AQUEOUS TWO PHASE EXTRACTION OF LECTIN FROM *PISUM SATIVUM* SEEDS

THESIS

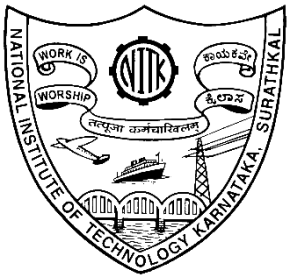
Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

RASHMI B S

Register Number: 148020CH14F08



DEPARTMENT OF CHEMICAL ENGINEERING

NATIONAL INSTITUTE OF TECHNOLOGY KARNATAKA,

SURATHKAL, MANGALORE - 575 025

JUNE - 2019

D E C L A R A T I O N

I hereby *declare* that the Research Thesis entitled **"AQUEOUS TWO PHASE EXTRACTION OF LECTIN FROM *PISUM SATIVUM* SEEDS"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in 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.

Name: Rashmi B S

Register number: 148020CH14F08

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 **"AQUEOUS TWO PHASE EXTRACTION OF LECTIN FROM *PISUM SATIVUM* SEEDS"** submitted by **Rashmi B S (Register Number: 148020CH14F08)** as the record of the research work carried out by her, *is accepted as the Research Thesis submission* in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy**.

Chairman - DRPC

Research Guide
Dr. I. Regupathi
Associate Professor
Department of Chemical Engineering
NITK, Surathkal

ACKNOWLEDGMENT

This thesis arose in years of research that has been undertaken since I came to this prestigious institute, NITK. By that time, I had an opportunity to work with number of people whose contribution in assorted ways to the research and the making of the thesis deserves a special mention. It is a pleasure to convey my gratitude to all of them in my humble acknowledgment.

First and foremost, I wish to express my sincere gratitude to my Research Guide, Dr. I. Regupathi, Associate professor, Department of Chemical Engineering, NITK-Surathkal. Without his valuable guidance, support and encouragement, I could never finish my Doctoral work. His wide knowledge of Chemical engineering, downstream processing and his logical way of thinking have been of great value to me. I thank him for graciously taking the time to read and offer the required improvements to the manuscripts and thesis.

I am extremely thankful to the RPAC committee members, Dr. P.A. Jagadeesh Babu, Department of Chemical Engineering and Dr. Jeyaraj P , Department of Mechanical Engineering, for their valuable advice and suggestions to improve the quality of my research work.

I express my sincere gratitude to The Director, NITK-Surathkal. I wish to thank the former H.O.Ds of Chemical Engineering, Dr. K. Vidya Shetty, Professor, Department of Chemical Engineering, NITK-Surathkal, Dr. B. Raj Mohan, Professor, Department of Chemical Engineering, NITK-Surathkal and our present H.O.D Dr. Hari Mahalingam, Associate Professor, Department of Chemical Engineering, NITK-Surathkal for providing the necessary support during the phase of this research work. I would also like to thank all the faculty members of the Department of Chemical Engineering, NITK-Surathkal for their valuable support and encouragement. I would also like to express my sincere thanks to Mr. Sadashiva, Mrs. Thrithila, Mrs. Bhavyashree, and Ms. Vijetha and all other non-teaching staffs for their helpful suggestions and timely maintenance of the laboratory equipment.

I am also grateful to all my former and current student colleagues, Ms. Swapnali, Mr. Basavaraj, Ms. Shwetha, Mrs. Smitha, Ms. Smruthi, Ms. Gayathri, Ms. Sushma, Mr. Sumit and all those who have helped me during my research work directly or indirectly.

I thank my other friends Ms. Supriya Kulkarni, Ms. Chaitra Kowshik, Mrs. Archana Satheesh and Mrs. Priyanka Biluve, for their care, support and encouragement.

I could not complete my thesis without the support and constant encouragement of my parent's Sri. Shashidhara B. S., Mamatha R. C., my in-laws Nagarajappa C. S., Renukamma H., my brother and sister, Ramu B.S. and Sowmya B.S., my bother-in-law and sister-in-law, Siddesh C.N. and Sheela S. I take the privilege to thank my husband, Naveen, for his unconditional and unwavering support throughout my studies.

Above all, I would like to thank to the almighty God for giving me the strength, wisdom and health to carry out and accomplish this research work.

RASHMI B S

DEDICATED TO MY FAMILY

ABSTRACT

The diversified properties of the lectins from different sources and their applications in biotechnological and pharmaceutical industries demand the economically feasible and scalable purification strategy. *Pisum sativum* seed lectin (Psl) has specific properties like anti-HIV reverse transcriptase, anti-HCV, anti-cancer, and mitogenic activities. The chromatographic process, which was commercially applied for the purification of this lectin, has number of limitations to implement in industrial scale. The Aqueous two phase extraction (ATPE) technique, a modified liquid-liquid extraction, was adopted for the recovery and partial purification of Psl from its seed crude extract. The PEG 6000-trisodium citrate system (ATPS) was identified as a suitable system by analyzing the influence of type of citrate salt, PEG molecular weight, phase components concentration, pH, additive (NaCl) and phase volume ratio on the partitioning behavior of Psl from the aqueous solution of pure Psl. The maximum partitioning coefficient of 14.5 was obtained for 18% PEG 6000-16% trisodium citrate system with 1% NaCl at pH of 8 and volume ratio of 1.32. Further, the optimized condition for the selective partitioning of Psl to the top phase from the crude extract of *Pisum sativum* seed was achieved in the ATPS formed with PEG 6000-sodium citrate at 41.01% TLL, 2% NaCl at pH of 7.5 with crude load of 20% and volume ratio of 0.76. The optimum value of crucial variables (PEG and salt concentration, NaCl, and pH) and their individual and interactive impact on the recovery and purity was identified using Response surface methodology. The optimum process conditions for different goals of extraction and purification were obtained by employing the desirability-based optimization tool and were validated experimentally. The feasibility of continuous operation of the extraction process has also been demonstrated in the Rotating disc contactor. Modeling of the equilibrium characteristics and the partitioning of the Psl was attempted using Artificial neural network.

Keywords: *Pisum sativum* lectin, aqueous two phase systems, Peg 6000-tri-sodium citrate, Rotating disc contactor.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ATPE	Aqueous Two Phase Extraction
ATPS	Aqueous Two Phase Systems
ANN	Artificial Neural Network
BCA	Bicinchoninic Acid Assay
BSA	Bovine Serum Albumin
CCD	Central composite design4
EEV	Effective Excluded Volume
IgG	Immunoglobulin G
mg	milligrams
mL	milliliter
PEG	Poly Ethylene Glycol
Psl	Pisum sativum lectin
pI	Isoelectric point
ppm	Parts per million
RDC	Rotating Disc Contactor
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
RPM	Rotation Per Minute
TFA	Tri-Fluoro Aceticacid

TLL	Tie Line Length
PF	Purification Factor
HA	Hemagglutination Activity
SHA	Specific Hemaggltiation Activity Partition coefficient
MSE	Mean Square Error
SC	Sodium Citrate Salt
PC	Potassium Citrate Salt
AC	Ammonium Citrate Salt

Symbols

ρ	Density (kgm^{-3})
μ	Viscosity of the liquid (mPas)
σ	Surface tension (mN/m)
H	Hold up
K_L	Partition Coefficient of Lectin
K_Da	Volumetric Mass Transfer Coefficient
V_r	Volume ratio
K_{SHA}	Specific Activity Partition Coefficient
nD	Refractive Index
K_P	Total protein content
Y_{HA}	Hemagglutination Activity Yield.

Scripts

t	Top phase
b	Bottom phase

CHAPTER 1

1. INTRODUCTION

Lectin, a group of protein or glycoprotein, has the ability to select and bind specifically to carbohydrate structures without altering the three-dimensional structure of the carbohydrate molecule. They are of non-immune origin and the interaction of lectin with carbohydrates is highly specific, non-covalent and reversible (Liener et al. 1986). Lectins are sometimes referred as "agglutinins" as they agglutinate the cells. William Boyd, in 1954, coined the term "lectin" which was derived from the Latin word 'legere' which means 'to select' (Boyd and Shapleigh 1954). Initially, lectins were called as phytoagglutinins or phytohaemagglutinin. However, it came to light that the proteins from other sources like animals, microorganisms also agglutinate the cells (Landsteiner and Raubitschek 1907). Thereafter the term lectin is generally used irrespective of the source.

The crosslinking of several RBCs (Red blood cells) by lectins leads to the precipitation of RBCs and the phenomenon is known as Hemagglutination (Sung et al. 1985). Hemagglutination is a classical and routine method used for detection and quantification of activity of the lectin. Landsteiner recorded the hemagglutination (agglutination of RBC) activity of lectins from different sources when tested on RBC of different animals (Landsteiner and Raubitschek 1907). However, the blood group specificity of lectins was identified by Renkonen (1948), Boyd and Reguera (1949). Subsequently, hundreds of blood group specific and nonspecific lectins were studied extensively from legume plants. Sumner holds the credit of isolating the first purified lectin, Concanavalin A (Con A) from jack bean. He also demonstrated the sugar specificity of this lectin (Sumner 1919). The progress in biochemistry and bioanalytical field lead to an improvement in the research pertaining to the structure, function and specificity relationship of lectins. The

researches explored the unique biochemical and biological properties of lectins and helped to extend their potential application to various fields such as biomedical field, biotechnological industry, food industry, and agricultural industry.

1.1 SOURCE OF LECTINS

Lectins are ubiquitously distributed in nature which can be found in plants, animals, and microorganisms. Animals are capable of producing varieties of lectin both intracellular and extracellular. Animal lectins discovered were found to be naturally multivalent; either because of their defined multi subunit structure or by virtue of having multiple carbohydrate-binding sites within a single polypeptide. The mass production of animal lectins is not feasible as it demands bulk quantities of raw materials. For example, the lectin purified from coral (*Acropora millepora*) plasma yields 0.7 mg per 100 mL plasma (Kvennefors et al. 2008), 0.35 μg/100g from *Nemopilema nomurai* (jellyfish) (Imamichi and Yokoyama 2010) and 0.97 mg/100g from *Bubalus bubalis* (Buffalo) heart tissue (Ashraf et al. 2010).

Fungi, bacteria, and Viruses express an enormous number of glycan-binding proteins or lectins. Lectins can be found in fungus where 82% arising from mushrooms, 15% from micro fungi and 3% from yeast (Singh et al. 2010). Fungal lectins were purified from *Fusarium solani* and *Macrophomina* with a yield of 26.7 and 3.3%, respectively. Yields of lectins from fresh mushrooms are low and around 2.6 mg of lectin was obtained from 100 g of fresh fruiting bodies of *Pleurocybella porrigens* (Suzuki et al., 2009). Most of the bacterial strain produces surface lectins commonly in the form of fimbriae. Best characterized lectin is mannose specific fimbrial lectins of *Escherichia coli.* The first microbial hemagglutinin identified was in the influenza virus, and it was shown by Alfred Gottschalk in the early 1950s (Nizet et al. 2017)

Plants are most widely explored and investigated source for lectin. Legume plants are one of the naturally rich sources of lectin (Sharon et al. 1990). Plant lectins are of heterogeneous group of proteins. Relatively large varieties of lectins with higher

concentration are distributed in many parts of the plant like seed, root, bulb, tuber, leaves, bark, stem and rhizomes (Peumans and Van Damme 1995). The amount of lectins present in different parts of plant will vary like, seeds contain about 50% of total seed proteins, and vegetative tissue contains 20% of total vegetative proteins. The seeds of legume plants contain very high amount of lectin in comparison with other parts of the plant. Lectin purified from French bean yields 1100 mg per 100 g of seeds, dark red kidney bean yields 107 mg per 100 g and 75 mg of the purified lectin was recovered from 100 g *Astragalus mongholicus* roots (Yan et al. 2005). Many of the legume seed lectins three dimensional structures were elucidated and thus their unique characteristics were well studied.

1.2 TYPES OF LECTINS

The unique characteristic which distinguishes lectin from other proteins is the carbohydrate binding property (Peumans and Van Damme 1995). The recent researches are mainly focused on the molecular characterization of the purified lectins from different plant sources. This includes sequencing of amino acids in polypeptides of lectins and elucidating their three dimensional structures (Ambrosi et al. 2005). More than 100 three-dimensional structures of plant lectins can be found in 3D lectin data bank (URL: http://www.cermav.cnrs.fr/databank/lectine). Based on the amino acid sequences of available lectins, it is deduced that the carbohydrate binding property of most lectins resides in a polypeptide sequence which is termed as carbohydrate-recognition domain (CRD) (Santos et al. 2014). Most of the lectins contain two or more CRDs which can interact with the sugars present on the surface of the RBCs.

Plant lectins are majorly classified in three ways based on their (a) overall structure (b) carbohydrate specificity (c) structural and evolutionary relationship. The classification scheme for the plant lectins is depicted in Fig. 1.1. Based on the overall structure of the mature lectins, they can be classified as merolectins, hololectins, chimerolectins, and superlectins (Van Damme et al 1998). Lectins with a single CRD is called as merolectins. These lectins are unable to agglutinate the cells due to their monovalent nature. A typical example for merolectin is Hevein, a chitin-binding lectin from the latex of *Hevea*

brasiliensis (Van Parijs et al., 1991). Hololectins possess two or more CRDs which are very similar or homologous, due to which they bind to structurally same or similar sugars. Hololectins exhibit the property of agglutination of cells due to their multivalency. Most of the plant lectins fall under this category. Chimerolectins are the fused proteins which contain one or more CRD fused to an unrelated domain. The latter domain has a well-defined "catalytic (or another biological) activity but acts independently of the carbohydrate binding domain". Depending upon their carbohydrate recognition sites they can behave as merolectins (class I plant chitinases) or as hololectins (type 2 ribosome inactivating protein). Superlectins are basically chimerolectins which have at least two CRDs which differ in structure and function, and have specificity towards structurally different sugars, like the lectin from a tulip bulb (Van Damme et al., 1996)

Another scheme of classification is based on the specificity of the lectins towards particular carbohydrates. Based on this concept, they are classified as mannose (*Canavalia ensiformis* lectin), mannose/glucose (*Pisum sativum* lectin), mannose/maltose (*Calystegia sepium* lectin), galactose (Gal)/ N-acetylgalactosamine (GalNAc) (*Ricinus communis* Agglutinin), N-acetylglucosamine (GlcNAc), fucose (*Ulex europaeus* agglutinin) sialic acid (wheat germ agglutinin), and complex glycan group (Lis & Sharon, 1986).

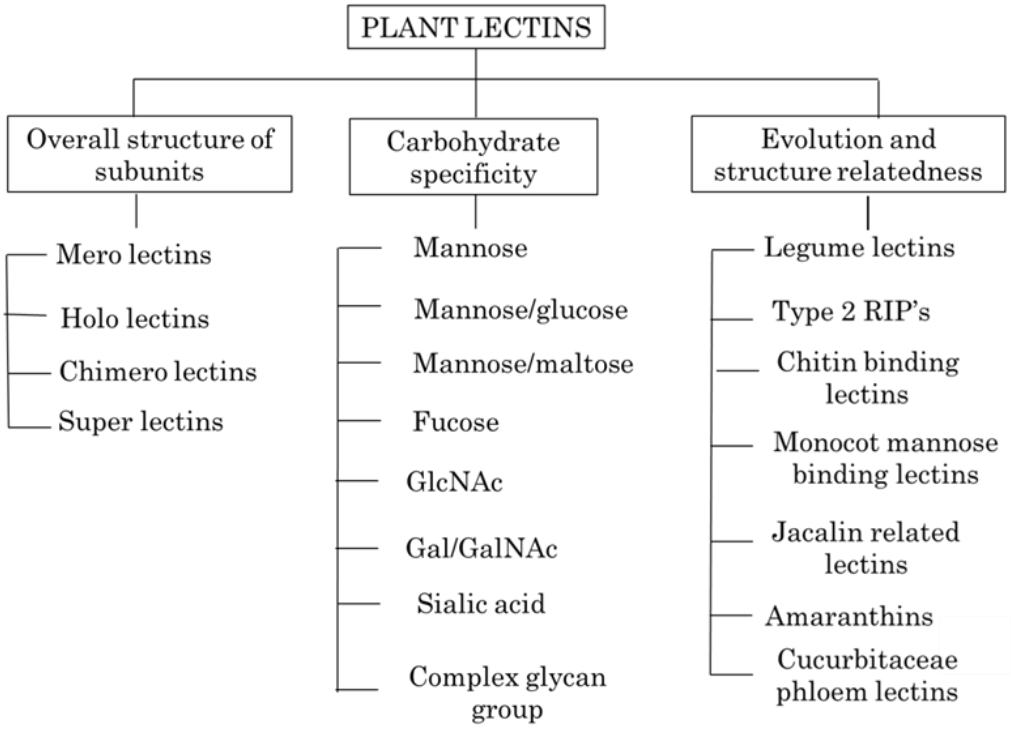


Fig. 1.1 Schematic representation of classification of plant lectins

Plant lectins are also categorized based on their evolution and structural relationship (Van Damme et al. 2008) as follows: (a) Legume lectins: are well-characterized families of lectins which can be exclusively found in the family of Leguminosae. Con A was the first purified legume plant lectin. (b) 'Monocot mannose-binding lectins' are the superfamilies' of lectins having specificity towards mannose. These lectins are found in six monocot families like *Araceae, Alliaceae, Amaryllidaceae, Bromeliaceae, Liliaceae, and Orchidaceae.* (c) Chitin-binding lectins: lectins which possess "Hevein" domain are classified as chitin-binding lectins. Lectins which belong to this family have similar carbohydrate specificity due to the conservation of amino acid sequences of hevein domain. (d) 'Type 2 ribosome- inactivating proteins (RIP)' are cytotoxic proteins which inactivate the eukaryotic ribosome catalytically. Ricin and abrin from *Ricinus communis and Abrus precatorius* respectively are the well-known lectins from this group. (e) 'Jacalin related lectins' are the galactose-specific lectins found in *Artocarpus integrifolia* (jackfruit). Lectins which are structurally and evolutionarily related to *jacalin* lectin fall under *jacalin*-related lectins. Amaranthine lectins are the GalNAc specific lectins found

in the seeds of *Amaranthus caudatus* family. These lectins are mainly involved in the plant defense. (f) 'Cucurbitaceae phloem lectins' are found in the phloem sap of the Cucurbitaceae plants. These are a small group of chitin-binding lectins, but they are devoid of hevein domain.

1.3 APPLICATION OF LECTINS

The availability of lectin in huge number in nature and their amenability to manipulation by chemicals, their inhibition by simple sugars and importantly the carbohydrate recognition and binding property made this protein as an attractive biotechnological tool in various fields of research. The property of specificity of a lectin towards particular carbohydrate is the main determinant of its application.

1.3.1 Antimicrobial and anti-insecticidal:

The antibacterial effect of lectin occurs through the interaction of lectin with carbohydrates (teichoic and teichuronic acids, peptidoglycans and lipopolysaccharides) present on the cell wall of the bacteria (Hamid et al. 2013). The cell surface glycans were utilized as receptors by many human pathogens to initiate the adhesion and infection (Zem et al. 2006). Lectins from plants and other source will display antimicrobial effect by interfering in this process showing anti-adherence agents (Islam *et al.,* 2009). The antifungal activity of lectin occurs through the binding of lectin to hyphas thus lead to the poor absorption of nutrients and reduces the spore germination (Lis and Sharon, 1981). Some of the lectins which exhibits antifungal and antibacterial properties are *Artocarpus incisa* seed lectin (*Fusarium moniliforme, Saccharomyces cerevisiae), Triticum vulgaris* seed lectin (*Fusarium graminearum, F. oxysporum*) and *Araucaria angustifolia* seed lectin (*Clavibacter michiganensis*), *Eugenia uniflora* seed lectin (*Bacillus subtilis, Escherichia coli*), respectively. The antimicrobial and anti-insecticidal properties of lectin can be used in agricultural sector to control the pathogens affecting the plant growth. Lectin usually increases the mortality rate of insects or delays the development of insect. Some of the insecticidal lectins are *Arisaema jacquemontii* (acts on *Bactrocera*

cucurbitae larvae) (Kaur et al. 2006), *Allium sativum* lectins (acts on *Spodoptera littoralis* larvae) (Sadeghi et al., 2008).

1.3.2 Anti-tumor and Antiviral activity

The antitumor activity is one of the most important properties of several lectins. Lectins elicit apoptosis (programmed cell death) process through various mechanisms which usually involves the activation of various caspases (called as cysteine aspartases, activation of these enzymes ensures the degradation of cellular components). There was a considerable evidence for the lectin induced apoptosis in several cancer cell lines, which includes the following examples; B16-BL6 melanoma cells (Park et al. 2001), and human A253 cancer cells were treated by Korean mistletoe lectin (Choi et al., 2004). HeLa cells were treated by *Agrocybe aegerita* lectin (Yang et al., 2009), Abrus agglutinin (Bhutia et al. 2008) and *Sophora flavescens* lectin (Liu et al. 2008). Breast cancer MCF-7 cells were treated by French bean lectin (Lam and Ng. 2010). A number of lectins which possess antitumor activity are in preclinical and clinical trials to use as a potential drug for cancer treatment (Ernst et al. 2003).

Lectin inhibits the viral replication by inhibiting the genome integration of HIV (human immunodeficiency virus) in the target cell (Chan et al. 2000). Banana lectin was reported to block the entry of virus by binding to the gp120 (glycoprotein 120) receptor on the cell envelope (Swanson et al., 2010). *Russula delica* lectin and autumn purple bean lectin were found to inhibit the HIV reverse transcriptase activity (Zhao et al., 2009, Fang et al., 2010). Concanavalin A, *Vicia faba* lectin, *Pisum sativum* lectin, *Lens culinaris* lectin, and *wheat* germ agglutinin are some of the lectins which were reported to exhibit anti HIV activity (Lam and Ng 2011). Due to the potential application in inhibiting the HIV infection, lectins were considered as the promising agents for the treatment of AIDS (Autoimmune disorder syndrome).

1.3.3 Lectin based techniques

Selective targeting of drugs to the proposed site of action provides therapeutic advantages such as reduced toxicity and smaller dose levels. In contrast to classical mucoadhesion,

which relies on nonspecific interactions of polymer chains and mucins, the lectin interactions with mucins are very specific. The unique carbohydrate specificities of plant lectins can facilitate mucoadhesion and cytoadhesion of drugs to specific targets and thus find its application in drug delivery (Lehr 2004).

Lectin-affinity chromatography has been widely used for the purification and analysis of oligosaccharides, glycopeptides, and glycoproteins (Hunter and Pines 1994). The advent of lectin microarray paved the possible way to study glycan structures of a specific protein, histochemistry of cells and tissues, biomarker screening (focusing on glycan structure changes), stem cell characterization, quick monitoring of therapeutic protein glycan structure and quick and easy glycan profiling of infectious virus and intestinal bacteria (Hu and Wong 2009). Lectin biosensor developed using lectin as a biorecognition element serves its utility in clinical diagnostics, food technology and environmental monitoring (Rahaie and Kazemi 2010).

1.4 SEPARATION AND PURIFICATION OF LECTINS

Owing to the popularity of lectin, the extraction and purification of lectins have gained importance. Initially, lectins were extracted using the conventional method of precipitation by salts and the solvents (Sattsangi & Sattsangi 1984). This method uses the harmful solvents and the purity of the desired molecules could not be assured. A major breakthrough in the field of purification of lectin came with the use of affinity chromatography which exploits the unique property of sugar specificity of the lectin (Sharon et al., 1974). Then onwards, most of the lectins are purified using chromatographic procedures; mainly affinity chromatography. Other chromatographic techniques such as gel filtration chromatography (Suseelan et al. 2007), ion exchange chromatography (Pajic et al. 2002), and hydrophobic interaction chromatography (Thakur et al., 2007) are also employed by a few researchers to purify lectin. The main drawbacks of chromatographic techniques such as the use of costly affinity matrices or adsorbents**,** scale-up issues, and the short lifespan of the columns due to the use of complex sources like the plant or seed extract, limit their application as an efficient downstream process

for high-value proteins (Alves et al. 2000). In this context, a few research groups tried the extraction and purification of lectin using membrane technology (Guo and Ruckenstein 2011), magnetic separation (Banerjee and Chen 2007), affinity precipitation (Teotia et al. 2006), and reverse micellar extraction (He et al. 2015). However, recovery of proteins with these methods has major disadvantages like fouling and cost of membranes in the membrane separation process, complexity in designing the magnetic particles in magnetic separation, a high cost of affinity ligands in affinity precipitation, and the denaturation of the protein in cationic and anionic surfactants in reverse micellar techniques. Further, the chemicals used in these separation processes may denature the sensitive biomolecules and the fragmented peptides may reduce the selectivity of the separation process (de Oliveira et al. 2009). This paved way for the inquisition of a new separation process that would use less harmful chemicals and provide a hydrophilic environment for the recovery of protein from seed extract. Lectin being a high-value protein demands the use of mild and economically feasible extraction and purification technique. The aqueous two-phase separation process is the one such technique which overcomes all the above constraints.

1.5 AQUEOUS TWO-PHASE EXTRACTION (ATPE)

ATPS (aqueous two-phase systems) is formed by combining aqueous solutions of two incompatible phase components (polymer-polymer, polymer-salt or carbohydrate-solvent etc.) above a critical concentration. One of the phase forming components predominates in one phase while the other predominates in the other phase. The partitioning of the biomolecule in ATPS depends on the net affinity of the desired biomolecule to any one of the phases. The phenomenon of aqueous two-phase formation was first reported by Beijerinck in 1986, but the prospective use of these systems in the separation of biomolecules was unveiled by Albertsson in 1986 (Albertsson 1986). Since then, ATPE has been successfully established as a feasible technique for the separation of biomolecules in their native state. The unification of recovery and fractional purification in a single step with considerable yield grabs the attention of researchers and make use of this technique for the lab-scale purification of biomolecules.

The complex biological samples such as animal extracts (Boland et al. 1989), plant extracts (Srinivas et al. 2002), and the industrial effluents (Iyyaswami et al. 2012) can be loaded directly into the ATPS without pretreatment and the partially purified biomolecules can be further purified by high-resolution techniques like chromatography (Raghavarao et al. 1995). Different types of components have the ability to form the ATPS in combination such as polymer-polymer, polymer-salt, ionic liquid-salt, thermo separating polymers, alcohol-salt, carbohydrate-alcohol (Azevedo et al. 2009). For the scale-up operation, polymer/salt systems are more preferable as they offer lower viscosity, lower interfacial tension, higher density difference between the phases, low chemical cost and faster phase separation (Raja et al. 2011). These factors are beneficial during potential execution at the commercial level. The commonly used polymer in the ATPS is polyethylene glycol (PEG) and it is available in various molecular weights. PEG's are non-toxic, non-immunogenic, non-antigenic, and highly soluble in water and solvents (Sedlak 2005). FDA (Food and Drug Administration) has approved PEG for biotechnical and medical applications. The stabilizing effect of PEG on the protein structure also helps to preserve the biological activity of protein (Azevedo et al. 1999). Initially, inorganic salts with cations such as sodium, potassium and ammonium and anions such as carbonates, phosphates, and sulfates were used along with the polymer as a phase component. These systems were not encouraged further due to environmental problems like the accumulation of sulfates and phosphates in effluents and water bodies. Organic salts such as citrates emerged as a promising eco-friendly alternative in ATPS due to their non-toxic and biodegradable properties (Verna and Kula 1990). Since then, PEG/citrate salt combination has been used to separate several proteins such as antibodies (Azevedo et al. 2009), penicillin acylase (Marcos et al. 1999), glycomacropeptide (da Silva et al. 2009).

ATPE is highly selective for specific biomolecules when compared to the other processes due to the tunable system properties like the hydrophobic/ hydrophilic interaction of proteins with polymers and the electrical potential difference between the

phases. ATPS is an attractive choice over traditional separation techniques at industrial scale for the extraction of a specific biomolecule from complex mixtures.

1.6 PARTITIONING OF BIOMOLECULES IN ATPS

The partitioning of a biomolecule in an ATPS depends on the surface properties of the biomolecule such as surface hydrophobicity, charge, the isoelectric point, molecular weight and biomolecule interaction with the phase forming components (PFCs) (Goja et al. 2013). Biomolecule interacts with the PFCs with a number of non-covalent interactions like hydrophobic force, hydrogen bonds, van der Waals interaction, electrostatic interaction etc. These interactions vary among both the phases which form a basis for partitioning of the target biomolecule (Hachem et al. 1996). The magnitude of the interactive forces between the PFCs and the biomolecule can be varied by varying the system parameters like pH, temperature, polymer, and salt concentration. Thus the partition of target biomolecule is made to concentrate in any one of the phases (Wongmongkol et al. 2006). Owing to the involvement of many variables and the complex nature of the source, development of a unique model to predict the partition coefficient of different biomolecules from diverse sources is less feasible.

The affinity of a biomolecule to a particular phase due to their surface properties is the major factor influencing the partitioning. To achieve maximum recovery of the desired biomolecule in a particular system, the knowledge of the interactive effect of variables on the partitioning is highly important and that can be analyzed through a statistical approach. Response surface methodology (RSM) is the widely applied statistical approach to study the interactive effect of the variables on the responses. RSM was successfully applied to optimize the ATPE process for the extraction of various proteins such as such as α-amylase using PEG/citrate (Zhi et al. 2005), thaumatin using PEG/sodium sulfate (Ahmad et al. 2008), papain using PEG/ammonium sulfate (Ling et al. 2010), recombinant *Bacillus halodurans* xylanase using PEG/phosphate (Rahimpour et al. 2007).

1.7 CONTINUOUS EXTRACTION OF BIOMOLECULES IN ATPS

The batch mode studies on ATPE of biomolecules were well studied and documented by several researchers. The high operational and maintenance cost, high processing time, lack of efficient and cheaper recycling method of PFC, and low mass transfer associated with the ATPE made the industries reluctance to adopt for large scale operation. By considering the facts, many research groups came out with the concept of continuous aqueous two-phase extraction process which has many advantages such as lower cost, high mass transfer, process automation, and continuous recycling of phase forming components (Rito-Palomares 2004, Vázquez-Villegas et al., 2011). The extractors used in liquid-liquid extraction became a basis for the implementation of continuous extractors in ATPS. The column extractors, specifically the agitated contactors are exploited for the ATPE. The Rotating disc contactor (RDC), rotary annular column, pulsed cap columns and vanes agitated column etc. are some of the agitating column extractors used for the liquid-liquid extraction (LLE) (Biazus *et al.,* 2007). The selection of extractor for a particular extraction process depends on biomolecule diffusivity, viscosity and interfacial tension of the system, energy consumption of the extractor, and cost of the extractor. Among the different types of column extractors, RDC has gained popularity due to its simplicity, high throughput and less power consumption (Moris et al. 1997). RDC has been used extensively at the industrial level. Some of the proteins include cutinase (Cunha et al. 2003), bovine serum albumin (Porto et al. 2000), and ascorbic oxidoreductase (Porto et al. 2004) were extracted continuously using the RDC by employing the ATPS. The column performance of the RDC is improved by the action of rotating discs, which disturbs the drop motion and thereby increases the interfacial mass transfer by size reduction of the drops. Operational variables such as disperse and continuous phase flow rate and rotor speed affect the hold-up capacity of the column and mass transfer coefficient which in turn defines the efficiency of the column. The knowledge of hydrodynamic and mass transfer studies is essential for the designing and scale-up of RDC for a specific biomolecule. The design of RDC for efficient lectin extraction may help to establish the technique at the commercial level.

1.8 MODELING OF THE AQUEOUS TWO-PHASE EXTRACTION PROCESS

The prediction of the phase equilibrium of the ATPS and the partitioning characteristics in the system may reduce the waste experimentation and improve the application of ATPS. Phase equilibrium studies are associated with the determination of the concentration of phase components in the two phases that are in equilibrium. The direct measurement of phase equilibrium is often difficult, time-consuming and requires expensive instruments which are not available in all laboratories. In this context, researchers came up with the concept of modeling of phase equilibria. Many thermodynamic models and empirical models were used for the correlation and prediction of the binodal and tie line data (Ghanadzadeh et al. 2011). But an attempt at modeling the partition of a biomolecule from complex sources is very rare. However, some of the thermodynamic models such as UNIQUAC, lattice model techniques, scaling thermodynamic techniques and virial expansions were used to highlight the influence of several molecular-mechanisms on protein partitioning (Hatti-Kaul 2001). The exact mechanism governing the protein partitioning in an ATPS is complex and not well understood (Raghavarao et al. 1995). So the conceptual equations developed to model the partition of a protein in an ATPS system is not a good choice. Alternatively, soft-computing methods (Sanchez et al. 1997), which concern with the computation of an imprecise environment, have gained significant attention. The main components of soft-computing, namely fuzzy-logic, neural network, and genetic algorithms have shown great ability in solving problems of complex nonlinear systems. ANN (Artificial neural network) is an algorithm based modeling technique which was inspired by the human brain framework and function (Asfaram et al. 2016). ANN is highly flexible and its nonlinear models have been successfully used in estimating complicated systems such as VLE (vapor-liquid equilibria) and LLE (liquid-liquid equilibria) data (Bogdan et al. 1995, Ganguly 2003). ANN was also implemented by a few researchers to model the aqueous two phase extraction of a biomolecule from complex sources such as Bromelain from pineapple (Coelho et al. 2015) and Alkaline active xylanase from recombinant E.coli (Rahimpour et al. 2016).

CHAPTER 2

2. LITERATURE REVIEW

The basic characteristics of lectins, their availability in nature and potential area of applications were consolidated and explained in detail. The extent of research progressed with respect to the purification of lectin for their characterization and application were also discussed based on the information available in the literature. The techniques/ methods adopted for the purification of lectins including chromatographic, membrane, reverse micelle processes and the combination of these methods were consolidated and analyzed in detail. The fundamental details and method of operation of Aqueous Two Phase Extraction (ATPE) along with the effect of variables on the equilibrium and partitioning characteristics of biomolecules in the system were also discussed. The optimization method employed to optimize the variables of ATPE, specifically the Response Surface Methodology (RSM) and the ANN modeling of the ATPE was discussed. The equipment's utilized to implement the ATPE in continuous process were also discussed. Based on the literature survey and the critical analysis, the research gaps were identified and scope for the present research was established.

2.1 LEGUMES LECTINS

Legume lectins (L-type lectins) are one of the large families of sugar binding protein. They are found in legume plants. They belong to a family of Leguminosae (or Fabaceae). The mature seeds of leguminous plants are a major source of lectin, where they represent 10% of the total protein content in the seed. A small content of lectin can also be found in other parts of the plant like leaves, root, and bark (Yanagi et al. 1990). Seed lectin can be found in organelles called protein bodies in the cotyledons along with the other storage proteins (Etzler 1986). Beans, peas, peanuts, lentils are some of the popularly known legumes from which lectin can be extracted. The primary sequence of most of the legume lectins is known, and it was shown that they have remarkable homology with some of the invariant amino acid residues. Although legume lectins belong to the same taxonomic

group and have similar physicochemical properties, they exhibit variation in their quaternary association and carbohydrate specificity (Loris et al. 1998). Legume seed lectins serve as a model system to investigate biological processes such as microbial infection, cancer metastasis, growth and differentiation, targeting the cells, etc. due to their known three-dimensional structures and varied sugar specificity (Sharon and Lis 1990). Legume lectins consist of two or more subunits where each subunit is characterized by a carbohydrate binding site. Metal ions such as Ca^{2+} and Mn^{2+} or other transition metal ions are required for the interaction of lectin with carbohydrates. This metal ion binding site is well conserved in legume lectins, and these were described in detail for the first time in Concanavalin A lectin (Hardman et al. 1982). Many of the legume lectins crystallographic coordinates are deposited in the PDB (protein data bank). Some of these include lectin from Concanavalin A (Edelman et al. 1972), *Pisum sativum* lectin (Einspahr et al. 1986), soyabean lectin (Dessen et al 1995, Loris et al. 1994), peanut agglutinin (Banerjee et al. 1996), and *Phaseolus Vulgaris* Leucoagglutinin (PHA-L) (Hamelryck 1986).

2.2 HEMAGGLUTINATION AND HEMAGGLUTINATION INHIBITION ASSAY

Though the ability of lectins to agglutinate red blood cells (RBCs) was known as early as in the 1900s, the reason for the agglutination was discovered by researchers in the 1950s. The link between the recognition of carbohydrates on the surface of RBCs by lectins and the process of agglutination was first demonstrated by Watkins and Morgan in 1952 (Watkins and Morgan 1952). Hemagglutination assay is a simple procedure followed to determine the activity of the lectin based on the agglutination of RBC cells. The main principle of the assay relies on the interaction between the carbohydrate binding site of lectin and carbohydrate moieties present on the RBCs. This interaction results in an extensive network formation as shown in Fig. 2.1a. Hemagglutination is generally assayed by “serial dilution using human or rabbit erythrocytes”. The estimation of lectin activity is possible by adding a constant quantity of RBC to a serially diluted lectin

sample in 96-well microtiter plate (Correia et al. 2008). Detailed procedure to measure lectin activity is given in 3.5.4.

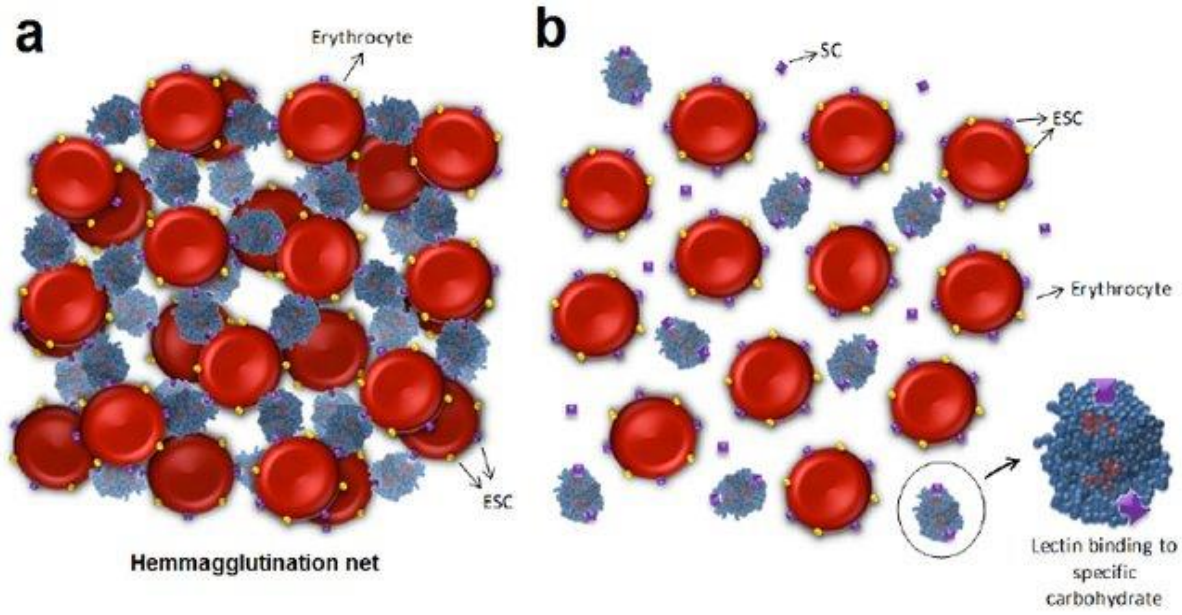


Fig. 2.1 Scheme of hemagglutinating activity (HA) and inhibition of HA assays. (a) The lectin sample induces hemagglutination due to lectin linkage to erythrocyte surface carbohydrate (ESC). (b) HA inhibition occurs when lectin sample is incubated with carbohydrate prior to addition of erythrocytes; binding of a specific carbohydrate (SC) to lectin sites extinguish net formation **(Santos et al. 2013).**

The lectin specificity towards specific carbohydrates was obtained by hemagglutination inhibition assay (HIA). The main principle of HIA assay is that the lectin-carbohydrate binding sites will be occupied by the carbohydrates present in the sugar solution and no free sites will be available on the lectin to make interaction with the RBC cells. As a result, no network formation takes place and RBC cells settle to give a red button (Fig. 2.1b). Serially diluted specific sugar solution with phosphate buffer saline was prepared in a microtiter plate, where a constant quantity of RBCs were added, incubated and visualized for results. The HIA is expressed as the "lowest concentration of the

carbohydrate solution that completely inhibits hemagglutination" **(**Sano and Ogawa 2014).

2.3 *PISUM SATIVUM* PLANT (PEA PLANT)

2.3.1 Plant Description

Pea plant is an annual crop with tendril climbing stem. It usually grows in the cool season. It belongs to the Leguminosae family. *Pisum sativum* (*P. sativum*) seeds are usually round, soft and green in color (Fig. 2.2). Seeds are enclosed in a pod which contains two seed valves (Pavek 2012).

Fig. 2.2 Plant and seeds of *Pisum sativum*

Seeds are enclosed in a pod which contains two seed valves (Pavek 2012). It originated from the Mediterranean basin and near east. Pea stands in the fourth position after peanut, soyabean and dry bean (Vidal-Valverde et al. 2003) in the production of legume foods. The major pea producing countries are China, India, Canada, Russia, France, and the United States (Food and Agriculture Organization, 2012). In mature pea seeds, the lectin is localized within protein bodies along with the major storage proteins such as legumin and vicilin (Higgins et al. 1983).

Table 2.1 Taxonomical classification of *P. sativum* based on the 'United States Department of Agriculture'

Kingdom	Plantae	**Subclass**	Rosidae
Subkingdom	Tracheobionta	**Order**	Fabales
Superdivision	Spermatophyta	**Family**	Fabaceae
Division	Magnoliophyta	**Genus**	*Pisum*
Class	Magnoliopsida	**Species**	*Pisum sativum*

2.3.2 Structure of Pea Lectin

Pisum sativum lectin (Psl) is a β barrel protein (β sheets twists and coils to form closed structure). It is composed of two different polypeptide chains, a α-chain of about 6,000 D and a β-chain of about 18,000 D (Ng et al. 2015). The quaternary structure of the lectin is dimeric, consisting of two αβ monomers non-covalently linked together making up a molecular weight of 49 kDa. The three-dimensional structure of other legume lectin monomers is similar to Psl monomer in spite of their differences in processing and multimerization. It was reported that Psl exists as a dimer, where each monomer built up to form two β sheets of seven (curved front face) and six (flat back face) anti-parallel strands interconnected by turns and loops. A third smaller β-sheet made of five short strands, also referred to as the S-sheet, helps to keep the front and back sheets together (Barre et al. 2001).

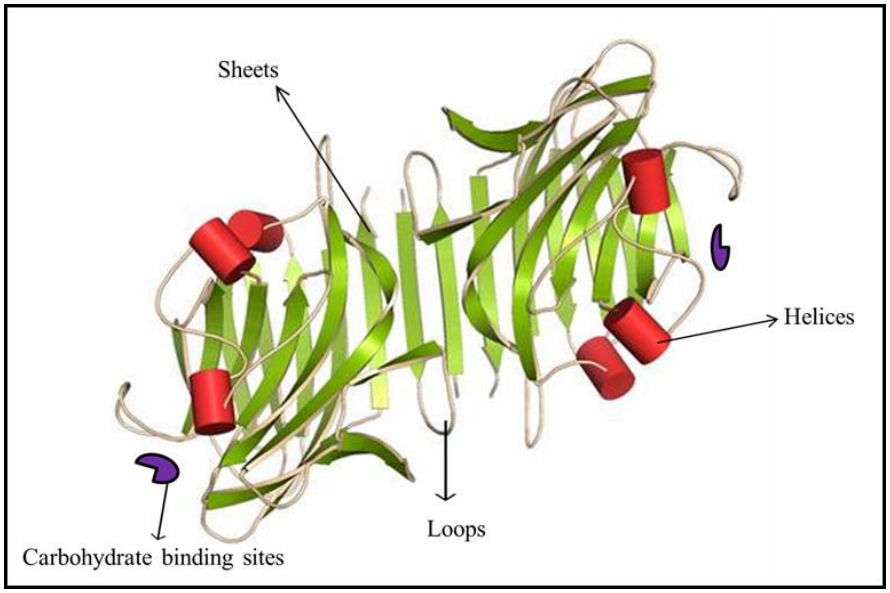


Fig. 2.3 Dimeric structure of *Pisum sativum lectin* which shows Canonical twelve - stranded beta - sandwich structure.

The structures resemble flattened bell-shaped domes containing a shallow pocket at their apex, which forms the carbohydrate-binding site. The bottom of the pocket contains binding sites for bivalent metal ions (Barre et al. 2001). Fig. 2.3 represents the Canonical twelve - stranded β - sandwich structures which is exhibited by almost all mannose/glucose binding lectin. Pea lectin is a metalloprotein which requires Mn^{2+} and Ca^{2+} to maintain its structural integrity and stability. It has metal binding sites, which are conserved in mannose/glucose specific lectins. The amino acid composition of pea lectin reveals that the lectin is rich in hydroxyl amino acids and lacks sulfur amino acids like cysteine and sulfur (Higgins et al. 1983). The hydrophobic sites of pea lectin are mainly made of hydrophobic amino acids such as valine, isoleucine, and phenylalanine (Liener 2012). The crystallographic structure of pea lectin up to 2.6 Å has been elucidated (Rini et al. 1983).

2.3.3 Applications of *Pisum sativum* lectin

The therapeutic application oriented properties possessed by pea lectin made researchers to explore the protein for various applications in different fields such as biomedical,

biotechnology industry, agricultural and food technology. The important properties possessed by the pea lectin are discussed below.

2.3.3.1 Anti-HIV-1 Reverse transcriptase activity and anti-HCV activity

There are two types of Human immunodeficiency viruses (HIV), namely HIV-I and HIV-II, HIV-II being less contagious. The replicative cycle of HIV-I virus depends on various macromolecules including viral receptors and enzymes (*e.g.*, HIV-Reverse transcriptase, HIV-Protease, HIV-Integrase) (Pommier et al. 2005). HIV-1 Reverse transcriptase catalyzes the synthesis of a double-stranded proviral DNA using the viral genomic RNA (Ribonucleic acid) (Peliska and Benkovic 1992). *Pisum sativum* agglutinin (PSA) binds to the envelope glycoprotein gp120 to inhibit fusion of HIV-infected cells with CD4 cells by a carbohydrate-specific interaction. It was reported that 0.1 mg/ml concentration of pea lectin can inhibit nearly 64% of the activity of HIV-1 reverse transcriptase (Ng et al. 2015). Psl have also been evaluated for its ability to inhibit Hepatitis C virus replication. The inhibition activity was found at 20 and 40 microgram/mL and it was suggested that the lectin can be used as an effective drug in designing safe anti HCV therapy **(**Al-Sohaimy et al. 2007).

2.3.3.2 Anti-cancer activity

Multiplication of cancer cells was stopped by several kinds of plant lectin and due to the differences in their sugar specificity, each lectin exhibits differences in their anti-proliferative effect against tumor cell lines.

Pea lectin was analyzed for its ability to act as an anticancer agent on SW480 and SW48 cell lines. Apoptosis was induced by Psl in both cell lines which was further confirmed by the increased level of caspase-3 and caspase-9. Also, the increased expression of p53 and p21 (protein that regulates cell cycle progression) proteins was observed with the reduction in the level of PARP1 (involves in repairing the breaks in single-stranded DNA) protein expression (Islam et al. 2018). Psl showed 64-68% of inhibition in these cell lines. At 1mg/ml dosage, Psl showed nearly 62% and 63% of cell growth inhibition in SW48 and SW480 respectively. Psl was evaluated for its anticancer property on

Ehrlich ascites carcinoma (EAC). Mice were made to develop EAC and Psl was injected in different doses. It showed 63% of inhibition of growth against EAC cells when mice were injected with 2.8mg of Psl/kg/day. It was noticed that Psl induced the cell arrest and there was an increased level of Bax gene expression (induces apoptosis) and much reduced level of Bcl-2 and Bcl-X gene expression (encode proteins that inhibit cell arrest) (Kabir et al. 2013).

2.3.3.3 Mitogenic activity

Mitogenic activity of the lectins and their derivatives was assayed in mouse spleen cell suspensions which are prepared from spleens of hybrid mice 2-8 months old. The mitogenic activities of native pea lectin and its chemically modified derivatives were studied and compared with that of Con A. The dose-response curve of native pea lectin was similar to that of Con A displaying maximum mitogenic activity at low concentrations of lectin and inhibition of the response at high concentrations of lectin (Sitohy et al. 2007). The mitogenic activity of Psl was analyzed on cell suspension of mouse spleen cells. The binding of Psl to glycoproteins present on the spleen cells transfers the mitogenic signal from the cell membrane to cytoplasm and makes cell to activate and differentiate. The maximum stimulation of spleen cells occurred at 2 microgram/mL (Trowbridge 1973).

2.3.3.4 Anti-microbial activity of Psl

The binding of Psl to N-acetylglucosamine sugar moieties on the cell wall of the fungi affects the fungal activity and the viability and finally inhibits the growth. The growth of *Aspergillus flavus, Trichoderma viride*, and Fusarium oxysporum are also inhibited by Psl. Growth inhibition of *Fusarium oxysporum*, *Aspergillus flavus*, and *Trichoderma viride* was noticed in the presence of Psl at several serial dilutions, where the original concentration was (100 mg/ml). Psl inhibited the growth of *Aspergillus flavus*, *Trichoderma viride* and *Fusarium oxysporum* with LIC of 0.1, 0.01, and 0.01 mg/ml respectively (Sitohy et al. 2007). Psl binds to mannose associated receptors on the bacteria and inhibits its growth. It was reported that Psl inhibited the growth of bacteria

such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa* (Nair et al. 2013). Also, when the pollen beetle (Meligethes aeneus) larvae fed on mannose/glucose specific lectin expressed transgenic oilseed rape, the growth of the larvae reduced which shows the insecticidal activity of lectin (Melander et al. 2003).

2.3.3.5 Diagnosis

Enterococcus faecalis which belongs to the group of Streptococcus is a gram positive bacterium which inhabits the human gastrointestinal tract. This can cause life-threatening infections in humans, especially in the nosocomial (hospital) environment. Peroxidase-conjugated pea lectin was used to identify fucosyl residues in the cell walls of Enterococcus faecalis with Western blotting (Shimura et al. 1987).

2.4 EXTRACTION AND PURIFICATION STRATEGIES TOWARDS LECTIN PURIFICATION

The exploitation and detailed study of unique properties of lectin and their applications in various fields emphasize on the downstream processing of lectins. Various separation methods are employed to extract and purify lectins. The primary step in any protein purification is the preparation of the crude extract from the selected source. An aqueous solution is regarded as a suitable extraction medium for lectins, as lectins are completely soluble in water, buffer, and saline solutions (Araujo et al. 2012). The obtained extract was evaluated for protein concentration and lectins' activity prior to selecting it as a starting material for lectin purification. Salting out process was majorly used to fractionate the soluble proteins from the crude extract. High concentration of salts captures the water from hydration layer of the protein and helps to precipitate out protein. Ammonium sulfate is a widely used salt for the salting out process of proteins. Dialysis is used to remove the salts from the fractionated proteins. Acid precipitation is also employed in lectin extraction process to remove abundant storage proteins from the crude extract and acetic acid is widely used for acid precipitation. The details of separation techniques employed for the extraction and purification of various legume lectins, their

carbohydrate specificity, and the main outcome of the studies are represented in Table 2.2.

2.4.1 Chromatographic techniques

Even though the presence of lectin in legume plants was recognized long back, very few lectins were purified until the 1970s. Initially the conventional methods of extraction involving a series of precipitation with salts and solvents were used. One of the dramatic changes in the lectin purification happened with the advent of affinity chromatography. Goldstein and workers used cross-linked dextran gels to capture the lectins (Agrawal and Goldstein, 1967). Since then, most of the purification strategies include affinity chromatography which exploits the most important property of the lectin i.e., the specificity towards sugar. The application of this technique to purify lectin depends mainly on the interaction of the lectin to be purified with the ligand of the stationary phase in a specific and in reversible manner. Based on the lectins carbohydrate specificity, a variety of affinity matrices are employed such as guar gel (dos Santos Nunes et al. 2011), agarose (Sun et al. 2007), affi-gel blue gel (Shao et al. 2011) and many others as listed in Table 2.2.

Many researchers have demonstrated the loading of crude extract directly to the affinity column without any pre-purification steps such as precipitation. Single step affinity chromatography was employed for the purification of various legume lectins. However, some of the chromatographic purification strategies include acid and salt precipitation prior to actual column loading. This was done to concentrate the proteins by eliminating excess contaminants from the crude extract. The combination of acetic acid and ammonium sulfate precipitation or any one of the process was usually employed as pre-purification step before the application of the sample to the column (Naeem et al. 2007). The purification of lectins was carried out by ion exchange chromatographic technique. Many of the lectins were successfully purified by employing ion exchange matrices like DEAE (diethylaminoethyl)-cellulose (Katre et al. 2008), DEAE-Sephadex (Silva et al. 2012), carboxymethyl (CM)-Sepharose (Katre et al. 2008) and using others mentioned in Table 2.2.

Size exclusion chromatography has also been employed to purify lectins based on molecular size (Kennedy et al. 1995). Some of the molecular exclusion matrices used was Superdex 75 (Silva et al. 2012), Sephadex G-100 (Costa et al. 2010) and Sephacryl S200 (Shao et al. 2011). However, other than affinity chromatography, all other chromatographic techniques were not used as a single step technique; instead, they were used in combination with other chromatographic techniques to purify a lectin.

Even though the chromatographic techniques were successful in the purification of various lectins, the limitations associated with these techniques restrict their commercial establishment. The major limitations of the techniques are: the typical chromatographic media which is made up of complex sugars of high economic value, which increases the cost of the purification process. The implementation of a series of chromatographic techniques to purify a lectin increases the overall production cost and it is economically not feasible. The direct loading of crude extract to column limits the life of the column. The number of purification steps reduces the efficiency of recovering the target product. It can only be operated in batch mode and difficulty in scaling up limit their application at the industrial level. Low handling capacity and longer time consumption were also major drawbacks.

Table 2.2 Chromatographic techniques to purify legume lectins

Legume seed lectin	**Purification strategy**	**Carbohydrate specificity**	**Inference**	**Reference**
Erythrina speciosa	Single step affinity chromatography on lactose–Sepharose column	D-galactose	SHA=773 U/mg, PF=386, Y=66%	Konozy et al. 2003
Dioclea lasiophylla	single step affinity chromatography on a Sephadex - G-50 column	mannose and and glycoprotein (ovalbumin and fetuin)	SHA=5390 HU/mg, PF=27.5	Pinto-Júnior et al. 2013
White tepary Bean	Ammonium sulfate followed by affinity chromatography 6on an immobilized Fetuin matrix.	N-glycans	SHA= 226376.51, PF=26, Y=0.586%	Valadez-Vega ET AL.2011
Cicer arietinum L.	ammonium sulfate fractionation and affinity chromatography on an N-acetyl-D-galactosamine-linked agarose column	N-acetyl-D-galactosamine	SHA=24.38, Y=34.40%	Qureshi et al. 2006
Trigonella foenumgraecum	acid precipitation, salt fraction ation, and affinity chromatography on mannan cross-linked agarose	glucose/mannose	SHA=324.1, PF=10.1, Y=0.04%	Naeem et al. 2007
Bauhinia forficata	Ammonium sulfate fractionation, dialysis,	Fetuin, asialofetuin,	SHA=7876.9 U/mg	Silva et al. 2012

	DEAE-Sephadex ion exchange chromatography, Sepharose-4B and chitin affinity chromatography and Superdex 75 size exclusion chromatography.	thyroglobulin and azocasein	23.5%, PF= 93.2	
Phaseolus vulgaris cv.	Affinity chromatography on Affi-gel blue gel, fast protein liquid chromatography (FPLC)-ion exchange chromatography on Mono S, and FPLC-gel filtration on Superdex 200.	glucose/mannose	SHA=4267 titer/mg, Y=26mg/g, PF=15.80	Sharma et al. 2009

SHA=Specific hemagglutination activity, Y=Yield, PF=Purification factor

2.4.2 Non-chromatographic approaches for lectin purification

The limitations of the chromatographic approaches to purify high-value proteins like lectins made researchers to investigate analogous processes. Various non-chromatographic techniques implemented by several researchers to purify lectin are discussed below.

