3 Purification strategies

The purification of a particular enzyme involves the removal of other substances (proteins as well as non-proteins) present in the preparation. Thus purification is a multi-step process exploiting a range of biophysical and biochemical characteristics such as the relative concentration of the enzyme in the source, solubility, charge, size (molecular weight), hydrophobicity/ hydrophilicity of the target protein. Depending on the properties, downstream processing is planned for the purification of biomolecules. The steps in purification are either performed individually or integrated for better productivity. In general, chromatography is the method of choice for minor protein purification.

Lactoperoxidase in milk was first isolated and purified using the precipitation method in 1943 by Theorell and Akeson. Since then, most purification protocols have concentrated on chromatographic methods. Industrial purification protocols employ chromatography and membrane filtration. Ion-exchange chromatography techniques involving DEAE-Sephadex, CM-Sephadex, and CM-cellulose have been tried by various researchers. Carboxymethyl-Sepharose and sulfopropyl-Sepharoseare commonly used resins. Membrane processes like microfiltration and ultrafiltration are integrated into chromatography. Cationic membrane chromatography is also later used in the dairy industry (Clovis K et al. 1997; Plate et al. 2006) to purify LP.

Combined methods by coupling chromatography and membrane separations are also researched. It is economical to simultaneously purify Lactoferrin and Lactoperoxidase because of their similar physio-chemical properties, yet again only a few researchers have concentrated on this aspect (Fee and Chand 2006; Plate et al. 2006). Though widely used, limitations of chromatographic processes include the requirement of large columns and slow throughput, expensive adsorbents, leaching of the ligand, etc., makes affinity chromatography undesirable. Membrane processes can result in the

denaturation of proteins due to varied thermal and acidic treatments (Voswinkel and Kulozik 2011).

Convenient and easy to scale-up extraction and purification technologies are the need of the hour. Of late non-chromatographic methods like aqueous two-phase systems (ATPS), aqueous micellar two-phase systems (AMTPS), and reverse micellar systems are being explored for the selective extraction and purification of biomolecules. These systems are economically feasible, non-tedious, and provide a milder environment for proteins. Being aqueous in nature, the solubility of the protein can be tuned conveniently to partition into either of the phases. Polymer systems though old, are revisited because of the ease of operation, lesser complexity, and relatively better yields. Surfactant systems are new and gaining a lot of attention in the recent past. Many proteins, peptides, and enzymes have been purified using the said methods. Lately, few researchers have concentrated on the extraction of whey proteins using these methods. These processes being amenable for continuous regimes are an excellent replacement for chromatography and membrane-based methods.

1.4 Liquid-Liquid extraction systems for protein purification

In the light of increasing interest in the usage of purified enzymes and proteins in the food industry, more reliable strategies like aqueous two-phase extraction are employed for maximizing the recovery and purity of the protein. An aqueous two-phase system is formed by mixing two different water-soluble incompatible polymers or polymers and low molecular weight salts above certain critical concentrations with water. The partitioning of the desired component selectively into one of the phases is the basis of separation, and it can be tuned by manipulating the properties that govern the distribution. Charge, affinity, hydrophobicity are also taken into account to improve the partition characteristics. Since its first use by Albertsson for bioseparation; Polymer/Polymer and Polymer /salt systems are most commonly used (Glyk et al. 2015). The gentle water environment for biomolecules, ease of operation and scale-up, economical and relatively good yields make them an attractive option in large-scale industrial downstream processing.

The difficulty of protein recovery from phase-forming components in polymer systems paves the way for other ATPS. One such system is alcohol/ salt ATPS(Ooi et al. 2009; Amid et al. 2012), where hydrophobic interaction and salting-out effect leads to a desirable partitioning of the proteins. The target protein, usually in the top organic phase, can easily be recovered by evaporation. The system is proven to be effective for both low and high molecular weight proteins (Sala et al. 2014). The ionic liquid has opted as a safer green choice in place of organic solvent as they have high thermal and chemical stability. Ionic liquids are mild when compared to the denaturing organic solvent; also they are less viscous, non-flammable, and exhibit faster phase separation.

Similarly, surfactant-based extraction methods are also a recent development. Surfactants being amphiphilic in nature, normally arrange themselves in an oil-water interface. When their concentration is high enough, they form aggregates called micelles, and the onset concentration for micelle formation is named critical micelle concentration (CMC). The hydrophilic and hydrophobic balance of the surfactant dictates its nature. At a specific surfactant concentration and temperature (above cloud point), some micellar solutions separate into two-phase, like the micellar rich phase and the poor micellar phase. This system offers selective partitioning of hydrophilic and hydrophobic entities, and this approach is termed an aqueous micellar two-phase system. Aggregates of surfactant in an organic solvent are Reverse micelles. In the presence of water, they exist as a ternary system of organic solvent (~80-90%), water (~10%), and surfactant (<1%) with a "water pool" (water-in-oil emulsions) at the center of aggregation. These water pools can solubilize hydrophilic proteins providing a safe environment during separation/extraction processes. Reverse micelles are thermodynamically stable structures but dynamic in nature. Hence reverse micelles can be used as both separation and purification methods in downstream processing (Regalado et al. 1996; Tonova and Lazarova 2008;Krei and Hustedt 1992; Chaurasiya and Umesh Hebbar 2013).

The reverse micelle extraction (RME) cycle in the purification comprises two processes: Forward and backward extraction. In the former step, the protein is

transferred from the aqueous phase into the organic phase, where proteins are solubilized in the water pockets. During the latter step, the protein is transferred back to a fresh aqueous phase. Here, conditions that negate the driving force for forward extraction are utilized for the successful back extraction of the extracted protein. Both surfactant systems are controlled by factors like type and concentration of the surfactant, co-surfactants used, shape and size of the micelle formed, charge on the protein, temperature, pH, presence of salts, and its type. The solubilization of protein into the RM is mediated by electrostatic, bioaffinity, and hydrophobic forces between the protein and surfactants. In addition to these forces, the steric and van der Waals interaction also plays a minor role in the formation of RM. The water core provides a more favorable environment for proteins than the organic phase surroundings (Chia et al., 2019).

Further, advanced partitioning systems involving affinity ligands are developed. At appropriate concentrations, these ligands bind specifically to the target protein, thus increasing the purification yield. Ligands are either freely added (de Gouveia and Kilikian 2000;Teotia et al. 2001) or coupled to one of the phase forming components by chemical modification(Birkenmeier et al. 1991; Rosa et al. 2007). Apart from substrates(Teotia et al. 2004) and inhibitors (Andrews et al. 1990), certain metal ions (Wuenschell et al. 1990;Jiang et al. 2015) and dyes (Rathnasamy and Kumaresan 2014; Malpiedi et al. 2015) are used as ligands too. Microwave (Dang et al. 2014) and Ultrasound (Qin et al. 2017; Dordevic and Antov 2017) assisted techniques are some of the new developments in extraction processes. Biosurfactants are also increasingly used in surfactant-based extraction processes. These natural surfactants offer the advantage of being mild and are required in very few concentrations in comparison to synthetic surfactants (Xu et al. 2011). Biosurfactants are also mild, unlike synthetic surfactants. They form mild interaction and thus do not denature the proteins (Madsen et al. 2015). The characteristics of the biosurfactants make it a very attractive replacement to synthetic surfactants.

1.5 Organisation of thesis

Chapter 1: Introduction

This section presents an overall view of the thesis via a generic introduction to the topic. The section focuses on Food spoilage and the effect of synthetic antimicrobial usage. Biopreservation using Lactoperoxidase is discussed as a suitable solution to microbial food spoilage with its various uses. A gist of the available purification methods for proteins from various sources is discussed. A brief overview of the Liquid-Liquid extraction methods highlighting the importance of reverse micellar extraction is provided in this section.

Chapter 2: Literature Survey

This chapter explains in detail the literature available on the sources, characteristics, and applications of LP. Also, different purification strategies employed to separate LP from whey are discussed, along with their drawbacks. The method employed in the present research work, i.e., Reverse micellar extraction, is discussed in this section with emphasis on different process and system parameters that affect a successful extraction process. Further, new research developments in the RME are highlighted. Based on the literature review, research gaps have been identified, and the scope of the present work is detailed.

Chapter 3: Materials and Methods

This chapter gives insight into the different materials used throughout the research work. All the methods followed during the experiments are detailed, along with the instruments used.

Chapter 4: Results and Discussion

The observations and interpretations from the experiments performed based on the objectives are discussed in detail in this section. The chapter is divided into four parts:

Part I: Screening of the compatible extraction process for Lactoperoxidase

Part II: Reverse micellar extraction of Lactoperoxidase from aqueous Lactoperoxidase solution

PartIII: Mixed surfactant-based Reverse micelle extraction of Bovine Lactoperoxidase from whey

Part IV: Biosurfactant based reverse micelle extraction of Lactoperoxidase

Chapter 5: Summary and Conclusion

Significant findings and major conclusions drawn from the present work are summarised in this section. Also, a short note on the prospective of future work is presented.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Lactoperoxidase

Lactoperoxidase (LP) is a natural preservative. The enzyme with its reaction products forms the natural defense system in mammals. Since the reaction products are safe for humans, there is ongoing research on theirusage in various fields. In recent year's manufacturers have focused on the application of this as a preservative in the food, pharma, and cosmetic Industry products**.**

2.1.1 Sources of LP

Lactoperoxidase belongs to a family of mammalian peroxidase that includes myeloperoxidases (MPO), eosinophil peroxidases (EPO)(Bafort et al. 2014). LP is a well-characterized component of secretions from mammary and salivary glands in mammals. It is regarded as an important constituent of innate immunity. LP activity in human airway secretions is about 0.65±0.09 µg/mg secreted protein and is active against *Pseudomonas aeruginosa, Burkholderiacepacia,*and*Haemophilus influenza* (Wijkstrom-Frei et al. 2003). Human saliva also has significant amounts of antimicrobial and immunomodulatory proteins that include mucins, histatins, agglutinin, lysozyme, lactoferrin, lactoperoxidase, myeloperoxidase etc. Major peroxidase activity is contributed by leukocyte released myeloperoxidase (3.6 micrograms/mL) which is present in twice the amount of Lactoperoxidase (1.9 micrograms/mL). LP is present in human milk throughout the lactation period and is likely to contribute to the protective effects oninfants. Human milk contains about 0.77 ± 0.38 mg/L and activity is highest in colostrums(Shin et al. 2001).Bovine milk is richer in peroxidase activity and contains 1.2 to 19.4 units per ml compared to human milk with 0.06 to 0.97 units (Wolfson and Sumner 1993). Amongst all the sources of LP, Guinea pig milk is the richest source of LP with 20 fold higher than human milk at 22 units /ml (Stephens et al. 1979).

Whey, by-product of cheese manufacturing, is a good source of LP and other minor proteins. Whey proteins are a mixture of globular proteins that includes beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin (BSA), and glycomacropeptide. The actual concentration of these whey proteins depends on the whey type (acid or rennet),milk source, feed type, lactation period, processing quality(Madureira et al. 2007). The value of the global whey protein market was 9.7 billion USD in 2018 and is expected to reach 15.4 billion USD in 2024 (Shahbandeh, 2018). Most processes such as ultrafiltration and industrial chromatography are capable of producing whey protein concentrates but are not able to isolate and fractionate the specific whey proteins.

Table 2.1: Whey Proteins and their properties (Guiziou 2010; Wang and Gu, 2016;Ramos et al. 2017)

Protein	**App. Conc(w/wt%)**	**Mol. Wt.(kDa)**	**Isoelectric Point**	**Number of amino acids residues**
beta-lactoglobulin	0.3	18.4	5.35-5.49	162
alpha-lactalbumin,	0.07	14.2	4.2-4.5	123
Immunoglobulins	0.06	150-900	5.5-8.3	-
Bovine serum albumin	0.03	69	5.13	582
Lactoferrin	0.003	78	7.8-8.0	700
Lactoperoxidase	0.002	78	9.2-9.9	612

2.1.2 Bovine Lactoperoxidase and its characteristics

Lactoperoxidase EC 1.11.1.7 belongs to the peroxidases of class oxidoreductase. It is a calcium containing glycoprotein with 8-10% carbohydrate. Bovine LP consists of 612 aminoacids and heme in a single polypeptide chain with approximately 78 kDa (Rombauts, Schroeder and Morrison, 1967) molecular weight.

It is basic in nature with an isoelectric point between 9.2-9.9. The active site, heme is linked to polypeptide chain via a disulphide bond (Kussendrager and van Hooijdonk, 2000). Maximum optical absorbance is at 412nm and its theoretical purity ratio A412/A280 is 0.95. The enzyme is highly heat stable under pasteurization conditions and found to be inactive at temperatures higher than 78˚C(Wolfson and Sumner 1993). Bovine LP is relatively stable against proteolytic enzymes but insensitive to light in presence of riboflavin. Being surface-active it adheres to surfaces of glass, enamel (Seifu, Buys and Donkin, 2005). It is resistant to acidic pH in-vitro and even to human gastric juice(Reiter and Härnulv, 1984). The substrate channel is hydrophobic, narrow, long, and less exposed to the surrounding environment (Sheikh et al. 2009).Calcium ion imparts stability to the enzyme (Wit and Hooydonk, 1996). Cationic surfactants (for example benzalkonium chloride) are known to stabilize and increase the activity of bovine lactoperoxidase(Marcozzi, Domenico and Spreti, 1998). The enzyme is amenable for both electrostatic and hydrophobic interaction. LP loses its activity in whey in less than 2 weeks. The best storage conditions for the whey to retain LP activity include dialysis and freeze-drying. Dialyzed and freeze-dried(stored at -20ºC) samples retained activity upto 4 weeks(Al-Baarri, Ogawa and Hayakawa, 2011).

The unstable short-lived products of thiocyanate oxidation mediated by lactoperoxidase impart the antimicrobial effect. The components of the LPS are ubiquitous in milk. The presence of thiocyanate anion, SCN^-, in cow's milk varies depending on feeding strategies and external addition may be required to activate the LPS. Hydrogen peroxide is produced under aerobic conditions by many bacterial species. For preservation purposes exogenously sodium percarbonate and magnesium peroxide areadded. Optimum concentrations of about 8 and 12ppm are generally advised for H_2O_2 and SCN- respectively (Junejaet al.2012). However such concentrations are also known to affect the clotting of milk, inactivate the other enzymes and denature the proteins (Yingling 2016).The other hydrogen peroxide producing systems like glucose-glucoseoxidase, hypoxanthine-xanthine oxidase, beta-galactosidase are also used (Juneja et al. 2012, Garci et al. 1995). In the absence of microbes, the products of these enzymes like gluconic acid are not toxic.

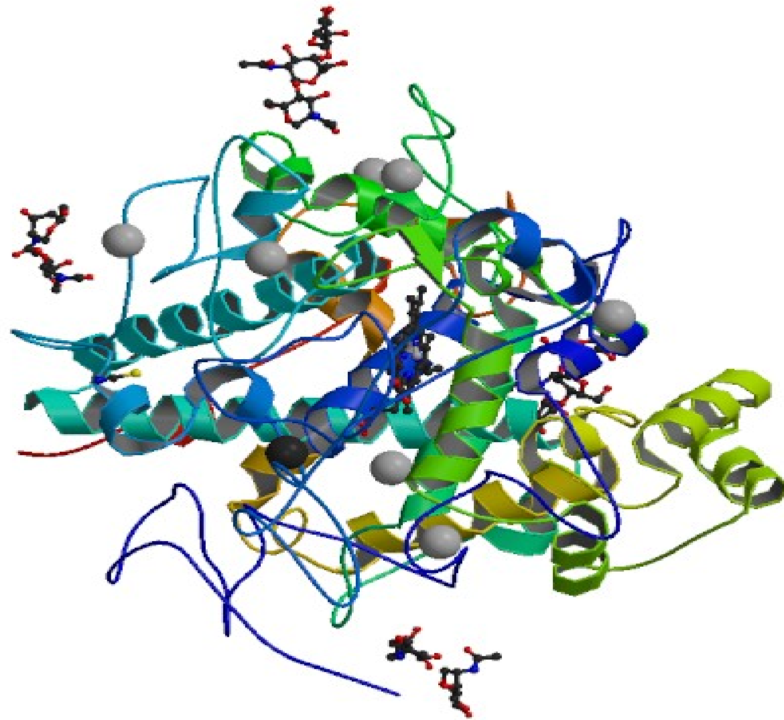


Figure 2.1: Crystal Structure of Lactoperoxidase(Singh et al. 2010)PDB number-3GC1

2.1.3 Application of Lactoperoxidase

The antimicrobial activity of Lactoperoxidase is an advantage in the food, pharma, and cosmetic industry. Since LP itself does not have antimicrobial activity all the components of the LPS should be used for effective antimicrobial activity. The LP system used in food and pharma preparations usually consists of pure LP, Sodium or Potassium thiocyanate, and the aqueous solution of hydrogen peroxide or sodium percarbonate (Jasour et al., 2015). Sometimes Glucose oxidase and Glucose are used instead of hydrogen peroxide or sodium carbonate to make the preparation more chemical free. In pharmaceutical applications, LP is effectively being used in dental care products and ophthalmic solutions. Orally administered tablets containing Lactoferin & Lactoperoxidase were effective on periodontal conditions (Kobayashi et al., 2011). In the cosmetic industry, it is used as a natural alternative to preservation. Many cosmetic related US patents propose the usage of Lactoperoxidase. Many

topical skincare products use LP/Glucose oxidase preparations. The main use includesthe preventionof acne-type symptoms, use as antimicrobial handwashes, and also to prevent the cosmetic product itself from spoilage(Ahsan, 2019). Recently LP was tested for its anti-cancer properties and synergistically with Lactoferrin it proved to be were cytotoxic against Caco-2, HepG-2, MCF-7, and PC-3 cells. The cell death was reasoned to be due to apoptosisand the added enzymes did not harm the normal cells (Abu-Serie and El-Fakharany, 2017).

Studies on assessment of the antimicrobial activity of Bovine Lactoperoxidase have been performed on both microbial cultures and directly on food products. The main advantage of LPS is in extending the shelf-life of raw milk and many microbial challenge tests have been performed. This is particularly applicable in tropical countries to extend the shelf life of milk during transportation from the collection center to the processing center where there is a lack of cold storage facilities. LP also proved the best preservative when added externally to milk. LP activity was tested on raw milk at refrigeration temperatures against *Listeria monocytogenes*with the external addition of sodium thiocyanate and hydrogen peroxide(Gaya et al. 1991). LP was active up to 7 days and proved bactericidal. Milk samples were challenged with *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Streptococcus thermophilus* and pasteurized at 72°C and 80°C (Marks et al. 2001). The activity of LP decreased, had almost no effect in milk samples pasteurized at 80°C. LPS in ovine, bovine, and caprine milks were activated by the addition of thiocyanate ions and hydrogen peroxide, and storage studies were performed at 4 °C, 22°C,and 30 °C and found that LPS was effective against all temperatures(Haddadin et al. 1996). LP in combination with reuterin was bactericidal against *Salmonella enterica, Campylobacter jejuni, Aeromonashydrophila Yersinia enterocolitica* and *E. coli* at 4 and 8°C(Arques et al. 2008a,b).

LPS are used alone or in conjunction with other antimicrobials like nisin, lysozyme, reuterin etc. LP and Nisin synergistically reduced *Listeria* counts in skimmed milk by three log units (Zapico et al., 1998). Nisin alone proved bactericidal with regrowth of *Listeria* but combined with LP,the bactericidal effect lasted upto 15days(Boussouel et al., 2000). *In vitro* studies on the susceptibility of *Candida* to LP

weretested with two substrates iodine and thiocyanate (Ahariz and Courtois, 2010). The iodine peroxidase system inhibits the biofilm and authors claim its refined medium can be used as an antifungal. The combined effect of lactoferrin and lactoperoxidase on the viability of *Streptococcus mutans*was accessed and they were found to be very effective at low pH (Soukka et al. 1991). High hydrostatic pressure treatment combined with LP or activated lactoferrin were tested against *Listeria monocytogenes, Salmonella enteritidis,* and *Escherichia coli* on cured beef (Bravo, de Alba and Medina, 2014).Combining treatments of LP with high hydrostatic pressure reduced the intensity of pressurization. Inactivation of *E. coli* and *Listeria* in milk was studied using combined high hydrostatic pressure and Lactoperoxidase system. LP alone induced bacteriostatic effect and the synergistic effect of LP with high hydrostatic pressure proved better(Garcia-Graells et al. 2000).

Coatings of LP with whey (Shokri, Ehsani and Jasour, 2014; Yıldız and Yangılar, 2016; Shokri and Ehsani, 2017) chitosan (Cissé et al., 2015), sodium alginate (Barkhori Mehni et al., 2019) have been tested on different food like Mango and fish products. LPS activities against a variety of both gram-positive and gram-negative microbes were tested on beef cubes. It was observed that LPS did not inhibit the growth of native lactic acid bacteria in beef but prevented the growth of *Listeria* and *Salmonella* species(Elliot et al., 2004). Lactoperoxidasehas been immobilized in nanoparticles(Sheikh et al., 2018),developed into edible coatings (Yener, Korel and Yemenicioğlu, 2009; Molayi, Ehsani and Yousefi, 2018). These coatings are tested on fish and beef products. Edible whey protein isolate films with Lactoferrin & Lactoperoxidase inhibited *Penicillium commune*, a fungus that affects meat and cheese(Min and Krochta 2005).The combined effect of apolactoferrin(iron-free) and lactoperoxidase can be used in oral care products with a pronounced decrease in hypothiocyanite (reaction product). LPS activity tested in combination with high-pressure homogenization resulted (Vannini et al., 2004; Tribst, Franchi and Cristianini, 2008) in conformational changes on LP leading to improved activity.

Table 2.2: Commercial products containing LP

Product name	Other ingredients	Product	Manufacturer
Biovert™	Glucose and Glucose oxidase	Personal care	Lonza
ZYMOX® Shampoo	Vitamin E, Lysozyme, Lactoferrin, Vitamin A, Vitamin B5,	Pet care	PKB
Zendium	amyloglucosidase, glucose oxidase	Oral care	Scandinavian Researcher
Biotene	Glucose oxidase, Lysozyme	Oral care-toothpaste,gel, chewinggum,mouth rinse	Laclede
Bioxtra	Lactoferrin, Colostrum whey extract,	Oral care	Codibel
Sebomine SB12™	Lactoferrin , Glucose oxidase	Personal care	Croda
Galatea™	Lactoferrin, vitamin C&E	Cosmetic – anti ageing	Galactic
Total Skin Rejuvenation Cream	Glucose, Glucose oxidase	Cosmetic	Lectro life technologies
Flaminal® Forte	Glucose oxidase	Personal care-wound care	Flenpharma

2.1.4 Lactoperoxidase purification methods

Lactoperoxidase (LP) has been isolated from various milk sources including human milk. After the first attempt on isolation (Theorell et al.1943) using

ammonium sulphate precipitation from bovine milk, many researchers tried chromatographic purification. LP was isolated using carboxylic acid resin as an adsorbent in both batch and column mode(Morrison et al. 1957). Amberlite CG 50 H^+ resin, CM sephadex C-50 resin were used in ion-exchange chromatography and Sephadex G-100 in gel filtration chromatography. Every chromatography step followed salt precipitation and dialysis. The research group followed the same protocol for Buffalo milk as well.

Sulphanilamide, an inhibitor of lactoperoxidase, was used in Affinity chromatography coupled to Sepharose to purify Lactoperoxidase in a single step(Atasever et al., 2013). The method gave 409-fold purification with 62.3% yield. Further, improvised usage of sulphanilamide through its derivatives was taken up as inhibitor ligand for affinity chromatography.5-amino-2-methylbenzenesulfonamide and 2-chloro-4-sulfamoylaniline were used as ligands for purification of LP from bovine, buffalo, cow, and goat milks. The derivatives gave better purification factors than sulphanilamidealone (Koksal et al. 2017). Similarly, the affinity of triazene dyes was tested towards LP by immobilizing them on Sepharose6B matrix. The work (Urtasun et al., 2017) showed good adsorption capabilities of triazene dyes which was later used as an affinity ligand for lactoperoxidase purification in batch and packed-bed columns.

Many researchers have explored the identical properties of LP and LF for their simultaneous purification. Cation exchange membrane Sartobind™ was used for isolation of Lactoferrin and Lactoperoxidase from raw cheese whey with 0.3&0.9 M NaCl as elution buffer(Doultani, Turhan and Etzel, 2004). The authors concluded that the commercially available membrane modules were useful in rapid fractionation with continuous usage. Sartobind S nano was used for fractionating Lactoperoxidase from acid whey with other proteins(Voswinkel and Kulozik, 2011). 0.35M sodium chloride at pH 4.8 was used to elute LP. Carboxymethyl-Toyopearl-cation exchange chromatography was also employed for simultaneous isolation of LF and LP(Yoshida, 1991).Similarly, continuous simulated moving bed column chromatographic process (Andersson and Mattiasson, 2006). Elution was performed using sodium phosphate buffer of pH 6.5 with 0.42M for LP and 1.25M for LF respectively. To prevent

significant losses of minor proteins during pre-processing, capture of LF and LP fromraw untreated milk using chromatography column packed with SP Sepharose Big Beads™ of volume 10ml was demonstrated(Fee and Chand, 2006). More than 100 column volumes could be processed and the results indicated the feasibility of processing 500L raw milk in 5L column volume on an average yield of 275g of LF and 28g of LP.

Large-scale purification of LP involves integrated protocols and fractionation techniques. Commercial-scale processes include selective precipitation, membrane filtration, selective adsorption, and selective elution. The protocol involves simultaneous concentration and fractionation of the desired protein. Lactoperoxidase and Lactoferrin aremarketed as specialty proteins and are usually fractionated along with other major whey proteins. Sepragen Corporation, USA has developed a patented process called Sepralacfor whey protein isolation. The process consists of lowering the pH of whey giving all proteins a positive net charge. This aid in their binding Sepra Prep-S resin column and washed with patented ‘Opposing Salt and pH’ solutions. The fractions are concentrated in ultra diafiltration units and 25-27% solids were obtained. The concentrates are then spray dried. Though the purity is not considered 250,000 liters of whey per day could be processed by the system. Technology licensees include major dairy producers from New Zealand and Ireland. Upfront Chromatography, Denmark developed Rhobust® Whey Refinery-Large-scale chromatography platform. The technology combines Expanded Bed Adsorption chromatography with membrane filtration resulting in high purity isolates. The technology is applied in a Dairy farmers’ company in Australia processing 200000 L of cheese whey. Novasep, France provides a similar integrated process with Applexion® ion exchange technology for selective elution of Lactoperoxidase and other whey proteins followed by nanofiltration. The technology is based on ion exclusion rather than ion exchange. Advances in membrane technology led the industries to explore new membranes. Glanbia Nutritionals, use combined proprietary microfiltration/ ultrafiltration to obtain whey protein isolates with more calcium and less sodium(Smithers and Augustin, 2013). Armor proteins, France is involved in a commercial sale of LP under the brand name Vitalarmor™ LP

Table 2.3: Lactoperoxidase purification methods available in the literature

Source used	Method adopted	Process conditions	Final purity/ Concentration	Reference
Camel milk	carboxy methyl-celluose exchange resin	Low salt molarity	----	Elagamy et al. 1996
Bovine milk	Simulated moving bed	Elution in phosphate buffer, pH 6.5, 0.42M NaCl	>90% purity,6.6 fold	Andersson and Mattiasson 2006
Pooled milk from Holstein cows	Carboxymethyl-Toyopearl	0.10 to 0.15M NaCl	41 mg from 1 L milk	Yoshida 1991
Bovine milk	Sulphanilamide-cyanogen bromide activated-Sepharose 4B matrix	1M NaCl / 25 mM Na_2HPO_4 (pH 6.8)	409-fold/62.3% yield	Atasever et al. 2013
Bovine milk whey	Reverse micellar extraction		86.60% and 3.25-fold, 127.35% and 3.39	Nandini and Rastogi 2010
Bovine sweet whey	Cation exchange membrane technology	sodium chloride gradients ((0 .1 M; 0.2 M NaCl)	85%	Plate et al. 2006
Raw bovine milk	SP Sepharose Big	0-0.4 M	48.6 mg/mL of	Fee and

	Beadscation exchange	NaClstep elution	resin	Chand 2006
Homogenised,skim & whole milk	cryogel monoliths column-cationic polyacrylamide	0-0.4 M NaCl step elution	-----	Billakanti and Fee 2009
Bovine whey	Dye(reactive red)-Sepharose 6B chromatograp hy	20 mM acetate buffer, pH 5.0	Yield6.5 %, purification factor 46.1	Urtasun et al. 2017
Cheddar cheese whey	immobilized sulfonic acid moieties in Membrane chromtography	0.3M NaCl in Sodium phosphate buffer (0.01M, pH 6.5)	8-fold ,73%	Clovis et al. 1997

2.2Modified Liquid-Liquid extraction of Proteins

In contrast to the new trends in upstream /fermentation processes, the tools/processesused in downstream processing have not significantly changed over the decades. Downstream processing (DSP) is the major bottleneck in the bioprocess industry. In this regard, traditional extraction methods are modified with different solvents and experimented (D'Souza et al. 2013) to improve the purification of biomolecules.

2.2.1 Aqueous two phase extraction

Aqueous two phase extraction is a liquid–liquid extraction technique researched both at a large scale (Kepka et al. 2003; Selber et al. 2004) and nano scale- miniaturization (Negrete et al. 2007; Soares et al. 2016) for a variety of biomolecule purification and

the scalability of this method is advantageous in Bioprocess industry. Moreover, they are amenable to continuous operations(Espitia-Saloma et al. 2014).Water being the main component in both phases, provides agentle environment for sensitive biomolecules. It provides single-step simultaneous concentration and purification of the target molecule. Poor understanding of the mechanism behind the partitioning of the target molecule is the only reluctance for its widespread commercial use. ATPS can revolutionize the bioprocess industry, provided the system is studied and selected carefully(Iqbal et al. 2016). ATPS is formed from components that are hydrophilic yetincompatible with each other. PEG with different molar mass is mostly used as one of the components with advantages of low cost and ability to form two phases with other neutral polymers and salts(Hatti-Kaul 2001).

Amongst the ATP systems, two polymer systems, single polymer(smart polymer) systems(using Thermoseparating polymers), polymer/ salt system, alcohol/salt are commonly used. Albertsson specifies six different possibilities-Size, charge, hydrophobicity, affinity, and conformation(Albertsson 1995) to describe partitioning of proteins in ATPS and this stays true for both proteins and phase components.The phase behavior is affected by the presence of salts, type of polymer, and concentration. Polymer/ Polymer systems are more viscous and can sometimes result in problems for phase separation. Moreover, the two polymer systems are quiteexpensive. Polymer/ salt systems are less expensive can form stable two phases. Salts change the hydrophobic interactions between protein molecules and change their partition behavior. The disadvantages of polymer systems with respect to viscosity, high ionic strength, and complexity of recovery of the target molecule from phase forming components (often accomplished by ion-exchange chromatography, diafiltration, crystallization, or ultrafiltration), recycling issues led to experimentation with other phase forming components like alcohol, surfactant, and ionic liquids. In alcohol/salt ATPS both sating out and hydrophobic interaction work to separate the target molecule(Yau et al. 2015). Target biomolecules can be easily extracted from the top alcohol phase by evaporation(Guan et al. 1996). Some of the alcohols used are mild ethanol and propanol. Though economical & less toxic to the environment, they are highly volatile and can denature labile proteins.Ionic liquids are salts

withproperties likenon-flammability, low viscosity, high solubility,thermal stability, and at temperatures below 100˚C they exist as liquids(Oppermann et al. 2011). Their physical properties of polarity and hydrophobicity can be tuned based on the combinations of cations and anions(Pei et al. 2009).Imidazolium, phosphonium, guanidinium-based ionic liquids are popular choices. Ionic liquids are found to stabilize the protein by maintaining three-dimensional structures even at high temperatures(Dreyer and Kragl 2008). It can solubilize large amounts of proteins and well aid in crystallization by retaining activity in comparison to a pure aqueous solution(Patel et al. 2014).

Table 2.4:ATPS employed for the separation of various biomolecules

Protein/ Enzyme	**Source**	**Phase components**	**Purity/ yield**	**Reference**
Polymer/ Polymer systems				
Monoclonal antibody	Chinese hamster ovary cell culture	PEG/hydroxypropyl starch	Purity 97.6,yield 86.8	Wu et al. 2014
IgG	Mouse hybridoma cells	PEG/dextran	84 ± 6.5%	Silva et al. 2014
L-glutaminase production	*Bacillus cereus* MTCC 1305	PEG 4000(8.5%)/ dextran 1500(9.5%)	----	Singh and Banik 2012
Polymer/ salt systems				
xylanase	recombinant	6% PEG 6000/ 20% phosphate	6.7/78%	Rahimpour et al. 2016
bovine lactoferrin	commercial	14%PEG/10%sodiu m citrate		Costa et al. 2015
Thermoseparating polymer systems				
Lysozyme	Hen Egg	EOPO/potassium phosphate	85 % yield / 16.9 PF	Dembczyński et al. 2013
Bromelain	pineapple peel	EOPOEO 2000-2300/ potassium phosphate	6.53/68.6%	Han et al. 2017
Laccase	*Peniophoracinerea*	UCON/ potassium	Upto 81 ± 5	Moreira et

		phosphate		al. 2013
Alcohol/salt systems				
IFN-α2b	Escherichia coli	2-propanol18% /22% ammonium sulfate	16.24/74.64%.	Lin et al. 2013
Amylase	Mango	19% ethanol/25% Sodium phosphate	13.3/88.4%	Amid and Manap 2014
Ionic Liquid base systems				
Lipase A	*Candida antarctica*	1-ethyl-3-methylimidazolium butyl sulphate/ ammonium sulfate	99%	Deive et al. 2012
Horseradish Peroxidase	Commercial	alkylimidazolium chloride/Dipotassium phosphate	80%	Cao et al. 2008

2.2.2 Surfactant-based two phase extraction

Similar to polymer-based extraction systems, surfactant-based Aqueous two phase extraction fall into two categories: Cloudpoint extraction and Coacervate extraction. In both these variants, a surfactant rich and surfactant poor phase is formed. However, the factors responsible for the phase separation in both cases are different. Cloudpoint extraction relies on temperature and is a characteristic feature of nonionic and zwitterionic surfactants(Yazdi 2011). These surfactants in aqueous solutions above their critical micelle concentration can solubilize hydrophobic compounds. Upon increase in temperature,the aqueous solutions of these non-ionic surfactants start to form acloudy phase or turbidity and above this cloudpoint temperature, the aqueous solution separates into micelle/surfactant rich and poor phases. However, the addition of salts and other additives can lead to better and faster clouding and phase separation. This can collectively reduce the cloudpoint temperature and make it useful in the extraction of heat-labile proteins and enzymes. On the other hand, coacervate extraction can be performed using both cationic and anionic surfactants.Here, unlike cloudpoint extraction, the temperature is not used for phase separation.Dehydratingfactors like higher concentrations of salts, hydrophobic counter ions, pH changes induce the phase separation in aqueous solutions of ionic

surfactant systems.The micelle/surfactant-rich phase is usually termed the coacervate phase. The mixture of cationic and anionic surfactants gives better separation and extraction efficiencyof proteins. The mixture of surfactants also leads to the usage of lower concentrations of surfactants. Moreover,the native structure of the protein is maintained in mixed surfactant systems as strong interactions with a single surfactant are reduced(Lu et al. 2005, 2007).

Reverse micellar extraction (RME), a surfactant-based liquid–liquid extraction process, has gained a lot of attention recently. Protein such as lactoferrin (Pawar et al., 2019), antibiotics such as amoxicillin and erythromycin (Chuo et al., 2014), and enzymes such as L-asparaginase (Jayachandran et al., 2019) have been purified using reverse micellar systems. RME is a nontedious and economically feasible two-step process that is amenable for continuous operations (Wan et al., 2016) and can be applied to crude protein feedstocks with minimal pretreatment. Recentresearch shows that RME provides a higher yield and purity than many other primary recovery bioseparation processes (Hu et al. 2017). RME has been successfully used for the extraction of whey proteins such as α–lactalbumin (Naoe et al., 2004), β-lactoglobulin (Lee and Dungan, 1998), and lactoferrin (Pawar et al., 2019) without any pretreatment.

2.2.3 Nonconventional purification methods for LP

Non-conventional extraction techniques like aqueous wo phase extraction (Nandini and Rastogi 2011; Kandasamy et al. 2014) and surfactant-based reverse micelle extraction (Nandini and Rastogi 2010) were researched for the separation of LP from various sources. The anionic surfactant-AOT and cationic surfactant - CTAB based methods were studied. A slightly modified method of RME isreverse micelle-assisted extraction.. This single-step process involves the extraction of proteins by adjusting the pH of whey such that all contaminating proteins are extracted into the reverse micelles leaving onlyLP in the initial feed or aqueous phase(Nandini and Rastogi 2010). This was unlike the standard RME procedure where usually the target protein is solubilized into the reverse micelles and extracted. The procedure was achieved by using CTAB reverse micelles which could solubilize all the major whey proteins at

pH 6. The method resulted in 127. 35% LP activity recovery and 3.39 fold LP purification.

ATPS using classic polymer/ salt and the ionic liquid was attempted and a comparison was drawn (Kandasamy et al. 2014). It was observed that the ionic liquid system gave better yield and purity of 94% and 35.6 respectively when compared to the polymer/ salt system at 54% yield and 2.72 fold purification. Similarly, intermittent ultrasound-assisted ultrafiltration can recover LP that partitions to the bottom phase in ATPS. This improves permeate flux without hampering the LP activity or recovery (Nandini and Rastogi 2011). Integration of salt precipitation with strong cation exchange chromatography for LP extraction (Li et al. 2019) results in improved LP recovery. The non-conventional method of concentrating the proteinusing salts can remove all non-proteinaceous contaminants. This prevents back pressure problems in the succeeding chromatography procedure.

2.3 Reverse micellar systems for protein Extraction

Relatively new surfactant-based liquid-liquid extraction, namely Reverse Micellar Extraction (RME) is gaining a lot of attention in the recent past. Reverse micelles (RM) are the nanosized ternary system of water, surfactant, and organic solvent. In the presence of the water, the hydrophilic head group of the surfactant arranges themselves around water forming a water core. This is ideal for selective solubilization of hydrophilic proteins. The rapid Brownian motion of the RM in the system facilitates the exchange of solutes between RM or between RM and the bulk aqueous phase (Pileni, 2006).

In the phase transfer method of protein solubilization, the organic phase consisting of the surfactant is mixed with the aqueous protein solution, and the target protein is solubilized into the water core of the RM that is formed in the organic phase. The amount of water solubilized in a water core during the forward extraction depends on the surfactant type, organic solvent, and ionic strength of the aqueous protein solution. The solubilization of protein into the RM is mediated by electrostatic, bioaffinity, and hydrophobic forces between the protein and surfactants. In addition to these forces, steric and van der Waals interaction also plays a minor role

in the formation of RM. The water core provides a more preferable environment for proteins than the organic phase surroundings (Chia et al., 2019). The solubilized proteins are back-extracted from the RM by careful isolation of the RM (organic) phase and contacting it with a fresh aqueous phase. Here, conditions that negate the driving force for forward extraction are utilized for the successful back extraction of the extracted protein. The change of pH and increase of ionic strength in the fresh aqueous phase reduces the electrostatic interactions between protein and surfactant during the back extraction of the solute. The exchange of proteins between bulk aqueous/organic phases in these two steps occurs mainly at the liquid–liquid interface. The protein diffuses from the bulk aqueous phase to the interface of RM formed during the forward extraction. The RM from the bulk organic phase fuse with the surfactant monolayers at the liquid–liquid interface and proteins are released after fusion occurs due to the reduced driving forces during back extraction (Krishna et al., 2002).