2.4.2.1 Membrane separation

The principle of affinity binding and membrane separation was combined to develop an affinity membrane separation process to purify lectins. The affinity of lectin towards specific sugar was kept as a basis for the development of affinity membranes and the ligands were selected based on the lectin to be purified. An affinity membrane was first developed in 1984, by Mattiasson and Ramstorp with a combination of affinity binding and ultrafiltration to purify Con A lectin from the crude extract of *Canavalia ensiformis.* The heat killed yeast (*Saccharomyces cerevisiae*) cells were used as the macromolecular ligand. Con A interacts with the exposed carbohydrate residues on the surface of the

yeast cells forming a high molecular weight complex. Lectin from the complex was separated by providing free ligand (glucose). A few years later, in 1998, Zeng and Ruckenstein developed macroporous chitin membrane which has the advantages of high porosity, high surface area, and more accessibility to binding sites (made of numerous N-acetyl-D-glucosamine (GlcNAc) moieties) for the separation and purification of wheat germ lectin (specific to N-acetyl-D-glucosamine) from wheat germ. The complexes of lectin and sugar moieties were separated by 1 M acetic acid. Guo and Ruckenstein in 2001 developed macroporous cellulose membrane (activated by 1, 4-butanediol diglycidyl ether) which was immobilized with maltose (ligand) to purify Con A from crude extract of jack bean. The major yields and purity of all the membrane affinity techniques are given in Table 2.2. Some commercial lectins were used to evaluate the affinity membrane adsorption capacity towards specific lectin and they are represented in Table 2.2. However, the use of affinity membrane technology is not feasible due to the associated higher cost and decreased efficiency by fouling, protein-protein interaction and protein adsorption.

2.4.2.2 Magnetic separation

The affinity membranes were prepared by using magnetic-based material (usually iron oxide) as a support to which adsorbents were attached. Ligands were immobilized or attached to adsorbent to adsorb the specific proteins. In 2004, Heeboll-Nielsen and coworkers demonstrated the development, testing and uses of HMGF (High gradient magnetic fishing) technique for the purification of Con A directly from the crude extract of Canavalia brasiliensis. Various magnetic adsorbents were prepared by using super magnetic iron oxide core as a support. The affinity membrane was prepared by incorporating various ligands such as mannose, glucose, 6-amino-4-hydroxy-2-naphthalene sulphonic acid (AHNSA), D-(+)-glucose, 2, 5-Dimercapto-1, 3, 4-thiadiazole (DMTD) and dextran-derivatized adsorbents. The two lectins, Con A and Lens culinaris which have specificity to glucose were selected as model proteins in small-scale studies to characterize the adsorbents. Among all the magnetic affinity adsorbents dextran derivatized adsorbent showed high binding capacity of 240 mg/g of adsorbent.

A few years later, in 2007, Shashwat and his group evaluated two different magnetic nanoparticle-based affinity membranes to evaluate their specific adsorbent capacity for Con A (specific to glucose/mannose). The nanoparticles used were gum arabic modified magnetic nanoparticle (GAMNP) and glucose grafted gum arabic modified magnetic nanoparticle (G-GAMNP). Con A was adsorbed specifically to G-GAMNP but not to GAMNP. Gum Arabic is a polysaccharide which has galactopyranose units whereas Con A is glucoside-binding protein due to which it adsorb to G-GAMNP. The effect of pH on adsorption of lectin was studied. Acidic pH leads to the leaching of metal ions and pH above 7 leads to the denaturing of the lectin. Maximum adsorption specific capacity was seen around pH 7 where Con A exists as a tetramer (Banerjee and Chen 2007).

Con A lectin, specific to mannose/glucose was purified from the crude extract of jack bean using glucosylated magnetic nano matrix (GMNM). The glucose moiety which was immobilized on the magnetic nano matrix (MNM) acts as a binding site for the Con A. Binding efficiency of the Con A on GMNM was evaluated by using 20-1000µg of commercially available Con A and 2mg of GMNM. Efficient binding of Con A to GMNM occurred at 20 µg and the binding efficiency reduced above this concentration due to limited binding sites and limited available surface area. Later, Con A from crude extract was purified with GMNM in batch studies (Table 2.3). Magnetic separation technique suffers from the high price of the current generations of commercial adsorbents. The automation required would be rather complex and expensive, negating the initial advantage of simplicity and low cost. A large number of separation steps are needed in the purification protocol. Designing of magnetic particles is a complex process.

2.4.2.3 Affinity precipitation

Affinity precipitation is yet another purification strategy involving a combination of affinity binding and precipitation. Suitable affinity ligands were selected based on the protein to be purified. Guar gum (composed of galactose and mannose) linked alginate was used as an affinity ligand to purify peanut lectin (specific to galactose carbohydrate residues) from peanut crude extract. Dialyzed crude extract was stirred with guar gum

linked alginate solution. Alginate was precipitated by adding 2M $CaCl_2$ and 30mM $CaCl_2$ to remove unbound proteins. Galactose was used to elute bound ligand from the alginate. The purified lectin was analyzed on SDS PAGE which gave a single band of 29000 D. This method yields about 46mg of lectin/100g of peanut seeds (Tyagi et al. 1996).

Teotia and his coworkers studied affinity precipitation and aqueous two-phase affinity extraction (ATPAE) to purify a lectin from the crude extracts of potato, tomato, and wheat germ (all the three lectins were specific to N-acetyl glucosamine) by using chitosan (composed of D-glucosamine and N-acetyl-D-glucosamine) as a smart ligand. In affinity precipitation, chitosan was used as a smart affinity ligand, where it is soluble at pH below 6.5 and insoluble above pH 6.5. When the crude extract of lectin was incubated (by stirring) with chitosan solution, the lectin-chitosan complex was formed which was precipitated by raising the pH to 7. The dissociation of the lectin from chitosan was carried out using $MgCl_2$. Wheat germ, potato, and tomato lectin activity recovery were found to be 84%, 88% and 82% with purification fold of 24, 15 and 16 respectively. ATPAE method was also tested to purify all the three lectins mentioned above. Aqueous two-phase system composed of PEG (Polyethylene glycol)/salt was prepared with chitosan as a free ligand (which partition to PEG phase). WGA (wheat germ agglutinin) which was partitioned to PEG phase forms complex with chitosan. The complex was precipitated and WGA was recovered as explained in affinity precipitation. 90%, 85%, and 75% activity yield with 25, 37, and 22 fold purification was obtained for wheat germ, potato and tomato respectively. Purified lectin was observed as a single band (of subunit molecular weight 50 kDa) on SDS-PAGE (Teotia et al. 2006). The high cost of affinity ligands and matrix limits the use of affinity precipitation as an efficient separation technique.

2.4.2.4 Reverse micellar extraction (RME)

Reverse micellar extraction is a liquid-liquid extraction which uses reverse micelles in the extraction process. Reverse micellar extraction generally consists of two steps: forward and back extraction. The transfer of biomolecules from the aqueous phase to organic

micellar phase takes place in the forward extraction, whereas the release of protein from reverse micelles to stripping aqueous phase takes place in the back extraction (Anjana et al. 2010). Factors that affect the protein transfer in reversed micelles are water content in reversed micelles, aqueous phase pH, ionic strength and type of ion, protein charge, surfactant and critical micellar concentration (CMC), volume ratio, and shape and size of reversed micelles (Kilikian et al. 1999).

In 2002, Nascimento et al. implemented the RME separation process to purify lectin from *Cratylia mollis* seed extract. Sodium di (2 - ethylhexyl) sulfosuccinate (AOT), an anionic surfactant in isooctane, was used to prepare the reverse micellar phase. Initially, the extraction was carried out using commercial Cratylia mollis lectin and obtained 100% forward (at pH 5) and back-extraction (at pH 10). Same conditions were applied to extract lectin from *Cratylia mollis* seed extract to achieve 38% of forward-extraction and 100% of back-extraction. It was reported that the lower extraction in comparison with pure lectin was mainly due to contaminants in the crude extract that can reduce the radius of the reverse micelle. He et al. in 2013 studied the purification of *Phaseolus vulgaris* (black kidney bean) from its seed crude extract. Later, the same research group has implemented RME for the purification of the lectin from the crude extract of black turtle bean which belongs to the same species i.e. *Phaseolus vulgaris*. Box–Behnken Design (BBD) in response surface methodology (RSM) was used to optimize the forward and backward extraction process. Table 2.3 gives the details of parameters optimized with yield and purity. The use of anionic or cationic surfactants has the problem of denaturation of proteins as the solubilization of proteins involves electrostatic interactions and these methods often yield lower recovery. The protein may denature or lose its biological activity in a volatile organic solvent like isooctane.

Table 2.3 Non chromatographic techniques used to purify lectins

Lectin and source	study	Inference	Reference
Affinity precipitation			
peanut crude extract	Using Guar gum linked alginate as affinity ligand	Yield of about 46mg of lectin/100g of peanut seeds was obtained	Tyagi et al. 1996
potato, tomato and wheat germ crude extract	Both affinity precipitation and ATPAE methods were used Using Chitosan as a ligand	Affinity precipitation: activity recovery - 84,88 and 82 % with purification fold of 24,15 and 16 for wheat germ, potato and tomato lectins ATPAE: 90, 85 and 75% of activity yield with 25, 37 and 22 fold purification was obtained respectively for wheat germ, potato and tomato lectins	Teotia et al. 2006
Reverse micellar extraction			
Cratylia mollis crude extract	System used: AOT/isooctane	38% of forward and 100% of backward extraction was achieved	Nascimento et al. in 2002
Crataeva tapia bark	System used : AOT/isooctane Forward extraction factors: Surfactant concentration, pH and ionic strength Backextraction factors: pH,	Forward extraction: maximum recovery of 70% at pH 5.5 and in 5mM AOT Backward extraction: pH 5.5, 5% butanol and 500mM KCl gave 80% recovery	Nascimento et al. 2008

	butanol and KCl concentration		
black kidney bean crude extract	System used: AOT/isooctane Forward extraction: water content, NaCl concentration, pH, Extraction time Backextraction: pH, KCl	Forward extraction: 100Mm NaCl, pH 5.5, extraction time of 15min Backextraction: pH of 8.01 and 592.97 mM of KCl. overall protein recovery of 53.28% and purification factor of 8.2 was obtained	He et al. 2013
Magnetic separation			
Con A directly from the crude extract of *Canavalia brasiliensis*	High gradient magnetic fishing technique	Yield: 280 mg/g of support with high affinity	Hubbuch et al. 2001.
Commercial Con A	Gum arabic modified magnetic nanoparticle (GAMNP) and Glucose grafted gum arabic modified magnetic nanoparticle (G-GAMNP) were evaluated for their specific adsorbent capacity for Con A lectin The effect of pH on adsorption	G-GAMNP was able to adsorb Con A and GAMNP was unable to adsorb Maximum adsorption specific capacity was seen around PH 7.	Shashwat et al. 2007

	of lectin was studied		
Con A lectin, specific to mannose/glucose was purified from crude extract of jackbean	glucosylated magnetic nano matrix (GMNM) was evaluated for specific adsorption of Con A	Purity of Con A lectin was analyzed using SDS PAGE. Purified lectin showed single band of 26.5 kDa molecular weight and reveals that the Con A was specifically separated from jackbean	Kim et al. 2016
Membrane separation			
Con A lectin from crude extract of *Canavalia ensiformis*	Developed affinity membrane (using Saccharomyces cerevisiae cells were used as ligand) in combination of affinity binding and ultrafiltration	The overall yield of the process was reported as 70%. SDS PAGE	Mattiasson and Ramstorp 1984
Wheat germ lectin from wheat germ extract.	Developed macroporous chitin membranes	WGA with high purity of 99% and yield of 50 mg/100g was achieved in the process.	Zeng and Ruckenstein 1998
Con A lectin from jackbean	Macroporous cellulose membrane	The purified Con A gave single band on SDS PAGE which confirms the quality of purification.	Guo and Ruckenstein in 2011

Momordica charantia lectin , Ricinus communis agglutinin and peanut agglutinin	The two types of cellulosic matrices (cellulose membrane made of whatman-541, sartobind epoxy matrices) and ligands (N-Acetyl-d-galactosamine, Arabinogalactan) were evaluated	The affinity matrices with whatman filters were proved to have faster binding kinetics in comparison with sartobind epoxy matrices Arabinogalactan exhibited highest binding capacity.	Boi et al. 2006
Commercially available Con A and PNA	Glycosylated Nano fibrous membrane was evaluated for the specific adsorption of Con A and PNA	glycosylated nanofibrous membrane (GNM) showed high specificity to Con A by showing no affinity towards PNA	Che et al. 2010
commercially available crude Con A	Various activators used to prepare macroporous Cellulose membrane were evaluated	Affinity membrane prepared using 1,4-butanediol diglycidyl ether with pore size 1.33micro meter gave maximum adsorption capacity of 1.05 mg/ml	Guo and Ruckenstein in 2011
Commercially available Con A and WGA	Glycopolymer based porous membrane	Prepared affinity membranes exhibited specific adsorption of Con A with D-mannose and WGA with GluNac. Con A was adsorbed fivefold and WGA was twofold when compared to BSA	Ogata et al. 2013

2.4.3 Separation and characterization study of Pea seed lectin

Pea (*Pisum sativum*) lectin was extracted and purified by employing a chromatographic technique; it is extracted using a single step affinity chromatography or pre-purification steps followed by affinity chromatography or by a series of chromatographic techniques. The purification steps, columns used to purify lectins, some partial characterization of purified lectin and the main outcome of the studies are given in the Table 2.4.

Table 2.4 Techniques employed for purification and characterization of *Pisum sativum* lectin

Source	purification steps	Partial characterization	Inference	Reference
Pea seeds	Crude extract, isoelectric precipitation, ammonium sulfate fractionation, dialysis, column of hydroxyapatite, dialysis, DEAE-cellulose column, Sephadex G-I50	The two isolectins purified has a pI lie between 7.7-7.9 pH. Molecular weight of phytohemagglutinin I and II were found to be 54 and 53 KDa respectively	Yield of phytohemagglutinin I and II was 1.0 and 1.22 respectively.	Entlicher et al. 1970
Garden pea	Crude extract, acid precipitated (using HCL), ammonium sulfate fractionation, affinity column Sephadex G-100	Amino acid analysis: β subunit contains high content of, phenylalanine, aspartic acid, isoleucine, lysine and low amount of serine, valine, and histidine in comparison with α subunit. Lectin was proved to have mitogenic property	The molecular weight of the β and α subunits were found to be 17000 and 7000 Dalton respectively 75% of β protein and 5-10% of α protein was recovered in pure form.	Trowbridge 1974

Pea seeds	Crude extract, acetic acid precipitation, ammonium sulfate fractionation, dialysis, Sephadex G-100 column	SDS PAGE analysis reveals that pea consists of isolectins. Specificity: D-glucose		Kato et al. 1979
Egyptian *Pisum sativum seed*	Crude sample, Defatted, dialyzed, ion exchange chromatographic column (DEAE (Diethylaminoethyl)-Sepharose column), Sephadex G100	Molecular mass of the purified lectin was estimated as 120 KDa by gel filtration. SDS PAGE analysis showed single band of 32 KDa. Specificity: D-glucose and D-mannose Amino acid analysis: high content in alanine, glycin, asparagin, glutamine and poor in sulfuric amino acids		Al-Sohaimy et al. 2007
Pea seed lectin	Crude extract preparation, ammonium sulfate fractionation, dialysis, Sephadex G column	SDS PAGE: two bands of molecular weight 17 and 6 kDa. Purified sample reveals two bands of molecular weight 17 and 6 kDa.		Higgins et al. 1983
Egyptian Pisum sativum seeds	Crude extract, acetic acid precipitation, Sephadex G-100 affinity column	The molecular weight of the two fractions was found to be 19.3 kDa and 5.53 kDa by reducing SDS PAGE Specificity: mannose Antifungal activity: inhibited *Aspergillus flavus, Trichoderma viride*	SHA: 320HU/mg, PF: 213 Yield: 80%. Amino acid analysis: lack sulfur amino acids and rich in hydroxyl amino acids	Sitohy et al. 2007
Fresh grand	Crude extract, 0-80% ammonium	Anti-insect study: toxic	Fresh green peas: SHA: 143.82	Kaur et al.

dried peas (India)	sulfate fractionation	against melon fruit fly	HU/mg, Purity: 34.40% Recovery: 68%. Dried seeds: SHA: 177.16HU/mg SHA Purity: 38.18% Recovery : 96%	2014
Green Split Peas	Crude extract, DEAE-cellulose column, dialysis, cation-exchanger SP-Sepharose column, dialysis, FPLC-Superdex 200 HR 10/30 column	The molecular weight was observed as 6 KDa for alpha chain and 19 KDa for beta chain. Specificity: glucose, mannose, sucrose.	SHA: 4958HU/mg, PF: 15, Recovery: 16%.	Ng et al. 2015

SHA: specific hemagglutination activity, PF: purification factor

2.5 AQUEOUS TWO-PHASE EXTRACTION

After the invention of ATPE by Beijerinck, from 1950 till today, rapid progress has happened in the field of aqueous two phase extraction. Liquid-liquid extraction from the classic chemical engineering technique has not been well recognized in the field of biotechnology due to the denaturing effect on biomolecules by organic solvents. However, ATPS has the potential to overcome the limitations of conventional organic-aqueous extraction by offering a favorable environment to biomolecules by virtue of its aqueous based phase components which can extract functionally active biomolecules in their native state. ATPE was applied to recover a wide variety of molecules such as dyes, antioxidants, metal ions, proteins, pigments (Raja et al. 2011). The ease of integration of ATPE with other techniques such as aqueous two-phase affinity precipitation (Teotia et al. 2006), extractive fermentation using ATPE (Ooi et al. 2011) and extractive bioconversion using ATPE (Zijlstra et al. 1998) has broadened its application in various fields.

2.5.1 Phase forming components

Various components are used to obtain two-phase systems in the aqueous solution. The aqueous two-phase system of polymer and dextran were the most common and well-recognized polymer/polymer systems earlier. These systems offer good stability for biomolecules and also aid in affinity partition where specific ligands can be attached to any one of the polymers (Ruiz-Ruiz et al. 2012). However, these systems didn't gain much traction due to the very expensive nature of dextran. Hence, a number of cost-effective polymers such as hydroxypropyl starch, methyl cellulose, cashew-nut tree gum, maltodextrin have been proposed as alternatives to dextran (Antov et al. 2006). It was noticed that the charge based separation cannot be achieved in these systems. Owing to this, many charged polymers were investigated such as polyethylene oxide–maleic acid copolymer (Kajiuchi et al. 2002), polyethylenimine–PEG (Gupta et al. 2002) etc. However, the high viscosity and lower density difference between the phases limits its use in large-scale continuous operation. PEG/salt systems emerged as efficient systems which offer less viscosity, highly tunable electrochemical properties, easy handling, and suitability to establish at large scale. Initially, inorganic salts (sulfates and phosphates) were widely explored for the recovery of biomolecules. Later, the unfriendly environment nature of inorganic salts was realized and as an alternative to inorganic salts, organic salts emerged as an environmentally friendly system (Raja et al. 2011). The only drawback of PEG/salt systems is the high ionic strength.

Alcohol/salts systems came into the light where these were explored for the partitioning of hydrophobic or partially hydrophobic molecules. Some of the alcohols such as ethanol and propanol (Ooi et al. 2009, Amid et al. 2012) were combined with salts as the phase forming components. Even though these systems offer low viscosity, reduced settling time, and easy recovery, the instability of sensitive molecules like proteins in alcohols limit their application.

The ability of surfactants to form two phases such as micellar rich phase and micellar poor phase (micellar ATPS) over certain temperature was investigated to recover some of

the biomolecules. Ionic surfactant based micellar system cannot be applied for sensitive biomolecules; instead, a non-ionic surfactant can be a good choice for biomolecules which are sensitive to the ionic environment (Liu et al. 1998). Mixed surfactant systems are also employed to extract many biomolecules (Xiao et al. 2000). But again, the surfactant used was not feasible for all the proteins. ATPS formed with green solvents such as ionic liquids were studied to separate organic molecules, metal ions, amino acids, and proteins (Li et al. 2010). ATPS can be formed with two ionic liquids or an ionic liquid and salt. Some of the combinations used were 1-Butyl-3-methylimidazolium tetrafluoroborate and trisodium citrate dihydrate (Shao et al. 2014), Imidazolium – dipotassium phosphate (Shu et al. 2016) etc. These are highly suitable for low volume and high value compounds like recombinant technology products, pharmaceutical, aromatic compounds, and enzymes. Further, these ATPS are not suitable for large-scale application due to the higher price of the ionic liquids when compared to the polymers. Recently, the ATPS formation with acetonitrile and carbohydrates were investigated, where the system was successfully applied to partition antioxidant vanillin (de Brito Cardoso et al. 2013). The capability of this system is not yet fully explored for other sensitive biomolecules. The formation of two phases is also possible by using thermo-separating polymers which are temperature dependent. The critical solution temperature (CST) of these polymers is very low (47ºC for 50% Ethylene Oxide - 50% Propylene Oxide) compared to that of polyethylene glycol (PEG 20,000 (95ºC)). When the solution temperature is increased above its CST, these polymers show two phases where one of the phases is enriched with polymer and the other is polymer depleted (Jonsson et al. 2003). Temperature-induced phase separation limits its use in the separation of biomolecules which are temperature sensitive.

Among different types of ATPS, polymer-salt systems are being used widely; several partitioning studies have been reported using these systems. Diamond and Hsu (1989) stated that the extraction in these systems resulted in higher yield and purity of biomolecule along with the advantage of large sample loading. Physical properties of the polymer-salt system such as density, viscosity, and less interfacial tension between the

phases allow the ease of mass transfer between the phases and also allow the continuous operation of ATPE process in the existing commercial extractors (Yucekan and Onal 2011).

2.5.2 Phase diagram of ATPS

Aqueous two-phase extraction is modified liquid-liquid extraction (LLE). In LLE, one of the components is water based and the other is organic solvent based. Generally, the polar aqueous solutions are immiscible with nonpolar organic solvent (Gu. 2000). However, both immiscible phases are water based in aqueous biphasic systems. The distinct phase formation is affected by temperature, pH, ionic strength, and critical concentration of polymer and salt (Mazzola et al. 2008).

In a ternary system (PEG + salt + water), each PEG molecule and the salt ions are surrounded by a layer of water molecules called hydration layer (Sadeghi and Golabiazar 2010). When there is an increase in the concentration of salt or PEG or both, the ether oxygen atoms of PEG interact with the salt cations. Moreover, there is repulsion between the salt anions and ether oxygen atoms. This repulsive effect is more for small multivalent anions and ether oxygen atoms which eventually results in the formation of two clear and distinct phases (Hatti-kaul 2000). Both PEG and salt have a high affinity for water and their mutual exclusion results in the formation of salt-rich bottom phase and polymer rich top phase (Sadeghi and Golabiazar 2010).

The phase formation mainly depends on the critical concentration of the phase components. In PEG/salt systems, the molecular weight of the polymer and type of the salts affects the phase formation. The molecular weight of the polymer affects the position of the binodal curve. As the molecular weight increases, the binodal curve moves towards the origin. Therefore, less concentration of PEG is required to form two phases. Various researchers studied the effect of salt on phase formation. The salting out ability of salts differs with different salts. Hofmeister series gives the order of salt ions based on their salting out ability (Saravanan et al. 2007). The contributions of salt anion are more effective than cation in phase separation. The multivalent anions with higher

charge density limit the interaction with the polymer chain and form salt depleted phase and consequently lead to the phase formation. The phase formation is also affected by water structuring and water breaking salt ions (Bolar et al. 2013). pH changes the ratio of ionic species in the system and thus affects the binodal location. The increase in pH expands the two-phase region. It was observed that the hydrogen bond interactions of PEG and water decrease with an increase in pH (Zafarani et al. 2000). Therefore, increase in pH reduces the attraction between water and PEG and thus water is driven to the bottom phase increasing the volume of the bottom phase which in turn dilutes the salt concentration (Rahimpour and Baharvand 2009). On the other side, PEG concentration increases and the phase becomes more hydrophobic. The solubility of the salts increases with an increase in the temperature which attributes to the increase in biphasic area (Perumalsamy and Murugesan 2009).

Liquid-liquid equilibrium data for an aqueous two-phase system constituted by polymer/polymer and PEG/salt are extensively reported in the literature. The binodal curve can be determined using three methods namely cloud point method, the turbidimetric method, and node determination method. In the cloud point method, a known amount of one phase component is titrated against another phase component and the appearance of turbidity indicates the endpoint where the two-phase formation takes place. Water is added until the disappearance of turbidity and the procedure is repeated to get other binodal points. This is the common method used to generate the binodal curve. In the turbidimetric method, a series of systems with known compositions are prepared and diluent was added until two-phase disappears. The transition point is noted down and this procedure is repeated many times to get maximum binodal points (Kaul 2000). The determination of tie lines involves the measurement of phase components in the top and bottom phases by using sophisticated instrumental methods which are listed in the Table 2.5.

Table 2.5: Analytical instruments used to measure phase components

Analytical instrument	Phase component	Reference
Flame photometer	Salt	Murugesan and Perumalsamy 2005
Conductivity meter	Salt	Regupathi et al. 2009
Atomic absorption spectrometry (AAS)	Salt	de Oliveira et al. 2008
Flame Atomic Absorption Spectrometer (FAAS)	Salt	Rodríguez et al. 2007
Turbidimeter	Salt	Gonzalez-Tello et al. 1996
Titration	Salt	Amaresh et al. 2008
Enzymatic method	Salt	Tubío et al. 2006
Gravimetric method	PEG and Salt	Cunha et al. 2009
Refractometer	PEG	Amaresh et al. 2008
High-performance liquid chromatography (HPLC)	PEG	de Belval et al. 1998

2.5.3 Phase formation

Aqueous two-phase extraction is an alternative to liquid-liquid extraction (LLE). In LLE, one of the components is water based and the other is organic solvent based. Generally, the polar aqueous solutions are immiscible with nonpolar organic solvent (Gu. 2000). However, both immiscible phases are water based in aqueous biphasic systems. The distinct phase formation is affected by temperature, pH, ionic strength, and critical concentration of polymer and salt (Mazzola et al. 2008).

In a ternary system (PEG + salt + water), each PEG molecule and the salt ions are surrounded by a layer of water molecules called hydration layer (Sadeghi and Golabiazar 2010). When there is an increase in the concentration of salt or PEG or both, the ether oxygen atoms of PEG interacts with the salt cations. Moreover, there is repulsion

between the salt anions and ether oxygen atoms. This repulsive effect is more for small multivalent anions and ether oxygen atoms which eventually results in the formation of two clear and distinct phases (Hatti-kaul 2000). Both PEG and salt have a high affinity for water and their mutual exclusion results in the formation of salt-rich bottom phase and polymer rich top phase (Sadeghi and Golabiazar 2010).

The phase formation mainly depends on the critical concentration of the phase components. In PEG/salt systems, the molecular weight of the polymer and type of the salts affect the phase formation. The molecular weight of the polymer affects the position of the binodal curve. As the molecular weight increases, the binodal curve moves towards the origin. Therefore, less concentration of PEG is required to form two phases. Various researchers studied the effect of salt on phase formation. The salting out ability of salts differs with different salts. Hofmeister series gives the order of salt ions based on their salting out ability (Saravanan et al. 2007). The contributions of salt anion are more effective than cation in phase separation (Silverio et al. 2013). The multivalent anions with higher charge density limit the interaction with the polymer chain and form salt depleted phase and consequently lead to the phase formation. The phase formation is also affected by water structuring and water breaking salt ions (Bolar et al. 2013). pH changes the ratio of ionic species in the system and thus affects the binodal location. The increase in pH expands the two-phase region. It was observed that the hydrogen bond interactions of PEG and water decrease with an increase in pH (Zafarani et al. 2000). Therefore, increase in pH reduces the attraction between water and PEG and thus water is driven to the bottom phase increasing the volume of the bottom phase which in turn dilutes the salt concentration (Rahimpour and Baharvand 2009). On the other side, PEG concentration increases and the phase becomes more hydrophobic. The solubility of the salts increases with an increase in the temperature which attributes to the increase in biphasic area (Perumalsamy and Murugesan 2009).

2.5.4 Factors Affecting the Partition of Protein in PEG/salt systems

The partition of a biomolecule in a system depends mainly on the surface properties of the target biomolecule and the phase forming components (PFC). Biomolecule interacts with the PFC with a number of non-covalent interactions like hydrophobic force, hydrogen bonds, van der Waals interaction, electrostatic interaction etc. These interactions vary among phases which form a basis for partitioning of the target biomolecule into any one of the phases. The factors influencing the partition of the protein in the PEG/salt system are discussed below:

2.5.4.1 Polymer molecular weight

The partitioning of biomolecules to the PEG-rich top phase was mainly affected by PEG-molecular weight. As the molecular weight (MW) increases, the binodal curve approaches origin where phase formation needs less concentration of polymer to form. In general, PEG of low molecular weight requires a high concentration of PEG and PEG of high molecular weight requires a low concentration of PEG for phase formation (Voros et al. 1993). The differences in physical properties such as density, refractive index, and viscosity between the phases also increase with an increase in PEG MW or PEG concentration (Raghavarao et al. 1995). Several authors studied the effect of polymer molecular weight on the partitioning of the biomolecule in PEG/salt systems. In 2011, Mehrnoush et al. evaluated the effect of PEG molecular weight on pectinase extraction in a PEG-salt system. The effect of PEG MW ranging from 2000-10000 was studied on the pectinase partition and yield. It was noticed that a high amount of pectinase was concentrated in the top phase at PEG 4000 whereas; both lower and higher MWs than PEG 4000 do not yield favorable results. This trend was also agreed with the study of serine protease from mango peel, where the author had chosen different molecular weights of PEG ranging from 3000-8000; in this study, PEG 6000 showed higher partition coefficient (Amid et al. 2012). Bradoo et al. (1999) reported that the lipase partition in PEG/phosphate system was majorly influenced by the PEG molecular weight. At a very low PEG molecular weight (PEG 600 g/mol), all the proteins in the extract partitioned to the PEG phase whereas, at a high PEG molecular weight (PEG 20000

g/mol), most of the proteins partitioned to the salt phase. However, the differential partition of desired and undesired proteins to top and bottom phase respectively was achieved at an intermediate molecular weight of PEG 6000.

Increase in MW increases the PEG chain length which in turn increases the viscosity and hydrophobicity of the polymer-rich phase. As PEG molecular weight increases, effective excluded volume increases in the top phase due to higher molecular size in the top phase which leads the protein partitioning in the bottom phase (Nalinanon et al. 2009). This results in poor protein–polymer interaction and finally leads to low recovery (Raghavarao et al. 1995). Conversely, the polymer with low molecular weight has shorter chain length with hydrophilic ends which reduces the hydrophobicity, thereby protein polymer interaction takes place and protein partitions into upper polymer rich phase (Raghavarao and Nair 2011). Very low molecular weight tends to reduce excluded volume which attracts both the desired protein and contaminants and reduces the partition of the biomolecule. Therefore, suitable intermediate molecular weight polymer is preferred rather than very low and high molecular weight.

2.5.4.2 Effect of type and concentration of phase forming salt

In order to obtain a high partition coefficient and yield of target biomolecule, the selection of phase forming salt type and concentration plays a major role. In PEG-salt systems, the hydrophobic interactions of the protein and the phase components were majorly influenced by the type and concentration of salt. The interaction of salt ions with the protein surface charges forms a double electric layer. The hydration effect of salt ions dehydrates the protein which gradually exposes the hydrophobic sites. This enhances the hydrophobic interaction of protein and PEG (Porto et al. 2011). The ionic strength of the salt phase influences the solubility of protein. Thus the screening of different concentrations of salt which creates varied ionic strength in the system is very important.

The efficiency of salts in promoting phase separation is characterized by the position of the salt ions in the hofmeister series (a classification of ions based upon salting out ability) (Chaiwut et al. 2010). The effect of salts on the solubility of proteins according to

hofmeister series are given below: The salting out power of anions is in the order of "$HC_6H_5O_7^{2-}$ > SO_4^{2-} > HPO_4^{2-} > CH3COO$^-$ > F^- > Cl^- > Br^-" and the salting power of cations as follows "NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}". Anions have a larger effect on solubility of proteins than a cation (Bolar et al. 2013). Xueqiao et al. (2010) reported that salting out capacity of anions with high valency is greater than anions with fewer valences. The cations with less ionic radius (K^+: 0.133nm, Na^+: 0.098 nm, $NH4^+$: 0.15nm) and more gibbs free energy of hydration (Na^+: 375 kJ/mol, K^+: 304 kJ/mol, $NH4^+$: 285 kJ/mol) have more salting out ability.

Most of the ATPS studies make use of citrate, phosphate and sulfate salts where all these enhances the hydrophobic interaction of polymer and proteins (Franco et al. 1996). The PEG/phosphate system was widely used to extract biomolecules from fermentation broth (Madhusudhan et al. 2008), plant crude extracts (Nascimento et al. 2003) etc. and system pH lies in the range of 6.7-7.3. Sulfate salts have more affinity towards water molecules. Thus enhances the hydrophobic interaction of protein and PEG. Sulfate salt based systems were widely utilized for recovery of proteins and value added compounds from various animal and plant tissues (Saravanan et al. 2006 and 2007 and Imelio et al. 2008). Polyethylene glycol/organic salts ATPS is considered as potentially useful over PEG/inorganic systems because of their environmentally friendly nature, biodegradability and non-toxicity (Kashyap et al. 2002, Azevedo et al. 2009). The organic citrate salts possess good salting out property, biodegradable, and nontoxic characteristics. The PEG/citrate system offers a system pH of around 8. PEG/citrate salts combination was studied for the extraction of various biomolecules such as Lectin (Soares et al. 2011), alpha -amylase (Zhi et al. 2004), alkaline protease (Sarangi et al. 2011), Bovine serum albumin (Yan-Minn et al. 2010), Lysozyme (Lu et al. 2010).

2.5.4.3 Effect of system pH

The change in pH alters the surface properties of the protein and the ionic composition of the system and thus influences protein partition behavior (Yang et al. 2008). In acidic pH, proteins amine group protonated and protein acquires a positive charge; in alkaline pH

carboxyl group is deprotonated and protein acquires a negative charge. The net surface charge of the protein depends on its pI. If pH is below pI, protein acquires a positive charge and accumulates in the bottom phase and if pH is above PI, protein acquires a negative charge and moves to the PEG-rich top phase.

The influence of pH on protein partition is reported by several researchers. Sindra Virtuoso and his coworkers studied the partition of BSA in PEG/salt system with pH more and less than its isoelectric point. BSA became more negative and partitioned to PEG-rich phase when pH was increased to 7 - 8 (pI of BSA=4.8); also, more partition coefficient was obtained at this pH when compared to pH 3.5 and 4 (Sindra Virtuoso et al. 2010). Wongmongkol and Prichanont studied the effect of pH on alkaline protease partition in PEG/potassium phosphate system. Increase in pH increased the enzyme partition coefficient (K_e) and gave maximum K_e at pH 10.5 (pI of alkaline protease is 9). The similar behavior has been observed and reported for β-galactosidase (Silva et al. 2002), endo-pectinase (Pericin et al. 2004). In above-reported observations, protein acquires net negative charge at pH above its isoelectric point. As the negativity increases, K also increases due to the affinity of the negatively charged protein to ether oxygen atom of PEG which tends to interact with PEG in top phase and derives high partition towards top phase (Kalaivani et al. 2014).

Another study reveals that the partition of cheese whey to top phase decreases with increase in the pH of the system. Even though cheese whey acquires a negative charge above its pI, it has a higher affinity for lower salt phase. This is due to the salting out effect and ratio of trivalent to divalent citrate ions in bottom phase at high pH and also due to reduced hydrophobic interaction between the protein and the PEG as cheese whey is hydrophilic in nature (Perumalsamy and Murugesan et al. 2012). The working system pH depends on the isoelectric point of the biomolecule to be partitioned. The system pH can also be altered by the addition of neutral salts as additives or by varying the ratio of mono hydrogen and dihydrogen salt. The consideration of the isoelectric point of contaminating proteins in case of protein extraction from the complex feed helps to

understand and manipulate the system pH for differential partitioning of desired and contaminating proteins.

2.5.4.4 Effect of tie line length (TLL)

Tie line length describes the concentration of phase forming components present in the top and bottom phase. As variation in TLL varies the system composition, it's very important to study and optimize the value of TLL in order to obtain better partition and yield of the target molecule. The effect of TLL on the protein partition is well documented in the literature. Mehrnoush et al. (2012) studied the effect of TLL on serine protease (from mango waste) partition coefficient. The range of TLL from 6.25 - 35.27% w/w was evaluated, where enzyme partition coefficient increases with increase in TLL up to 17.2% (maximum partition was observed at this TLL) and found to decrease above this TLL. A similar phenomenon was also observed by Bolar et al. (2012), where the partition coefficient of glutaminase increases with increase in TLL but reduces at very high and low TLL. These results were also in accordance with the findings of Nagaraja and Iyyaswami et al. (2014) where the partition of fish proteins increases with an increase in TLL. Increase in TLL increases the PFC concentration in respective phases and also increases the density difference between the two phases (Yaun et al., 2015). The increased concentration of PEG in the top phase enhances the number of hydrophobic interactions of polymer and protein (Lan et al., 2013). The increase in salt concentration reduces the free volume in the bottom phase and increases the salting-out effect. This tends a protein to move towards the top phase which in turn increases the partition coefficient. Very high TLL reduces the water content in both the phases and affects the available free volume for target biomolecule to accommodate in the phases. The excluded volume effect shown by polymer and salting out effect exhibited by salt makes protein to precipitate at the interface (Rito-Palomares and Hernández 1998). Viscosity and interfacial tension of the phases increases at very high TLL which makes the process operation more tedious (Narayan et al., 2011). At very low TLL, there is no clear-cut resolution between the phases because of minimal difference in density and other physical properties.

Other authors (Perulmelsamy and Murugesan 2012) reported that the partition of cheese whey protein decreases with an increase in TLL. The reason behind this observation was, as TLL increases, the concentration of PEG in the top phase increases which in turn increases the hydrophobicity of the PEG phase. Cheese whey being hydrophilic in nature, transfers to the bottom phase due to effective excluded volume and hydrophobic nature of the PEG phase. By considering the above-documented results it is very important to notice that very high and very low TLL result in poor partition coefficient. One can achieve a high partition at optimum TLL value, where there is a well-established balance between the hydrophobicity of PEG and salting out nature of salt.

2.5.4.5 Effect of additives

The addition of salts as an additive to the aqueous two phase system has appreciable effect on the partition of the desirable biomolecule to any one of the phases. Salts like NaCl, KCl, $MnCl_2$, $MgCl_2$, $MgSO_4$ and sodium perchlorate was used to study their effect on solutes partition (Yucken et al. 2011). Different salts have different affinity towards the phases and gives rise to an electrostatic potential difference. Monatomic ions such as chloride, partition equally between the phases, whereas unequal partition occurs in the case of diatomic (sulfate) or triatomic (phosphate) ions (Hemavathi and Raghavarao 2011). The interfacial potential difference created by the distribution of ions between the phases alters the overall partition coefficient of the biomolecules which is directly proportional to the net charge of the protein at that system pH. The charged protein will carry a sufficient number of counter-ion and co-ions with it to maintain the phase electro neutral. These salts are classified based on their interaction with water, as water structure making (sulfate, phosphate and sodium) and water structure breaking ions (nitrate and potassium) (Zaslavsky et al. 1983,1989, Gupta et al. 2002). Cations such as Li^+, Na^+, NH^{4+}, Ca^{2+}, Mg^{2+} and anions F^-, SO_4^{2-}, CO_3^{2-}, PO_4^{3-}, CH_3COO^- are structure making while the structure breaking ions are K^+, Rb^+, Cs^+, Cl^-, Br^-, I^-, SCN^- etc. (Gupta et al. 2002).

The neutral salt, NaCl was widely used as an additive in partitioning of many proteins such as Human antibodies (Azevedo et al. 2005), lipase derived from *Burkholderia pseudomallei* (Ooi et al. 2009), penicillin acylase from Escherichia coli (Marcos et al. 1999) and also in the partitioning of *Canavalia brasiliensis* lectin (Nascimento et al. 2010), ricin B (subunit of ricin) (Zhang et al. 2005). In all the above examples the addition of NaCl increases the partitioning of target protein to PEG rich phase.

The NaCl addition to the system increases the hydrophobic differences between the phases and thus increases the partition of more hydrophobic proteins predominantly to PEG rich phase by enhancing the hydrophobic protein - PEG interaction. The protein shift from salt phase to PEG phase happens due to increased salting out effect as well as the shielding of surface ions of the protein by Na^+ and Cl^-. Furthermore, the increase in NaCl salt concentration reduces the volume of the PEG phase in comparison with salt phase and thus helps in the concentration of the desired product and consequently increases the hydrophobicity (Rosa et al. 2007).

Some authors reported the opposite trend in comparison with above experimental results. The addition of NaCl to the system favors the partition of protein towards bottom phase. Based on the Hofmeister series Cl^- are water breaking ions (chaotropes) and partition predominantly into top phase. Due to which the top phase become more negative with increase in NaCl concentration (higher concentration of chloride ions), thus partitioning the positively charged and more hydrophobic proteins to PEG phase. In contrast the salt phase becomes positively charged which attracts negatively charged and hydrophilic proteins towards it (Glyk et al. 2017). The addition of NaCl can influence the transfer of protein from top or bottom phase to opposite phase depending up on the differences in hydrophobicity of the aqueous phases or surface charge acquired by the protein or by its own hydrophobicity.

2.5.4.6 Effect of volume ratio

Volume ratio is the ratio of the volume of the top phase to the volume of the bottom phase. An efficient purification process should combine both lower volume of the

'interested' phase (Solute partitioned phase) and high yield and purity with a higher partition coefficient of the target molecule. In ATPS, this could be easily achieved by altering the volume of the phases, where the target molecule is partitioned into one particular phase. Altering the volume ratio may be helpful in large-scale processing with higher profit. The volume ratio is varied by changing the volume of the individual phases at fixed total volume in which the phase compositions (PEG and salt) of the top and bottom phases remain the same with respect to their tie lines.

The effect of different range of volume ratio (1-4) on fish protein partition in PEG 2000/Sodium sulfate system was studied by Nagaraja and Iyyaswami (2014) where there was an increase in the partition coefficient of fish proteins with the increase in volume ratio up to a certain value and above that partition coefficient and yield of the protein decreased. The similar result was obtained by Kalaivani et al. (2013) where the partition of α-lactalbumin in PEG/citrate system increases with the volume ratio. A similar trend was also obtained in the volume ratio study for penicillin acylase (Marcos et al. 1999). This phenomenon was observed because, as the volume ratio increases, the top PEG phase volume increases and protein solubility limit is reached in the bottom phase which in turn increases the hydrophobic interaction of polymer and protein (Chetana et al. 2007) and thus the protein tends to transfer towards PEG-rich phase. Furthermore**,** there was a more space for accommodation of protein which in turn increased the partition coefficient of the protein. The increase in partition coefficient was observed till saturation limit is attained by top PEG-rich phase and thereafter proteins precipitated at the interface (Madhusudhan et al. 2008). The increase in volume ratio above a certain limit may accommodate other contaminating proteins along with the target protein resulting in lower partition coefficient and yield of the target protein. Finding the optimal volume ratio for a particular system is of utmost importance to achieve enhanced protein coefficient and good yield of the target protein.

2.6 OPTIMIZATION OF THE PROCESS

2.6.1 Response surface methodology

The study of cause-effect of variables on the responses is very important to know the role of an independent variable on a response. As responses are multivariate, the interactive studies among the variables and their effect on responses give valuable insight into the inter-relationship between the independent variables and the dependent responses. The use of Design of Experiment (DoE) has grown rapidly and been adapted for many applications in different areas. Response surface methodology (RSM) is one of the best-known types of DoE design; the concept of RSM was first introduced in the early 1950s by Box and Wilson (Box and Wilson 1951). Since then, many researchers have used the RSM procedures in different disciplines. RSM is a set of mathematical and statistical techniques that are useful for modeling and predicting the response of interest affected by a number of input variables with the aim of optimizing the response. RSM also specifies the relationships among one or more measured responses and the essential controllable input factor. The statistical analysis of the model is used to analyze the significance of the variables on the responses. Many of the researches exploited RSM for process optimization and modeling of the process variables in the field of aqueous two-phase extraction which is discussed below:

Environment friendly polyethylene glycol/citrate system was adopted to partially purify α-amylase from white pitaya peel. The behavior of response variables, Yield (Y), partition coefficient (K) and purification factor (PF) based on the independent variables such as PEG molecular weight and concentration, sodium citrate and NaCl concentration was accessed by designing the experiments based on CCD (central composite design). The R^2 value of all response variables was more than 0.8, which proves the better fineness of the models. The interaction of PEG molecular weight and its concentration, PEG molecular weight with sodium citrate concentration and NaCl concentration were the most significant factors on Y, K and PF respectively. The global optimized conditions of 14% PEG 6000/16% sodium citrate with 5% NaCl gave 6.62 partition coefficient,

89.12% amylase yield and PF of 4.43. It was also reported that more than 5% of NaCl denatures the enzyme (Shad et al. 2018).

In 2015, Chavan et al (2015) studied the ATPS composed of PEG 4000 and potassium phosphate for the extraction of protease from *Bacillus licheniformis* NCIM 2042. Central composite rotatable design (CCRD) was followed where the effect of independent factors such as PEG 4000 (A), potassium phosphate concentration (B), pH (C) and NaCl (D) was studied on the dependent variables like partition coefficient (K), yield and purification factor (PF). The R^2 and adjusted R^2 were more than 97% and 96% respectively for all the three responses. The interactive effect of AB on K, AD, and BC on PF and AB, AC, AD, BC, BD, and CD on Y were significant. 15.93% PEG 4000, 22.36% potassium phosphate, 1.08 mM of NaCl at pH 10.06 gave a K of 11.95 with maximum Y of 89.5% and PF of 2.2.

The extraction of enzyme glutaminase from *Zygosaccharomyces rouxii* was optimized with the approach of RSM by considering the experimental design given by CCRD in PEG – sodium sulfate system. The effect of independent factors such as TLL (tie line length), pH, Broth loading, volume ratio, and NaCl concentration on the yield and partition coefficient of a glutaminase enzyme was studied. MINITAB was used for the statistical and graphical analysis. All the linear terms and the interaction of pH and TLL were found to be the most significant terms in maximizing the extraction parameters. The partition coefficient of 12.99 and yield of 95.12% was achieved in the process (Bolar et al. 2015).

Purification studies of Luciferase, an oxidative enzyme, from *Photinus pyralis* was carried out in PEG 1500 – sodium sulfate system (Priyanka et al. 2013). A set of 20 experiments were designed by considering phase components concentration and system pH as the independent variables and specific activity (Y1), activity recovery (Y2), purification fold (Y3), volume ratio (Y4), partition coefficient of protein (Y5) and enzyme (Y6) as the dependent responses. The polynomial response models fitted well with R^2 ranging from 89%-95%. Optimum conditions of all the responses were obtained

by overlay plots. The predictability of the models was also verified by conducting experiments at different conditions (Priyanka et al. 2013).

Raja and Murthy (2013) adopted fractional factorial design to choose the significant variables for the partitioning of proteins from tannery wastewater. Phase components (PEG/sodium citrate) concentration, temperature, pH and NaCl were considered as independent factors and recovery as the response. From the normal plots, they concluded that the significant variables affecting the partitioning were temperature, pH, and NaCl. The significant variables were further optimized using RSM. A maximum of 94% recovery was reported. However, the effect of wastewater loading on the partitioning was not considered which is very important for commercialization of the process.

Azevedo et al. (2007) optimized ATPS conditions for the separation of therapeutic human antibody from the supernatant of simulated serum medium. 28 experimental runs were performed by considering pH, PEG, potassium phosphate and NaCl concentration as independent factors. Based on ANOVA analysis, they stated that purity of the partitioned protein depends solely on the additive (NaCl) concentration in the system. Likewise, the yield is affected by the interaction of NaCl with other independent variables. Response surface plots suggest low potassium phosphate concentration and high PEG, NaCl, and pH obtain the maximum yield and purity of immunoglobulin. Further, they compared the results of pure protein studies with the extraction process from a complex source and reported that the results are in agreement with each other.

Platis et al. (2009) implemented a full factorial CCD to optimize the purification process of anti- HIV monoclonal antibodies 2G12 (mAb 2G12) and 4E10 (mAb 4E10) from an unclarified crude extract of transgenic tobacco. The desirable combinations of independent variables to obtain maximum bottom phase yield and purity of antibodies was indicated by contour plots and response optimization. A differential partitioning of cell debris to the top phase and mAbs to the bottom phase was achieved under 13.1% w/w PEG 1500/12.5% w/w phosphate with a volume ratio of 1.3 at pH 5 and at the crude

load of 8.25% w/w. Under these conditions, maximum purification factor of 2.4 and 2.1 and the yield of 85% and 84% were found for mAb 2G12 and mAb 4E10 respectively.

The statistical approach of central composite design and response surface analysis was applied to optimize the purification of trypsin from pancreas homogenate using PEG/sodium citrate ATPS. Through ANOVA analysis, it was confirmed that the developed second-order model was efficient to describe the relationship between independent and dependent variables. The optimized variables, 3.34% NaCl, a volume ratio of 6.37 at biomass load of 9.30% in PEG 3350/sodium citrate system resulted in maximum purification factor of 2.55 and a yield of 99.7% in the PEG-rich phase (Pérez et al. 2015).

2.7 AQUEOUS TWO-PHASE EXTRACTION OF LECTINS

Along with other non-chromatographic techniques, ATPE was also employed by few researchers, mainly to recover *Canavalia* species lectins such as *Canavalia brasiliensis*, *Canavalia grandiflora* benth, *Canavalia ensiformis*, and *Cratylia mollis* seed lectin. In 1975, Flanagan and coworkers reported the partition of Con A in PEG/dextran system. Dextran played the dual role of a phase forming component and as a specific ligand for Con A. Partition coefficient of Con A was found to be 0.04 which suggests the affinity of Con A towards dextran rich bottom phase. This was further confirmed by adding D-mannose and L-fucose, a competitor and a non-competitor respectively, for Con A to bind to dextran. The addition of D-mannose increased the partition coefficient whereas not much effect was seen with L-fucose (Flanagan and Barondes, 1975). Later in 2005, Zhang et al. evaluated two systems, PEG/potassium phosphate, and PEG/sodium sulfate, to recover a non-toxic chain called Ricin from the castor bean. PEG/sodium sulfate showed superior performance by giving partition coefficients ranging from 4 to 14 whereas PEG/potassium phosphate gave lesser partition coefficients. NaCl and sodium sulfate concentration were identified as prominent factors in the process. Response surface methodology was implemented to optimize the factors to concentrate ricin in the PEG phase. The selectivity of 2.8 was obtained at optimized conditions such as 9% PEG

8000/7.17 % sodium sulfate and 0.48M NaCl. The partition of protein to the PEG phase increases the stability due to the stabilizing nature of PEG (Zang et al. 2005). In 2010, Nascimento et al. purified Con Br (Canavalia brasiliensis lectin) from a crude extract of Canavalia brasiliensis seeds. Various salts were evaluated with PEG-600 for their performance in the partitioning of Con Br. Among the systems studied, PEG/phosphate system was selected as an efficient system for the purification of Con Br. Factors (PEG, salt, NaCl concentration, and pH) affecting the Con Br partition in ATPS were optimized using a central composite design. The study has shown that the low molecular weight PEG and high ionic strength created by NaCl played a prominent role in selective partitioning. Low value of PEG (16.5 %) and central value of phosphate (15%) and NaCl (4.5 %) at pH 7.5 gave maximum extraction yield of 100% with 73.04% purity.

In 2011, an eco-friendly ATPS, PEG/sodium citrate was used by Porto and his group to partition lectin from Canavalia grandiflora benth. Con GF (Canavalia grandiflora lectin) was selectively partitioned to PEG phase. The effect of PEG molecular weight and concentration, salt concentration, and pH were studied on the partitioning of Con GF. Citrate concentration prominently affected the partition coefficient (K_L). Maximum recovery yield of 104% with 8.67 partition coefficient was achieved in 20% (w/w) PEG 400 and 20% (w/w) citrate, at pH 6.

Table 2.6 Aqueous two phase extraction of lectins

Lectin	**ATPS**	**Extracted Phase**	**Specifications**	**Reference**
Commercial Con A	PEG/dextran	Bottom dextran phase	Partition coefficient=0.04	Flanagan and Barondes, 1975
Ricin B	PEG/sodium sulfate	PEG-rich top phase	K=14 Selectivity=2.8	Zhang *et al.* 2005
Canavalia brasiliensis lectin	PEG/Salt	PEG-rich top phase	Yield=100% Purity=73.04%	Kelany *et al.* 2010
Canavalia grandiflora lectin (Benth)	PEG/Sodium citrate	PEG-rich top phase	Yield=104% Partition coefficient =8.67	Camila *et al.* 2011
Canavalia ensiformis	PEG/Citrate	Salt-rich bottom phase	Purification factor=11.5	Paulo *et al.* 2011
Cratylia mollis seeds	PEG/Sodium citrate	Salt-rich bottom phase	Activity yield= 125%,PF=13.28	Cynthia *et al.* 2013
Phaseolus vulgaris	PEG/Ammonium Sulfate	PEG-rich top phase	Yield=42.32%	Jiang et al. 2017

Soares et al. (2011) studied and optimized the system parameters like PEG molecular weight, PEG concentration, citrate concentration, and pH for commercial Con A. Lectin preferentially partitioned to the bottom phase due to size exclusion of PEG 8000. The optimal conditions to partition lectin were 22% (w/w) PEG 8000, 12% (w/w) citrate, and pH 6.0. Same conditions were applied for the extraction of the lectin from Canavalia ensiformis crude extract and achieved 100% recovery in the bottom phase with 11.5 purification factor. By using the same concept of volume exclusion of PEG 8000 and the isoelectric point of lectin, Nascimento, and group successfully partitioned Cratylia mollis seed lectin to the bottom phase and contaminants to the top phase. Maximum activity recovery of 125% and a purification factor of 13.28 was obtained by 22% (w/w) PEG

8000/20% (w/w) citrate at pH 5. Very recently in 2017, Jiang and his coworkers purified Phaseolus vulgaris lectin in PEG 600–Ammonium Sulfate system. It was reported that the lectin partitioned selectively to the top phase with hemagglutination activity which was 3.08 times than in the crude extract and a yield of about 42.32% was obtained. Table 2.6 shows the aqueous two phase extraction of lectins in ATPS.

2.8 ARTIFICIAL NEURAL NETWORK MODELING

Artificial Neural Networks (ANN) is computing systems roughly influenced by neural networks in animal brains. They are a framework of machine learning algorithms designed to process complex input data. ANN "learns" to perform tasks based on the input examples given to them. ANN is a collection of connected units called artificial neurons. A neuron can have a linear or a non-linear transformation function based on the complexity of the system. When a neuron is given an input, it processes the input and signals the next neuron. The connections between the neurons are called edges. Both the neurons and the edges typically have a weight which adjusts as the learning proceeds. The weight increases or decreases the contribution of a signal for the function. Typically, artificial neurons are aggregated into layers. Each layer typically has a different transformation function to perform on its input. Once the transformation function is applied to the input on layer one, the signal is sent to the second layer; and from the second layer to the third layer and so on till the last layer.

ANN has applications in multiple fields like image processing, character recognition, and forecasting, biomedical, etc. Traditional methods have their limitations and render themselves less useful when the influencing factors become complex. However, ANN applied correctly can give a robust alternative to these relations. ANN can model and extract features in unseen relationships between the inputs. Research is going on across different fields to improve machine learning using ANN to find more applications for ANN.

2.8.1 Working principle of ANN

The formulation of a feed-forward neural network with back propagation algorithm is considered to understand the working of an ANN. A feed-forward network is a network without any loops. A simple feed-forward neural network with backpropagation is shown in Fig. 2.4.

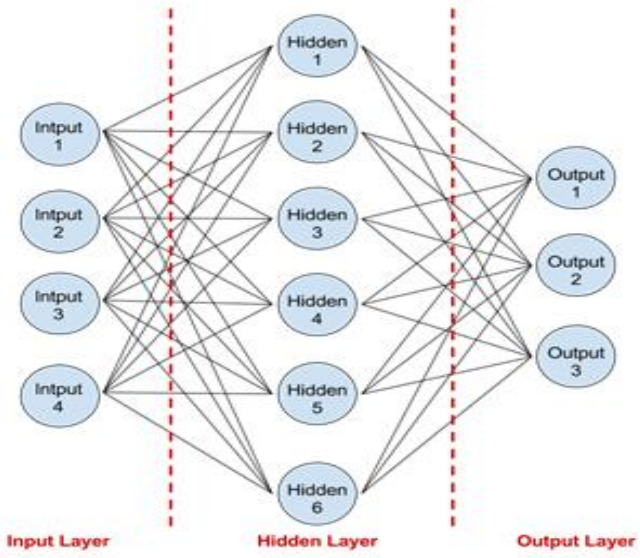


Fig. 2.4 Three layer feed forward neural network design

The neural network consists of an input layer, two hidden layers, and an output layer. The nodes in each of the layers are called neurons. A layer is made up of many such neurons. The input layer neurons receive the input signals and apply their transformation function to produce an output. The neurons in the input layer are connected to the neurons in the next hidden layer through edges. Each of the edges is given weight at the beginning calculated using a stochastic gradient. The edges apply their respective weights to the output of the input layer and feed it to the hidden layer. The data is again processed in each of the hidden layers and forwarded to the next layer until it reaches the output layer. After the output layer processes the data, it produces the output. After the output is produced, the back propagation comes into the picture. The output data is compared to the standard values, and an error is computed. This error is propagated through the

network in the direction opposite to that of the input. At each of the layers, the error is used to adjust the gradient of the edges in order to reduce the error. This process is repeated for a sufficient number of input data to reduce the overall error of the system.

2.8.2 ANN Modeling of phase equilibrium

Kan and Lee (1996) employed the ANN with feed forward network with backpropagation algorithm to model the phase equilibrium of PEG/potassium phosphate. Different range of PEG molecular weight of 600, 1500, 3400, 8000, and 20 000 with phosphate salts were considered for the training process where PEG 400 and 1000 were employed for testing purpose. The characteristic phase diagram, binodal curves and tie lines were constructed based on experimental and ANN values. The responses obtained by the ANN have an excellent agreement with the experimental values. The developed ANN model successfully predicted phase equilibrium values from PEG 400 to PEG 20000 and the author reported that ANN can be used as a powerful design oriented modeling tool.

Lv and Tian (2017) studied the performance of three dimensional ANN by using polyvinyl pyrrolidone (PVP)/phosphate/water as a model system. Along with the PVP/phosphate system, valency of the ions and the temperature of the systems were considered as a input data. The experimental data was collected from the literature and the data was divided into training and validation sets. The three dimensional neural network was optimized by weight comparison method. It was reported that the current model gave better accuracy in comparison with traditional ANN and other thermodynamic equations.

Lv and Zheng (2018) developed a three dimensional neural network using six different molecular weight of PEG and inorganic salts as a input data. The predictive ability of the model was checked by categorizing a data into training, validation and testing. This model reflects the effect of molecular weight, temperature and type of salt on equilibrium composition successfully. The error comparison between traditional and the three dimensional model was made and it was shown that the three dimensional model gave slightly higher accuracy than traditional model.

2.8.3 ANN simulation of ATPE of biomolecules

The artificial neural networks to simulate the extraction and purification process for a few biomolecules are implemented in the area of ATPE. The superiority of ANN over other modeling techniques like RSM is proved in predicting the complex process. The Table 2.7 contains the information about input and output data considered, type of ANN employed, algorithm and network used, statistical parameters' values which help to evaluate the model and the optimized hidden neurons in the hidden layer.

Table 2.7 Application of ANN in simulation of aqueous two-phase extraction of biomolecules

Biomolecule	Type of modeling	Inputs and outputs considered	Results	Reference
Invertase from baker's yeast	Hybrid group method data handling neural network	**Input:** PEG (1500-6000 Da), pH (4.0-7.0), PEG percentage (10.0-20.0 w/w), MgSO4 (8.0-16.0 w/w), cell homogenate (10.0-20.0 w/w) and MnSO4 (0-5.0 w/w) **Output**: Partition coefficient	HN=9 RMSE = 0.0160, MSE=2.258x10-, AARD = 32.752 %	Padilha et al. 2016
Bromelain from pineapple	Artificial neural network	**Input:** Temperature, PEG's molar mass, Concentration of PEG and ammonium sulphate and dilution factor of sample **Output:** Total protein and activity partition coefficient, and Purification factor	HN=30 R^2=0.99 MSE=0.02	Coelho et al. 2015
Polyhydroxy alkanoates from bacterial cells	Bootstrap neural network modeling	**Input:** potassium phosphate and EOPO concentration, pH and NaCl addition **Output:** Protein partition coefficient, Yield % and Purification factor	HN= 30, MSE=1.04×10^{-7}, RMSE=3.22×10^{-9}, R^2=1	Leong et al. 2018

Alkaline active xylanase from recombinant E.coli	Artificial neural network	**Input:** pH, PEG MW, PEG %, NaCl % and Salt % **Output:** Enzyme and protein partition coefficient, yield % and specific activity.	HN=7,9,6,6 for Partition coefficient of enzyme and total protein, yield and specific activity	Rahimpour et al. 2016
Alkaloids from alkaloids (caffeine and nicotine)	Hybrid group method of data handling neural network	**Input:** feed's weight percent compositions along with slope of tie-line, tie-line length (TLL) and difference of molecular weight of salt and Ionic liquid **Output:** Partition coefficient	AARD = 8.78%	Abdolrahimi et al. 2014
Biomolecules (amino acid and protein)	Artificial neural network model	**Input:** Temperature, biomolecular MW, MW of PEG and dextran and difference between weight fractions of PEG and dextran in the top and bottom phases **Output:** Partition coefficient	HN=1	Pazuki et al. 2010

HN-Hidden neuron, RMSE-Root mean square, AARD- Average absolute relative deviation, MSE-Mean square error

2.9 CONTINUOUS EXTRACTION OF PROTEINS IN ATPS

Continuous extraction of biomolecules in ATPE is done using different types of extractors. The extractors employed are classified as column extractors, mixer-settler units, and other extractors. Among these, the most studied and preferred extractors are column extractors due to reasonably low capital and operational cost. The redispersion of the biomolecules in the multistage operation is advantageous to achieve high recovery and purity of partitioned biomolecules. Column extractors are categorized as agitated and non-agitated extractors.

2.9.1 Non-agitating extractors

Spray column and packed column extractors fall under non-agitating extractor type, where there is an absence of agitator for mixing and drop formation and mainly the internal geometrical design such as orifice distributor in spray column and packing materials in packed column increases the mixing efficiency of the column.

2.9.1.1 Spray column

The efficiency of the spray column is evaluated in terms of hold up (H) and dispersed phase mass transfer coefficient (K_{Da}). The major factors affecting these extraction parameters in the spray column was studied for continuous extraction of pure proteins. Jafarabad et al. 1992 investigated the effect of column height, phase composition and variation in the number of sparger holes, and dispersed phase velocity on H and K_{Da} of BSA and amyloglucosidase using PEG/potassium phosphate and PEG/sodium sulfate. It was found that the increase in sparger holes and dispersed phase velocity increases the H and K_{Da} whereas an increase in phase composition decreases H and K_{Da}. It was also observed that the extraction parameters were not influenced by the column height. Pawar et al. (1997) adopted modified spray column to study the dispersed phase holdup and mass transfer coefficients for amyloglucosidase and alpha-galactosidase using PEG/sodium sulfate system. Increase in dispersed phase velocity increases the holdup and mass transfer coefficients of dispersed and continuous phases. The increase in the concentration of sulfate and PEG decrease the extraction parameters studied. Srinivas et al (2002) studied the effect of NaCl concentration, orifice size, and dispersed phase

superficial velocity on K_{Da} and H for horseradish peroxidase using PEG/potassium phosphate. The increase in dispersed phase velocity, orifice size, and NaCl concentration increased the K_{Da} and Hold up.

Arsalani et al. (2005) implemented a spray column for the continuous extraction of Lipoxygenase-1 from soyabean seed crude extract. The influence of dispersed phase flow rate and composition of the phases on dispersed phase holdup (H) and volumetric mass transfer coefficient (K_{Da}) was studied. It was reported that H and K_{Da} increased with the dispersed phase flow rate and decreased with the increasing phase composition. Correlations were developed for the estimation of H and K_{Da} by nonlinear regression.

2.9.1.2 Packed column

The continuous extraction of amyloglucosidase in packed bed column was studied by Patil et al. in 1991. The influence of the type of packings and their geometries, phase composition and superficial dispersed phase velocity on hold up and mass transfer coefficient was evaluated. Among the packings studied, ceramic rasching ring was selected as it gave high dispersed phase hold up values. From the experimental study, it was revealed that the values of HD and K_{Da} were independent of column height and the increase in phase composition decreases the values of extraction parameters considered. The decrease in bed voidage and increase in surface area of packings increases the HD and $K_{D}a$.

Igarashi et al. (2004) investigated the performance of packed column in xylanase extraction using PEG 4000 and dipotassium phosphate. The behavior of xylanase extraction under the influence of dispersed and continuous phase velocities and in the presence of different packings such as raschig rings, glass spheres, and polystyrene rings was studied. Irrespective of packing material, K_{Da} was increased with the increase in salt phase velocity. The mass transfer efficiency depends on the size of the packing material and the efficiency of extraction increases with the decrease in the size of the packing material. The extraction of xylanase increased with polystyrene rings of 3mm (75%) in comparison with 6 mm rasching rings and 5mm glass spheres.

The continuous extraction of human immunoglobulin from the supernatant of Chinese hamster ovary (CHO) was studied using PEG 3350/phosphate aqueous two-phase system. The wetting studies were conducted based on which stainless steel was selected as a material for column packing bed. The mass transfer process was explained in terms of Whitman's two-film theory. Hydrodynamic studies were conducted and the experimental data was related to the holdup values using the Richardson–Zaki, and Mísek equations, generally used for the organic-solvent systems. Pump mixer-settler combination with packed column and the high flow rates favors the mass transfer of IgG (Rosa et al. 2012).

2.9.2 Agitating Extractors

Rotating disc contactor, rotary annular column, raining bucket contactor, york – Scheibel column etc. are the agitating column extractors which consist of a series of discs, turbines, caps or vanes attached to a central shaft which are responsible for dispersion and coalescence of drops.

2.9.2.1 Perforated rotating disc contactor (PRDC)

For the first time, PRDC was implemented by Porto et al. (2000) for the aqueous two-phase continuous extraction of pure protein (BSA) using PEG 550 and 1000/ potassium phosphate. The effect of dispersed phase velocity and composition of the systems were studied on dispersed phase holdup and mass transfer coefficient. It was found that the dispersed phase holdup increased with increasing dispersed phase velocity and decreasing TLL for both the systems. The increase in the concentration of phase components/molecular weight increases the drop size and drop velocity which in turn reduces the residence time of drops in the column and leads to decreased hold up values. In this study, it was observed that the mass transfer coefficient values were independent of the dispersed phase velocity.

The continuous extraction of BSA was also studied in PRDC by Sarubbo et al. (2003) using PEG–cashew-nut tree gum. The effect of dispersed phase velocity, rotor speed and composition of the system (two tie lines were studied) on dispersed phase holdup and mass transfer coefficient was evaluated. Increase in rotor speed increases the H and $K_D a$

due to coalescence of the drops at discs' edge thereby decreasing the drop velocity. The effect of phase composition and dispersed phase velocity was the same as observed by Porto et al. (2000).

Cunha et al. (2003) studied the performance of PRDC for continuous extraction of extracellular *Fusarium solani pisi* cutinase expressed in Saccharomyces cerevisiae. The 60% fermentation broth containing enzyme was added to the continuous phase (dipotassium phosphate phase) and the hydrophobic protein partitioned to PEG-rich phase (PEG 3350). The separation efficiency was found to be two times of the batch process; however, the obtained protein was in diluted form when compared to the batch process. The maximum recovery was achieved at a flow velocity of 1 and phase velocity of 3.67×10^{-5} m/s.

Citrinin was continuously extracted in PRDC from *Penicillium citrinum* fermentation broth using 20% PEG 4000 /16.7% phosphate salt at pH 3.4. The concentration of citrinin in fermentation broth was maintained at 4-5 µg/mL. The flow rates of 1 and 2 mL/min for continuous and dispersed phase was tested with constant disc rotational speed of 140 rpm. Maximum recovery of 98.44% was reported at the flow rate of 1 mL/min (Pimentel et al. 2013).

The continuous aqueous two-phase extraction of ascorbic oxidoreductase from the crude extract of *Cucurbita maxima* was studied in PEG/potassium salt system using PRDC. The crude extract was mixed in the PEG-rich phase and the enzyme preferentially partitioned to salt-rich phase. The effect of dispersed phase velocity (1, 2, 3 mL/min) on mass transfer coefficient and separation efficiency with constant continuous phase velocity of 2 mL/min was evaluated. It was reported that hold up and mass transfer coefficient increases with dispersed phase velocity, whereas separation efficiency decreases with the increase in dispersed phase velocity. The maximum enzyme recovery of 236% with a purification factor of 34.3 was achieved in 1 mL/min dispersed phase velocity (Porto et al. 2004).

Cavalcanti et al. (2008) studied the performance of PRDC to extract α-toxin from the fermentation broth of *Clostridium perfringens* Type A using 15% PEG and 25% phosphate at pH 8.0. Statistical analysis was used to design the experimental runs. The effect of independent variables dispersed phase flow rate (1–3 mL/min), the continuous phase flow rate (1–3 mL/min) and the disc rotational speed (35 – 140 rpm) on hold up and mass transfer coefficient were investigated. The separation efficiency and the purification factor were considered as the response variables. The maximum dispersed phase hold up and mass transfer coefficient values were obtained at optimized operating conditions of 3.0 mL/min dispersed phase flow rate and a 140 rpm of disc rotational speed.

2.9.2.2 Rotating disc contactor

Conventional rotating disc contactor was implemented by Kalaivani et al. (2016) for the differential partitioning of α lactalbumin (α-La) and β lactoglobulin (β-Lg) to PEG-rich and salt-rich phase respectively. The hydrodynamic and mass transfer studies were carried out in an aqueous two-phase system of PEG 1000/sodium citrate. The effect of operating variables such as dispersed and continuous phase velocities and rotor speed were evaluated on dispersed phase hold up, mass transfer coefficients, recovery and separation efficiency. The maximum dispersed phase velocity (9.86×10^{-5} m/s) and minimum continuous phase velocity (1.34×10^{-5} m/s) with high rotor speed (above 5 1/s) yields 86% of recovery and 85.5% of purity of α-lactalbumin in PEG phase.

2.9.2.3 Raining bucket contactor

Coimbra et al. in 1994 demonstrated the continuous extraction of α-La and β-Lg in graesser raining bucket contactor using PEG 1500/potassium phosphate salt system. Hydrodynamic characteristics (hold up, axial mixing, residence time distribution) were studied at different operating conditions. At the optimum condition of rotor speed of 2 rpm and residence time of 1h, 660g of whey proteins were processed to obtain 8.8 g/L of α-La per day. It was also reported that the lower rpm (avoid the inactivation of protein) and a higher solvent to feed ratio (increases the interface area per unit time) increases the extraction efficiency. However, for the efficient continuous extraction, the study of the

distribution of other whey proteins and back mixing hydrodynamic parameters is necessary which were not included in the study.

2.9.2.4 York – Scheibel column

Jafarabad et al. (1992) investigated the continuous extraction of Bovine serum albumin (BSA) and amyloglucosidase in York – scheibel contactor using PEG 4000/potassium phosphate and sodium sulfate systems. The effect of column height, phase composition, impeller speed and dispersed phase velocity on H and k_{Da} was studied. It was observed that the values of H and k_Da were independent of column height and increase with the impeller speed but decrease with the phase composition.

2.9.3 Novel extractors

The continuous extraction of α and β amylase from Zea mays malt was carried out in microextraction column using PEG 4000/$CaCl_2$. Response surface methodology was used to design and optimize the enzyme recovery in the process. The effect of independent variables like flux rate, the free area of vane and vane rotation was studied on the purification factor of the enzyme in both the phases. It was reported that the high area of vane with low vane rotation and low flux rate resulted in the maximum purification factor. At the optimum condition of 75 rpm, 1:1 flow rate and 18% free area of vane gave purification factor of 130 in calcium chloride phase (Biazus et al. 2007).

Vazquez-Villegas in 2011 designed a novel separator device for the continuous recovery of whey proteins (α-La and β-Lg) from whey protein extract. The ability of the novel separator (consists of static mixer before tubular separator) in the partitioning of biomolecules in continuous extraction was evaluated using dyes where the tubular length and static mixer configurations were optimized. The model protein (BSA) partition behavior was also studied and then whey protein extraction was conducted. Both the proteins have different affinities for phase systems where α -Lg has more affinity towards the PEG phase and β -La towards salt-rich phase. The maximum recovery of 90% of the proteins was observed in top and bottom phases with minimal precipitation at the interface. It was reported that the length of the tubular separator increases the interfacial

contact between the phases and thereby increases the efficiency of separation. SDS-PAGE profile confirms the mass transfer of α -La and small portion of β-Lg to the top phase and β -Lg with the traces of other proteins in salt rich bottom phase.

Rosa et al. (2013) attempted to purify human immunoglobulin from the recombinant cells in pilot plant mixer settler device. The set up consists of a number of mixer settler units for multi-stage, back extraction and washing. Cell culture supernatant was added to the phosphate phase and immunoglobulins were extracted to the PEG phase. Washing was done to remove low molecular weight contaminants from the back extracted salt phase. This increases the immunoglobulin purity in the salt phase. The extracted immunoglobulins from different cell types resulted in more than 80% recovery and 99% purity.

Muendges and coworkers studied the continuous extraction of immunoglobulin G1 in PEG/phosphate salt using multistage mixer settler unit. Experiments were conducted with and without the addition of NaCl where, without NaCl the extraction was performed as a washing step (washing of bottom phase repeatedly transfers contaminants towards top phase) whereas with NaCl extraction was performed as a stripping step. The immunoglobulin G1 has affinity to salt phase without NaCl and yields nearly about 90% recovery and 32% purity. Whereas with the addition of NaCl, immunoglobulin G1 showed affinity to top phase. The performance of the extractor was analyzed by varying the factors such as throughput, phase ratio, and stage number. The purity of the immunoglobulin G1 increases with the increase in number of stages from one to five and with the increase of the product phase volume and yields nearly 90% recovery and purity of 32% (without NaCl). Extraction without NaCl helps to remove the low molecular weight contaminant proteins and extraction with NaCl able to remove high molecular weight contaminants from the immunoglobulin-G1. Finally, it was concluded that the combination of salt rich and poor (with respect to NaCl) extraction can be used to achieve better extraction performance (Muendges et al. 2015).Table 2.8 represents the various biomolecules extracted in continuous extractors.

Table 2.8: Continuous ATPE of biomolecules

Biomolecule and source	ATPS	Operational variables	Inference	Reference
Non agitated column extractors				
Spray column				
BSA and Amyloglucosidase	PEG 4000-sodium sulfate/ potassium phosphate	Dispersed phase flow velocity (0.14- 8 mm/s) Different diameter of columns and distributor	PEG/potassium phosphate: BSA H: 0.032, $K_D a$: $7.2x10^{-5}$ (s^{-1}). PEG/sodium sulfate: AMG H: 0.06, $K_D a$:2.75 $x10^{-5}$ (s^{-1})	Jafarabad et al. 1992
Horse radish peroxidase	PEG - 6000/potassium phosphate at 2% NaCl	Dispersed-phase flow rate : 2.98×109 - 9.73×10-9 m^3/s, Orifice diameter: 0.5-1.32 mm, NaCl concentration : 0 - 5%w/w	$K_D a$: 390×10^{-5} (s^{-1}) H: 0.03	Srinivas et al. 2002
Lipoxygenase	PEG/sodium sulfate	Dispersed-phase flow rate : 0.16-1.12mm/s Phase composition : 3 systems	$K_D a$:10×10^{-4} (s^{-1}) H: 0.8	Arsalani et al. 2005
Packed column				
xylanase	PEG 4000-potassium phosphate	Type of packing: raschig rings, glass spheres, polystyrene rings. Superficial velocity ratios of both the phases :	$K_D a$: $0.33min^{-1}$ Recovery:94%	Igarashi et al. 2004

		0.05-0.19 mm/s		
Xylanase	PEG-4000/K_2HPO_4	Dispersed phase velocity: 0.03-0.18 mm/s Column height: (75, 161 and 246 mm)	K_Da:0.066m^{-1} RE:72%	Igarashi et al. 2004a
Agitated column extractors				
Perforated rotating disc contactor				
Ascorbic oxidoreductase	PEG-550/ Phosphate salts	Dis (1 – 3 mL/min) Con (2 mL/min) Rotor speed: 220 rpm	K_Da: 0.073min^{-1}, SE: 95% PF: 35	Porto et al. 2004
BSA	PEG 550/1000-potassium phosphate	Dis (1 – 3 mL/min), Con (1 mL/min), Rotor speed 140 rpm	H: 0.45 K_Da: 0.125 min^{-1}	Porto et al. (2000)
BSA	PEG-4000/Cashew-nut tree gum	Dis (2 – 5 mL/min) Con (1 mL/min) Rotor speed: 60, 140 and 220 rpm	H: 0.56 K_Da: 0.28 min^{-1} S.E : 96%	Sarubbo et al. 2003
α-toxin	PEG-8000/phosphate salts	Dis (1 – 3 mL/min) Con (1 – 3 mL/min) Rotor speed: 35, 90 140 rpm	K_Da: 0.003 min^{-1} H: 0.80 PF: 2.4	Cavalcanti et al. 2008
Rotating disc contactor				
α-lactalbumin and β-lactoglobulin from acid whey	PEG 1000/sodium citrate	Dis (1.64×10^{-5} to 9.87×10^{-5}) Con (1.64×10^{-5} to 9.87×10^{-5}) Rotor speed: 1-6 rpm	Rec: 86%, Purity: 85.5% of purity of α-lactalbumin in PEG phase.	Kalaivani et al. 2016

Raining bucket contactor/Graesser contactors				
α-lactalbumin and β-lactoglobulin from whey powder	PEG 1550 – potassium phosphate	Dispersed phase flow rate (20 – 80 mL/min) Continuous phase flow rate (20 – 80 mL/min)	H: 0.06	Giraldo-Zuniga et al. (2006)
York – scheibel				
BSA and amyloglucosidase	PEG 4000/ potassium phosphate and PEG 4000/ sodium sulfate System	Rotor speed (0, 0.75 and 1.27 1/s) Height of the column (300, 660 and 900 mm)	PEG/Potassium Phosphate:BSA H:0.95, K_Da:1.4 x10^{-5} (s^{-1}) PEG/sodium sulfate – AMG H: 0.17, K_Da: 5.5 x10^{-4} (s^{-1})	Jafarabad et al. 1992.
Novel extractors				
Extraction of amylase from maize malt in micro column	PEG 4000 – calcium chloride	Vane rotation-75 RPM Free area of vane-18% Flow rate-1:2	PF:132	Biazus et al. 2007
Whey proteins from whey protein isolate using static mixer	PEG 1000 – potassium phosphate	Tubular length ((3.0 and 5.0 m) and static mixer configurations (single beads-filled tube, zig-zag, 3 glass beads filled and tube Spiral/single	Recovery of 90% with minimal precipitation at the interface.	Vázquez-Villegas in 2011

		beads-filled tube		
Immunoglobulin G using counter current pilot plant mixer-settler battery	PEG 3350 – potassium phosphate	Flow rate, cell supernatant concentration	Recovery:80% Purity:99%	Rosa et al. 2013
Monoclonal Antibody (Immunoglobulin G1) from cell supernatant	PEG 2000/phosphate	Throughput (200–600 mL/h), phase ratio, and stage number (1-5).	Purity of 32.6% and yield of nearly 90% was achieved.	Muendges et al. 2015

Dis - dispersed flow rate, Con - Continuous flow rate, H - Holdup, PF- Purification factor, k_Da - mass transfer coefficient, k – partition coefficient.

2.10. RESEARCH GAPS

It is well established from a number of research works that legume plants are a rich source of lectin; to be specific the seeds of legume plants are a very rich source of lectins. *Pisum sativum* lectin is a legume lectin, whose structural and functional properties are well researched. Its well-known properties include anti-HIV, anti-HCV and mitogenic. These properties give an excellent opportunity to exploit this lectin in a number of biomedical applications. Researchers are also suggested that, lectin can be used as an effective drug in designing safe anti HCV therapy.

The available separation process for lectin has a number of disadvantages like high cost, non-scalability and requirement of number of unit operations etc. It is highly essential to find cost effective process for the extraction of biomolecule in functionally active state. Few researchers have suggested ATPE has an alternative for the extraction of lectin from its crude extract. However very few lectins, particularly from the beans family were extracted. As literature suggests that the properties of lectin vary with the source, the study of mechanism of separation of pea lectin from its contaminant proteins in ATPS is highly essential.

It is essential to develop a suitable aqueous two phase system and study the effect of various operating parameters on the selective partitioning of lectin in identified system. Continuous extraction of lectin is required for the commercial establishment of the ATPE process. Till now no attempt has been done in this regard. Based on the literature survey, the implementation of conventional extractor (Rotating disc contactor) in extracting sensitive proteins was recognized and considered. To avoid the extensive experimental work and to improve the process robustness, the development of effective simulation tool is necessary. Artificial neural network was found as an effective soft computing method to simulate the extraction process. The research methodology for the present work was designed by considering all the above mentioned factors.

2.11 SCOPE AND OBJECTIVES OF THE PRESENT WORK

The Plant lectins have gained vast attention in the biomedical and biological field as they are readily available and safe to develop as a drug. Pisum sativum is a legume which contains mannose/glucose specific lectin which has anti-HIV-1 reverse transcriptase, mitogenic activity, and anti-cancer properties and finds its application majorly in the biomedical and therapeutic field.

The demand for these lectins is increasing due to their diversified applications. The extraction of lectins from conventional methods such as precipitation, dialysis, and chromatographic methods suffer from a high production cost with longer time consumption, low handling capacity, and difficulty in scaling up. These limit their application at the industrial level. This enunciates the need to develop alternative lectin extraction methods that are rapid, efficient, and economically viable. In this context, the present study aims at studying the aqueous two-phase extraction of the lectin from *Pisum sativum* (Psl) seeds. The ability of the aqueous two-phase system composed of biodegradable polyethylene glycol and citrate salt for extraction of the desired biomolecule has been evaluated. The ATPE of Psl can be optimized through statistical approach to obtain a best combination of variables, for the implementation of continuous extraction to develop the process for large scale production and commercialization.

In this perspective, the following objectives were formulated to study the purification of Psl.

1. To select a suitable aqueous two-phase system by considering different phase forming components and to study the influence of process parameters on the partitioning of commercially available *Pisum sativum* lectin in the selected system.
2. To study the influence of significant variables on the aqueous two-phase extraction of the lectin from the crude extract of *Pisum sativum* seeds.
3. Study and optimization of the crucial factors for the selective extraction of Psl from P.sativum seeds.

4. To implement the continuous aqueous two-phase extraction of lectin in the Rotating disc contactor and study the effect of operating variables of the extractor.
5. Artificial neural network modeling of equilibrium characteristics of ATPS and partitioning coefficient of *Pisum sativum* lectin in the ATPS.

2.12 ORGANISATION OF THESIS

The thesis is organized as five chapters. **'Chapter 1'** presents the generic introduction of the thesis which discusses the background of lectin research, their classification and application. This section also introduces the necessity and importance of ATPS along with the merits and demerits of other purification techniques. **'Chapter 2'** presents the detailed **Literature Review.** This chapter summarizes the relevant literature pertaining to the current study and highlighting the research gaps observed by critical review of the literature.

'Chapter 3' explains the **'Materials and Methods'** adopted in the present work. It explains the experimental methodologies, analytical methods and instruments used to achieve the stated objectives. The results obtained in different experiments were consolidated, analyzed and discussed in detail and presented in the **'Chapter 4'** as **'Results and discussion**'. It comprises of four parts such as Selection of system and investigation of the key factors on the partitioning of pure *Pisum sativum* lectin, Investigation and optimization of the crucial factors for the selective extraction of Psl from its crude seed extract, Continuous extraction in Rotating disc contactor and Artificial neural network modelling of the ATPE of Psl. The important results are summarized, and the significant conclusions drawn in the research work is consolidated in the **'Chapter 5'** as **'Summary and conclusion'**.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 MATERIALS AND INSTRUMENTS

The present research involves the use of poly (ethylene) glycol [HO-($CH_2CH_2O)_n$-H] of different molecular weight, analytical grade salts, pure proteins and HPLC grade solvents. Molecular weight, purity and the manufacturer of the chemicals are listed in Table 3.1. The procured materials were used without any further purification. The experiments were carried out using the deionized water obtained from Siemens, Lobostar ultra-pure water purification system.

Automatic digital refractometer (RX-5000α, ATAGO CO., Ltd, Japan, accuracy of ± 0.00004 and a precision of ± 0.00002), Refrigerating bath circulator (JEIO Tech, RW – 0525G, accuracy of ± 0.05°C), Digital viscometer (Rolling –ball viscometer Lovis 2000 M/ME, Anton paar) with an accuracy of ± 0.005 mPa.s, Digital densitometer (DDM 2911, Rudolph, USA, accuracy of ± 0.00005 $g.cm^{-3}$ and 0.03°C, precision ± 0.00001 $g.cm^{-3}$ and 0.01°C), Flame photometer (Elico Ltd., model CL 378), Electronic weighing balance (Shimadzu, Japan) with a precision of ± 0.01mg, High performance liquid chromatography (HPLC) (Shimadzu, prominence) equipped with C-18 column (Shodex, C-18, RSpak RP 18-415), UV/Vis Spectrophotometer (Double beam UVD3500, Labomed, USA), Refrigerated cooling centrifuge (Kubota 6930, Japan) and magnetic stirrer are some of the equipment's used for the study. Software like MS Excel, Origin 9.1, Minitab, Matlab, Neural network toolbox and Design expert were used for mathematical calculations, analysis of results through plotting graph, ANOVA analysis, modeling and designing the experimental trials.

Table 3.1. Details of list of chemicals used in the present study

S.No	chemicals	Grade	Molecular weight	purity	Manufacturer
1	Polyethylene glycol (PEG)	Analytical	2000, 4000,6000 and 8000 $g.mol^{-1}$	99%	Sigma-Aldrich, USA
2	copper sulfate ($CuSo_4.5H_2O$)	Analytical	159.609 g/mol	99%	Spectrum chemicals, India
3	Sodium hydroxide	Analytical	39.997 g/mol	95%	Merck, India
4	Hydrochloric acid	Laboratory	36.46 g/mol	99.8%	Merck, India
Salts					
5	Trisodium citrate	Analytical	294.10 $g.mol^{-1}$	99%	Merck, India
6	Tri-potassium citrate	Analytical	342.42 $g.mol^{-1}$	99%	Merck, India
7	Tri-ammonium citrate	Analytical	243.22 $g.mol^{-1}$	98.5%	Merck, India
8	Sodium chloride (NaCl)	Analytical	58.44 $g.mol^{-1}$	99%	Spectrum chemicals, India
9	Citric acid monohydrate	Laboratory reagent	210.14 $g.mol^{-1}$	99.5%	Spectrum chemicals, India
10	Potassium chloride	Analytical grade	74.5513 g/mol	99%	Merck, India
11	Di-sodium hydrogen phosphate	Analytical grade	141.96 g/mol	99%	Merck, India
12	Potassium dihydrogen phosphate	Analytical grade	172.114 g/mol	99%	Merck, India
Solvents					
13	Acetonitrile	HPLC	41.05 $g.mol^{-1}$	99.9 %	Merck, India
14	Tri-fluoro Acetic acid (TFA)	HPLC	114.02 $g.mol^{-1}$	99.9 %	Merck, India
Pure proteins					
15	*Pisum sativum* lectin	L5830	50 KDa	99%	Sigma-Aldrich, USA
16	Bovine serum albumin	Analytical	66 KDa	98%	Merck, India
Reagent					
17	Bicinchoninic acid reagent (BCA)	Analytical	B9643	99%	Sigma-Aldrich, USA

3.2 EXPERIMENTAL METHODS AND CALCULATIONS

3.2.1 Binodal curve preparation

The cloud point method was followed to develop the binodal curves. The experiment was carried out in a jacketed glass vessel. The temperature of the working solutions was maintained at 25°C by using a refrigerating bath (JEIO Tech, model RW-0525G), by circulating water through the external jacket. Aqueous stock solutions of 50% PEG (w/w) of different molar mass and 30% salt (w/w) were prepared and used for the experiments. To ensure uniform concentration of the constituents in the jacketed vessel, constant stirring was maintained using a magnetic stirrer. A salt solution of known concentration was titrated against the polymer solution (or vice versa) until the clear solution turned turbid and then water was added until turbidity was eliminated. The titrated procedure was repeated to get more number of binodal points. The complete binodal curve was obtained by interchanging the burette and pipette solutions. Volume to mass conversion was done using the density of the solutions, which were measured at 25°C in automatic digital density meter (Kaul 2000).

The binodal curves for PEG 6000 - sodium citrate, PEG 8000-sodium citrate/potassium and ammonium citrate ATPS were constructed at 25°C using the cloud point method and the data are presented in the Table A1.1 (Appendix).The binodal data for PEG 4000 trisodium/ tripotassium/ triammonium citrate system and PEG 2000/sodium citrate was referred from the research work of Kalaivani and Regupathi (2013). PEG 2000/trisodium and potassium citrate were retrieved from Murugesan and Perumalsamy (2014) and PEG 2000-ammonium citrate was taken from Govindarajan and Perumalsamy (2013). PEG 6000-Potassium and ammonium citrate binodal data was retrieved from Zafarani and Hamidi (2003) and Regupathi et al. (2009).

3.2.2 Measurement of phase components concentration

3.2.2.1 Salt concentration measurement

Salt concentration in the top and bottom phase was measured using flame photometer (Elico Ltd., CL-378, India). The bottom and top phases were diluted appropriately and analyzed for sodium and potassium concentrations using flame photometer and NH_3 concentration using conductivity meter. Salt calibration curve was prepared with known concentration of salt with suitable dilutions. Salt calibration curves considered for the present work are presented in Fig. A1.1 (Appendix), A1.2 (Appendix) and A1.3 (Appendix).

3.2.2.2 PEG Concentration measurement

PEG concentration in the samples was measured using the refractive index (RI) method (Digital refractometer RX-500, ATAGO Co. Ltd, Japan). Calibration curves were prepared with known concentrations of different PEG and salt combinations in the homogeneous region based on the binodal curve of the selected systems. Refractive index of ternary homogenous solution of known weight fractions of PEG and salt were measured at 25°C (Fig. A1.6 (Appendix) - A1.14 (Appendix)). The relation between the refractive index (n_D) and the mass fraction of polymer (w_p) and salt (w_s) in the solution is developed (Eq. 3.1.) through regression analysis and used for the determination of PEG concentration in the samples of top and bottom phases with the known the salt concentration.

$$n_D = a_0 + a_1 w_P + a_2 w_S \tag{3.1}$$

where, a_0, a_1 and a_2 are the fitting parameters, a_0 value is refractive index (1.3325) of water at 25°C. Salt concentration of the phases obtained from the flame photometer (tri-sodium citrate and tri-potassium citrate) and conductivity meter (ammonium citrate), refractive index of the phases and the constant values were substituted in Eq. 3.1 to determine the weight fraction of PEG in the phases. The constant values of the various systems considered in the study are listed in Table 3.2.

Table 3.2 Fitting parameters for Eq. 3.1

Systems	**a_1**	**a_2**	**Regression coefficient**
PEG 2000 - trisodium citrate	0.1741	0.1865	0.979
PEG 2000 - tripotassium citrate	0.1542	0.1547	0.986
PEG 2000 - triammonium citrate	0.1365	0.1358	0.978
PEG 4000 - trisodium citrate	0.1524	0.1341	0.992
PEG 4000 - trisodium citrate	0.1432	0.1617	0.985
PEG 4000 - triammonium citrate	0.1314	0.1423	0.993
PEG 6000 - trisodium citrate	0.1212	0.1521	0.987
PEG 6000- tripotassium citrate	0.1222	0.1654	0.985
PEG 6000- triammonium citrate	0.1634	0.1427	0.976
PEG 8000 - trisodium citrate	0.1152	0.1745	0.942
PEG 8000- tripotassium citrate	0.1357	0.1652	0.9851
PEG 8000- triammonium citrate	0.1482	0.1684	0.9654

3.2.3 Tie line length (TLL) determination

The tie lines were developed by mixing the appropriate weight of PEG and salt stock solutions. The phase components were dissolved thoroughly using vortex mixer. The phase separation was achieved by centrifugation for 5 minutes at 2000 RCF. The systems were then incubated at 25°C for 5 hours in the thermostat. Later, volumes of the clear phases were noted down and equilibrated phases (top and bottom phases)

were separated carefully. The refractive index (refractometer) and salt concentration (flame photometer and conductivity meter) was measured for each phase as explained in section 3.2.3. From the obtained PEG and salt concentrations for each phase, TLL was calculated using Eq. 3.2. (Regupathi et al. 2011 and Kalaivani et al. 2012)

$$\text{TLL}\ (\%) = \sqrt{\left[W^{T}_{Salt} - W^{B}_{Salt}\right]^{2} + \left[W^{T}_{PEG} - W^{B}_{PEG}\right]^{2}} \qquad (3.2)$$

where W^{T}_{Salt} and W^{B}_{Salt} refer to the concentration of salt in weight percentage in top and bottom phases and W^{T}_{PEG} and W^{B}_{PEG} refer to the concentrations of PEG in weight percentage in top and bottom phases.

3.3 PARTITIONING STUDIES WITH PURE LECTIN

Psl partitioning behavior in different ATPS was analyzed initially to identify a suitable citrate salt by preparing the ATPS with PEG 4000 and three different citrate salts namely, trisodium/ tri-potassium/ tri-ammonium citrate at different concentrations. Further the effect of PEG molar mass on the Psl partitioning was studied by preparing the ATPS with trisodium citrate salt and PEG molar mass of 2000, 4000, 6000 and 8000. The ATPSs are formed by adding the appropriate amount of PEG and salt solution from stock solutions (PEG 50% and salt 30%) with 1ml of Psl solution having the concentration of 0.5 mg Psl/mL on 5 g basis by adding deionized water in a 10 ml graduated conical tubes. The prepared systems were subjected to vortex for uniform mixing for 1min and centrifuged at 2000 RCF for 5min. The samples were incubated for 5 hour at 25°C for complete phase separation. After the appearance of fine separation between the phases, phase volume was recorded and phases were separated by using micropipette and used for further analysis.

The effect of pH on the partitioning coefficient was studied by preparing the ATPS at different pH (5, 6, 7, 8 and 9) by mixing the sodium citrate stock solution, whose pH was adjusted by the addition of either citric acid (adjust to pH of 5, 6, 7, 8) or sodium hydroxide (adjust to pH of 9), and the required quantity of PEG 6000 solution, deionized water and the protein was added in appropriate quantity to the total weight of 5g. The pH

values of the solutions were measured precisely with a digital pH meter. The crystal NaCl of known weight was directly added to the ATPS to study the effect of additives (NaCl) on the partitioning coefficient.

3.3.1 Protein estimation

The concentration of Psl in the top and bottom phases were estimated by Bicinchoninic acid assay (BCA) in UV/Visible spectrophotometer at 562nm (Smith et al.1994). The absorbance of protein in the phases were measured by considering the similar phase (only with PEG and salt solution) without protein as a blank to nullify the interference of phase forming components on the absorbance measurement. The quantification of protein in both the phases was performed and the mass balance of protein was analyzed by considering the initial amount of protein added to the system. The calibration curve of Psl developed is given in Fig. A1.4.

The Psl partition coefficient (K_L) (Eq. 3) and % yield (Eq.4) were calculated.

$$K_L = \frac{C_{LT}}{C_{LB}} \tag{3.3}$$

The yield of the Psl (Y_L) was defined as the ratio of total concentration of lectin present in the top phase (C_{LT}) and the total concentration of lectin present in the initial feed (C_{LF}), expressed as percentage as shown in Equation (3.4).

$$Y_L\,(\%) = \frac{C_{LT} \times V_T}{C_{LF} \times V_F} \times 100 \tag{3.4}$$

where, V_T and V_F represent the volume of the top phase and the volume of feed protein solution respectively.

3.3.2 High performance liquid chromatography (HPLC)

Reverse phase HPLC (Shimadzu, LC-20AD, Japan) analysis was done to confirm the stability of Psl during the ATPS extraction using C18 (Capcell Pak C18 MG II, Shiseido, Japan) column by comparing the chromatogram obtained before and after ATPE. Mobile phases A (0.1% trifluoroacetic acid in water) and B (100% Acetonitrile) was used.

Column was equilibrated with mobile phase for 15minutes before injecting the sample. Column temperature and the flow rate were maintained at 25°C and 0.5mL/min, respectively. Binary gradient was used in order to monitor the elution rate. The binary gradient mode was adopted and maintained as 5% solvent B from 0.01 to 2min, and 80% solvent B was linearly increased till 21 to 23min, kept constant for 2min and re-equilibrated from 27 to 35min by 5% of solvent B. The total run time was 35min. Absorbance was recorded using a UV detector at 214nm. The top and bottom phase samples were diluted appropriately to prevent the blocking of chromatographic column due to their higher viscosity and concentration.

Table 3.3 The variables and their range studied for partitioning of pure Psl

Variables	**Range of values**
PEG molecular weight	2000-8000 g/mol
PEG concentration (wt %)	14-22%
Salt concentration (wt %)	12-20%
Tie line length (wt %)	30-44%
Additive concentration (wt %)	0-5%
pH	5-9
Volume ratio	0.34-2.88

3.4 SELECTIVE PARTITIONING OF PSL FROM CRUDE EXTRACT OF *PISUM SATIVUM* SEEDS

3.4.1 Preparation of crude extract

Pisum sativum seeds were purchased from local market of Mangalore city, Karnataka state, India. Collected seeds were freeze-dried and powdered in an ice-cold mixer. Powdered seed sample (10% w/v) was mixed with phosphate buffer saline (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 , 137 mM NaCl, and 2.7 mM KCl) having the pH of 7.4 and stirred overnight at 4°C. The extract obtained was filtered using a muslin cloth and

centrifuged at 10000 rpm for 30 min at 4ºC. The supernatant was collected and used as a crude sample for all the experiments.

3.4.2 ATPS preparation for selective partitioning of Psl

Aqueous two phase systems were prepared by adding the required weight of phase forming components (PEG and salt) with 20% w/w of the crude sample as a feedstock for all the experiments except crude loading studies and deionized water. Total weight of the system was maintained at 10g by adding the required weight of phase forming components (PEG and salt), 20% w/w of the crude sample as a feedstock for all the experiments except crude loading studies and deionized water. The systems were incubated at 25°C (5 hours) to reach equilibrium after vortexed for 1 min and centrifuged for 2000 RCF. Phase volume was recorded after the fine separation of the phases and the phases were separated by using micropipette for further analysis.

3.4.3 Partitioning of Psl from the crude extract of *Pisum sativum* seed

The experiments were designed to deduce the effect of individual variables on the total protein partition coefficient (Kp) and selective partitioning of Psl which was accessed through the specific hemagglutination activity partition coefficient (K_{SHA}), hemagglutination activity yield (Y_{HA}) and purification factor (PF) by varying one variable at a time approach. The effect of salt type was examined with varying concentrations of salt at a constant PEG 4000 concentration of 20% by considering the respective binodal. The effect of PEG molar mass on the selective partitioning of Psl from its seed crude extract was studied in the ATPS formed by 20% PEG of different molar mass (2000, 4000, 6000 and 8000) and 16% trisodium citrate (SC) system. However, the effect of PEG concentration was studied by varying PEG 6000 concentration from 14% to 22% with a constant SC concentration of 16% and salt concentration effect was studied at constant 18% PEG by varying SC from 12 to 20%. The effect of pH on the extraction parameters was studied at the pH of 5, 6, 7, 8 and 9 by adjusting the pH of the stock solution of SC using citric acid monohydrate or NaOH. The effect of electrolyte additives on extraction parameters was studied by adding the NaCl

crystals directly while preparing the system. The effect of crude concentration and loading capacity of the system was analyzed by adding a different amount of weighed crude to the system. All partitioning studies were repeated thrice for reproducibility. The effect of TLL on the partitioning characteristics of Psl was analyzed by calculating the TLL (Eq.3.1) using the equilibrium composition of the phase components in both the phases (measured as explained in section 3.4.5).

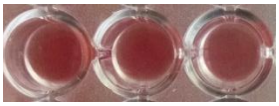
The concentration of total protein in the top and bottom phase were determined using Bicinchoninic acid assay (BCA) in UV/Visible spectrophotometer (UV3000+, Labindia) at 562nm (Smith et al. 1994) Interference of phase components was nullified by measuring all the systems against blank which contains only phase components without the crude sample. The quantification of protein in both the phases was done using the calibration curve developed with the standard BSA (Fig. A1.5).

The total protein partition coefficient (K_p) was calculated as;

$$K_P = \frac{C_{TP}}{C_{BP}} \qquad (3.5)$$

where, C_{TP} and C_{BP} are the total protein content in the top phase and bottom phase, respectively.

The hemagglutination activity of Psl in the samples of top and bottom phases obtained from each experiment was analyzed using the Hemagglutination assay (HA) according to the procedure described by Correia and Coelho (1999). Phase samples (50 µl) were 2-fold serially diluted in U shaped bottom microtiter plate with PBS of pH 7.2. 2% w/w of O^{+ve} human erythrocytes (Informed consent was obtained from the donors) was added to this diluted mixture and the plate was slightly tilted and allowed for 1-hour incubation. Wells without samples and only with PBS and erythrocytes which were diluted in 2 fold serial dilution fashion was maintained as 'control'. The appearance of a diffused mat of erythrocytes indicates positive results and the appearance of a red dot inside the well indicates negative results (Fig. 3.1 (a) (b)).



Positive results: Diffused mat of erythrocytes **Negative results:** Appearance of red dot

Fig. 3.1 Positive (a) and negative results (b) of hemagglutination assay

Hemagglutination unit (HU) was defined as 'the reciprocal of the highest dilution which shows visible agglutination' and specific hemagglutination activity (SHA) was defined as 'hemagglutination unit per mg of protein (HU/mg)' and SHA of the top phase (SHA_T) and the bottom phase (SHA_B) were calculated (Eq.3.6). Further, the Specific hemagglutination activity partition coefficient (K_{SHA}) was calculated using Eq. (3.7). The hemagglutination activity yield (Y_{HA}) was calculated and expressed as percentage (Eq 3.8).

$$SHA_T = \frac{HA_T}{C_{TP}} \quad \text{(or)} \quad SHA_B = \frac{HA_B}{C_{BP}} \qquad (3.6)$$

$$K_{SHA} = \frac{SHA_T}{SHA_B} \qquad (3.7) \qquad Y_{HA} = \frac{HA_T \times V_T}{HA_C \times V_C} \qquad (3.8)$$

where, HA_T and HA_B are hemagglutination activity in the top and bottom phase respectively. V_T and V_C represent the volumes of the bottom phase and the crude extract, respectively.

The purification factor (PF) was calculated as 'the ratio of specific activity in the top phase (SHA_T) to the initial specific activity in the crude extract prior to partition (HA_C/C_{CP})', expressed as follows:

$$PF = \frac{SHA_T}{SHA_C} \qquad (3.9)$$

where HA_C (HU/mL) and C_{CP} (mg/mL) represent the hemagglutination activity and total protein concentration in the crude extract, respectively.

3.4.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the extracted Psl was analyzed by the SDS-PAGE. Discontinuous polyacrylamide gel comprising of 12% resolving gel and 5% stacking gel was prepared (Laemmli 1970). All the samples were dissolved in sample buffer of pH 6.8; containing 1 % SDS and incubated at 90 ^{0}C for 5 min. Phase samples were dialyzed overnight at 40 ^{o}C against deionized water to minimize the interference of phase components. The interface was collected from the system and mixed with PBS and subjected to centrifugation; settled cell debris was discarded and the supernatant was used as a sample. Marker, standard Psl, *Pisum sativum* seed crude extract, samples from top phase, interphase and bottom phase of optimized ATPS were loaded to respective wells and electrophoresis was carried out at a constant current of 50 v for 3 h. The gel was stained with Coomassie brilliant blue R-250 and destained with a destaining solution.

3.4.5 High performance liquid Chromatography (HPLC)

Reverse phase HPLC (Shimadzu, LC-20AD, Japan) analysis was done to confirm the purity of Psl during the ATP extraction of the lectin from *Pisum sativum* crude extract using C18 (Capcell Pak C18 MG II, Shiseido, Japan) column. Mobile phase A (0.1% trifluoroacetic acid in water) and B (100% Acetonitrile) was used. The column was equilibrated with mobile phase for 15 minutes before injecting the sample. Column temperature and the flow rate were maintained at 25^{0}C and 0.5mL/min, respectively. The binary gradient mode was adopted and maintained as 5% solvent from 0.01 to 2 min and 80% solvent B was linearly increased till 21 to 23 min, kept constant for 2 min and re-equilibrated from 27 to 35 min by 5% of solvent B. The total run time was 35min. Absorbance was recorded using a UV detector at 214 nm. The top and bottom phase samples were diluted appropriately to prevent the blocking of the chromatographic column due to their higher viscosity and concentration.

3.4.6 Response surface methodology (RSM)

RSM was used to elicit the optimal concentrations of the most significant independent variables to maximize the lectin production. 30 trials were run with the four factors at

five discrete levels (-α, -1, 0, +1, +α) in a central composite design (CCD). The factors PEG concentration, salt concentration, NaCl and pH were considered as independent variables and Hemagglutination activity Yield (Y_{HA}), Specific Hemagglutination activity coefficient (K_{SHA}) and Purification factor (PF) were taken as the response. Design expert software 11 (Trial version) was used for multiple regression analysis of the experimental data and to construct the response surface plots. The data obtained from RSM on lectin extraction were subjected to analysis of variance (ANOVA). The relationship between the variables and their effect on the response was developed by fitting the data in a second order polynomial equation (Uy and Telford 2009).

$$Y = \beta_0 + \sum_{i=1}^{p} \beta_i X_i + \sum_{i=1}^{p} \sum_{j=1}^{p} \beta_{ij} X_i X_j + \sum_{i=1}^{p} \beta_{ii} X_i^2 \qquad (3.10)$$

Where, β_0 denotes response of overall mean

β_i denotes the each factor main effects (i =1, 2, …. p)

β_{ij} denotes the two-way interaction between the ith and jth factors

β_{ii} denotes the quadratic effect for the ith factor.

X_i and X_j denotes the coded independent variables.

The statistical significance of the developed quadratic model equation and model terms was evaluated by using analysis of variance (ANOVA).

Table 3.4 Levels of independent factors with their coded and uncoded values.

Factors	**Factor code**	**levels**				
		-α	**-1**	**0**	**+1**	**+α**
PEG concentration %	A	14	16	18	20	22
Salt concentration %	B	12	14	16	18	20
NaCl %	C	0	1	2	3	4
pH	D	6.5	7	7.5	8	8.5

3.5 CONTINUOUS EXTRACTION OF *PISUM SATIVUM* LECTIN IN ROTATING DISC CONTACTOR

3.5.1 Configuration of RDC

The continuous extraction of Psl was studied in Rotating disc contactor (RDC). The column of the RDC was made of glass. All the column internals (shaft, stator ring, rotating discs and connecting nozzles) are made up of stainless steel. The column consists of cylindrical vessel with flanges at the top and bottom ends. The dimensions of the extractor used in the study are given in the Table 3.5. The compartments were formed by placing the stator rings (horizontal doughnut- shaped) at equal distance and a rotating disc is provided within each compartment which is mounted on a central shaft. The central shaft was driven by electric DC motor with gearbox and the rotation of the central shaft was controlled by voltage regulator. The speed of rotation was measured and digitally displayed by electronic tachometer with higher precision. The column top and bottom flange were installed with the necessary pipe connections for the inlet and outlet of the dispersed and continuous phase solutions. The phase flow rates of dispersed and continuous phase were maintained by two peristaltic pumps. Continuous phase outlet was fitted with an adjustable limb to control the position of the

interface at the top of the column. The schematic representation of RDC was given in Fig. 3.2.

Table 3.5 Experimental rotating disc contractor (RDC) dimensions.

Column internals	Dimensions (cm)
Diameter of column	2.54
Height	51
Rotor diameter	1.524
Stator ring diameter	1.178
Compartment height	2.54
Number of compartments	5
Volume of column (mL)	320

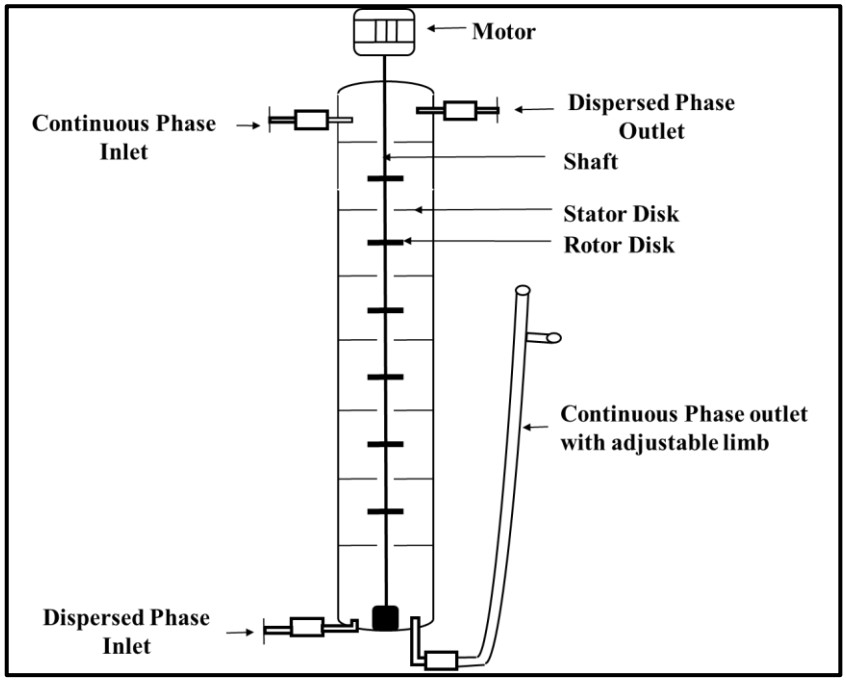


Fig. 3.2 Schematic representation of rotating disc contactor (RDC)

3.5.2 Operation of RDC

Initially, the column was filled with continuous phase, which contains 20% of the *Pisum sativum* seed crude extract through inlet provided at the top of the column. Then both continuous (Salt solution with crude *Pisum sativum* seed crude extract) and dispersed phase (PEG solution) were continuously fed into the column in upward and downward manner through top and bottom inlet of the column, respectively at the desired flow rates. The interface level was maintained just above the top stator ring by adjusting the continuous phase outlet limb. The extract phase enriched with Psl and the raffinate phase with exhaust salt solution was collected from top and bottom outlets respectively. The column was operated in counter current mode at varied dispersed and continuous phase velocities in the range of 2.91×10^{-5} to 9.32×10^{-5} m/s and at the varied rotor speed of 2.5 to 7.5 rps. The column was operated until the steady state was reached which was indicated by the stationary interphase.