Extraction of the target biomolecule is mediated by various forces in reverse micellar extraction. The major dominating force is the electrostatic interactions between the ionic head groups on the surfactants and the charged amino acids on the target proteins. Depending on the dominant charges on the amino acids of the proteins oppositely charged ionic surfactants can be chosen for the extraction. Similarly, hydrophobic interaction between the surfactant and the organic bulk phase determines to some extent the size and curvature of the reverse micelles(Kadam, 1986). Hydrophobic interactions between the surfactant and the non-polar amino acids also aid the transfer of protein from the bulk aqueous phase to the interior of the reverse micelles(Hebbar and Raghavarao, 2007). Apart from these steric and Van der Waals interactions also play minor roles in maintaining the stability and integrity of reverse micelles structure (Carvalho and Cabral, 2000).The water content of the reverse micellar organic phase defined by (W_0 = [H_2O]/[surfactant]) depends on the solubility of surfactant in an aqueous or non-aqueous solvent, also governs the solubility of the protein. The charge on the surfactant head group and its interaction with protein at various pH affects the protein's solubilization (Goklen and Hatton, 1987).

Table 2.5:RME systems employed for the separation of few biomolecules

Protein/ Enzyme	Source	Phase components	Purity/ yield	Reference
Lipase	*Pseudomonas*	AOT - isooctane	80% recovery and 2.5-fold purity	Gaiakwari et al. 2012
Lectin	Black turtle bean	AOT - isooctane	63.21 mg protein/g bean meal yield	He et al. 2015
Bromelain	Pineapple peel	gemini surfactants	160 % recovery with 2.7 fold purity	Guo et al. 2018
Lipase	*Aspergillus niger*	CTAB/isooctane/hexanol	82.72% recovery with 4.09 fold purity	Nandini and Rastogi et al. 2009
Plasmid DNA	pharmaceutical grade	TOMAC-isooctane	--	Streitner et al. 2007
BSA	Pure	CTAB/alkyl halides/hexanol	84% recovery	Zhang et al. 2002
Ovalbumin	pure	CTAB/hexanol	14–25% recovery	Ding et al. 2015

2.3.1Components of reverse micelle system

2.3.1.1 Surfactants

The surfactants are amphiphilic molecules containing hydrophobic tail and hydrophilic head groups.They form the phase boundary between the organic and aqueous phases. Above a minimum concentration called critical micelle concentration(CMC), they form structure micelles in water or reverse micelles in organic solvent(as shown in Fig 2.2). Surfactants can be either chemically derived- Synthetic surfactants or biologically derived- Biosurfactants. Further, depending on the charge of the headgroups they are classified as anionic (negatively charged), cationic(positively charged), neutral and zwitterionic(dual charged) (Anarbaev et al., 2009). The cationic surfactants require a co-surfactant (alcohol) to form reverse micelles or to increase the water uptake into the reverse micelle (Melo et al.

2001).Ionic surfactants mainly take part in electrostatic interactions whereas non-ionic surfactants involve hydrophobic interactions with the target protein. Sometimes a cosolvent like alcohol or buffers/salts reduces the ionic interaction of surfactant head groups & enables their close packing(Krishna et al., 2002). The cosolvents arrange themselves at the interface of the ionic surfactants and ions form salts from ionic interactions with the surfactants headgroups. Both of these phenomena result in reduced repulsions between ionic head groups of surfactants and results in closer packing of reverse micelles.

Of the many synthetic surfactants, AOT (Sodium bis(2-ethylhexyl) sulfosuccinate) and CTAB(cetyltrimethylammonium bromide) are well studied for protein solubilization and used for extraction purposes. AOT in Isooctane is known to solubilize maximum water, thus capable of solubilizing more protein. But, these come with limitations. AOT can cause serious denaturation of the solubilized proteins and some of these are not reversible (Steinmann 1986; Ternstrom et al. 2005; Kaur and Mahajan, 2014; Goto et al. 1995). However, these limitations can be overcome by the addition of non-ionic surfactants and/ or using surfactants similar to AOT but with less denaturing characteristics. Sodium bis(2-Ethylhexyl) phosphate (NaDEHP) is an anionic that is similar to AOT, withthe same hydrocarbon tail, but different polar headgroup (Hu and Gulari, 1996; Li et al. 1998; Li et al. 2001). The effect of different surfactants and theirconcentration during the reverse micelle extraction of proteins is discussed in section2.3.2.1

2.3.1.2Organic solvent

The lack of suitable solvent that can result in stable reverse micelles, that is not harmful to the labile proteins and that can solubilize maximum water into RM to house the extracted protein, has limited the extensive implementation of RME for protein recovery (Goklen and Hatton, 1985). Solvent type and its structure affectthe size, shape, structure, and water content of the subsequent RM formed (Li et al. 1998; Yu and Neuman 1994). More polar the solvent is, it penetrates deeply into the surfactant monolayer and thus into the water of the RM creating less room for protein solubilization(Mat and Stuckey, 1994). Hence, non-polar solvents particularly

aliphatics, aromatic, and cyclo-aliphatic hydrocarbons are chosen for RM formation. Some surfactants need an additional solvent, called the co-solvent that enhances surfactant dissolution in the organic solvent (Mat and Stuckey, 1993). The popular solvents chosen for extraction purposes are n-octane, isooctane, hexane,cyclohexane, and heptane. Halogenated alkanes such as chloroform are also used. Many researchers over years have used most or all of these solvents and demonstrated that the best solvent that can be used is isooctane as RMs formed solubilizes more water(Dekker et al., 1986; Chang and Chen, 1995).

2.3.1.3Water pool

The water inside the RM is termed as water pool. It has been observed that the characteristics of the water in the water pool are completely different than that of the bulk phase water (Melo et al. 2001). Even the pH of the water inside the RM is different than the bulk water. Most surfactants are known to causing a buffering effect on the water pool creating a different pH for the solubilized water (Marques et al. 2014). The water pool in the RM is measured as water content (W_o) and calculated as the ratio of the concentration of water to the surfactant in the organic phase. At lesser water content, the water molecules in the water pool are strongly bound to the surfactant headgroups and are immobile. Proteins solubilized in these RMs are usually denatured(Larssson and Pileni 1993). As the water content increases the mobility of the solubilized water increases. However, this mobility is still less than that of the bulk phase water(Riter et al. 1998a; Riter et al. 1998b). Since RMsare dynamic in nature, the extraction of proteins from the bulk aqueous phase into the RM and vise versa is achieved via the transfer through these water pools. And within the organic phase, RMs can exchange the contents of the water pool by fusion-fission mechanism which can result in bigger RMs with larger water pools or smaller RMs with lesser water pools (Melo et al. 2001).

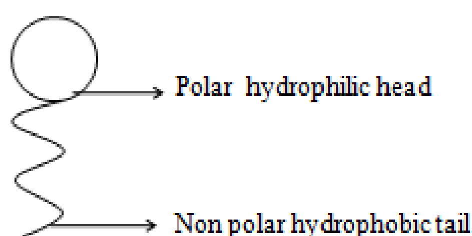
2.3.1.4Aqueous phase

The aqueous phase carries the target protein to be extracted along with the other contaminating proteins. The aqueous phase in RME can be diverse from direct crude fermentation broth to pre-processed or partially processed mixture of proteins. One of

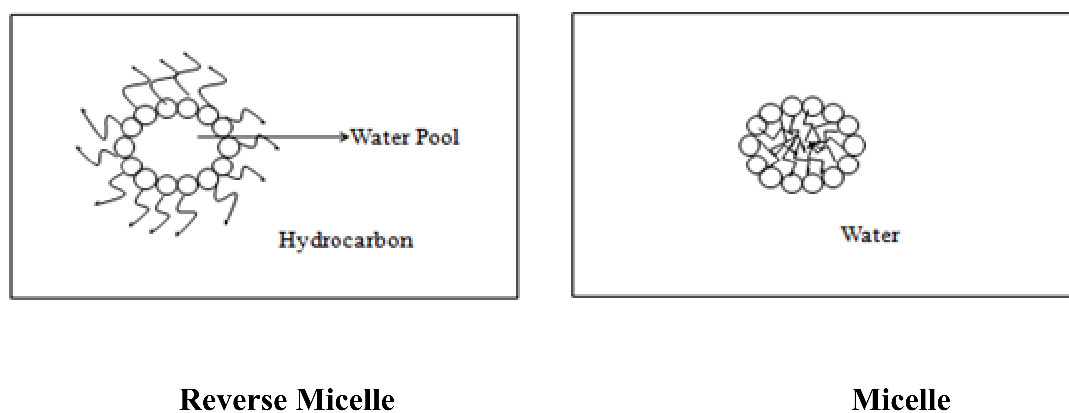
the determining factors in a successful RME process is the pH and ionic strength of the aqueous phase which forms a deciding factor for the choice of the surfactant to be selected. It is best to consider the nativity of the target protein while setting the pH of the aqueous phase.

2.3.1.5 Protein uptake and location inside the reverse micelles

The uptake of protein from the aqueous phase into the reverse micelles occurs via the interface between the aqueous and organic phase as represented in Fig 2.4 with reference to a mixed surfactant RM extraction. The target protein is solubilized in the RM adopting one of the ways represented in Fig 2.3.



Surfactant - amphiphilic nature



Reverse Micelle **Micelle**

Figure 2.2: Representation of Surfactant, Micelles and Reverse micelles

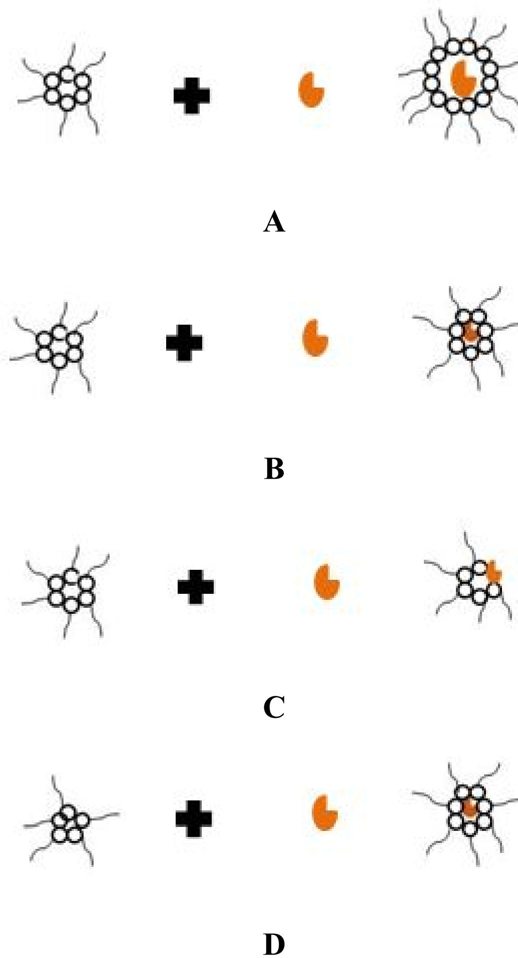


Figure 2.3: Hypothetical structures of reverse micelles with solubilized protein

(Carvalho and Cabral, 2000; Pileni et al. 1985)

Sometimes a hydration layer is formed around the target protein but the amount of water inside the micelle would be the even after solubilization of the protein (A). Also, water could be expulsed from the RM after protein uptake and the RM rearranges itself to fit the solubilized protein (B). Hydrophobic proteins may prefer localization at the interface of the water pool(C),thus the water pool volume remains constant. Solubilized protein can also induce an increase in the water pool volume by increasing the size of the RM to accommodate protein and its hydrous layer (D). Most commonly the last phenomenon is observed in the phase transfer method of protein extraction.

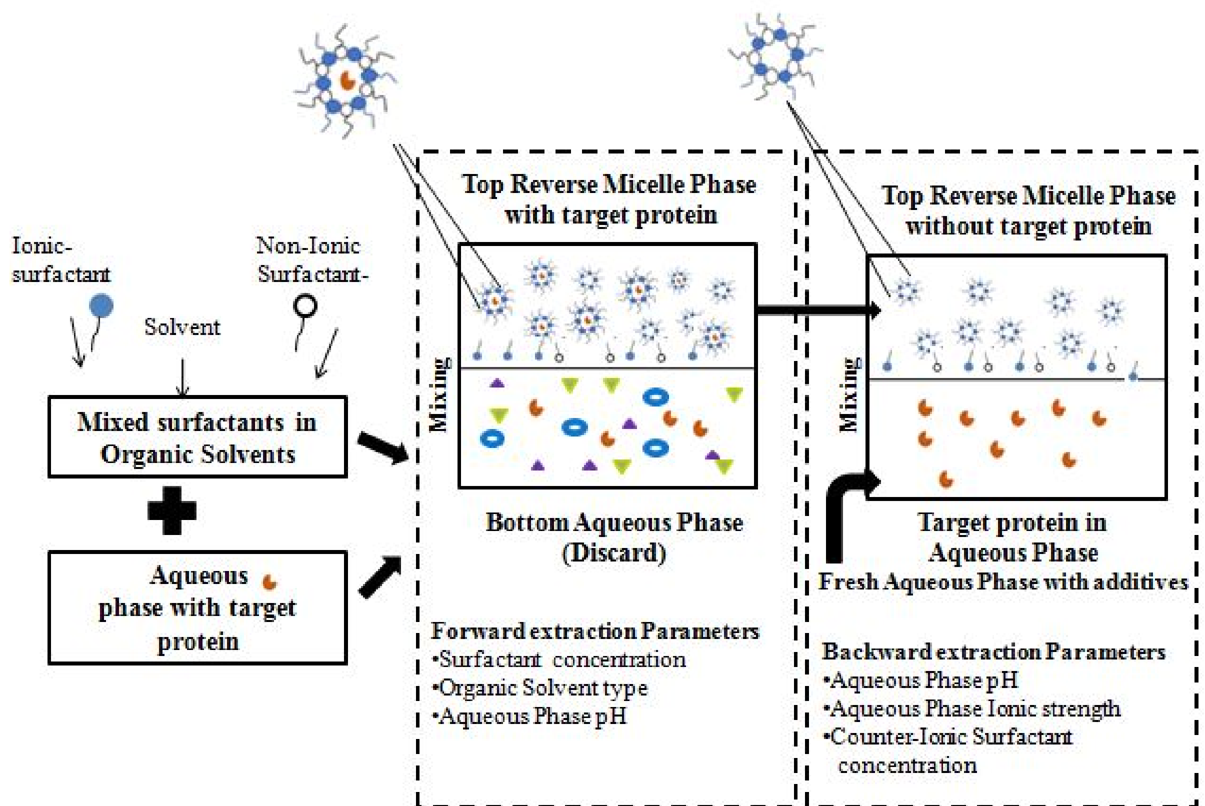


Figure 2.4: Schematic representation of Reverse micelle extraction

2.3.2 Variables for protein extraction usingreverse micelle systems

The number of intrinsic and extrinsic parameters affects the extraction of a solute molecule into reverse micelles. Ionic nature and concentration of surfactant, aqueous phase pH, protein load and its nature, Ionic strength induced by the added electrolytes, micelle size, and water content are some of the important factors. The extrinsic factors like the concentration of salts, alcohols added as co-surfactants are interrelated and sometimes affect the intrinsic parameters too. Other minor factors include temperature, cosurfactant, Phase volume ratio, nature of contaminating proteins, contact time.

2.3.2.1 Effect of system and process parameters on forward extraction of Proteins

Effect of aqueous phase pH

The surface charge of the protein is decided by the aqueous phase pH.At pH above isoelectric point, the amino acids are predominantly negatively charged, and below it is positively charged. This facilitates their interaction with the ionic groups on the surfactant headgroups. The charge density of the amino acids on the protein determines the extent of the transfer of protein (Andrews and Haywood. 1994). It also forms the basis for the selection of ionic surfactants. Sincethe major driving force for forward extraction is electrostatic interactions between the surfactant headgroups and amino acids on the target protein. Cationic surfactants interact with negatively charged proteins (Jayachandran et al. 2019) and solubilize them within the RM while positively charged proteins are solubilized by the anionic surfactants (Murugesan et al. 2017). It is also observed that the maximum forward extraction occurs at aqueous pH sufficiently away from pI where maximum numbers of amino acids exhibit the required charge (Chaurasiya et al. 2015).Some studies have reported higher extraction efficiency in an anionic system with anionic proteins. This is attributed to the hydrophobic interactions dominating the electrostatic interactions. Proteins that contain glutamate amino acids in the side chains possess higher hydrophobicity at the pH above the pI value (Zhao et al. 2010).The addition of non-ionic surfactants also shifts the pH range for extraction of target proteins (Dekker et al., 1989). Apart from the aqueous phase pH, some surfactants like AOT have been shown to induce a buffering effect on the water core of the RM. The aqueous solution of AOT has an initial pH of 6.5 to 8; while pK_a values are in the range of 3−4. Irrespective of the initial aqueous phase pH, the AOT offers a buffering effect to the water core of the RM and the pH of the water core remains different than the bulk aqueous phase (Marques et al. 2014). This can affect the conformation of the solubilized proteins.

Effect of aqueous phase ionic strength

Reverse micelles are formed when the aqueous phase consists of small amounts of salts. This minimum ionic strength is required for the formation of reverse micelles or water in oil emulsions. This minimum concentration reduces the repulsion between

surfactant headgroups bringing about a stable reverse micelle formation. The ionic strength also decides the solubilization of the surfactant in the organic or aqueous phase. Thus,protein solubilization and extraction follow a bell-shaped curve over the range of increasing ionic strength. Type of ions- either cationic or anionic and size of ions plays important role in water/protein uptake. The salt types that are “structure forming” are more useful than “structure breaking” types (Hancer, Celik and Miller, 2001). K+ is named the water structure breaking ion and Na+ is named the water structure forming ion.Salts also decide the concentration of co-surfactant alcohols. Higher salt concentration with lower alcohols induces maximum water uptake and vise-versa(Mathew and Juang, 2007).Smaller ions like Na^+ induceless electrostatic screening effect than larger ions like K+ (Andrews and Haywood. 1994). Here, the atomic radii of the ion crucial role. Likewise, monovalent ions like Na^+, K+have a lesser screening effect than divalent ions(Pang et al.,2016). Cations greatly influences the extraction than anions and the order goes K^+<Rb^+ /<Cs^+/<Na^+ /<Li^+ in monovalent ions and Ba_2^+<Sr_2^+ <Ca_2^+ in divalent ones (Kinugasa et al. 2003).

Effect of surfactant type and concentration

Surfactants offer solubilization of target proteins in two ways; Ionic interaction between surfactant head groups and proteins and Exchange of proteins between reverse micelles and/ or simple encapsulation or engulfing of the target protein along with aqueous phase(Chaurasiya, Hebbar and Raghavarao, 2015).Ionic surfactants are commonly used as they offer the required electrostatic interaction. Anionic surfactants are used for the extraction of cationic proteins and vise versa. AOT/ Isooctane is the commonly studied anionic surfactant system as this system can solubilize more water and concurrently more protein. Similarly, CTAB is the commonly studied cationic surfactant system. The technique of solubilizing contaminating proteins into the reverse micelles leaving behind target proteins is also explored. This method utilizes ionic surfactants with charge groups similar to the target protein to minimize interaction between the target protein and ionic surfactant thereby reducing its extraction (Nandini and Rastogi, 2010). Non-ionic surfactants induce hydrophobic interaction between non-polar amino acids and surfactant tails leading to the internalization of proteins. Through zeta potential studies it was observed that most

non-ionic surfactants exhibit a negative charge at neutral pH (Vasudevan and Wiencek, 1996). This was further explored for extraction of other proteins by ionic interaction (Naoe et al., 1998). Non-ionic surfactants are also blended with ionic surfactants to reduce strong electrostatic interactions per reverse micelles. This is discussed in detail in section 2.6.

Similarly, zwitterionic surfactants are unique surfactants with the positive and negative head group. These are also easily biodegradable, biocompatible, exhibit improved water solubility and temperature stability (Mohamad-Aziz, Zularisam and Mimi Sakinah, 2019). However, their effectiveness in selectively extracting a target protein due to their dual ionic nature is still ambiguous. Both non-ionic and zwitterionic surfactants have been modified and made into single ionic headgroup systems using ionic dyes(Sun et al., 1999). These have been successfully used for the extraction of proteins(Liu, Dong and Sun, 2007). However, these are long procedures, and the leaching of these toxic triazene dyes over time into the aqueous phase is most commonly observed. This renders the aqueous phase unfit for further processing to harvest biomolecules.

Lately, biosurfactant-based reverse micelle systems are researched for the extraction of biomolecules. Biosurfactants are advantageous over chemical surfactants as they have lower CMC and also are readily biodegradable. Rhamnolipid, Sophorolipid(Chuo et al., 2018), Surfactin(Peng et al., 2018) are the studied biosurfactant systems. Since they have lower CMC in organic solvents the surfactant concentration required during extraction processes is about 10 times lesser than chemical surfactants(Peng et al., 2012). Surfactant concentrations are chosen much above the critical micelle concentration of the particular surfactant and solvent. An increase in surfactant concentration can result in two possible outcomes. It can lead to an increase in the size of reverse micelles(Wanget al., 2018)and/or an increase in the number of reverse micelles. This can help in solubilizing more amount of protein which is also reflected in the increase in water content. Simultaneously, increased surfactant concentration during forward transfer can reduce the backward extraction efficiency. Small proteins get easily extracted using low surfactant concentration (Kilikian et al., 2000).

Effect of organic solvents

The nature of oil or the organic solvent has shown important roles in the size and shape of reverse micelles formed. It is known that large molecular volume organic solvent penetrates less into the surfactant monolayer. The chain length of the alkanes also determines the number of reverse micellar aggregates that are formed in the organic phase. As the length of the alkane increases, it becomes more difficult for solvent molecules to take up the space in the interfacial film resulting in closely packed AOT molecules. This increases the aggregation number which also increases the size of the micelles(Lang, Jada and Malliaris, 1988). As this micellar size increases the micellar collisions increases due to the increase in surfactant tail overlap/interactions. Similarly, reverse micelles' shape also changes upon changing the chain length in alkanes. Micellar shapes range from spherical to rod-like to cylindrical. In a less penetrable solvent or alkanes, cylindrical micelles are formed (Shioi, Harada and Tanabe, 1993).

Generally, alcohols from medium to large chain are used as co-surfactants or co-solvents in the reverse micellar extraction process.Alcohols serve two purposes during reverse micellar extraction. Small chain alcohols act as co-surfactants and are arranged in between AOT molecules in the reverse micelles. This aids in stable reverse micelle formation. Some cationic surfactants cannot form reverse micelles without the aid of co-surfactant alcohols. The alcohol chainlength affects the orientation of surfactants in the organic phase. Generally, small to medium-chain alcohols are used in back extraction processes and medium to large chain alcohols are used for reverse micelle formation.C_2-C_6 alcohols get arranged at the interface thereby improving water/protein solubilization and C_6-C_{10} are less arranged at the interface and are known to destabilize the reverse micelles with increasing concentration.

Effect of time

Though electrostatic interactions are quick to take place, sometimes due to the presence of impurities the extraction efficiency may increase with time(Gaikaiwari, Wagh and Kulkarni, 2012). In other cases when protein is sensitive to the strong electrostatic interaction between surfactant headgroups and protein longer extraction

time can lead to denaturation. In such cases, a very short extraction time of about 5-10 minutes ismore beneficial(Liu et al., 2004). Generally, forward extraction is quicker and the back extraction process is a rate-limiting step in the complete extraction process. Back extraction can take anywhere between 15-60minutes to achieve about 90% recovery in proteins (Chen et al., 2014; Chuo et al., 2014).

Effect of temperature

Temperature can play an important role in the extraction process of RM. But, it has garnered little attention by the researchers. Temperature plays an important role in surfactant solubilization/dissolution in either of the phases i.e. in forming different emulsion systems. AS temperature increases the water content of the RM also decreases. This can affect the amount of target protein being solubilized (Dekker et al. 1991).They also would probably be less effective while extraction is considered. This is because both little and extreme temperature changes can adversely affect the properties ofthe target protein.

Effect of added affinity ligands

To improve the selectivity in the RM extraction process and achieve better extraction efficiencies various affinity ligands are employed. Affinity ligands have been successfully used in other protein purification protocols like chromatography. In the RME context, they can be added freely into the aqueous phase protein mixture (Kumar et al. 2011) or can be tagged along with the surfactants in the organic phase (Liu et al. 2006). These affinity ligands are selected based onthe properties that are specific to the target protein. For example, if the target protein is glucose or mannose containing glycoprotein, they can be selectively extracted from non-glycosylated protein using affinity ligand- lectin concanavalin A (Con A). Con A has been used for selective extraction of proteins like horseradish peroxidase (Paradkar and Dordick, 1991), Bromelain (Kumar et al. 2011). Other affinity ligands include octyl β-D-glucopyranoside (Woll et al., 1989), avidin (Coughlinand Baclaski, 1990). Triazene dyes have also been reported to form non-specific strong interactions with various proteins and this is exploited in RME as well.Cibacron Blue 3GA, has been used in few RME processes both in free form (Zhang et al. 1999) or coupled to the surfactants

(Liu 2006). When the coupling is considered, usually non-ionic surfactants are a choice. However, the triazene dyes used are not environmentally friendly and their usage showed be considered with caution. Also, protein denaturation possibilities in the presence of a higher concentration of dye-ligands should not be ruled out (Puri and Roskoki 1994).

2.3.2.2 Effect of system and process parameters on backward extraction of Proteins

Effect of aqueous phase pH

Aqueous phase pH is also important during the backward extraction. Generally, a pH opposite to that used in forward extraction is selected to reduce the electrostatic interaction between the amino acids on the protein and surfactant headgroups (Andrews and Haywood. 1994). Usually, for negatively charged proteins solubilized in cationic surfactant RM, pH below pI is chosen wherein the majority of the amino acids on the solubilized proteins become positively charged and loses their interaction with the cationic surfactant. Similarly, for the positively charged proteins solubilized in anionic surfactant RM, pH above pI is chosen. Sometimes hydrophobic interactions may be the dominating force during the forward extraction and release of proteins from the RM can be difficult. The addition of salts to the aqueous phase can enhance the recovery process (Andrews and Haywood. 1994).

Effect of aqueous phase ionic strength

Similar to aqueous phase pH, the aqueous phase ionic strength during back extraction also follows conditions opposite to that in forward extraction. As the ionic strength of the aqueous phase is increased, the water uptake capacity in the RM decreases dramatically creating a “salting out” effect. The increasing salt concentration shields the surfactant head groups with counterions. This causes surfactant molecules to be attracted towards each other thus reducing the water content in them. Higher salt concentration induces more screening reducing the repulsion between the surfactant headgroups (Andrews, Pyle and Asenjo, 1994). Larger ions like K^+ induce a more electrostatic screening effect than smaller ions like Na^+ (Andrews and Haywood. 1994). Likewise, divalent ions have more screening effects than monovalent

ions(Pang et al.,2016). Similar to the forward extraction trend, increasing salt concentration in the aqueous phase usuallyfollows a bell-shaped curve for activity recoveries of enzymes. Higher salt concentrations could damage or denature the enzymes leading to a loss in activity (Kim,2014).

Effect of alcohols

Long-chain alcohols from C6-C10 are less arranged at the interface and are known to destabilize the reverse micelles with increasing concentration. Thus hexanol and octanol improve the back extraction and butanol and propanol reduces the back extraction (Lee et al., 2004). Alcohols with a higher alkyl chain improve back extraction with increasing concentration. Also, the hydrophobic hydrocarbon groups on alcohols suppress the micellar-micellar interaction(percolation behavior) proportionally to their chain number, while a hydrophilic hydroxyl group enhances it.Sometimes they drive the surfactant to an aqueous phase wherein the protein is still attached to the surfactants, thus resulting in back extraction of protein to the fresh aqueous phase(Mathew and Juang, 2007). However, additions of alcohol can sometimes result in loss of enzyme activity. The alcohol type and concentration play acrucial role in retaining the activity of the recovered enzyme. Higher concentrations of long-chain alcohols are detrimental to enzyme activity. Usually, 15%v/v can lead to the recovery of 100% protein(Carlson and Nagarajan, 1992). It is also observed that alcohols can be used stand-alone protein recovery method in RME without salts(Lee et al., 2004). Recovery phase pH has to be carefully considered when alcohols are used, as here again extreme pH combined with alcohols can result in activity losses(Mathew and Juang, 2007).

Effect of temperature

Temperature effects during back extraction of the extracted protein are more pronounced than during the forward extraction process. As mention earlier, rising the temperature of the system during the extraction process can result in smaller RMs with lesser water content, this can significantly improve the back extraction efficiency (Paradkar and Dordick 1993; Yu et al. 2003). However, extreme temperatures where

the target protein loses its properties irreversibly should be avoided. This depends on the property of the temperature stability of the protein.

2.3.2.3.Unique strategies for backward extraction of protein

Back extraction of the solubilized protein from reverse micelle is the final and most important step in reverse micellar extraction. Many back-extraction strategies are tried over the years. They aim at reducing the protein surfactant interaction and/or breaking up the micelles. As a thumb rule pH and ionic strength opposite to the one that induces protein solubilization in forward extraction areused for back extraction. Higher alkanols (pentanol, hexanol, octanol) are found to suppress the formation of reverse micelle clusters whereas lower alkanols (propanol & butanol) are efficient cluster enhancers (Mathew and Juang. 2007). Apart from these other unique protein back extraction strategies are explored. Using counterionic surfactant, DTAB and TOMAC (Jarudilokkul, Poppenborg and Stuckey, 1999), cytochrome c, ribonuclease A& lysozyme were back-extracted from the AOT-isooctane system. Electrostatic interaction between surfactant molecules that are oppositely charged leads to the collapse of the reverse micelles. This aids in releasing the solubilized protein into the aqueous phase. The addition of counter-ionic surfactants during the recovery process enables usage of mild pH and low to nil salts in the recovery aqueous phase. Similar back extraction of papain was employed from AOT/ isooctane in using counter ionic surfactant TOMAC and DTAB (Mathew and Juang. 2005). The two surfactants can form a complex and precipitate at the interface of the two phase system. The hydrophobic complex of AOT and TOMAC (1:1 ratio) formed in the organic phase could be efficiently recovered by adsorption using montmorillonite and reuse(Jarudilokkul, Poppenborg and Stuckey, 1999). Silica gel was used to dewater the organic phase after forward extraction. The organic phase RMs is stripped of the water content in them and the solubilized target protein is retained on the surface. These silica gels are simply washed with an alkaline solution to strip them off the attached protein (Leser et al. 1993).Destabilization of the RMs in the organic phase after forward extraction was also achieved by using mechanical shear force imparted by glass beads. This method eliminates the second step(back extraction process) in RME (Dhaneshwar et al. 2014). Organic solvent from the organic phase after forward

extractioncan bevaporized while the remaining content redissolved in KCl to recover the extracted target protein.This method has been shown to significantly improve back extraction efficiency from 57% to 80% (Sun et al. 2009)

2.3.3 Mixed surfactantreverse micelle systems

Though electrostatic interactions are the key force of RME process for the selective extraction, occasionally strong electrostatic interactions between the protein and surfactant head groups denature and/or inactivate the biomolecules. This has been observed in proteins like BSA, lysozyme, hemoglobin (Goto et al. 1995; Kaur and Mahajan 2014), and enzymes like cutinase and LP (Ternström 2005; Marcozzi et al. 1998).To mitigate such effects, the non-ionic surfactants are used in conjunction with ionic surfactants to reduce the strong electrostatic bonds (Shioi et al. 1997) and modify the interfacial forces of reverse micelles. The non-ionic surfactant mixed with the ionic surfactants reduces the surface charge density in each reverse micelle, thus reducing the strong interactions(Peng et al. 2016).It can minimize the concentration of ionic surfactants for the successful formation of reverse micelles and thereby extraction of target protein (Ma et al. 2015). The addition of nonionic surfactants improves the solubilization of proteins as they modify the physio-chemical properties of the reverse micelles(Das et al. 2013). The addition of nonionic surfactants significantly alters the extraction efficiency due to their larger head group area than AOT. The synergistic effect of two different types of surfactants also improves the water solubilization behaviour (Das et al. 2013). This, in turn, can affect protein uptake, stabilize enzyme activity and reduce denaturation (Hossain et al.1999). Such instances of improved extraction and reduced denaturation of proteins have been reported for IgG1 (Daliya and Stuckey, 2010) and β-glucosidase (Hemavathi et al., 2010). A similar effect was also observed for the RME of the antibiotic amoxicillin (Chuo et al., 2014). Non-ionic surfactantscan act as cosurfactant arranging themselves at the interface of AOT micelles. The changes induced by the non-ionic surfactant in the micelle aggregates can help protein to solubilize at extreme pH ranges(Wolbertet al., 1989). Such instances have been reported in IgG1 (Daliya et al. 2010), β-

glucosidase (Hemavathi et al. 2010), horseradish peroxidase (Shome et al. 2007) extraction using reverse micelles.

Table 2.6: Mixed surfactant based reverse micelle extraction of proteins

Target Protein	**RM system**	**Optimized conditions**	**Extraction Efficiency**	**Reference**
Amoxicillin	AOT/Tween85(total surfactant concentration 102.57 g/L)	F.E. pH 1.90, KCl-8.54 g/L. B.E. pH 6.58, KCl-11.02 g/L,	>90% efficiency	Chuo et al. 2014
α-Chymotrypsin	AOT-DOLPA (dioleyl phosphoric acid)(4:1 ratio and total surfactant at 10 Mm)	F.E. isobutyl alcohol 10% , pH 6.8 B.E. pH 8.1	97.1	Goto et al. 1998
*Chromobacter iumviscosum*Lipase, α-Chymotrypsin	AOT/Tween85, AOT/Span 60	Only F.E. experiments were performed pH 6-6.5, 0.5 M NaCl	--	Yamada et al. 1994
β-glucosidase	AOT/Tween series, 4.5:1.0 ratio	F.EpH 8.0, 0.15M KCl , B.E.pH 9.5,300µl hexanol	95.18%	Hemavathi et al. 2010
Rhamnolipid/ Tween series (mixed RM)	Rhamnolipid/ Tween series (mixed RM)	F.E-pH7 B.E.pH8,ethanol 4%	90% activity recovered	Peng et al. 2016
Bovine serum	CTAB/alkyl	F.E-pH9.10,0.1	>84%activity	Zhang et al.

albumin	halides(50mM CTAB/4% halide/20% hexanol(v/v)	M KCl, B.E. pH 4.3, 1M KCl	recovered	2002
Erythromycin	AOT/lauryl sulfobetaine, 60.0 g/L AOT & 0.1 fraction of lauryl sulfobetaine	F.E. pH 5, B.E.-pH8, 30g/L NaCl,5% Isopopanaol	94.2% B.E. efficiency	Norazimah et al. 2019
Bovine serum albumin	CTAB/ TBP (CTAB - 50 mM)/TBP 5.0% hexanol/ 15% hexane v/v)	F.E. pH 4.57,0.1 M KBr B.E. pH 4.5, 0.1 M KBr, 24.1% hexanol	about 82% B.E efficiency	Zhang etal. 1999
MAb (IgG1)	AOT/Tween-85(100 mM AOT/10Mm Tween)	F.E. pH-5.2; NaCl-0.15 M, B.E 0.3 MKCl; pH -11.5	75 and 80%	George and Stuckey, 2010

2.3.4 Biosurfactant based reverse micelle extraction

Biosurfactants(BS) are amphiphilic surface-active agents produced by natural sources. The sources include microorganisms, animals, and plants. The microbial sources include bacteria, yeast, and fungi that tend to synthesize biosurfactants using very cheap substrates like agricultural residues, sugars, oils, dairy waste. The BS produced from these varied sources and organisms differ widely in their characteristics. For example, the BS produced by a single species of microbe on one substrate may vary from that produced in another substrate (Ron and Rosenberg 2002; Nitschke and Costa 2007; Xu et al. 2011). Lately, there is increased attention tothese naturally derived compounds as they offer advantages over synthetic surfactants. Their production is very diverse i.e. the characteristics of the produced BS depend on varied factors like organism, substrate source,growth conditions, etc. They can be produced from industrial wastes and byproducts, they are environmentally friendly, biodegradable, they show very low toxicity, and exhibit excellent foaming

propertiesand high selectivity. Theyare active at extremes of pH, temperature, and salinity (Velioglu and Urek et al. 2016). In the protein purification context, it is observed that biosurfactants do not denature the proteins unlike anionic synthetic surfactants (Madsen et al. 2015). Also, the antimicrobial-film and antimicrobial properties exhibited by these BS can be very advantageous in the food Industry (Sharma 2016). Owing to theirnatural production biosurfactants come in various shapes and forms and unlike synthetic surfactants, they do not have neat and separated hydrophilic and hydrophobic groups. Biosurfactants exhibit mosaic nature with complex structures. The four classes of biosurfactant include Glycolipids, lipopeptides, Saponins, and the rest all polymeric compounds in class 4. Amongst these Glycolipids are commonly used and as the name suggests they include a sugar moiety in the structure. The two major sugar groups are Rhamnose and Sophorose and accordingly, the biosurfactants are named Rhamnolipid (RL) and Sophorolipid(SL). Rhamnolipids are produced by *Pseudomonas aeruginosa* and Sophorolipids by yeast *Candida* species (Otzen 2017).

Rhamnolipids are produced in monorhamnolipids and dirhamnolipidforms(Sinumvayo and Ishimwe 2015).Monorhamnolipid includes RhaC10C10, RhaC10C12-H2, RhaC10C12, and dirhamnolipids include Rha2C10C10, Rha2C10C12-H2, and Rha2C10C12 (Mendes et al. 2015). The microbially produced RLs are usually mixtures of these and the dominant form differences in the mixture composition can influence the physico-chemical properties of RL.The rhamnosyl and one or two β-hydroxy fatty acids constitute the hydrophilic and hydrophobic moieties of RL molecules, respectively. The rhamnosyl group endows hydrophilic nature to RLs while carboxylic moiety performs the functional control of the amphipathic properties (non-ionic to anionic) of RL depending on the sub-phase conditions such as the presence of electrolytes and pH. The carboxyl groups dissociate to form carboxylate groups in the absence of electrolytes (Helvac et al. 2004).The dissociation constant (pKa) is around 4.3 for the monomer and about 5.5 for the micelle andsurface-adsorbed state (Paler et al. 2006). RLis completely protonated at pH 5 making it non-ionic in nature and becomes anionic at pH 6.8 and above (Ikhwani et al. 2017).

Rhamnolipid is exempted from the requirement of a tolerance residue limit when used as a fungicide on all foods. After its successful applications in environmental remediation, more patents are being filed using the new generation of biosurfactants in the cosmetic sector (Rikalovic et al. 2015).Various studies demonstrate the benefits of RL as biologically active compounds in Biomedicine. The properties exhibited by RL include antimicrobial, anti-inflammatory,immunomodulatory, antiviral, anti-tumor-anticancer activity, and cellular differentiation agents(Banat et al. 2010). Due to their non-toxic and safe nature, they can be a good bet as they move from their nascent trial stage. RL has also been employed in oil recovery. They are as effective if not better than the synthetic surfactants in the oil industry for extraction, transportation, and storage (Silva et al. 2014).