3.5.3 Dispersed phase holdup

The dispersed phase holdup was measured by the volume displacement method at the steady state operation. The initial position of the interface was marked at specific operating condition. Both the continuous and dispersed phase inlet and outlets were closed concurrently. The interface level was reduced due to the accumulation of dispersed phase above the continuous phase. Meanwhile the rotor was made to rotate for further 10 minutes and the reactor was left undisturbed for 30minutes. Then the accumulated dispersed phase was collected by admitting the continuous phase slowly till the interface reaches the initial position (steady state position). Thus collected dispersed phase volume was measured and the dispersed phase holdup was expressed as the ratio of volume of dispersed phase to effective volume of the column (from bottom of the column to interface) (Murugesan and Regupathi 2004). The dispersed phase holdup was measured for various combinations of phase flow velocities and rotor speed.

$$\text{Dispersed phase holdup} = \frac{\text{Volume of the dispersed phase}}{\text{Effective volume of the coulmn}} \qquad (3.11)$$

3.5.4 Mass transfer coefficient (K_Da)

The continuous phase was prepared with predetermined quantity of *Pisum sativum* seed crude extract according to the tie line. The transfer of Psl takes place from continuous to dispersed phase. The phase flow rates and the rotor speeds were varied to understand their effect on mass transfer coefficient. The reactor was operated in counter current mode to determine the mass transfer coefficient. After allowing a continuous and dispersed phase at desired velocities and rotor speed, the samples from extract and raffinate phases were collected for every 15minutes. The activity of the Psl was measured using hemagglutination assay (explained in section 3.6.4) and the total protein content was measured using BCA assay (section 3.6.4). The activity and protein concentration obtained was found to increase with time and reaches a constant value after some time, which was considered for calculation. In the study, at studied range of flow velocities and rotor speeds, the time taken to achieve steady state was nearly 2 hours. The following equation was used to calculate the K_Da

$$K_Da = \frac{F_{salt}}{V_d}\left[\left(\frac{1}{1-\frac{F_{salt}}{K_{HA}\times F_{PEG}}}\right) \times \ln\left(\frac{HA_{salt\ in}-\left(\frac{HA_{PEG\ in}}{K_{HA}}\right)}{HA_{salt\ in}-\left(\frac{HA_{PEG\ out}}{K_{HA}}\right)}\right)\right] \quad (3.12)$$

where K_{HA} = hemagglutination activity partition coefficient at equilibrium, V_d is the volume of the dispersed phase, F_{salt} and F_{PEG} are the flow rates of continuous and dispersed phase velocities.

Recovery, purification factor and separation efficiency were calculated using Eq. (3.13), (3.14) and (3.15) respectively.

$$\text{Recovery \%} = \left(\frac{F_d \times HA_{PEG\ out}}{F_c \times HA_{crude\ extract}}\right) \times 100 \quad (3.13)$$

$$\text{Purification factor, PF} = \frac{SHA_{PEG\ out}}{SHA_{Crude\ extract}} \quad (3.14)$$

$$\text{Separation efficiency, E} = \frac{HA_{crude\ extract} - HA_{raffinate}}{HA_{crude\ extract}} \quad (3.15)$$

where F_D and F_C are flow rates corresponds to dispersed phase and continuous phase respectively. SHA is specific hemagglutination activity.

3.6 MODELING OF EXTRACTION PROCESS OF PSL BY ARTIFICIAL NEURAL NETWORK (ANN)

The modeling of the aqueous two phase extraction process of Psl is carried out in the present study to predict the equilibrium characteristics of the phase forming components and the solute Psl. The influence of sodium, potassium and ammonium citrate and the influence of PEG molar masses on phase behavior and Psl extraction and the selective extraction of Psl was modeled using artificial neural network (ANN). Feed forward multilayer ANN with back propagation algorithm was used to develop a model. PEG and salt concentrations of three different citrate salts namely sodium, potassium and ammonium citrate their respective cationic radius and PEG molar masses from 2000-8000 was considered as inputs. PEG and salt concentrations in the top and bottom phases, partition coefficient of pure Psl was considered as outputs. The number of neurons in the hidden layers was optimized to obtain an efficient topology.

Further the selective extraction of Psl from its seed crude extract was modeled by using central composite experimental design and by considering the inputs and outputs as independent factors and dependent factors considered in the RSM respectively. Inputs and outputs selected for the corresponding studies were represented in Table 3.6. A three-layer ANN model using a back-propagation (BP) algorithm was implemented to predict the responses. An optimization study to determine the optimal network structure was conducted using BP algorithm. Finally, the outputs obtained from the ANN modeling were compared with the experimental data using statistical parameters. All ANN calculations were carried out using neural network toolbox of MATLAB version 7.9 (R2009b).

Table: 3.6 Inputs and Outputs considered for the ANN modeling of *Pisum sativum* lectin

Input	**Output**
ATPE modeling of selection of suitable citrate salt	
Type of salt (sodium, potassium, ammonium citrate), Radius of Cation (RoC), PEG molar mass (2000, 4000, 6000 and 8000 g/mol), PEG and salt concentrations (%,w/w).	PEG concentration in top phase (%,w/w) PEG concentration in bottom phase (%,w/w) Salt concentration in top phase (%,w/w) Salt concentration in bottom phase (%,w/w) Partitioning coefficient of Psl
Modeling of Selective ATPE of Psl	
PEG concentration (%,w/w) Sodium citrate concentration (%,w/w) NaCl (%,w/w) pH	Hemagglutination activity Yield, % (Y_{HA}), Purification factor (PF) Specific activity partition coefficient (K_{SHA})

CHAPTER 4

4. RESULTS AND DISCUSSION

The development of a suitable aqueous two phase extraction process for the recovery of Psl from *P.sativum* seeds crude extract was attempted in the eco-friendly ATPS through a methodical approach. It is well established that the separation of desired biomolecules from the complex sources can be achieved in Aqueous Two Phase Systems (ATPS) by thoughtful selection of phase components wherein the contaminants and the desired biomolecules have affinity towards different phases. Further, the recovery and purity can be enhanced by appropriate manipulation of the other key factors such as phase components concentration, addition of the additive, system pH and volume ratio of the system. Hence, a systematic strategy was adopted to separate Psl from the other contaminants present in the crude extract. Initially the suitability of chosen phase components for the partitioning of desired proteins were analyzed and thereafter, the effect of process variables on the partitioning ability were studied using pure protein solution. Commercial implementation of the process necessitates scale-up and continuous operation which has been endeavored in Rotating Disc Contactor (RDC). The experimental results of partitioning studies in batch and continuous operation are discussed in four parts of this chapter along with the possible facts behind those observations.

Part one: Selection of system and investigation of key factors on the partitioning using pure Psl

This part describes the selection of suitable ATPS by considering the effect of different variables on the extraction process based on the partitioning studies using commercially available Psl. Suitable PEG-citrate salt ATPS was identified based on the partition coefficient of Psl in the systems of varying cation of citrate salt and PEG molecular weight. Further, the influence of phase components concentration, additive concentration,

pH and volume ratio on the partitioning of Psl was studied and reported. Efficacy of the system was discussed in terms of partition coefficient and yield of Psl.

Part two: The selective partitioning of Psl from *P.sativum* seeds

This part conveys the selective partitioning of Psl from the P.sativum seed crude extract and the optimization of significant variables for the desired output. The performance of the various process variables in the selective extraction of Psl was assessed by hemagglutination activity yield (Y_{HA}), purification factor (PF), specific hemagglutination activity partition coefficient (K_{SHA}) and total protein partition coefficient (K_p).The range and the process variables used in the pure protein studies were considered in the present study as the contaminant proteins and non-protenacious substances present in the crude extract may change the behavior of the partitioning of Psl and also to access the influence of these variables on the behavior of extraction parameters (Y_{HA}, PF,K_{SHA} and K_P) in each step. This part also explains the multi-variate multi-objective optimization of the crucial factors to achieve the desired goals through response surface methodology (RSM). The experiments were designed using central composite design (CCD) and the responses were represented by polynomial equations. Moreover, the interaction effect of the independent variables on the responses was analyzed using response surface plots. Optimum values of the process variables to achieve the desired goals were also obtained through desirability based numerical optimization approach and were validated experimentally.

Part three: Continuous extraction in Rotating disc contactor (RDC)

The continuous ATPE of Psl from P.sativum seed crude extract was attempted in a continuous counter current Rotating Disc Contactor. The resulted extraction efficiency and mass transfer characteristics of the RDC were analyzed at various disc rotational speed, continuous and dispersed phase velocities. In the range of experimental conditions, holdup and mass transfer coefficient were obtained for the process and the effect of variables on holdup and mass transfer coefficient also discussed in this section.

Part four: Artificial neural network modeling of equilibrium characteristics of ATPS and partitioning coefficient of *Pisum sativum* lectin in the ATPS.

The aqueous two phase extraction process of Psl was modeled starting from the phase equilibrium to selective extraction of Psl from its seed crude extract. Modeling of the extraction of pure Psl partition study, where the effect of three different citrate salts namely sodium citrate, potassium citrate and ammonium citrate and the effect of four types of PEG molecular weight (2000, 4000, 6000 and 8000 g/mol) on phase equilibrium and the partition behavior of Psl were modeled using Artificial neural network (ANN). Further the selective extraction of Psl was also modeled using ANN with selected ATPS i.e., PEG 6000 - sodium citrate salt.

4.1 PARTITIONING STUDIES OF PURE *PISUM SATIVUM* LECTIN (Psl) IN PEG/CITRATE ATPS.

Partitioning studies of pure protein reveals information about the suitability of a specific ATPS through the interactive forces with the specific biomolecule due to the effect of process variables like phase forming components and their concentrations, pH, TLL, volume ratio etc. on partition coefficient. Further, the influence of these variables on the partitioning of specific biomolecule may be realized through the partitioning studies with individual pure protein solution. The pure protein studies may reveal the extent of specific interactive forces exist between the phase system and biomolecule in the absence of interferences caused by other molecules and impurities which are actually present in the real-time crude. The magnitude of the major non-covalent forces like vanderwaals force of attraction and hydrophobic interaction involved in the partitioning characteristics differs for different ATPS and thus the partition coefficient, recovery and purification fold of the biomolecules present in the system are found to vary. Based on the information obtained from pure protein studies, the process variables can be further tuned to recover more desired proteins with higher yield and purity from the complex sources like plant crude extracts. Hence the partition behavior of pure *Pisum sativum* lectin (Psl) was studied in PEG- citrate ATPS. Initially the binodal curves for all the four systems

were obtained and the most suitable salt was selected by conducting the partitioning study at constant concentration of PEG 4000 with varying salt concentration. Further, the experiments were conducted to study the effect of process variables like PEG molar mass and their concentration, salt concentration, tie line length (TLL), additive concentration, pH, and volume ratio in ATPS.

4.1.1 Phase forming ability of citrate salts

Organic citrate salts were chosen as phase forming salts in the present study since the citrate salts possess good salting out, biodegradable and non-toxic characteristics. Three different citrate salts such as sodium citrate (SC), potassium citrate (PC) and ammonium citrate (AC) were considered. Binodal curve denotes the critical concentration of phase components required to form two phases. The amount of salts required to form the two phases in ATPS will varies with the type of salts. To study the phase forming behavior of all the three citrate salts, the binodal curves with PEG 4000 was considered. The binodal for PEG 4000-SC/PC/ and AC (Fig. 4.1a) were retrieved from the research work of Kalaivani and Regupathi (2013). These citrate salts have similar anion but differs in cation. The salting out ability of the salts varies with the properties of the associated cations of the salts having similar anion, citrate.

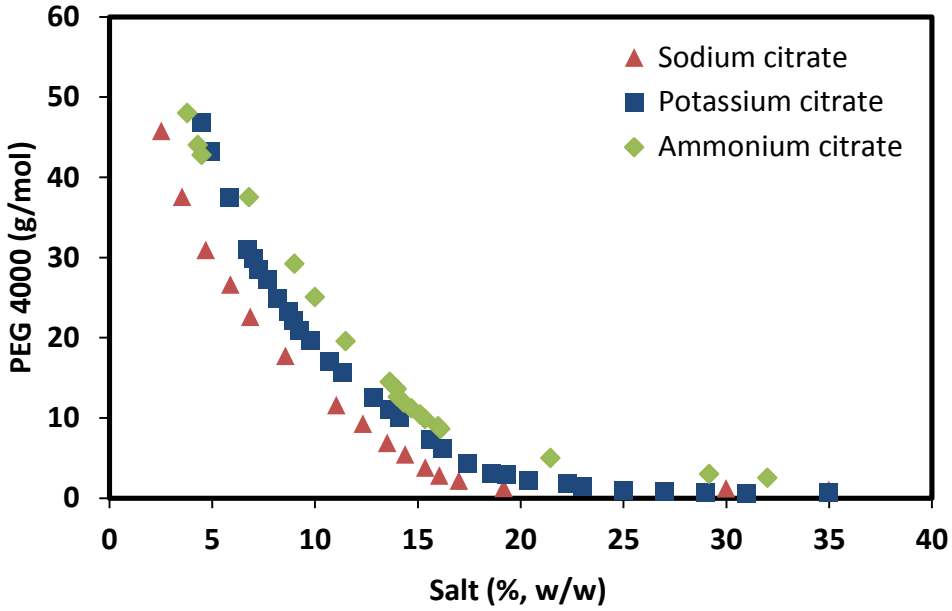


Fig. 4.1a Binodal curves developed for PEG 4000/sodium (▲), potassium (■) and ammonium (♦) citrate at 25^{0}C.

Salting out ability of different salts were analyzed based on the Gibbs free energy of hydration ($\Delta G_{hydration}$), size of the cation and effective excluded volume (EEV). It was reported that the salt which contains more negative Gibbs free energy of hydration ($\Delta G_{hydration}$) has higher salting out ability. The sodium citrate forms more biphasic region since the sodium citrate (Na^+: -375kJ/mol) has more negative $\Delta G_{hydration}$ than potassium citrate (K^+: -304kJ/mol) and ammonium citrate (NH_4^+: -285kJ/mol) (Raja and Murthy 2012). Further, it is reported that the salting out capacity of the cation also differ with their ionic radius and cations with lesser ionic radius has more salting out capacity (Zafarani-Moattar and Hamidi 2003). Na^+ (0.102 Å) has lesser ionic radius compared to K^+ (0.138 Å) and NH_4^+ (0.143 Å).The effective excluded volume (EEV) of the system, which depends on the molar mass of the PEG and type of the phase forming salt, also affects the salting out capacity of salt. EEV of particular PEG/salt system can be calculated from the binodal points of that particular system. The salt with higher EEV values has higher salting out ability. Salting-out strength of the salt could be related to effective excluded volume (V_{123}), as proposed by Guan et al. 1993

$$\ln\left(V_{123}^{*}\,\frac{w_p}{M_p}\right)+\left(V_{123}^{*}\,\frac{w_s}{M_s}\right)=0$$

where V_{123}^{*} is the EEV, W_P and W_S are the weight fractions of the polymer and salt, respectively. M_P and M_s are the molar mass of polymer and salt respectively. The EEV values are already reported in the literature for PEG 4000-SC/PC and AC (Kalaivani and Regupathi 2013). The salt which has high EEV has more salting out ability. The sodium citrate has more salting out ability, since the EEV of PEG 4000 and sodium citrate salt (49.57 g/mol) is higher than the potassium (48.11 g/mol) and ammonium (37.56 g/mol) citrate salts. EEV of the system was observed to increase with decrease in the radius of the cation. The minimum amount of salt required to form a two phase decreases with the increase in EEV of the system, $\Delta G_{hydration}$ of the cation and with lesser cationic radius.

4.1.1.1 Selection of suitable citrate salt

The partitioning characteristics, partition coefficient (K_L) and yield (Y_L) of Psl, in the PEG-salt ATPSs formed with three different citrate salts namely, sodium citrate, potassium citrate and ammonium citrate were studied (Fig.4.1b). As the properties of the citrate salts vary with associated cations and their concentration, two different ionic environments formed in the top and bottom phases of ATPS and subsequently their specific interaction with the protein differs during the partitioning. The charged protein experiences a chemical potential difference between the phases and resulted to a net partitioning. The effect of citrate salts in the ATPS was studied by conducting the experiments at a constant concentration of 20%, w/w PEG 4000 with three different salts concentration between 10-20% (w/w). It was observed that maximum K_L varied in the order of 3.23± 0.05 > 2.81 ± 0.06> 2.23± 0.10 and maximum Y_L varied in the order of 77.04± 1.05% > 75.07± 1.29 % > 71.42 ± 1.58 %, respectively for $Na^+ > K^+ > NH_4^+$ (Fig.4.1b).

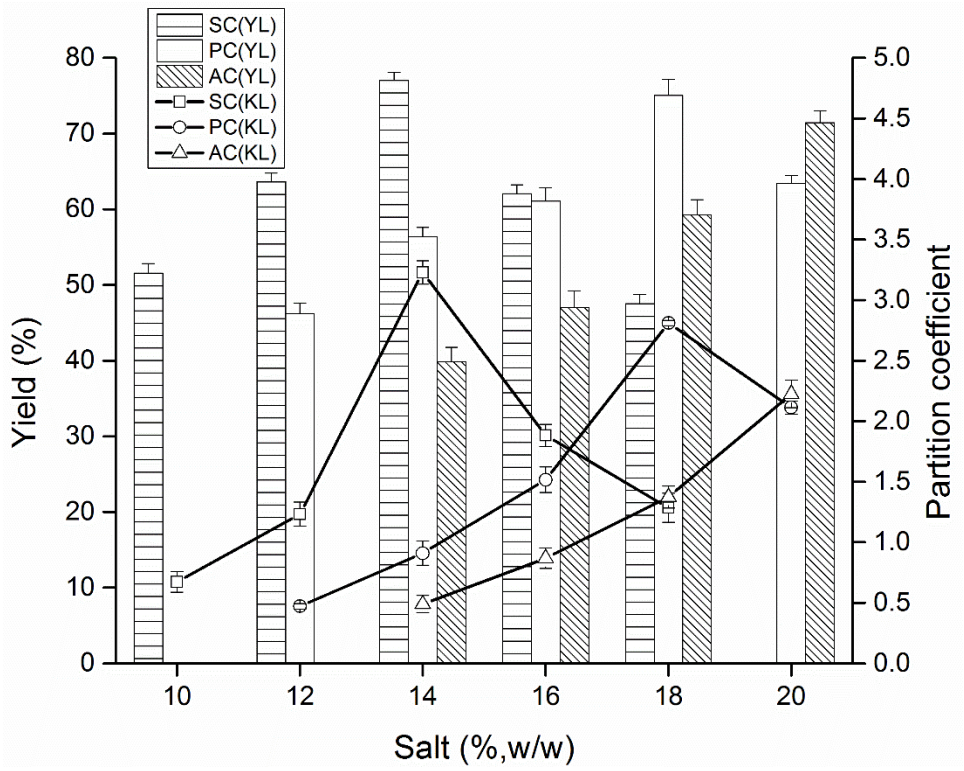


Fig. 4.1b Effect of type of salts at different concentration (SC- sodium citrate, PC - potassium citrate, AC- ammonium citrate) with 20 wt% PEG 4000 on partition coefficient (K_L) and Yield % (Y_L) of the lectin.

The higher salting out characteristic was observed for sodium citrate due to the favorable $\Delta G_{hydration}$, and smaller ionic radius, which limits the solubility of the lectin in salt rich phase and eventually, expelled it to PEG rich top phase through salting out process. The hydrophobic domains of the Psl are exposed and interact with the PEG molecules. 20% PEG 4000 and 16% sodium citrate provide a maximum Psl partitioning.

The decreasing salting out ability and EEV of potassium citrate and ammonium citrate indicate the requirement of larger concentration of salt to achieve a favorable partitioning and hence the ammonium citrate was unable to form two phases at 10 and 12% concentration (Fig. 4.1b). However, the maximum partitioning for potassium citrate and ammonium citrate was achieved at a concentration of 18% and 20%, respectively. The PEG - sodium citrate system was successfully employed not only for the partitioning of *Canavalia brasiliensis* (ConBr) lectin (Nascimento et al. 2010) and also for different

other proteins like, α-La from whey (Kalaivani and Iyyaswami 2015) and glycomacropeptide (Da Silva et al. 2009). The sodium citrate salt was selected as a phase forming salt for further experiments, as it gave maximum Psl partition coefficient and yield when compared to other two citrate salts.

4.1.2 Effect of PEG molar mass on partition of Psl

The multiple binding interactions of polymer and protein are a prime factor along with the other factors for the proteins like Psl, which get partition in the PEG phase. However, it is difficult to form multiple binding sites with protein for too shorter or longer chain length of PEG (Wu et al. 2017) and hence, the experiments were designed to screen the appropriate PEG molar mass which consists of optimum chain length to interact with the Psl and enhance its partition into the top phase. The partitioning experiments were carried out with constant total phase composition of PEG 20% (w/w) and sodium citrate 16% (w/w) with different molar mass of PEG from 2000 to 8000 (Fig. 4.2). The Psl partition in to the top phase was found to increase with increasing molar mass up to 6000 and further increase in molar mass to 8000 resulted in lesser Psl partitioning. As the molar mass increases, the hydrophobicity of the phase also increases due to the increase in the length of the PEG chain with increasing repeating nonpolar CH_2 group in polymer chain. The partitioning of protein to either of the phases depends on proteins intrinsic factors such as protein size, hydrophilic/hydrophobic characteristics, and conformational structure and extrinsic factors such as temperature, pH, ionic strength, and interaction between protein molecules. The isoelectric point of Psl is in the range of 6.1-7.2 (Hoedemaeker et al. 1994). The maximum partitioning of Psl by retaining its functionality was observed at PEG 6000 when compared to other molar mass of the PEG (Fig. 4.2) due to the optimum chain length and the hydrophobicity which enhances the interaction between the PEG and Psl.

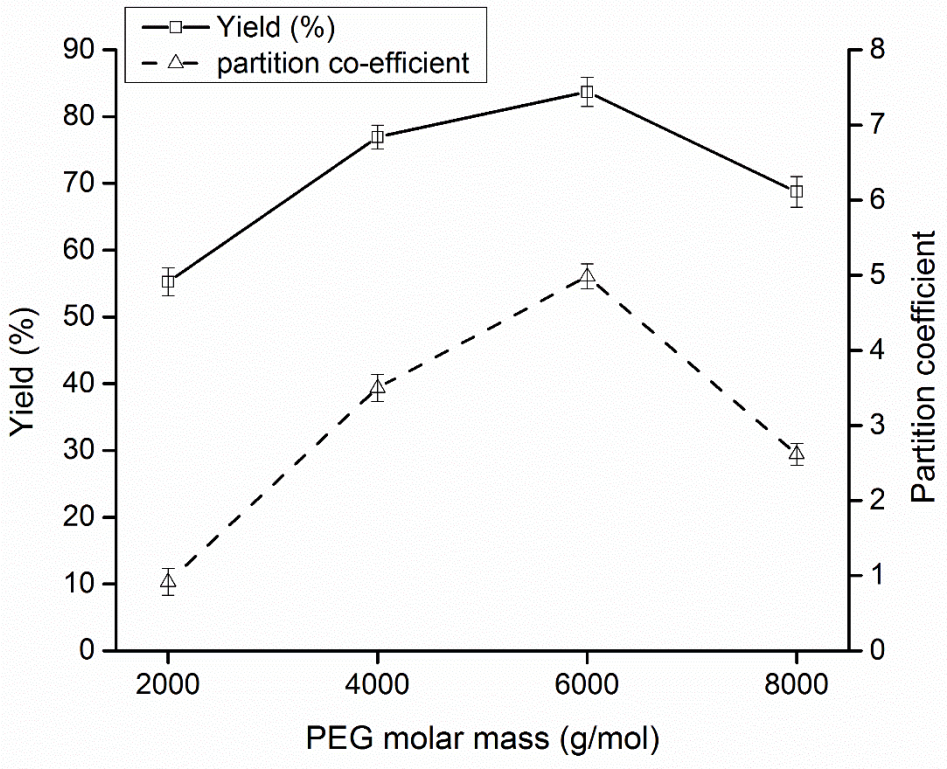


Fig. 4.2 Effect of molecular weight of PEG on partition coefficient (K_L) and Yield % (Y_L) of the lectin in the ATPS of PEG 20% (w/w) and sodium citrate 16% (w/w).

The lower partitioning of Psl was observed at PEG 2000 molar mass may be due to the lower hydrophobicity of the top phase. The protein molecular weight also influences the accommodation of proteins in top phase. The molecular weight of Psl is 50 KDa, which is intermediate when compare to most of the other proteins in general. Accordingly, its steric exclusion rate is also low when compared to very high molecular weight glycoproteins. Interface precipitation of Psl in higher molar mass (PEG 8000) decreases the partition coefficient due to volume exclusion effect. However, lectin from *Cratylia mollis* (Nascimento et al. 2013) (molecular weight (MW = 60 KDa) and *Canavalia ensiformis* (MW=50 KDa) (Soares et al. 2011) were partitioned to the bottom phase of ATPS formed with PEG 8000 – sodium citrate by utilizing the volume exclusion effect of the PEG 8000. In contrary, *Canavalia brasiliensis* (MW= 60 KDa) lectin partitioned to top phase with a recovery yield of 70% in the ATPS formed by very low molar mass of

PEG 600 with citrate salt (Nascimento et al. 2010). The maximum partitioning characteristic was reported for ATPs formed by PEG 6000 for other proteins like serine protease (MW = 65 KDa) from mango waste (Mehrnoush et al. 2013) and lysozyme (MW = 14.4 KDa) partition from chicken egg where in the partitioning is governed by hydrophobicity (Su and Chiang 2006). Molecular weight of a PEG has a significant effect on K_L and Y_L and the higher yield and partition coefficient of Psl was observed in the PEG 6000 - sodium citrate system, PEG 6000 was selected for further studies.

4.1.3 Effect of Concentration of PEG on Psl partitioning

The experiments were conducted to acquire the effect of PEG 6000 concentration with constant sodium citrate concentration of 14% (w/w) on the partition behavior of Psl. The K_L and Y_L were found to increase with increasing PEG 6000 concentration and maximum K_L and Y_L was observed at 18% PEG (Fig. 4.3). The increased hydrophobicity with increasing PEG concentration promotes the PEG-Lectin interaction and resulted in higher partitioning and yield. The presence of hydrophobic binding site in legume lectins was identified by using hydrophobic ligands (Roberts and Goldstein 1983) and reported that the amino acids like, valine, isoleucine and phenylalanine were the major amino acids present in the hydrophobic site (Liener 2012) which has binding affinity towards hydrophobic molecule such as PEG. The hydrophobic interaction was further confirmed by Rathnasamy et al. (2014) with the partitioning studies of lysozyme from quail egg in 25% PEG and 18% ammonium sulfate and reported that the increase in partitioning of enzyme to top phase was mainly because of hydrophobic interaction. The similar effect was further reported for the partitioning studies of *Canavalia grandiflora* lectin by Porto et al. (2011). The lesser K_L and Y_L was observed at 14 and 16 % PEG (Fig. 4.3) due to the insufficient interactive force between the PEG and Psl, including the lower hydrophobicity of the PEG phase at lower PEG concentration.

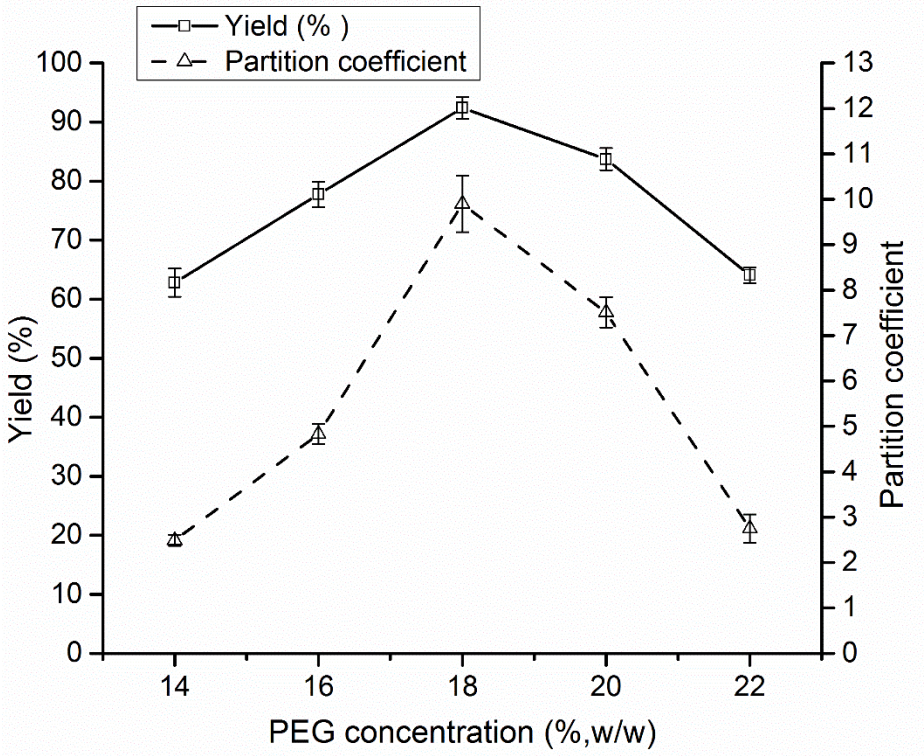


Fig. 4.3 Effect of PEG concentration on partition coefficient (K_L) and Yield (Y_L %) of the lectin at constant sodium citrate concentration of 14% (w/w).

The K_L and Y_L found to be less even at higher concentration of PEG (20 %) (Fig. 4.3) due to intermolecular hydrophobic bonding takes place between the PEG molecules and resulted in the compact structure of PEG which occupies more space. Hence, the free volume available for the Psl was reduced and consequently excluded from the PEG phase and may be forced to the interface or bottom phase. The ATPS with PEG 6000 concentration of 18% was selected for further studies.

4.1.4 Effect of concentration of sodium citrate on partition of Psl

The influence of salt concentration on Psl partitioning was studied with different salt concentration by keeping constant PEG 6000 concentration of 18%. Salts affect the protein partitioning in PEG/salt systems by changing the distribution of charged amino acids by imparting electrostatic effect. A gradual improvement in the partition of Psl was observed for increasing salt concentration from 12% to 16% and the partition of Psl was

found to decrease for the salt concentrations of 18 and 20% (Fig. 4.4). As the citrate concentration increases, the solubility of the Psl decreases in the bottom phase due to the salting out process. Further the hydration effect of ion increases with increasing salt concentration and resulted in the dehydration of proteins with the exposure of hydrophobic sites which in turn favors the interaction of Psl with PEG and facilitating the migration of Psl towards top phase. The increase in sodium citrate concentration also increases the ionic strength in the system which facilitates the movement of negatively charged proteins to top phase by electrostatic repulsive effect (Porto et al. 2011).

However, the higher salt concentration (18 and 20%) fails to partition the Psl to top phase due to the reduction in volume ratio at higher salt concentration (Fig. 4.4). The desired protein gets concentrated in the PEG phase and reaches the maximum possible concentration in the reduced volume. The combined effect due to the non-availability of the free solvent in the PEG phase and the salting out characteristics of the bottom phase promoted the interphase precipitation. The similar trend was observed in Immunoglobulin-G (IgG) partition in PEG / phosphate system and a significant loss of IgG at interface was reported due to increased salting out process (Rosa et al. 2009). Porto et al. (2011) also reported that the *Canavalia grandiflora* lectins partition coefficient increasing to 8.67 in the system composed of 20% PEG 600 and 20% sodium citrate and concluded that the salting out capacity of salt was the main reason for increasing yield of lectin in the top phase. Based on the observations, 16% sodium citrate was selected as an optimum concentration for further experiments. It was noticed from the study that the salting out dependent hydrophobic interaction was playing prominent role in the partitioning of lectin towards top phase. 16% of sodium citrate was considered as an optimum concentration for further studies as it gives maximum K_L and Y_L.

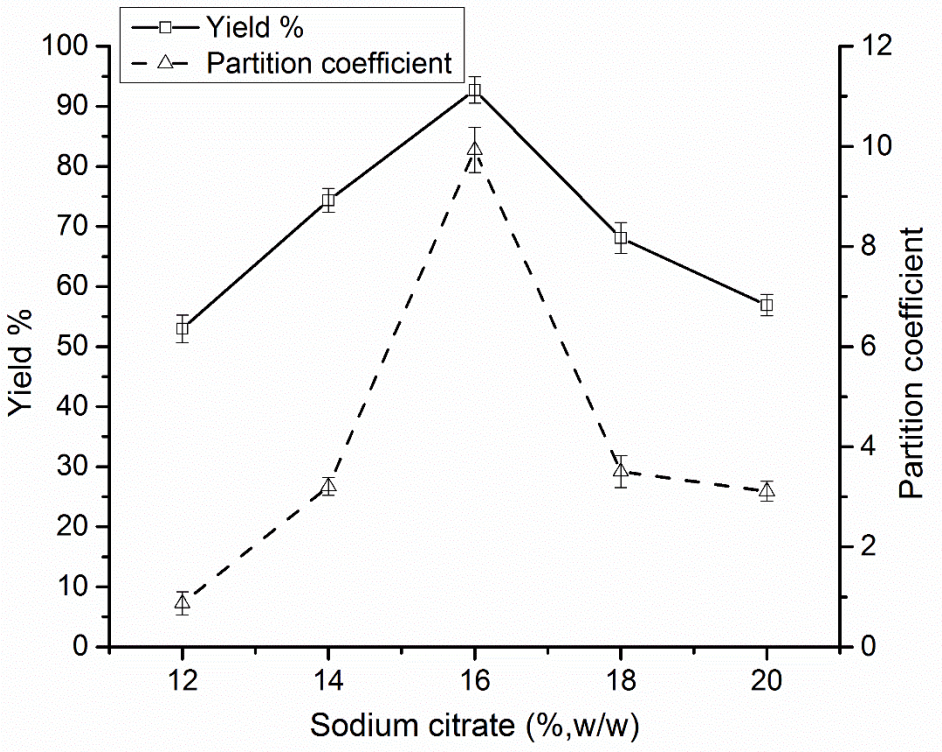


Fig. 4.4 Effect of sodium citrate salt concentration on partition coefficient (K_L) and yield % (Y_L) of the lectin at constant PEG concentration of 18% (w/w).

4.1.5 Effect of Tie line length (TLL) on Psl partitioning

Tie line is the line connecting the equilibrium compositions of the components present in the two phases of ATPS formed by mixing the defined quantity of phase forming components. As the partition characteristics of the solute is governed by the equilibrium composition of the phase forming components (PFC), it is very important to obtain the effect of TLL in order to obtain better partition and yield of target molecule. Partition of protein in the system can be well understood with the equilibrium composition of phases rather than feed composition (Kalaivani and Iyyaswami 2015).

The effect of TLL was evaluated by considering different composition of phase forming components which result the TLL in the range of 30-48%. The phase diagram of the system which indicates the binodal and tie lines with total and equilibrium concentrations of the present system was represented as Fig. 4.5a.

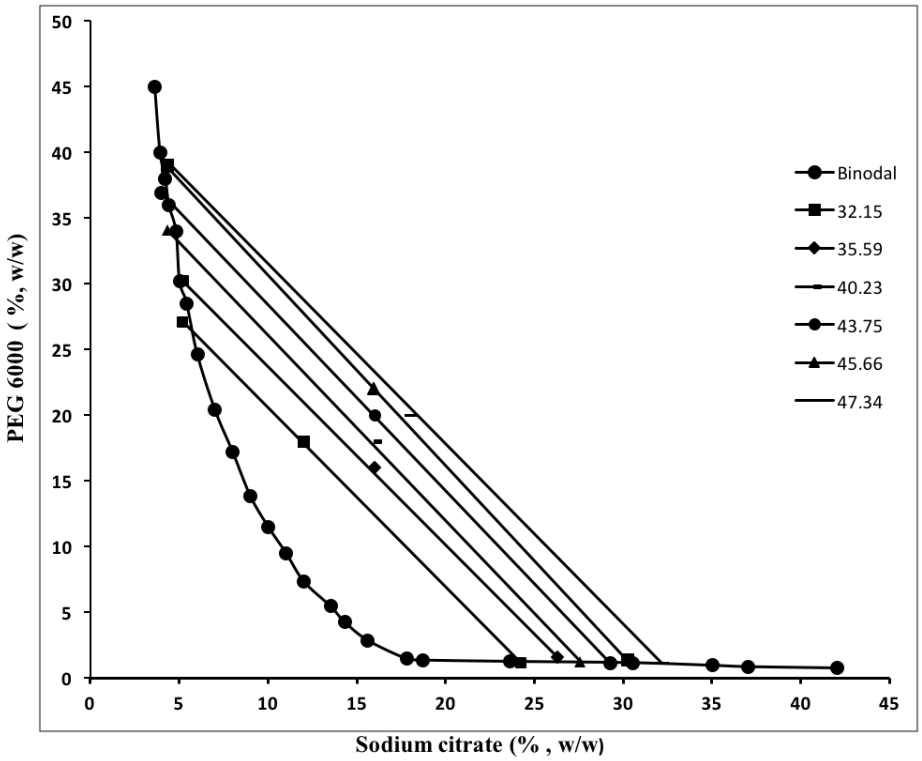


Fig. 4.5a Binodal curve and Tie lines for PEG 6000-sodium citrate system with Psl.

The hydrophobicity and salting out ability of the PEG/salt system can be varied with TLL. The TLL of 40.23% provided maximum K_L and Y_L of Psl (Fig. 4.5b), in which the combination of hydrophobic interaction and salting out effect played a major role for the efficient partitioning of Psl. The optimum equilibrium concentration of PEG in top phase (34.1%) enhances the number of hydrophobic interaction with the exposed hydrophobic sites of Psl and favors its movement to top phase; consequently optimum concentration of salt (27.56%) creates high ionic strength which favors the partition of Psl to top phase due to electrostatic repulsion. However, the increase in TLL to 47.34% increases the concentrations of PEG in top phase and salt in bottom phase to 39.43% and 32.14%, respectively and consequently K_L and Y_L were found to reduce to 3.11 and 56.91%, respectively (Fig. 4.5b) due to the reduction in the water content and free volume of phases to accommodate the Psl. The denaturation and interface precipitation of Psl was observed due to the higher PFC, particularly the salt concentration. Further the increased

density and viscosity of the phases with TLL also makes the system more tedious for the process operation (Narayan et al. 2011). However, the lower TLL fails to provide good resolution in the distribution of Psl due to the lesser salting out and hydrophobic properties, which were not enough to push the Psl to a particular phase.

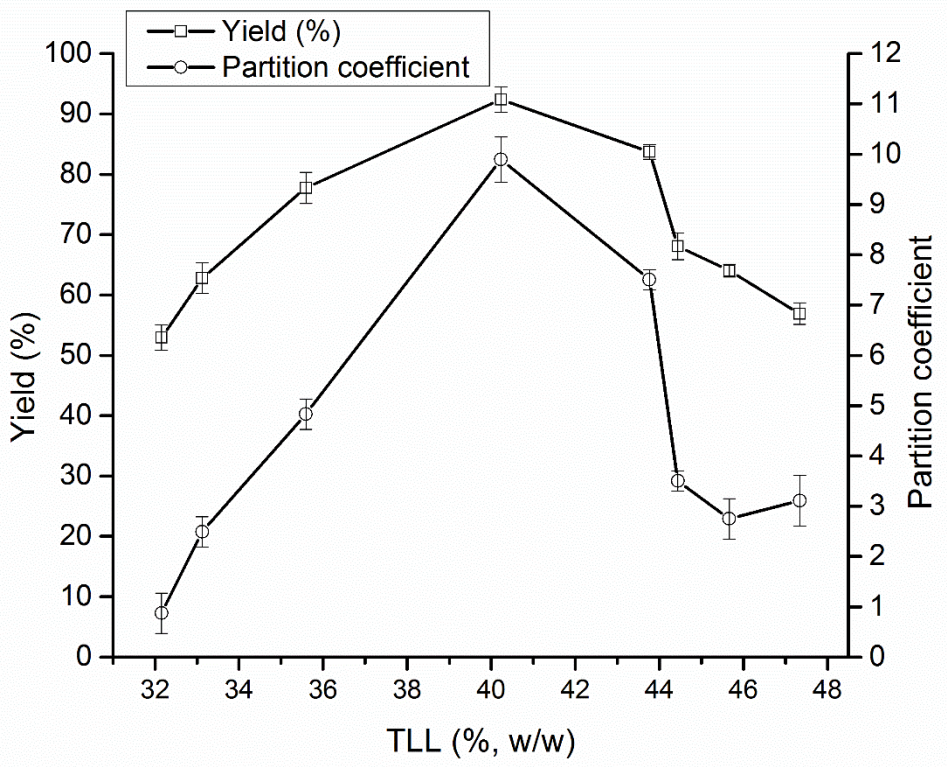


Fig. 4.5b Effect of TLL on Yield and Partition coefficient of Psl in PEG6000-sodium citrate system.

4.1.6 Effect of NaCl on partition of Psl

The addition of electrolyte salts as additives in the ATPS system increases the hydrophobic resolution between the PEG and salt phases due to the change of surface properties of the proteins like exposing the hydrophobic interactive sites (Goja et al. 2013). The larger hydrophobic difference between the phases improves the selective partitioning of desired proteins to any one of the phase. The improvement of partitioning characteristics and subsequent increase in the yield and purity by the addition of

electrolyte salt was reported for several proteins like, antibodies (Azevedo et al. 2007), α-amylase (Zhi et al. 2004) and bovine serum albumin (Gunduz and Korkmaz 2000). Hence, the electrolyte salt, NaCl was considered as an additive because of its ability to enhance the partitioning of protein to hydrophobic phase. Varied range of NaCl from 0 to 5% (Fig. 4.6) was considered to study its influence on partitioning of Psl in the system consist of 18% PEG 6000, 16% sodium citrate with a TLL of 40.23%. Maximum K_L and Y_L was achieved at 1% NaCl and above which there was a slight decrease in yield up to 3% of NaCl. However, a gradual decrease in both yield and partition coefficient was observed beyond the addition of 3% NaCl.

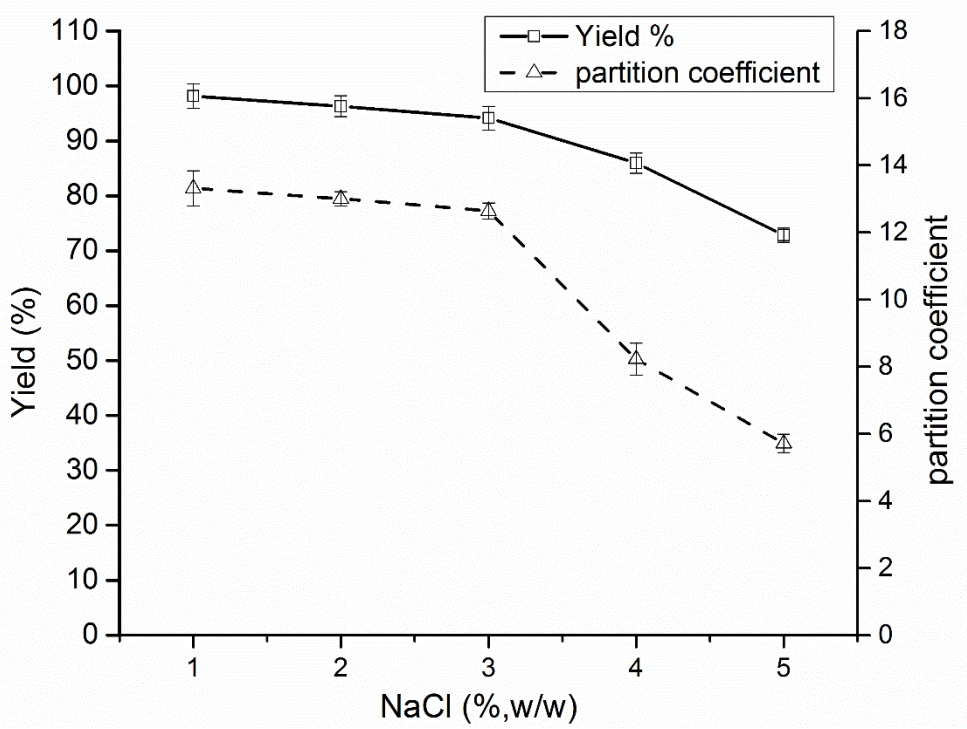


Fig. 4.6 Effect of NaCl as additive on the partitioning of Psl in the ATPS system made-up of 18% PEG 6000, 16% sodium citrate

The accumulation of electrochemical species among the two phases creates difference in electrostatic potential which acts as a driving force for the movement of protein to a phase along with the increase in hydrophobic resolution between the phases. Similar

increase in partitioning coefficient up to the NaCl concentration of 5% was observed in the partitioning of Canavalia brasiliensis lectin to top phase in PEG 600/sodium citrate system (Nascimento et al. 2010). The addition of NaCl has improved the partitioning of Zihua snap-bean lectin toward PEG phase, evidencing the role of NaCl in partitioning of proteins to top phase (Jiang et al. 2017). It was confirmed by the behavior of K_L and Y_L that NaCl of 1% drive the lectin toward top phase and 1% of NaCl was used for further studies.

4.1.7 Effect of pH on Psl partitioning

The change in the pH controls the ionization state of the amino acids present on the surface of a protein. The partition of the desired protein can be fine-tuned by manipulating the pH. In the present study, the effect of pH was studied at different range of pH from 5 to 9 on the partition coefficient and Yield of Psl in a system composed of 18% PEG 6000 and 16% sodium citrate with a TLL of 40.23% and the addition of 1% NaCl. The surface property of the protein and also composition of ions in the phases vary with pH. The amine groups present on the surface of the proteins get protonated and the proteins acquire positive charge at acidic pH. Conversely, protein becomes negatively charged in the alkaline pH by deprotonation of the carboxyl group. The net surface charge of the protein depends on its pI. When the pH changes, generally the protein acquire positive charge below its pI and partitioned in the salt rich bottom phase. But the proteins gain negative charge above its pI and moves to the PEG rich top phase (Benavides and Rito-Palomares 2008). Accordingly, the maximum partition of Psl in the PEG rich phase was noticed at a system pH of 8, which is slightly higher than the pI of the Psl (6.8 to 7.2) (Hoedemaeker et al. 1994), since the Psl acquires negative charge and thus partitions to positively charged PEG rich phase with maximum K_L (Fig. 4.7). In addition to that the increasing pH also increases the trivalent citrate concentration in the bottom phase and increases the salting out process there by Psl expelled to top phase. Similar observation was reported for many proteins like lipase (pI of 6.3) gave maximum partition in pH 7-8 (Ooi et al. 2009) and xylanase (pI of about 4.5) gave more partition coefficient with increasing pH to 6 (Rahimpour et al. 2016).

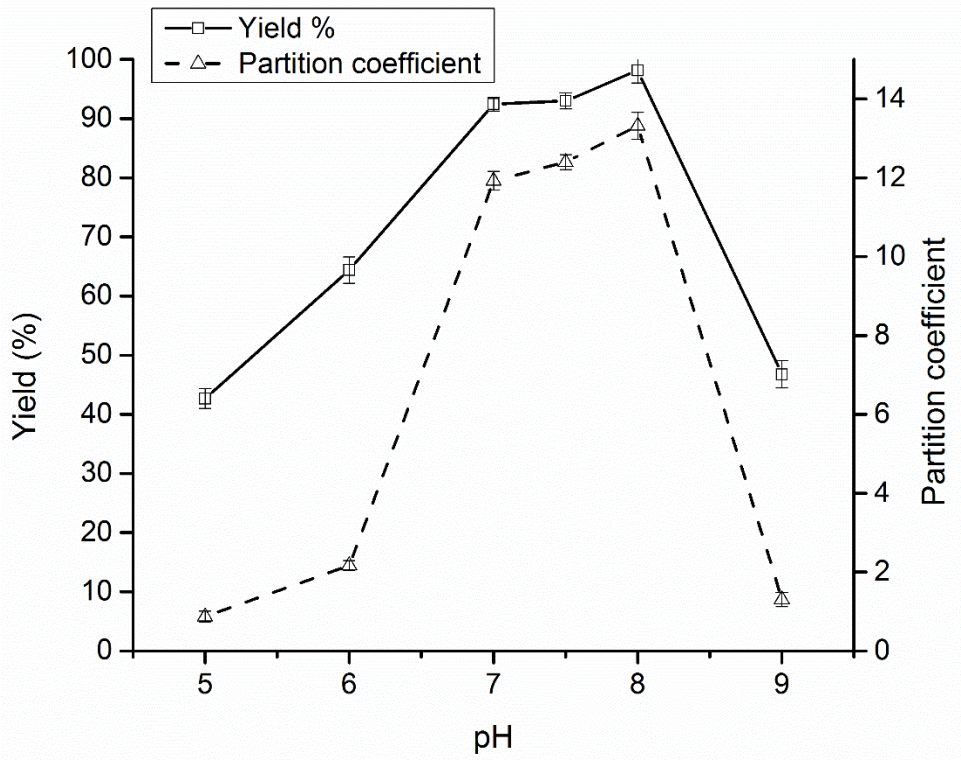


Fig. 4.7 Effect of pH on the partitioning of Psl in the ATPS system made-up of 18% PEG 6000 and 16% sodium citrate.

However, lower partition was observed in acidic pH because of the dissociation of α and β subunits of the Psl and destabilization of its structure. Leaching out of divalent metal ions also takes place at acidic pH where metal ions are essential for the stability of the lectin. pH less than 6 and more than 8 gave lesser partition coefficient and yield which shows that lectin prefers a slightly basic pH than acidic and or too basic. Hence, pH 8 was selected as an optimum pH for further studies.

4.1.8 Effect of Volume ratio on Psl partition

The accommodation of target biomolecule in particular phase depends on the free volume of solvent available in that particular phase. It is possible to manipulate the extraction performance of the system by altering the volume ratios of individual phase by keeping the total volume constant where the equilibrium composition of both the phases remain constant with respect to their tie line. The extractive performance of the system based on

phase volume ratio was evaluated by considering different compositions of phase components on tie line (40.23%) and the volume ratios varied from 0.34 to 2.88. The effect of V_r on K_L and Y_L were shown in Fig. 4.8. Partition coefficient of a Psl remains almost constant for different volume ratios along the tie line and there was a slight decrease in yield at the volume ratio of 0.34 and 0.59 because of decrease in volume of top phase which may lead to precipitation of lectin (Ooi et al. 2009). K_L and Y_L tend to increase as volume ratio increases, and the maximum K_L of 14.5 ± 0.65 and Y_L of 98.66 ± 0.82% was observed at the volume ratio of 1.32 after this there was a very less decrease in the Y_L and K_L and at 2.88 Vr there was a decrease in both the parameters, this may be due to the dilution of the protein by increased top phase.

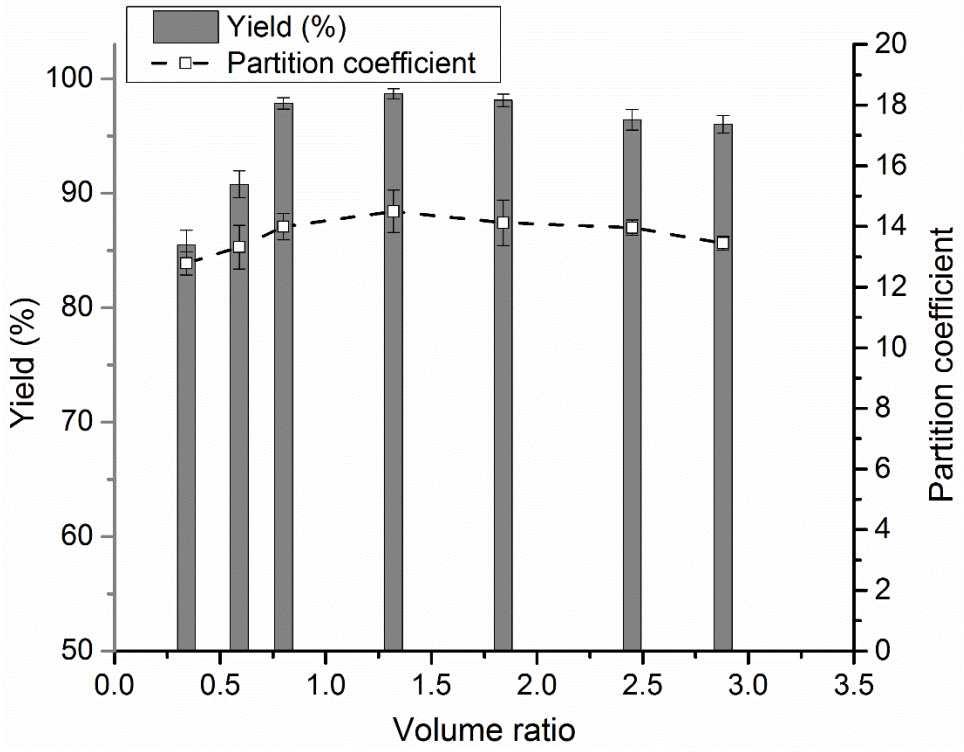


Fig. 4.8 Effect of volume ratio on the partitioning and Yield of Psl in the ATPS system along a same tie line (40.23).

4.1.9 Qualitative Analysis of Extracted Lectin

The Psl subjected to ATPS was analyzed in the RP-HPLC to infer the effect of phase forming component and operating condition on the stability of Psl by comparing it with the commercially available Psl. Peak elution was observed at 27.41 (Psl1) and 27.72 (Psl2) minutes at appropriate chromatographic conditions for pure pea lectin (Fig. 4.9a). At the same chromatographic conditions peak elution for extracted lectin (Fig. 4.9b) was found at 27.44 and 27.76 minutes for Psl 1 and Psl 2, respectively. A slight variation in the peak of extracted Psl was observed due to the presence of few ions in the sample, but the peak structure is similar when compared to pure Psl. Maximum intensity was observed at 214 nm.

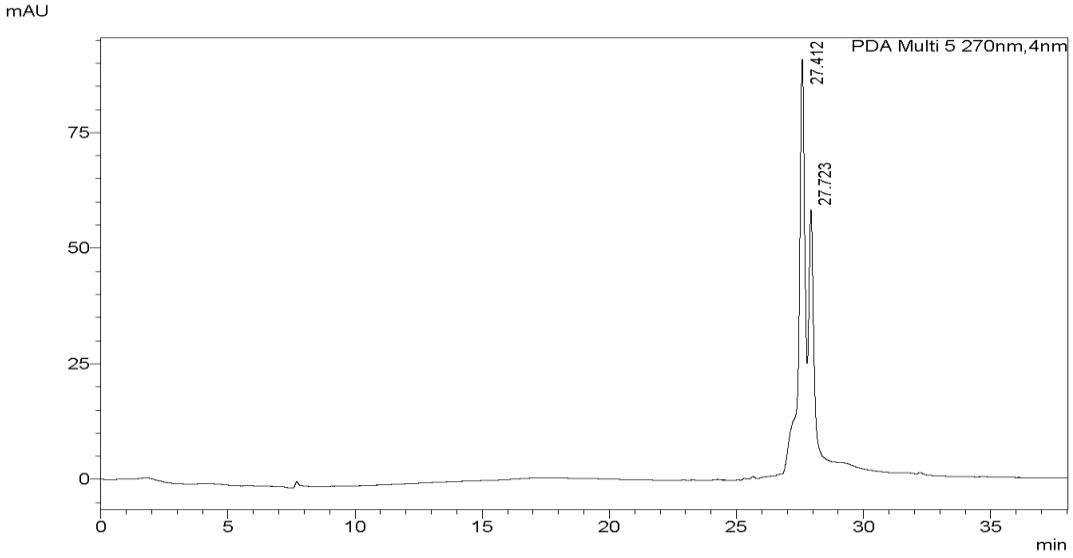


Fig. 4.9a Elution pattern of pure Psl at the flow rate of 0.5min/ml in binary gradient mobile phase (acetonitrile and trifluoroacetic acid) and in column: RP-C18.