BShas been used in reverse micelle formation for protein extraction and esterification studies.BSis advantageous over synthetic surfactants as the latter offers lower CMC. The HLB of RLwas reported as 22–24 and thus it is easily soluble in water than in organic solvents. It requires co-solvent(alcohols) for solubilization to form reverse micelles. Protein extraction and esterification studies with biosurfactants usually employ the lab-produced BS. Since BS characteristics can differ depending on the strain of producing organisms, the minimum concentration of BS required for RM formation also varied in each of these cases. Literature shows concentrations ranging from 0.055 mM (Peng et al. 2015) to 50 mM (Ramirez et al. 2017) used for RM formation from different forms and types of RL.The table below lists various literature that use biosurfactant reverse micelles.

2.3.5 Reuse of Phase components

Affordable and economical RME can be developed when the phase-forming components are reused to the maximum possible extent. This also helps to cap environmental concerns with respect to the synthetic substances used in the RME.The release of surfactants and solvents into the environment directly is not usually accepted. The possible alternative is to reuse the whole organic phase after back

extraction by mixing with fresh crude to start the second cycle of RME. This way both surfactants and organic solvent can be put to recycle and reuse for several cycles. But the accumulation of surfactants in the aqueous phase during the forward and backward extraction steps was observed. This mass transfer of surfactants into the aqueous phase will eventually result in a lower concentration of surfactants in the organic phase than that was initially present. This usually reduces the forward extraction efficiency. Lactoferrin extraction efficiency reducing from 97.5% in the first cycle to 53% after four cycles (Pawar et al. 2019), β-glucosidase recovery from 100% to 90% after one cycle during (Hemavathi et al. 2010), and laccase recovery from 92.7% to 70.8% after three cycles of using biosurfactant Rhamnolipid (Peng et al. 2012) were reported. The other option is to mix some volume percentage of the first organic phase with a fresh organic phase (Nandini and Rastogi 2010). This newly added fresh organic phase replenishes and makes up for the lost surfactants thereby keeping the extraction efficiency same as that observed in the first cycle.

2.4 Application of reverse micelles for the purification of whey proteins

Like any other waste, whey was also subjected to protein extraction using reverse micelles in many studies. Since whey is a source of multiple proteins each with different physical and chemical characteristicsextraction protocols generally attempted also variesfor each protein.Lactoferrin is one of the whey proteins that have properties similar to that of LP and competes in most purification processes. Properties that LP shares with other proteins include carbohydrate contents, cationic nature, basic isoelectric point, high molecular weight, iron contents.

The separation of LP from whey was investigated earlier using RM systems composed of single ionic surfactants. However, LP is not stable in acidic pH phases in the presence of anionic surfactants (Marcozzi et al., 1998; Okazakiet al., 2000). This result seems to contradict results from the previous studies, where LP extraction with aerosol-OT RM resulted in 86.60% recovery of activity at pH 6.0 (Nandini and Rastogi, 2010). It is also noteworthy that the previous studies have mentioned that most of the protein denaturation and enzyme activity loss occurred during forward extraction (Dekker et al., 1989). Such pH-dependent denaturation and inactivation of

protein and enzyme has been observed for other proteins and enzymes as well when ionic surfactants were used for RME (Dekker et al., 1989; Jones et al., 1982).

Table 2.7: Biosurfactant based Reverse micelles used for various purposes

Biosurfactant	Organic solvent and Co-solvent	Used for	Biosurfactant concentration	Recovery and Purification fold	Reference
Sophorolipid	Isooctane	Erythromycin and Amoxicillin extraction	0.6-0.8g/L and 0.2-0.4 g/L	--	Chuo et al. 2018
Rhamnolipid	Isooctane	laccase	3.3.mM	92.7% and 4.79 PF	Peng et al. 2012
Rhamnolipid	n-Hexanol/Isooctane(1:1 to 1:3)	Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) by Laccase	0.075 mM	<50 % degeradation	Peng et al. 2015
Rhamnolipid	isooctane/tert-butanol (1:1)	Enzymatic(Lipase) hydrolysis of waste frying oil	50 mM	>57% degree of hydrolysis	Ramírez et al. 2017
Rhamnolipid	isooctane/n-hexanol	Enzymatic reaction of ethanol and oleic acid by lipase and lignin peroxidase	60 CMC RL/ isooctane	Ester content 18/106g/hr	Shan et al. 2015
Rhamnolipid/ Tween series	isooctane/n-hexanol	Cellulase	Surfactant ratio 0.4	90% activity recovered	Peng et al. 2016

Table 2.8: Whey protein extraction using Reverse micelles

Whey protein	RM system & conditions	Source	Extraction efficiency	Reference
α- lactalbumin	AOT-isooctane at pH 6	Pure Protein	100% F.E. and 90% B.E.	Naoe et al., 2004
Immunoglobulin -G	50mMAOT-isooctaneat pH 6.5	Bovine Colostral Whey	>90% remained in aqueous phase	Su and Chiang, 2003
Lactoferrin	100Mm CDAB, pH9 sodium borate buffer with 50mM KCl	Whey protein Isolate	96% remained in aqueous phase	Noh, Rhee and Imm, 2005
α- lactalbumin& β lactoglobulin	100 MmAOT isooctane with 1mg/ml protein	Pure proteins	100% α- la in organic phase and 60% β la in aqueous phase	Kawakami and Dungan, 1996
Lactoferrin	50 mM CTAB in n-heptanol, pH10 , 1 M NaCl	Pure protein	100% F.E. & 98% B.E. n-butanol and n-propanol	Pawar, Iyyaswami and Belur, 2017
Lactoperoxidase	AOT & CTAB in Isooctane at pH6	Bovine milk Whey	86.6% and 127.35% in organic phase and aqueous phase	Nandini and Rastogi, 2010
Bovine serum Albumin	glucosylammonium (GA) and lactosylammonium	Pure protein	86 & 50% F. E. in GA and LA with	Chen, Dong and Guo, 2017

	(LA) surfactants with n-octane & 1-hexanol		100&70% B.E in LA	
α- lactalbumin& β lactoglobulin	100 mMAOT isooctane with 1mg/ml protein	Pure protein mixture(50: 50)	80% β Lg in aqueous phase and >85% α-La in organic phase at pH9	Lee and Dungan, 2006
Bovine serum Albumin	AOT&TX-100 in toluene and isooctane at pH5.5	Pure protein	100%F.E& B.E. at 0.75 &2M NaCl	Hebbar and Raghavarao, 2007
Bovine serum Albumin	AOT in presence of KCl, NaCl, $CaC1_2$ and $MgC1_2$ at pH5.5	Pure protein	100%F.E& B.E at pH 5.5 in KCL and 9 in NaCl	Shiomori et al., 1998
Bovine serum Albumin	AOT 160mM at pH 4 with 0.8M NaCl	Pure protein	95% back extraction with 1M NaCl at pH 7.5	Pawar, Regupathi and Prasanna, 2017

Note: F.E is Forward extraction, B.E is Backward extraction

2.5 Antimicrobial studies

Antimicrobial activity of the LP system i.e. LP along with its reaction substrates i.e. hydrogen peroxide and thiocyanate has been evaluated by many studies over the years. The effect of LPS directly (Dajanta et al. 2008) or encapsulated (Jacquot et al. 2000) has been explored in various systems like raw milk (Dajanta et al. 2008) and other dairy products (Goyal and Goyal, 2012), media (Kamau et al. 1990) and directly on the food product like lettuce (Telmoudi and Hassouna 2017).

Studies with milk were performed in two ways. One by challenging the milk with test organism (Faweja et al. 2008) and the other by treating the inherent milk microbes to

LPS (Garibay et al.1995). LPS is effective against both gram-positive and gram-negative bacteria. Gram-negative bacteria like *E.coli, Pseudomonas aeruginosa*and Gram-positive bacteria like *Staphylococcus aureus,Bacillus cereus, and Listeria monocytogenes*have been used to test the antimicrobial activity of LP (Faweja et al. 2008;Kamau et al. 1990; Ndambi et al. 2008). LP is found to be both bacteriostatic and bactericidal. It is bactericidal against gram-negative bacteria but is only bacteriostatic towards gram-positive bacteria (Kamau et al. 1990). Antimicrobial activity has been accessed over varied hours. Some studies end with 8hour trials and some have been extended upto 48hours.All studies involved intermittent sample collection to understand the growth trajectory of the test organism. Studies are usually employed at room temperatures or the optimum temperature for test organism growth. However, studies also include refrigeration temperatures for test organisms like *Listeria and S. aureus*(Kamau et al. 1990).

2.6 Summary and Research Gap

The food industry always looks for low-cost preservatives, which makes chemical preservatives an easy choice. However, the risk involved in their usage is very evident through many medical surveys. Though few antimicrobials are available from microbial sources its growth and related upstream is an additional step for industries. The available literature justifies the importance of the natural anti-microbial system, LP. Its inherent characteristics of heat stability, activity at low pH, broad-spectrum activity havebeen proven by its application in various industries. The added advantage of the harmless reaction products makes it an ideal antimicrobial agent in Food and Pharma sector.

Milk contains considerable amounts of LP. Though few researchers have concentrated on the direct isolation of LP from milk, it is not a good choice with the growing scarcity of food worldwide. Whey is a cheap source of LP from the dairy Industry. Most dairy industries employ complex,tedious, time-consuming, multi-step chromatography and membrane processes to purify whey proteins.These processes sometimes harm the native structure of protein leading to loss of its activity. The widely used chromatography requires large columns that consume a lot of energy, this can reflect in the cost of the end product. Even membrane processes are tedious with

the problems of fouling and membrane polarization. Moreover, most dairy industries do not isolate valuable whey proteins. Instead, whey is usually desalted and whey proteins are concentrated to be sold as whey protein isolates and concentrates in the market. Some dairy industries don't extract these proteins from waste and let them as effluent because of the complexity and cost of its purification process. Except for few dairy industries in Europe, US, and Australia not many industries employ extraction strategies for why proteins. SinceLP purification involves high costs, its use in food and pharma industry is limited.

Similarly, Liquid-Liquid extraction has been an attractive method for the extraction of many valuable enzymes and proteins. The availability of various phase forming components and with novel components being explored, makes it an ideal choice for preliminary extraction of proteins. As these systems can be modified in many ways to match the properties of protein and/or to retain their stability, they suit well the needs of the downstream processing industry.Though LLE has been used for the extraction of other major whey proteins (β-lactoglobulin and α-lactoalbumin), very few works have concentrated on LP extraction using LLE. Moreover, most of these studies involved using pure proteins which are not reflective of the conditions from whey. The idea in the present study is to address these research gaps in the purification of LP.

2.7 Scope of the Work and Objectives

Growing concerns over the use of synthetic antimicrobials and exhaustive use of antibiotics have prompted manufacturers to move towards natural products.The demand for natural antimicrobials is tremendous in the food, pharma, and cosmetic sector. Natural products either as a whole or in the purified form are effective against many pathogenic microbes. Most naturals antimicrobials like Lysozyme, Lactic acid bacteria-derived peptides, Lactoperoxidase are generally regarded as safe (GRAS). Of these, Lactoperoxidase is popular, Food and Drug Administration(FDA) approved,and proven to be an effective antimicrobial. They are already used in various commercial entities like personal care, pet care, oral care, and hygiene products (Table 2.2).

LP secreted by mucosal glands in mammals is an inherent antimicrobial system in milk, tears, and saliva. Industries are concentrating on exploring the chances of using this system against pathogenic microbes. Economically the isolation and purification of LP should be easy and from a cheap source. LP from milk is left out in whey along with other whey proteins during the cheese and paneer-making process. Whey, which was once considered waste, is now termed a byproductof the dairy industry. Whey is a cheap yet complex source of LP, with many important proteins like α-lactalbumin,β-lactoglobulin, lysozyme,lactoferrin, immunoglobulins. The complexity increases with large concentrations of different ions present in whey.These hinder the purification process and makethe purification of LP from whey quite challenging. LP is available in minute quantities (approx 1%) and remains as one of the minor proteins in whey. Hence, not much attention is given to its purification.Moreover, Lactoferrin, another important minor protein in whey has similar properties and competes with LP in all purification processes(Table 2.1). The other whey proteins are also commercially important and possess various good biological activities. Hence, selective isolation and purification of these whey proteins are very promising.

Most whey protein fractionation methods concentrateon using expensive,labour intensive, and tedious chromatography and membrane processes.Since most downstream processes involve harsh conditions, gentler processes that retain the activity with maximum purity should be developed. Reducing the downstream cost can in turn reduce the end product price. Thus, simple, inexpensive, effective purification strategies that focus on the selective extraction of Lactoperoxidase giving better yields with lesser loss have to be explored.

2.7.1Objectives

In this regard, it is proposed to extract and purify Lactoperoxidase from whey using non-conventional extraction processes. The following objectives are proposed to achieve higher purity of Lactoperoxidase by employing the Liquid-Liquid extraction,

1. To select the compatible phase forming components of the modified Liquid-Liquid extraction systems (Aqueous Two-phase systems and Reverse micellar system) and select the suitable extraction process for the partitioning of LP.

2. To study the effect of process and system variables for Reverse micellar extraction of LP from the aqueous LP solution using mixed surfactant systems including ionic, non-ionic, and bio-surfactants.
3. To implement the established extraction procedure and study the effect of variables for the selective extraction of LP from unpasteurized bovine whey.
4. To study the antimicrobial activity of the purified LP at different conditions

CHAPTER THREE

3. MATERIALS AND METHOD

3.1 Materials

Non-ionic surfactants used for the study i.e. Tween 20(Polyoxyethylenesorbitan monolaurate), Tween 60 (Polyethylene glycol sorbitan monooleate), Tween 80(Polyoxyethylenesorbitan monooleate), Span 60(Sorbitan monostearate), span 80(Sorbitane monooleate), Triton X-100(Polyethylene glycol tert-octylphenyl ether), Triton X-45 were procured from Sigma-Aldrich, USA.Ionic surfactants include AOT [sodium bis(2-ethyl hexyl) sulfosuccinate] and Aliquat 336 were obtained from LobaChemie, India. CTAB (cetyltrimethylammonium bromide),Bicinchoninic acid (BCA)solution, copper sulphate pentahydrate,and sodium hydroxide, Span 85, and Sodium dodecyl sulphate from Sigma Aldrich. Sodium bis(2-ethylhexyl)phosphoric acid (NaDEHP) was prepared by mixing equimolar quantities of sodium hydroxide and bis(2-ethylhexyl)phosphoric acid. The solution was left to evaporate in a vacuum evaporator at 60˚C till complete evaporation of water. The gummy anionic surfactant material thus obtained was left in a desiccator for further use(Li et al., 2001).Organic solvents Isooctane,Heptane, Hexane, Cyclohexane were obtained from Merck,India. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, Copper (II) Sulfate Pentahydrate and Bicinchoninic Acid solution werefrom Sigma Aldrich, USA. Bovine serum albumin was procured from Himedia, India. Sugars and polyols were obtained from Himedia, India.

Pure Lactoperoxidase obtained from MP Biochemicals, USA was used directly for all the experiments by dissolving it in 0.1M phosphate buffer at pH 6. The enzyme stock was stored at -20˚C until use. Lyophilized LP(≥35 units per mg dry weight)was also obtained from Abnova Corporation, Taiwan, and used without further purification for the experiments concerning biosurfactant-based extraction. Rhamnolipid- AX grade 80% Rhamnolipid in solid acid form (avg molecular weight:

600) was a kind gift from Natsurfact, USA. Buffers used in aqueous phase were citrate buffer (pH 5- pH 5.5), potassium phosphate buffer (pH 6- pH 8), and sodium carbonate (pH 9- pH10.5) buffer at 0.1 M concentration. Salts for the buffersand hydrogen peroxide (30% w/v) were obtained from Loba Chemie, India.Reagents for Karl Fischer titration were obtained from Honeywell Fluka.

Acid whey was prepared using undiluted fresh raw milk. Raw milk was adjusted to pH 4.6 using 2N HCl and the resulting curd was separated. This was further centrifuged at 13000rpm for 20 minutes at 4 ºC. The supernatant was filtered through Whatman filter paper grades 1 and 49. This was further syringe filtered using a 0.2micron filter. pH of freshly prepared whey was adjusted to the desired level and refrigerated at -20ºC until further use. The crude was desalted for the RME of LP with Rhamnolipid usingAmicon® Ultra-15 centrifugal filter unitsby spinning at 5000RCF for 40 minutes. The conductivity of the desalted whey was measured using Hanna edge® multiparameter meter with conductivity electrode.

3.2 Methods

3.2.1 Stability studies with different phase forming components

Aqueous solutions of different phase-forming components were tested for LP stability. Known quantity (phase forming compositions) was used to check the stability of LP in phosphate buffer. Three different polymers PEG, PVP, PPG at 15%w/w were tested.The salts Phosphate, sulphate, and citratesalts of Sodium and Potassiumat 15%w/w were also tested. Non-ionic surfactants TritonX-45, Triton X-100, Triton X-114, Tween 20, Tween 60, Tween 80, Tergitol MN6, Tergitol 15s7, Brij 35, Span 60, Span 80, Span 85 were considered for the study. Anionic surfactants AOT, SDS, Rhamnolipid at pH 6 and 8 were also used. Non-ionic surfactants were tested at 2% concentrations whereas ionic surfactants were tested at 50mM concentrations. Small chain alcohols viz. ethanol, 1-propanol, 2-propanol, butanol, and hexanol were tested at 10%w/w concentration. Carbohydrates viz. glucose, arabinose, sucrose, mannosewere tested at 10%w/w concentration, and sugar alcohols or polyols viz. sorbitol and xylitol were tested at both 10%w/w and 30%w/w

concentration for their stability towards LP.25µg/ml of LPwas mixed with each of the above said phase forming components using a vortex mixer. The mixture was incubated for 20 minutes at room temperature. After the incubation 100µL of the mixture was subjected to LP assay to access the stability of LP in each phase forming component.

Table 3.1: List of surfactants used and their properties

Surfactant	**HLB**	**Molecular weight, g/mol**	**Ionic group**	**CMC in water**
Sodiumdodecylsulfate /Sodium lauryl sulphate (SDS)	40	288.4	Anionic	8.2m
Bis-(2-ethylhexyl)sulfosuccinate sodium salt (AOT)	10.5	444	Anionic	2.1mM
Rhamnolipid (90%)	22-24	567.46	Anionic abovepH 6.8	0.2
Polyoxyethylenesorbitan monolaurate (Tween 20)	16.7	1227.5	Non-ionic	0.0499mM
Polyoxyethylenesorbitan monostearate (Tween 60)	14.9	1309	Non-ionic	0.0167 mM
Polyoxyethylenesorbitan monooleate (Tween 80)	15.0	1228	Non-ionic	0.015 mM
Sorbitan monostearate (Span 60)	4.7	430	Non-ionic	-
Sorbitan monooleate (Span 80)	4.3	428.6	Non-ionic	-
Sorbitan trioleate (Span 85)	1.8	958	Non-ionic	-
Triton x 45 (TX 45)	9.8	426	Non-ionic	0.018 mM
Triton x 100 (TX 100)	13.4	647	Non-ionic	0.024 mM
Triton x 114 (TX 114)	12.3	537	Non-ionic	0.021 mM
Tergitol TMN6	13.1	540	Non-ionic	52ppm
Tergitol 15S7	12.1	508	Non-ionic	0.073 mM
Polyoxyethylene lauryl ether(Brij 35)	16	1199.55	Non-ionic	-

3.2.2 Reverse micellar extraction of LP

The organic phase was prepared by mixing appropriate concentrations of surfactants(synthetic, synthetic mixed, biosurfactant) in an appropriate organic solvent. The organic phases were prepared immediately before the use to prevent loss due to evaporation.The aqueous phase during RME experiments using pure LP consisted of 5μL of stock LP(5mg/ml concentration) in 0.1 M phosphate buffer at different pH. This corresponded to 25μg/ml of LPcontent available in the milk. For forward extraction, the aqueous solution was mixed with an equal volume of organic phase in a 10 mL beaker of dimension 26 mm OD × 35 mm height. Mixing was facilitated by a magnetic stirrer at 500 rpm for 30 min. This was followed by centrifugation (Remi R8C-BL, India) at 1500g for 5 minutes. The two phases were carefully separated. The bottom aqueous phase was subjected to LP assay (section 3.2.3a Lactoperoxidase assay) to access the amount of LP solubilized in reverse micelles in the organic phase. During back-extraction, the organic phase from forward extraction was mixed with an equal volume of fresh aqueous stripping solution consisting of electrolyte and buffer at appropriate pH.The mixture was stirred followed by centrifugation to separate the phases under similar conditions followed during forward extraction. The aqueous phase here too was subjected to LP assay to access the back-extracted LP and its activity.Visual representation of the RME process is provided in Fig 3.1.% LP solubilized/extracted and % LP recovered was measured using the expression (eq.1 and 2) and reported. The protein concentration of LP solubilized into the organic phase was estimated through eq 3 and the purification fold was calculated as per eq 4.Both forward and backward extraction experiments were carried out at room temperature and in triplicate.

$$\%\ \text{LP solubilized/extracted} = \frac{\text{LPA}_\text{f} - \text{LPA}_\text{aq}}{\text{LPA}_\text{f}} \times 100 \ldots\ldots\ldots\ldots\ldots.(1)$$

$$\%\ \text{Activity Recovered /LP recovered} = \frac{\text{LPA}_\text{faq}}{\text{LPA}_\text{f}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots(2)$$

Where, LPA_f (Units mL^{-1}) and LPA_{aq} (Units mL^{-1}) are the concentration of LP in the feed and aqueous phase after forward extraction, respectively and LPA_{faq} (Units mL^{-1}) is the concentration of LP in the striping phase after back extraction.

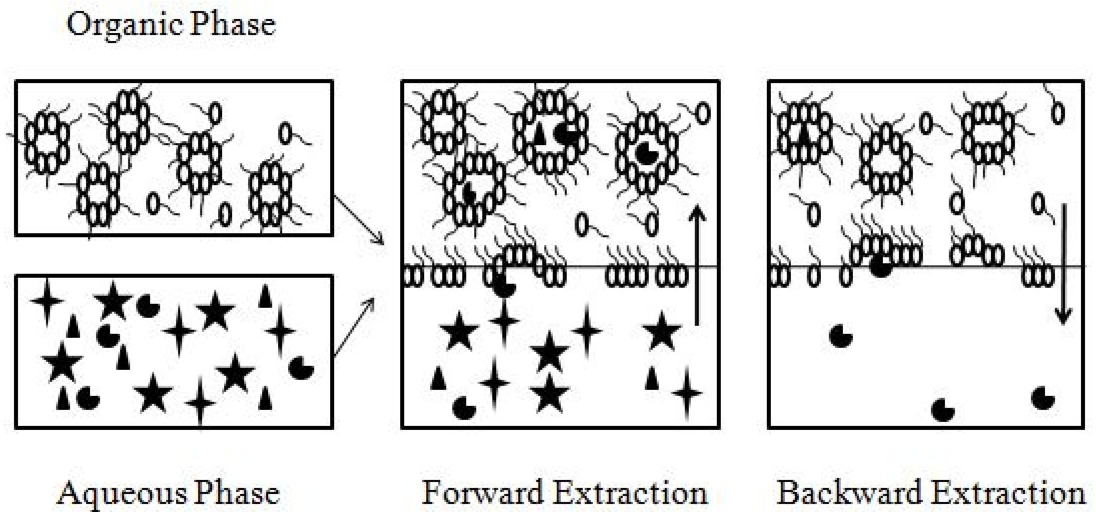


Figure 3.1: Visual representation of reverse micellar extraction

Similarly, Lactoperoxidase extraction from acid whey was studied using the same protocol with theexception that in forward extraction acid whey was used at desired pH. The total protein content was quantified according to BCA method(section 3.2.3b Protein Estimation)in the bottom aqueous phase after the back extraction process.

$$\text{Back extraction efficiency}(\%) = \frac{P_{aqf}}{P_{org}} \times 100 \;\ldots\ldots\ldots\ldots\; (3)$$

$$\text{Purification fold} = \frac{\text{Specific activity of LP after back extraction}}{\text{Specific activity of LP in feed}} \ldots\ldots\ldots\ldots\ldots.(4)$$

where, ‘aqf’ and ‘aqb’ indicate the aqueous phase after forward and back extraction, respectively. Similarly, feed whey is indicated with ‘f’. P_{aqf} and P_{org} (mg mL^{-1}) are the protein concentrations of the resultant bottom aqueous phase and top organic phase after back extraction, respectively.

3.2.3 Analytical Methods

a. Lactoperoxidase assay

LP quantification was performed by ABTS assay (Kumar and Bhatia 1999). Fifty-five mg of ABTSwas dissolved in 0.1 M phosphate buffer at pH 6.0 andthe volume was made up to 100 ml giving 1mM solution. The solution was prepared freshly before use. From the commercially available hydrogen peroxide solution of 30 % v/v a stock solution of 100 mM was prepared by titrating against standard potassium permanganate solution. The stock solution was diluted to 3.2 mM concentration immediately before use.1mM ABTS solution was prepared in potassium phosphate buffer (pH 6). The assay mixture contained 3.0 ml of ABTS and 0.1 ml of the sample. The reaction was started by adding 0.1 ml of 0.1mM hydrogen peroxide. The absorbance was recorded at 412 nm for over 2 min in a spectrophotometer (Labomed UV 3000+, India). ABTS and enzyme solution without hydrogen peroxide was considered as a blank. One unit of activity (U) is defined as the amount of enzyme that catalyzes the oxidation of 1µmol of ABTS per min, in 0.1 Molar phosphate buffer pH 6.0, using a concentration of 1 mM ABTS and 0.1 mM hydrogen peroxide in the reaction mixture assuming a molar extinction coefficient of 32,400 M^{-1} cm^{-1} for ABTSat 412 nm.

$$2ABTS + H_2O_2 \longrightarrow 2ABTS^{+} + 2\,H_2O$$

$$\frac{\Delta A_{412} \times 3.2 \times 1}{32.4 \times 0.1 \times 1 \times 2} = 0.4938 \times \Delta A_{412}/\text{min Units of LP /mL of sample}$$

The sample calculation is provided in Appendix III

b.Protein Estimation

Protein estimation was performed by bicinchoninic acid assay.BCA solution constituted the reagent A and 4% solution of Copper (II) Sulfate Pentahydrate was used as reagent B. Standard working reagent (SWR) was prepared by mixing 100

volumes of reagent A with 2 volumes of reagent B. The resulting solution was apple green in colour and was stable at room temperature for 1 week.

100µL of aqueous sample/protein was added to 2 mL of standard working reagent. Each tube was vortexed to ensure adequate mixing.Tubes were incubated at 37°C for 30 minutes, followed by cooling to room temperature before measuring the absorbance. The absorbance of standard and samples were measured at 562 nm and where necessary dilutions were performed and dilution factors were used for calculations (Walker, 2002).Pure LPwas used to prepare a standard curve preparation and used for RME experiments concerning extraction of pure LP and Bovine serum albumin was used toprepare standard curve for RME experiments concerning LP extraction from whey(Appendix II). The standards were prepared between 0.2mg/mL to 1mg/mL(BSA) and 5mg/L to 25mg/L(LP) concentrations in deionised water.

c. Water Content

The water content of the organic phase reverse micelles is given as the molar ratio of water to surfactant. This was measured by obtaining the amount of water entrapped in the core of reverse micelles using Karl Fischer titrator 899 coulometer, Metrohm, Herisau, Switzerland. These tests were performed for all the organic phases. The analysis was performed in duplicates and the average was noted as ppm. This was converted to mM/L by dividing with a molecular weight of water i.e.18. The water content values are obtained using the formula: $[M]_{water}/[M]_{surfactant}$. A sample calculation is provided in Appendix IV.

d. RP-HPLC for Lactoperoxidase analysis

The qualitative determination of LP was performed by Reverse Phase- HPLC. The unit consisted of a solvent delivery unit - quaternary pump (LC-20AD), a detector with wavelength range 190-700nm (SPD-20A), a column oven (CTO-10ASVP), a degassing unit DGU-20A. The whole unit was controlled by Labsolutions software. Reversed-Phase column (C4) from Phenomenex called Jupiter with 250 * 4.6 mm I.D, particle size 5µm, pore size 300 Å was used. Chromatographic conditions followed a

slight modification from Liang et al. (2011). Solvent A: Water with 0.1% trifluoroacetic acid, Solvent B: Acetonitrile with 0.1 % trifluoroacetic acid was used. All solvents were of HPLC grade. The column was washed with 100% methanol and equilibrated with 5% Solvent B. 20 μL sample was injected in manual mode. The column temperature was set to 35ºC and the detector to 40ºC. The detector wavelength was set to 280 nm and 412 nm. Elution was performed with gradient procedure for 24 minutes with the first 4 minutes from 5-10% Solvent B, 4-8 minutes from 10-25% solvent B, 8-12 minutes from 25-55% solvent B, 12-20 minutes from 55-25% solvent B and last 4 minutes from 25-5% solvent B.

e. CD spectra analysis

Secondary structure changes of LP in the presence of surfactants also before and after reverse micellar was studied in the far UV range were analyzed by CD spectra. Spectral studies in the far UV range were performed on Jasco J-810 Circular Dichroism Spectropolarimeter from Jasco Mary's Court Easton, MD, USA. The parameters employed were bandwidth^{-}1 nm, spectral range from 200 to 250 nm, data pitch 0.5 nm, number of scans 5, and scan speed— 100 nm min−1.

f.Dynamic light scattering (DLS) measurements

The size of the reverse micelles was determined using Anton Paar Particle size analyzer Lite sizer-500. Six repetitive runs were performed on each sample for analysis. The measurement angle was kept on automatic mode. Measurements were performed at 22ºC, and the dispersant solvent for the measurements was Isooctane with arefractive index of 1.391.

g. Ion content measurements using ICP-OES

Whey consists of many important ions. The concentrations of these ions were measured using ICP-OES from Agilent Technologies. The instrument was calibrated with standards at appropriate wavelengths for important ions viz. Ca^{+}, Na^{+}, K^{+}, and Mg^{+}. The samples were appropriately diluted and used without any further processing.

h. Statistical analysis

The extraction was performed in triplicates, the mean and Standard deviation were analysed on Origin Pro Software. The data were subjected to oneway analysis of variance on Microsoft excel, and the significance was reported to be $p < 0.05$.

3.2.4 Antimicrobial assay using the recovered LP

The standard plate count method was followed for quantitative estimation of antibacterial activity of recovered LP. *Staphylococcus aureus subsp. aureus* ATCC® 11632 was procured from Himedia India. Assay tubes of 10ml were prepared from 16-18 hr cultures at an initial concentration of 104 cells/ ml. Two sets of assay tubes were maintained; one at 30 ºC and one at 9 ºC. Each assay set consisted of control without addition of LP system, blank with all LP system components except LP, and sample with LP system components and LP. Studies were performed with pure LP from an external provider, LP recovered after AOT/Tween 80 based extraction, and LP recovered after biosurfactant-Rhamnolipid based extraction. 250µM sodium thiocyanate and 250µM hydrogen peroxide were used as activators of LP i.e. LP system components. 100µL from these assay sets was plated onto TSA plates with appropriate dilutions for convenient manual counting of colonies. Counts were recorded at different time intervals viz. 0, 2, 4, 6, 8, and 24 hrs for both sets i.e. at 30 ºC and 9 ºC.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

Part one: Screening of the compatible extraction process for Lactoperoxidase

4.1 Stability studies with different phase forming components

Proteins differ in their stability based on their various inherent properties as well as environmental factors. Also, proteins are sensitive and mostly require aqueous environment to retain their structure.Lactoeproxidase (LP) is an antimicrobial enzyme and its economical value depends on the activity of the purified protein. The different phase forming components used in liquid-liquid extraction viz. polymers, salts, surfactants, alcohols, and sugars affect the stability and activity of proteins and enzymes. However, this also depends on the concentration of these phase-forming components. To decide on the best liquid-liquid extraction procedure, LP was evaluated for its stability in various phase-forming components at concentrations usually employed in the extraction processes.The enzyme was incubated at room temperature (30-32ºC) for 30 minutes using Phosphate buffer at pH8 in each of the phase forming component. The LP activity was measured after incubation and compared with that of the LP activity obtained from LP in Phosphate buffer at pH8 without any phase forming components.

4.1.1 Effect of polymers on LP activity

PEG is the most commonly used phase forming component in ATPS. PEG is non-denaturing towards proteins solubilized. Most proteins are better stabilized in the presence of PEG. However, in the present study upto 40% loss in the activity was found in both low and high molecular weight Polyethylene glycol. This loss in the activity could be because of two reasons a. Enzyme precipitation b. Interaction of

enzyme substrate with PEG. Activities of glucose-6-P dehydrogenase, glutamate dehydrogenase, and pyrroline-5-carboxylate reductase reduced with increasing weight % of PEG. The reason for such inactivation could be due to the unfavorable interaction of the PEG with surface charges on the protein. PEG is known as the best stabilizer of peroxidase activity. In Industrial processes, peroxidase stability is lost by autooxidation and even in presence of small amounts of co-substrate hydrogen peroxide. PEG and PVP are used in many peroxidase enzyme extraction protocols to prevent its oxidation by phenolic compounds present in extracts. PEG, PVP, PPG of all molecular weights form complexes with phenolic compounds (Guttman and Higuchi, 1956) and prevent oxidation by oxidative enzymes. In one of the recent studies by (Juarez-Moreno, Ayala and Vazquez-Duhalt, 2015) antioxidant capacity of PEG was tested in different substrates pyrogallol(phenolic substrate for peroxidase), ABTS(non–phenolic substrate for peroxidase). It was found that both low and high molecular weight PEG was able to scavenge the radicals harmful to protein. PEG stabilized peroxidase enzyme in the reactions involving oxidation of the phenolic compound (Mao et al., 2013).

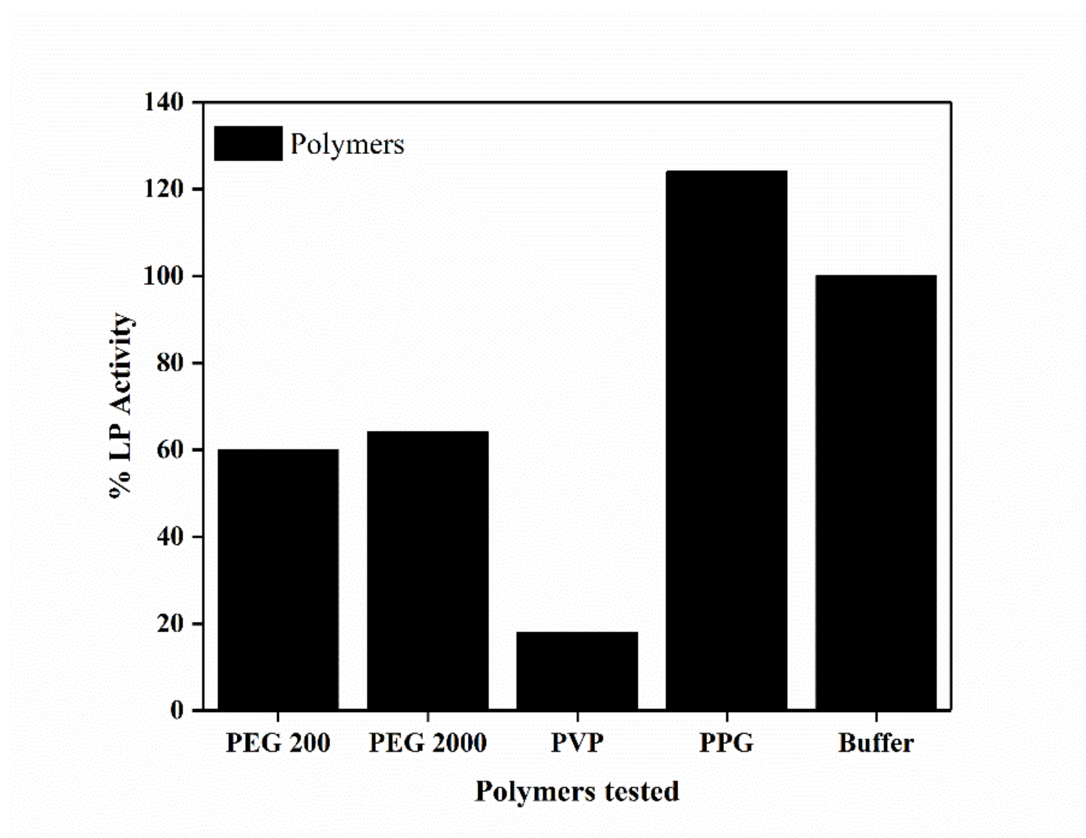


Figure 4.1: Effect of polymers on LP activity

PEG being partially hydrophobic in nature is known to solubilize hydrophobic tryptophan amino acids residues. This could lead to an unfolded state of LP which consists of hydrophobic residues in the catalytic centerof the protein. Further, PEG is known to stabilize the unfolded state by interacting with exposed hydrophobic residues. This could result in lowering of melting temperature of the protein leading to protein denaturation(Lee and Lee, 1987). LP is rather a thermally stable protein with irreversible denaturation only at 70˚C(Ludikhuyze, Claeys and Hendrickx, 2001;Boscolo et al., 2007).This could be reduced in the presence of PEG leading to denaturation at much lower temperatures. The change in the monomer residues in PEG i.e. PEG 200 and PEG 2000 did not improve the LP activity(Fig 4.1). Similarly, PVP also showed poor LP activity. PPG on the other hand showed enhanced activity but could not form stable two phase systems in any of the salts tested.

4.1.2 Effect of salts on LP activity

Salts have either "salting out" or "salting in" effect on proteins that decides their solubilization and precipitation characteristics commonly following the Hofmeister series. They also affect the thermal transition temperature such as the chaotropic salts that destabilize the protein structure. In contrast, kosmotropes enhance the native state of the enzyme (Endo, Kurinomaru and Shiraki, 2016). Salts that induce the salting-out effect increase thermal transition temperature and stabilize the protein conformation. The effect of the individual ions of the salt is varied. They can be stabilizers as well as destabilizers. However, when in combination their effects cancel out(Fonteh, Grandison and Lewis, 2005).

Amongst the salts tested sodium sulphate, sodium citrate were neutral salts, sodium phosphate & potassium phosphate were acidic salts, potassium citrate was alkaline salt (Fig 4.2). Phosphate and sulphates are protein stabilizers. In the present study, phosphate salts reduced the LP activity and this is because of the higher concentrations. At higher concentrations, salt could destabilize the normal tertiary structure of the protein as well as remove water from the enzyme surrounding making it inactive. Citrate salts in the contrast increased the LP activity by about 54%. Sodium Chloride and Potassium chloride did not greatly affect the LP activity. The effect is predominantly with the anions of the salt that could change the cationic LP conformation.