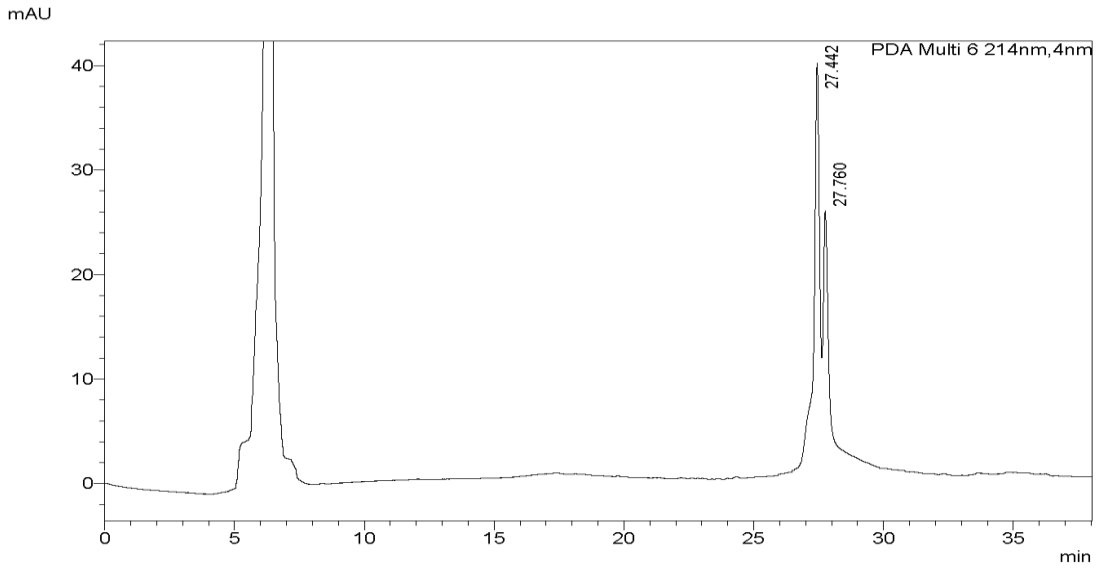


Fig. 4.9b Elution pattern of aqueous two phase extracted Psl at the flow rate of 0.5min/ml in binary gradient mobile phase (acetonitrile and trifluoroacetic acid) and in column: RP-C18.

4.2 SELECTIVE EXTRACTION OF Psl FROM CRUDE PEA SEED EXTRACT.

The partitioning characteristic of the Pure Psl in the PEG 6000- sodium citrate system from the PEG/sodium citrate reported a maximum partition coefficient and yield of 14.5 and 98.66%, respectively in the PEG phase. However, the partition behavior of protein may alter with the influence of other proteins and non proteinaceous substances in the crude extract. Hence, the present study was organized to analyze the effect of process parameters like, molecular weight of PEG, type of salt, the concentration of PEG and salt, the effect of tie line length, and NaCl, pH, crude load and volume ratio on the selective partition of Psl. The experiments were conducted by changing one-factor at a time to study and analyze the individual effect of variables on the partitioning. The performance of the various process variables in the selective extraction of Psl was assessed by hemagglutination activity yield (Y_{HA}), purification factor (PF), specific hemagglutination activity partition coefficient (K_{SHA}) and total protein partition coefficient (K_p).

4.2.1 Effect of type of citrate salts.

Phase separation in PEG/salt varies with the type of salt which affects the water structure and hydrophobic interaction differently (Settu et al. 2015). To determine the most applicable type of ATPS for selective extraction of the lectin from the crude extract of *P. sativum* seed, three citrate salts (sodium citrate (SC), potassium citrate (PC), and ammonium citrate (AC)) were considered and studied. The performance of the various salts was assessed by hemagglutination activity yield (Y_{HA}), purification factor (PF) (Fig. 4.10a) and specific hemagglutination activity partition coefficient (K_{SHA}) (Fig. 4.10b). The Y_{HA} and PF varied respectively in the range of 14.19 ± 0.13% to 56.87 ± 14 0.62% and 0.48 ± 0.02 to 1.55 ± 0.08 for SC and 6.8 ± 0.09% to 52.50 ± 1.25% and 0.27 ± 0.02 to 1.37 ± 0.07 for PC. The SC and PC systems exhibited relatively good performance in terms of Y_{HA} and PF. However, more salt concentration (18%) of PC was required to give nearly same Y_{HA} and PF than SC (16%). In contrast to these results, AC yields comparatively lesser Y_{HA} (46.23 ± 18 1.91%) and PF (1.12 ± 0.07) even at a higher concentration of AC (20%). The concentration of salt required to form the two phases depends on its salting out ability, and thus SC and PC require less concentration while

AC requires more concentration (Fig. 4.10a & 4.10b). The salting out effect of citrate salts used in the study can be evaluated by the concept of EEV (effective excluded volume), Gibbs free energy of hydration $\Delta G_{hydration}$, and the ionic radius of the cation (Pimentel et al. 2017). Na^+ being highly kosmotropic has more negative $\Delta G_{hydration}$, and has more EEV and less cationic radius in comparison with potassium and ammonium ions. Thus sodium citrate salt exhibited more salting-out ability (also maximum Y_{HA}, PF, and K_{SHA}) when compared to the other two salts. The concept and detail are explained in section 4.1.1.

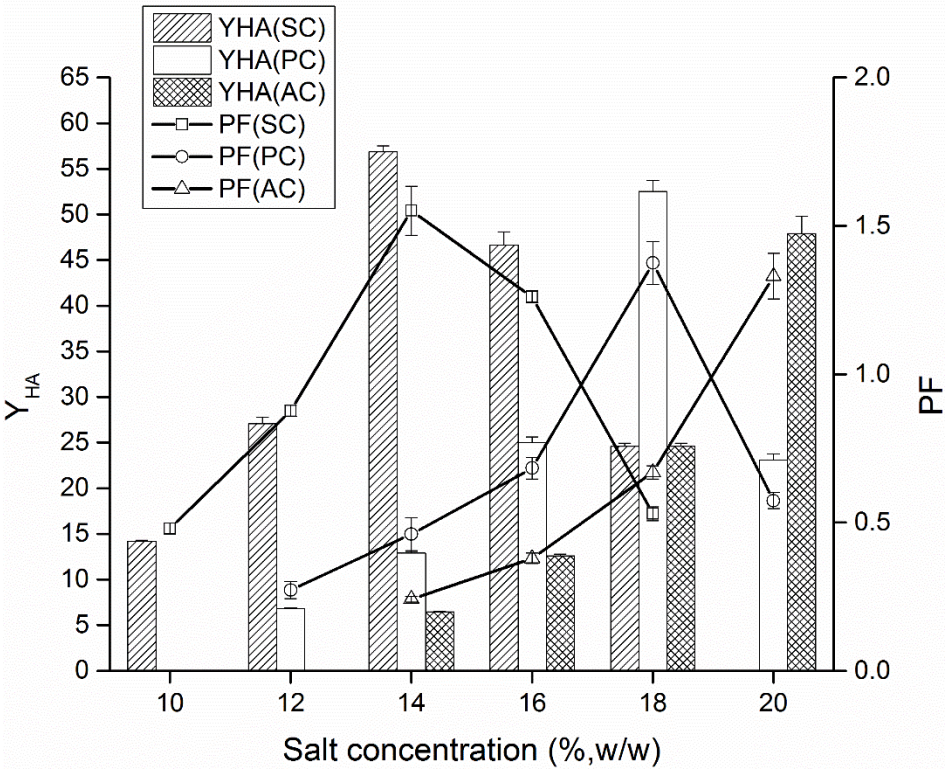


Fig. 4.10a Effect of type of salts at different concentration (SC: sodium citrate; PC: potassium citrate; AC: ammonium citrate) with 20% (w/w) PEG 4000 (g/mol) on hemagglutination activity yield (Y_{HA}) and Purification factor (PF) of Psl.

The specific activity of the lectin was maximum in the top phase for SC, PC, and AC which was evidenced by K_{SHA} of 10.37 ±0.42, 9.22 ± 0.32 and 8.42 ± 0.4 respectively. The differential partitioning of Psl and other proteins was assessed by K_{SHA} and K_P. The

high K_{SHA} with low K_P indicates that the target protein was successfully partitioned to top phase (Khayati et al. 2015) and maximum K_{SHA}, PF and yield was given by sodium citrate.

The native solution pH of the system was around 8.1-8.2 for the ATPS formed by all the three salts, which was favorable for Psl to preferentially partition to the top phase as it has an isoelectric point (pI) of 6.8-7.2 (Hoedemaeker et al. 1994). 'Salting out' property of salts excluded protein from the bottom phase to the top phase by limiting its solubility.

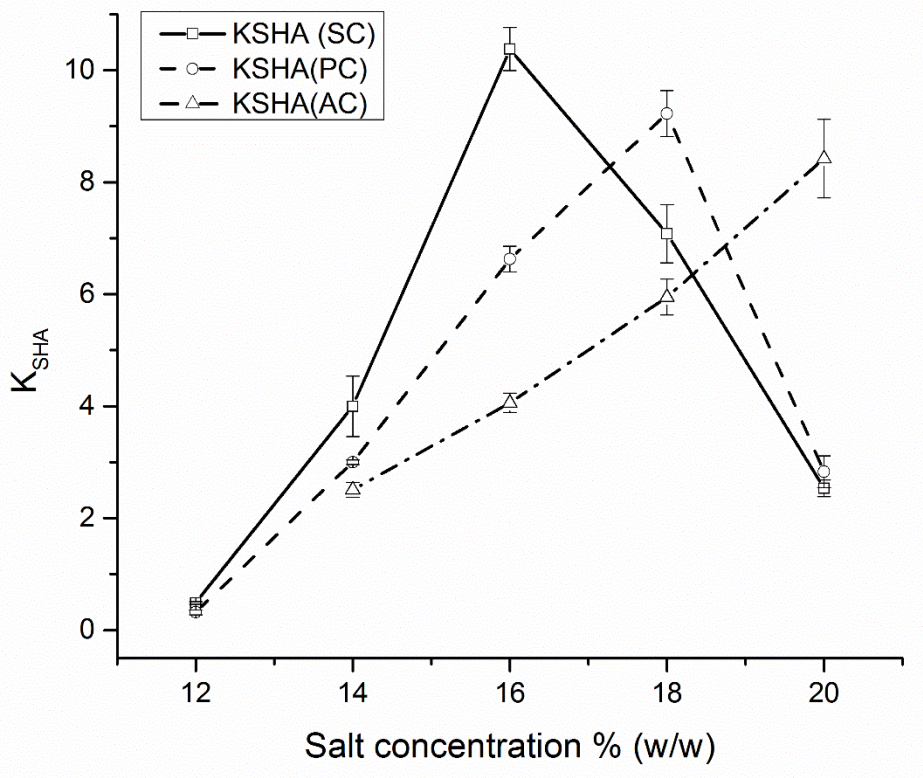


Fig. 4.10b Effect of type of salts at different concentration (SC: sodium citrate; PC: potassium citrate; AC: ammonium citrate) with 20% (w/w) PEG 4000 (g/mol) on hemagglutination specific activity partition coefficient (K_{SHA}) of Psl.

The distribution of ions in the system alters with the different type of salts which affects the partition of lectin in the system (Andrews et al. 2005). Many complex sources such as bovine pancreatic homogenate (Perez et al. 2015), fermentation broth of *Aspergillus*

tamarii URM4634 (da Silva et al. 2018) and B. cereus (Ng et al. 2011) were explored to extract individual biomolecules like trypsin, protease, cyclodextrin glycosyltransferase respectively by employing PEG/sodium citrate combination as a phase forming component. In the current study, sodium citrate salt outperforms than the potassium and ammonium salts with better purification factor and hemagglutination activity recovery. Hence the PEG/sodium citrate ATPS was selected for further studies as it gave Psl Y_{HA}, PF, and K_{SHA} when compare to other two citrate salts.

4.2.2 Effect of molar mass of PEG

Hydrophobicity is one among the main parameter which affects the extent of partitioning of proteins in PEG/Salt systems. The hydrophobicity of the system varies mainly with the molar mass of PEG which affects the PEG-protein interaction. As the molar mass of PEG varies, there will be a variation in the count of the ethylene oxide group per PEG, which is the main determinant of hydrophobicity (Andrews and Asenjo 2010). Multiple fold increase in the specific activity and purification factor for increased PEG molar mass from 2000 to 6000 g/mol (Fig. 4.11a and b) was observed due to the significant increase in the hydrophobicity with increasing PEG molar mass, which helped to selectively partition the lectin towards the PEG phase. ATPS aims to achieve not only a good partitioning coefficient for a protein but also the enrichment of the desired protein towards a particular phase by discriminating contaminant proteins to another phase. The specific hemagglutination activity partition coefficient (K_{SHA}) increases with the increase in molar mass up to 6000. The specific activity of the crude was 6.44 ± 0.03 U/mg, and the maximum specific activity achieved in the top phase at PEG 6000 was 22.53 ± 0.65 U/mg. The K_{SHA} of 0.42 ± 0.05, 4.80 ± 0.32, 9.60 ± 0.26, 3.53 ± 0.35 obtained in the current study for PEG 2000, 4000, 6000 and 8000, respectively and corresponding decrease in the K_P for PEG 2000 to 8000 proves that the Psl was selectively partitioned towards the top phase with the increase in molar mass.

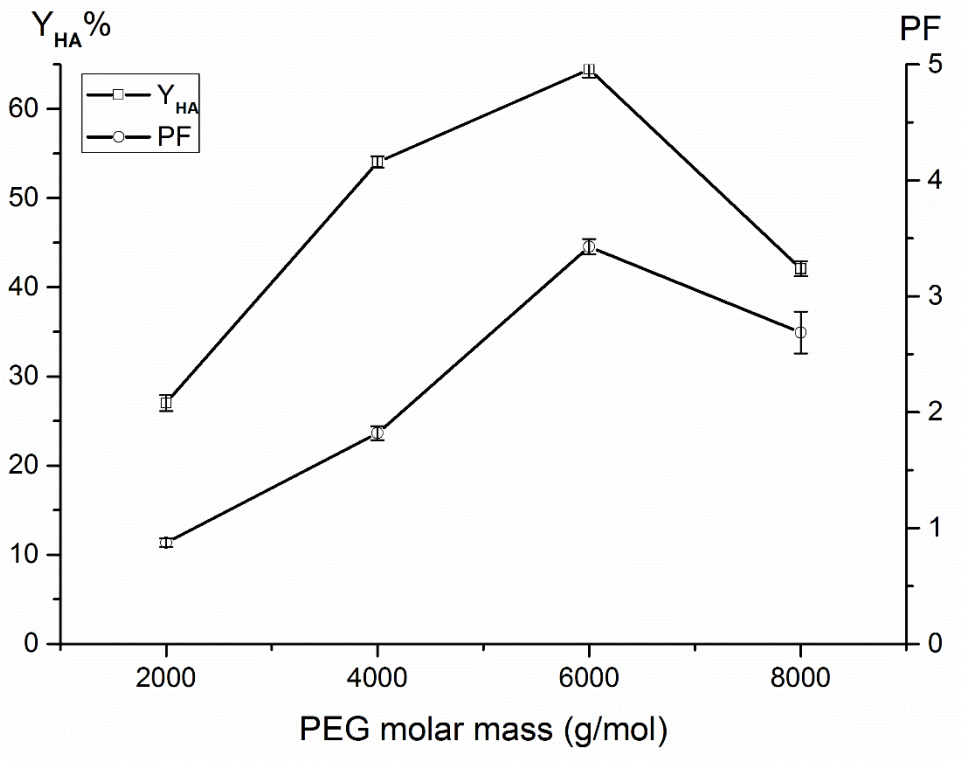


Fig. 4.11a Effect of molar mass of PEG (g/mol) on hemagglutination activity yield % (Y_{HA}) and purification factor (PF) in the ATPS of PEG 20% (w/w) and sodium citrate 16% (w/w).

However, Y_{HA}, PF, and K_{SHA} increased till PEG 6000 and decreased at PEG 8000 due to the unavailability of free water solvent. Maximum specific activity (22.53 ± 0.65 U/mg) was achieved with low total protein content in top phase (0.62 mg/mL), which is the desirable condition for the selective extraction, for PEG 6000-SC ATPS. The simultaneous increase in enzyme activity with molar mass was also reported by Mohammadi and his coworkers where recombinant phenylalanine dehydrogenase was produced using E.Coli and the enzyme concentrated towards the PEG phase. It was observed that the enzyme partition coefficient and enzyme recovery increased with increase in molar mass from 2000 to 6000 and then gradually decreases above 6000 (Mohammadi et al. 2008). The results observed by Ooi et al. was also in agreement with the current trend where the selectivity of *Burkholderia pseudomallei* lipase increased

with increasing PEG molar mass from 3000 to 6000 (Ooi et al. 2009). The enzyme recombinant neutral protease (Loc et al. 2013) has also been successfully partitioned to the PEG-rich phase at PEG 6000. The hydrophobic interaction of Psl and PEG play the main role and thus the specific activity increases with the increase in molar mass. However, the K_{SHA} decreases at PEG 8000 with the loss of protein at the interphase; this behavior may be due to the volume exclusion effect exerted by the PEG 8000.

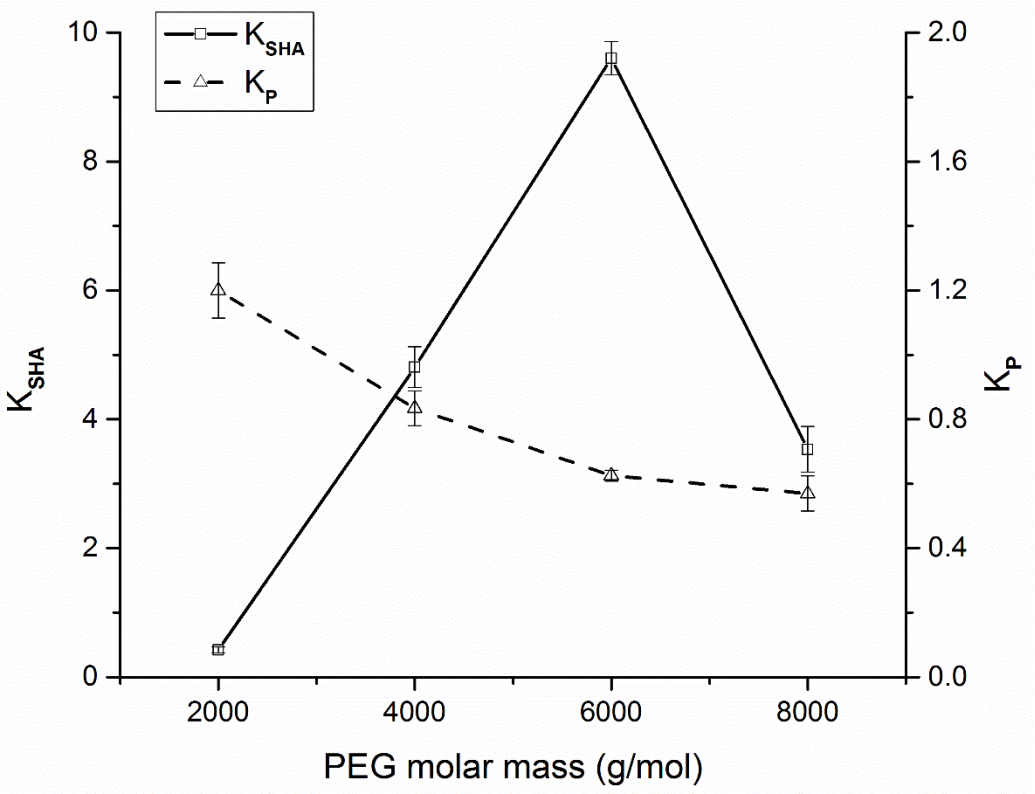


Fig. 4.11b Effect of PEG molar mass on Hemagglutination specific activity partition coefficient (K_{SHA}) and total protein partition coefficient (K_P) in the ATPS of PEG 20% (w/w) and sodium citrate 16% (w/w).

Polymers with high molar mass have a greater difference in density and interfacial tension because of which they tend to induce phase separation with a lower amount of phase components. Accordingly, in the present study, PEG 2000 and 4000 require more concentration for effective phase separation when compared to PEG 6000. There was a

poor resolution in the partitioning of lectin and contaminating proteins towards opposite phases in low molar mass PEG such as 2000 which leads to lowest Y_{HA} and PF of 27.02 ± 0.92% and 0.87 ± 0.03 respectively. Hence, PEG 6000 was selected as an optimal molar mass for further studies as it was able to give good selectivity with maximum Y_{HA} and PF of 64.43 ± 0.94% and 3.42 ± 0.06 respectively. Hence, PEG 6000 was selected as an optimal molar mass for further studies as it was able to give good selectivity with maximum Y_{HA} and PF of 64.43 ± 0.94% and 3.42 ± 0.06 respectively.

4.2.3 Effect of PEG Concentration

The effect of PEG concentration on selective partitioning of the target protein was studied by varying PEG 6000 concentration from 14% to 22% with a constant SC concentration of 16% (Fig. 4.12). The partition coefficient for total protein (K) was < 1 at all the PEG concentration and the Y_{HA} and PF were maximum in the top phase, which indicates that most of the contaminant proteins accumulated in the bottom phase and the Psl were preferentially concentrated in the top phase. However, Nascimento et al observed the partitioning of *Cratylia mollis* lectin into the bottom phase with high activity partition coefficient and low total protein partition coefficient in the PEG/citrate system (Soares et al. 2011). Increase in the PEG concentration from 14% to 18% leads to a maximum Y_{HA} and PF of 83.75 ± 1.25 % and 6.3 ± 0.20 respectively (Fig. 4.12). The total protein content increased linearly in the top phase with the PEG concentration. However maximum PF was achieved at 18% and gradually decreased there onwards. The behavior observed may be due to the migration of contaminant proteins along with the Psl. This indicates that there was no efficient separation of the lectin from other proteins at higher concentrations of PEG, which was confirmed by the increasing profile of K_P (K_P>1). It was reported in the literature that all legume lectins are characterized by a hydrophobic cavity other than the carbohydrate binding domain (Hamelryck et al. 1999). 18% PEG at the molar mass of 6000 creates a favorable hydrophobic environment in the top phase which facilitates the interaction of PEG with the hydrophobic cavity of Psl. This hydrophobic interaction of PEG - Psl was mainly responsible for the enhanced migration of Psl towards the PEG phase. However, the increased hydrophobicity due to

the increase of PEG concentration over and above 18% attracts the other proteins and the selectivity of the separation was lost. A similar trend was observed by Bim and Franco (Bim and Franco 2000), where alkaline xylanase extracted in PEG 6000/dipotassium phosphate system from *Bacillus pumilus* showed increased activity partition coefficient (of 46.9) with decreased K_P (0.1) when the PEG concentration was increased from 16 to 22% and decreases thereafter. The decrease in purification factor and Y_{HA} above 18% PEG 6000 was mainly due to the attraction of other contaminant proteins towards the top phase as evidenced by an increased total protein partition coefficient.

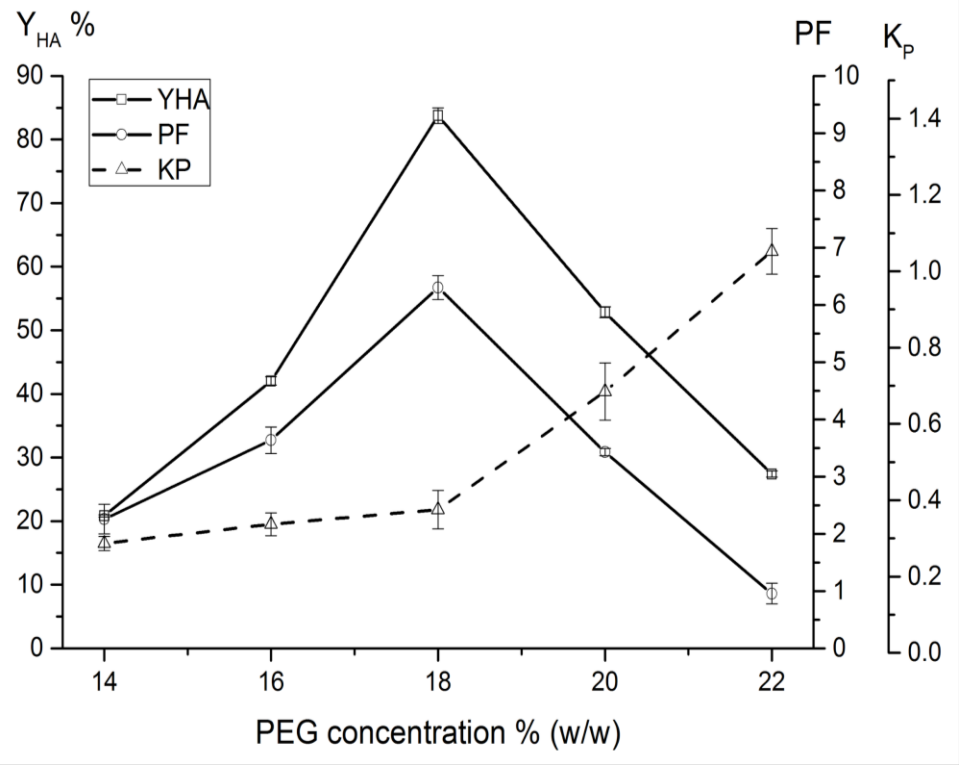


Fig. 4.12 Effect of PEG 6000 (g/mol) concentration on Hemagglutination activity yield (Y_{HA}), purification factor (PF) and total protein partition coefficient (K_P) at constant sodium citrate concentration of 16% (w/w).

At very high concentration (22%), the volume exclusion effect of PEG molecule pushes the Psl to the bottom phase and leads to low Y_{HA} and PF. A decrease in the bromelain partition coefficient was reported with increasing PEG concentration due to volume

exclusion effect of PEG (Ketnawa et al. 2011). The high extraction rate with better purification factor was achieved at 18% PEG and this concentration of PEG was used as an optimal concentration for further studies.

4.2.4 Effect of Sodium citrate concentration

The influence of SC concentration on the partitioning of Psl from its crude extract was studied in the ATPS formed with 18% PEG 6000 at different concentration of SC (Fig. 4.13). The solubility of the protein diminishes in the salt phase with an increase in the salt concentration thereby expelling the proteins to the top phase, which was observed by the increasing K_P. However, a reasonable degree of separation between Psl and other proteins was observed with a gradual increase in activity recovery and PF, leading to higher purification factor till 16% of SC due to the selectivity of the phases. This was further confirmed by K_P <1 and K_{SHA} >1.

The movement of protein towards the top phase was mainly due to the interaction of the salt ions with the hydration layer of the protein which forms a double electric layer; the layer makes protein to dehydrate with the exposure of hydrophobic sites and allows its interaction with other hydrophobic molecules (Bonomo et al. 2006). Increased sodium citrate concentration in the bottom phase strengthens the electrostatic repulsion between the anion and the negatively charged Psl which facilitates the movement of Psl to the PEG phase (Cavalcanti et al. 2006). A similar pattern was observed in the extraction of luciferase from fireflies where the enzyme activity coefficient increased several times more than the total protein concentration with increase in salt concentration from 12 - 16% (Priyanka et al. 2013). The trend of increasing activity and PF with increasing salt concentration was also observed in aqueous two-phase extraction of bromelain from *Ananas comosus* pulp (Wu et al. 2017). It was observed in the present study that an increase in salt concentration above 16% increases the partitioning of undesired protein towards top phase leading to a lesser degree of separation between contaminating proteins and the target protein where K_P>1 and PF reduced from 6.3 ± 0.2 to 2.68 ± 0.18.

High lectin activity with less K_P was achieved at 16% SC which was used as an optimum salt concentration for further studies.

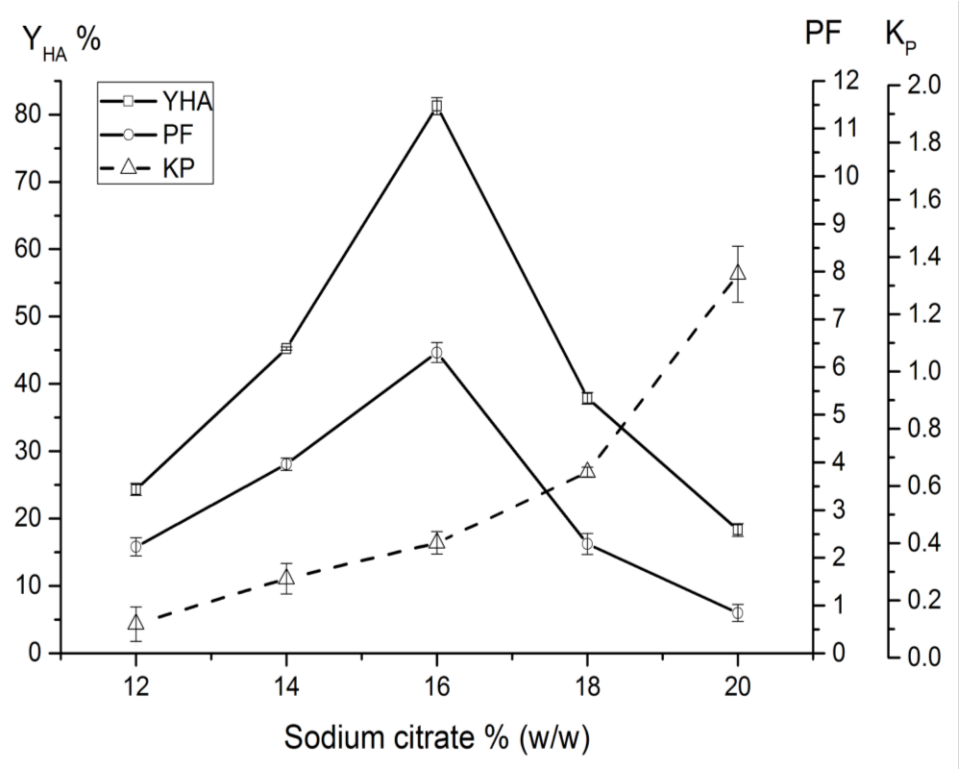


Fig. 4.13 Effect of sodium citrate salt on Y_{HA} (Hemagglutination activity yield), PF (purification factor) and K_P (total protein partition coefficient) at constant PEG 6000 concentration of 18% (w/w).

4.2.5 Effect of tie line length

Experiments were conducted at different TLL (30 to 50 %) to study the net effect of the equilibrium concentration of phase forming components on selective extraction of Psl in PEG 6000 – sodium citrate system. The equilibrium phase compositions at six different tie line and the corresponding extraction performance parameters like Y_{HA}, PF, K_{SHA}, and K_P are presented in Table 4.1. Manipulating the TLL is one among the way to alter the magnitude of phase hydrophobicity. The favorable magnitude of hydrophobic force exerted by PEG and salting out ability exerted by sodium citrate for the selective partitioning was achieved at the TLL of 41.01%. It was noticed that both K_P and K_{SHA}

were gradually increasing till the TLL of 41.01%. The equilibrium composition of 34.1% PEG in the top phase and 28.5% of SC in the bottom phase gave maximum K_{SHA} of 19.96 at low K_P of 0.40 mg/mL. The specific activity was raised to 41±1.10U/mg when compared to crude extract (6.44 ± 0.03 U/mg) at this TLL.

Table 4.1 Equilibrium concentration of phase components on the extraction parameters in the ATPS of 18% PEG 6000 and 16% sodium citrate.

Feed (%, w/w)		Top phase (%, w/w)		Bottom phase (%, w/w)		TLL %	Y_{HA}	PF	K_P	K_{SHA}
PEG 6000	TSC	PEG 6000	TSC	PEG 6000	TSC					
18	12	27.12	1.20	5.20	24.23	33.3	20.8 ± 0.2	2.26±0.3	0.25 ± 0.03	4.00±0.50
16	16	30.32	1.50	5.14	27.50	36.5	42.04±0.2	3.63±0.1	0.32± 0.01	12.29±0.5
18	16	34.1	1.31	3.86	28.50	41.0	81.25±1.2	6.30±0.2	0.40 ± 0.02	19.96±1.0
20	16	37.12	1.18	3.86	30.32	44.6	64.44±0.6	3.43± 0.1	0.62 ± 0.02	9.60±0.30
22	16	39.07	1.28	4.12	30.95	46.3	27.39±0.5	0.95± 0.1	1.16 ± 0.06	1.74±0.22
18	20	40.03	0.80	4.18	32.86	48.6	18.31±0.2	0.84± 0.02	1.34 ± 0.05	0.74±0.05

Note: TSC = Trisodium citrate

The balanced environment at TLL of 5 41.01% facilitates an enhanced interaction of PEG –lectin and leads to a maximum Y_{HA} and PF. At the lower TLL (33%), the lesser equilibrium concentration of phase forming components (PFC) in either of the phases (27.12% of PEG in the top phase and 24.23% of SC in the bottom phase) were not enough to facilitate the movement of more Psl towards top phase, which was witnessed with low K_P of 0.25 ± 0.02 mg/mL along with low Y_{HA} and PF. There was a decrease in both the extraction parameters (Y_{HA} and PF) as the TLL exceeded 40.01%, where K_P increased and K_{SHA} decreased. Higher equilibrium composition of 40.03% PEG in top phase and 32.86% of SC in bottom phase limits the Psl solubility in both the phases and

slight precipitation was observed at the interface at extreme TLL of 48.59%. Further, increased concentration of salt in the bottom phase increases the concentration of Psl in the top phase due to volume reduction of the top phase and more of the contaminant proteins partitioned into the comparatively larger volume of bottom phase. TLL of 41.01% was considered as optimum as it gave better performance in terms of Y_{HA}, PF, and K_{SHA}. A similar phenomenon was observed by Bolar and his coworkers (Bolar et al. 2013), where enzyme partition coefficient and activity yield of glutaminase increases with increase in TLL but reduced at very high and low TLL.

4.2.6 Effect of NaCl

The addition of neutral salts as an additive into the ATPS influences the preferential responses of individual proteins present in the extract. The effect of NaCl concentration on the partitioning characteristic of Psl was studied by adding 1 to 5% NaCl in the ATPS consisting of 18% PEG 6000/16% sodium citrate at 41.01% TLL (Fig. 4.14). It was observed that the addition of NaCl enhances the partition of Psl towards the PEG-rich phase. The prominent effect on Y_{HA} and PF was observed at 1 to 2% of NaCl addition, where both the factors increased rapidly from 79.41 ± 0.84% to 119.5 ± 1.8% and 7.4 ± 0.7 to 14.5 ± 0.8, respectively due to the increased relative hydrophobicity and electrostatic property differences between the phases (da Silva et al. 2014). The same trend was observed by Ng et al. Where the addition of NaCl at 4% to PEG/sodium citrate ATPS increased the CGTase (Cyclodextrin Glycosyltransferase) partition towards PEG-rich phase and exhibited highest purification factor of 16.3 at 4% NaCl (Ng et al. 2011). Similarly, the increasing lipase activity partition coefficient was observed with the addition of 1% NaCl in the PEG/potassium phosphate system (Mohammadi et al. 2008). The storage proteins present along with the Psl can bind to Psl through ionic interactions; however, they may be unbound at higher ionic strength (Wenzel and Rüdiger 1995). Hence, the sweeping increase in Y_{HA} and PF was observed initially with increasing NaCl concentration. The K_P was found to increase with increasing concentration of NaCl which was prominent beyond the NaCl concentration of 2%. The selectivity for the Psl partitioning was lost at the higher NaCl concentration since other proteins are also

attracted towards the top phase which was confirmed by K_P>1. The addition of more NaCl increases the ionic strength of the phases and attracts low molecular weight proteins and small hydrophobic molecules to the PEG phase which in turn reduces the activity and purification of a specific protein (Benavides and Rito-Palomares 2008).

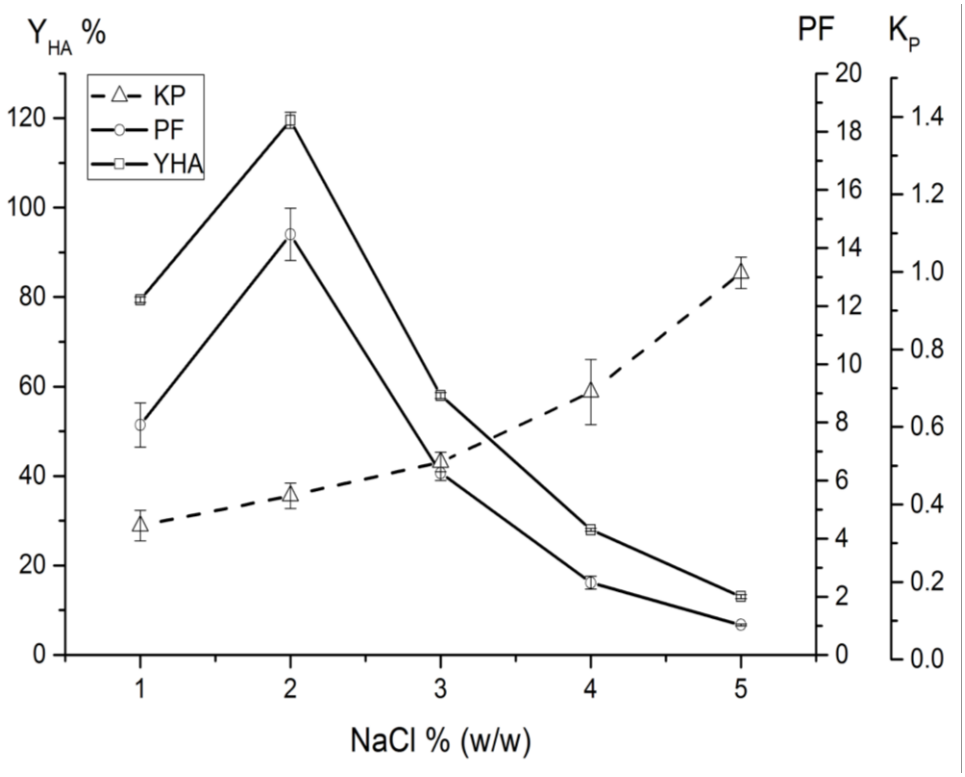


Fig. 4.14 Effect of NaCl as additive on the partitioning of Psl in the ATPS system made-up of 18% PEG 6000, 16% sodium citrate at TLL of 41.01%.

Moreover, the hemagglutination activity was found to reduce drastically at higher NaCl concentration even with the increased K_P due to the dissociation of Psl subunits. High concentration of NaCl above 3% leads to precipitation of Psl along with the other proteins (visually observed). The maximum Y_{HA} and PF with lower K_P was observed at 2% NaCl and the subsequent experiments were conducted with the addition of 2% NaCl

4.2.7 Effect of pH

The pH of the system is responsible to preserve the native structure of a protein which in turn enhances the activity. The impact of pH on Y_{HA}, PF, and K_P in a system composed of 18% PEG 6000, 16% sodium citrate at 41.01% TLL with 2% NaCl was analyzed in the pH range of 6 to 9 (Fig. 4.15). The biomolecules acquire specific charge on their surface based on the isoelectric point (pI) and ionic composition of the system at different pH. In general, protein acquires negative charge above its pI and positive charge below its pI (Hatti-Kaul 2000).

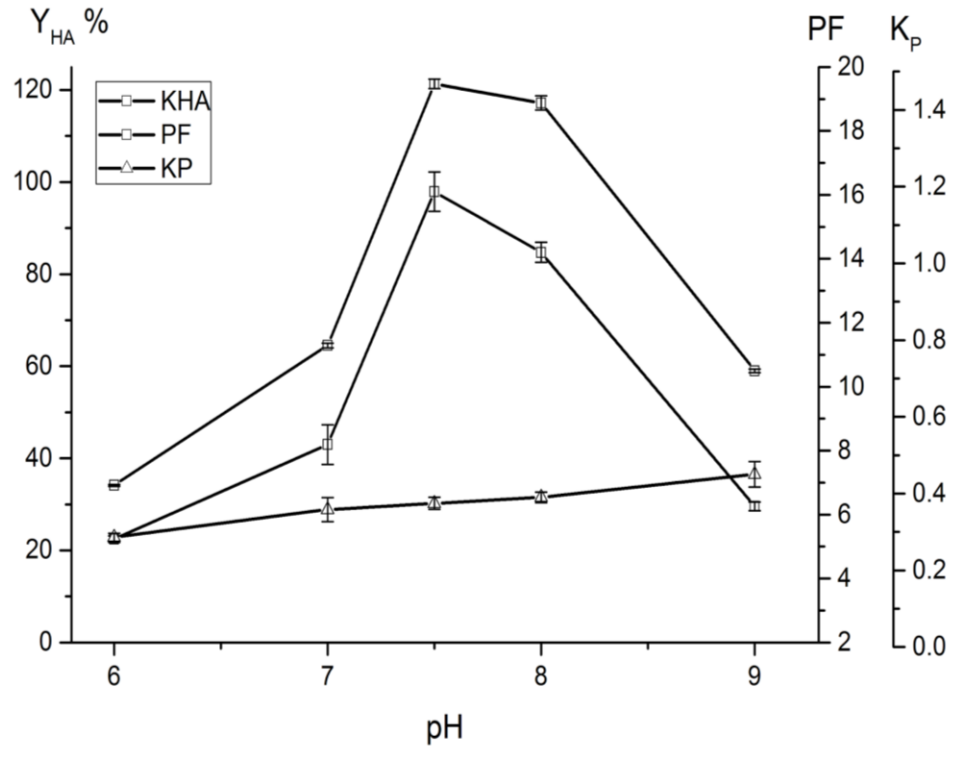


Fig. 4.15 Effect of pH on the partitioning of Psl in the ATPS system made-up of 18% PEG 6000 and 16% sodium citrate and 2% NaCl at 41.01% TLL.

The pI of Psl is around 6.8-7.2 and hence the Psl acquired a negative charge in the PEG/sodium citrate system, whose native pH was 8.1- 8.2. The specific hemagglutination activity was found to increase until pH 7.5 and Y_{HA} reached a maximum of 121.33%; accordingly, the PF also found to increase which proves the prominent effect of pH on

studied responses (Fig. 4.15). The dimer form of lectin is essential to exhibit hemagglutination activity and Psl maintains its dimer form in slightly basic pH of around 7-8. Very acidic or basic pH leads to dissociation of the alpha and beta subunits which in turn leads to the loss of hemagglutination activity (Sitohy et al. 2007).

Further, the major contaminant proteins in Pea seeds extract are legumin and vicilin where they associate to form hexamer and trimer (Barac et al. 2015) at slightly basic pH and thus transfer to bottom phase due to volume exclusion effect. Hence, the higher purity was achieved at a pH of 7.5. However, the total protein content in the top phase gradually increased as the system pH increased. The increase in pH from 7.5 to 8 enhanced the K_P from 0.26 ± 0.006 to 0.38 ± 0.01 mg/mL besides, the gradual decrease of PF (Fig. 4.15). This behavior may be due to the migration of other contaminant proteins which have a similar isoelectric point as Psl. The pH influences not only the Psl surface property but also the surface properties of other contaminating proteins in the extract which might attract them to the top phase at higher pH. When pH is reduced to 7.5 from 8 and above, these contaminants can be discriminated and maximum PF and Y_{HA} can be achieved. It's always good to maintain pH slightly more than pI rather than increasing too much. The salting out of proteins due to the dominance of citrate ions with an increase in pH also helps to partition all the proteins to the top phase and resulted in higher K_P and lower PF. These behaviors depict the distinct influence of pH on the solute partition due to electrostatic interactions. The system pH was maintained at 7.5 for the efficient partitioning where Psl partitioned to the top phase with maximum Y_{HA} of 121.33 ± 1.52 % and PF of 16.10 ± 0.61 with a minimum total protein partition coefficient of 0.37 ± 0.02 (Fig. 4.15).

4.2.8 Effect of crude load

The effect of crude load on 18% PEG 6000 and 16% sodium citrate at 2% NaCl was studied by varying crude load from 10-50% at the pH of 7.5 (Fig. 4.16). The maximum uptake capacity of the Psl in the top phase and the other impurities in the bottom phase of the ATPS was realized by increasing the crude concentration in the system. As the crude

concentration varies, the partition behavior of the desired protein and the physical properties of the phases also altered. It was observed from the Fig. 4.16 that the Y_{HA} and PF increase till the addition of 20 % (volume) crude.

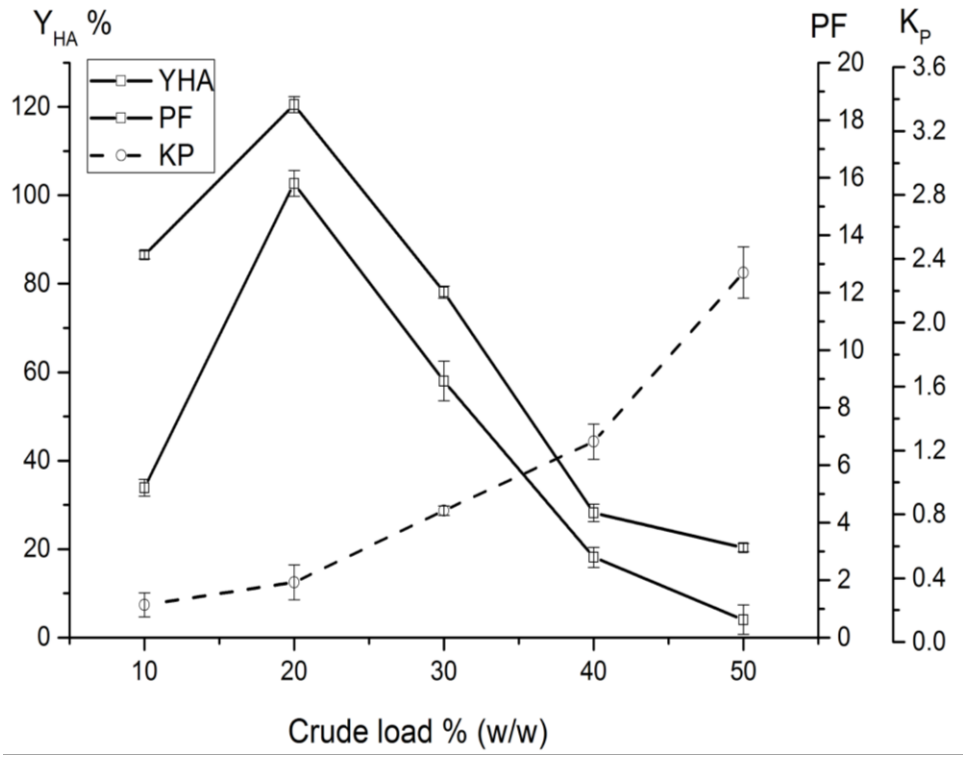


Fig. 4.16 Effect of crude load on hemagglutination activity yield (Y_{HA}), purification factor (PF) and total protein partition coefficient (K_P) in the ATPS of 18% PEG 6000 and 16% sodium citrate and 2% NaCl at pH of 7.5 and at 41.01% TLL.

The accumulation of precipitate at the interphase was observed as the crude load increased above 20% due to the saturation of both the phases, which lead to a loss of Psl along with the non-target proteins. The salting out effect of bottom phase and the excluded volume effect of the top phase are the major reasons for the rejection of proteins from the respective phases. Further, the results indicate that the higher concentration of impurities associated with the crude had a direct effect on the partitioning of the Psl and the ATPS may provide higher Y_{HA} with higher PF if the major impurities are removed

from the crude before subjecting to the ATPS partitioning. Crude load of 20% was used in further experiments as it gave maximum Y_{HA} and PF.

4.2.9 Effect of volume ratio (V_r)

The volume ratio of the phases (top by bottom phase volume) along a specific tie line provides the constant equilibrium characteristics of the system. The maximum possible solute recovery with higher concentration may be achieved by varying the phase volume ratio of the system. Five different volume ratios were considered along the same tie line to study the influence of volume ratio on the partitioning of Psl with 20% crude load. The recovery and PF were observed to increase initially with increasing volume ratio from 0.4 to 0.76, however, most of the unwanted proteins were accommodated at the increased volume ratio (K_P>1). The highest PF of 16.10 ± 0.63 with a recovery of 122.12 ± 1.36 % was achieved at the volume ratio of 0.76 (Fig. 4.17). The obtained result indicated that the top phase was able to attract the maximum concentration of Psl at the volume ratio of 0.76. Higher volume ratio decreases the degree of purification as it promotes the partitioning of contaminants into the larger top phase, due to the solubility of impurities in the free solvent available in the top phase and the rejection of proteins from the salt phase through salting out effect at the reduced salt phase volume. The decrease in volume ratio below 0.76 resulted in the lower recovery and PF with precipitation at the interface due to the solubility limit of the phases. The reduced top phase volume with maximum Psl concentration obtained at lower phase volume ratio also help to handle subsequent purification process with reduced volume. By considering the maximum PF, the volume ratio of 0.76 was selected as optimum volume ratio, even though the volume ratio of 1 gave high activity yield of 130.17 ± 1.95 % (Fig. 4.17).

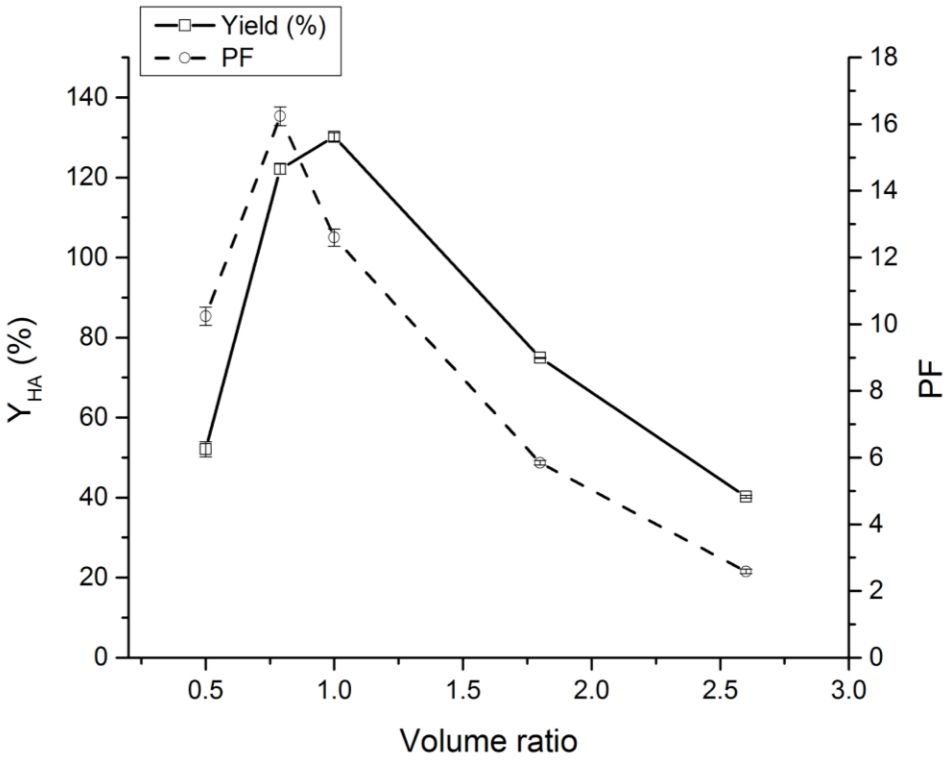


Fig. 4.17 Effect of volume ratio on hemagglutination activity yield (Y_{HA}) and purification factor (PF) in the ATPS composed of 20% crude load, 18% PEG 6000 and 16% sodium citrate and 2% NaCl at pH of 7.5 and at 41.01% TLL.

4.2.10 Optimization of ATPE of Psl using Response surface methodology

The study of clear cause effect of variables on the responses is very important to know the role of an independent variable on a response. Even though this can be achieved through one factor at a time (OFAT), the interaction effect of the factors on the responses could not be revealed by OFAT. As responses are multivariate dependent the interactive studies among the variables and their effect on responses gives valuable insight on the interrelationship between the independent variables and the dependent responses. For this purpose, the concept of RSM was introduced in the early 1950's by Box and Wilson (Box and Wilson 1951). Since then, many researchers have used the RSM procedures in different disciplines. Further, the number of experimentations required for the RSM study may be obtained by designing the experimental points using the Design of Experiment

(DoE), which was developed based on the factorial design concept without missing the significant effects of the variables on the responses. The application of DoE has grown rapidly and been adapted for many processes in different areas. The extraction and partial purification of Psl from its seed crude extract was studied and optimized using RSM (response surface methodology). The interactive effect of independent variables on the responses were analyzed for the ATPS formed by PEG 6000 and sodium citrate during the partitioning of Psl from crude extract. The multivariable effect of process variables such as PEG concentration, sodium citrate concentration, NaCl and pH on multiresponses like specific hemagglutination partition coefficient, hemagglutination activity yield and purification factor was studied in detail.

4.2.10.1 Development of model equation and their analysis

The factors PEG concentration (A), salt concentration (B), NaCl (C) and pH (D) were considered as independent variables and hemagglutination activity Yield (Y_{HA}) (Y_1), Specific Hemagglutination activity coefficient (K_{SHA}) (Y_2) and Purification factor (PF) (Y_3) were taken as the response. The central composite design of DoE was adopted to design the experiments and the experimentally recorded responses were used to develop quadratic model. The developed quadratic model which was fitted by regression analysis was tested for its statistical significance by using analysis of variance (ANOVA) as shown in Table 4.3. The model significance was decided based on the probability value (P value). The quality of fit of the second –order polynomial model equation was estimated via the coefficient of determination (R^2), the adjusted R^2 and the predicted R^2. Final quadratic model was reduced by removing the insignificant model terms. The higher significance of model was suggested by the low probability ($p < 0.05$) and high Fischer's (F-test) value. The R^2 values near to 1 suggested that the formulated predictive models were statistically very significant. The reliability and precision of the model was suggested by the insignificancy of lack of fit which was evident by $p > 0.05$ and less F value. The developed model equation was used to describe the effect of independent variables on the response, and to find out the interrelationship among the variables and to determine the combined effect of the independent variables on the responses. The model

fitting showed that there was a significant effect of selected independent variables (A, B, C and D) in the lectin extraction process on the dependent variables or responses (Y_1, Y_2, and Y_3). The regression analysis revealed that the model terms such as linear, interactive and quadratic effects of process variables has a significant effect on the responses. The interactive effects of the process variables were graphically depicted by 3D response surface plots. 3D plots shows the three dimensional view of relationship between three variables i.e. two independent factors and one response. The optimum levels of different factors for maximum lectin production and the final responses predicted by the model were validated by experimental trials.

The quantitative assessment of the responses such as K_{SHA}, Y_{HA} and PF for central composite design points was reported in Table 4.2. The statistical evaluation of the obtained responses revealed that the lectin showed preference towards PEG rich phase in almost all runs. This was evidenced by the K_{SHA} value of more than 1 (except few runs). Most of the contaminant protein partitioned to bottom phase. Thus, the specific activity increased with low total protein content in top phase when compared to increased total protein content with decreased specific activity in bottom phase. Similar trend was reported by Bim and Franco et al. (2000) where alkaline xylanase produced by *Bacillus pumilus* extracted to PEG rich phase with higher enzyme activity partition coefficient of 46.9 with low total protein content of 0.1. The maximum specific activity obtained in the top phase was 107.56U/mg after partial purification of *Pisum sativum lectin* (Psl) in ATPS from the specific activity of crude extract of 6.44 ± 0.03 U/mg, which gave maximum K_{SHA} of 42.32.