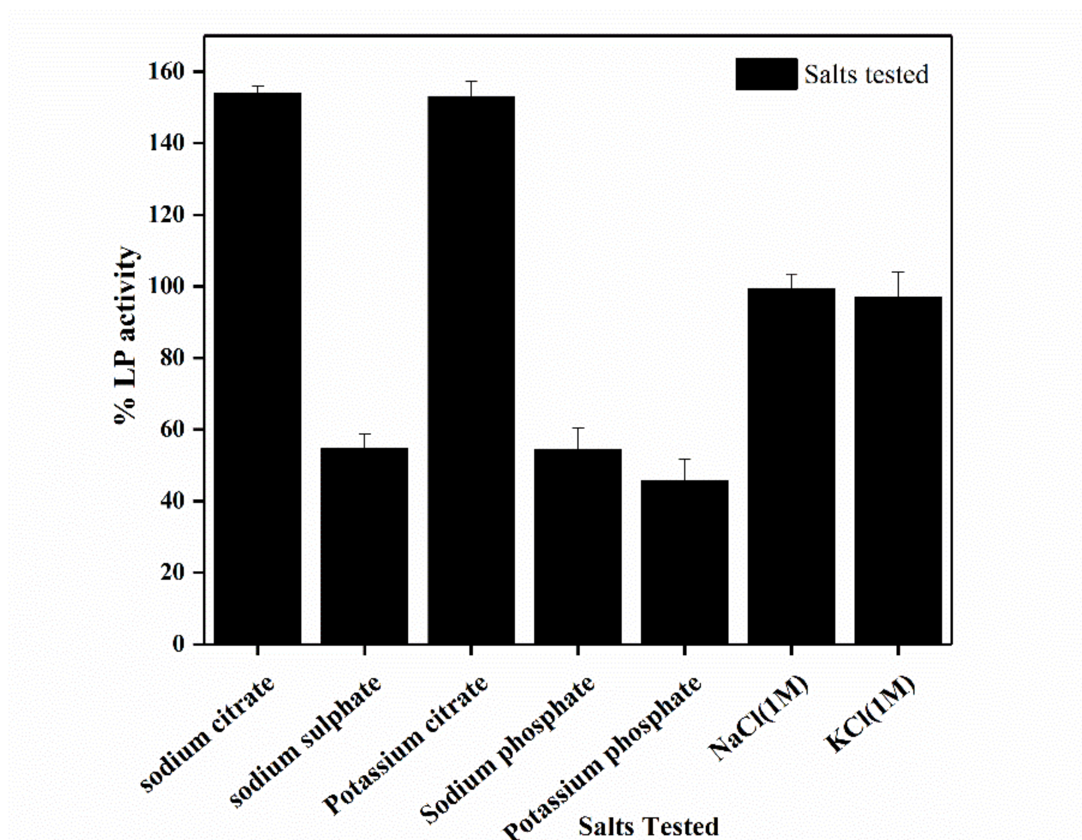


Figure 4.2: Effect of Salts on LP activity

4.1.3 Effect of sugars and sugar alcohols on LP activity

LP activity in the presence of sugars like glucose, arabinose, sucrose, and mannose, and sugar alcohols like sorbitol and xylitol was tested and the obtained results are presented in Fig. 4.3. It was observed that sugars reduce the activity of LP drastically since they act as inhibitors of the enzyme by blocking the substrate channel. Sugars are known to be inhibitors of cellulase and β-glucosidase during hydrolysis of cellulose (Xue et al. 2015). Similar effects of sugars on LP antimicrobial activity were studied in mono and disaccharides. The authors reported that loss in antimicrobial activity of LP in presence of sugars is mainly due to loss in catalytic activity (Al-Baarri et al. 2011). However, LP activity was found to increase from 7% to 42% with increasing concentrations of sugar alcohols from 10% to 30% for both xylitol and sorbitol.

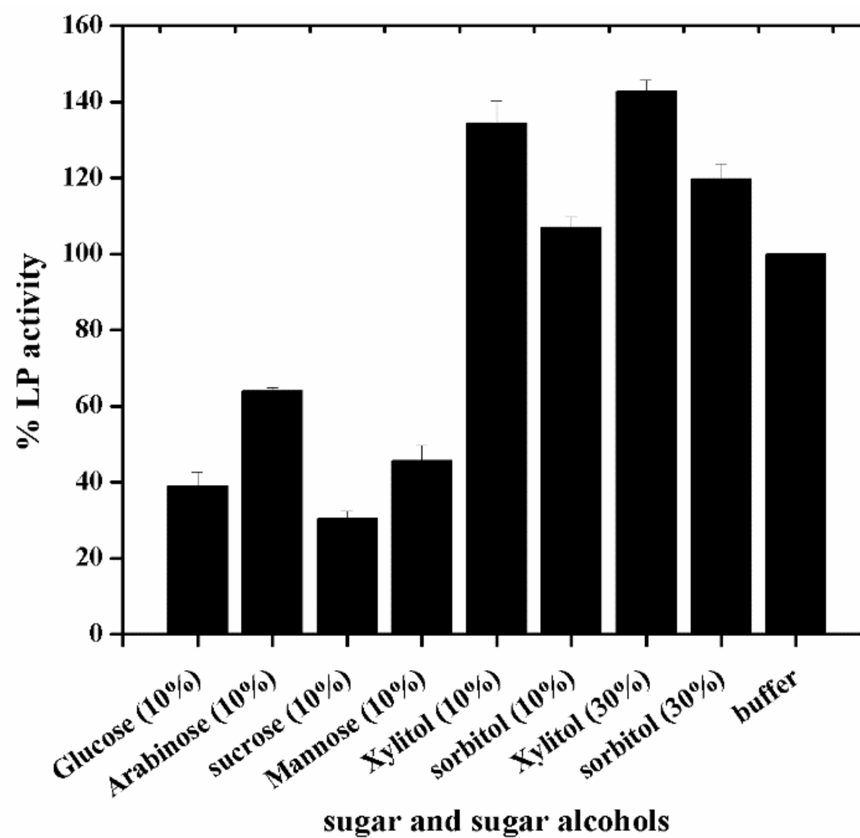


Figure 4.3: Effect of sugars and sugar alcohols on LP activity

4.1.4 Effect of alcohols on LP activity

Alcohols like ethanol, propanol, and butanol were tested for LP activity and the results obtained are shown in Fig.4.4. The LP activity was found to increase linearly with respect to the polarity of alcohol. Ethanol, the most polar amongst the alcohols reduces the activity to 31%. The short-chain alcohols, which are water-miscible, alter the active site of the enzyme and catalytic efficiency (Singh, Prakash and Shah 2012). The LP activity improves as the water miscibility of the alcohol reduces. Thus, 85-92% of the LP activity was retained in the presence of medium-chain alcohol, butanol, and hexanol as it is slightly miscible with water.

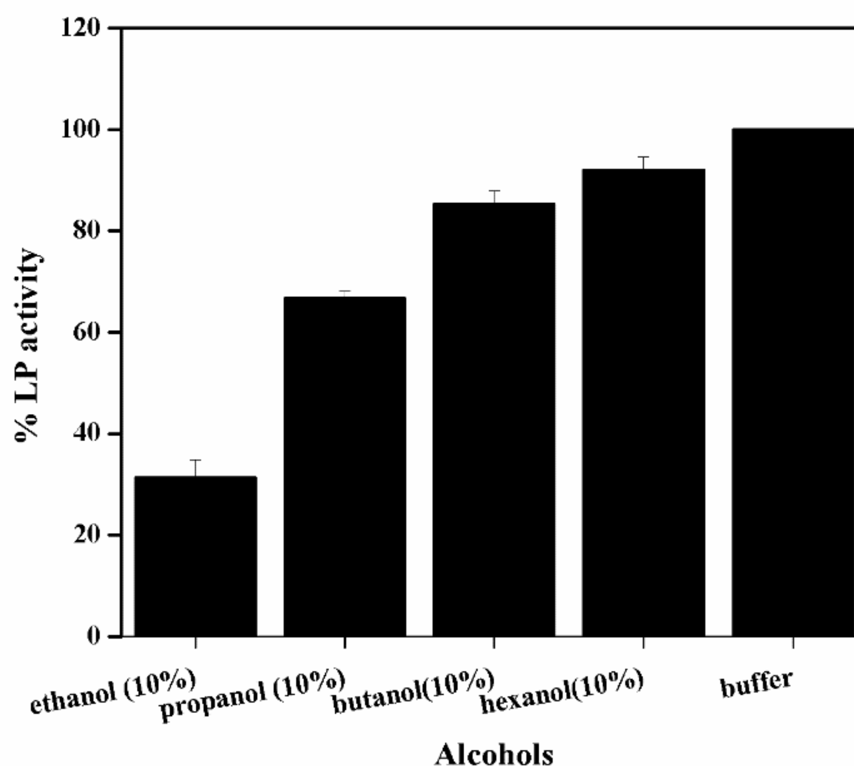


Figure 4.4: Effect of alcohols on LP activity

4.1.5 Effect of Non-ionic surfactants on LP activity.

Non-ionic surfactants, triton, tween, span, tergitol series, and Brij 35 were used in the present study at 2% concentration and their effect on LP activity is shown in Fig.4.5.Non-ionic surfactants are mild in nature due to the absence of ionic head groups for electrostatic interaction with protein. However, the hydrophobic interactions are feasible and can affect the activity of the enzyme by competing with a substrate for the active site. The surfactant environment can also change the folding patterns of the enzyme leading to the change in the activity. Reports on both increased and decreased activity of enzymes with non-ionic surfactants are available (Rubingh 1996). LP activity decreased with increasing concentration of Igepal CO-720 (Grudzinska and Gebicka 2005) and also varied with the substrates used for activity measurements.

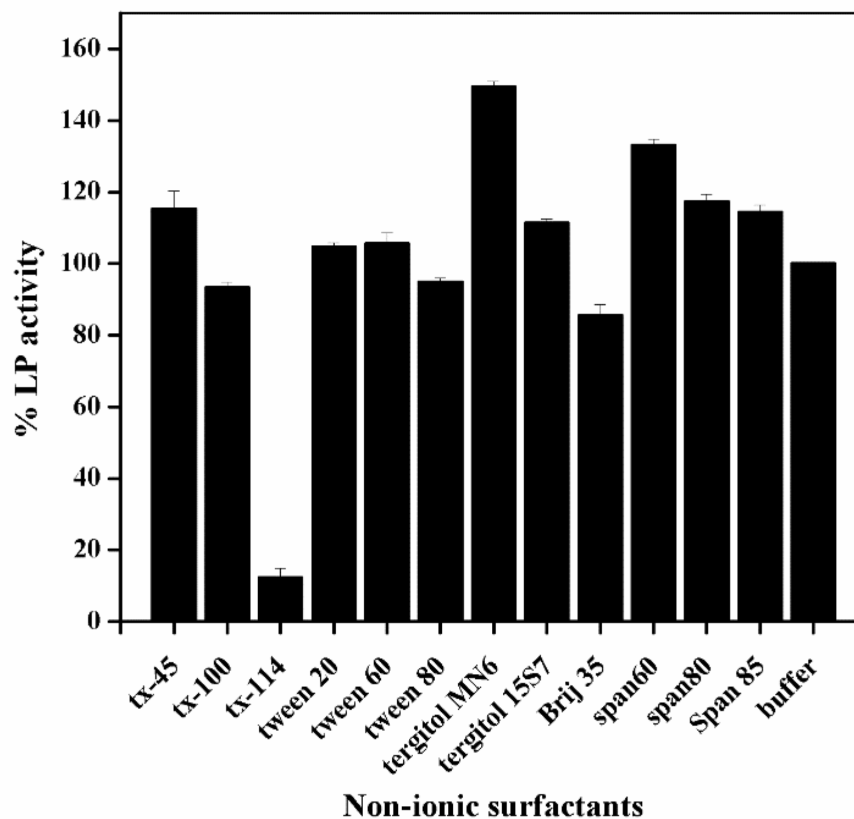


Figure 4.5: Effect of non-ionic surfactants on LP activity

However, LP activity in that study could be retained after dilution (500 times) of the sample. In the present study, the surfactants showed varied results from super activity to loss in activity for LP in aqueous solutions. LP activity in the presence of TX 114 was reduced by a staggering 88%. It was observed that the activity in the presence of TX 45 was higher and slightly lower for TX 100, whereas TX 114 had the least activity amongst the triton series tested. Hence, a correlation between HLB (Table3.1) or average ethylene oxide unit in triton series and LP activity could not be made. Similar studies with TX 45 showed improved activity for lipase (Yamada, Kuboi, and Komasa 1993) and decreased activity in TX 114 for α-chymotrypsin. Tween series surfactants did not show much difference in the activity of LP compared to that in the buffer. Span series of surfactants showed higher activity with a 32% increase for span 60, 19% for span 80, and 15% for span 85. Brij 35 showed up to 16% loss in the activity and the tergitol series surfactants showed better activity with a 10% increase for 15-S-7 and a 46% increase for TMN6.

4.1.6 Effect of Ionic surfactants on LP activity

Many researchers have reported the denaturation of proteins in the presence of ionic surfactants due to theirstrong electrostatic bonds with proteins. Irreversible denaturation of protein due to protein micellar interaction has been observed previously (Rubingh 1996). In the present study three anionic surfactants- SDS, AOT, and Rhamnolipid were studied for their effect on LP at 50 mM concentrations and the results are shown in Fig. 4.6. The experiments were conducted at different pH for each surfactant to access the effect of the ionic nature of the surfactant on the enzyme activity over changing aqueous phase pH. The structure of surfactants, their chemical nature havea significant effect on theirinteraction with proteins. Both the tails form hydrophobic interaction with hydrophobic residues on protein at the same time surfactant headgroup forms electrostatic interactions with positively charged amino acids on protein. The effect of ionic surfactants seemed to be pH-dependent. At pH 6 both SDS and AOT resulted in the loss of LP activity, whereas pH 8 showed no loss in LP activity. This is primarily because of the strong interaction between the anionic head group of the surfactants and positively charged amino acids on protein. LP is a basic protein with pI between 9.2-9.9 and it is predominantly positively charged below pI and negatively charged above pI. Thus, the positive charge density of amino acids is high at pH 6 compared to that of at pH 8. This could affect the protein interaction with surfactants and thus its activity. Moreover, the catalytic centerof LP is situated in the hydrophobic core; the interaction seems to be strong and resulting in the conformational changes on LP. This in turn resulted in the loss of activity. Rhamnolipid is a biosurfactant that is anionic at pH 6.8 and hence, it had no effect on LP at pH 6. However, even at pH 8 rhamnolipid did not show the major loss in LP activity. Synthetic surfactants have different chemical structures compared to biosurfactants and they interact weakly with proteins owing to their mosaic distribution of head and tail groups (Otzen 2017). Hence, less conformational changes and denaturation wereobserved for rhamnolipid. Moreover, it is reported that twin-tailed synthetic surfactants have destabilizing and denaturing effects on proteins (Kaur and Mahajan 2014). SDS used in the present study is a single-tailed anionic surfactant whereas AOT is twin-tailed. It is noted that single-tailed surfactant interacts

differently with proteins than twin-tailed surfactants. The behavior of the three anionic surfactants differs depending on their structural properties as well.

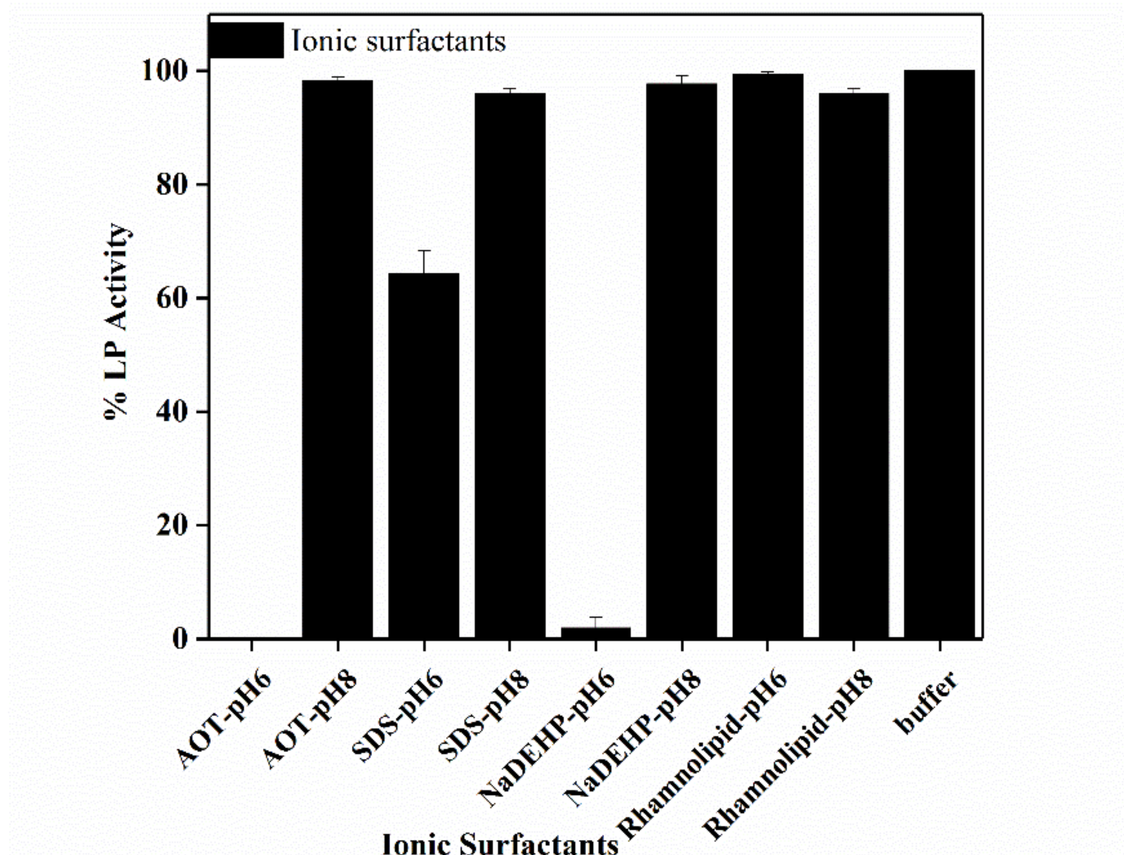


Figure 4.6: Effect of ionic surfactants on LP activity

4.1.7 Conformation studies of LP in the presence of surfactants

Circular dichroism studies for LP with and without surfactant were performed in phosphate buffer at pH 6. The measurements were performed in the far UV region to study the secondary structure of the protein and the results obtained are as shown in Fig 4.7. The defined negative peak at 208 nm that corresponds to the alpha helical structure present in LP (Marcozzi, Domenico and Spreti. 1998) confirms the non-structural modification for LP at pH 6 without surfactant. The reduced alpha helix of LP at pH 6 in the presence of surfactant clearly shows the denaturation of LP. Such denaturation of proteins was observed for β-lactoglobulin, insulin, and transferrin. The authors also mention about concentration-dependent denaturation with as little as 4.3×10^{-4} M for β-lactoglobulin and transferrin, and for insulin 2.3×10^{-4} M could

cause structural changes (Jelińska et al. 2017). This low concentration is lesser than critical micelle concentration and such low concentrations are not useful for encapsulation by stable emulsion. Thus, the concentration of surfactants and pH of the media are critical parameters to be considered when encapsulating LP using surfactants.

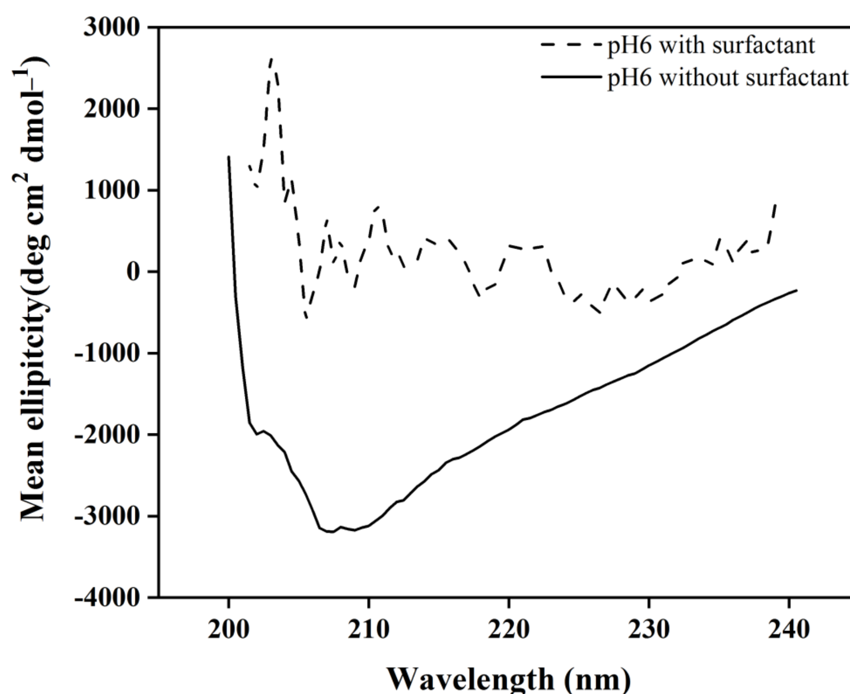


Figure 4.7: CD spectra of lactoperoxidase with and without surfactants

Part Two: Reverse micellar extraction of Lactoperoxidase from aqueous Lactoperoxidase solution

4.2 Reverse micelle extraction of pure Bovine Lactoperoxidase

RME experiments were conducted to identify suitable surfactant systems that can maximize the extraction of LP activity (forward extraction), and recovery of LP from the organic RM-rich phase to a fresh aqueous phase through back extraction. Surfactant systems composed of cationic and anionic surfactants were initially chosen for RME of LP. It was observed that cationic surfactants failed to extract LP at pH 8 due to the lack of electrostatic attractive driving force between positively charged LP and cationic surfactant. Employment of an anionic surfactant, neat AOT, was able to extract 38% of the LP activity at pH 8. The % LP extracted through forward extraction was maximized by employing mixtures of nonionic and anionic surfactants and recovery was maximized using a cationic surfactant during the back-extraction process. Various processing factors affected the forward and back extraction of LP, viz. type and concentration of surfactants, solvent type, pH, and ionic strength.

Table 4.1 LP extracted and recovered using RM system with neat AOTand isooctane

pH of the aqueous solution with neat AOT in the Organic Phase	% LP extracted	% LP recovered
pH 6	100	nil
pH 7	100	2
pH 8	38	6
pH 9	24	2.8

4.2.1 Effect of Nonionic Surfactants on Forward Extraction

The structural nature of the surfactants played a significant role in controlling LP stability by modulation of hydrophobic, hydrophilic, and ionic interactions. Preliminary studies indicated that LP was inactive in the RM phase resulting from RME using neat AOT at pH ≤6. This result seems to contradict results from the previous studies, where LP extraction with aerosol-OT RM resulted in 86.60%

recovery of activity at pH 6.0 (Nandini and Rastogi, 2010). It is also noteworthy that the previous studies have mentioned that most of the protein denaturation and enzyme activity loss occurred during forward extraction (Dekker et al., 1989). Such pH-dependent denaturation and inactivation of protein and enzyme has been observed for other proteins and enzymes as well when ionic surfactants were used for RME (Dekker et al., 1989; Jones et al., 1982). Thus, the current study was performed to expand the existing knowledge of RME of LP using mixtures of anionic and nonionic surfactants, including the factors that affect pH-dependent denaturation and inactivation.The highest LP extraction was observed at pH 8 since LP was more stable and underwent lower inactivation. However, not all of the LP activity was transferred from the aqueous solution into the RM phase at pH 8. On closer observation, it was seen that the effect of AOT on LP activity seemed to be pH-dependent (Table 4.1). At pH 6, LP is positively charged (pI of 9.2–9.9) (Yafei et al., 2011), resulting in strong electrostatic interaction between LP and AOT that can change the conformation of LP, leading to exposure of its hydrophobic catalytic site and hence to inactivation and denaturation. Though LP was positively charged at pH 8, the positive charge density of the protein is lower than at pH 6, hence reducing the extent of denaturation. Such pH-dependent loss of LP activity has been observed earlier for RME using SDS (Marcozzi et al., 1998) and dioleyl N-Dglucono- L-glutamate (Okazaki et al., 2000) as surfactant.

Similarly, at pH 8 no losses are reported in the literature; but, in our studies, only 38% extraction efficiency wasobserved at pH 8. The addition of nonionic surfactants improved the forward extraction of proteins by modifying the structural properties of the RM such as the hydrodynamic radius of RM due to increased water solubilization (Kundu and Paul, 2013). Thus, mixtures of AOT with Tween, Triton, and Span series non-ionic surfactants were investigated for RME of LP at pH 8. Initial organic phases contained AOT at 100 mM and non-ionic surfactants at 2–10 mM. The extraction efficiencies increased with the increasing concentration of Tween and Triton series surfactants and decreased with the increasing concentration of Span series surfactants (Fig. 4.8a). Tween or Triton surfactants are believed to selfassemble at the oil-water interface of the AOT RM (Yamada et al., 1993), thereby reducing the charge density

of AOT, or equivalently, the density of anionic sulfonate head groups at the RM interface. Thus, increasing concentration of the nonionic surfactants in the system results in a greater number of smaller RM due to the incorporation of nonionic surfactants in between the head groups of AOT, which increases the interfacial curvature. As the charge density of AOT reduces with the addition of nonionic surfactants, the hydrophobic forces may significantly increaseand contribute to the extraction of LP. Of note, 50% of amino acids of bovine LP are nonpolar (Pruitt, 2003).The nonpolar amino acids interact with the hydrophobic tails of nonionic and ionic surfactants through hydrophobic interactions (Marcozzi et al., 1998), while the positively charged amino acids interact with the AOT molecules through electrostatic interactions.

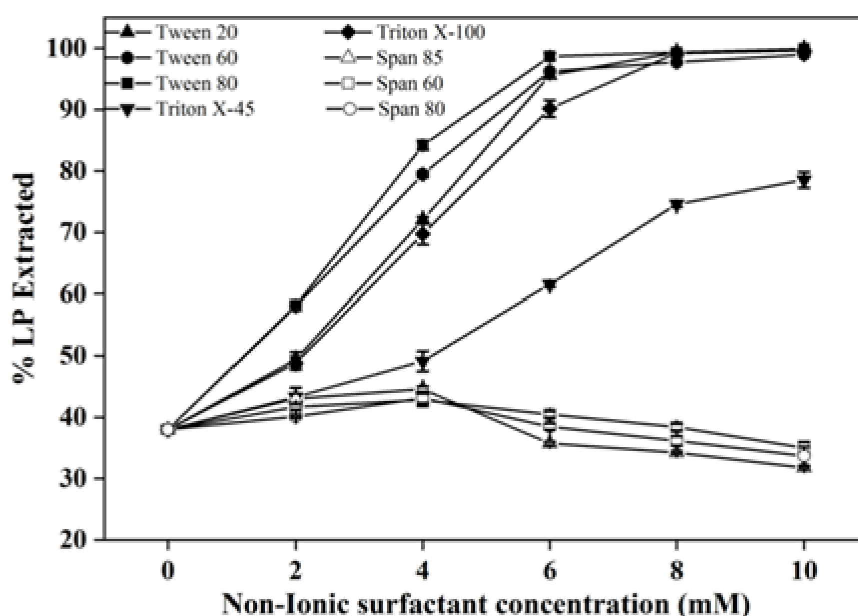


Figure 4.8a: Effect of nonionic surfactant concentration for RME of pure LP

The combined effect of hydrophobic and electrostatic forces may be responsible for the increased extraction ofLP into the RM through the employment of a mixture of AOT and Tween or Triton series compared to neat AOT. Differences in the Hydrophilic–Lipophilic Balance(HLB) of the nonionic surfactants may explain the difference in the performance of RME that wasobserved. It was observed that the use of Triton X-45, with 4.5 units of ethylene oxide (HLB of 10.4), led to lower LP

extraction compared to that of Triton X-100, with 9.5 units of ethylene oxide (HLB of 13.5). Moreover, it was stated that an increase of HLB enhances the solubilization of LP in RM (Kundu and Paul, 2013; Yamada et al., 1994). A similar result was also observed during the RME of cellulose by AOT-Tween series mixtures (Peng et al., 2016). Even though a similar extraction efficiency was observed for RME conducted using mixtures of AOT with Tween 80 and TritonX-100, the maximum solubility of LP (99.97%) was observed for 8 mM Tween 80, which is more hydrophilic than Triton X-100 (HLB of 15 and 13.5, respectively) (Fig. 4.8a). Similarly, Span series surfactants, which are more lipophilic than the Tween orTriton series surfactants(HLB of 1.2 to 8.6 for Span), yield lower extraction efficiency due to the lower concentration of RM that form in the top phase (Yamada et al., 1994). Further, extraction of LP decreased withincreasing concentration of Span as the water content of the top phase is not affected by the presence of the additional amount of Span molecules. It was observed that an increase of forward extraction efficiency for LP is coincident with an increase of water content for the RM in the top phase (Fig. 4.8a). Moreover, thewater content of the RM phase increased with the HLB of the nonionic surfactant. In addition, the forward extraction of water increased with the concentration of Tween series and Triton series surfactants. For example, the decreasing order of water-surfactant ratios achieved (44 mol/mol for the Tween series >38 mol/mol for the Triton series>10 to 20 mol/mol for the Span series) parallels the relative order of forward extraction efficiency (Fig. 4.8b).Also, the water-surfactant ratio increased from 24 to 44 mol/mol as the Tween80concentrations increased from2 to 8 mM (Fig. 4.8a). The increased water content in the RMcore enhances the extraction of LP.

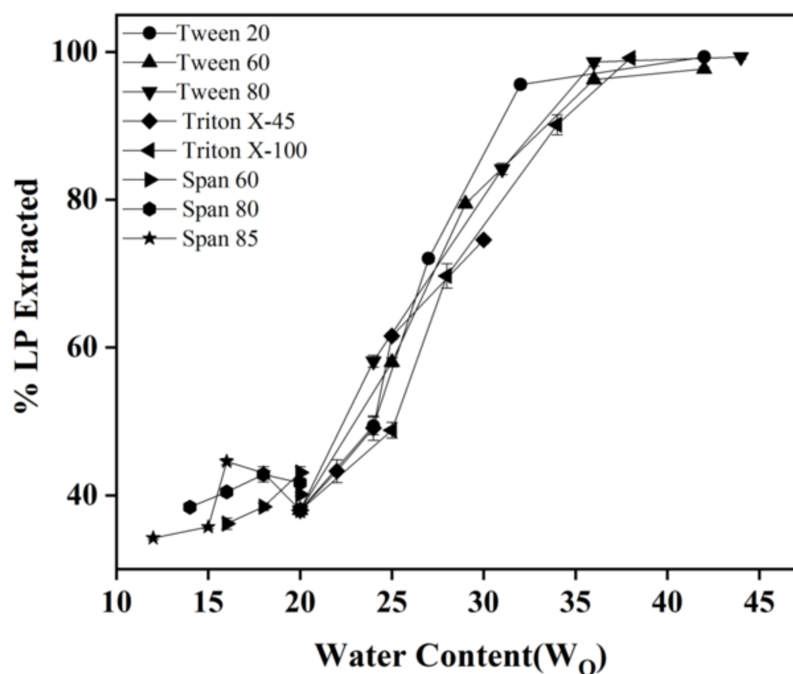


Figure4.8b: Effect of water content on the LP extraction efficiency at different nonionic surfactants and their concentration

4.2.2 Effect of Organic Solvent Type on Forward Extraction

The stability of the protein during RME is affected bythe type of organic solvent used which in turn affects theresultant water content in the RM. The water content ofRM changes with the carbon number of the solvent. Specifically,when branched alkanes are used to form the RM, the RMs tend to solubilize more water than straight-chainalkanes (Caroline, 1991). LP extraction efficiencywas compared between surfactant mixtures of AOT (100 mM) and nonionic surfactants (Tween 80, Triton X-100, or Span 85; 8 mM) and solvents viz. n-hexane,n-heptane, and isooctane (Fig. 4.9).

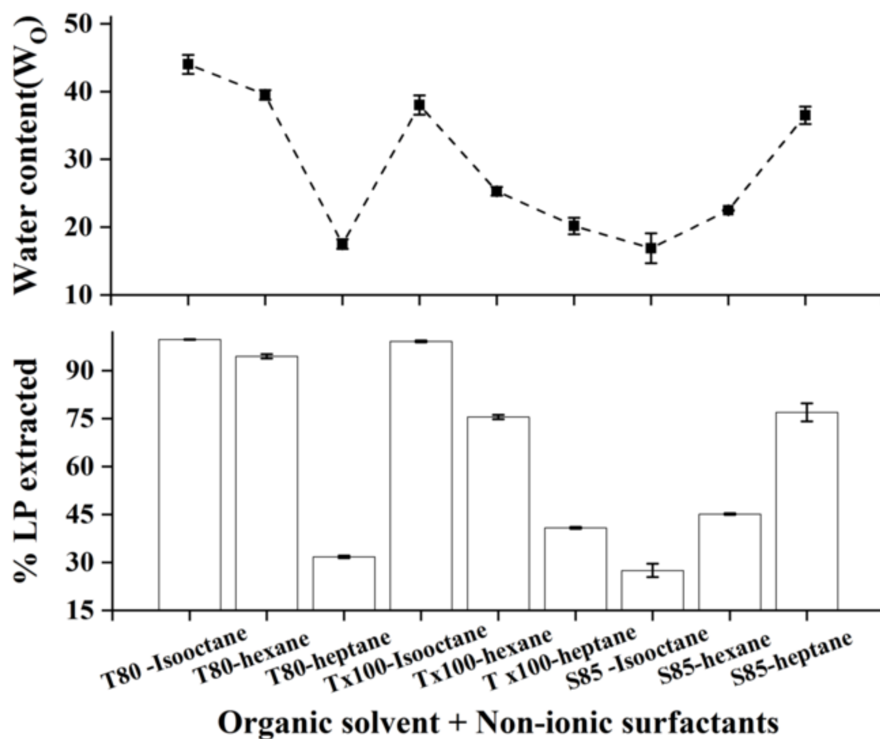


Figure 4.9: Effect of organic solvent for RME of pure LP

The maximum extractionof LP was observed for isooctane and both Tween80 and Triton X-100. However, for the AOT/Span85 mixture, LP extraction was maximized for n-hexane.Among the solvents tested, isooctane has the largestmolecular volume, and hence it is the least penetrable into the alkyl chains of surfactant (Gomes et al., 2017).As a result, isooctane promotes an increased naturalradius of curvature for the surfactant monolayer at theliquid–liquid interface relative to the other solvents investigated.The increased natural radius of curvature enablesan increase of water solubilization in the RM, henceincreased extraction of LP, as discussed above. Similarly,as the water content was decreased in the RM, the extractionefficiency also decreased accordingly (Fig. 4.9). Insummary, isooctane as oil maximized the forward extractionof LP for mixed surfactant systems of AOT with Tween 80 or Triton X-100.

4.2.3 Effect of Ionic Surfactant Type and Concentration onForward Extraction

The anionic surfactants AOT and NaDEHP at several concentrations (50–100 mM) were compared for theLP extraction in the presence of 8 mM Tween 80 at pH 8 (Fig.

4.10). NaDEHP was chosenbecause of its similar structural properties to AOT, i.e., twoalkyl tails. The employment of NaDEHP/nonionic surfactantmixtures for RME has not been reported to date. The denaturationof LP was observed at pH ≤ 6 for RME using neatNaDEHP.

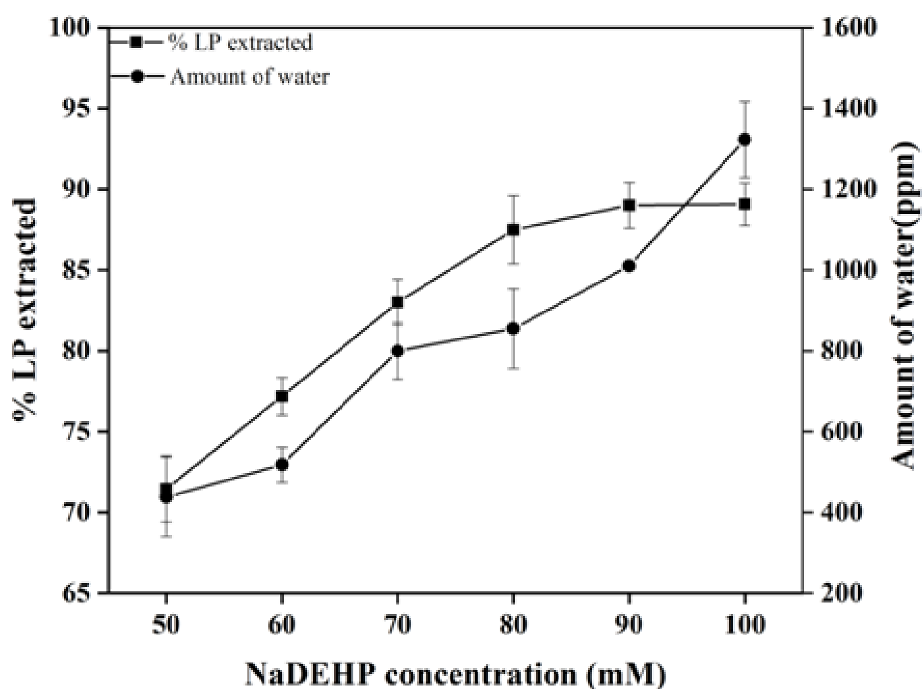


Figure 4.10a: Effect of ionic surfactant NaDEHP concentration for RME of pure LP

The complete extraction of LP was observed forthe surfactant system that employed 90 mM AOT; however,for NaDEHP-based surfactant mixtures, the maximumextraction efficiency was only 89% (100 mM NaDEHP)(Fig. 4.10a).
Though both surfactants have similar hydrocarbon tails, their aggregation behavior differs due to the differentinteraction of their dissimilar polar head groups with water(Li et al., 1998; Quintana et al., 2012), as observed throughthe higher water content of the top phase for AOT over NaDEHP-based surfactant mixtures (Fig. 4.10a,b). Of note, watersolubilization increased with increasing surfactant concentrationfor both anionic surfactants (Yu andNeuman, 1994). Yet, a fair amount of LP was extracted into the RM phase using 100 mM NaDEHP and 8 mM Tween 80.

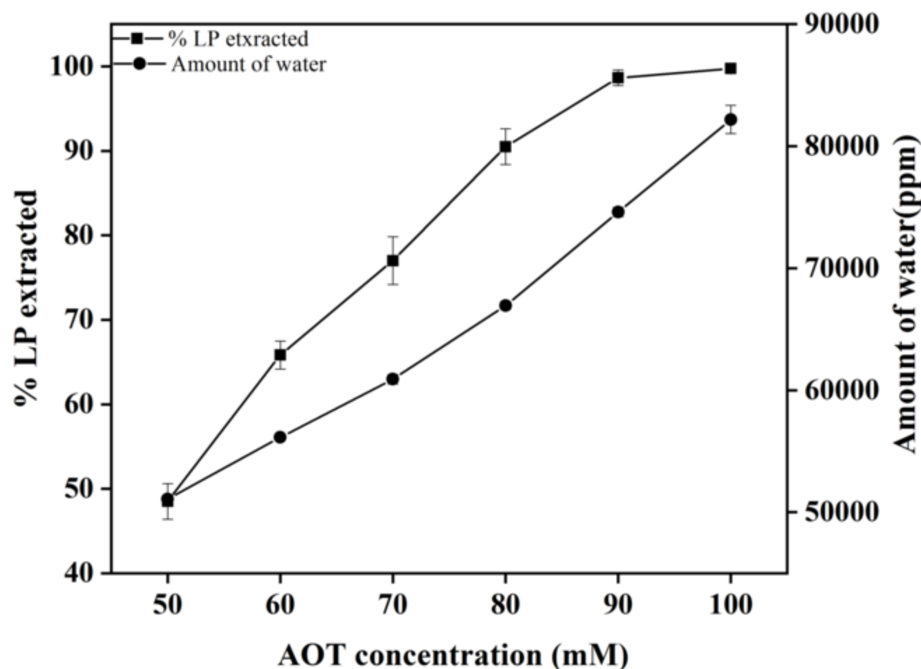


Figure4.10b: Effect of ionic surfactant AOT concentration for RME of pure LP

4.2.4 Effect of Aqueous Phase pH on Forward Extraction

In addition to the surface charge density of the surfactant monolayer, the surface charge of the protein may also be asignificant factor governing the RME of proteins. Generally,proteins are extracted via RME using anionic surfactants, such as AOT, at a pH below the protein's isoelectricpoint (pI), i.e., when the protein surface is positivelycharged. The maximum extraction of LP in the RM formedby AOT was reported at pH 6 (Nandini and Rastogi, 2010).It was also observed that very little protein is extracted byAOT for pH >pI (Andrews et al., 1994). However,Wolbert et al. (1989) observed that large proteins could beextracted by AOT when pH >pI. It was also reported thatthe addition of nonionic surfactants to ionic surfactants mayshift the pH range where RME can successfully occur(Dekker et al., 1989). Hence, RME of LP was investigatedin the pH range 7.0–10.5 to determine the optimal pH using90 mM AOT in combination with 8 Mm Tween 80.The extraction efficiency was >95% across the pH range7.5–9.5 (Fig. 4.11) due to the modified surface charge of theRM

by the addition of nonionic surfactants. The addition of Tween 80 shifted the optimal pHrange for LP forward extraction from 6.0–8.0 to 7.5–9.5.The synergistic effect of hydrophobic forces, electrostaticinteractions, and the positive charge distribution on the surfaceof the protein may be responsible for the extractionbehavior of LP (Wolbert et al., 1989).

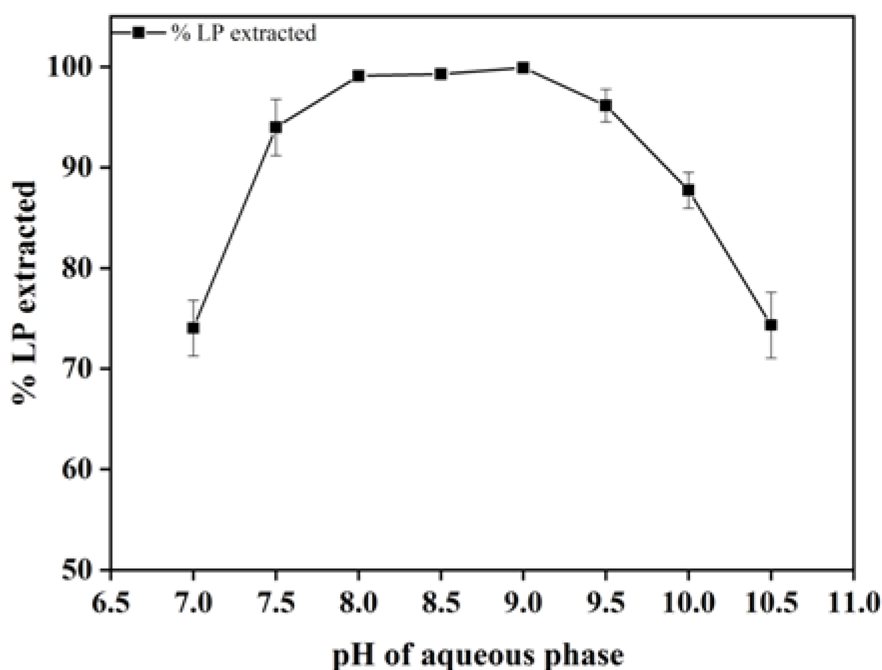


Figure 4.11: Effect ofaqueous phase pH during the forward extractionof LP for the RME of pure LP.

However, LP extraction decreased for pH > 9.5 due to theincrease of a negative surface charge for the protein, whichnegates the electrostatic attractive driving force between LPand the AOT head groups. As the pH approaches the pI ofLP, the positive charge density of LP is slightly reducedleading to a reduction in electrostatic interactions. Thus, aslightly lower LP extraction was observed below pH 8.0(Fig. 4.11).

Recovery of LP from Reverse micelles by Back extraction

The recovery of solubilized proteins from RM is often difficult.Strong electrostatic and hydrophobic interactionsbetween surfactants and proteins hinder the recovery ofLP. In many cases, a low yield of activity was observeddue to slower mass transfer

rates during back extractionthan forward extraction (Hebbar et al., 2011). Back extraction experiments probed the effect of the pH and ionicstrength of the aqueous stripping solution and the additionof alcohol (cosurfactant) and oppositely charged surfactantto the organic phase. The former reduced the electrostaticattractive driving force while the latter destabilized the RM.

4.2.5 Effect of the Stripping solution's pH on Back extraction

The stripping phase pH was adjusted so that pH >pI,thereby reducing the strength of electrostatic interactionsbetween the RM AOT head groups and LP. Since the pI ofbovine LP is high (9–9.5) and LP is inactivated at pH ≥ 12 (Boscolo et al., 2009), the pH range for the stripping phasewas restricted to pH 7–11 using 0.1 M buffers. It wasobserved that the back-extraction efficiency was sharplyincreased as the aqueous phase pH was increased from 9 to10.5, with the latter pH providing the highest backextractionefficiency (Fig. 4.12). The increase of backextractionefficiency was coincident with an increase of thepurification factor due to the larger mass of LP recovered.Recovery of active LP from RM was very minimal atpH 7–8 (Fig. 4.12). The residual electrostatic interactionbetween AOT and LP beyond pI of LP could be the reasonfor such low recovery (i.e., 12%). It could be due to thestrong hydrophobic interactions between Tween and LPalso, which is independent of aqueous phase pH.

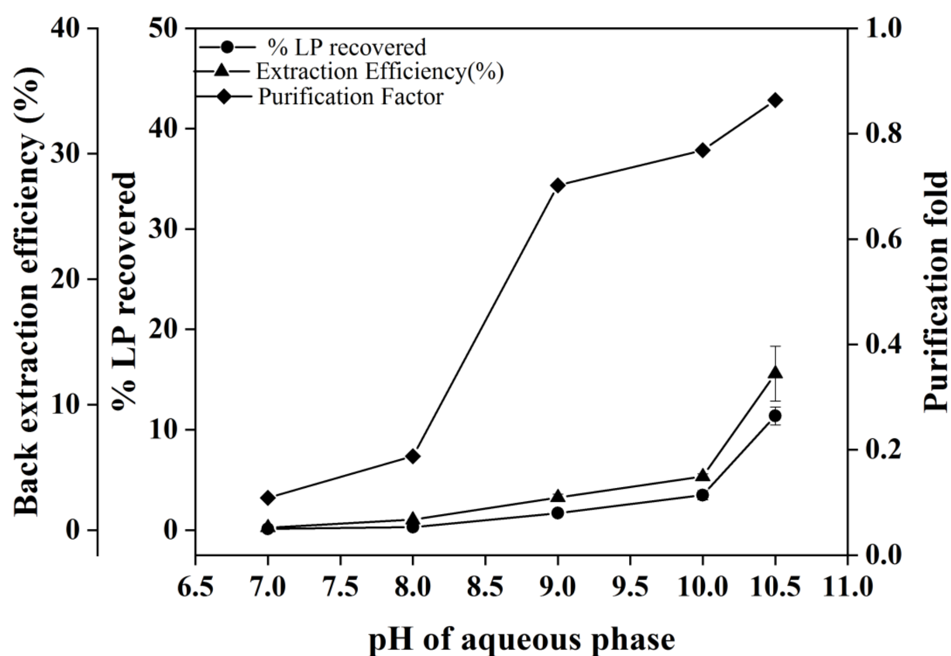


Figure 4.12: Effect of pH on the recovery of LP and purification fold during the back extraction for the RME of pure LP.

4.2.6 Effect of Inorganic Salt Type and Concentration onBack extraction

The increase of ionic strength for the stripping phaseenhances back extraction by reducing the electrostatic interactionof LP to AOT sulfosuccinate head groups through Debye shielding. Consequently, the water content of RMand retention of LP are expected to decrease (Andrewset al., 1994). The type of ions also affects the solubility ofwater in the RM. The role of cation being significantlyhigher than that of anion for anionic surfactants (Chaurasiyaet al., 2015) due to its water structure breaking or forming characteristics. Smaller cations (e.g., Na^+) induce a lowerscreening effect than larger cations (K^+) (Andrews andHaywood, 1994). The effect of monovalent salts, KCl and NaCl, at different concentrations (0.5–2.0 M) in the aqueous stripping solution on back-extraction efficiency wasstudied, with the pH held constant at 10.5 (Fig. 4.13). Of note,the pH of the stripping phase was not altered due to theaddition of KCl and NaCl.The recovery of LP activity and protein content increased with

increasing concentration of KCl and NaCl; however, the activity decreased for KCl and NaCl concentrations above 1.0 and 0.75 M, respectively (Fig. 4.13). The maximum recoveryof LP activity occurred when using 1 M KCl (38%) and 0.75 M NaCl (11%). Such increased back-extraction efficiency and reduced activity with an increase of salinity forthe stripping solution have also been observed for papain(Mathew and Juang, 2005) and lipase (Yu et al., 2003). The lower recovery observed for NaCl compared to KCl is due to the lower water structure breaking characteristic of Na^+. K^+ ions are better water structure breaking ions than Na^+. Even though the water content of the RM was found to decrease drastically with increasing salinity, the recovery of the LP was not significant, suggesting that LP strongly interacts with surfactant molecules. Hence, an alternate approach for backextraction was pursued.

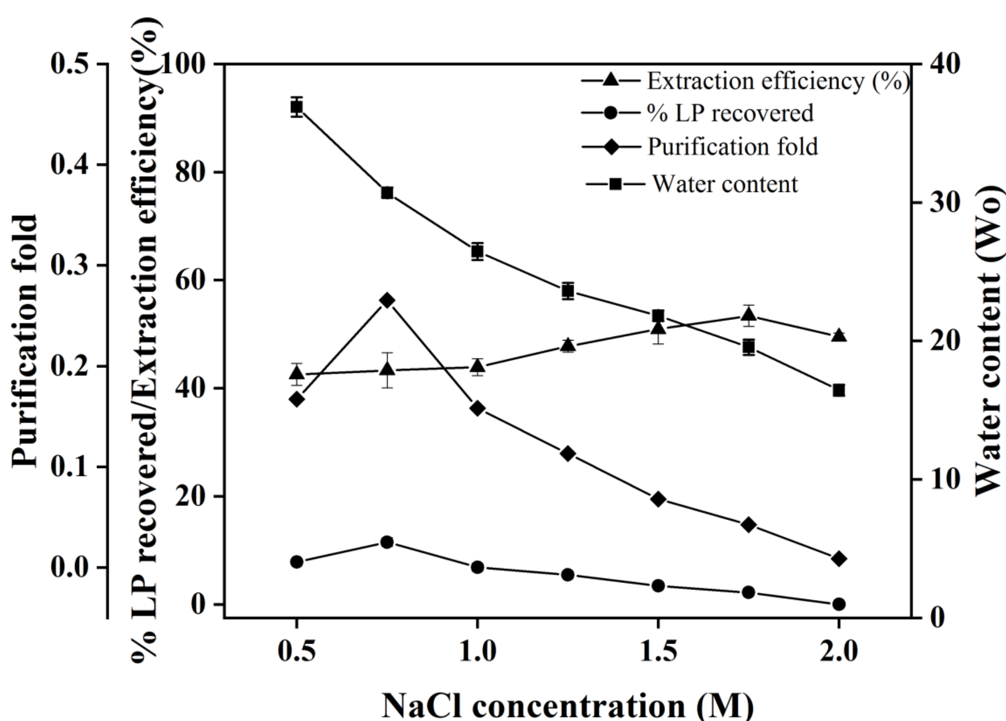


Figure4.13a: Effect of the stripping solution's NaCl concentration on back-extraction efficiency, LP recovery, water content, and purification fold

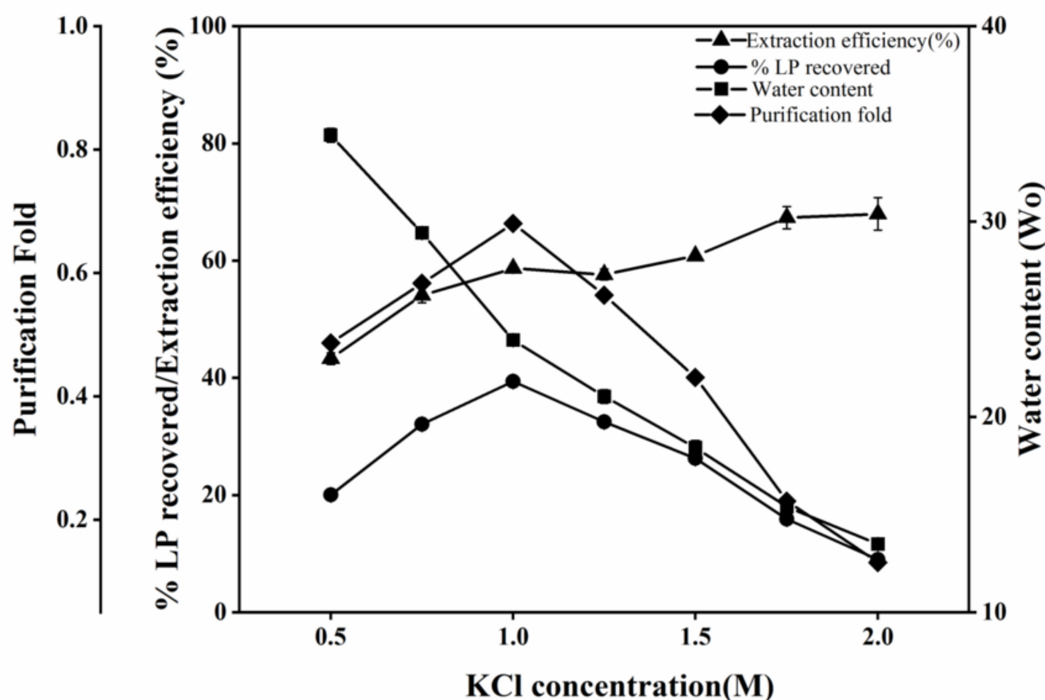


Figure4.13b: Effect of the stripping solution's KCl concentration on back-extraction efficiency, LP recovery, water content, and purification fold

4.2.7 Effect of counterion surfactant concentrationon on Back extraction

Attempts were made to improve the back extraction of LP by incorporating both short- and long-chain alcohols such as isopropyl alcohol, ethanol, hexanol, and octanol to destabilize the RM (Mathew and Juang, 2007). But, loss ofactivity was observed for LP in the presence of alcohol (Fig. 4.14), as was also observed for papain (Mathew andJuang, 2005). Hence, the addition of cationic surfactantswas chosen as an alternative approach, as employed previously(Jarudilokkul et al., 2000). AOT head groups will bind with cationic surfactants through electrostatic attractiveforces, resulting in the destabilization of RM, thereby leading to the release of LP and water. The cationic surfactants TOMAC and CTAB were added at different concentrations (millimoles per L of RM phase) along with 10% hexanol (to aid cationic surfactant solubility) to the organic phase obtained from the forward extraction (using 90 mM AOT and 8 mM Tween 80) and an aqueous stripping solution with a pH of 10.5 and containing 1.0 M KCl. LP was not recovered by adding 100 mM TOMAC to the RM phase since LP was inactivated by strong

electrostatic interactions between TOMAC and LP at pH 10.5. However, LP was successfully back-extracted through the addition of CTAB, with the LP recovery increasing with an increase of CTAB concentration.

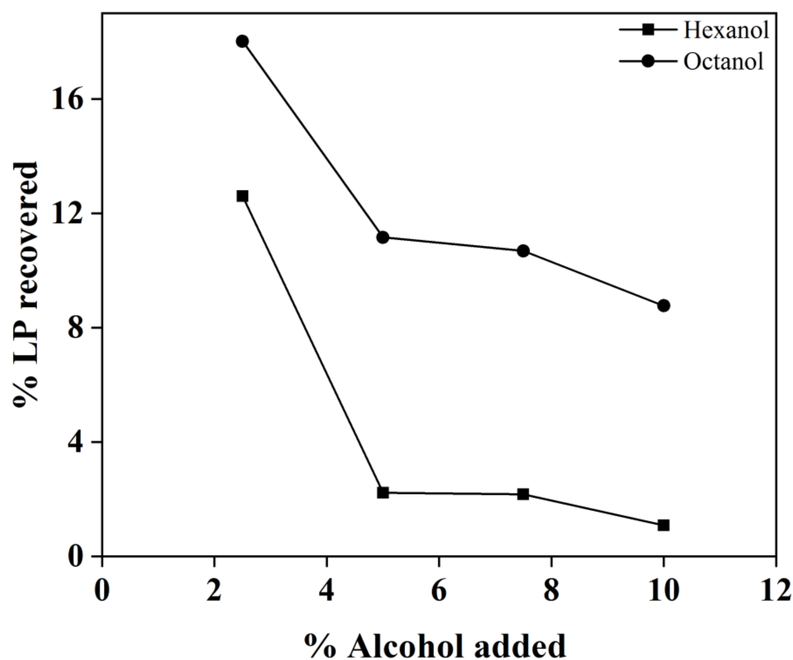


Figure 4.14: Effect of alcohol concentration in RM phase on LP recovery during back-extraction.

Maximum back-extraction efficiency of 95.5% and LP activity recovery of 66% was achieved upon the addition of 60 mM CTAB to the RM phase, and a significant amount of CTAB AOT surfactant precipitate was collected at the liquid–liquid interface for 50–70 mM CTAB. However, the purification factor remained at <1.0 for all of the back-extraction experiments due to the loss of LP-specific activity during the process. Precipitation was reduced when using CTABat concentrations >70 mM; however, an increase in CTAB concentration lowered the recovery of LP. Simultaneously, the water content of the organic phase also was reduced as the CTAB concentration was increased up to 80 mM (Fig. 4.15), but underwent an increase when the CTAB concentrationwas increased above 80 mM. Higher concentrations of CTAB may have increased RM formation, thereby leading to an increase in water contentand

increased retention of LP. A favourable electrostatic attractive force between CTAB and LP under the conditions used for back extraction would further hinder the release of LP from the RM.

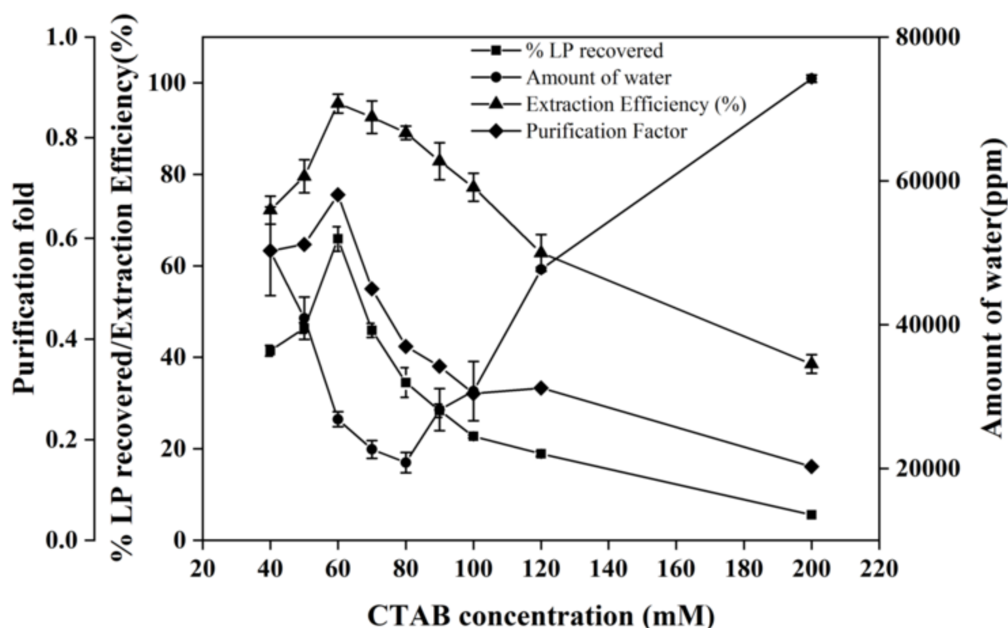


Figure 4.15: Effect of CTAB concentrationin RM phase on extraction efficiency, LP recovery, water content and purification fold during back extraction.

4.2.8 CD Spectral Analysis of LP Recovered through RME

CD analysis in the far-UV region of LP recovered from RME and in the original aqueous solution (0.10 M phosphatebuffer, pH 8) was performed to identify changes of secondary structure that may have occurred as a result of RME. Since bovine LP is a helical protein with 20 alpha-helices and two short beta-strands (Sharma et al., 2013), CD spectra of LP before and after extraction had a well defined negative band at 208 nm arising from the helices(Watanabe et al., 2000); but, LP recovered through RME had a lower ellipticity (Fig. 4.16). In addition, RME treatment resulted in a red, or bathochromic shift of LP's CD spectrum, a result also observed for LP in the presence of small amounts of surfactant (Miles and Wallace, 2016).

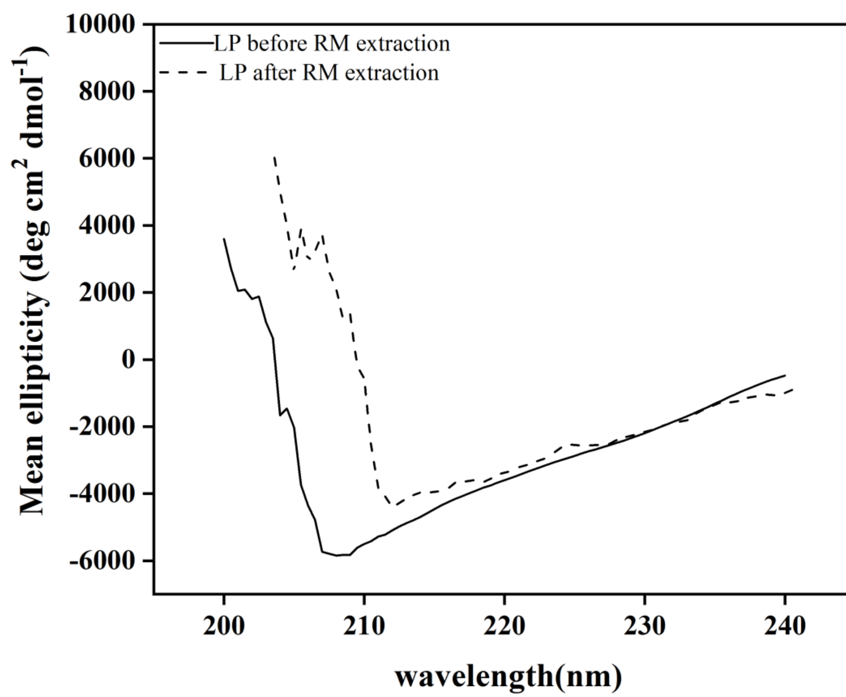


Figure 4.16: Far-UV CD spectra of LP before and after RME of pure LP.

Part three: Mixed surfactant-based Reverse micelle extraction of Bovine Lactoperoxidase from whey

4.3 Mixed surfactant-based Reverse micelle extraction of Bovine LP from whey

The extraction of LP using a pure aqueous solution is not reflective of the process using whey. As whey contains other proteins and ions that can interfere and affect the extraction process, the parameters/variables for extraction used in whey would be different. The objective is to selectively extract the LP leaving behind other proteins. This was achieved by studying the various important intrinsic and extrinsic variables of the RME process.

The extraction experiments were initially performed withAOT alone in the organic phase, and whey was adjusted to pH 6. Similar to pure LP studies,here too it was found that LP was denatured/inactivated at pH 6 in the presence ofAOT. This is because of the strong electrostatic interaction between AOT head groups and positively charged amino acid species on LP at pH 6. Thus, extraction studies were carried out with acid wheyat pH 8 by the addition of non-ionic surfactants.

4.3.1 Effect of Triton series on forward extraction

Three different Triton series surfactants, namely, TritonX-45 (TX-45), Triton X-100 (TX-100) were used. The increase in the concentration of TX-45 and TX-100with AOT increases the LP extraction efficiency (Fig. 4.17). The water content in theorganic phase increased with the increasing concentration of Triton series surfactants and also showed marked differences corresponding to the POE chain lengths. The reverse micelles formed with TX-45 showed lesser water content as compared with TX-100. The differences inthe polar head of Triton surfactants are reflected in LP extraction efficiency as well. Triton series surfactants adsorb at AOT micelle interfaces due to the interaction between the AOT and POE chain(Yamada at al.1993). They reside in the interfacial region by either immersion of the polar headgroup into the water/aqueous pool or their polar headgroup lies in the vicinity of polar head groups of AOT (Kundu Paul 2013). Both these sites of immersion of nonionic surfactants result in the expansion of the micellar interface and anincrease in the hydrodynamic radius of reverse micelles.Changes in hydrodynamic radius upon

the addition of anionic surfactant (Triton) have been observed in earlier studies, (Yamada at al.1993) which were further correlated to the water content and extraction efficiency. Accordingly, the increase in water content was observed in the present study (Table 4.2) and resulted in a larger number of reverse micelle aggregates and increased LP extraction efficiency. Moreover, the interfacial location of Triton series surfactants reduces the repulsion between AOT moleculesthat might help in stable mixed reverse micelles formation. However, beyond certain concentrations of Triton, this reduction of repulsive forces can result inthe formation of smaller reverse micelles with lesser LP extraction capabilities. Thus, beyond 20 mM, LP extraction decreases. TX-45 with a smaller polar headforms lesser rigid reverse micelles as compared withTX-100 (Dhar et al. 2009). Hence, TX-45 has lesser extraction efficiency than TX-100.

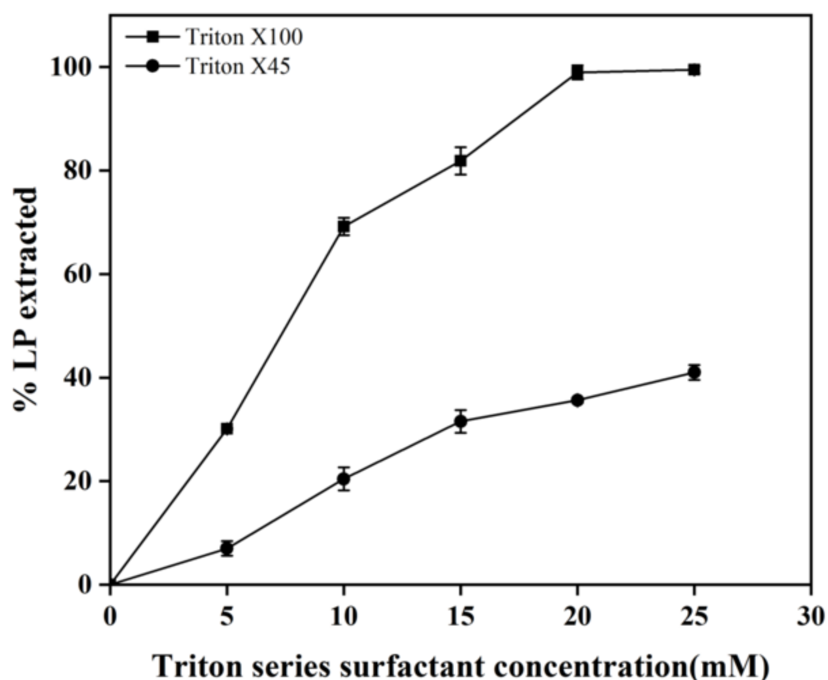


Figure 4.17: Forward extraction of Lactoperoxidasefrom acid whey at pH 8 with the reverse micelles formed AOT 100mM and Triton series non-ionic surfactants

4.3.2 Effect of Span (sorbitan esters) series on forward extraction

Among the Span series of surfactants, Span 60 (Sorbitanmonostearate) and Span 80 (Sorbitan monooleate) werestudied for LP extraction characteristics. Span series of surfactants are hydrophobic surfactants with identicalpolar head groups that consist of sorbitan. The Spanseries differ only in their hydrophobic alkyl ester tail, andthey do not contain the POE chain. The Span series of surfactants, though hydrophobic,are known to be solubilized into the water pool of the reverse micelles, because the aqueous pool ofthe reverse micelles is in a hydrophobic/structured state(Yamada et al.1994) and differs from the bulk aqueous phase. The increasingconcentrations of Span resulted in decreasing water/aqueous content of reverse micelle (Table 4.2), and also,lesser AOT is exposed to electrostatic interactions withLP. The solubilization of Span into the water pool resultsin lesser room for LP solubilization or its accommodationat the interface and thus reduces its extraction. Reduced extraction of proteins in the presence of Span 60 has beenreported for lipase and α-chymotrypsin(Yamada et al.1994)with constant water content before and after extraction when compared with the Tween series.However, in the present study,water content was reduced with an increasing concentrationof Span with a simultaneous decrease in extraction capacities (Fig 4.18). This could be because of the differencesin extraction procedures followed. The former study was performed using only pure proteins with NaClas an electrolyte and free of impurities, whereas LP extraction in the present study was performed using natural sources with contaminating substances. These contaminating substances can further occupy spaces available for LP solubilization; had it been the only proteinin the aqueous phase.

4.3.3 Effect of Tween (Polysorbates) series on forward extraction

Tween series of surfactants are derived from Spanproducts by adding polyoxyethylene chains to the head. Polyoxyethylene (20) sorbitan monolaurate (Tween 20),Polyoxyethylene (20) sorbitan monostearate (Tween 60),and Polyoxyethylene (20) sorbitan monooleate (Tween80) were the Tween series used in the study.

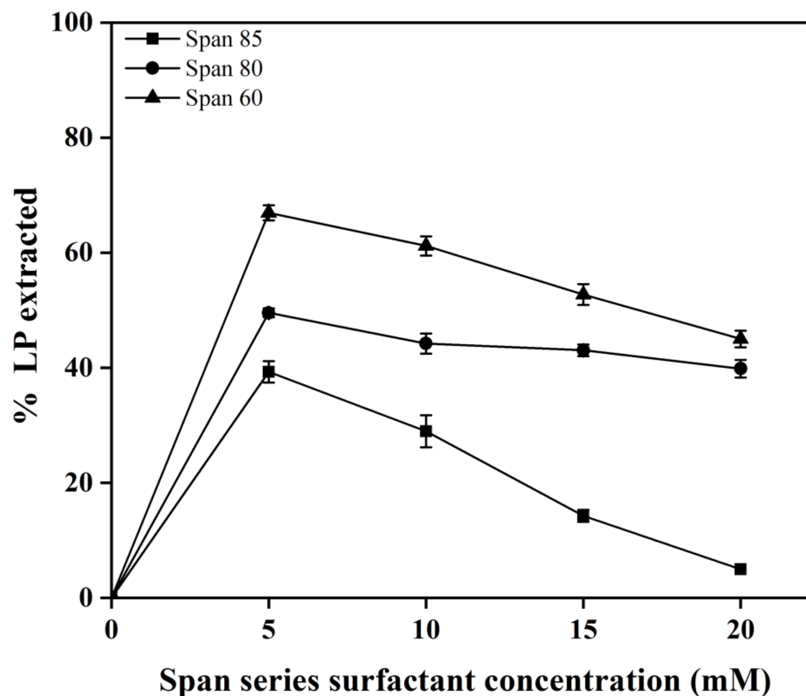


Figure 4.18: Forward extraction of Lactoperoxidase from acid whey at pH 8 with the reverse micelles formed using AOT 100 mM and span series non-ionic surfactants

These surfactantshave the same head group but have different lengths of hydrocarbon chain on their tail, that is, thefatty acid ester tail. The similarity in the head group should ideally give the same LP extraction efficiency asthe head groups interact more with the solubilized protein/water(Chatterjee et al.2006). However, the LP extraction efficiency increased with an increasing tail length of the Tween series (Fig. 4.19). Similarly, water content also increased with increasing hydrocarbon moiety in the hydrophobictail of the Tween series surfactants (Table 4.2). Theincreased water solubilization was observed with increasing hydrocarbon chain length from Brij 56(C16) to Brij76(C18) as well,(Paul and Mitra 2005) which concludes that the contributionof hydrophobic moiety cannot be neglected in water solubilization capabilities. The increasing water content of the reverse micelles with the Tween series also increases the LP extraction (Fig. 4.19). Tween series arrange themselvesat the interface of AOT surfactants as Triton seriessurfactants and perform the extraction

similar to thereverse micelles formed by Triton series. Tween 20 iscomposed of medium-chain carbon tail (12), that is,lauric acid, whereas Tween 60 and Tween 80 have longcarbon chains (18) on their hydrophobic end. Thus,Tween 60 and Tween 80 perform better than Tween20 during LP extraction. A similar better performance ofTween 80 over Tween 60 and Tween 20 has beenobserved for the cellulase extraction in the presence of Rhamnolipid anionic surfactant (Peng et al. 2016). The significant differencesin extraction efficiency of Tween 60 and Tween80 may be attributed to the presence of unsaturatedcarbon–carbon double bond on Tween 80, as oleic acid is an unsaturated fatty acid. Tween 80 is known for its strong stabilization characteristics and thus offers better LP extraction capabilities. The double-bonded carbon tailcan alter its packing at the interface leading to stablereverse micelles with enhanced water solubilization characteristics (Paul and Mitra 2005). Moreover, the presence of this double bondimposes stereochemical constraints on the system resulting in hydrocarbon chain bends. This increases thevolume of the hydrocarbon chain while decreasing itslength.

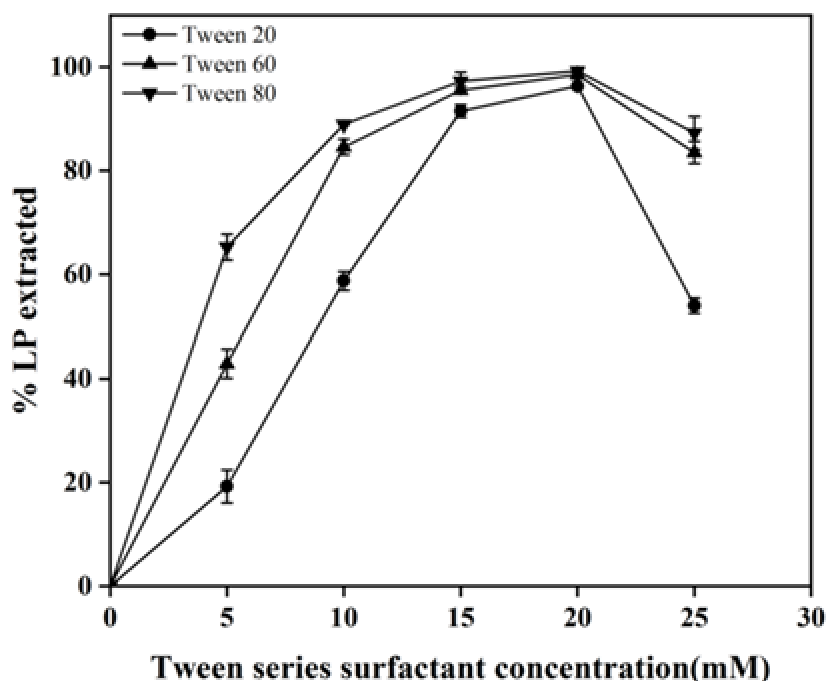


Figure 4.19: Forward extraction of Lactoperoxidase from acid whey at pH 8 with AOT 100mM and Tween series non-ionic surfactants

These factors result in decreased interaction between hydrocarbon chains of surfactants and showed repulsion with increased water solubilization.The LP extraction capabilities of mixedreverse micelles were found to increase with an increasingchain length of the head and tail of the surfactantsas observed for Triton and Tween series surfactants. Both the hydrophilic head group and hydrophobic tail of the added nonionic surfactants equally affected the water solubilization and LP extraction capabilities.However, the extent of packing of nonionic surfactantsat the interface is the major driving factor forprotein extraction. Though the Span series have a lesser hydrophilic-lipophilic balance (HLB) value than the Tweenseries, Tween series performed better than other non-ionic surfactants. Tween 80 has a better ability to undergobiodegradation due to the presence of the carbon-carbon double bond, which made them environmental-friendly system. Since the Tween 80 outperformscompared with other nonionic surfactants tested for LP extraction, Tween 80 is chosen as a non-ionic surfactant to be mixed with AOT to form mixed surfactant-based reverse micelles for the extraction of LP from acid whey.

Table 4.2: Water content in Reverse micelles formed with non-ionic surfactants and AOT for LP extraction

Reverse micelle system	Water content (W_o) at 5mM	Water content (W_o) at 10mM	Water content (W_o) at 15mM	Water content (W_o) at 20mM
AOT/ Tween 20	34	48	63	82
AOT/ Tween 60	36	50	67	105
AOT/ Tween 80	46	58	71	112
AOT/ Tx45	19	22	35	40
AOT/ Tx100	26	30	39	46
AOT/ Span 60	21	16	15	13
AOT/ Span 80	20	18	16	15
AOT/ Span 85	19	16	16	15

Note: Water content of AOT (100 mM) alone = 20

4.3.4 Effect of acid whey pH on LP forward extraction

The whey pH is an important factor that affects the LP extraction into the reverse micelles. The whey pH was adjusted between 7 and 10.5 and tested for LP extraction using 100mM AOT and 20mM Tween 80. The concentrations of ionic and non-ionic surfactants were fixed based on the literature. The LP extraction efficiency, activity recovered, purification fold achieved, and water content in the organic phase during theforward extractionis represented in Fig 4.20. In general, the proteins may be get extracted into the reverse micelles till the pI of the proteins. However, the addition of non-ionic surfactants can shift the optimal pH required for protein solubilization, i.e protein can be extracted beyond its pI (Dekker et al., 1989). It is also reported that the addition of non-ionic surfactants can change the width of optimum pH for enzyme activity in reverse micelles (Hossain et al.1999). LP is a basic protein with a pI of about 9-9.5. Initially, as the pH increases, LP extraction efficiency was found to increase and reach 100% at pH 8. But, the contaminating proteins were also extracted into the reverse micelles resulting in lower purification folds at lower pH. Even though major acidic whey proteins like α-lactalbumin, β-lactoglobulin, bovine serum albumin areretained in the aqueous whey phase due to their negative charge, the Lactoferrin and Immunoglobulins may get extracted along with LP due to their positive charge at higher pH and cause the interference to the extraction and purity of LP into the reverse micelles. The Lactoferrin (pI: 8.0 to 8.5) and Immunoglobulins (pI 5.5 to 6.8) remain positively charged above pH 8 and forms an electrostatic interaction with negatively charged AOT.The addition of non-ionic surfactants induces an increase in hydrophobic forces that improves the extraction of LP. 50% of non-polar amino-acids that constitute LP interact with hydrophobic tails of non-ionic and ionic surfactants through hydrophobic interactions.The LP extraction efficiency was found to decrease as the pH of whey approaches the isoelectric point of LP and beyond.As the pH rises above 8, the positive charge density on LP, which was responsible for the interaction with the RM of AOT, reduces. Although the maximum LP extraction efficiency was observed at pH 8, the highest purity of 5.8 was achieved at pH 9.5. The purification fold improves as the lesser amount of contaminating proteins being extracted into the reverse micelles due to their reduced interaction with

the AOT/Tween 80 RM. Farther away from its pI (pH >9.5), LP extraction efficiency significantly decreases along with purity, since LP becomes negatively charged and cannot form a stable electrostatic interaction with AOT.The water content in the organic phase also follows the trend of extraction efficiency, reaching a maximum at pH 8 and reducing beyond pH 8.The size of the reverse micelles also increases corresponding to the water content and LP extraction. The larger size protein extraction into the water core of RM requires the adaptation of reverse micelle size to that of protein (Hilhorst et al. 1992, George and Stuckey, 2010). It was also observed that the size of the RM increases from 8.2 nm for empty RM without protein solubilization to a maximum size of 146.77 nm at pH 8. The increasing water content justifies the increasing LP solubilization into the water core of the reverse micelles and thereby its extraction. Such an increase in reverse micelle size with increasing water content has been previously observed during solubilization of lipase in AOT/ Tween85 system (Hossain et al.1999). The size and water content results obtained are similar to the studies conducted on the extraction of peanut protein(Limin et al. 2018). LP recovery also follows forward extraction characteristics with maximum recovery at pH 8 and decreasing to 17.98% at pH 9.5 and 4.10 % at pH 10.5.