Table 4.2 Central composite design of Experimental points for four factors and the corresponding responses

RUN	Factors				Experimental responses			RSM Predicted responses		
	PEG (w/w) (A)	Salt (w/w) (B)	NaCl (C)	pH (D)	Y_{HA} % (Y1)	PF (Y2)	K_{SHA} (Y3)	Y_{HA} % (Y1)	PF (Y2)	K_{SHA} (Y3)
1	20.00	14.00	1.00	7.00	55.62	4.23	14.00	54.05	4.02	13.32
2	16.00	14.00	3.00	8.00	50.21	2.80	6.00	48.89	2.82	5.74
3	18.00	20.00	2.00	7.50	30.16	0.41	0.80	31.12	0.46	0.91
4	20.00	14.00	1.00	8.00	75.68	6.12	15.42	75.93	6.51	15.25
5	18.00	16.00	2.00	7.50	124.0	16.55	41.00	124.07	16.04	41.08
6	20.00	14.00	3.00	7.00	115.0	7.54	13.50	115.54	7.55	13.59
7	16.00	14.00	3.00	7.00	46.23	2.61	5.89	46.47	2.76	5.81
8	18.00	16.00	2.00	7.50	116.0	15.12	42.00	117.51	15.94	41.18
9	20.00	18.00	3.00	7.00	59.75	1.20	0.70	59.47	1.34	0.68
10	18.00	16.00	0.00	7.50	81.31	8.45	21.23	81.94	9.95	20.87
11	20.00	18.00	1.00	7.00	48.95	2.22	4.00	48.14	2.21	3.79
12	16.00	18.00	3.00	8.00	36.00	0.30	0.52	35.68	0.28	0.56
13	20.00	18.00	3.00	8.00	60.03	4.20	4.20	59.89	4.25	4.22
14	22.00	16.00	2.00	7.50	59.54	1.13	1.80	60.57	1.03	1.57
15	16.00	18.00	1.00	7.00	50.00	2.86	8.52	50.34	3.05	8.85
16	18.00	16.00	2.00	7.50	126.1	16.85	40.00	125.51	16.33	40.45
17	16.00	14.00	1.00	7.00	49.00	3.45	11.02	50.34	3.49	10.58
18	16.00	18.00	1.00	8.00	58.78	4.50	13.56	57.12	4.13	13.81
19	18.00	16.00	2.00	8.50	63.00	4.56	11.89	64.22	4.45	12.59
20	18.00	16.00	2.00	7.50	115.0	16.75	41.05	115.14	16.2	41.08
21	18.00	16.00	4.00	7.50	84.00	3.58	11.30	85.69	3.99	10.79
22	18.00	16.00	2.00	7.50	122.0	15.86	42.32	121.51	16.12	41.96
23	14.00	16.00	2.00	7.50	18.95	0.20	0.73	17.98	0.22	0.67
24	18.00	12.00	2.00	7.50	65.34	3.10	7.215	66.09	3.14	7.56
25	20.00	14.00	3.00	8.00	135.0	7.50	15.12	134.82	7.22	14.74
26	16.00	14.00	1.00	8.00	55.00	3.13	13.37	51.88	3.03	13.05
27	20.00	18.00	1.00	8.00	40.12	3.50	7.500	40.14	3.49	7.53
28	16.00	18.00	3.00	7.00	40.12	0.50	0.68	40.15	0.59	0.74
29	18.00	16.00	2.00	6.50	55.3	2.45	12.1	53.52	2.31	11.67
30	18.00	16.00	2.00	7.50	126	16.5	39.55	126.13	15.98	40.54

The effect of independent variables and the interaction between these variables on the responses were best represented by following second order polynomial equations (4.1-4.3).

$$Y_1 = 121.52+11.92A-10.76B+4.77C+2.56D-9.81AB+11.85AC-7.07BC-19.94A^2-17.81B^2-9.09C^2-14.96D^2. \quad (4.1)$$

$$Y_2 = 16.27+0.76A-0.98B-0.54C+0.48D-0.65AB+0.75AC-0.65BC-3.81A^2-3.54B^2-2.47C^2-3.10D^2. \quad (4.2)$$

$$Y_3 = 40.99+0.71A-2.81B-2.53C+0.71D-1.79AB+1.62AC-0.88BC-9.93A^2-9.25B^2-6.18C^2-7.25D^2. \quad (4.3)$$

The results obtained by the regression analysis of the experiments shown that most of the model terms affected the Y_{HA}, PF and K_{SHA}. All the quadratic terms A^2, B^2, C^2, D^2 has very prominent effect on the Y_{HA} ($P<0.0001$), PF ($P<0.0001$), and K_{SHA} ($P<0.0001$). Y_{HA} was predominantly affected by linear model terms such as PEG and salt concentration ($p<0.0001$). PF was affected very significantly by sodium citrate concentration ($P< 0.0008$) and significantly by PEG concentration ($P<0.001$). Sodium citrate concentration ($P<0.0001$) and PEG concentration ($P<0.004$) has significant effect on K_{SHA} .

The combination of PEG (A) and sodium citrate (B) concentration was the most influenced interactive term for Y_{HA} ($P<0.0001$), PF (0.004) and K_{SHA} ($P<0.0001$) which was graphically depicted by 3D response surface plots as A1, B1, C1 in Fig. 4.18, respectively. The positive influence of sodium citrate was observed initially. Salt ions in the bottom phase interact with the water ions surrounding the protein; as a result, protein dehydrates with the exposure of hydrophobic sites (Porto et al 2011). This facilitates the interaction of PEG and protein. Thus the increase in salt concentration in the bottom phase eventually raises the hemagglutination activity (HA) of Psl in the top phase. However negative effect was noticed when sodium citrate concentration exceeds 16% (w/w) (except run 6 and 14), as the high amount of salt salted out the contaminant proteins along with the Psl towards top phase.

The increase in PEG 6000 concentration improved the responses at the beginning. The increase in PEG concentration associated with the increase in hydrophobicity of the top phase, which in turn enhances the interaction of hydrophobic PEG molecule with hydrophobic sites of the lectin (Ooi et al. 2008). However, the opposite trend was observed above the concentration of 18% (except run 6 and 16). The partial purification of lectin in ATPS other than Psl such as Conbr (*Canavalia brasiliensis*) lectin studies (Nascimento et al. 2010) reported that the important property of legume lectins of having hydrophobic cavity and hydrophobic sites markedly influences their partition in PEG/salt systems. These characteristic properties of lectin were extremely influenced on the movement of lectin towards hydrophobic PEG rich phase through PEG and lectin interaction.

The hydrophobicity exerted by PEG and salting out process of salt lead to an increase in the Y_{HA}, PF and K_{SHA} showing the synergism between these two process variables. The maximum responses were obtained when both the factors increase to their center level (run 26-30). The behavior noticed in the present study was in accordance with response surface methodological study of partition of lysozyme from crude hen egg white, where the mid-range values of the central composite design of 18% of PEG, 16% of potassium citrate salt yielded the maximum response of 104% activity yield, 31200 U mg−1 of specific activity and 21.15 of purification factor (Lu et al. 2013). The purification factor of α-Amylase from white pitaya peel was also significantly affected by the interaction between PEG and sodium citrate concentration where the synergism between these factors gave maximum PF of 4.59 at their center level (Shad et al. 2018).

NaCl exhibited prominent influence on K_{SHA} ($P<0.0001$), more significant influence on PF ($P<0.0008$) and significant influence on Y_{HA} ($P<0.009$). In most of the aqueous two phase extraction of protein studies addition of NaCl drives the desired protein towards top phase (Asenjo et al. 2005). The difference in hydrophobicity between the two phases increases with the addition of NaCl. The volume of the top phase reduces with increase in NaCl concentration which reduces the water content in top phase and concentrates the

PEG and in turn increases the hydrophobicity (Azevedo et al. 2007), thus increases the partition of Psl to top phase through hydrophobic interaction between Psl hydrophobic sites and PEG. The prominent influence of NaCl was also observed in the partitioning of ricin in PEG/sodium sulfate system. The addition of 0.47M NaCl almost doubles the partition of ricin towards PEG rich phase (Zhang et al. 2005).

Table 4.3 ANOVA of models for the prediction of responses

	DF	**SS**	**MS**	**F**	**P**
Hemagglutination activity yield %					
Model	14	32143.02	2295.93	37.74	<0.0001
Lack of fit	10	792.11	79.21	3.29	0.1005
Residual	15	912.52	60.83		
Pure error	5	120.41	24.08		
R^2 = 0.9724, Adj R^2 = 0.9466, Pred R^2 = 0.8567					
Purification factor					
Model	14	908.38	64.88	60.01	<0.0001
Lack of fit	14.03	10	1.40	3.20	0.1053
Residual	15	16.22	1.08		
Pure error	5	2.19	0.4377		
R^2 = 0.9825, Adj R^2 = 0.9661, Pred R^2 = 0.9092					
Specific activity partition coefficient					
Model	14	5873.27	419.42	165.89	<0.0001
Lack of fit	10	32.09	3.21	2.74	0.1383
Residual	15	37.93	2.53		
Pure error	5	5.85	1.17		
R^2 = 0.9936, Adj R^2 = 0.9876, Pred R^2 = 0.9673					

DF: Degree of Freedom, SS: Sum of Squares, MS: Mean of Squares, F: F-factor, P: Probability of Error.

The simultaneous increase in sodium citrate salt (B) and NaCl (C) till 16% (w/w) and 2% (w/w) respectively lead to an increase in the PF and K_{SHA} (Fig. 4.18 B3, C3). Psl synthesized along with the other storage proteins in the seed and stored in the vacuoles (Higgins et al. 1983). It was reported that some of the storage protein like vicilin may interacts with the Psl. The increased ionic strength established by the sodium citrate and NaCl facilitates the detachment of interacted storage protein like vicilin from the Psl

(Wenzel, M., & Rüdiger) due to which Psl specific activity increase and in turn enhanced the PF and K_{SHA}.

The combined effect of PEG 6000 (A) concentration and NaCl (C) concentration significantly affected the responses and maximum PF and K_{SHA} was achieved around 18% (w/w) PEG and 2 %(w/w) NaCl (Fig. 4.18 B2, C2).The increased value of NaCl ended with less purification factor and K_{SHA}. Similar behavior was noticed by Nascimento et al. where the purity of *Canavalia brasiliensis* (ConBr) gradually increases with the increase in NaCl up to 5% and the addition of NaCl above 5% attracted most of the contaminant proteins towards top phase and resulted in the reduction of the purity of ConBr lectin (Nascimento et al. 2010).

The interactive effect of sodium citrate concentration and pH significantly affected Y_{HA} ($P<0.10$). The combined effect of these two process variables may facilitate the transfer of protein to PEG rich phase as the isoelectric point of Psl is 6.8-7.2 (Hoaddmaker 1994). The electrostatic interaction between PEG and Psl played a significant role in driving Psl towards PEG phase (Asenjo et al. 1994). Similar phenomenon was noticed by Salmon et al. (2014) in the aqueous two phase extraction of phytase from *Schizophyllum commune* where concentration of PEG and pH interactively influenced the phytase activity yield. Rahimpour et al (2016) also noticed that the interactive effect of phosphate salt and pH significantly affected recombinant alkaline active xylanase activity yield. The PF ($P<0.01$) and K_{SHA} ($P<0.04$) also significantly influenced by pH.

pH played a significant positive effect up to center level value. The higher and lower level of pH leads to decrease in Y_{HA}, K_{SHA} and PF (run 5, 13). Protein acquires negative charge above its pI and positive charge below its pI (Shang et al. 2004). Psl attains negative charge above pH 7.2 at basic pH (pI of Psl 6.8-7.2), thus at slightly basic pH Psl gave better extraction performance than in acidic pH. As Psl is a metalloprotein, it requires metal ions like Ca^{2+} and Mn^{2+} for their hemagglutination activity (Sithoy et al. 2013). At acidic pH the leaching out of these metal ions takes place which results in the loss of hemagglutination activity. Dimer form of the lectin was essential requirement for

the pea lectin to function and this state was maintained at slightly basic pH. Hence, too acidic and too basic pH condition reduces the activity of lectin by dissociation of subunits (Sithoy et al. 2013). The high level value of pH i.e. pH 8 exhibited low PF and $K_{SHA;}$ this may be due to the migration of storage proteins towards top phase which have pI nearly same as Psl.

Run 16 gave a maximum Y_{HA} of 135% at 20% PEG, 14% salt, 3% NaCl and at pH 8. Even though maximum Y_{HA} was observed, the obtained PF and K_{SHA} were low at this particular run. Whereas run 26 achieved the maximum Y_{HA} with maximum PF and K_{SHA} at 18% PEG, 16% sodium citrate salt, 2% NaCl and at pH 7.5. The purification factor of the protein increases with the separation of the target protein from other contaminant proteins. Hence the type and intensity of contaminant proteins present in the crude extract is a deciding factor for PF. *Canavalia brasiliensis* lectin partitioned to top phase in PEG/potassium phosphate ATPS was recorded a PF of 1.7. *Cratylia mollis* seed lectin was partitioned to bottom phase by yielding a PF of 13.28. 11.5 PF was achieved for *Canavalia ensiformis* lectin partitioned in PEG/citrate system. In present study, Psl partitioned to top phase with the PF of 16.24 which was slightly more than PF obtained for *Cratylia mollis* seed lectin.

Few of the beans family lectin partition studies in ATPS such as *Canavalia brasiliness* achieved more than 100% activity yield (Nacimento at al. 2011). The research groups reported that the activation of active site of the protein by stabilizing effect of PEG was one of the reasons to achieve maximum yield. Meyrnanoff also stated that the separation of inhibitors from the desired protein after partial purification of lectin enhances the HA activity and may lead to increase of activity yield more than 100% (Mayerhoff et al. 2004).

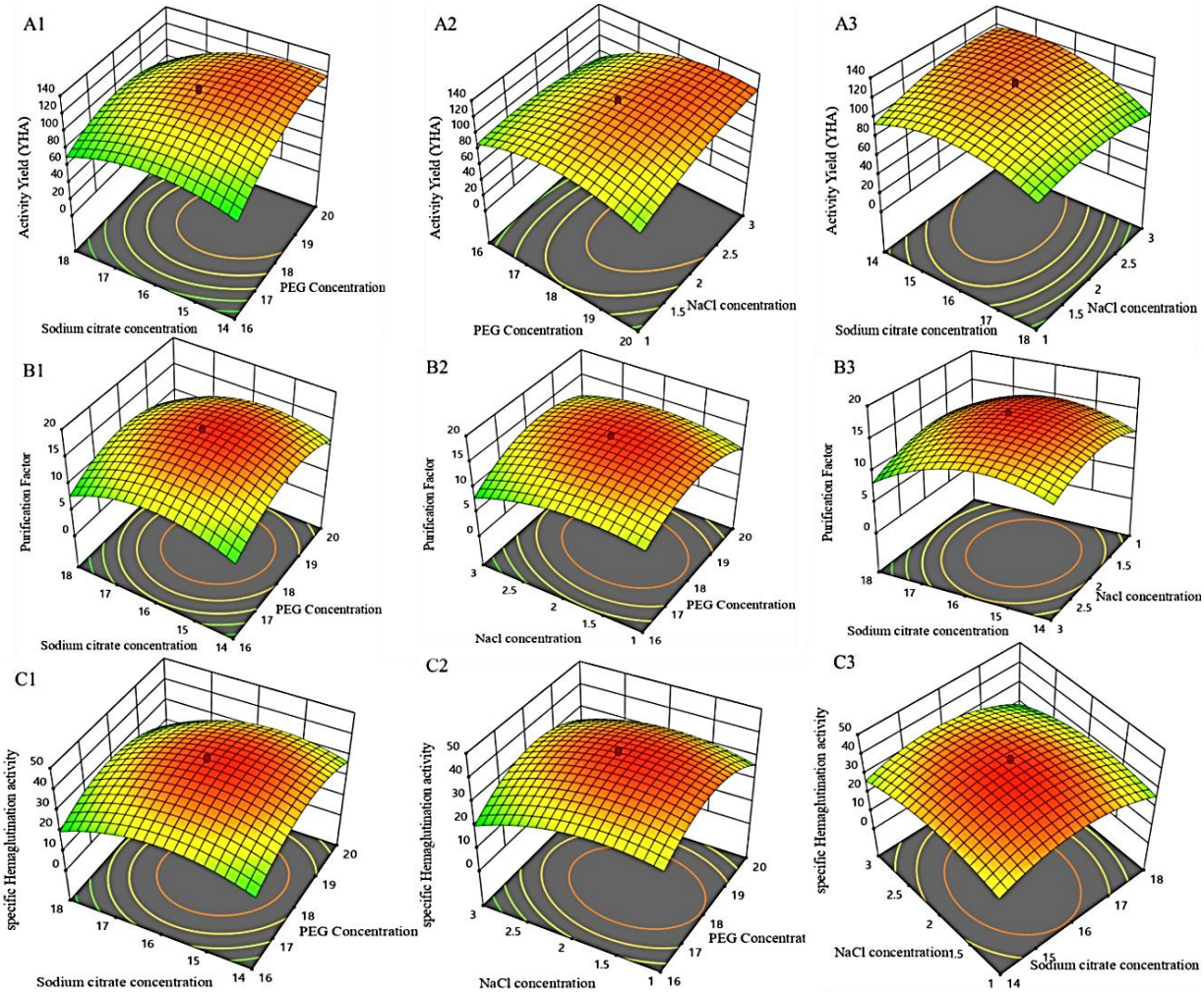


Fig. 4.18 Response surface plots showing the interaction effects of a PEG and sodium citrate concentration, PEG and NaCl concentration, Sodium citrate and NaCl concentration on Hemagglutination activity yield (A1, A2, A3) Purification factor (B1, B2, B3) and Specific Hemagglutination activity coefficient (C1, C2, C3) respectively.

4.2.10.2 Optimization of the variables and validating the optimum responses

When multiple responses depend on the same process variables, optimization of the process variables for any one of the responses often results in the condition that varies disproportionally from the optimal condition of other responses. Even though the

responses are interdependent, it is mandatory to find a single feasible optimal condition based on the requirement of the process industry. In the purification of the biomolecules from a complex source, it is essential to find optimum conditions (either to enhance or minimize) for different responses; at the same time keeping an eye on the other responses is necessary. The multi-response optimization was performed through numerical optimization approach which is based on the desirability objective function. The goal of optimization may vary depending on the requirement of the process implementation, whether the desired protein has to be partitioned with maximum purity or yield. The optimum conditions for the given constraints were obtained based on the desirability approach. The optimum condition is the point where the desirability of the said goal is maximum within the design space. Desirability based optimization is a powerful, efficient, and relatively new method for the optimization of multi-response processes (Arumugam et al. 2014). For a specific goal, the quadratic model developed for each response should be considered to obtain a particular desirability function (di) (Eq. 4.4). The inputs are the model equations, weight, target value or the upper and lower bounds. The desirability (D and d) ranges from least to most (zero to one) desirable.

$$D = (d_1 \times d_2 \times \ldots\ldots\ldots d_n)^{1/n} \qquad (4.4)$$

where, n is the number of responses in the process. The overall function (D) becomes zero when any of the responses or factors fall outside their desirability range (Mehri 2014). The shape of the overall desirability function for different goals may be modified by assigning different weights to the individual responses and variables. In the present work, partitioning of Psl into PEG rich phase was targeted and accordingly the activity yield and purification factor of this protein in the respective phase was considered as responses. Three responses (Y_1, Y_2, and Y_3) were considered to obtain the optimum process conditions for three different process goals namely,

I. Maximize the hemagglutination activity yield % (Y_1)
II. Maximize the purification factor (Y_2)
III. Maximize both Y_1 and Y_2

In the optimization tool of design expert, optimum conditions were searched for maximization of the particular responses, whereas other responses were set within a range. Constraints given for optimization and optimum values are reported in Table 4.4 and 4.5 respectively. Ramp graph and bar graph that gives detailed information on the given constrains and the optimized values are given in Fig. A2.1 (Appendix) - A2.9 (Appendix).

The responses are represented as circular contours and the predicted optimum condition is given as flag in Fig. 4.19. Feasibility of these optimized conditions and the predicted responses were validated experimentally (Table 4.5) and the experimental values are found to be in agreement with the predicted values. The Psl partitioning in PEG 6000-trisodium citrate system process has been successfully described by the response equations (Eq. 4.1-4.3), using which the process can be modified and operated for the preferred goals. Based on the preceding discussion of the results, it was concluded that the PEG 6000- trisodium citrate ATPS is most appropriate for the selective extraction of Psl from crude extract of seed. Therefore, the suggested ATPS and the optimum conditions of the variables could be useful to study the scale-up parameters and industrial scale application.

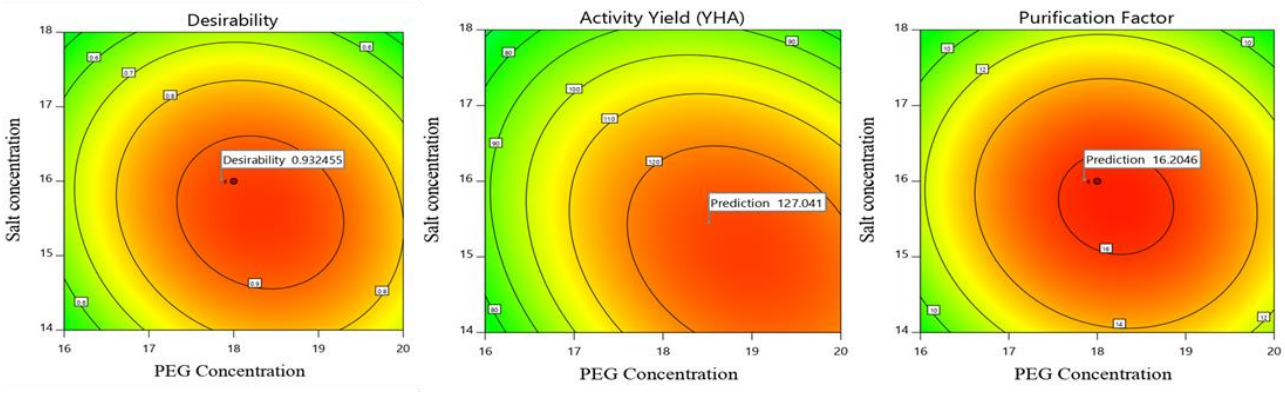


Fig. 4.19 Estimated desirability Contour plot (A) and estimated contour plots for the maximization of both Hemagglutination activity (B) and Purification factor (C)

Table 4.4 Constraints given for different goals.

Sl. No		Factors				Responses		
		PEG Conc (w/w)	SALT Conc (w/w)	NaCl (w/w)	pH	Activity yield (%)	PF	K_{SHA}
	Maximize activity yield							
1	Constraints	In range	In range	In range	In range	Maximize	In range	In range
	Values	16-20	14-18	1-3	7-8	135	0.2-16.85	0.52-42.32
	Maximize Purification factor							
2	Constraints	In range	In range	In range	In range	In range	Maximize	In range
	Values	16-20	14-18	1-3	7-8	18.95-135	16.89	0.52-42.32
	Maximize activity yield and Purification factor							
3	Constraints	In range	In range	In range	In range	Maximize	Maximize	In range
	Values	16-20	14-18	1-3	7-8	135	16.89	0.52-42.32

Table 4.5 Optimum conditions obtained for different goals.

Sl no		**PEG Conc,** (w/w) **(A)**	**SALT Conc,** (w/w) **(B)**	**NaCl,** (w/w) **(C)**	**pH (D)**	**Y_{HA} (%) (Y_1)**	**PF (Y_2)**	**K_{SHA} (Y_3)**	**Desirability**
1	**Maximize activity yield**								
	Predicted	19.56	14.53	3	7.59	133.22	11.75	26.58	0.985
	Experimental					134.23	10.86	28.56	
2	**Maximize Purification factor**								
	Predicted	18.21	15.72	1.92	7.54	123.4	16.42	41.34	0.974
	Experimental					122	16.87	40.75	
3	**Maximize activity yield and Purification factor**								
	Predicted	18.53	15.44	2.16	7.55	127.233	16.17	40.18	0.946
	Experimental					128.54	16.24	40.21	

4.2.11 Purity analysis of extracted Psl

4.2.11.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the partitioned pea lectin in PEG/sodium citrate ATPS was analyzed by SDS-PAGE and shown in Fig. 9. Lane 1 represents the molecular markers used and lane 2 represents the standard Psl separated into two bands in the presence of β-mercaptoethanol at 6 and 18 kDa correspond to smaller α and larger β chains, respectively. Hence, the bands that were seen in the SDS–PAGE image is most likely representing the subunits of a dimeric lectin. Lane 3 was loaded with the crude sample which shows the dense protein bands including Psl. Lane 3, 4, 5 was loaded with top phase sample of optimized ATPS at a different concentration, where similar electrophoretic pattern and molecular weight of standard Psl was observed for all the three lanes. The other contaminant proteins were concentrated in bottom phase (lane 8) and interphase (lane 7). The obtained image clearly indicated the presence of Psl with smaller α and larger β chains (lane 4, 5 and 6) as similar to that of pure standard (lane 2). Further, it is evident that most of the contaminants are either accumulated in the bottom phase (lane 8) or interphase (Lane 7). The bands of Psl corresponding to 6 and 18 kDa were not visible in the Lane 7 and 8 confirm the selective partitioning of Psl in the top phase.

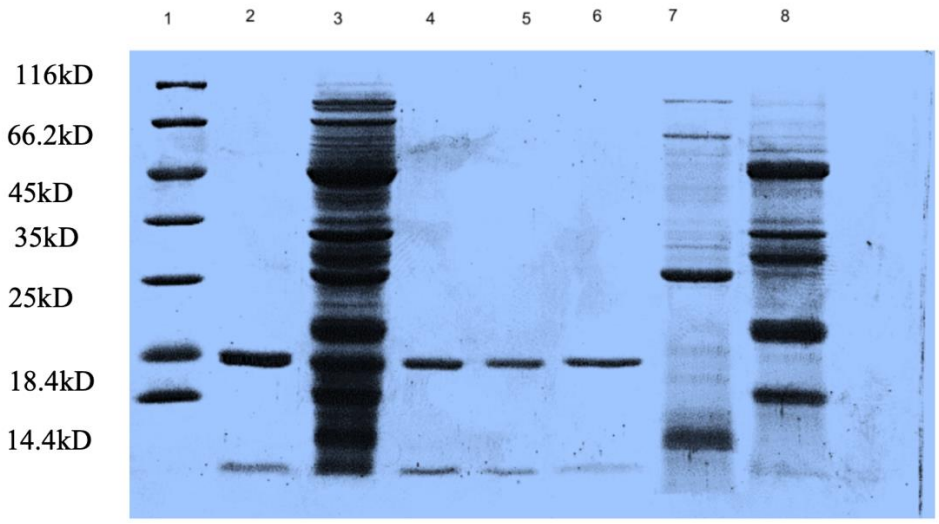


Fig. 4.20 SDS–PAGE analysis of different samples. Lane 1: molecular weight marker (116KD-14.4KD), Lane 2: pure Psl, Lane 3: crude extract of *P. sativum* seeds, Lane 4, 5,6: Extracted Psl to top phase, Lane 7: Interface, Lane 8: Bottom phase of ATPS.

4.2.11.2 Chromatographic analysis

High performance liquid chromatography (HPLC) was used to analyze the purity of protein qualitatively. The chromatogram of a crude extract of Psl is presented in Fig. 10 (a). The two isoforms of the Psl; larger β and smaller α were separated at a retention time of 27.434 and 27.755 minutes, respectively along with the other protein peaks. Fig.10b represents the chromatogram of the top phase which was partially purified. The extracted Psl also showed the peaks of two isoforms (27.452 and 27.783 minutes) similar to crude Psl chromatogram without any other peaks corresponding to contaminant proteins. This proves that the ATPE successfully partitioned the Psl selectively from other contaminant proteins while maintaining the stability of the Psl.

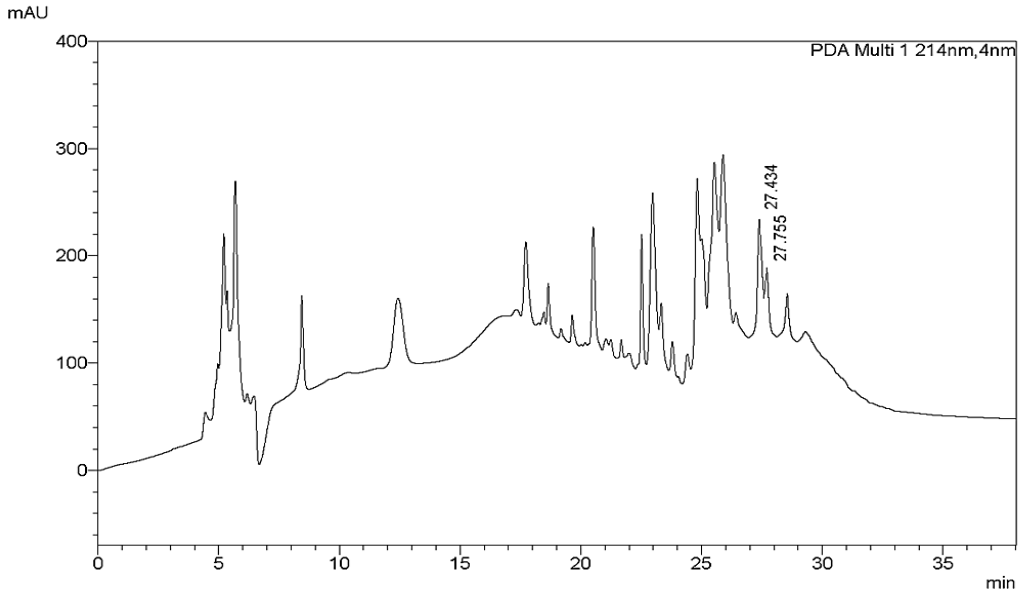


Fig. 4.21a Elution pattern of proteins in seed crude extract of *Pisum sativum* at the flow rate of 0.5min/mL in binary gradient mobile phase (acetonitrile and tri-fluoroacetic acid) in column: RP-C18.

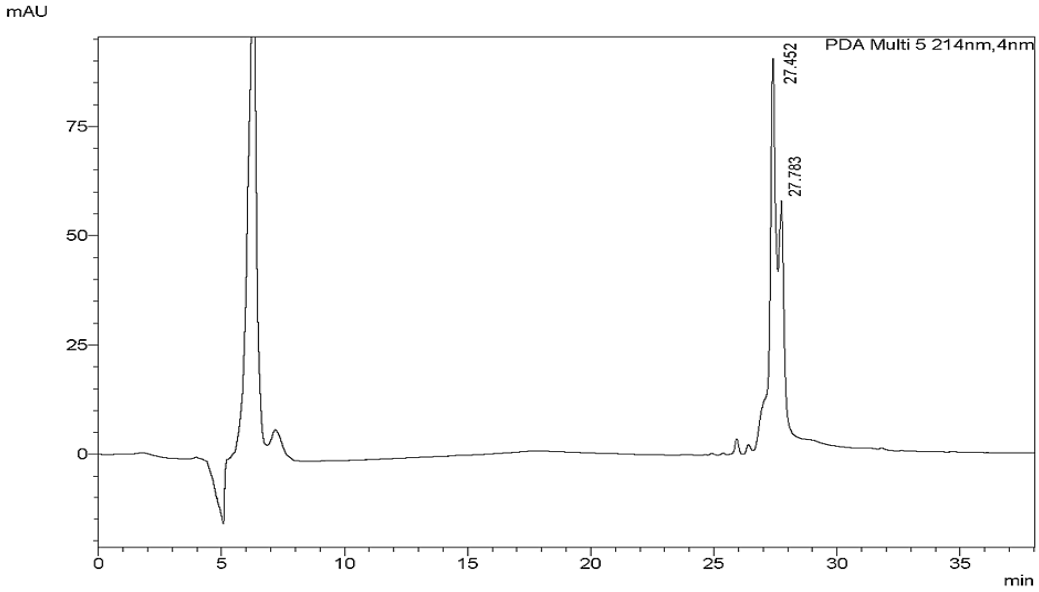


Fig. 4.21b Elution pattern of top phase extracted Psl at the flow rate of 0.5min/mL in binary gradient mobile phase (acetonitrile and tri-fluoroacetic acid) in column: RP-C18.

4.3 CONTINUOUS ATPE OF Psl IN ROTATING DISC CONTACTOR

The conventional RDC column, which consists of six stator rings and the rotating discs attached with a central shaft and placed at the center of each compartment, was considered as a continuous extractor for the extraction of *Pisum sativum* lectin in the current study. The performance of the extraction column was evaluated by analyzing the hydrodynamic and mass transfer behavior at different operating conditions. Salt phase (heavier phase) was used as the continuous phase and PEG phase (lighter) is dispersed in the form of droplets in the continuous phase. The dispersed phase holdup is the most important characteristic in hydrodynamic studies. Holdup refers to the amount of dispersed phase available in the column to remove the target protein from the feed at any instant during the steady state operation. The holdup and size of the drops were directly proportional to the interfacial area. Correspondingly, the mass transfer efficiency is the function of the drop size as well as the holdup of the dispersed phase. Thus, the holdup measurements of the column under specified conditions were used to evaluate the mass transfer capability of a contactor. The knowledge of holdup is very important as it does not only influence the mass transfer coefficient but also limiting the column operation at flooding condition (Aravamudan, 1999). The extent of mixing and separation of the phases governs the rate of partition and recovery of protein. The rate of protein transfer from the continuous phase to the dispersed phase depends on the mass transfer coefficient. The turbulence created by the movement of the rotor discs placed at the center of each compartment enhances the dispersion and thereby increases the mass transfer of the solute from one phase to another phase (Gavhane 2006). For an efficient and reliable extractor design, the information on the dispersed phase holdup, mass transfer coefficient at various conditions, and physical properties of the phase components such as density, viscosity, and interfacial tension are necessary. The physical properties of the phase components used in the present study are given in Table 4.6.

The optimization of the ATPE of Psl in batch studies showed a better yield and purification factor in 39.86 wt% TLL of PEG 6000-trisodium citrate system with 20% crude load and pH of 7.55. Hence, the system was chosen to study the continuous

extraction of Psl from pea seed crude extract. The phases were prepared at the equilibrium concentration corresponding to the selected TLL. The crude was mixed with salt phase while preparing the equilibrated (by phase forming components) phases. In the present study, the effects of dispersed phase velocity, continuous phase velocity, and the rotor speed on the holdup, volumetric mass transfer coefficient, recovery, purification factor, and separation efficiency were evaluated.

Table 4.6: Physical properties of the PEG 6000 – trisodium citrate system

System composition	**Density (Kg/cm^3)**	**Viscosity (mPa.s)**	**Surface tension (mN/m)**
PEG rich phase (PEG 6000 34.86 wt %, TSC -2.5 wt %)	1065.51	22.29	53.54
Salt rich phase (PEG-6000 4.2 wt%, TSC 25.36 wt%, Pea seed crude extract -20 wt%)	1190.80	2.994	46.23
Pea seed crude extract	1106.82	0.910	42.40

4.3.1 Effect of dispersed phase velocity

The influence of dispersed phase velocity on dispersed phase holdup, overall volumetric mass transfer coefficient based on dispersed phase (K_Da), and recovery and purification factor were studied under different continuous (2.91×10^{-5}, 6.41×10^{-5} and 9.32×10^{-5} m/s) and dispersed (2.91×10^{-5}, 6.41×10^{-5} and 9.32×10^{-5} m/s) phase velocities and at varied rotor speeds (2.5, 5 and 7.5 s^{-1}). The dispersed phase holdup and K_Da were found to increase with an increase in the dispersed phase velocity at constant rotor speed as shown in Fig. 4.22 (a) and (b) respectively. As dispersed phase velocity increases, the size of the drops decreases and the number of the drops increases due to the increased kinetic and buoyancy forces that act on the droplets. The formation of smaller drops increases the retention time of the dispersed phase droplets in continuous phase due to

their low raising velocity. This leads to the increase in dispersed phase holdup with the increase in dispersed phase velocities, which was significantly high at higher rotor speed. The available interfacial area for mass transfer in a counter-currently operated column depends on the dispersed phase holdup of the column and the mean drop size. Therefore, the decrease in drop size at higher dispersed phase flow rates give higher holdup which facilitates the higher interfacial area for mass transfer and thus increases the mass transfer coefficient (Sawant and Sikdar 1990). A similar trend of positive effect of dispersed phase velocity on dispersed phase holdup and mass transfer coefficient was noticed in PRDC column (Porto et al. 2000) in the continuous extraction of BSA in PRDC using PEG/phosphate system, continuous extraction of α-toxin in PEG/phosphate system (Cavalcanti et al. 2008) and also in the continuous extraction of α – lactalbumin (Kalaivani and Regupathi et al. 2016). Similar results were also noticed in other columns such as spray column in the extraction of horseradish peroxidase enzyme, York – scheibel column in the extraction for BSA and amyloglucosidase (Jafarabad et al. 1992).

The increase in dispersed phase velocity has a positive effect on activity-recovery, separation efficiency and the purification factor of *Pisum sativum* lectin. The variation in these with the dispersed phase velocity is shown in Fig. 4.22 (c), 4.22 (d), and Fig. 4.23 respectively. As stated earlier, the increase in rotor speed with the increase in dispersed phase velocity creates high turbulence which aids the formation of smaller drops whose up-rising velocity decreases and retention time of drops in the column increases. The high interfacial area offered by the smaller drops enhances the mass transfer and consequently enhances the activity recovery, purification factor and separation efficiency. Porto et al. (2004) also reported a similar trend of increasing activity recovery. The authors reported that the activity recovery and purification factor of oxidoreductase increased to 236% and 34.3 respectively with the increase in dispersed phase velocity. The higher lectin activity and purification factor observed in the present study was mainly due to the structural

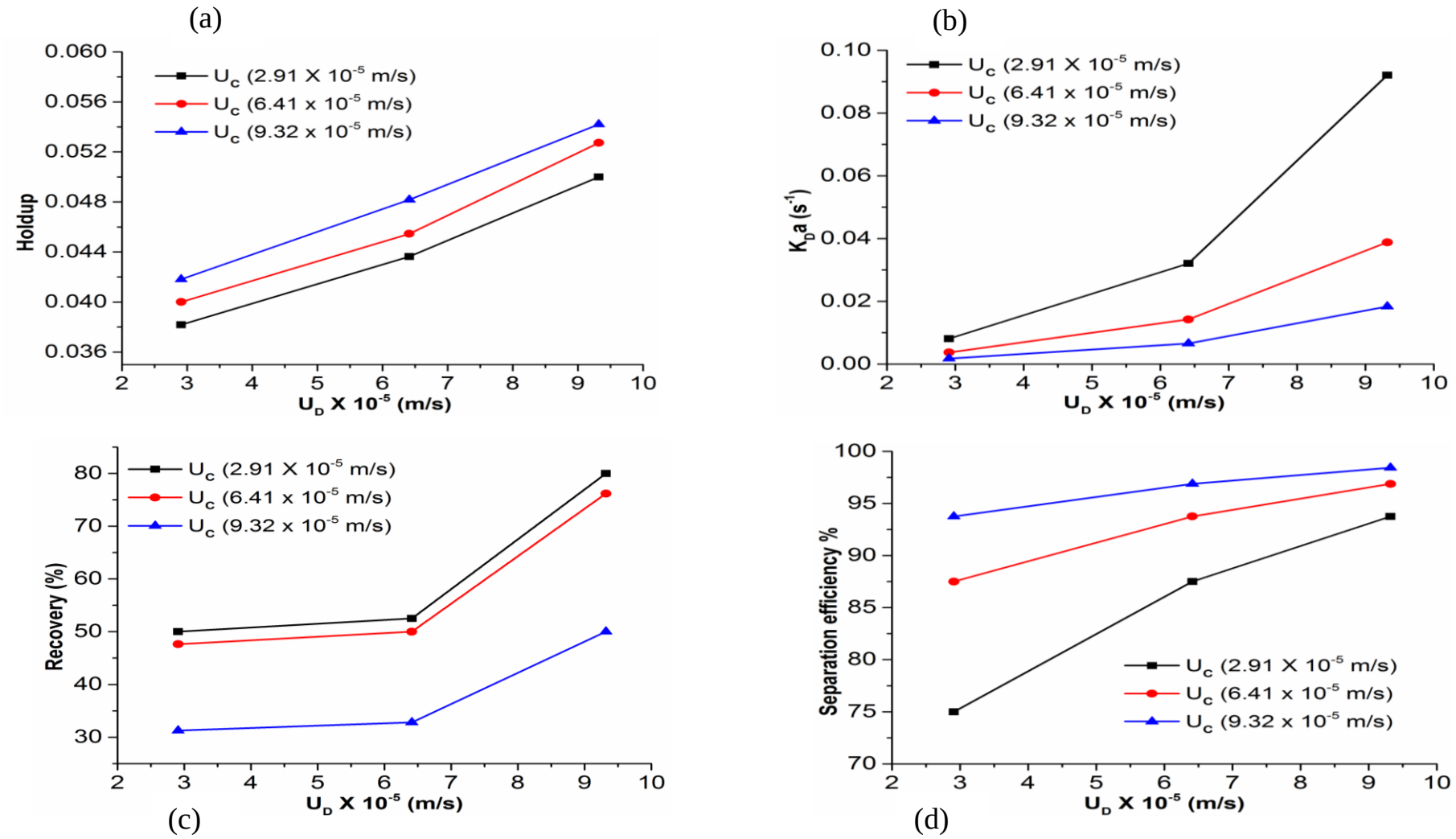


Fig. 4.22 Effect of dispersed phase velocity (U_D) on (a) holdup (b) mass transfer coefficient (K_Da) (c) recovery and (d) separation efficiency at different continuous phase velocities (U_C) at constant rotor speed of 5$s^{-.}$

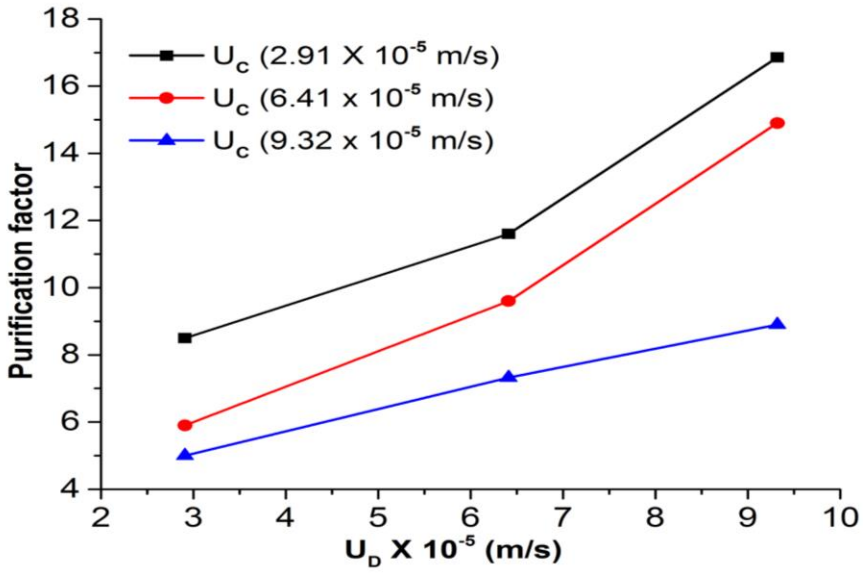


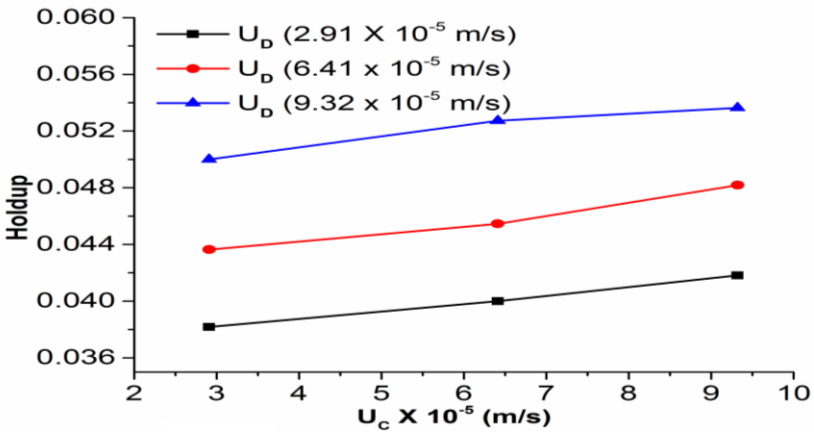
Fig. 4.23 Effect of dispersed phase velocity (U_D) on purification factor at a constant rotor speed of 5s^{-1} and at various continuous phase velocities.

stability offered by the PEG towards lectin (Albertsson 1986) and the separation of contaminants from the lectin during the extraction. The maximum Psl activity recovery of 160% with the purification factor of 20.42 was achieved at dispersed phase velocity of 2.91×10^{-5} m/s.

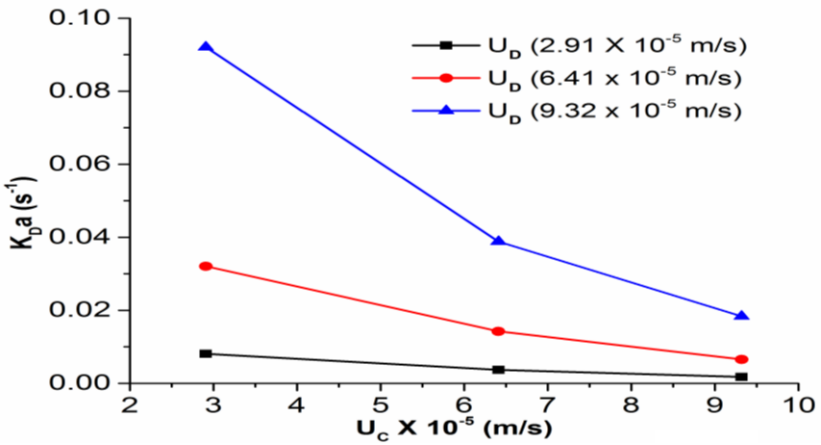
4.3.2 Effect of continuous phase velocity

The effect of continuous phase flow rate at different rotor speed (2.5, 5 and 7.5 s^{-1}), dispersed phase flow rate (2.91×10^{-5}, 6.41×10^{-5} and 9.32×10^{-5} m/s), and continuous phase flow rate (2.91×10^{-5}, 6.41×10^{-5} and 9.32×10^{-5} m/s) was evaluated on the performance of the column with respect to holdup, volumetric mass transfer coefficient, recovery, purification factor, and separation efficiency. The increase in the continuous phase flow rate increases the dispersed phase holdup. The influence of continuous phase velocity on holdup at 5 s^{-1} rotor speed and at different U_d is shown in Fig. 4.24 (a). By increasing the continuous phase velocity, the drag force between the dispersed drops and

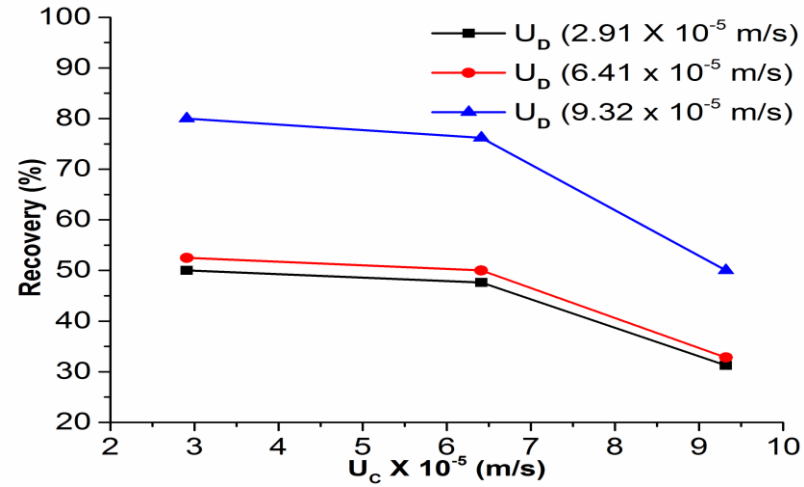
the continuous phase increases, which increases the residence time of the drops in the system due to the restricted movement of the drops. Hence, a number of smaller drops are formed and retained for longer duration in the dispersion zone due to the lesser gravitational force on the smaller droplets at higher continuous phase flow rates. For higher flow rates, the effect of drag force becomes more prominent than the gravitational force on drops and results in the increase in holdup. This effect was observed to be more pronounced at the higher dispersed phase flow rate at all rotor speeds. The obtained trends are in accordance with the previous reports (Kumar and Hartland 1995, Kalaichelvi and Murugesan 1998 and Regupathi 2005) of RDC and asymmetric rotating disc contactor (ARDC) (Kadam et al. 2009). The mass transfer coefficient decreases with the increase in continuous phase velocity (Fig. 4.24 (b)); this trend was observed due to the decrease of the mean residence time of continuous phase in the column with the increase in continuous phase velocity. The similar result of decreasing mass transfer coefficient with increasing U_c was also observed by Kalaivani et al (2016) in the extraction of alpha-lactalbumin and beta-lactoglobulin in PEG 1000/sodium citrate system in RDC column and also by Cavalcanti et al. (2008) in the extraction of alpha-toxin in PEG 8000/potassium phosphate system. As explained above, the increase in continuous phase velocity leads to the decrease in mass transfer coefficient and in turn leads to the decrease in the activity-recovery, separation efficiency and purification factor. The variation in the activity-recovery, separation efficiency, and purification factor at different conditions are shown in Fig. 4.24 (c), (d) and Fig. 4.25 respectively. In the current study, the minimum continuous phase velocity of 2.91×10^{-5} gave higher recovery, purification factor, and separation efficiency.



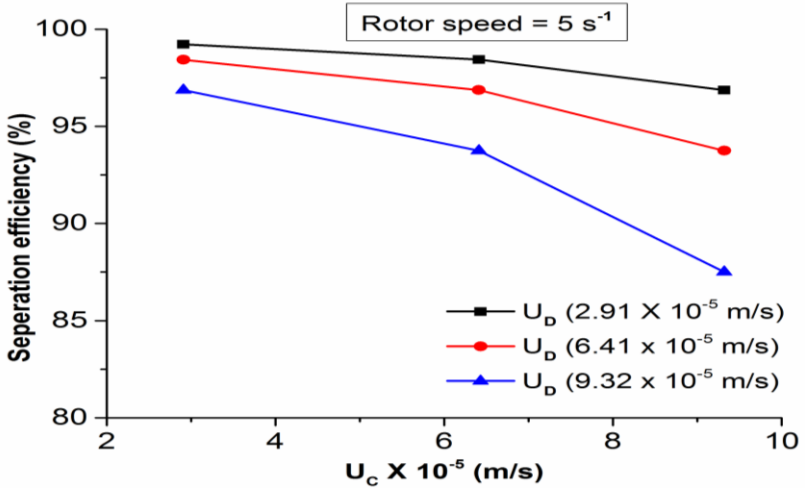
(a)



(b)



(c)



(d)

Fig. 4.24 Effect of Continuous phase velocity (U_C) on (a) holdup (b) mass transfer coefficient (K_Da) (c) recovery and (d) separation efficiency at different dispersed phase velocities (U_C) at constant rotor speed of $5s^{-}$

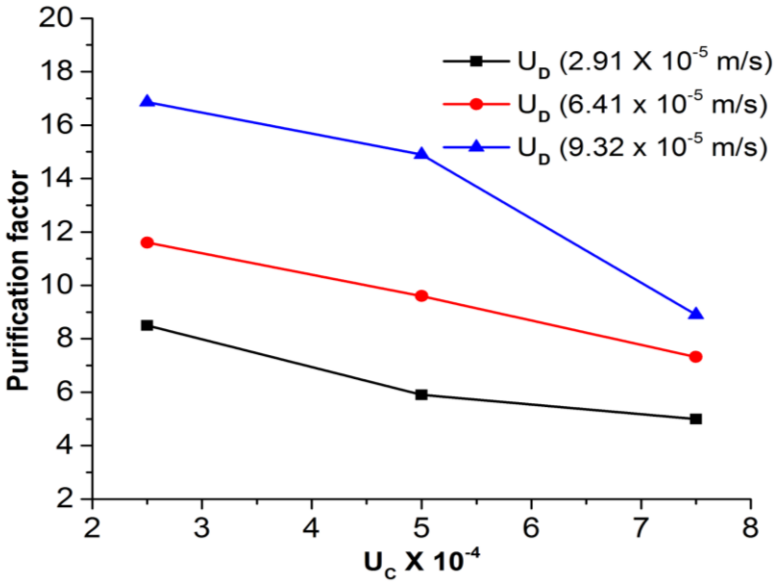


Fig. 4.25 Effect of continuous phase velocity on purification factor at a constant rotor speed of $5s^{-1}$ and at various dispersed phase velocity.

4.3.3 Effect of rotor speed

Rotor speed is one among the prominent variable which affect the performance of agitating column extractors. The effect of rotor speed on dispersed phase holdup, volumetric mass transfer coefficient, recovery, purification factor, and separation efficiency was assessed by conducting experiments with different rotor speeds (2.5, 5 and 7.5 s^{-1}) at varied phase velocities of dispersed phases (2.91×10^{-5}, 6.41×10^{-5} and 9.32×10^{-5} m/s) and continuous phases (2.91×10^{-5}, 6.41×10^{-5} and 9.32×10^{-5} m/s). The phenomenon of drop breakage and coalescence, which affects the dispersed phase holdup and mass transfer coefficient, was controlled by the rotor speed. The dispersed phase holdup was observed to increase with the increase in rotor speed irrespective of the phase velocities of both the phases. At a given rotor speed, the holdup was observed to increase with the increase in dispersed and continuous phase velocity. The observed holdup values for dispersed and continuous phase velocities at $5s^{-1}$ are shown in Figure 4.22 (a) and

4.24 (a). The increase in rotor speed provided the higher phase dispersion. The higher rotor speed provides higher shear stress and increases the drop breakage near the edge of the discs. Hence, a number of drops are formed with a smaller diameter. The settling velocity of these smaller-size drops was lesser since the net gravitational force is lesser on the drops and the drops passes through a large number of obstacles. The decrease in the drop rise velocity increases the number of droplets in the column. Thus the increasing rotor speed increased the dispersed phase hold-up (Sarubbo et al. 2003).

In agitating columns like RDC, turbulence was created when the liquid came in contact with the rotor and the effect increased with an increase in rotor speed. The increase in k_Da with the increase in rotor speed may be attributed to the increased degree of turbulence generated by increasing rotor speed. Thus, the generated turbulence increases the rate of protein mass transfer between the two phases by the following effects:

(i) The thickness (δ) of the diffusion layer around the drop in dispersed phase reduced with the generated turbulence and consequently leads to increase in kDa, KDa= D/ δ

(ii) The generated turbulence created the high shear stress which in turn increases the rapid breakup and coalescence of drops of the dispersed phase. The frequent coalescence and redispersion of drops increases the mass transfer rate.

(iii) The created turbulence helps to form smaller droplets by breaking the larger dispersed droplets. The smaller sized droplets revolve several times inside the compartment along a toroidal path. Thus, increases the interfacial area (A) for the mass transfer according to the Eq 4.1:

$$A = \frac{6\Psi}{d} \qquad (4.5)$$

where, d is the diameter of the drops and Ψ is the dispersed phase holdup.

Contrarily, the lower rotor speed results in a larger drop size which rises quickly through the contact zone thereby giving less interfacial area and low mass transfer of the protein.

The increase in holdup and mass transfer indicates that the turbulence created by the high speed of rotating discs facilitated the extraction of lectin from continuous phase to dispersed phase.

The activity recovery, purification factor and separation efficiency increases with increase in rotor speed and this effect was more pronounced at high dispersed phase velocities. The variation of activity recovery, purification factor and separation efficiency at varied rotor speed and dispersed phase velocities at constant continuous phase velocity were depicted in Fig. 4.26, 4.27 and 4.28, respectively. As explained, the formation of smaller drops and their higher retention time in the dispersion zone which provides higher interfacial area for solute mass transfer by the virtue of turbulence created by the increased rotor speed. The rotor speed is the main parameter which is responsible for the increase in the extraction parameters. In the present study, a maximum recovery, PF and separation efficiency was observed at the dispersed phase of 9.32×10^{-5} and continuous phase flow velocity of 2.91×10^{-5} and at the rotor speed of 7.5 s^{-1}. The maximum rotor speed and dispersed phase velocity with minimum continuous phase velocity favors the efficient of Psl in the RDC implemented in the present study.