Table 4.3: Size of Reverse micelles formed with acid whey at different pH with AOT 100mM and 20mM Tween 80

Reverse micelle system with whey pH	**Size(nm)**
Empty Reverse micelles(without protein)	8.23nm
pH 7	38.90
pH 7.5	98.92
pH 8	146.77
pH 8.5	75.44
pH 9	57.71
pH 9.5	40.59

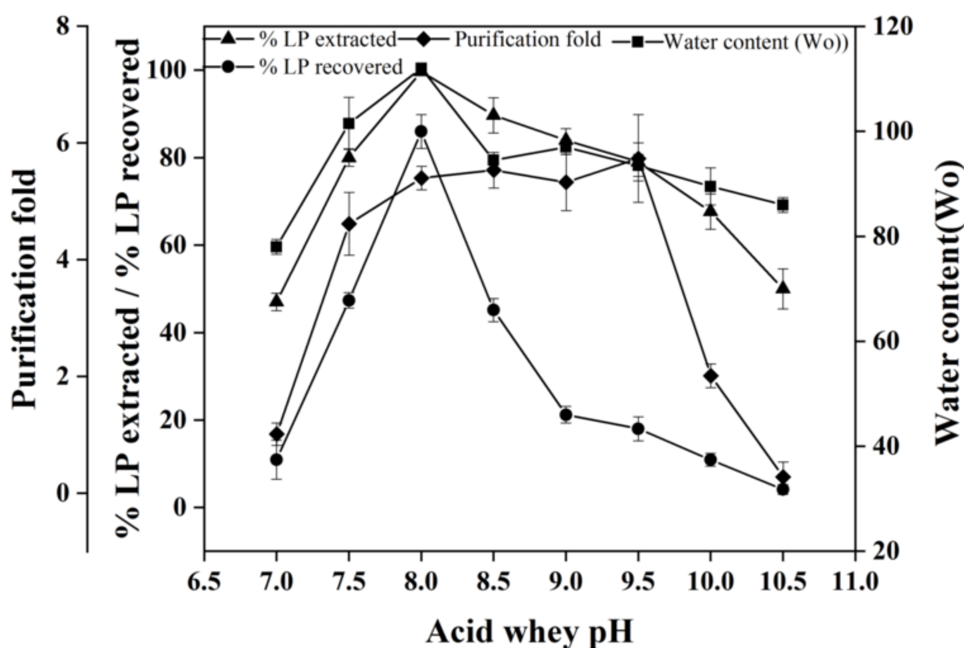


Figure 4.20: Effect of acid whey pH on Lactoperoxidase forward extraction, activity recovery and purification fold using the RM of 100 mM AOT, 20 mM Tween 80 in isooctane system.

4.3.5 Effect of acid whey ionic strength on LP forward extraction

Protein extraction into the reverse micelles is also dependent on the ionic strength of the aqueous phase. The ions aid in the formation of stable reverse micelles by reducing the repulsions between the surfactant headgroups (Chen et al. 2019) till a specific ionic concentration in the aqueous phase and helps to improve the forward extraction of the desired proteins. However, protein extraction capabilities are reducing beyond a critical concentration as the added ions bind onthe surfactant headgroups and result in the formation of smaller size reverse micelles (Andrews and Haywood, 1994). The effect of ionic strength and ion type on LP forward extraction was studied by the addition of 0.1- 0.7M NaCl and KCl to the acid whey at pH 9.5 and mixed with organic phase containing 100mM AOT and 20mM Tween 80. Back extraction was performed using carbonate buffer at pH 10.5 with 1M KCl for the protein analysis. Fig.4.21 represents the effect of KCl and NaCl on the forward

extraction parameters of LP. As the KCl and NaCl are neutral salts; they do not change the pH of whey upon their addition. The precipitation of surfactants was observed at the interphase with the addition of 0.1 and 0.2 M NaCl and also the addition of NaCl did not result in the formation of the reverse micelles. However, the stability of RM and the extraction efficiency were found to increase by the addition of 0.2 M KCl. The extraction efficiency was found to decrease with further increasing concentration of K^+ ions since the surfactant head groups are masked by K^+ ions and leaving less space for the LP interaction. The maximum LP extraction efficiency was observed with the addition of 0.2 M KCl.

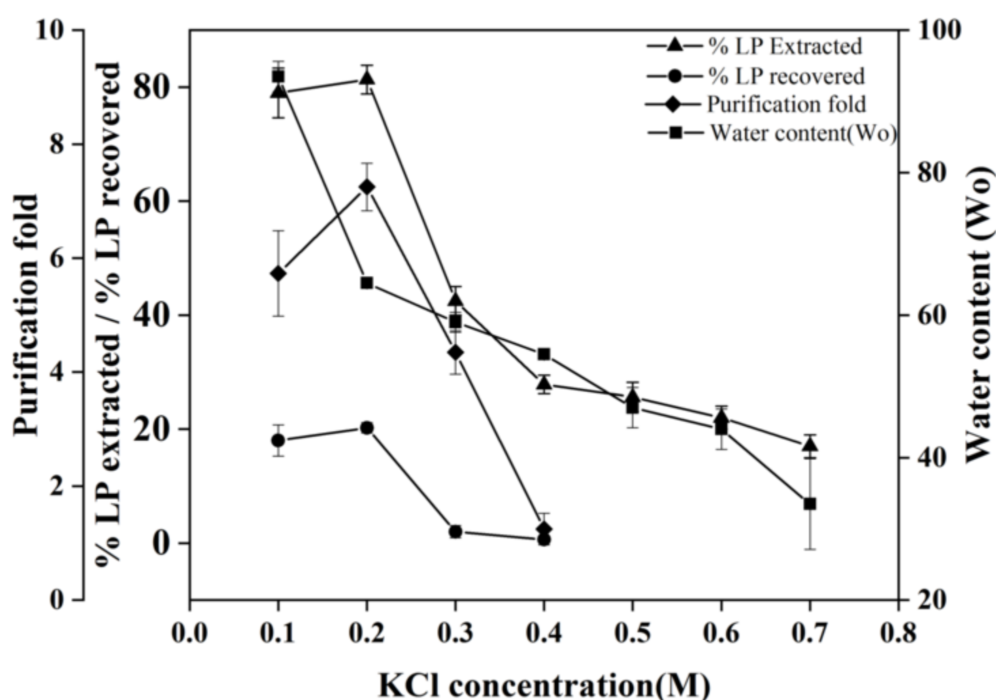


Figure 4.21a:Effect of KCl added to acid whey at pH 9.5 on Lactoperoxidase forward extraction, activity recovery, and purification fold using the reverse micelle system of 100mM AOT and 20 mM Tween 80 in isooctane

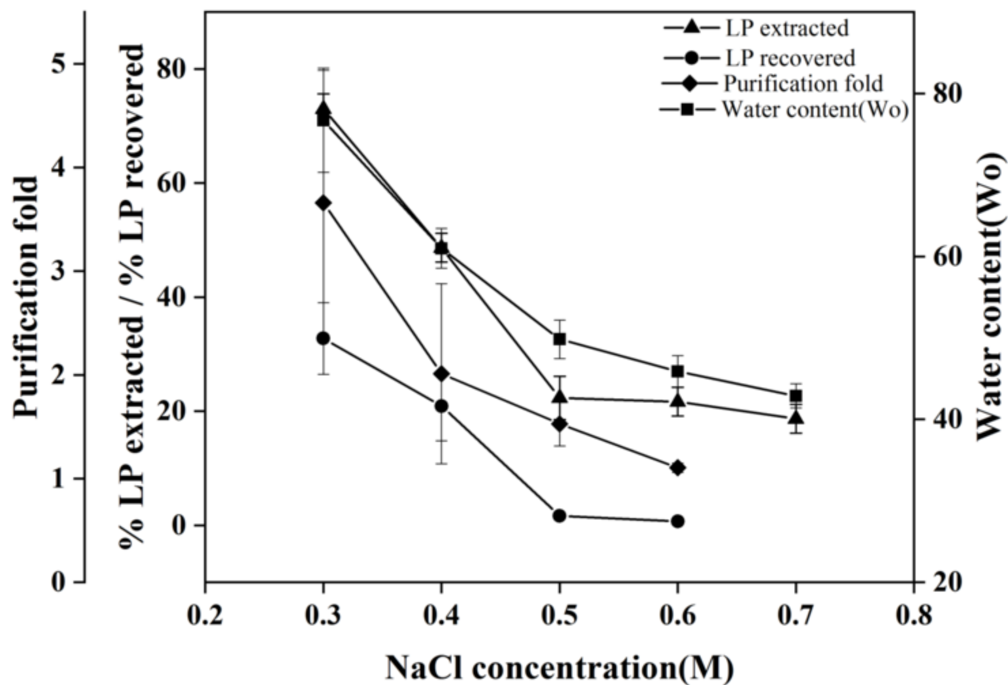


Figure 4.21b:Effect of NaCl added to acid wheyat pH 9.5 on LP forward extraction, activity recovery, and purification fold using the reverse micelle system of 100mM AOT and 20 mMTween 80in isooctane

The ion species of the added salt also greatly influence the extraction efficiency. Anion species, particularly chloride ions, have the least effect in improving extraction efficiency as no interaction occurs with anionic AOT. The effect of cationic species depends on the ionic radius of the cation, and Na^+ has smaller radii than K^+(Kinugasa et al, 2003). Thus Na^+ions have a lesser screening effect than K^+ ions. This is also evident from the water content as NaCl has more water content than KCl. The purification fold was also found to increase with the addition of 0.2MKCl due to the lesser water content with smaller-sized RM, which could not able to accommodate other proteins. The negatively charged other major whey proteins get extracted into the reverse micelles and result in lower purification folds, as more cationic species accumulated from the added salts block the anionic headgroups of AOT. However, the effect of ions on stable reverse micelle formation was observed at a very low concentration.

4.3.6 Effect of surfactant ratio on LP forward extraction

Even though maximum purification fold was achieved by increasing the aqueous phase pH to 9.5, the LP extraction efficiency was reduced beyond pH 8. Hence, an attempt was made to modify the RM surface forces at a whey of pH 9.5 with 0.2M KCl by increasing the surfactant concentration of both the ionic surfactant AOT and nonionic surfactant Tween 80 at the literature reported ionic to non-ionic surfactant molar ratio of 5 for the maximum LP extraction at pH 8. Thus, the ionic to non-ionic surfactant molar ratio was kept constant at 5, and an increase in the concentration of both the surfactants was studied to improve the LP extraction efficiency, and the results obtained were given in 4.22.

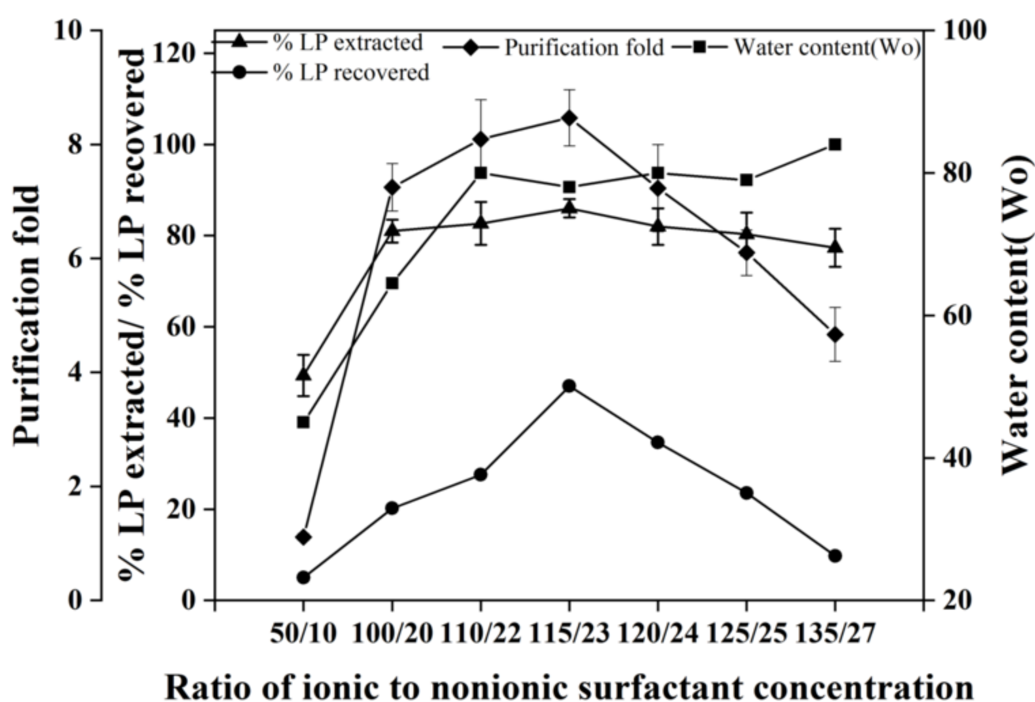


Figure 4.22:Effect of surfactant concentrationsin the organic phase on Lactoperoxidase forward extraction, activity recovery, and purification fold from acid whey at pH 9.5 with 0.2 M KCl.

The LP extraction efficiency was found to increase with the increasing concentration of both the surfactants. The least extraction capabilities were observed at 50:10, and a

maximum of 86% LP was extracted with 115:23 (mM) of AOT: Tween80. As the concentration of both the surfactants increased, the number of RM in the organic phase increase thereby increasing extraction. In addition to the electrostatic interaction of LP with AOT, the increasing concentration of non-ionic surfactants induces the hydrophobic interaction with the non-polar amino-acids of LP that further increases the LP extraction efficiency. Along with extraction efficiency, the purification factor was also found to increase till the surfactant ratio of 115:23 (AOT: Tween 80). A slight decrease in LP extraction efficiency was observed beyond 115:23, whereas a sharp reduction in activity recovery was observed due to the increase in the extraction of contaminating other proteins. The reduction in the LP extraction efficiency and purity with a slight increase in water content of the RM between the ratios of surfactant from 115:23 to 135:27 justifies the extraction and accumulation of protein impurities. Surfactant precipitation at the interface was observed beyond the surfactant ratios of 135:27.

4.3.7 Effect of phase volume ratio on LP forward extraction

The extraction and activity recovery of LP may be improved by adjusting the volume ratio of the organic phase RM phase to the aqueous whey phase. The effect of different volume ratios (organic phase to aqueous phase)was studied by increasing between 0.5 and 2, and their effects on LP extraction and activity recovery are shown in Fig 4.23. For the economical RM operation, the phase volume ratio should be small for forward extraction and larger for backward extraction. During forward extraction, the increasing volume of organic phase increases the number of RMs for the extraction of LP. Thus, increasing phase volume ratio resulted in increasing LP extraction. LP extraction efficiency was found to increase from 76 % at 0.5 ratio to 86% at 1,however, beyond phase volume ratio 1,the extraction efficiency did not improve greatly. A maximum of 92% LP was extracted at a phase volume ratio of 2 with 62 % recovery and about 88% extraction and 60% recovery from a phase volume ratio of 1.25. Hence, for economic reasons, the phase volume ratio of 1 was considered for further studies.

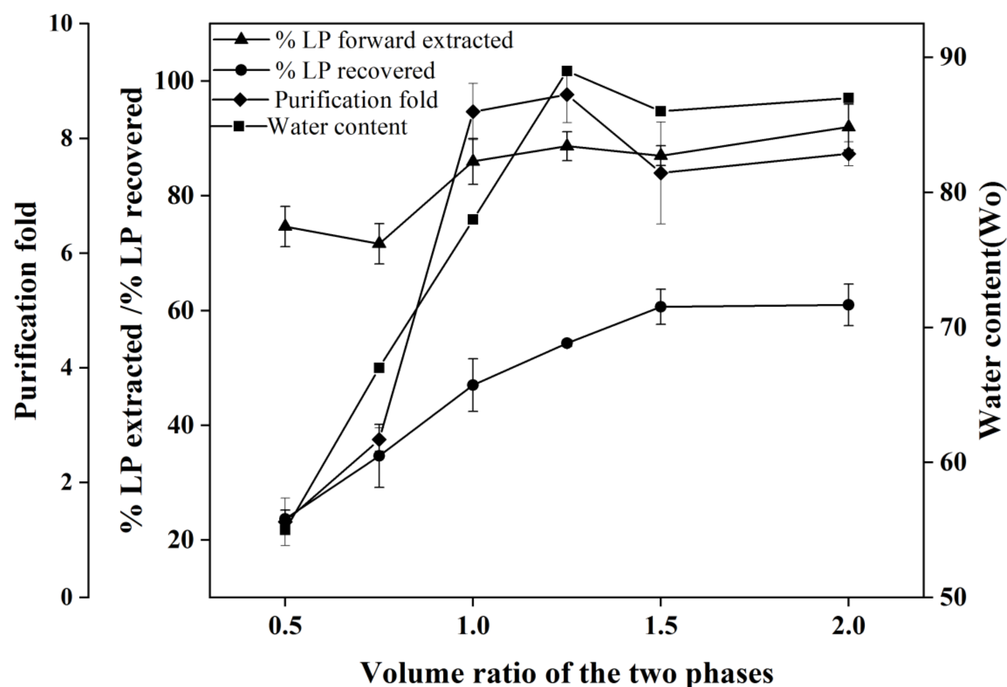


Figure 4.23:Effect of volume ratio on the forward extraction Lactoperoxidase, activity recovery, and purification fold for the reverse micelle system of 100 mM AOT and 20 mM Tween 80 in isooctane.

Back Extraction/LP recovery

Recovery of proteins that are solubilized from the RM termed as back extraction is often a rate-limiting step in the RM extraction process. The protein behaviour during the forward transfer at different conditions helps in deciding the conditions required for their recovery. The recovery during back extraction is usually facilitated by reducing the interactions that exist between the RM and the targeted solute that were initially responsible for the forward extraction. Often, changes in the pH and ionic strength of the new stripping aqueous phase can alter the interactions between protein and surfactants. This aids in the recovery of the solubilized protein from RM. Accordingly, the recovery of LP was performed by varying the pH, salt concentration

of the stripping phase. Further, the effect of counter-ionic surfactant for the back extraction of LP was studied to improve the back extraction efficiency.

4.3.8 Effect of aqueous phase pH on LP backward extraction

Forward extraction was performed with optimized parameters, i.e., 115 mM AOT and 23 mM Tween 80 in the organic phase and acid whey at pH 9.5 with 0.2M KCl by maintaining the organic phase to aqueous phase ratio at 1 (Fig 4.24). As the maximum forward extraction efficiency was observed at 8.0 pH and the extraction was found to decrease beyond pH 8.0, the pH effect on the stripping phase was studied by varying the pH between 7.0 and 11 using 0.1M buffers without the addition of KCl. This was done to understand the specific influence of pH alone on LP recovery.

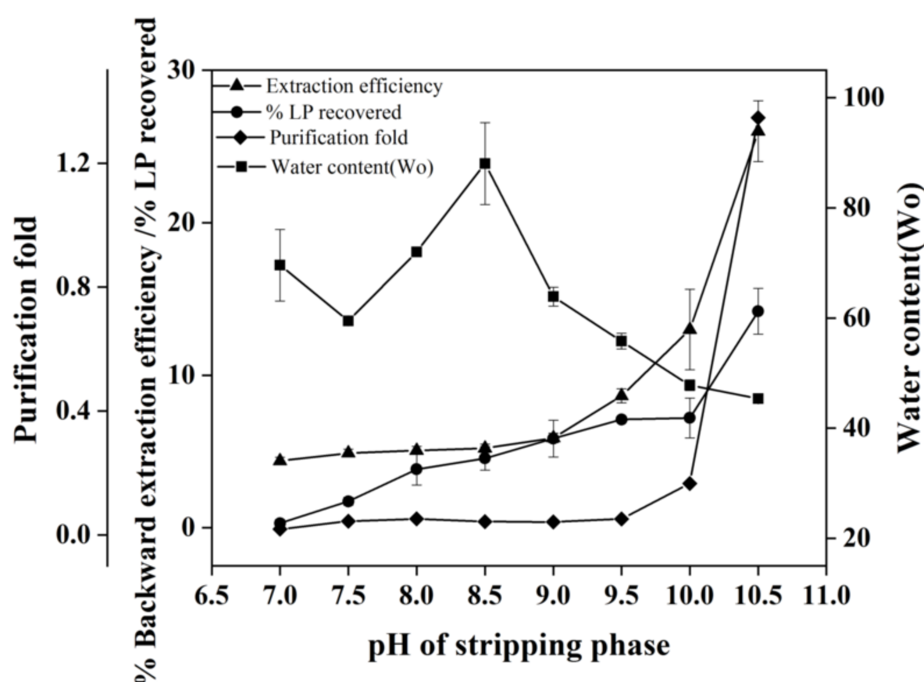


Figure 4.24:Effect of stripping phase pH by using various buffers with 1 M KCl on backward extraction efficiency, activity recovery, and purification fold of LP from the RM phase of 115 mM AOT, 23 mM of Tween 80 system.

The inactivation of LP was observed at pH 11 as reported in the literature (Boscolo et al. 2009). Since the solubilization and extraction of LP were favored at pH close to isoelectric point (pI); back extraction was majorly possible above the isoelectric point. The change in the aqueous stripping phase pH induces a change in the charged amino

acid species of LP. Above its isoelectric point, LP is predominantly negative, and this reduces the interaction between the negatively charged head groups of AOT and LP. Fig 4.24 shows the effect of stripping phase pH on LP recovery. The least amount of LP was recovered in the pH range 7.0 to 8.5 and increased beyond 8.5. Maximum recovery was observed at pH 10.5. Recoveries could not be achieved beyond 14.19%; as some residual electrostatic interactions could still exist between AOT and LP. Moreover, hydrophobic interactions between the surfactant mixture (AOT and Tween 80) and LP are independent of pH, and this interaction could be the major reason for lower recoveries. Further, the number of RMs in the system may reduce as the RMs lose their micellar structure, the proteins and water molecules are transferred from RMs to the stripping aqueous phase, which was evident from the reduction of water content with the improvement of back extraction efficiency and LP recovery.

4.3.9 Effect of aqueous phase ionic strength on LP backward extraction

The influence of increasing ionic strength in the stripping phase was studied using KCl at concentrations between 0.5 to 2M and the observed results are shown in Fig 4.25. The organic phase with solubilized protein obtained during the forward extraction (performed with 115 mM AOT and 23 mM Tween 80 in isooctane system with acid whey pH of 9.5 and addition of 0.2M KCl was considered for the back extraction. The pH of the stripping phase was maintained at 10.5 using carbonate buffer and the neutral salt, KCl at different concentrations were added to the buffer to study the effect of ionic strength during back extraction. As the KCl concentration increases in the stripping phase, the recovery of LP and purity were found to increase up to the KCl concentration of 1.5M. Beyond that, the LP activity was found to decrease whereas back extraction efficiency i.e. protein recovery increased. Higher salt concentration could hamper the activity of LP and leading to lower activity recoveries even though the back extraction efficiency increased. The presence of K^+ions reduces the repulsion between the AOT headgroups resulting in smaller reverse micelles with reduced water content in the core (Andrews et al. 1994).Thus, LP can be excluded from the water core of reverse micelles along with the reduction in the water content to the aqueous stripping phase. As the RM structure is highly dynamic, the addition of ions forms an electrostatic shield around the surfactant

(AOT) headgroups and may not allow the interaction of LP with AOT, hence the percentage of LP solubilized in the organic phase of RMs reduces. These two effects combined with the stripping buffer at pH 10.5 improve the back extraction of LP.

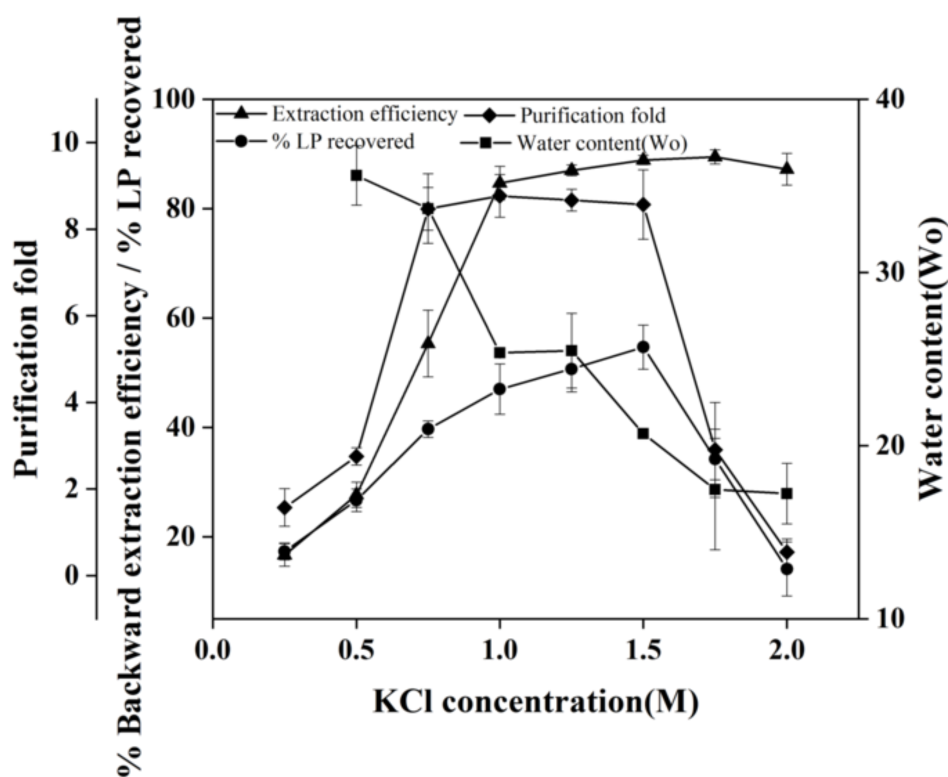


Figure 4.25:Effect of stripping phase(pH 10.5) ionic strength on the backward extraction efficiency, activity recovery, and purification fold of LP from the RM phase of 115 mM AOT, 23 mM of Tween 80in isooctane system.

4.3.10 Effect of counter-ion surfactant concentration on LP backward extraction

Most proteins can be successfully recovered from the reverse micellar phase after forward extraction by tweaking the pH and ionic strength of the stripping phase. However, satisfactory protein and LP activity recovery werenot achieved using the optimized parameters of both pH and ionic strength. Hence, the addition of cationic surfactant (CTAB) to the stripping phase was attempted to improve LP recovery from the reverse micellar organic phase. Forward extraction was conducted using 115 mM AOT and 23 mM Tween 80 as organic phase and aqueous phase consisted of acid whey at pH 9.5 with 0.2 M KCl. The CTAB was added at different concentrations to

the fresh stripping phase constituted carbonate buffer at pH 10.5 and 1.5M KCl.10% hexanol was added to the organic phase to aid in the solubility of cationic surfactant. The results obtained are shown in Fig 4.26.

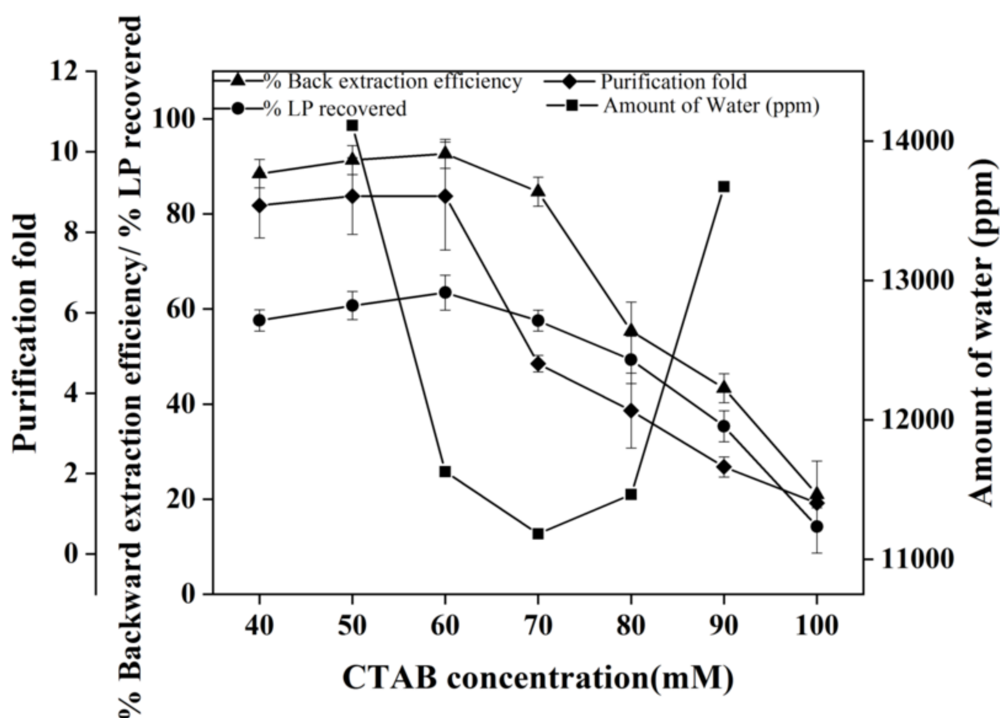


Figure 4.26:Effect of CTAB as counterion in the organic phase on the backward extraction efficiency, activity recovery, and purification fold of LP during back extraction using carbonate buffer at pH10.5 with 1.5 M KCl.

Negatively charged AOT headgroups will form an electrostatic interaction with the added positively charged CTAB. The back extraction efficiency and LP recovery were found to increase with increasing CTAB concentration upto60mM with a maximum back extraction efficiency of 92.6% and 64% LP recovery. Beyond 60mM, the LP recovery and back extraction efficiency decreased. The negatively charged LP interacts favorably with the positively charged CTAB at pH 10.5 of the fresh aqueous phase (stripping phase). At a higher concentration of CTAB (>60 mM), the excess CTAB could form reverse micelles of its own with or without Tween80 and thereby resolubilizing or hindering the back extraction of LP. Similarly, the amount of water solubilized in the organic phase after back extraction decreased upto 70mM CTAB concentration and thereafter increased; justifying the formation of CTAB RMs and LP

resolubilization. Such resolubilization of target protein has been observed in the case of DTAB counterion added to back extract cytochrome C from AOT RM system (Jarudilokkul et al. 1998). This could mean that 60-70 mM AOT is the actual concentrations remaining in the organic phase at equilibrium with the aqueous phase while the remaining AOT being solubilized in the aqueous phase over the course of extraction.

4.3.11 Effect of volume ratio on LP backward extraction

The goal of the back extraction of LP is to concentrate all the protein in the smallest volume during back extraction.This can be accomplished ata higher phase volume ratio, which results in effective concentration and recovery of LP. The effect of phase volume ratio was studied between 0.5 to 2 and the results are shown in Fig 4.26.The organic phase obtained from the forward extraction with LP (organic Phase: 115 mM AOT and 23 mM Tween 80 in isooctane; aqueous phase: acid whey at pH 9.5 with 0.2 M KCl)was considered for the back extraction of LP using the carbonate buffer at pH 10.5, 1.5M KCl and 60 mM CTAB as stripping phase.The extraction efficiencyand LP recovery were found to increase with increasing phase volume ratio and reach a maximum at a phase volume ratio of 1.5, however, the LP recovery does not improve beyond the ratio 1.5 though extraction efficiency was found toincrease.A maximum of 80% LP was recovered with 112% back extraction efficiency and purification fold of 11.266 at a phase volume ratio of 1.5.The available specific interactive forces offered due to the stripping phase pH, ionic strength, and counterion concentration to LP are limited in the aqueous stripping phase, as the volume of the aqueous stripping phase was found at smaller quantity in the higher volume ratios. However, the other proteins may be still extracted at higher volume ratios. Hence, the LP recovery and purification fold are not improved beyond the volume ratio of 1.5. Previous research on nattokinase back extraction from RM has shown that increasing contact time of the two phases from 5 to 20 minutes can lead to 8 times increase in the protein concentration in the aqueous phase (Liu et al. 2006). However, the maximum contact time of 30 minutes was employed for the back extraction of LP and the active enzyme recovery was affected for higher contact time.

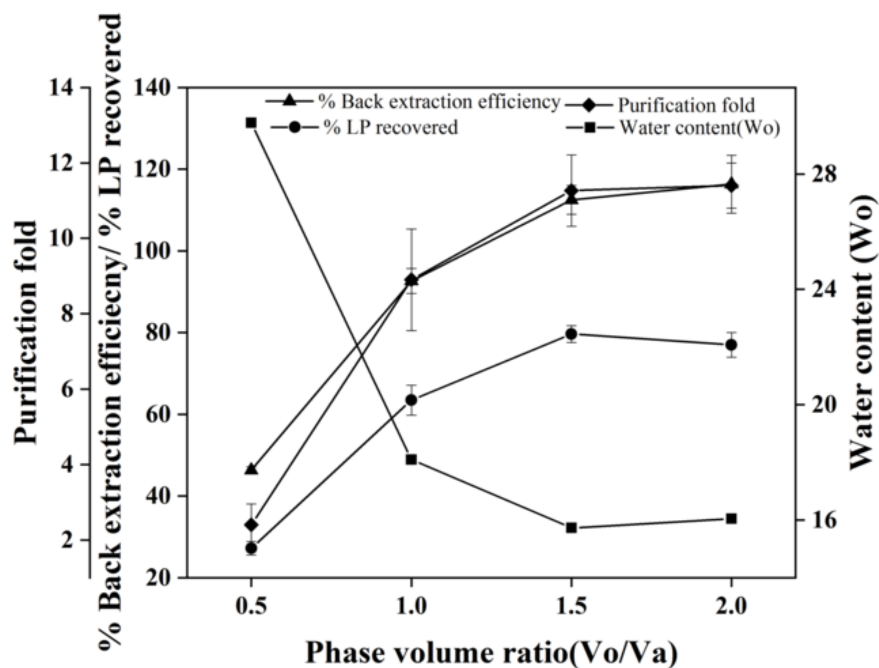


Figure 4.27: Effect of volume ratio on the back extraction efficiency, activity recovery, and purification factor for the back extractionof LP from the organic phase of forward extraction into the stripping phase at pH10.5 with 1.5 M KCl and 60mM of CTAB in the organic phase

4.3.12 RP-HPLC analysis of different samples

RP-HPLC was used to qualitatively analyze LP and other proteins at 412 nm and 226 nm, respectively in the samples of crude whey aqueous phase after forward extractionand aqueous stripping phase after backward extraction. The chromatogram of the aqueous phase after forward extraction clearly shows the major whey proteins left behind after forward extraction and is comparable to the chromatogram of crude whey. The chromatogram of the stripping phase clearly shows the absence of any major whey proteins justifying that the back extracted LP is devoid of most major and minor whey proteins. The chromatogram of acid whey shows LP elution at 11.5min. The chromatogram of the aqueous phase after forward extraction exhibits a small amount of LP which is not extracted. The chromatogram after forward extraction also

shows LP peak at 11.5 and shift peak at 14.5. The stripping phase chromatogram after back extraction shows the shift in retention time from 11.5 in crude whey to 14.5.

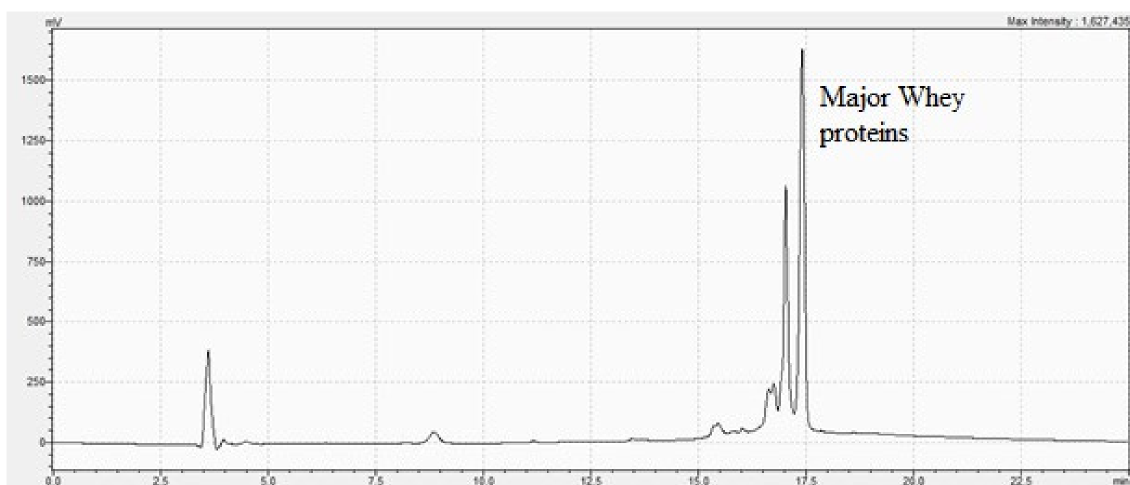


Figure 4.28a: RP-HPLC chromatograms of acid wheyat 226nm

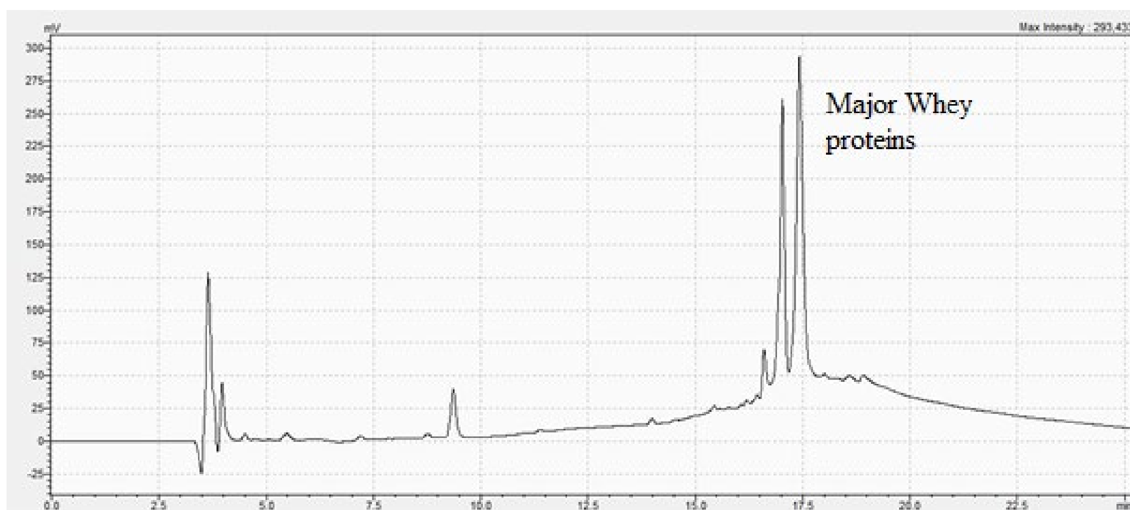


Figure 4.28b: RP-HPLC chromatograms of aqueous phase after AOT/Tween 80 based forward extractionat 226nm

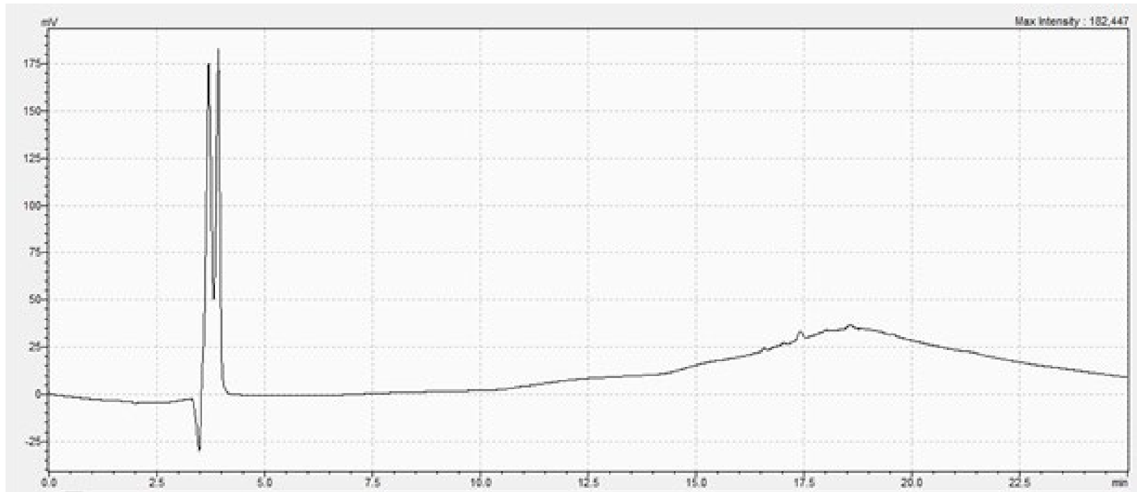


Figure 4.28c:RP-HPLC chromatograms of stripping phase after AOT/Tween 80 based back extraction at 226nm

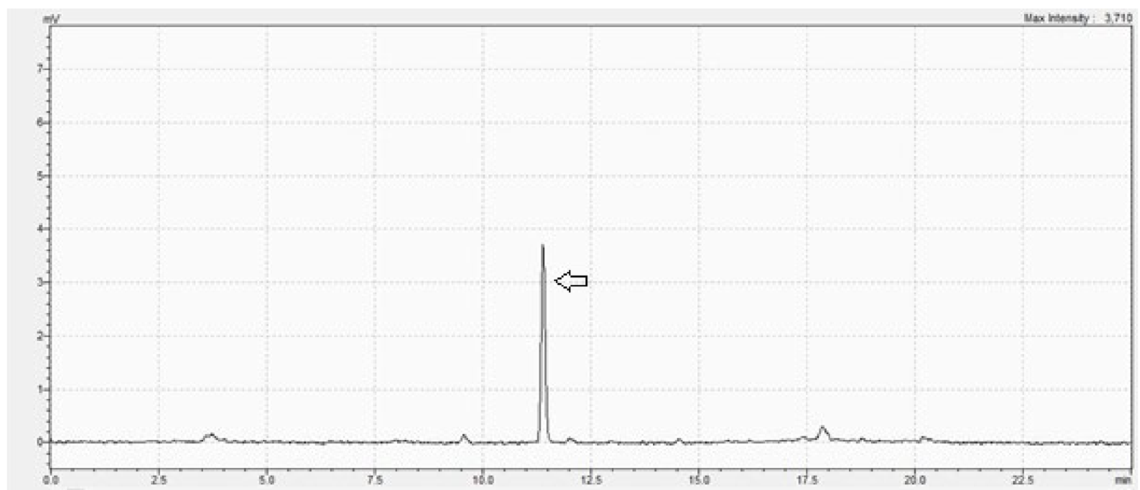


Figure 4.28d:RP-HPLC chromatograms of acid wheyat 412nm

This could be the result of changed pH and ionic strength in the stripping phase which is not the same as that in the whey. Added to this, the residual surfactants that could occur in the stripping phase due to the mass transfer over the course of the extraction process can also affect the conformation of LP. The salts, pH, and surfactants in the stripping phase would change the apparent hydrophobicity of the protein, which resulted in LP adopting a conformation that changed its interaction with the hydrophobic stationary phase and thus its elution time (Tripet et al. 2007; Wetlaufer

and Koenigbauer, 1986). Further, a change in the secondary structure of LP after RME using AOT/Tween 80 system has been previously established.