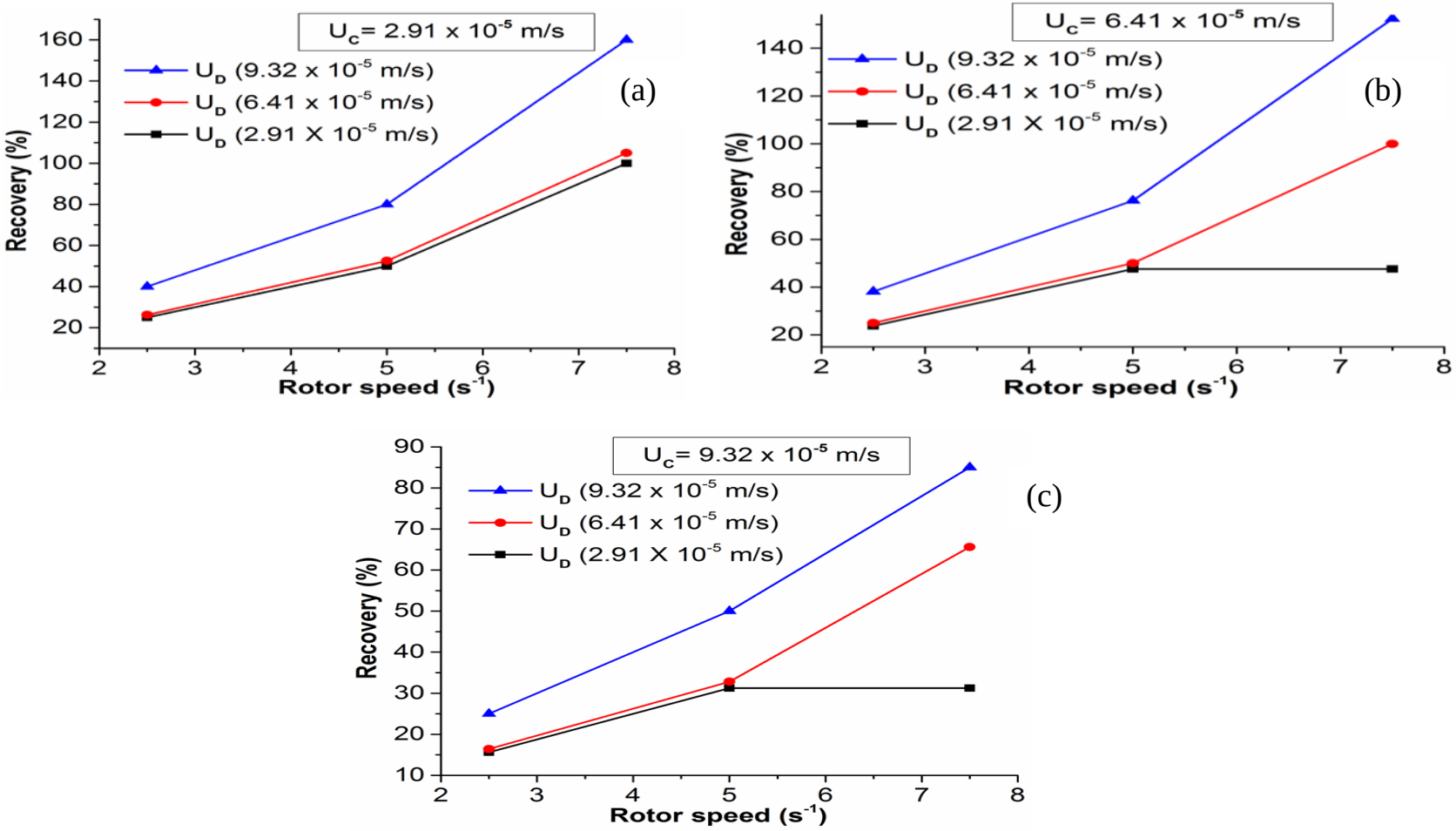


Fig. 4.26 Effect of rotor speed on recovery at different dispersed phase velocities (U_D) and at constant continuous phase velocities (U_C) of (a) 2.91 x 10^{-5} (b) 6.41 x 10^{-5} (c) 9.32 x 10^{-5}.

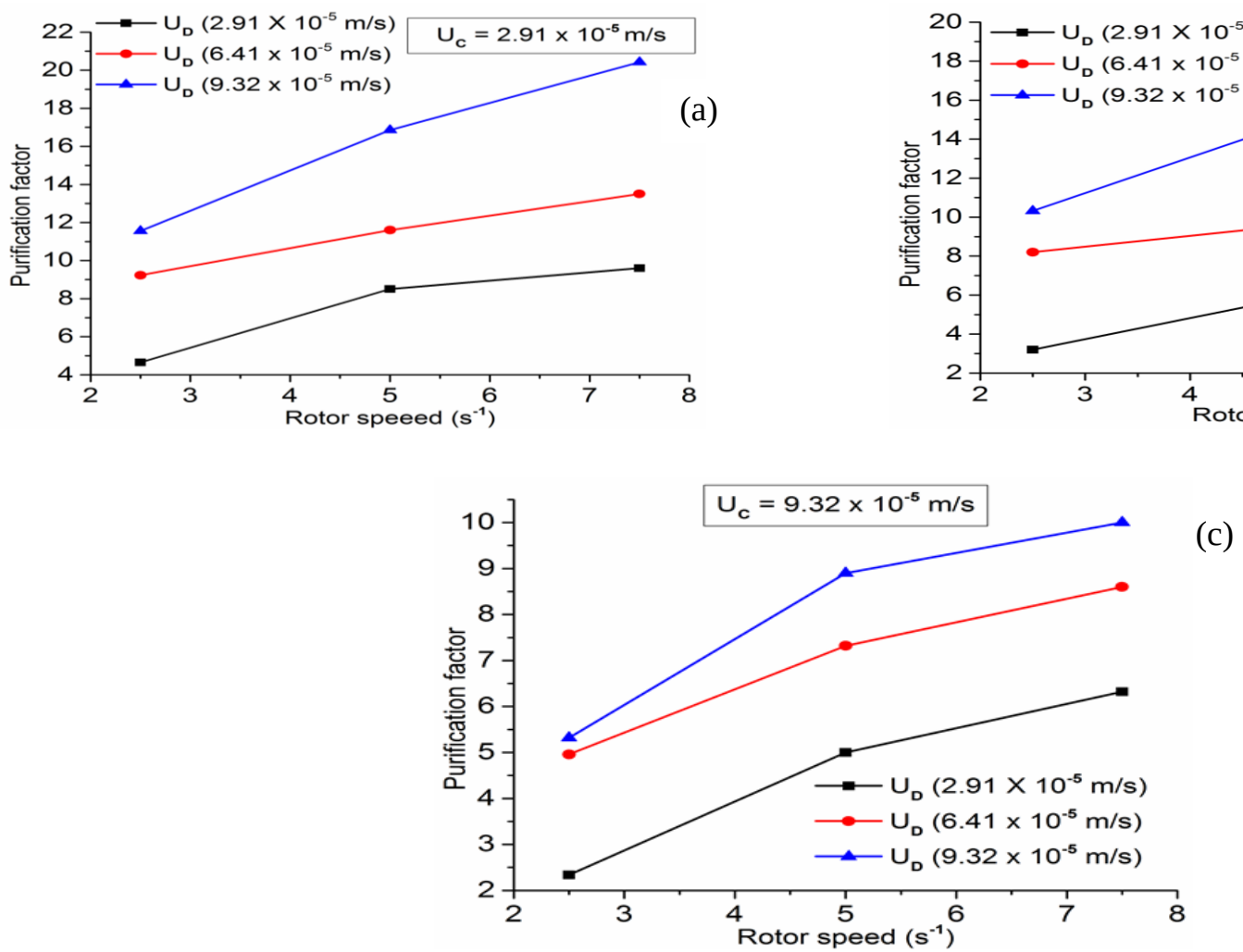


Fig. 4.27 Effect of rotor speed on purification factor at different dispersed phase velocities (U_D) at constant continuous phase velocities (U_C) of (a) 2.91 x 10^{-5} (b) 6.41 x 10^{-5} (c) 9.32 x 10^{-5}.

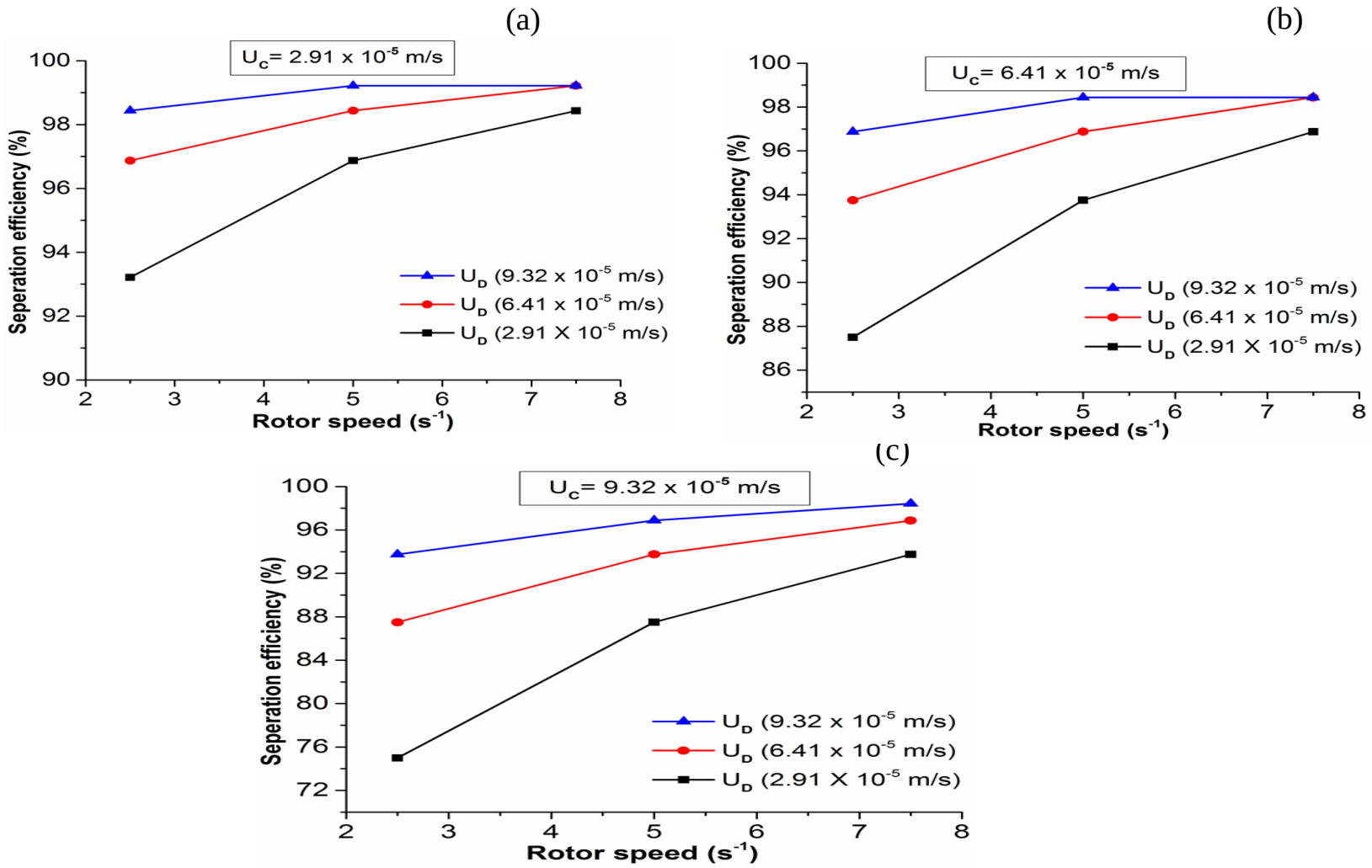


Fig. 4.28 Effect of rotor speed on separation at different dispersed phase velocities (U_D) at constant continuous phase velocities (U_C) of (a) 2.91 x 10^{-5} (b) 6.41 x 10^{-5} (c) 9.32 x 10^{-5}

4.4 ARTIFICIAL NEURAL NETWORK MODELING OF THE AQUEOUS TWO PHASE EXTRACTION OF *PISUM SATIVUM* LECTIN

First and foremost, step in the ATPE is the understanding of equilibrium characteristics of the phase forming components and the partitioning characteristics of the solutes in the phases. However, the equilibrium of the solute in a selected system will be explained through the partition coefficient which in turn depends on the equilibrium concentrations of phase components. Solubility of the desired solute in the phases is governed by the concentration of phase forming components; therefore, the partition prominently depends on the equilibrium concentration of the phases. The equilibrium concentration of phase components was obtained through direct measurement to construct the phase diagram. However, the experimental procedure will be tedious, time consuming and expensive. This motivates the researchers to develop reliable modeling processes which can be used to achieve required phase equilibrium information by reducing the number of experiments. Many attempts have been made in the area of ATPS to develop a suitable model to predict the phase equilibrium. The theoretical and empirical models were used to model the phase equilibrium data. The theoretical models were usually based on thermodynamics concept. In the literature, many theoretical predictive models are used to predict the phase behavior of the ATPS. The categorization of these models relies mainly on the basis of their development; Flory Huggins models based on the extension of lattice theory, Osmotic virial equations based on the solvents osmotic pressure, models developed based on integral equation theory, and other few models such as excluded volume approximations and group contribution schemes which do not fall under any of the above categories.

The modeling of phase equilibrium was studied by many researchers using the above said theoretical modeling procedures. However, the modeling of partition coefficient of a solute along with the phase equilibrium and the studies on modeling of the selective extraction process of desired protein from the crude source is very scarce. It's a very complex process to model the partition coefficient of the desired protein using theoretical modeling, where the partition depends on many relative complex forces. Hence the

application of ANN, a soft computing modeling method, was applied in the present study to model the extraction of Psl. Multilayer feed forward neural network was chosen as it has the capability to solve complete nonlinear data beyond the second order equations. The first step of ANN modeling was to optimize a neural network with the aim of obtaining an ANN model with minimal dimension and minimal errors during training and testing.

Modeling in the current study is divided into two sections. The first section included the modeling of pure protein studies which contains the effect of three different citrate salts and four different PEG molar mass effects on phase behavior and partition of Psl. Mainly, the phase diagram which includes equilibrium concentrations and binodal of all the three citrate salts (sodium, potassium and ammonium citrate) and molar mass of PEG (2000, 4000, 6000 and 8000) were modeled along with the partition of the Psl lectin in selected phase compositions. Once the influence of salts and molar mass of PEG were modeled from the first section, the second section includes the modeling of the selective extraction process of Psl from the crude pea seed extract. The independent and dependent factors of the central composite design were considered as input and output to the second model, respectively. The neural network tool in MATLAB R2018A was used for the modeling of ANN.

4.4.1 Modeling of the influence of citrate salts and PEG molar masses on phase behavior and partition of pure Psl.

The selection of appropriate inputs and outputs is very essential to build an application-oriented ANN model. The phase diagram is the blueprint to design any ATPE process, where it consists of the binodal curve and the equilibrium concentration data. The experimental data of phase equilibrium compositions were graphed to get a phase diagram. The partition of a biomolecule is prominently influenced by the phase equilibrium concentration of phase components. Hence, the factors on which the phase equilibrium compositions depend can be considered as input factors. Some of the factors which affect the phase diagram of PEG/Salt system were the type of salts, molecular

weight, pH, temperature (Silverio et al. 2012). In this study, the effect of type of salts and the PEG molar mass at 25°C were determined experimentally and the same experimental data were considered to simulate an extraction process of Psl. The data from one factor at a time (pure Psl partition study) was considered to simulate an extraction process of Psl. In order to investigate the influence of different salts and molar masses on the phase behavior and partition of a biomolecule, the modeling of the phase diagram is of at most importance. Three citrate salts, namely sodium citrate, potassium citrate, and ammonium citrate with four different PEG molar masses (2000, 4000, 6000, 8000 g/mol) were considered and the effect of these variables on phase equilibrium, and in turn, on the partition of Psl was considered to model the process using ANN.

In PEG/salt ATPS, salts affect the partition of a protein majorly through the salting out process. The salting out property differs with the type of salt based on the cation and anions it possesses. All the three salts chosen in the present study have similar anion and differ in their cation (Na^+, K^+, and $NH4^+$). Hence, the property possessed by the cation is majorly influencing the difference in salting-out capacity of the three salts. The research shows that the effect of salts on two phase formation can be attributed to the size and charge of the salts (Raja and murthy 2012). For ions which possess the same charge number, their charge density depends on the ionic radius of the ion. It was stated that the lesser the ionic radius of the cation, the higher the charge density; therefore, more ability to attract the water molecules through coulombic interaction (Zafarani-Moattar and Gasemi 2002). Thus the solubility of the PEG reduces and the phase formation happens more easily. In case of $Na_3C_6H_5O_7$ (sodium citrate), $K_3C_6H_5O_7$ (potassium citrate), and $N_3C_6H_{17}0_7$ (ammonium citrate), the ionic radius of the cations is in the order of Na^+ (0.098 nm) < K^+ (0.133 nm) < $NH4^+$ (0.137 nm) (Ananthapadmanabhan and Goddard 1987). Hence, the cationic radius of these ions can be considered as inputs.

The selection of suitable PEG molar mass also has a prominent role in the partitioning of the biomolecule. As stated earlier, the PEG molar mass has a prominent influence on the binodal curve and thus on the phase equilibrium concentrations, and in turn on the

partition of the biomolecule. As the PEG molar mass increases, the hydrophobicity of the PEG phase increases and hence it affects the Psl partition (Rathnasamy et al. 2014). Apart from these factors, the equilibrium composition varies with the feed compositions. Hence the phase compositions of different citrate salts and PEG molar masses were considered as input factors.

Finally, to teach the model about the influence of salts and molar masses on phase behavior and the partition of Psl, the extraction process was modeled by considering ionic radius of cation of SC,PC and AC, different molar masses (2000,4000,6000,8000 g/mol) and different feed compositions as input variables. phase equilibrium concentrations (PEG in top and bottom phase, salt in top and bottom phase) and partition coefficient of Psl (K_{Psl}) as output variables.

The schematic architecture of ANN considered for the modeling of ATPE process considering different citrate salts and PEG molar mass is depicted as Fig. 4.29.

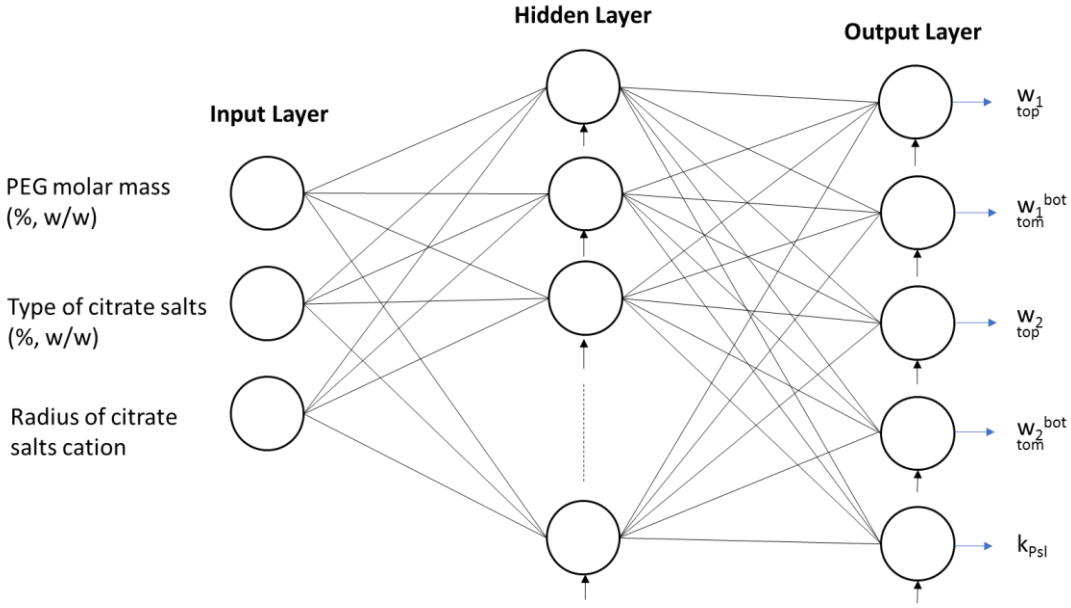


Fig. 4.29 Architecture of ANN for modeling of effect of citrate salts and molar mass on phase behavior and partitioning of Psl in PEG/salt system.

4.4.1.1 Categorizing the experimental data

The normalized input and output data sets were used to optimize the structure of an artificial neural network. The selected input and the output values were scaled to value between 0 to 1. Normalization process helps in fast learning process and computation. (Kumar et al. 2002). The Eq. 4.2 is used to normalize actual experimental value (Ismail et al. 2014)

$$X_n = \frac{X_{actual} - X_{min}}{X_{max} - X_{min}} \quad (4.2)$$

To denormalize the normalized value following Eq. 4.3 was used which was derived from the manipulation of Eq.4.2

$$X_{actual} = X_n (X_{max} - X_{min}) + X_{min} \quad (4.3)$$

where, X_{actual} is the actual value of the X, X_n is the value after normalization, X_{min} and X_{max} is the minimum and maximum value of X in the data.

The experimental phase-equilibrium data of various feed compositions of different molar masses and citrate salts were used to train the model about the relationship between the input and output data in order to obtain an optimal ANN architecture. Totally 48 sets (210 data points) of experimental data (which includes phase-equilibrium concentrations of PEG and citrate salts and partition coefficient of Psl) were considered. These datasets were divided into two sets, 60% of the data (126 data points) was used to train the modeling and 40% of data (84 data points) was used for validation. The former dataset was used to train the ANN model and determine the weights and biases; the latter was used to validate the predictive ability of the ANN model. While training, ANN can be disturbed by the over fitting problem thereby hindering its ability to accurately predict the data points excluded from the training set. In order to solve this problem, the test data can be provided to the network training in such a way that the training is terminated when the prediction of the test set tends to be “bad”. As a result, the initial validation set was further sub-divided into a test set (20% data sets) and validation set (20% data sets).

Moreover, to guarantee a high quality of learning, the dataset of all the three types of salts and molar mass of all the PEG was uniformly distributed among the three datasets. The experimental data along with the ANN predicted output was represented in the Table A3.

4.4.1.2 Training and validation of neural network to obtain the suitable structure

The number of neurons in the input and the output layer should correspond to those of the variables in these layers. Besides, the number of neurons in the hidden layer, and the system error are also important to build an effective ANN model. The model will fail to converge if there are too few neurons in the hidden layers. Conversely, if there are too many neurons in the hidden layers, the model will become a complicated structure of the network leading to slow convergence rather than high precision (Basheer et al. 2000). The system error is closely related to the calculation accuracy of the model and the generalization ability of the network (which is affected by the over fitting problem). For the phase equilibrium and partition coefficient data investigated, it was observed (Table 4.7) that the calculation accuracy of the predicted data monotonically increases with the decreasing system error. However, it was also observed that when the system error is very little, the accuracy of the test set starts to decrease, suggesting an over fitting problem. The convergence rate of the model is affected by a decrease in the system error. Hence, in order to obtain a moderate convergence rate, the topology which leads to over fitting was omitted. The mean square error (MSE) was used to assess the calculation accuracy. A lower MSE means higher accuracy.

The number of hidden layer neurons in the ANN model was studied based on the neurons in the input and output layers and the system error. From Table 4.4 it can be observed that a network with too few hidden layer neurons will not converge and a network with too many of them will not have high precision. The topology of the network can be represented with the corresponding neurons in the corresponding layers i.e. (neurons in input layer- neurons in hidden layer-neurons in output layer)**.** Table 4.7 depicts the topology of the network with corresponding MSE and R^2 value.

Table 4.7 Evaluation of the ANN predicted values by means of MSE and R^2

Network topology	W_{PEG}^{Top}		W_{PEG}^{Salt}		W_{Salt}^{Top}		W_{Salt}^{Bottom}		K_{Psl}	
	MSE	R^2	MSE	R^2	MSE	R^2	MSE	R^2	MSE	R^2
(3-3-1)	0.032	0.868	0.043	0.789	0.047	0.820	0.038	0.796	0.045	0.821
(3-4-1)	0.035	0.885	0.046	0.854	0.035	0.865	0.321	0.856	0.039	0.865
(3-5-1)	0.021	0.875	0.026	0.862	0.036	0.875	0.026	0.899	0.028	0.921
(3-6-1)	0.019	0.921	0.014	0.912	0.021	0.903	0.019	0.956	0.012	0.935
(3-7-1)	0.016	0.812	0.019	0.984	0.042	0.812	0.019	0.842	0.039	0.921
(3-8-1)	0.023	0.900	0.045	0.932	0.023	0.923	0.023	0.923	0.035	0.903
(3-9-1)	0.018	0.867	0.042	0.912	0.031	0.854	0.024	0.821	**0.006**	**0.989**
(3-10-1)	**0.009**	**0.987**	0.031	0.862	**0.016**	**0.978**	0.031	0.803	0.038	0.932
(3-11-1)	0.023	0.789	0.038	0.769	0.043	0.785	0.048	0.801	0.035	0.768
(3-12-1)	0.021	0.862	**0.012**	**0.965**	0.047	0.845	**0.010**	**0.972**	0.030	0.825
(3-13-1)	0.018	0.895	0.027	0.894	0.035	0.893	0.038	0.896	0.285	0.864
(3-14-1)	0.012	0.921	0.020	0.902	0.023	0.932	0.029	0.912	0.020	0.923
(3-15-1)	0.012	0.835	0.015	0.935	0.010	0.951	0.021	0.945	0.012	0.941
(3-16-1)	0.014	0.875	0.036	0.862	0.019	0.872	0.043	0.865	0.016	0.856
(3-17-1)	0.012	0.932	0.023	0.925	0.014	0.962	0.012	0.923	0.009	0.935
(3-18-1)	0.018	0.894	0.027	0.896	0.023	0.900	0.024	0.900	0.012	0.921

4.4.1.3 The effects of factors on the phase behavior of ATPS based on the suggested model

The influence of the type of citrate salts and PEG molar mass on the phase behavior of ATPS and the Psl partitioning behavior was investigated. The ANN models developed for the purpose were evaluated for their ability and accuracy to simulate the process. The simulated effect of sodium, potassium, and ammonium citrate on the phase behavior is graphically depicted in Fig. 4.30 (a), (b), and (c) respectively. The binodal and the tie

lines were successfully modeled for different type of citrate salts and PEG molar mass such as PEG 2000-SC/PC/AC, PEG 4000- SC/PC/AC, PEG 6000 – SC/PC/AC and PEG 8000- SC/PC/AC and the phase diagram of PEG 4000 – SC/PC/AC was depicted in the Fig. 4.30 (a), (b), and (c) respectively. It was observed that the SC was closer to origin followed by PC and AC. The two phase region was observed to expand in the order of $Na^+ > K^+ > NH_4^+$. The concept of increasing biphasic region with decreasing salt cation size, which also implies that the salting-out ability of salts increases with a decreasing cationic size, was successfully simulated by the model as shown in Fig. 4.33.

The influence of PEG molar mass on phase behavior was simulated and compared with the experimental data. The effect of various molar masses of PEG (2000, 4000, 6000 and 8000 g/mol) with sodium citrate salt was shown in Fig 4.31. The model developed was successful in simulating the effect of molar mass, where the two-phase region expanded with a high molar mass of PEG (Fig. 4.34). It was also observed from the figure that lesser concentration of phase components is required for the phase formation of a higher molar mass of PEG. The simulated phase diagram also follows the trend of higher molar mass PEG binodal being closer to origin and the lower molar mass binodal being away from the origin. It is evident from the (Fig. 4.34) that the ANN predicted binodal and tie line data were successful in portraying the nature of experimentally determined phase diagram with higher accuracy. The concept of the expansion of biphasic region by the movement of the binodal curve towards origin with increasing molar mass of PEG, and thus consuming less concentration of phase components for the formation of two phases, was also simulated successfully. The prediction accuracy of the partition coefficient of Psl was shown in Fig. 4.32 and observed a good agreement with the experimental K_{Psl}, as the predicted and experimental partition coefficient data lie almost on the line showing goodness of fit.

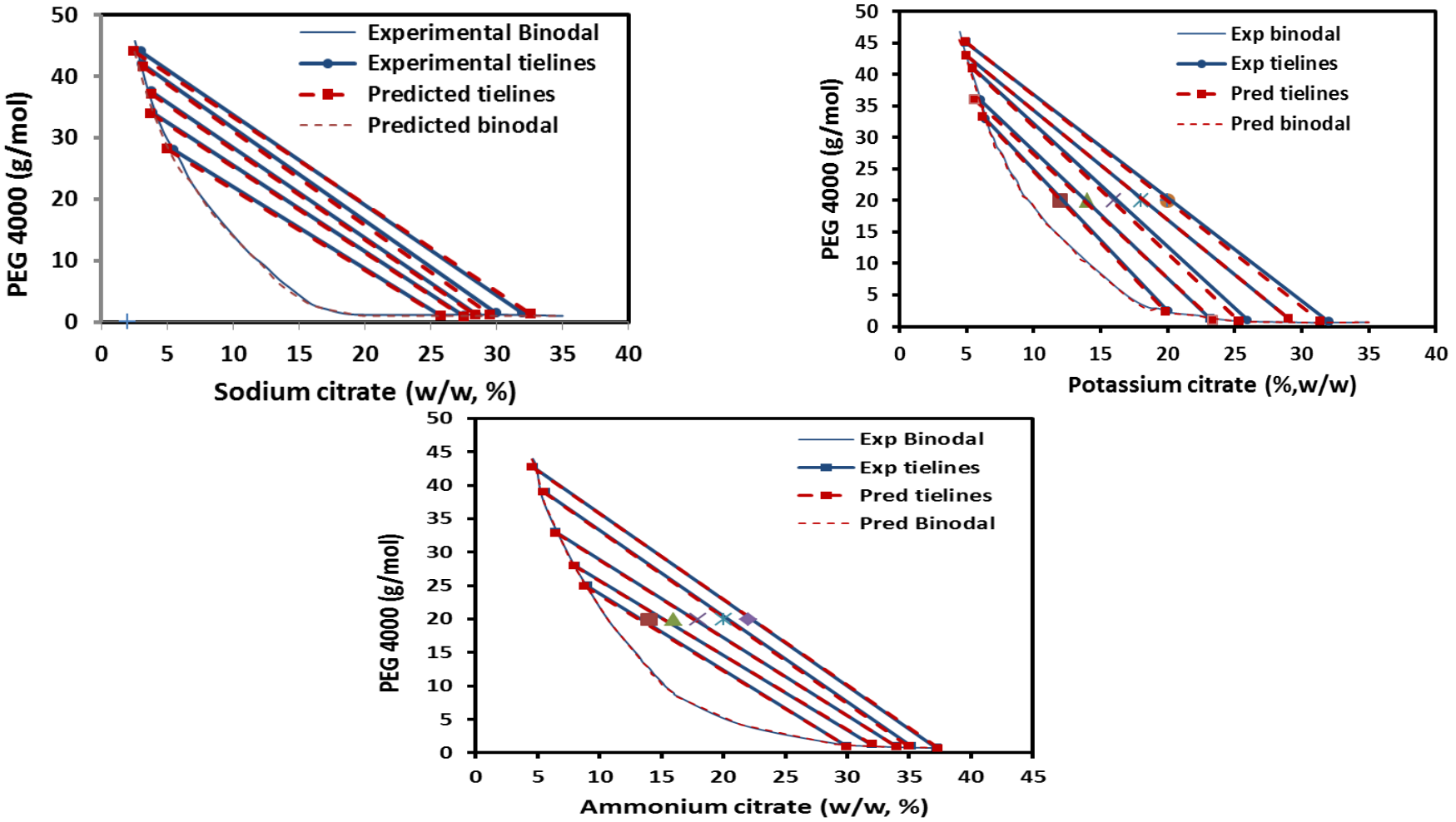


Fig. 4.30 Comparison of the experimental and ANN predicted binodal curves and tie lines (a) PEG 4000/Sodium citrate (b) PEG 4000/potassium citrate (c) PEG 4000/ammonium citrate.

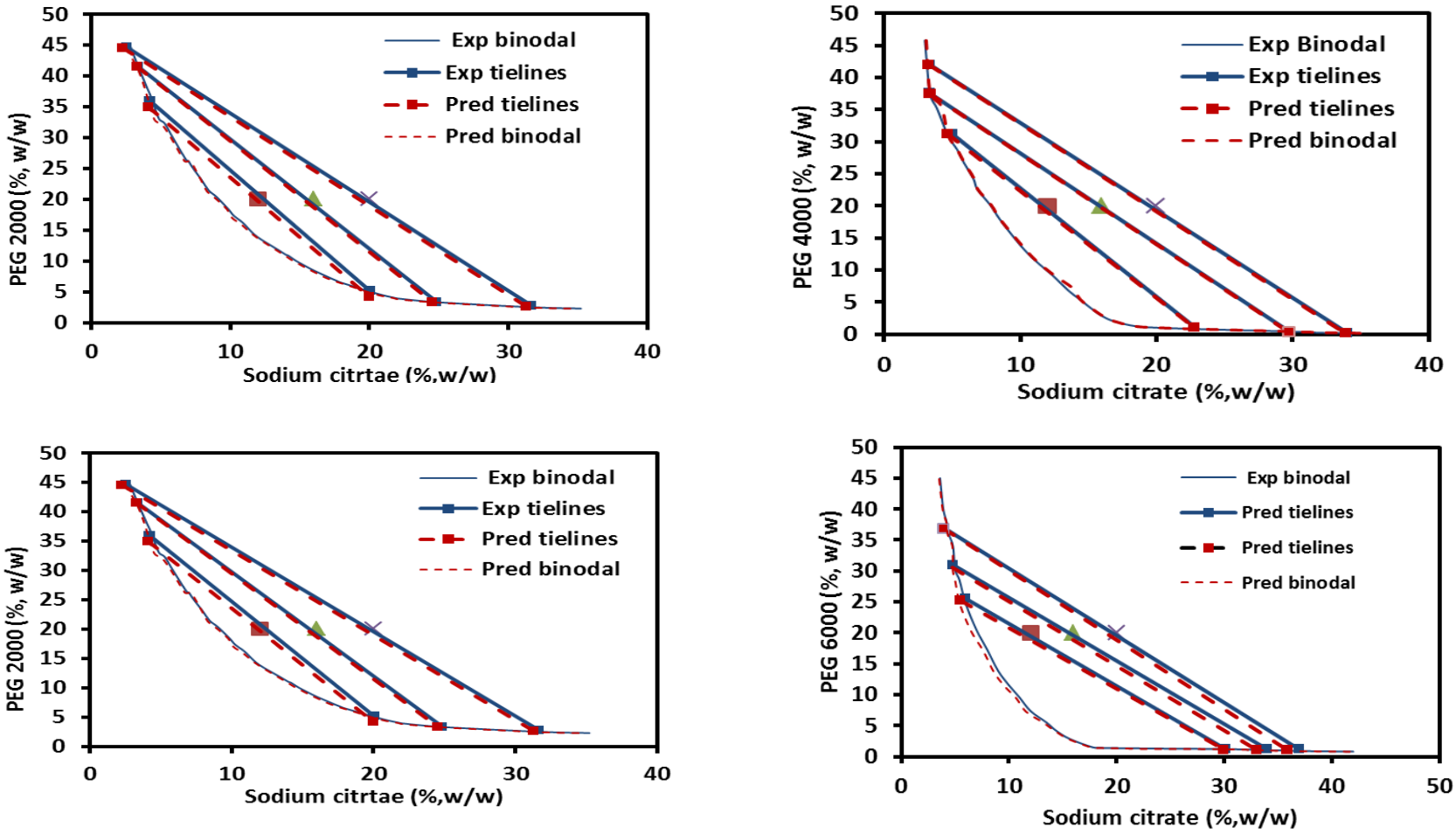


Fig. 4.31 Comparison of the experimental and ANN predicted binodal curves and tie lines (a) PEG 2000/Sodium citrate (b) PEG 4000/sodium citrate (c) PEG 6000/sodium citrate (d) PEG 8000/sodium citrate.

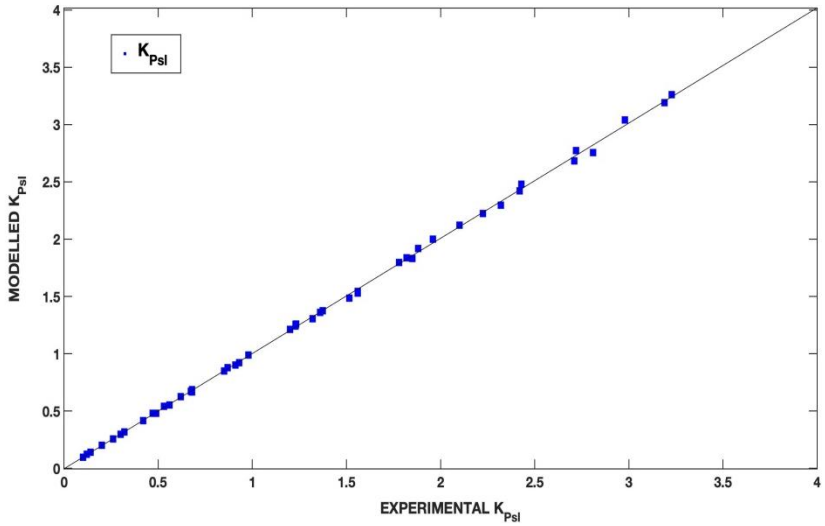


Fig. 4.32 Scattered plot showing the deviation of experimental and ANN simulated partition coefficient of Psl (K_{Psl}).

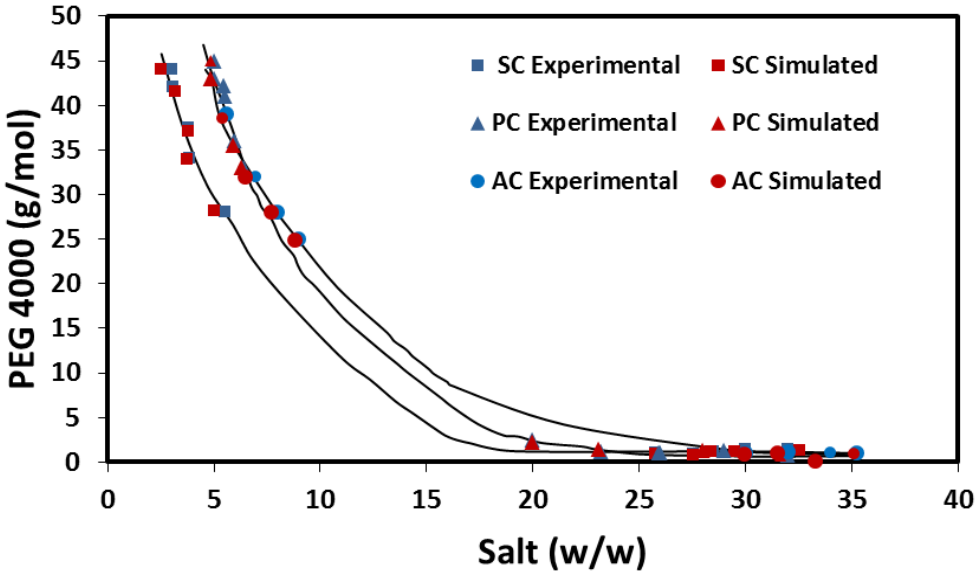


Fig. 4.33 Prediction of the behavior of binodal curves based on the Investigation of the effect of type of salts on binodal curves (using both experimental and predicted values.

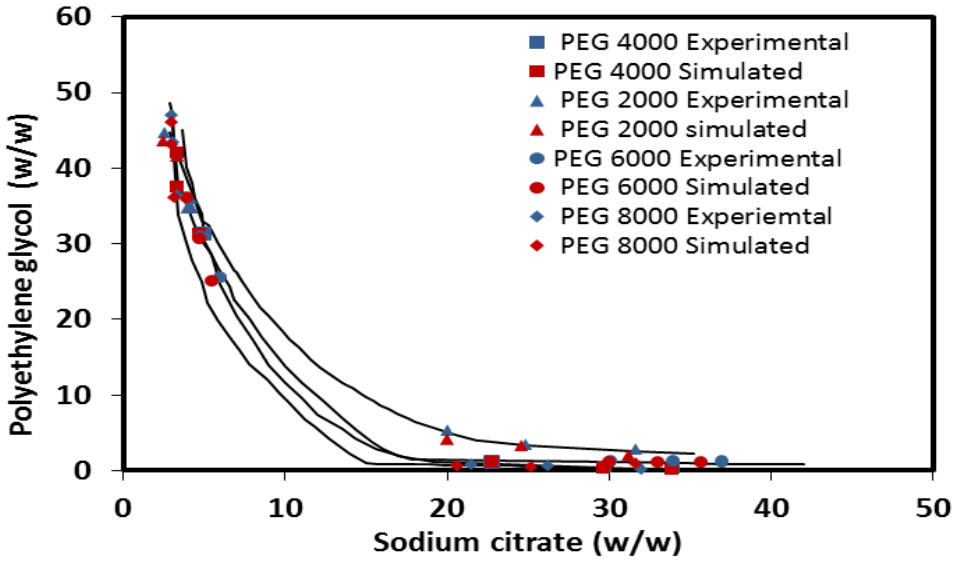


Fig. 4.34 Investigation of the effect of molar mass on binodal curves (using both experimental and predicted values)

4.4.2 Artificial neural network modeling of selective extraction of *Pisum sativum* lectin from its seed crude extract.

The design of experiments and their respective responses and input factors were used for training the network. The feed forward network has four input nodes and three output nodes (Fig. 4.35). The inputs chosen in this study are PEG 6000 concentration, sodium citrate salt concentration, NaCl and pH, while the outputs are activity yield, purification factor and specific activity partition coefficient. The data portioning (training set, testing test and validation set) had been done to avoid over-training and over-parameterization. Out of 30 experimental datasets, 70% of the data was considered for training, 15% of data was considered for validation and testing purpose. The Levenberg–Marquardt back-propagation algorithm (LMA) was applied for the network training. The optimal numbers of neurons in the hidden layer of the neural network are investigated by varying the number of neurons in the hidden layer and also for various combinations of ANN-specific parameters like learning rate and initialization. Each neuron in the hidden and output layers first calculates the weighted sum of its inputs and passes the result through

a transfer function to produce an estimate as output that corresponds to the input dataset. The result is compared to the corresponding desired values and the error is back–propagated through the network to adjust the connection weights according to the learning rule. The procedure is repeated iteratively, until the predetermined target MSE (mean square error) is reached.

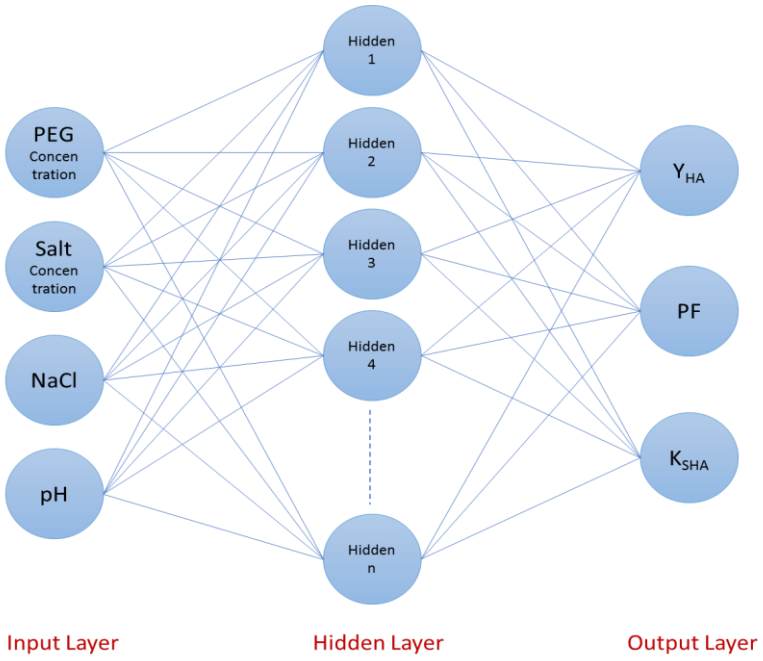


Fig. 4.35 Architecture of ANN used in present study

4.4.2.1 Artificial neural network analysis of extraction of Psl lectin

Although it is important to select the optimal number of hidden neurons carefully, depending on the type and complexity of the task, this usually has to be done by trial and error. An increase in the number of hidden neurons up to a point usually results in a better learning performance. Too few hidden neurons limit the ability of the neural network to model the process, and too many may allow too much freedom for the weights to adjust and thus, to result in learning the noise present in the database used in training. The effect of number of hidden neurons on the goodness of fit was tested. In examined cases, the optimum number of hidden neurons varies with the response. The best ANN chosen in

the present work has the topology of 4-9-1 for hemagglutination activity yield, 4-8-1 for purification factor and 4-9-1 for specific hemagglutination activity (Fig. 4.36).

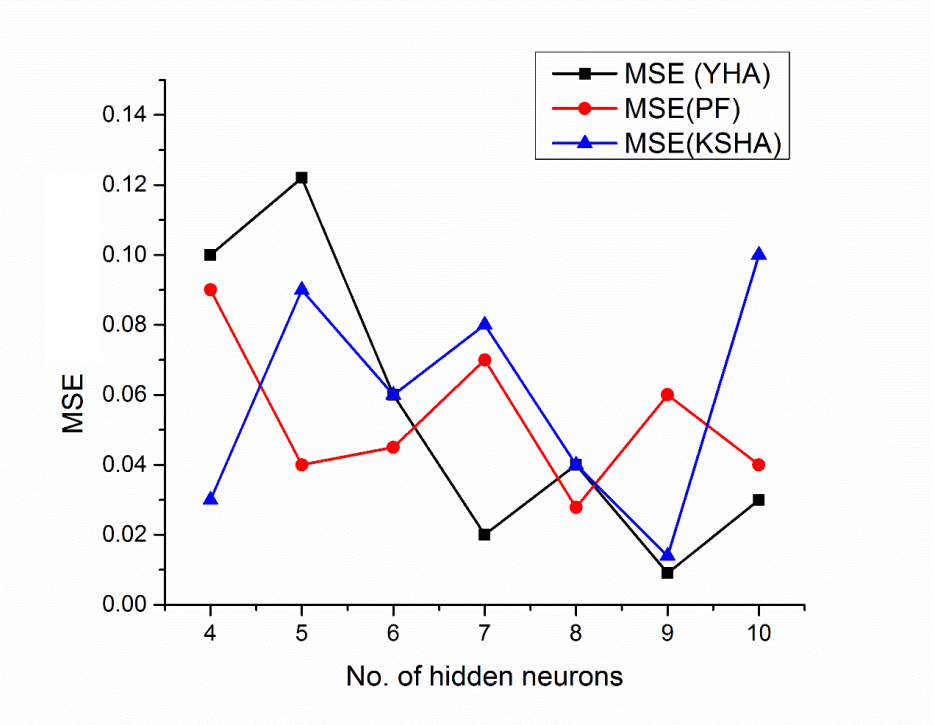


Fig 4.36 Effect of no. of hidden neurons on MSE (mean square error)

70% of the data was considered for training, 15% of data was considered for validation and testing purpose. Number of hidden neurons in the hidden layer were optimized by trial and error method based on the highest regression coefficient close to 1 and least mean square error nearer to zero during the modeling process. The considered responses such as activity yield was modeled with a high R^2 of 0.99 and MSE of 0.009, purification factor with 0.978 R^2 and 0.027 MSE and specific hemagglutination activity partition coefficient has simulated with R^2 of 0.989 with 0.014 MSE (Fig. 4.37). The scattered plot of all the responses was plotted and it was observed that all the responses gave an overall good fit. (Fig. 4.38).

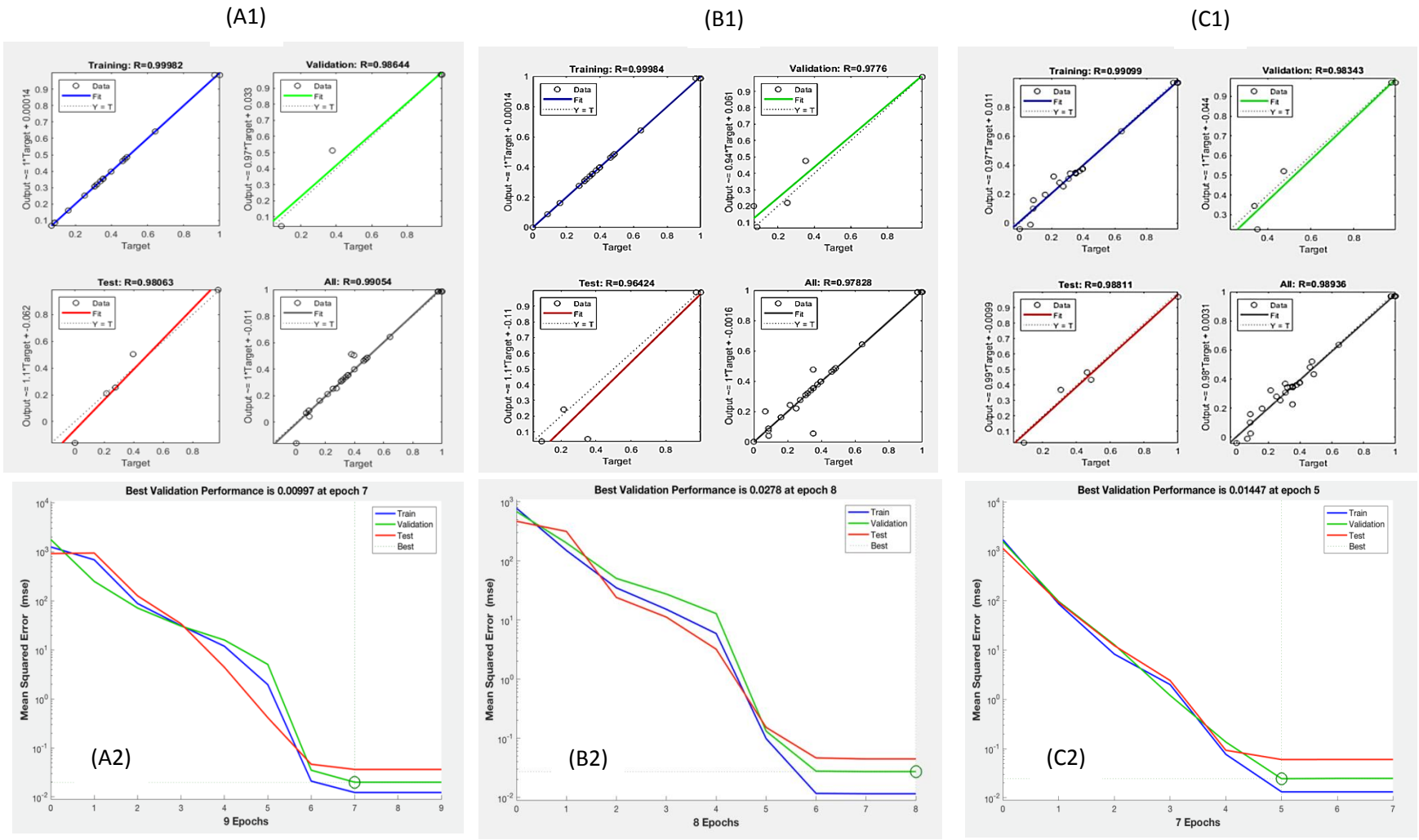


Fig. 4.37 Regression plot and Performance plot for Y_{HA} (A_1), PF (B_1), K_{SHA} (C_1) and Y_{HA} (A_2), PF (B_2) and K_{SHA} (C_2) for training, validation and testing respectively.

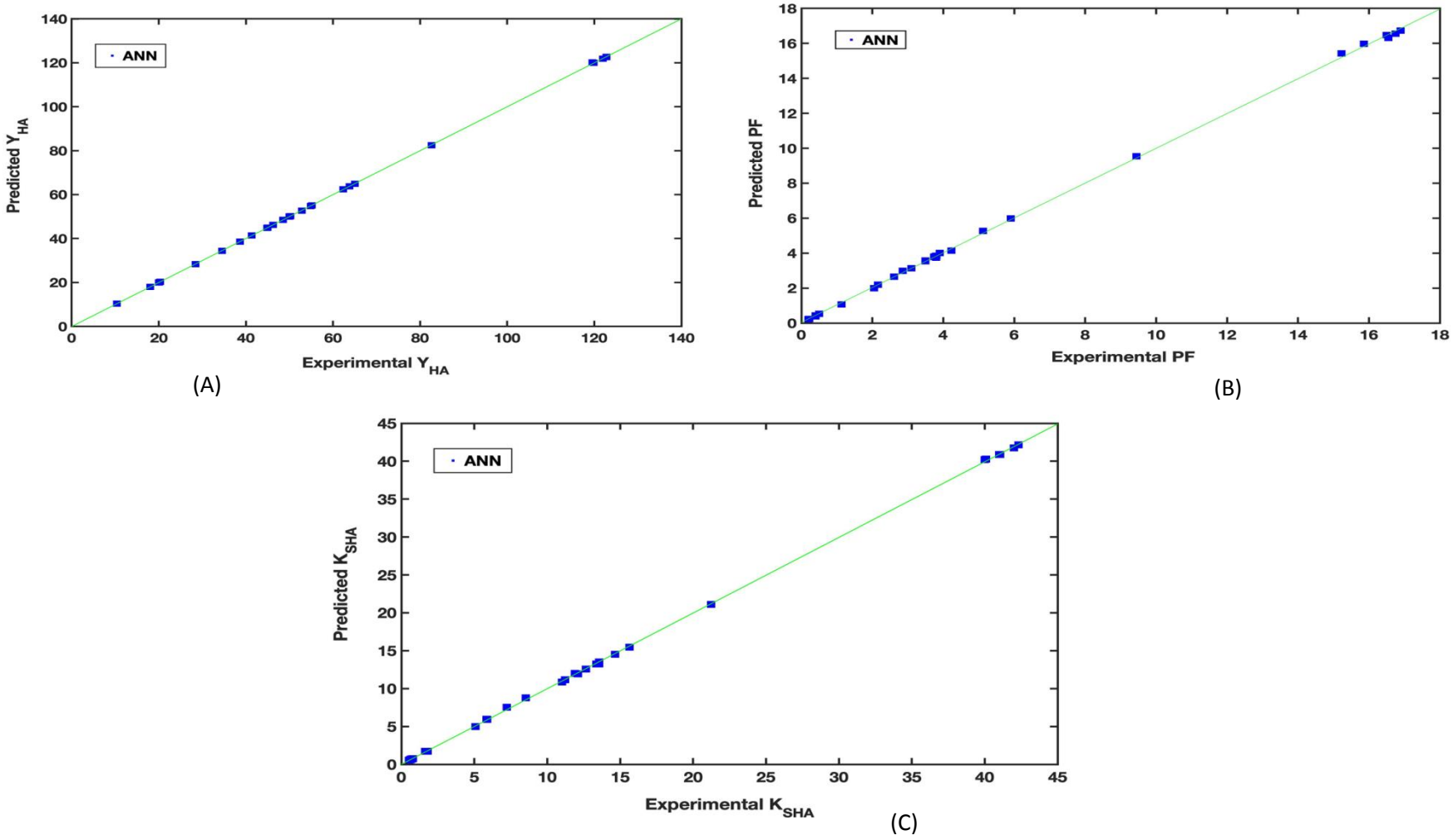


Fig. 4.38 Comparison of the experimental and predicted results in scatter plots(A) Hemagglutination activity yield (Y_{HA}), (B) Purification factor (PF), (C) Hemagglutination specific activity partition coefficient (K_{SHA}).