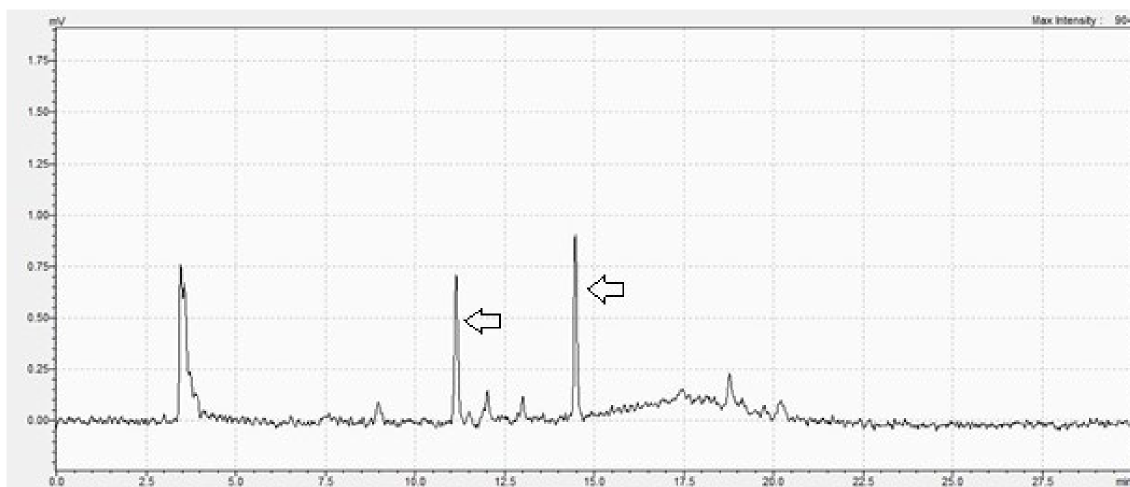


Figure 4.28e:RP-HPLC chromatograms ofaqueous phase after AOT/Tween 80 basedforward extractionat 412nm

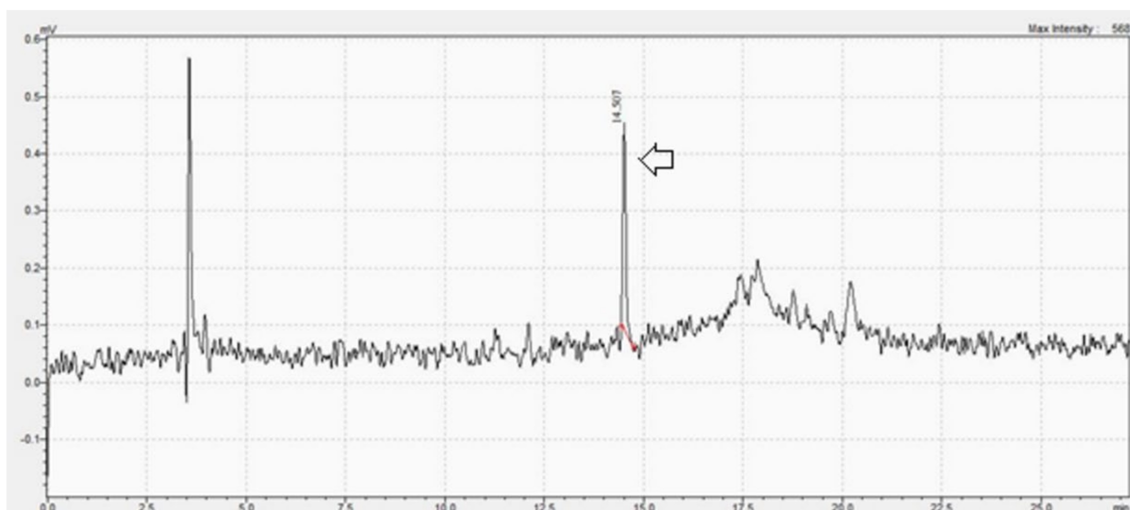


Figure 4.28f:RP-HPLC chromatograms ofstripping phase after AOT/Tween 80basedback extraction at 412nm

4.3.13 Antimicrobial assay using recovered LP.

LP is known to be bactericidal to gram-negative and bacteriostatic togram-positive bacteria. The effectiveness of the recovered LP against the pathogen has to be analysed for the antimicrobial application, as the LP was exposed to various surfactants (AOT, Tween 80, CTAB), solvents (Isooctane and hexanol), salts at different concentrations (KCl), different pH other than the native pH and buffers during the RME of LP. The antimicrobial activity of recovered LP was performed by analyzing the growth of *Staphylococcus aureus*in terms of the colony-forming units(cfu). Studies have reported that LP can effectively inhibit the growth of *S. aureus* at both 37°C and 30°C, but more effectively at 10°C. (Kamau et al. 1990; Garibay et al. 1995).

Table 4.4:Effect of pure LP on *Staphylococcus aureus*

Hours	***S. aureus* counts (cfu/ml)**		
	Control (10^7)	**Treated with Pure LP (10^7) at 30ºC**	**Treated with Pure LP (10^6) at 9ºC**
0	0.320	0.310	0.140
2	1.0	0.976	0.144
4	2.517	2.165	1.055
6	4.7	3.1	1.8
8	9.0	4.5	2.5
24	TNC	TNC	TNC

*TNC- too numerous to count

It was observed from Table 4.4 and 4.5 that the LP was effectively able to reduce the colony forming units(cfu) in all the samples. The effect of LP on reducing the number of colony-forming units/mL(cfu/mL) was better at 9ºC than at 30ºC. Notably, at 9ºC the multiplication of the organism was almost arrested even after 24 hrs. The lower temperatures could inherently arrest the growth of *S. aureus*resulting in lower cfu's. Also, at 30ºC there was a sharp dip in the cfu/mL from 0th hour to 2nd hr and continued upto 4 hrs. Such a decrease in the initial hours has been observed for LP treated samples of milk by Kamau et al. 1990. This could be because the organisms

were overwhelmed with the LP during the initial lag phase and areindicative of the initial bactericidal effect.

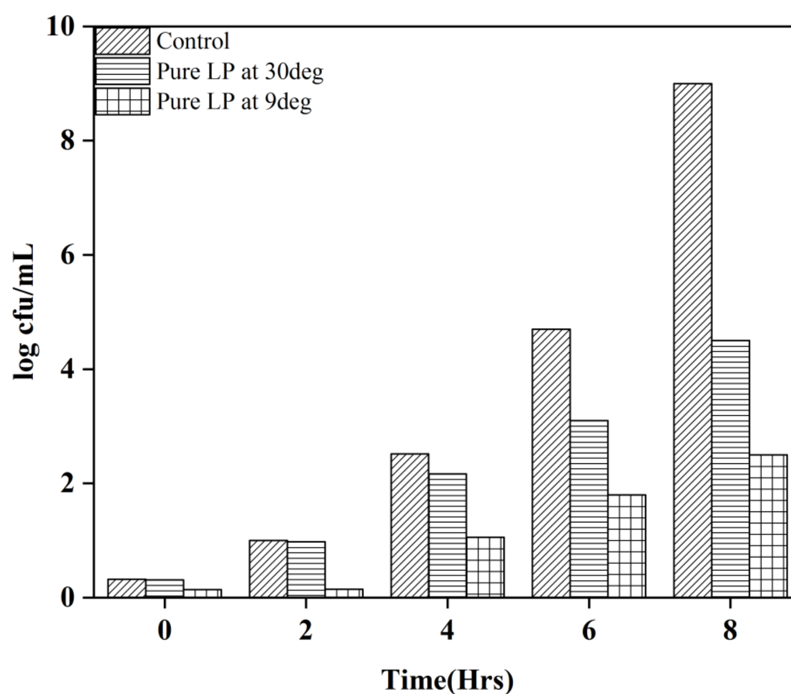


Figure 4.29: Effect of pure LP on *Staphylococcus aureus* assayedat 30ºC and 9ºC

It was reported that the bactericidal effect can be prolonged if the components of the LP system are changed by introducing glucose and glucose oxidase enzyme system for the continuous generation of hydrogen peroxide(Kamau et al. 1990). The results from treated samples were appreciably lower than blank that contained only surfactants suggesting good antimicrobial activity from the recovered LP (in the carbonate buffer).

Table 4.5: Effect of recovered LP after AOT/ Tween 80 based LP extraction on *Staphylococcus aureus*

Hours	*S. aureus* counts (Log number)					
	Temperature 30ºC			Temperature 9ºC		
	Control (10^7)	Blank (10^7)	Treated with recovered LP (10^7)	Control (10^7)	Blank (10^7)	Treated with recovered LP (10^7)
0	2.53	1.17	2.13	2.76	1.9	1.7
2	3.2	2.0	0.9	3.3	1.9	1.5
4	13.2	6.0	1.2	4.0	2.9	1.5
6	39.0	---	2.5	8.6	4.8	2.9
8	51.5	20.0	4.1	20.0	9.0	4.6
24	2600	47.0	11.2	130.0	49.0	6.6

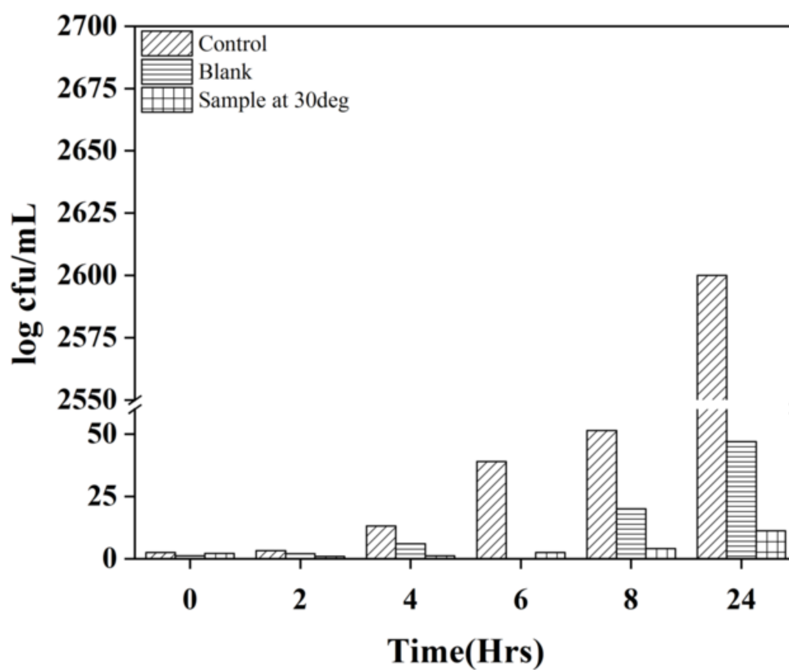


Figure 4.30a: Effect of recovered LP after AOT/ Tween 80 based LP extraction on *Staphylococcus aureus*at 30ºC

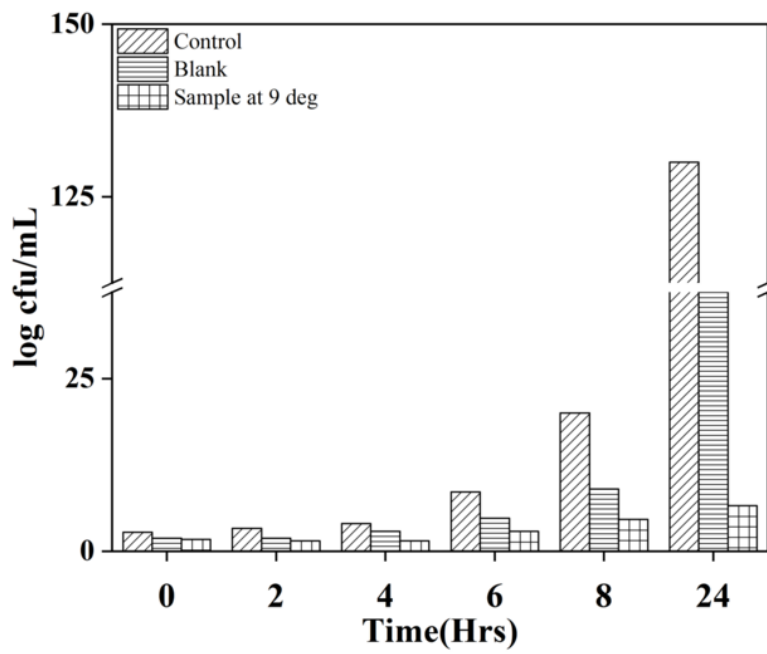


Figure 4.30b: Effect of recovered LP after AOT/ Tween 80 based LP extraction on *Staphylococcus aureus*at 9ºC

Part four: Biosurfactant based reverse micelle extraction of Lactoperoxidase

4.4 Rhamnolipid based Reverse micelle extraction using pure LP

The effect of solvents used for the organic phase and Rhamnolipid concentration used to form the reverse micelles were studied during the extraction experiments with pure LP solution. The effect of non-ionic surfactant addition, whey pH, and ionic strength of the whey werestudied for the forward extraction of LP from acid whey.The regular approach of back extraction by changing the pH of the tripping phase above the pI of the protein LP i.e. 10 and 10.5 failed to back extract the LP. However, the back extraction wasstudied based on the properties of the biosurfactant,Rhamnolipid, since the ionic moieties are distributed over the surface and also responsive to the external electrostatic forces. The surface properties of the Rhamnolipid were exploited to back extract the LP rather than the surface properties of the protein LP.The unique characteristics of Rhamnolipid such as the change of non-ionic to anionic nature beyond the solution pH 6.8 (Özdemir et al. 2004), was considered and the back extraction profile of LP was studied at pH 5.

4.4.1Effect of alcohols in the forward extraction

Different alcohols were chosen as co-surfactant in the preparation of the Rhamnolipid RM phase. The alcohols tested include n-butanol,n-pentanol, n-hexanol, n-heptanolbased on their HLB (Xie et al. 2005). 50 mM Rhamnolipid was dissolved in isooctane and the alcohols at various ratios to form the organic phase. This was mixed with 0.1M phosphate buffer containing 25mg/L of LP at pH 8 during forward extraction. Back extraction was performed using 0.1M citrate buffer at pH5 with 0.5MKCl. Rhamnolipid concentration in the organic phase was maintained at50 mM. Forward and backward extractionparameters were selected randomly for the experiments based on protein transfer capabilities of RME (Matzke et al. 1992), properties of LP and properties of Rhamnolipid (Özdemir et al. 2004). Fig 4.31 represents the effect of different co-solvent on LP extraction efficiency and activity recovery. Alcohols serve as both co-surfactant and co-solvent and their role is ambiguous. Alcohols with two to four carbon numbersimpart a co-solvent effect and decrease the surfactant-surfactant interaction. Similarly, alcohols with four to nine

carbons behave as co-surfactants and interact strongly with the oil while retaining adsorption at oil-water interface. Alcohols beyond eight essentially behave as oil (Sabatini et al, 2009). Alcohols help in better solubilization of Rhamnolipid onto the oilphase and provide a buffer to the repulsive ion-ion interactions between surfactants, thus aiding in their close packing and forming the inner core of reverse micelles (Peng et al. 2014). As the carbon number of the alcohol increased the LP extraction efficiency increased. Maximum extraction was achieved with both 50% n-pentanol and 50% n-hexanol. However, maximum LP was recovered with 50% n-pentanol. 10% co-solvent addition could not develop enough water content to accommodate and solubilize the LP. Earlier studies with 50 mM Rhamnolipid in tertiary- butanol /isooctane system also showed similar water content at 10% concentration and microemulsion formation only when co-solvent was increased to 50%(Ramirez et al. 2017).

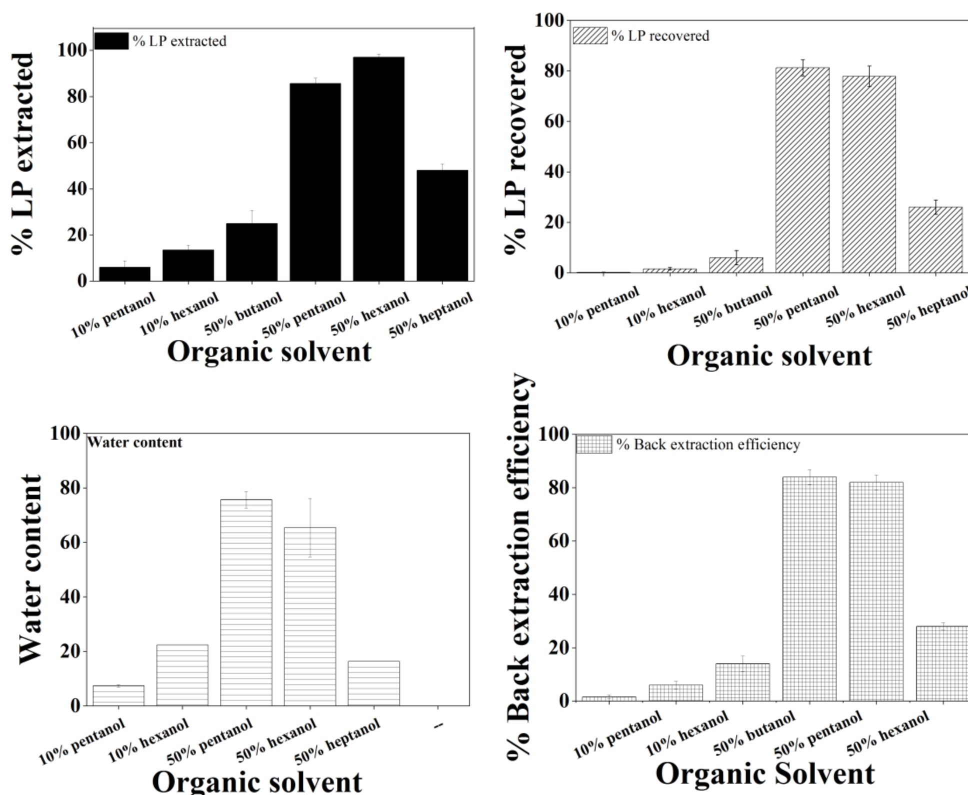


Figure 4.31:Effect of alcohols on the extraction efficiency, activity recovery, and purification factor of LP from the aqueous solution of pure LP in the RM system formed by 50mM Rhamnolipid in Isooctane/alcohol.

4.4.2 Effect of Rhamnolipid concentration during forward extraction

Literature shows the usage of a different range of concentrations of Rhamnolipid for RM extraction processes. They include concentrations ranging from as low as 1.65mM (Peng et al. 2014) to higher concentrations of 50mM (Ramirez et al. 2017). However, when low concentrations were used, Rhamnolipid was obtained directly from the microbial production and its fermented broth whereas when higher concentrations were used purified Rhamnolipid was procured from the external supplier. These differences in the source of Rhamnolipid impact the emulsifying properties of Rhamnolipid. Rhamnolipid used in the present study was procured from external sources and this makes a significant contribution. Rhamnolipid concentration between 25mM to 100mM was used to study the extent of LP extraction and recovery(Fig 4.32). The concentration of surfactant has little effect on reverse micelle size and shape but as the surfactant concentration increases the number of reverse micelles increases. This improves protein solubilization and extraction. At concentrations above a critical value, reverse micelles undergo interactions leading to interfacial deformation. This inturn results in micellar clustering and shape changes in reverse micelles. This clustering decreases the area available to accommodate protein and hence protein solubilization/ extraction decreases above the critical value of surfactant concentration (Krishna et al. 2002). As the concentration of Rhamnolipid increased, as expected LP extraction as well as recovery increased. However, LP extraction increased only upto 50 mM and thereafter LP extraction and also recovery was reduced. Maximum of 86% LP extraction was achieved 81% activity recovery was achieved. However, increasing Rhamnolipid concentration resulted in increasing water content in the organic phase. Beyond 50mM, inter micellar interactions and clustering occur leading to change in micellar shape and deformation. This, in turn, results in lesser LP extraction and hence lower recoveries. This is also reflected in the low water content in the organic phase as concentration increases.

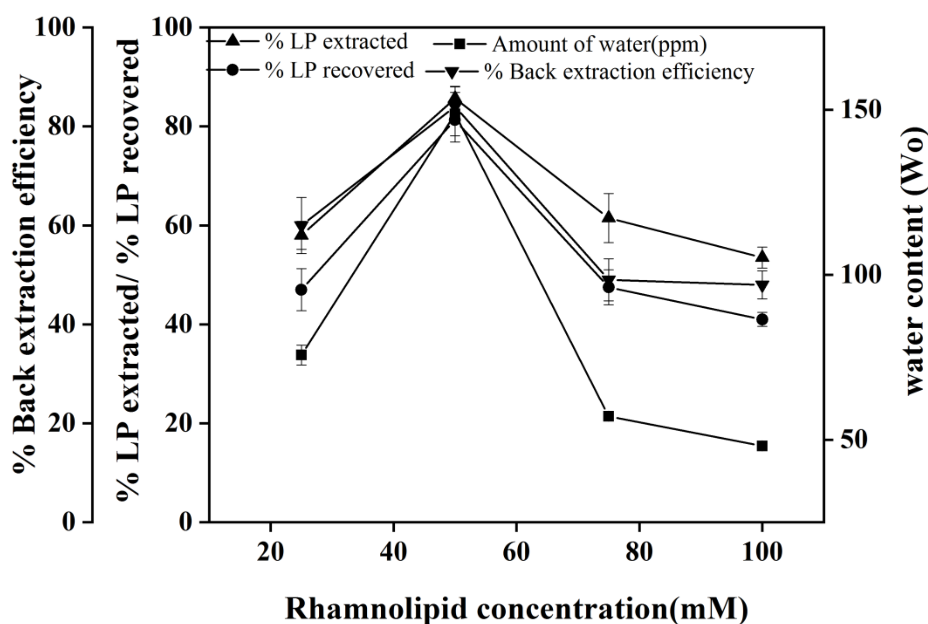


Figure 4.32:Effect of Rhamnolipid concentration on the extraction efficiency, activity recovery, and purification foldof LP from the aqueous solution of pure LP in the RM system formed by Rhamnolipid in Isooctane/50%alcohol.

4.5 Rhamnolipid based Reverse micelle extraction using whey

The extraction of LP from crude whey was performed at the process conditions used for the extraction of LP from the aqueous solution of pure LP (50mM Rhamnolipid in Isooctane/50%alcohol system) and observed a poor extraction efficiency. The ions present in the crude whey easily interact with anionic Rhamnolipidand leaving lesser room for the interaction of LP with Rhamnolipid. The predominant cations present in whey are Ca_2^+, Mg_2^+, Na^+ and K^+(Table 4.6). The carboxylate ions of the Rhamnolipid got neutralized by the cations present in the whey. It is also reported that the carboxylategroups interact with these smaller cations readily than the larger cations (Bala et al. 2007). However, a specific concentration of Ca_2^+ions in the whey is required to keep the LP intact. Hence the whey was subjected to dialysis to reduce the concentration of cations. With different trials, it was observed that the conductivity levels of about 0.8-1mS help in better extraction of LP. Dialysis beyond

these conductivity levels deprives LPdue to the non-availability of the necessary Ca^{2+} ions and affects the stability of LP. This results in activity losses in LP during the extraction process. 0.8 to1mS was decided as the optimum level of conductivity of whey to start the RME process. As theions present in the whey are sufficient to establish the RM formation, the external salts were not added to aid the RM formation that is generally followed in RME processes.

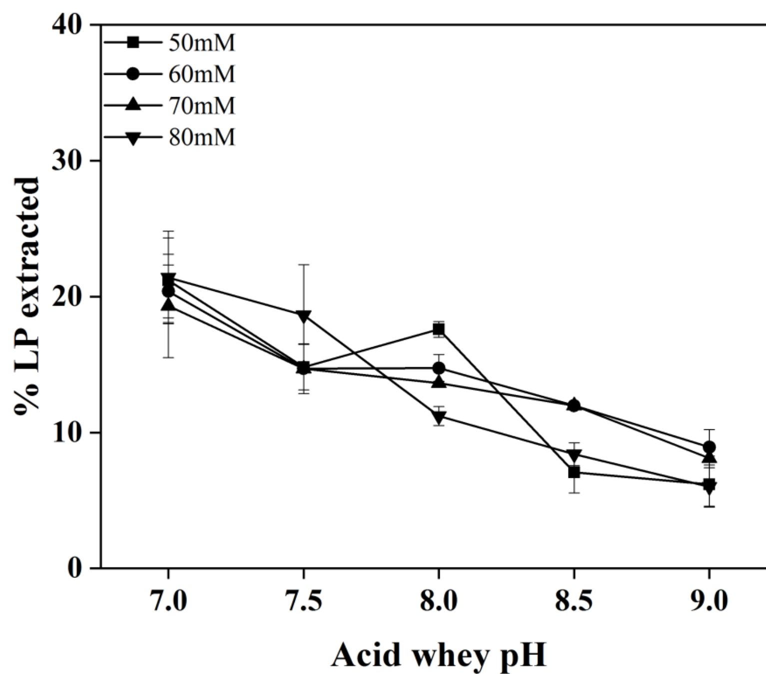
Table 4.6: Free metal Ion concentration observed in Acid Whey

	Ca ion (ppm)	**K ion(ppm)**	**Mg ion(ppm)**	**Na ion(ppm)**
Whey	1000.51	711.30	28.90	339.27
Buffer	1.07	6.71	0.77	33.29

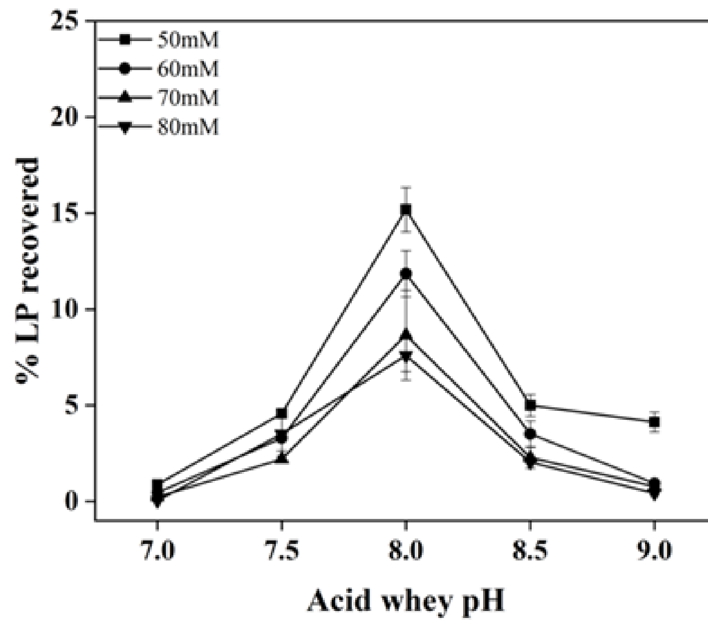
4.5.1 Effect of whey pH during forward extraction

The effect of whey pH at different Rhamnolipidconcentrations on the forward extraction efficiency of LP was analyzed by varying the pHbetween 7-9 using the dialysed whey with the RMS formed by the organic phase containing 50 mM Rhamnolipid in isooctane/ pentanol. The LP activity recovery was estimated by back extracting the LP using citrate buffer with 0.5M KClat pH 5 (Fig 4.33). Dialysed whey at 50 mM did not result in expected forward extraction efficiency as compared to LP extraction from the buffer. As seen from the water content in whey is very low during maximum LP extraction when compared to that in the buffer system(Fig 4.32). Thus, simultaneously Rhamnolipid concentration was studied for LP extraction capabilities. LP extraction increased with increasing concentration at pH7 & pH 7.5 with a maximum of 21.2% at 50 mM concentration using whey at pH7. However, the recoveries remained very negligible. At pH7-7.5 LP is farther from its isoelectric point and remains fairly positively charged. This leads to good interactions with the negatively charged Rhamnolipid at this pH and good LP extraction is observed. However, contaminant proteins like Lactoferrin (pI-7.8-8.0) and Immunoglobulins (pI-5.5-8.3) could also be extracted along with LP. These contaminant proteins are also back extracted resulting in lower LP recovery and lower purification fold. As the

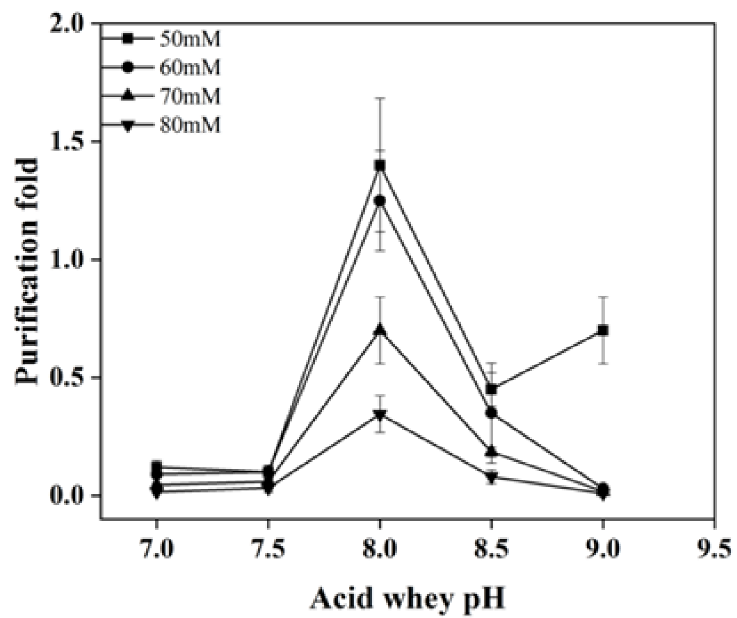
pH approached the pI of LP, extraction decreased but the purification fold improved. Between Ph8 and 9, LP is close to its isoelectric point and a significant number of amino acids is negatively charged as LP approaches the isoelectric point. Thus, the electrostatic interaction between LP and Rhamnolipid headgroups decreases, and the extraction decreases along with LP activity recoveries. It was also observed that Rhamnolipid concentration did not have a major effect on LP extraction from whey and 50Mm. Thus, based on the recovered LP activity, 50 mM Rhamnolipid in the organic phase and acid whey pH 8 were considered for further optimization.



(a)



(b)



(c)

Figure 4.33: Effect of acid whey pH on the (a) extraction efficiency, (b) activity recovery, and (c) purification foldof LP from the acid wheyin the RM system formed by Rhamnolipid in Isooctane/50%alcohol.

4.5.2 Effect of non-ionic surfactant addition during forward extraction

Even though the dialysis and the change of whey pH helped to extract the LP to a certain extent (15%), the entire LP was not extracted and the recovery remained very less due to the absence of suitable interactive force. The non-ionic surfactants that behave as co-surfactants may help in better solubilization of Rhamnolipid in the organic phase by increasing the hydrophobicity of the Rhamnolipid thereby modifying the interfacial properties of the RM microemulsion system.Hence, the effect of the addition of non-ionic surfactants viz. Tween 80, Tween 85, and Span 85 to the organic phase as cosurfactant were studied. The addition of hydrophilic surfactants Tween 80 and Tween 85 did not improve the recovery, whereas the lipophilic Span 85 significantly improved the recovery. The concentration of Span 85 was increased from 2 mM to 10 mM in the organic phase made up of 50% Isooctane / 50% Pentanol. The dialysed whey at pH 8 with the conductivity of 1mS was used as an aqueous phase. The extraction and activity recovery of LP was found to increase from 84.22% and 64% to 96.65% and 80 %, respectively atthe span 85 concentration of 4mM.The purification fold was also found to increase from 5.45 to 7.6 between 2 and 4 mM span 85(Fig 4.34). When a small amount of Span 85 is added to the organic phase with 50mM Rhamnolipid, they segregate near the Rhamnolipid tails and aid in extending the Rhamnolipid tails further into the organic phase, thereby helping in the solubilization of Rhamnolipid into the organic phase(Sabatini et al. 2003; Acosta et al. 2005). Theaddition of Span 85 is an added advantage that provides a similar effect of the pentanol i.e., helps to dissolve the Rhamnolipidin the organic phase. The pentanol and span 85 together behave as lipophilic linkers in the RM system and improve the number of RM in the organic phase. As the number of reverse micelles increases with increasingspan 85, the extraction and recovery of LP were found to increase. However, at span 85 concentration beyond 4 mM, these increased number of reverse micelles result in inter micellar clustering and deformation of the reverse micelles. This results in lower extraction, recoveries, and purification fold.

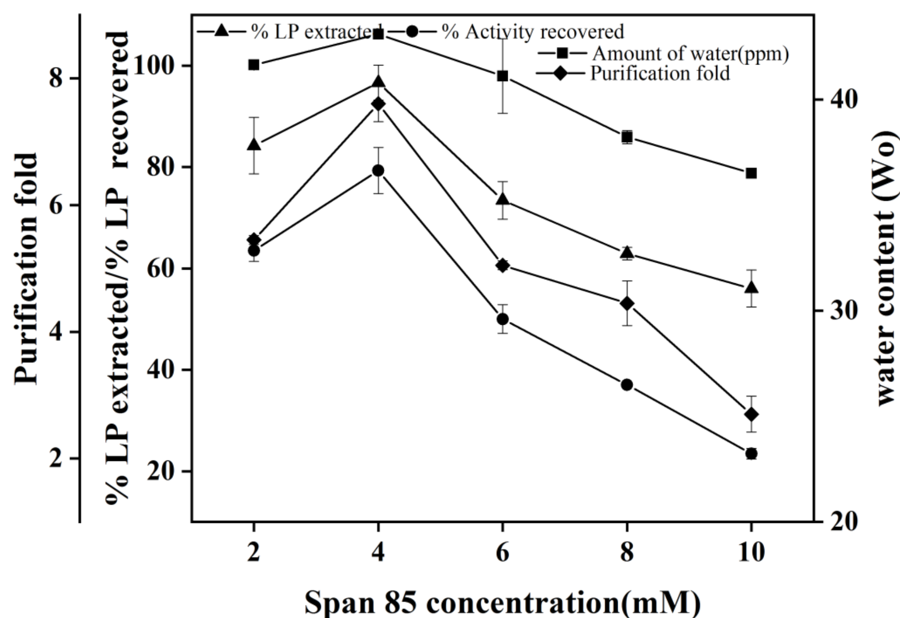


Figure 4.34: Effect of non-ionic surfactant addition on the extraction, activity recovery and purification fold of LP from the acid wheyin the RM system formed by Rhamnolipid in Isooctane/50%alcohol.

4.5.3 Effect of stripping phase pH during backward extraction

The LP extracted into the RM phase has to be recovered into a fresh aqueous phase while retaining maximum possible activity. The back extraction is found to be a rate-limiting step in the RME process.As a rule of thumb, all the reported anionic surfactants based RME processes employ apH higher than the isoelectric point (pI) of the target protein during back extraction. This reduces or does not facilitate the electrostatic interaction between the negatively charged target protein and negatively charged surfactant headgroups. However,mild interactions do remain as many aminoacids on the target protein could still exhibit a positive charge and hence not all of the forward extracted protein is successfully back-extracted.Same was observed for LP in the present study, and LP could not be recovered at pH higher than the pI of LP. The changes in the charged headgroups of the surfactant at different pH wereexploited to disintegrate the RMs. Rhamnolipid is completely protonated and remains non-ionic till the pH of 6.8 and becomesanionic in nature beyond the pH of 6.8 (Özdemir et al.

2004). This surfactant-specific feature of Rhamnolipid was explored for back extraction studies. Thus, back extraction studies were performed using citrate buffer between pH 5 and 6 and phosphate buffer at pH 6.5 without the addition of any salts.The RM organic phase obtained from the forward extraction of LP at 4 mM Span 85 and 50 mM Rhamnolipid in the organic phase (isooctane/pentanol) using dialysed whey at pH 8 was used for the back extraction(Fig 4.35).Though RL is usually protonated below pH 6.8, the electrostatic interaction between LP and RL from forward extraction could be stronger than the protonation of RL. Thus, as the pH of the aqueous phase is reduced back extraction capabilities of LP improve.

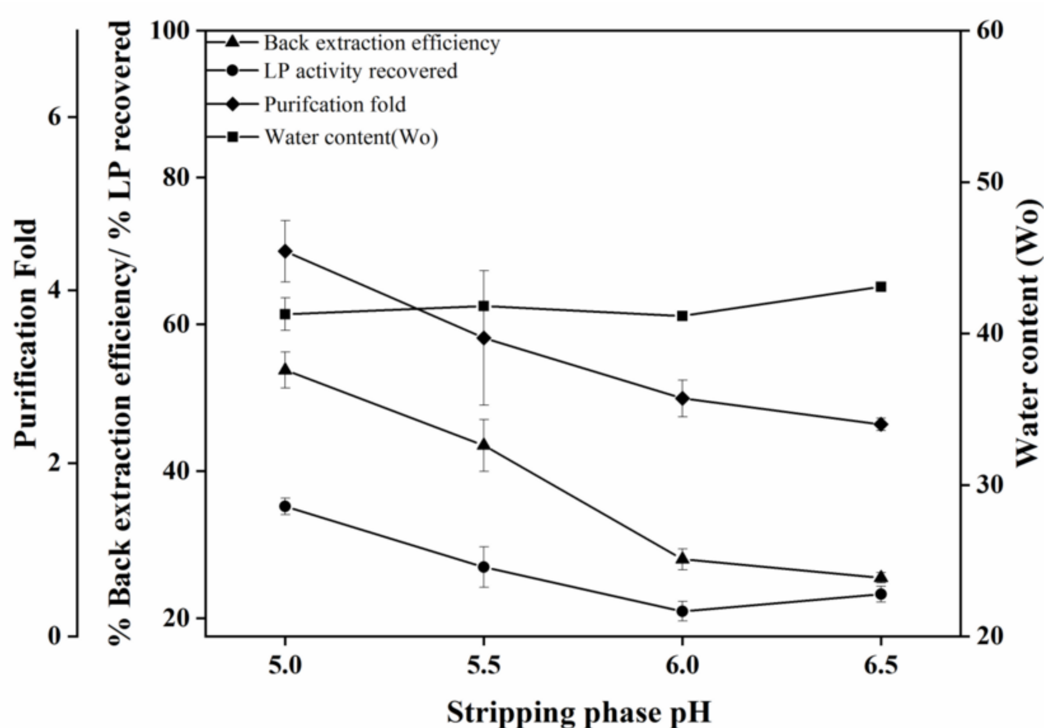


Figure 4.35: Effect of stripping phase pH on theback extraction efficiency, activity recovery and purification fold of LPfrom theRM phase (50% Isooctane/ 50% alcohol containing 50mM Rhamnolipid,4 mM span 85 and acid whey at pH 8) using buffers at various pH with 0.5 M KCl.

The maximum back extraction efficiency of 54% and LP activity recovery of 35% was observed at pH 5 whereas the least back extraction was observed at pH 6.5.The purification fold was also significantly lower. Hence, pH 5 remained the ideal pH for back extraction of LP in further optimization studies.

4.5.4 Effect of ionic strength of the aqueous phase during backward extraction

The recovery and back extraction efficiency of LP were further improved by studying the addition of neutral salt KCl at different concentrations from 0.5M to 1.5M in the stripping citrate buffer at pH 5. Though Rhamnolipid is neutrally charged and sufficiently protonated at pH 5; the added K^+ ions from KCl can further enhance the process by its interaction with the negatively charged head groups of Rhamnolipid i.e. COO- groups.

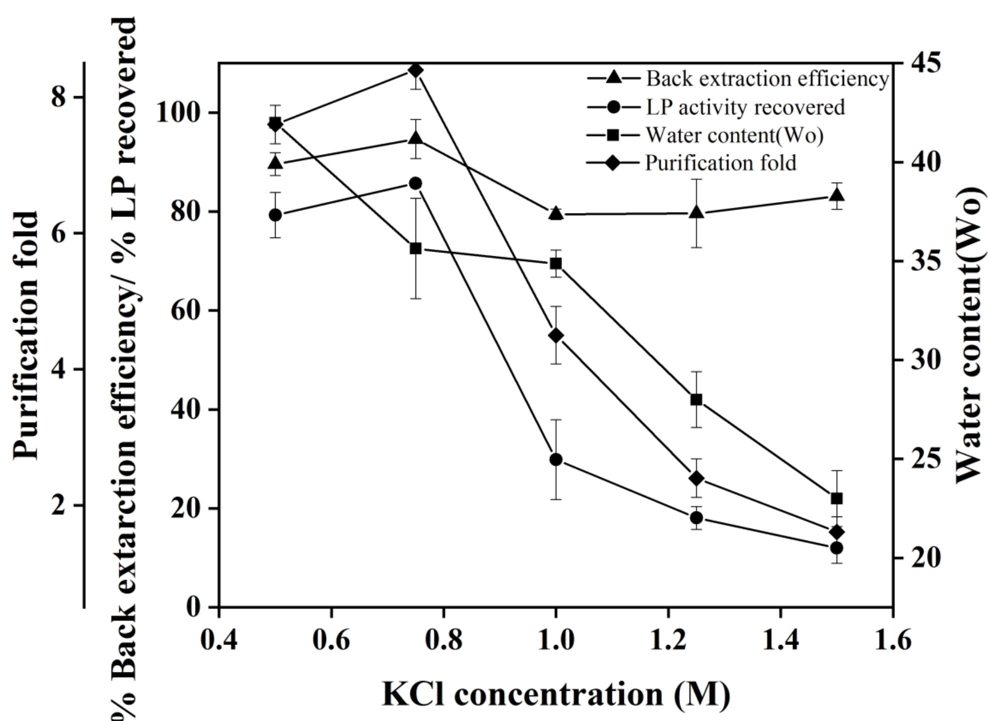


Figure 4.36: Effect of stripping phase ionic strength on LP back extraction, activity recovery and purification fold through mixing equal volumes of organic phase containing 50mM Rhamnolipid, 4 mM span 85 and acid whey at pH 8 followed by back extraction using citrate buffer at pH 5 with varying KCl concentrations

Increasing K^+ ion concentration reduces the repulsion between the like-charged Rhamnolipid headgroups resulting in smaller reverse micelles with reduced water content in the core (Andrews et al. 1994). The shrinking RM size with reduced water core due to the incorporation of K^+ ions and pH 5, exclude the LP fromthe RM phase

to the stripping phase. However, the recovery of LP was observed to increase with an increasing salt concentration in the stripping phase upto0.75M KCl. However, the LP activity decreased with fairly constant back extraction efficiency and protein recovery beyond 0.75M KCl. The presence of higher salt concentrations (0.75M KCl) in the stripping phase may hamper the activity of LP due to increased hydrophobicity and thus resulted in lower activity recoveries (Hemavathi and Raghavarao, 2010).A maximum of 85.71% active LP was recovered with 8.4 fold purification into the stripping phase consist of citrate buffer at pH 5 and 0.75 M KCl.

4.5.6 RP-HPLC analysis of different samples

The RP-HPLC chromatograms were obtained at two different wavelengths, 226nm and 412nm corresponding to total protein and specific to LP, respectively. The total protein and LP were qualitatively analyzed in the samples of crude whey, aqueous phase after forward extraction and back extractedstripping phase.The chromatogram of pure commercial LP shows retention time at 17.7 and the back-extracted LP elutes at 20.11 (Fig. 4.37a,b).

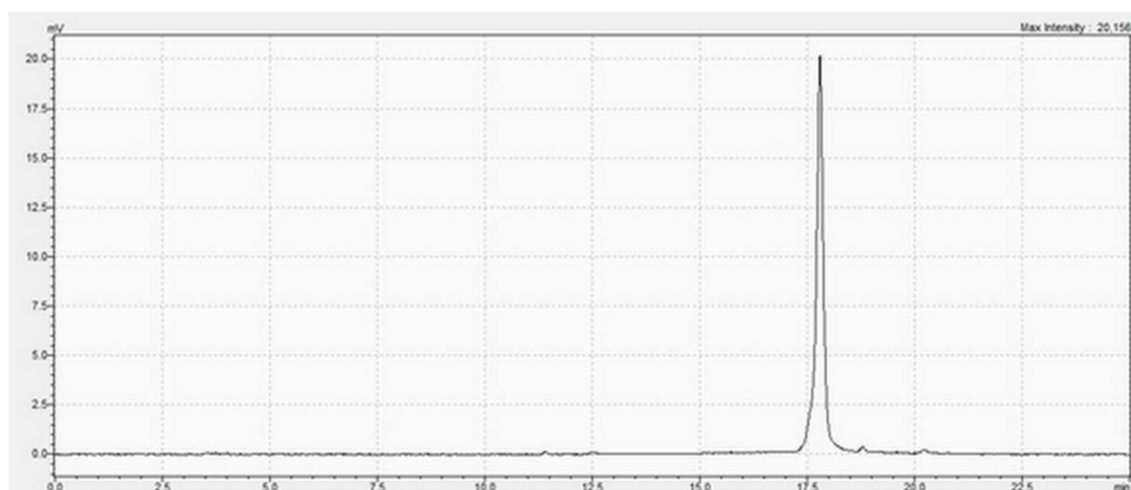


Figure 4.37a: RP-HPLC chromatogram of commercial pure LP at 412 nm

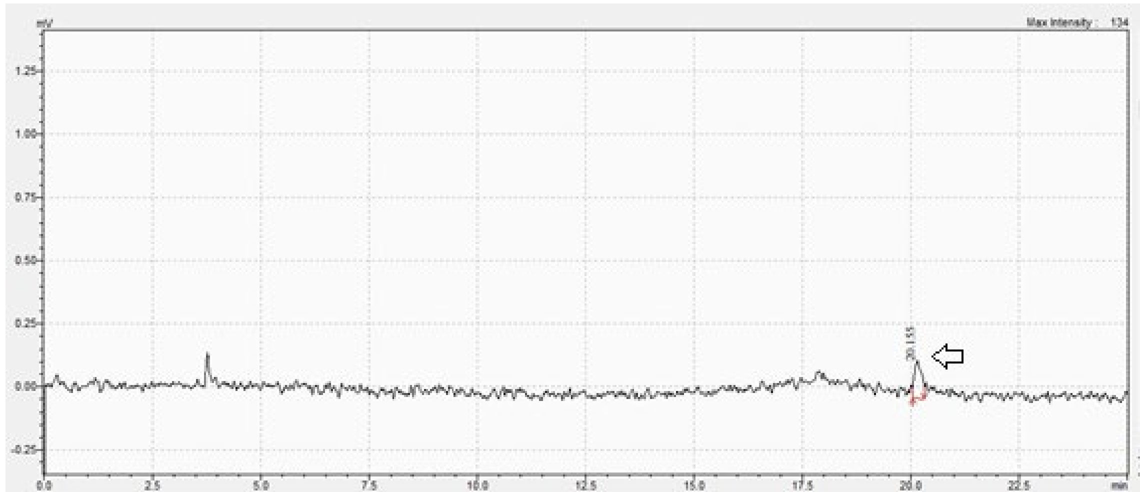


Figure 4.37b: RP-HPLC chromatogram of stripping phase after biosurfactant based back extraction at 412nm

The chromatogram of the aqueous phase of forwarding extraction indicated that the major whey proteins are left behind after the forward extraction and which is comparable to the chromatogram of crude whey (Fig 4.37c). The chromatogram of the stripping phase at 226nm clearly shows the absence of all the major whey proteins(Fig. 4.37d).

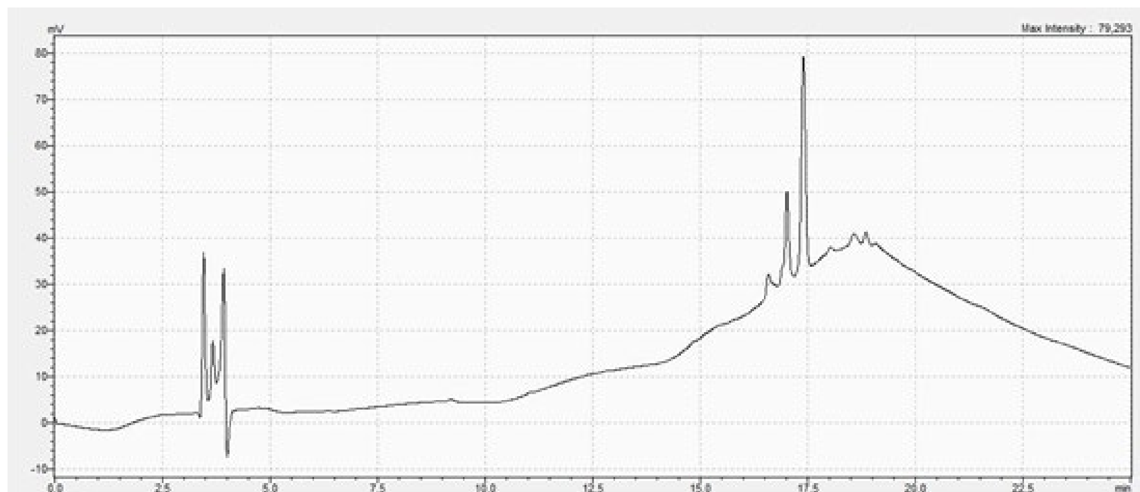


Figure 4.37c: RP-HPLC chromatograms of aqueous phase(whey) after biosurfactant based forward extraction at 226nm

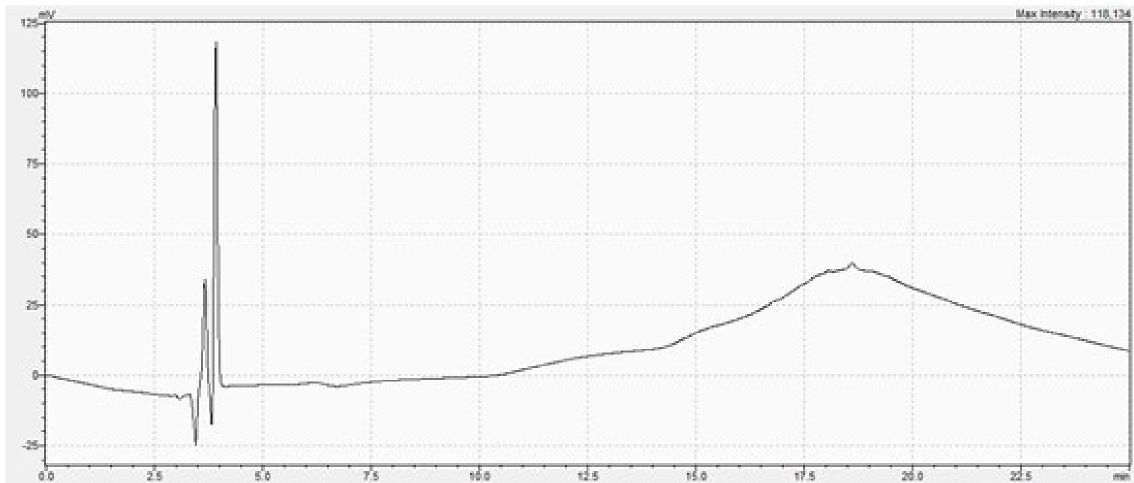


Figure 4.37d: RP-HPLC chromatogram of stripping phase after biosurfactant based back extraction at 226nm

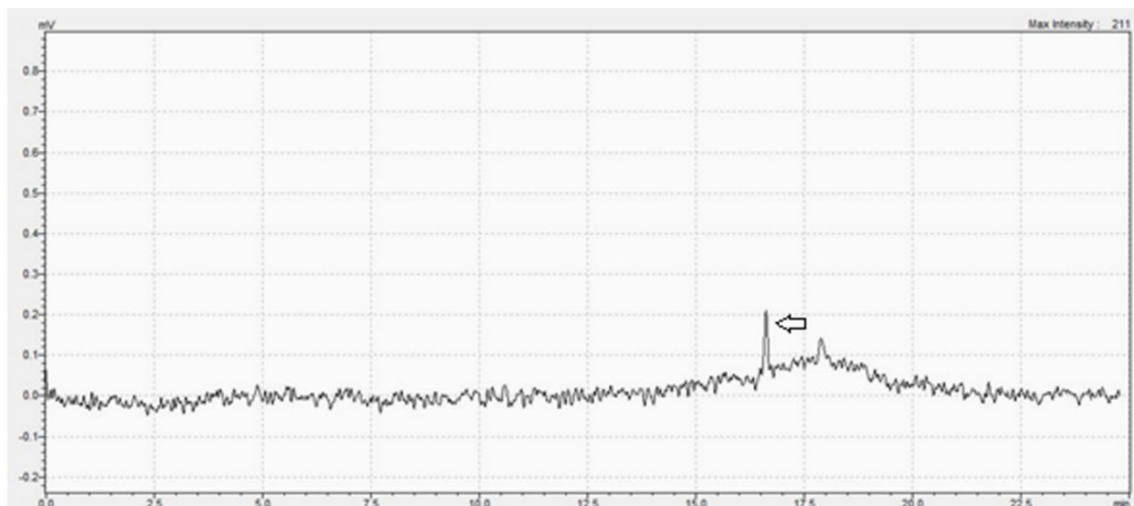


Figure 4.37e: RP-HPLC chromatograms of aqueous phase after biosurfactant based forward extraction at 412nm

A shift in the retention time from 11.5 min for LP in crude whey to 16.6 min for LP in the back extracted sample(Fig 4.37e) was observed in the chromatograms obtained at 412nm due to the conformational change of LP at the modified pH of stripping phase than the native pH of the crude. The residual surfactants present in the stripping phase, pH, and the added saltsaffect the conformation of LP and resulted in a modified interaction with the hydrophobicstationary phase due to the change in the apparent hydrophobicity of LP(Tripet et al. 2007;Wetlaufer and Koenigbauer, 1986).

Further, the changed retention time at 20.11 for pure LP studies and at 16.6 for whey studies, could be the effect of added Span 85 in the whey RME which was not used in pure LP studies.It is also interesting to note the peak at 11.5 in back-extracted LP from whey RME studies(Fig 4.37f), which indicates the protein can return to its original conformation after back extraction.

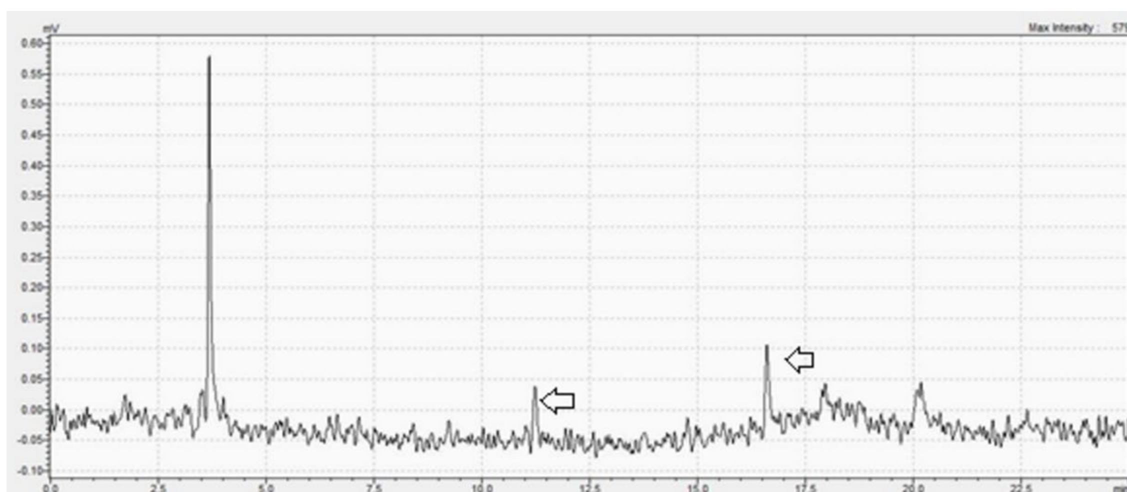


Figure 4.37f: RP-HPLC chromatogram of stripping phase after biosurfactant based back extraction at 412nm

4.5.7 Antimicrobial assay using recovered LP

LP induces bactericidal effects on gram-negative and bacteriostatic effects on gram-positive bacteria. The feasibility of using the recovered LP against microbial pathogens was accessed by performing the antimicrobial activity from the recovered LP. This helps in evaluating the effect of the extraction process on the antimicrobial activity of LP. Table 4.9 and Fig 4.38a,b below, shows the reduction in colony forming units (cfu) induced by LP in all the samples effectively. The recovered LP showed effective reduction in colony forming units/mL(cfu/mL) at 9ºC than at 30ºC.Notably, at 9ºC the multiplication of the organism was almost arrested even after 24 hrs. The lower temperatures inherently arrest the growth of organismsresulting in lower cfu's. There was a dip in the cfu/mL from 0th hour to 2nd hr andat 30ºC. A decrease in cfu in the initial hours has been observed for LP treated samples of milk as well (Kamau et al. 1990) and it is indicative of the initial

bactericidal effectinduced by LP. The cfu in LP treated samples were lower than blank which suggests good antimicrobial activity from the recovered LP.

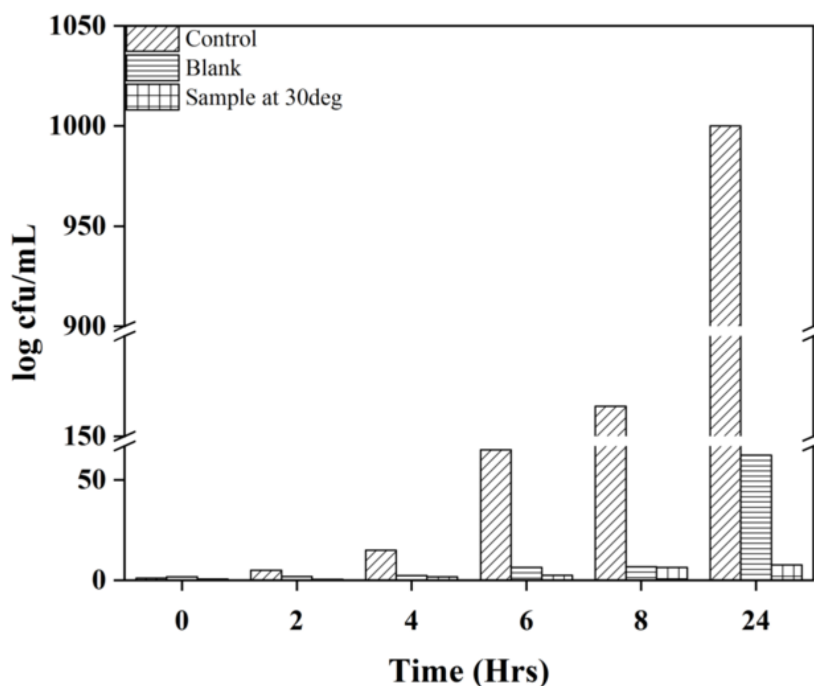


Figure 4.38a: Effect of recoveredLP after Rhamnolipid based LP extraction on *Staphylococcus aureus*at 30ºC

Table 4.7: Effect of recovered LP after Rhamnolipid based LP extraction on *Staphylococcus aureus*

Hours	***S. aureus* counts (Log number)**					
	Temperature 30ºC			**Temperature 9ºC**		
	Control (10^6)	**Blank (10^6)**	**Treated with recovered LP (10^6)**	**Control (10^6)**	**Blank (10^6)**	**Treated with recovered LP (10^6)**
0	1.12	1.75	0.59	1.16	1.3	0.79
2	5.0	1.82	0.38	1.67	1.2	0.59
4	15.0	2.25	1.7	2.0	0.85	0.34
6	65.0	6.4	2.4	2.12	0.8	0.15
8	165.0	6.7	6.4	2.36	1.33	0.3
24	10^3	62.4	7.7	21.5	5.4	0.5

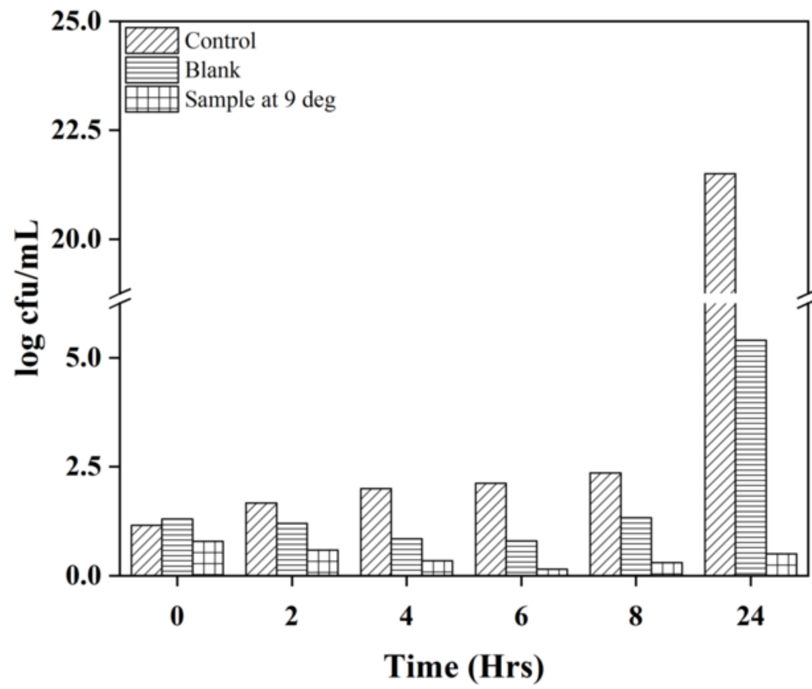


Figure 4.38b: Effect of recovered LP after Rhamnolipid based LP extraction on *Staphylococcus aureus*at 9ºC

CHAPTER FIVE

Summary and Conclusions

5.1 Summary

Though some recent procedures for LP purification have resulted in activity recoveries between 85% (Urtasun et al., 2017) to 88% (Andersson and Mattiasson, 2006), they involve limitations, such as toxic dye being used as affinity ligands and complexity of simulated moving beds in chromatography. Also, other single step chromatography procedures have resulted in lower recoveries 18.7% (Fweja et al., 2010) and 62% (Atasever et al., 2013).

The purpose of the current study was to selectively extract LP from acid whey with minimal losses. The components involved in the modified Liquid-Liquid extraction system were evaluated for their ability to retain the LP activity at various concentrations. By considering the stability and the physical characteristics of the LP, RME was chosen as the best LLE system to selectively extract the LP. Mixed synthetic and biosurfactant-based RMS are studied for the extraction of LP by analyzing the various system and process factors/variablesthat affected the forward and back extractionof LP, viz. type and concentration of surfactants, solvent type, pH, and ionic strength. The important results obtained in the present study are summarized in this section.

Part one: Screening of phase forming components and selection of the compatible extraction process for LP

- The stability of LP in different commonly used phase forming components of modified liquid-liquid extraction systems was studied. PEG and PVP/ salt systems could not be used for LP extraction. PVP /Salt failed to form two phases.

- Small chain alcohols and most salts reduce the activity of LP. Hence, the alcohol/salt system cannot be used for LP extraction. Medium-chain alcohols can be used as co-surfactants in RM systems
- Micellar two-phase systems formed with non-ionic surfactants triton and tergitolseries could not result in successful LP extraction. However, Non-ionic surfactants can be used in RME.
- The LP activity was retained above pH 6 only and hence the anionic surfactants showed a pH-dependent effect on LPand resulted in a loss in LP activitybelow pH 6 due tothe strong interaction with anionic headgroups of surfactantsresulted.

Part two: Study on reverse micellar extraction for LP purification using synthetic surfactants

- RME was the best non-conventional method for LP extraction. However, RME with AOT alone leads to only 38% LP extraction.
- The mixed RMs formed with anionic surfactant AOT and non-ionic surfactant increased LP solubilization and extraction. AOT with Tween 80 could solubilize the pure LP with widened pH window (pH 8-10).
- Complete extraction of LP from an aqueous phase of initial concentration 25 mg/L occurred with the RM formed by 90 mM AOT/8 mM Tween 80 in isooctane.
- Recovery of LP from RMs was maximum in stripping phase pH of 10.5 and was very minimal between pH 7–8 as this pH was above the pI (9.2-9.9) of LP.
- The increase in the ionic strength of the stripping phase enhances the back extraction by reducing the electrostatic interaction of LP to AOT. Amongst monovalent salts, KCl and NaCl, KCl induced LP recovery of 54.6%.
- The addition of CTAB improved the recovery slightlyto 63.45% and 80% LP recovery with 112% back extraction efficiency and purification fold of 11.266 was achieved at the phase volume ratio of 1.5.

Part three: Mixed surfactant-based Reverse micelle extraction of Bovine LP from whey

- Optimized conditions from pure LP RME were explored for selective extraction of LP from acid whey. However, poor extraction efficiency was observed due to the presence of other major and minor whey proteins.
- Acid whey at 9.5 pH with 0.2 M KCl and 115mM AOT, 23mM Tween 80 in Isooctane used in volume ratio 1 provided the maximum selective extraction of LP during forward extraction.
- A maximum of 112% LP was back extracted with an activity recovery of 80% andpurificationfoldof 11.26 using the aqueous stripping phase consisting of 1.5 M KCl along with addition of 60 mM CTAB into the organic phase at a pH of 10.5.

Part four: Biosurfactant based reverse micelle extraction of LP

- The forward extraction of LP from the aqueous LP solution was achieved with the RMs formed by 50 mM RL at pH 8 and the pH-specific protonation – deprotonation of RL headgroups was successfully utilized to recover the active LP at pH 5.
- Dialyzing the whey reduced the ion content present in whey and this aided in better extraction of LP.
- Dialysed whey at pH 8 and 50 mM RL combined with 4 mM Span 85 in Isooctane/Pentanol (50:50) formed the forward extraction parameters.
- This optimized forward extraction parameters resulted in 96.65% LP extraction while back extraction in citrate buffer at pH 5 using 0.75 M KCl resulted in 85.71% active LP recovery with 8.4 fold purification.

5.2 Conclusion

The LP can be selectively extracted from the complex crude, whey, without much denaturation by retaining the biological activity using the modified liquid-liquid extraction, RME.The RMs formed with the mixture of synthetic surfactants and

biosurfactant are capable of selectively extracting LP from whey with minimal loss in its activity. The pH-dependent inactivation of proteins during RME using only ionic surfactants was addressed by incorporating the nonionic surfactant as a co-surfactant. Further, a novel back extraction strategy was devised using the pH-specific protonation – deprotonation of biosurfactant (Rhamnolipid) and successfully used for efficient LP back extraction. The HPLC analysis along with the antimicrobial activity studies confirms the selective extraction and concentration of LP with the least denaturation. Overall, this study strengthens the idea that RMs can be used for selective extraction and concentration of the desired protein with least denaturation by tweaking certain parameters of the RME process.

5.3 Limitations of the work

- The RME process parameters discussed in the thesis is specific to LP However, they can be replicated to any other protein that shares similar physical and biochemical properties.
- The RME process parameters would vary in scale up studies.

5.4 Scope for future work

- Studies on the recycling and reuse of RM organic phase.
- Studies to improve the activity recovery of protein by reducing the concentration of salt and surfactant in the phases.
- Fractionation of other whey proteins from the aqueous phase after forward extraction.
- Pilot-scale studies along with cost analysis to make the process viable for industries.
- Studies to understand the combined antimicrobial effect of Rhamnolipid and Lactoperoxidase.

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Appendices

Appendix I

Buffers

Phosphate buffer (pH 6 to 8)

M potassium phosphate buffer was prepared using 0.2 M potassium phosphate, mono-potassium salt and 0.2 M potassium phosphate, di-potassium salt in the following ratios

Desired pH	Phosphate Mono Salt Solution(ml)	Phosphate di Salt Solution(ml)	Water (ml)
6	87.7	12.3	100
6.5	68.5	31.5	100
7.0	39	61	100
7.5	16	84	100
8.0	5.3	94.7	100

Carbonate-bicarbonate buffer (pH 9 to 10)

0.1M sodium bicarbonate and 0.1M sodium carbonate are mixed in the following ratios at 37˚C

Desired pH	Sodium bicarbonate(ml)	Sodium carbonate(ml)
9	80	20
9.5	60	40
10	30	70

Sodium citrate buffer (pH 4 to 5)

0.1M citric acid monohydrate and 0.1M trisodium citrate, 177on-ionic are mixed in the following ratios

Desired pH	Citric acid(ml)	Tri-sodium citrate 177 on-ionic(ml)
4	59	41
4.5	44.5	55.5
5	35	65
5.5	21	79

Na_2HPO_4 / NaOH buffer (pH 11)

100 mL 0.05 M Na2HPO4 + 0.1 M NaOH.

$NaHCO_3$ / NaOH buffer (pH 11)

100 mL 0.05 M NaHCO3 +0.1 M NaOH.

Appendix II

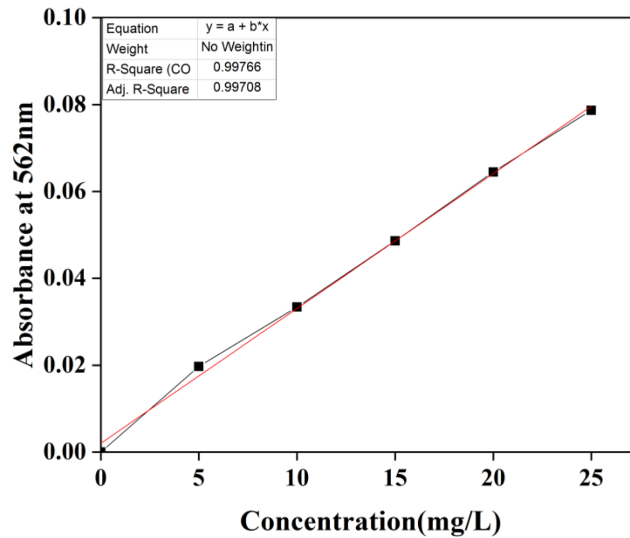

Figure A1.1 Protein calibration curve by BCA method using LP as standard

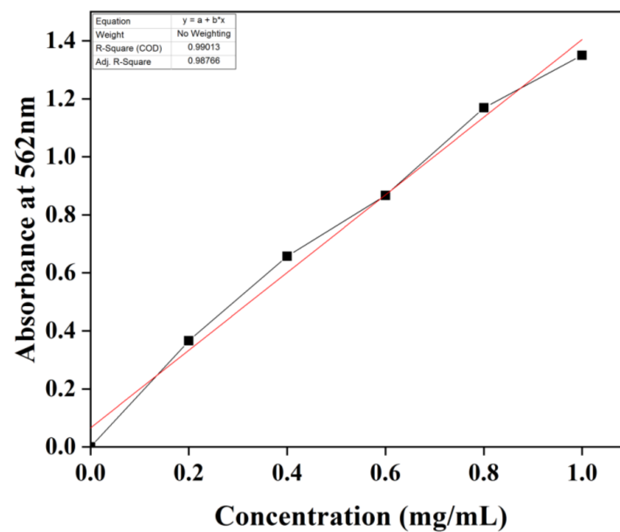

Figure A1.2Protein calibration curve by BCA method using BSA as standard

Appendix III

Lactoperoxidase assay

Pure Lactoperoxidase in 0.1M Phosphate buffer pH 6 (dilution 1:100)

Time (Seconds)	Absorbance at 412nm	Δ Absorbance
0	0.106	
10	0.113	0.007
20	0.120	0.007
30	0.126	0.006
40	0.133	0.007
50	0.139	0.006
60	0.145	0.006
80	0.151	0.006
100	0.156	0.005
120	0.162	0.006
140	0.168	0.006
160	0.173	0.005
180	0.179	0.006
	Sum	0.073
	Avg	0.006083

Activity calculation

=0.4938*(dilution factor)*(Δ Absorbance/min)

= 0.4938*100*0.006083

=0.300Units/ml

Appendix IV

Sample Water content calculation

Karl Fischer's titrator values are in ppm(mg/L). This is converted to mM/L by dividing with a molecular weight of water i.e.18.

AOT in isooctane -51160.3ppm i.e 2842 mM/L. 2842/(100mMSurfactant)=28

List of publications based on research work

Sl.No.	Title of the paper	Authors	Name of the Journal/ Conference/ Symposium, Vol., No., Pages	Month & Year of Publication	Category
1.	Mixed Surfactant-Based Reverse Micellar Extraction Studies of Bovine Lactoperoxidase	Shwetha Karanth, Regupathi Iyyaswami	Journal of Surfactants and Detergents, 24(2),255-267	March 2021	1
2	Analysis of ionic and 181 on-ionic surfactants blends used for the reverse micellar extraction of Lactoperoxidase from whey	Shwetha Karanth, Regupathi Iyyaswami	Asia-Pacific Journal of Chemical Engineering,16(2)	March/April 2021	1
3.	Stability analysis of bovine Lactoperoxidase in the presence of additives	Shwetha Karanth, Regupathi Iyyaswami	International Conference on Advances in Medical and Industrial Biotechnology (ICAMIB 2019)	March 2019	4
4.	Screening of mixed surfactants based reverse micellar system for Lactoperoxidase extraction from whey	Shwetha Karanth, Regupathi Iyyaswami	ICEF13 - 13th International Congress on Engineering and Food	September 2019	4

*Category:
1: Journal paper, full paper reviewed **2:** Journal paper, Abstract reviewed **3:** Conference/Symposium paper, full paper reviewed **4:** Conference/Symposium paper, abstract reviewed **5:** Others (including papers in Workshops, NITK Research Bulletins, Short notes etc

BRIEF BIO-DATA

SHWETHA KARANTH

LinkedInEmail: karanth.shwetha31@yahoo.co.in

EDUCATION:

PhD in Biochemical Engineering (Protein Purification)

National Institute of Technology Karnataka, (NITK) India

Thesis: Non-conventional Extraction of Bovine Lactoperoxidase from acid whey-Downstream Processing.

Supervisor: Dr. I. Regupathi

M.Tech, Chemical Engineering

Visvesvaraya Technological University, India

Thesis: Mass transfer studies in a fermenter with different impeller combinations

Center: Sartorius Stedim India- Integrated Solutions-Process Engineering

- Planned, Designed and Implemented New curved blade impellers to meet the high mass transfer requirements inFermentors.
- Performed extensive preliminary lab work on the same thereby benefitting the company to retrofit Rushton and Pitched bladeImpellers.

B.E., Biotechnology

Visvesvaraya Technological University, India

Final year project: Evaluation of antiproliferative activity of *Pavoniazeylanica* Cav. on mammarian Cancer Cells

PUBLICATIONS AND PRESENTATIONS:

- Karanth S, Iyyaswami R. Analysis of ionic and nonionic surfactants blends used for the reverse micellar extraction of Lactoperoxidase from whey. Asia-Pac J ChemEng.2020;e2590.
- Karanth S, Iyyaswami R. Mixed surfactant based reverse micellar extraction studies of bovine lactoperoxidase. Journal of Surfactants and Detergents.2021. DOI10.1002/jsde.12489
- “Effect of rushton impeller in stirred tank reactor on volumetric mass transfer coefficient” – Conferenceproceedings
- “Stability analysis of Bovine Lactoperoxidase in the presence of additives" at International conference on advances in Medical and IndustrialBiotechnology
- “Screening of mixed surfactants based reverse micellar system for Lactoperoxidase extraction from whey” at ICEF13 - 13th International Congress on Engineering andFood.