CHAPTER 5

5. SUMMARY & CONCLUSION

5.1. SUMMARY

PEG – Citrate salts based Aqueous two phase system was evaluated for the extraction of Psl from *P.sativum* seed crude extract. The suitable system components for the partitioning of the Psl to PEG phase was chosen based on the partition coefficient of pure Psl. The influence of type of citrate salts, PEG molar masses, concentration of phase components, additive concentration, pH and volume ratio on partitioning of pure Psl was studied. Further the one factor at a time experimental design similar to pure Psl studies along with the crude load was evaluated for the selective extraction of Psl from its crude extract of seed. The performance of the various process variables in the selective extraction of Psl was assessed by hemagglutination activity yield (Y_{HA}), purification factor (PF), specific hemagglutination activity partition coefficient (K_{SHA}) and total protein partition coefficient (K_p).The optimum conditions for the extraction of Psl were obtained from RSM studies. Continuous ATPE has also been done in RDC. Modeling of the extraction process of Psl was attempted using Artificial neural network. The important results obtained in the present study are summarized.

Part one: Partitioning studies of pure Pisum sativum lectin (Psl) in PEG/citrate ATPS

- The influence of phase forming citrate salts on the partitioning was studied in PEG 4000 – trisodium/tripotassium and triammonium citrate salt systems and the maximum partition of Psl to the PEG phase was observed in PEG-4000-sodium citrate.
- The Psl partition into the top phase was found to increase with increasing molar mass up to 6000 and a further increase in molar mass to 8000 resulted in lesser Psl partitioning. Addition of NaCl to system increases the partition of the Psl from bottom phase to top phase.

- The efficient partitioning of Psl to top phase was observed at slightly basic pH of 8 where Psl can maintain its dimer form. pH less than 6 and above 8 were not favorable for the efficient Psl partitioning due to the leaching of metal ions and dissociation of Psl dimers.
- The effect of volume ratio was varied along the tie line (TLL of 40.23%), which shows the maximum partition of Psl, and it was observed that K was almost constant and the decreased yield at very low volume ratio and increased yield with increasing volume ratio was observed.

Part two: Investigation and optimization of crucial factors for the selective extraction of *Pisum sativum* lectin from its seed crude extract.

- Among the three citrate salts studied, sodium citrate showed higher selectivity of Psl towards the PEG phase in comparison with other two citrate slats which was confirmed by the higher K_{SHA} (10.376) exhibited by sodium citrate.
- PEG molar mass of 6000 gave maximum K_{SHA} and PF in comparison to 2000, 4000 and 6000.
- The concentration of phase components was screened based on the Y_{HA}, PF, and K_P. 18% of PEG with 16% of sodium citrate was found to give a maximum Y_{HA} and PF with $K_P < 1$. The effect of TLL was also studied and it was found that the increase in TLL up to 41.01% increased the Y_{HA} and PF.
- The addition of NaCl of 2% gave a sweeping increase in the $Y_{HA,}$ however, the selectivity was lost at the higher NaCl concentration (more than 2%) since other proteins are also attracted towards the top phase which was confirmed by $K_P>1$ and low PF
- It was noticed that the present system can have a crude loading capacity of 20% and above which leads to the loss of selectivity.
- The interactive effect of the factors (PEG and sodium citrate concentration, NaCl, pH) on the desired responses (Y_{HA}, PF, and K_{SHA}) was analyzed using RSM.

- Optimum conditions for different goals were obtained through desirability approach using the developed regression models. Conditions obtained for maximum activity recovery of 126.54 and a purification factor of 16.15 for Psl are 18.53% PEG 6000/15.44% sodium citrate at pH 7.55 with the addition of 2.16% of NaCl.
- The models were validated by conducting experiments at the optimum conditions. The experimental values are comparable with the predicted ones.

Part three: Continuous extraction in Rotating disc contactor.

- Continuous ATPE was attempted in RDC and the effect of phase velocities and rotor speed on the activity recovery, purification factor and separation efficiency was studied.
- Increase in dispersed phase velocity was resulted in the increase of dispersed phase holdup, Psl activity recovery and volumetric mass transfer coefficient.
- Continuous ATPE was attempted in RDC and established that ATPE of Psl to the PEG phase can be enhanced by increasing the dispersed phase velocity and rotor speed while operating the reactor at less continuous phase velocity.
- The rotor speed plays a major role in improving the mass transfer of Psl to the PEG rich phase. At low rotor speed, activity recovery and purification factor increases moderately whereas at high rotor drastic increase was noticed.
- Maximum dispersed phase mass transfer coefficient (0.069 s^{-1}), recovery (160 %) and purification factor of 20.42 were achieved at U_d 9.32 x 10^{-5} m/s, U_c 2.91 x 10^{-5} m/s and at a rotor speed of 7.5 s^{-1}.

Part four: Modeling of extraction of psl using Artificial neural network

- The extraction process of Psl starting from the phase diagram to selective extraction of Psl was modeled using the ANN technique by utilizing the experimental data generated in the present study.

- The optimized topology of (3-10-1), (3-12-1) and (3-9-1) for equilibrium concentration of PEG and salt in top phase, equilibrium concentration of salt and

PEG in bottom phase and partition coefficient of pure Psl respectively gave low MSE values and high R^2 values which shows the better ability of prediction.

- The best ANN chosen in the present work has the topology of 4-9-1 for hemagglutination activity yield, 4-8-1 for purification factor and and 4-9-1 for specific hemagglutination activity. The considered responses such as activity yield was modeled with a high R^2 of 0.99 and MSE of 0.009, purification factor with 0.978 R^2 and 0.027 MSE and specific hemagglutination activity partition coefficient has simulated with R^2 of 0.989 with 0.014 MSE

- The validation of the model showed that the predicted values were almost near to the experimental values. The developed models showed the R^2 greater than 0.95 with the mean square error values less than 0.01.

CONCLUSION

The present investigation proclaims the eco-friendly PEG 6000 – trisodium citrate system for the selective separation of *Pisum sativum* lectin from its crude extract of the seeds. The optimum process condition obtained is useful to implement the process in the continuous mode of extraction. The demonstrations of continuous ATPE of Psl from the crude extract in RDC disclose the potentiality and suitability of the process for the continuous extraction and subsequent possibility of scaleup in the pilot scale capacity. The ANN based modeling provide the basis for the prediction of phase equilibrium of the ATPS along with partitioning characteristics of Psl. Thus, the present study contributes to identify an alternate bulk extraction process with relatively higher purity of Psl and extraction efficiency, which may be exploited for the recovery of similar high value proteins in their native form from the complex biological mixtures.

SCOPE FOR FUTURE WORK

Commercial implementation of the process requires further research in the following area.

- Development of separation strategies to separate the partitioned Psl from PEG phase and recycling the spent phase components.
- Identify the useful/valuable proteins in the crude extract other than Psl and devise the strategies to simultaneously purify them by employing multistep ATPE.
- Scale-up studies along with cost analysis in a pilot scale unit to make the process viable for industries.
- Studies on biological characteristics of the extracted Psl to confirm its natural state and study their effectiveness in various fields of application.

REFERENCES

Abdolrahimi, S., Nasernejad, B. and Pazuki, G. (2014). "Prediction of partition coefficients of alkaloids in ionic liquids based aqueous biphasic systems using hybrid group method of data handling (GMDH) neural network." *J. Mol. Liq., 191*, 79-84.

Agrawal, B.B.L. and Goldstein, I.J. (1967). "Protein-carbohydrate interaction: VI. isolation of concanavalin a by specific adsorption on cross-linked dextran gels." *Biochim. Biophys. Acta, Protein Struct.*, 147(2).

Ahmad, A.L., Derek, C.J.C. and Zulkali, M.M.D. (2008). "Optimization of thaumatin extraction by aqueous two-phase system (ATPS) using response surface methodology (RSM)." *Sep. Purif. Technol.*, 62(3), 702-708.

Albertsson, P.A. 1986. "Partitioning of cells and Macromolecules." 3rd eds, New York, Wiley.

Al-Sohaimy, S.A., Hafez, E.E., Abdelwahab, A.E., El-Saadani, M.A. (2007). "Anti-Hcv Lectin from Egyptian Pisum sativum." *Aust. J. Basic Appl. Sci.*, 1(3), 213-219.

Alves, J.G.L.F., Chumpitaz, L.D.A., Silva, L.H.M., Franco, T.T. and Meirelles, A.J.A. (2000). "Partitioning of whey proteins, bovine serum albumin and porcine insulin in aqueous two-phase systems." *J. Chromatogr. B: Biomed. Sci. Appl.*, 743, 235–239.

Amaresh, S.P., Murugesan, S., Regupathi, I. and Murugesan, T. (2008). "Liquid− Liquid Equilibrium of Poly (ethylene glycol) 4000+ Diammonium Hydrogen Phosphate+ Water at Different Temperatures." *J. Chem. Eng. Data*, 53(7), 1574-1578.

Ambrosi, M., Cameron, N.R. and Davis, B.G. (2005). "Lectins: tools for the molecular understanding of the glycocode." *Org. Biomol. Chem.*, 3(9), 1593-1608.

Amid, M., Shuhaimi, M., Sarker, M.Z.I. and Manap, M.Y.A. (2012). "Purification of serine protease from mango (Mangifera Indica Cv. Chokanan) peel using an alcohol/salt aqueous two phase system." *Food Chem.,* 132(3), 1382-1386.

Andrews, B.A. and Asenjo, J.A. (2010). "Theoretical and experimental evaluation of hydrophobicity of proteins to predict their partitioning behavior in aqueous two phase systems: a review." *Sep. Sci. Technol*., 45, 2165-2170.

Andrews, B.A., Schmidt, A.S. and Asenjo, J.A. (2005). "Correlation for the partition behavior of proteins in aqueous two- phase systems: Effect of surface hydrophobicity and charge." *Biotechnol. Bioeng*. 90, 380-390.

Anjana, P., Kumar, A.P., Sirivansh, P., Suryaprakash, P. and Kumar, R.S. (2010). "Reverse micelles extraction of lactoferrin using cationic surfactant from whey." *Int. J. Chem. Sci., 8*(5), 49-56.

Antov, M.G., Peričin, D.M. and Dašić, M.G. (2006). "Aqueous two-phase partitioning of xylanase produced by solid-state cultivation of Polyporus squamosus." *Process Biochem.*, 41(1), 232-235.

Aravamudan, K. and Baird, M.H. (1999). "Effects of mass transfer on the hydrodynamic behavior of a Karr reciprocating plate column." *Ind. Eng. Chem. Res., 38*(4), 1596-1604.

Arsalani, V., Rostami, K. and Kheirolomoom, A. (2005). "Lipoxygenase-1 mass-transfer coefficient in aqueous two-phase system using spray extraction column." *Ind. Eng. Chem. Res.*, 44(19), 7469-7473.

Arumugam, S., Sriram, G. and Rajmohan, T. (2014). "Multi-response optimization of epoxidation process parameters of rapeseed oil using response surface methodology (RSM)-based desirability analysis." *Arabian J. Sci. Eng.*, *39*(3), 2277-2287.

Asenjo, J.A., Turner, R.E., Mistry, S.L. and Kaul, A. (1994). "Separation and purification of recombinant proteins from Escherichia coli with aqueous two-phase systems." *J. Chromatogr. A*, 668(1), 129-137.

Asfaram, A., Ghaedi, M., Azqhandi, M.A., Goudarzi, A. and Dastkhoon, M. (2016). "Statistical experimental design, least squares-support vector machine (LS-SVM) and artificial neural network (ANN) methods for modeling the facilitated adsorption of methylene blue dye." *RSC Adv.*, 6(46), 40502-40516.

Ashraf, G.M., Rizvi, S., Naqvi, S., Suhail, N., Bilal, N., Hasan, S., Tabish, M. and Banu, N. (2010). "Purification, characterization, structural analysis and protein chemistry of a buffalo heart galectin-1." *Amino Acids*, *39*(5), 1321-1332.

Azevedo, A.M., Rosa, P.A., Ferreira, I.F. and Aires-Barros, M.R. (2007). "Optimisation of aqueous two-phase extraction of human antibodies." *J. Biotechnol.*, *132*(2), 209-217.

Azevedo, A.M., Fonseca, L.P. and Prazeres, D.M. (1999). "Stability and stabilisation of penicillin acylase." *J. Chem. Technol. Biotechnol*. 74, 1110–1116.

Azevedo, A.M., Gomes, A.G., Rosa, P.A., Ferreira, I.F., Pisco, A.M. and Aires-Barros, M.R. (2009). "Partitioning of human antibodies in polyethylene glycol–sodium citrate aqueous two-phase systems." *Sep. Purif. Technol.*, 65(1), 14-21.

Azevedo, A.M., Rosa, P.A., Ferreira, I.F. and Aires-Barros, M.R. (2007). "Optimisation of aqueous two-phase extraction of human antibodies." *J. Biotechnol.*, 132(2), 209-217.

Banerjee, R., Das, K., Ravishankar, R., Suguna, K., Surolia, A. and Vijayan, M. (1996). "Conformation, protein-carbohydrate interactions and a novel subunit association in the refined structure of peanut lectin-lactose complex." *J. Mol. Biol.*, 259(2), 281-296.

Banerjee, S.S. and Chen, D.H. (2007). "Glucose-grafted gum arabic modified magnetic nanoparticles: preparation and specific interaction with concanavalin A." *Chem. Mater.*, 19(15), 3667-3672.

Barac, M.B., Pesic, M.B., Stanojevic, S.P., Kostic, A.Z., Cabrilo, S.B. (2015). "Techno-functional properties of pea (Pisum sativum) protein isolates: A review." *Acta Period. Technol.*, 46, 1-18.

Barre, A., Bourne, Y., Van Damme, E.J., Peumans, W.J. and Rougé, P. (2001). "Mannose-binding plant lectins: different structural scaffolds for a common sugar-recognition process." *Biochimie*, 83(7), 645-651.

Benavides, J. and Ritoalomares, M. (2008). "Practical experiences from the development of aqueous two- phase processes for the recovery of high value biological products." *J. Chem. Technol. Biotechnol*., 83(2), 133-142.

Bhutia, S.K., Mallick, S.K., Stevens, S.M., Prokai, L., Vishwanatha, J.K. and Maiti, T.K. (2008). "Induction of mitochondria-dependent apoptosis by Abrus agglutinin derived peptides in human cervical cancer cell." *Toxicol. In Vitro*, 22(2), 344-351.

Biazus, J.P., Santana, J.C., Souza, R.R., Jordao, E. and Tambourgi, E.B. (2007). "Continous extraction of alpha- and beta-amylases from Zea mays malt in a PEG4000/CaCl2 ATPS." *J. Chromatogr. B*, 85, 227–233.

Bim, M.A. and Franco, T.T. (2000). “Extraction in aqueous two-phase systems of alkaline xylanase produced by Bacillus pumilus and its application in kraft pulp bleaching.” *J. Chromatogr. B: Biomed. Sci. Appl*., 743, 349-356.

Bogdan, S., Gosak, D. and Vasić-Rački, Đ. (1995). “Mathematical modeling of liquid-liquid equlibria in aqueous polymer solution containing neutral proteinase and oxytetracycline using artificial neural network.” *Comput. Chem. Eng.*, 19, 791-796.

Boi, C., Cattoli, F., Facchini, R., Sorci, M. and Sarti, G.C. (2006). “Adsorption of lectins on affinity membranes.” *J. Membr. Sci.*, *273*(1-2), 12-19.

Boland, M.J., Hesselink, P.G.M. and Hustedt, H. (1989). “Extractive purification of enzymes from animal tissue using aqueous phase systems.” *J. Biotechnol.*, 11, 337–352.

Bolar, S., Belur, P. D. and Iyyaswami, R. (2013). “Partitioning studies of glutaminase in polyethylene glycol and salt- based aqueous two- phase systems.” *Chem. Eng. Technol.*, 36(8), 1378-1386.

Bonomo, R.C., Minim, L.A., Coimbra, J.S., Fontan, R.C., da Silva, L.H.M. and Minim, V.P. (2006). “Hydrophobic interaction adsorption of whey proteins: effect of temperature and salt concentration and thermodynamic analysis.” *J. Chromatogr. B.*, 844, 6-14.

Box, G.E.P. and Wilson, K.B. (1951). “On the experimental attainment of optimum conditions.” J. R. Stat. Soc. Series B Stat. Methodol., 13, 1–45.

Boyd, W.C. and Reguera, R.M. (1949). “Hemagglutinating substances for human cells in various plants.” *J. Immunol.*, 62(3), 333-339.

Boyd, W.C. and Shapleigh, E. (1954). “Specific Precipitating Activity of Plant Agglutinins (Lectins).” *Science*, 119(3091), 419.

Bradoo, S.R.K.S., Saxena, R.K. and Gupta, R. (1999). “Partitioning and resolution of mixture of two lipases from Bacillus stearothermophilus SB-1 in aqueous two-phase system.” *Process Biochem.*, *35*(1-2), 57-62.

Cavalcanti, M.T.H., Carneiro-da-Cunha, M.G., Brandi, I.V., Porto, T.S., Converti, A., Lima Filho, J.L., Porto, A and Pessoa, A. (2008). “Continuous extraction of α-toxin from a fermented broth of Clostridium perfringens Type A in perforated rotating disc contactor using aqueous two-phase PEG–phosphate system.” *Chem. Eng. Process.*, *47*(9-10), 1771-1776.

Cavalcanti, M.T.H., Porto, T.S., de Barros Neto, B., Lima-Filho, J.L., Porto, A.L.F. and Pessoa Jr, A. (2006). “Aqueous two-phase systems extraction of α-toxin from Clostridium perfringens type A.” *J. Chromatogr. B.*, 833, 135-140.

Chaiwut, P., Rawdkuen, S. and Benjakul, S. (2010). “Extraction of protease from Calotropis procera latex by polyethylene glycol–salts biphasic system.” *Process Biochem.*, *45*(7), 1148-1155.

Chan, S.Y., Ma, M.C. and Goldsmith, M.A. (2000). “Differential induction of cellular detachment by envelope glycoproteins of Marburg and Ebola (Zaire) viruses.” *J. Gen. Virol.*, 81(9), 2155-2159.

Chavan, R.S., Avhad, D.N. and Rathod, V.K. (2015). “Optimization of Aqueous Two-Phase Extraction of Protease Produced from Bacillus licheniformis NCIM 2042 Using Response Surface Methodology.” *Sep. Sci. Technol.*, 50(1), 45-55.

Che, A.F., Huang, X.J. and Xu, Z.K. (2011). "Polyacrylonitrile-based nanofibrous membrane with glycosylated surface for lectin affinity adsorption." *J. Membr. Sci.*, *366*(1-2), 272-277.

Chethana, S., Nayak, C.A. and Raghavarao, K.S.M.S. (2007). "Aqueous two phase extraction for purification and concentration of betalains." *J. Food Eng.*, *81*(4), 679-687.

Choi, S.H., Lyu, S.Y. and Park, W.B. (2004). "Mistletoe lectin induces apoptosis and telomerase inhibition in human A253 cancer cells through dephosphorylation of Akt." Arch. Pharmacal Res., 27(1), 68.

Coelho, D.F., Silva, C.A., Machado, C.S., Silveira, E.C. and Tambourgi, E.B. (2015). "Use of artificial neural networks to predict aqueous two-phases system optimal conditions on bromelain's purification." *Chem. Eng. Trans.*, 43, 1417-1422.

Coimbra, J.D.R., Thömmes, J. and Kula, M.R. (1994). "Continuous separation of whey proteins with aqueous two-phase systems in a Graesser contactor." *J. Chromatogr. A*, *668*(1), 85-94.

Correia, M.T. and Coelho, L.C. (1995). "Purification of a glucose/mannose specific lectin, isoform 1, from seeds of Cratylia mollis Mart (Camaratu bean)." *Appl. Biochem. Biotechnol.*, *55*(3), 261-273.

Correia, M.T.S., Coelho, L.C.B.B. and Paiva, P.M.G. (2008). "Lectins, carbohydrate recognition molecules: Are they toxic." Recent Trends Toxicol., 37, 47-59.

Costa, R.M., Vaz, A.F., Oliva, M.L., Coelho, L.C., Correia, M.T. and Carneiro-da-Cunha, M.G. (2010). "A new mistletoe Phthirusa pyrifolia leaf lectin with antimicrobial properties." *Process Biochem.*, 45(4), 526-533.

Cunha, E.V. and Aznar, M. (2009). “Liquid− liquid equilibrium in aqueous two-phase (water+ PEG 8000+ salt): Experimental determination and thermodynamic modeling.” *J. Chem. Eng. Data*, 54(12), 3242-3246.

Cunha, M.T., Costa, M.J.L., Calado, C.R.C., Fonseca, L.P., Aires-Barros, M.R. and Cabral, J.M.S. (2003). “Integration of production and aqueous two-phase systems extraction of extracellular Fusarium solani pisi cutinase fusion proteins.” *J. Biotechnol.*, 100(1), 55-64.

da Silva, C.A.S., Coimbra, J.S., Rojas, E.E.G. and Teixeira, J.A.C. (2009). “Partitioning of glycomacropeptide in aqueous two-phase systems.” *Process Biochem.*, 44(11), 1213-1216.

da Silva, N.R., Ferreira, L.A., Mikheeva, L.M., Teixeira, J.A., Zaslavsky, B.Y. (2014). “Origin of salt additive effect on solute partitioning in aqueous polyethylene glycol-8000–sodium sulfate two-phase system.” *J. Chromatogr. A.*, 1337, 3-8.

da Silva, O.S., Alves, R.O. and Porto, T.S. (2018). “PEG-sodium citrate aqueous two-phase systems to in situ recovery of protease from Aspergillus tamarii URM4634 by extractive fermentation.” *Biocatal. Agric. Biotechnol.*, 16, 209-216.

de Araujo Padilha, C.E., de Oliveira Júnior, S.D., de Santana Souza, D.F., de Oliveira, J.A., de Macedo, G.R. and dos Santos, E.S. (2017). “Partition coefficient prediction of Baker's yeast invertase in aqueous two phase systems using hybrid group method data handling neural network.” *Chin. J. Chem. Eng.*, *25*(5), 652-657.

de Belval, S., le Breton, B., Huddleston, J. and Lyddiatt, A. (1998). “Influence of temperature upon protein partitioning in poly (ethylene glycol)–salt aqueous two-phase

systems close to the critical point with some observations relevant to the partitioning of particles." *J. Chromatogr. B: Biomed. Sci. Appl.*, 711(1-2), 19-29.

de Brito Cardoso, G., Mourão, T., Pereira, F.M., Freire, M.G., Fricks, A.T., Soares, C.M.F. and Lima, Á.S. (2013). "Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin." *Sep. Purif. Technol.*, 104, 106-113.

de Oliveira, F.C., dos Reis Coimbra, J.S., da Silva, L.H.M., Rojas, E.E.G. and da Silva, M.D.C.H. (2009). "Ovomucoid partitioning in aqueous two-phase systems." *Biochem. Eng. J.*, 47(1-3), 55-60.

de Oliveira, R.M., dos Reis Coimbra, J.S., Francisco, K.R., Minim, L.A., da Silva, L.H.M. and Rojas, E.E.G. (2008). "Equilibrium data of the biphasic system poly (ethylene oxide) 4000+ copper sulfate+ water at (5, 10, 35, and 45)° C." *J. Chem. Eng. Data*, 53(7), 1571-1573.

Dessen, A., Gupta, D., Sabesan, S., Brewer, C.F. and Sacchettini, J.C. (1995). "X-ray crystal structure of the soybean agglutinin cross-linked with a biantennary analog of the blood group I carbohydrate antigen." *Biochemistry*, 34(15), 4933-4942.

Diamond, A.D. and Hsu, J.T. (1989). "Fundamental studies of biomolecule partitioning in aqueous two- phase systems." *Biotechnol. Bioeng.*, *34*(7), 1000-1014.

dos Santos Nunes, E., De Souza, M.A.A., de Melo Vaz, A.F., de Sá Santana, G.M., Gomes, F.S., Coelho, L.C.B.B., Paiva, P.M.G., Da Silva, R.M.L., Silva-Lucca, R.A., Oliva, M.L.V. and Guarnieri, M.C. (2011). "Purification of a lectin with antibacterial activity from Bothrops leucurus snake venom." *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.*, 159(1), 57-63.

Edelman, G.M., Cunningham, B.A., Reeke, G.N., Becker, J.W., Waxdal, M.J. and Wang, J.L. (1972). "The covalent and three-dimensional structure of concanavalin A." *Proc. Natl. Acad. Sci. U. S. A.*, 69(9), 2580-2584.

Einspahr, H., Parks, E.H., Suguna, K.A.Z.A., Subramanian, E.S.W.A.R.A. and Suddath, F.L. (1986). "The crystal structure of pea lectin at 3.0-A resolution." *J. Biol. Chem.*, 261(35), 16518-16527.

Entlicher, G., Kostir, J.V. and Kocourek, J. (1970). "Studies on phytohemagglutinins. III. Isolation and characterization of hemagglutinins from the pea (Pisum sativum L.)." *Biochim. Biophys. Acta*, 221, 272-281.

Ernst, E., Schmidt, K. and Steuer- Vogt, M.K. (2003). "Mistletoe for cancer? A systematic review of randomised clinical trials." *Int. J. Cancer*, 107(2), 262-267.

Etzler, M.E. (1986). "Distribution and function of plant lectins - The lectins: Properties, Functions, and applications in Biology and Medicine." *Academic Press*, 371-435.

Fang, E.F., Lin, P., Wong, J.H., Tsao, S.W. and Ng, T.B. (2010). "A lectin with anti-HIV-1 reverse transcriptase, antitumor, and nitric oxide inducing activities from seeds of Phaseolus vulgaris cv. extra-long autumn purple bean." *J. Agric. Food Chem.*, 58(4), 2221-2229.

Ferreira, L., Madeira, P.P., Mikheeva, L., Uversky, V.N. and Zaslavsky, B. (2013). "Effect of salt additives on protein partition in polyethylene glycol–sodium sulfate aqueous two-phase systems." *Biochim. Biophys. Acta, Proteins Proteomics*, *1834*(12), 2859-2866.

Flanagan, S.D. and Barondes, S.H. (1975). “Affinity partitioning. A method for purification of proteins using specific polymer-ligands in aqueous polymer two-phase systems.” *J. Biol. Chem.*, *250*(4), 1484-1489.

Franco, T.T., Andrews, A.T. and Asenjo, J.A. (1996). “Use of chemically modified proteins to study the effect of a single protein property on partitioning in aqueous two-phase systems: Effect of surface hydrophobicity.” *Biotechnol. Bioeng.*, *49*(3), 300-308.

Ganguly, S. (2003). “Prediction of VLE data using radial basis function network.” *Comput. Chem. Eng.*, 27(10), 1445-1454.

Gavhane, K.A. (2006). “Chemical reaction engineering.” *Nirali Prakashan*.

Ghanadzadeh, H., Fallahi, S. and Ganji, M. (2011). “Liquid–Liquid Equilibrium Calculation for Ternary Aqueous Mixtures of Ethanol and Acetic Acid with 2-Ethyl-1-hexanol Using the GMDH-Type Neural Network.” *Ind. Eng. Chem. Res.*, 50(17), 10158-10167.

Giraldo-Zuniga, A.D., Coimbra, J.S., Minim, L.A. and Rojas, E.E.G. (2006). “Dispersed phase hold-up in a Graesser raining bucket contactor using aqueous two-phase systems.” *J. Food Eng.*, *72*(3), 302-309.

Glyk, A., Solle, D., Scheper, T. and Beutel, S. (2017). “Evaluation of Driving Forces for Protein Partition in PEG-Salt Aqueous Two-Phase Systems and Optimization by Design of Experiments.” *J. Chromatogr. Sep. Tech.*, 8, 389.

Goja, A.M., Yang, H., Cui, M. and Li, C. (2013). “Aqueous two-phase extraction advances for bioseparation.” *J. Bioprocess. Biotech.*, 4(1), 1-8.

Gonzalez-Tello, P., Camacho, F., Blazquez, G. and Alarcon, F.J. (1996). “Liquid− liquid equilibrium in the system poly (ethylene glycol)+ MgSO4+ H2O at 298 K.” *J. Chem. Eng. Data*, 41(6), 1333-1336.

Gottschalk, A. (1955). “Structural relationship between sialic acid, neuraminic acid and 2-carboxypyrrole.” *Nature*, 176, 881-882.

Govindarajan, R. and Perumalsamy, M. (2013). “Phase Equilibrium of PEG 2000+ Triammonium Citrate+ Water System Relating PEG Molecular Weight, Cation, Anion with Effective Excluded Volume, Gibbs Free Energy of Hydration, Size of Cation, and Type of Anion at (298.15, 308.15, and 318.15) K.” *J. Chem. Eng. Data*, *58*(11), 2952-2958.

Gu, T. (2000). “Liquid-liquid partitioning methods for bioseparations.” *Sep. Sci. Technol.*, 2, 329-364.

Gunduz, U. and Korkmaz, K. (2000). “Bovine serum albumin partitioning in an aqueous two-phase system: effect of pH and sodium chloride concentration.” *J. Chromatogr. B: Biomed. Sci. Appl.*, *743*(1-2), 255-258.

Guo, W. and Ruckenstein, E. (2001). ‘A new matrix for membrane affinity chromatography and its application to the purification of concanavalin A.” *J. Membr. Sci.*, 182(1-2), 227-234.

Gupta, V., Nath, S. and Chand, S. (2002). “Role of water structure on phase separation in polyelectrolyte–polyethyleneglycol based aqueous two-phase systems.” *Polymer*, 43(11), 3387-3390.

Hachem, F., Andrews, B.A. and Asenjo, J.A. (1996). "Hydrophobic partitioning of proteins in aqueous two-phase systems." *Enzyme Microb. Technol.*, 19(1996), 507-517.

Hamelryck, T.W., Loris, R., Bouckaert, J., Dao-Thi, M.H., Strecker, G., Imberty, A., Fernandez, E., Wyns, L. and Etzler, M.E. (1999). "Carbohydrate binding, quaternary structure and a novel hydrophobic binding site in two legume lectin oligomers from Dolichos biflorus." *J. Mol. Biol.*, 286, 1161-1177.

Hamid, R., Masood, A., Wani, I.H. and Rafiq, S. (2013). "Lectins: Proteins with Diverse Applications." *J. Appl. Pharm. Sci.*, 3, S93-S103.

Hardman, K.D., Agarwal, R.C. and Freiser, M.J. (1982). "Manganese and calcium binding sites of concanavalin A." *J. Mol. Biol.*, 157(1), 69-86.

Hatti-Kaul, R. (2000). "Aqueous two-phase systems - Aqueous two-phase systems: methods and protocols." 1-10, Humana Press.

Hatti-Kaul, R. (2001). "Aqueous two-phase systems." *Mol. Biotechnol.*, *19*(3), 269-277.

He, S., Shi, J., Walid, E., Ma, Y. and Xue, S.J. (2013). "Extraction and purification of a lectin from small black kidney bean (Phaseolus vulgaris) using a reversed micellar system." *Process Biochem.*, *48*(4), 746-752.

He, S., Shi, J., Walid, E., Zhang, H., Ma, Y. and Xue, S.J. (2015). "Reverse micellar extraction of lectin from black turtle bean (Phaseolus vulgaris): Optimisation of extraction conditions by response surface methodology." *Food Chem.*, 166, 93-100.

Heebøll- Nielsen, A., Dalkiær, M., Hubbuch, J.J. and Thomas, O.R. (2004). "Superparamagnetic adsorbents for high- gradient magnetic fishing of lectins out of legume extracts." *Biotechnol. Bioeng.*, *87*(3), 311-323.

Hemavathi, A.B. and Raghavarao, K.S.M.S. (2011). "Differential partitioning of β-galactosidase and β-glucosidase using aqueous two phase extraction." *Process Biochem.*, *46*(3), 649-655.

Higgins, T.J., Chandler, P.M., Zurawski, G., Button, S.C. and Spencer, D. (1983). "The biosynthesis and primary structure of pea seed lectin." *J. Biol. Chem.*, 258(15), 9544-9549.

Hoedemaeker, F.J., Richardson, M., Díaz, C.L., de Pater, B.S. and Kijne, J.W. (1994). "Pea (Pisum sativum L.) seed isolectins 1 and 2 and pea root lectin result from carboxypeptidase-like processing of a single gene product." *Plant Mol. Biol.*, 24(1), 75-81.

Hu, S. and Wong, D.T. (2009). "Lectin microarray." *Proteomics: Clin. Appl.*, 3(2), 148-154.

Hubbuch, J.J., Matthiesen, D.B., Hobley, T.J. and Thomas, O.R. (2001). "High gradient magnetic separation versus expanded bed adsorption: a first principle comparison." *Bioseparation*, *10*(1-3), 99-112.

Huddleston, J.G., Willauer, H.D. and Rogers, R.D. (2003). "Phase diagram data for several PEG+ salt aqueous biphasic systems at 25 C." *J. Chem. Eng. Data*, *48*(5), 1230-1236.

Hui Chao Lv, H.C., Tian, D.Y. (2017). “Modeling of the phase equilibria of aqueous two-phase systems using three-dimensional neural network.” *Korean J. Chem. Eng*., 34, 170-178

Hunter, T. and Pines, J. (1994). “Cyclins and cancer II: Cyclin D and CDK inhibitors come of age.” *Cell*, 79(4), 573-582.

Igarashi, L., Kieckbusch, T.G. and Franco, T.T. (2004). “Mass transfer in aqueous two-phases system packed column.” *J. Chromatogr. B, 807*(1), 75-80.

Imelio, N., Marini, A., Spelzini, D., Picó, G. and Farruggia, B. (2008). “Pepsin extraction from bovine stomach using aqueous two-phase systems: Molecular mechanism and influence of homogenate mass and phase volume ratio.” *J. Chromatogr. B, 873*(2), 133-138.

Islam, B., Khan, S.N., Naeem, A., Sharma, V. and Khan, A.U. (2009). “Novel effect of plant lectins on the inhibition of Streptococcus mutans biofilm formation on saliva-coated surface.” *J. Appl. Microbiol.*, 106(5), 1682-1689.

Islam, F., Gopalan, V., Lam, A.K.Y. and Kabir, S.R. (2018). “Pea lectin inhibits cell growth by inducing apoptosis in SW480 and SW48 cell lines.” *Int. J. Biol. Macromol.*

Iyyaswami, R., Belur, P.D., Girish, B. and Nagaraj, V.H. (2012). “Development and evaluation of PEG-lithium citrate salt based aqueous two phase system and its application in partitioning of proteins from fish industry effluent.” *Sep. Sci. Technol.*, 47(4), 591-598.

Jafarabad, K.R., Sawant, S.B., Joshi, J.B. and Sikdar, S.K. (1992). “Enzyme and protein mass transfer coefficient in aqueous two-phase systems—I. Spray extraction columns.” *Chem. Eng. Sci.*, 47(1), 57-68.

Jayapal, M., Regupathi, I. and Murugesan, T. (2007). "Liquid-liquid equilibrium of poly(ethylene glycol) 2000 + potassium citrate + water at (25, 35, and 45) °C." *J. Chem. Eng. Data*., 52, 56–59.

Jiang, B., Yuan, Y., Zhang, X., Feng, Z. and Liu, C. (2017). "Separation and Enrichment of Lectin from Zihua Snap-Bean (Phaseolus vulgaris) Seeds by PEG 600–Ammonium Sulfate Aqueous Two-Phase System." *Molecules*, *22*(10), 1596.

Jonsson, M. and Johansson, H.O. (2003). "Protein partitioning in thermoseparating systems of a charged hydrophobically modified ethylene oxide polymer." *J. Chromatogr. A*, 983(1-2), 133-144.

Kabir, S.R., Nabi, M.M., Haque, A., Zaman, R.U., Mahmud, Z.H. and Reza, M.A. (2013). "Pea lectin inhibits growth of Ehrlich ascites carcinoma cells by inducing apoptosis and G2/M cell cycle arrest in vivo in mice." *Phytomedicine*, 20(14), 1288-1296.

Kajiuchi, T., Shin- Ya, Y., Nagai, H. and Fujiwara, K. (2002). "Partitioning of chicken egg white proteins in polyelectrolyte/salt aqueous two- phase systems composed of polyethyleneoxide–maleic acid copolymer and potassium phosphate." *J. Chem. Technol. Biotechnol.*, 77(4), 495-499.

Kalaichelvi, P. and Murugesan, T. (1998). "Dispersed phase hold-up in rotary disc contactor." *Bioprocess Eng.*, *18*(2), 105-111.

Kalaivani, S. and Regupathi, I. (2013). "Partitioning studies of α-lactalbumin in environmental friendly poly (ethylene glycol)—citrate salt aqueous two phase systems." *Bioprocess Biosyst. Eng.*, *36*(10), 1475-1483.

Kalaivani, S. and Regupathi, I. (2016). “Continuous aqueous two-phase extraction of α-lactalbumin from whey in conventional rotating disc contactor.” *Sep. Sci. Technol.*, *51*(14), 2411-2419.

Kalaivani, S. and Regupathi, I. (2015). “Synergistic extraction of α-Lactalbumin and β-Lactoglobulin from acid whey using aqueous biphasic system: Process evaluation and optimization.” *Sep. Purif. Technol.*, *146*, 301-310.

Kan, P. and Lee, C. J. (1996). “A neural network model for prediction of phase equilibria in aqueous two-phase extraction.” *Ind. Eng. Chem. Res.*, 35(6), 2015-2023.

Kato, G., Maruyama, Y. and Nakamura, M. (1979). “Role of lectins and lipopolysaccharides in the recognition process of specific legume-Rhizobium symbiosis.” *Agric. Biol. Chem.*, 43(5), 1085-1092.

Katre, U.V., Suresh, C.G., Khan, M.I. and Gaikwad, S.M. (2008). “Structure–activity relationship of a hemagglutinin from Moringa oleifera seeds.” *Int. J. Biol. Macromol.*, 42(2), 203-207.

Kaul, A. (2000). “The phase diagram - Aqueous Two-Phase Systems: Methods and Protocols.” 11-21, Humana Press.

Kaur, A.P., Sohal, S.K., Kaur, M. and Singh, J. (2014). “Assessment of anti-insect potential of partially purified pea lectin against melon fruit fly, Bactrocera cucurbitae.” *J. Environ. Biol.*, 35(1), 159.

Kaur, N., Dhuna, V., Kamboj, S.S., Agrewala, J.N. and Singh, J. (2006). “A novel antiproliferative and antifungal lectin from Amaranthus viridis Linn seeds.” *Protein Pept. Lett.*, 13(9), 897-905.

Kennedy, J.F., Palva, P.M.G., Corella, M.T.S., Cavalcanti, M.S.M. and Coelho, L.C.B.B. (1995). "Lectins, versatile proteins of recognition: a review." *Carbohydr. Polym.*, 26(3), 219-230.

Ketnawa, S., Chaiwut, P. and Rawdkuen, S. (2011). "Aqueous two-phase extraction of bromelain from pineapple peels ('Phu Lae'cultv.) and its biochemical properties." *Food Sci. Biotechnol.*, 20, 1219.

Khayati, G., Anvari, M. and Shahidi, N. (2015). "Partitioning of b-galactosidase in aqueous two-phase systems containing polyethyleneglycol and phosphate salts." *Fluid Phase Equilib.*, 385, 147–152.

Kilikian, B.V., Bastazin, M.R., Minami, N.M., Gonçalves, E.M.R. and Junior, A.P. (2000). "Liquid-liquid extraction by reversed micelles in biotechnological processes." *Braz. J. Chem. Eng.*, *17*(1), 29-38.

Kim, H.M., Cho, E.J. and Bae, H.J. (2016). "Single step purification of concanavalin A (Con A) and bio-sugar production from jack bean using glucosylated magnetic nano matrix." *Bioresour. Technol.*, *213*, 257-261.

Konozy, E.H., Bernardes, E.S., Rosa, C., Faca, V., Greene, L.J. and Ward, R.J. (2003). "Isolation, purification, and physicochemical characterization of a D-galactose-binding lectin from seeds of Erythrina speciosa." *Arch. Biochem. Biophys.*, *410*(2), 222-229.

Kumar, A. and Hartland, S. (1995). "A unified correlation for the prediction of dispersed-phase hold-up in liquid-liquid extraction columns." *Ind. Eng. Chem. Research*, *34*(11), 3925-3940.

Kvennefors, E.C.E., Leggat, W., Hoegh-Guldberg, O., Degnan, B.M. and Barnes, A.C. (2008). "An ancient and variable mannose-binding lectin from the coral Acropora

millepora binds both pathogens and symbionts." *Dev. Comp. Immunol.*, *32*(12), 1582-1592.

Laemmli, U.K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature*, 227, 680.

Lam, S.K. and Ng, T.B. (2010). "First report of a haemagglutinin-induced apoptotic pathway in breast cancer cells." *Biosci. Rep.*, 30(5), 307-317.

Lam, S.K. and Ng, T.B. (2011). "Lectins: production and practical applications." *Appl. Microbiol. Biotechnol.*, 89(1), 45-55.

Landsteiner, K. and Raubitschek, H. (1907). "Beobachtungen uber Hämolyse und Hamagglutination." *Zentralbl. Bakteriol.*, 600–607.

Lehr, C.M. and Gabor, F. (2004). "Lectins and Glycoconjugates in Drug Delivery and Targeting." *Adv. Drug Delivery Rev.*, 56, 419–420.

Leong, Y.K., Chang, C.K., Arumugasamy, S.K., Lan, J.C.W., Loh, H.S., Muhammad, D. and Show, P.L. (2018). "Statistical design of experimental and bootstrap neural network modelling approach for thermoseparating aqueous two-phase extraction of polyhydroxyalkanoates." *Polymers*, *10*(2), 132.

Li, Z., Pei, Y., Wang, H., Fan, J. and Wang, J. (2010). "Ionic liquid-based aqueous two-phase systems and their applications in green separation processes." *TrAC, Trends Anal. Chem.*, 29(11), 1336-1346.

.Liener, I.E., Sharon, N. and Goldstein, I.J. (1986). "The Lectins: properties, functions, and application in Biology and Medicine." *Academic Press*, 600.

Liener, Irvin (2012). “The lectins: properties, functions, and applications in biology and medicine.” *Academic Press*.

Ling, Y.Q., Nie, H.L., Su, S.N., Branford-White, C. and Zhu, L.M. (2010). “Optimization of affinity partitioning conditions of papain in aqueous two-phase system using response surface methodology.” *Sep. Purif. Technol.*, 73(3), 343-348.

Lis, H. and Sharon, N. (1981). “Lectins in higher plants.” *Proteins and Nucleic Acids*, 371-447.

Lis, H. and Sharon, N. (1986). “Lectins as molecules and as tools.” *Annu. Rev. Biochem.*, 55(1), 35-67.

Liu, C.L., Kamei, D.T., King, J.A., Wang, D.I. and Blankschtein, D. (1998). “Separation of proteins and viruses using two-phase aqueous micellar systems.” *J. Chromatogr. B: Biomed. Sci. Appl.*, 711(1-2), 127-138.

Liu, Z., Liu, B., Zhang, Z.T., Zhou, T.T., Bian, H.J., Min, M.W., Liu, Y.H., Chen, J. and Bao, J.K. (2008). “A mannose-binding lectin from Sophora flavescens induces apoptosis in HeLa cells.” *Phytomedicine*, 15(10), 867-875.

Loc, N.H., Lien, H.T.T., Giap, D.V. and Quang, H.T. (2013). “Purification of recombinant neutral protease (NPRC10) by partitioning in aqueous two-phase systems.” *Eur. J. Exp. Biol.*, 3, 252-257.

Loris, R., Hamelryck, T., Bouckaert, J. and Wyns, L. (1998). “Legume lectin structure.” *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.*, 1383(1), 9-36.

Loris, R., Van Overberge, D., Dao- Thi, M.H., Poortmans, F., Maene, N. and Wyns, L. (1994). "Structural analysis of two crystal forms of lentil lectin at 1.8 Å resolution." *Proteins: Struct., Funct., Bioinf.*, 20(4), 330-346.

Lu, Y., Lu, W., Wang, W., Guo, Q. and Yang, Y. (2013). "The optimization of aqueous two phase extraction of lysozyme from crude hen egg white using response surface methodology." *J. Chem. Technol. Biotechnol.*, *88*(3), 415-421.

Lv, H. and Zheng, Y. (2018). "A newly developed tridimensional neural network for prediction of the phase equilibria of six aqueous two-phase systems." *J. Ind. Eng. Chem.*, 57, 377-386.

Madhusudhan, M.C., Raghavarao, K.S.M.S. and Nene, S. (2008). "Integrated process for extraction and purification of alcohol dehydrogenase from Baker's yeast involving precipitation and aqueous two phase extraction." *Biochem. Eng. J.*, *38*(3), 414-420.

Marcos, J.C., Fonseca, L.P., Ramalho, M.T. and Cabral, J.M.S. (1999). "Partial purification of penicillin acylase from Escherichia coli in poly (ethylene glycol)–sodium citrate aqueous two-phase systems." *J. Chromatogr. B: Biomed. Sci. Appl.*, 734(1), 15-22.

Mattiasson, B. and Ramstorp, M. (1984). "Ultrafiltration affinity purification: isolation of concanavalin A from seeds of Canavalia ensiformis." *J. Chromatogr. A*, *283*, 323-330.

Mayerhoff, Z.D., Roberto, I.C. and Franco, T.T. (2004). "Purification of xylose reductase from Candida mogii in aqueous two-phase systems." *Biochem. Eng. J.*, *18*(3), 217-223.

Mazzola, P.G., Lopes, A.M., Hasmann, F.A., Jozala, A.F., Penna, T.C., Magalhaes, P.O., Rangel-Yagui, C.O. and Pessoa Jr, A. (2008). "Liquid–liquid extraction of biomolecules: an overview and update of the main techniques." *J. Chem. Technol. Biotechnol.*, 83(2), 143-157.

Mehri, M. (2014). "Optimization of response surface and neural network models in conjugation with desirability function for estimation of nutritional needs of methionine, lysine, and threonine in broiler chickens." *Poult. Sci.*, *93*(7), 1862-1867.

Mehrnoush, A. and Yazid, A.M.M. (2013). "Purification and recovery of serine protease from mango (Mangifera indica cv. Chokanan) waste using aqueous two-phase system: Potential low cost of enzyme and purification method." *J. Food, Agric. Environ.*, 11(3), 0-4.

Mehrnoush, A., Mustafa, S., Sarker, M.Z.I. and Yazid, A.M.M. (2012). "Optimization of serine protease purification from mango (Mangifera indica cv. Chokanan) peel in polyethylene glycol/dextran aqueous two phase system." *Int. J. Mol. Sci.*, 13(3), 3636-3649.

Mehrnoush, A., Sarker, M.Z.I., Mustafa, S. and Yazid, A.M.M. (2011). "Direct purification of pectinase from mango (Mangifera Indica cv. Chokanan) peel using a PEG/salt-based aqueous two phase system." *Molecules*, *16*(10), 8419-8427.

Melander, M., Åhman, I., Kamnert, I. and Strömdahl, A.C. (2003). "Pea lectin expressed transgenically in oilseed rape reduces growth rate of pollen beetle larvae." *Transgenic Res.*, 12(5), 555-567.

Mohammadi, H.S., Omidinia, E. and Taherkhani, H. (2008). "Rapid one-step separation and purification of recombinant phenylalanine dehydrogenase in aqueous two-phase systems." *Iran. Biomed. J.*, 12, 115-122.

Moris, M.A., Diez, F.V. and Coca, J. (1997). "Hydrodynamics of a rotating disc contactor." *Sep. Purif. Technol.*, 11(2), 79-92.

Mourey, L., Pédelacq, J.D., Birck, C., Fabre, C., Rougé, P. and Samama, J.P. (1998). "Crystal structure of the arcelin-1 dimer from Phaseolus vulgaris at 1.9-Å resolution." *J. Biol. Chem., 273*(21), 12914-12922.

Muendges, J., Zalesko, A., Górak, A. and Zeiner, T. (2015). "Multistage aqueous two-phase extraction of a monoclonal antibody from cell supernatant." *Biotechnol. Prog., 31*(4), 925-936.

Murugesan, T. and Perumalsamy, M. (2005). "Densities and viscosities of polyethylene glycol 2000+ salt+ water systems from (298.15 to 318.15) K." *J. Chem. Eng. Data*, 50(4), 1290-1293.)

Naeem, A., Hasan Khan, R., Vikram, H. and Akif, M. (2001). "Purification of Cajanus cajan Root Lectin and Its Interaction with Rhizobial Lipopolysaccharide as Studied by Different Spectroscopic Techniques." *Arch. Biochem. Biophys.*, 396, 99–105.

Nagaraja, V.H. and Iyyaswami, R. (2015). "Aqueous two phase partitioning of fish proteins: partitioning studies and ATPS evaluation." *J. Food Sci. Technol., 52*(6), 3539-3548.

Nair, S.S., Madembil, N.C., Nair, P., Raman, S. and Veerabadrappa, S.B. (2013). "Comparative analysis of the antibacterial activity of some phytolectins." *Int. Curr. Pharm. J.*, 2(2), 18-22.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. (2009). "Partitioning of protease from stomach of albacore tuna (Thunnus alalunga) by aqueous two-phase systems." *Process Biochem., 44*(4), 471-476.

Narayan, A.V., Madhusudhan, M.C. and Raghavarao, K.S.M.S. (2011). "Demixing kinetics of phase systems employed for liquid–liquid extraction and correlation with system properties." *Food Bioprod. Process.*, 89(4), 251-256.

Nascimento, C.O., Coelho, L.C.B.B., Correia, M.T.S. and Carneiro-da-Cunha, M.G. (2002). "Liquid-liquid extraction of lectin from Cratylia mollis seeds using reversed micelles." *Biotechnol. Lett.*, *24*(11), 905-907.

Nascimento, C.O., Costa, R.M., Araújo, R.M., Chaves, M.E., Coelho, L.C., Paiva, P.M., Teixeira, J.A., Correia, M.T. and Carneiro-da-Cunha, M.G. (2008). "Optimized extraction of a lectin from Crataeva tapia bark using AOT in isooctane reversed micelles." *Process Biochem.*, *43*(7), 779-782.

Nascimento, C.O., Soares, P.A., Porto, T.S., Costa, R.M., Lima, C.D.A., de Lima Filho, J.L., Coelho, L.C., dos Santos Correia, M.T., da Cunha, M.D.G.C. and Porto, A.L. (2013). "Aqueous two-phase systems: new strategies for separation and purification of lectin from crude extract of Cratylia mollis seeds." *Sep. Purif. Technol.*, *116*, pp.154-161.

Nascimento, C.O., Soares, P.A., Porto, T.S., Costa, R.M., Lima, C.D.A., de Lima Filho, J.L., Coelho, L.C., dos Santos Correia, M.T., da Cunha, M.D.G.C. and Porto, A.L. (2013). "Aqueous two-phase systems: new strategies for separation and purification of lectin from crude extract of Cratylia mollis seeds." *Sep. Purif. Technol.*, *116*, 154-161.

Nascimento, K.S., Azevedo, A.M., Cavada, B.S. and Aires-Barros, M.R. (2010). "Partitioning of Canavalia brasiliensis lectin in polyethylene glycol–sodium citrate aqueous two-phase systems." *Sep. Sci. Technol.*, 45(15), 2180-2186.

Ng, H.S., Tan, C.P., Chen, S.K., Mokhtar, M.N., Ariff, A. and Ling, T.C. (2011). "Primary capture of cyclodextrin glycosyltransferase derived from Bacillus cereus by aqueous two phase system." *Sep. Purif. Technol.*, 81, 318-324.

Ng, T.B., Chan, Y.S., Ng, C.C.W. and Wong, J.H. (2015). "Purification and characterization of a lectin from green split peas (Pisum sativum)." *Appl. Biochem. Biotechnol., 177*(6), 1374-1385.

Ogata, Y., Seto, H., Murakami, T., Hoshino, Y. and Miura, Y. (2013). "Affinity separation of lectins using porous membranes immobilized with glycopolymer brushes containing mannose or N-acetyl-D-glucosamine." *Membranes, 3*(3), 169-181.

Ooi, C.W., Hii, S.L., Kamal, S.M.M., Ariff, A. and Ling, T.C. (2011). "Extractive fermentation using aqueous two-phase systems for integrated production and purification of extracellular lipase derived from Burkholderia pseudomallei." *Process Biochem.,* 46(1), 68-73.

Ooi, C.W., Tey, B.T., Hii, S.L., Kamal, S.M.M., Lan, J.C.W., Ariff, A. and Ling, T.C. (2009). "Purification of lipase derived from Burkholderia pseudomallei with alcohol/salt-based aqueous two-phase systems." *Process Biochem.,* 44(10), 1083-1087.

Pajic, I., Kljajic, Z., Dogovic, N., Sladic, D., Juranic, Z. and Gasic, M.J. (2002). "A novel lectin from the sponge Haliclona cratera: isolation, characterization and biological activity." *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.,* 132, 213–221.

Park, W.B., Lyu, S.Y., Kim, J.H., Choi, S.H., Chung, H.K., Ahn, S.H., Hong, S.Y.,Yoon, T.J. and Choi, M.J. (2001). "Inhibition of tumor growth and metastasis by Korean mistletoe lectin is associated with apoptosis and antiangiogenesis." *Cancer Biother.Radiopharm.,* 16(5), 439-447.

Patil, T.A., Jafarabad, K.R., Sawant, S.B. and Joshi, J.B. (1991). "Enzyme mass transfer coefficient in aqueous two phase system using a packed extraction column." *Can. J. Chem. Eng., 69*(2), 548-556.

Pavek, P.L.S. (2012). "Plant guide for pea (Pisum sativum L.)." *USDA-Natural Resources Conservation Service*, Pullman, WA.

Pawar, P.A., Veera, U.P., Sawant, S.B. and Joshi, J.B. (1997). "Enzyme mass transfer coefficient in aqueous two- phase systems: Modified spray extraction columns." *Can. J. Chem. Eng.*, 75(4), 751-758.

Pazuki, G.R., Taghikhani, V. and Vossoughi, M. (2010). "Prediction of the partition coefficients of biomolecules in polymer–polymer aqueous two-phase systems using the artificial neural network model." *Part. Sci. Technol., 28*(1), 67-73.

Peliska, J.A. and Benkovic, S.J. (1992). "Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase." *Science*, 258(5085), 1112-1118.

Perez, R.L., Loureiro, D.B., Nerli, B.B. and Tubio, G. (2015). "Optimization of pancreatic trypsin extraction in PEG/citrate aqueous two-phase systems." *Protein Expression Purif.*, 106, 66-71.

Pericin, D.M., Radulovic, L.M. and Curcin, S.S. (2004). "Extraction of endo-pectinase activity from the culture filtrate of Polyporus squamosus by aqueous two-phase systems composed of low molecular mass polyethylene glycol and phosphate salt." *Acta Period. Technol.*, (35), 215-224.

Perumalsamy, M. and Murugesan, T. (2009). "Phase compositions, molar mass, and temperature effect on densities, viscosities, and liquid− liquid equilibrium of polyethylene glycol and salt-based aqueous two-phase systems." *J. Chem. Eng. Data*, 54(4), 1359-1366.

Perumalsamy, M. and Murugesan, T. (2012). "Extraction of Cheese whey proteins (α-lactalbumin and β-lactoglobulin) from dairy effluents using environmentally benign aqueous biphasic system." *Int. J. Chem. Environ. Eng.*, *3*(1).

Peumans, W.J. and Van Damme, E.J. (1995). “Lectins as plant defense proteins.” *Plant Physiol.*, 109(2), 347.

Pimentel, J.G., Bicalho, S.F., Gandolfi, O.R.R., Verissimo, L.A.A., de Sousa Castro, S., Souza, E.A., Veloso, C.M., Fontan, R.D.C.I., Sampaio,V.S. and Bonomo, R.C.F. (2017).“Evaluation of salting-out effect in the liquid–liquid equilibrium of aqueous two-phase systems composed of 2-propanol and Na2SO4/MgSO4 at different temperatures.” *Fluid Phase Equilib.*, 450, 184-193.

Pimentel, M.C.B., Araújo, A.I., Figueiredo, Z.M.B., Silva, R.A., Cavalcanti, M.T.H., Moreira, K.A. and Porto, A.L.F. (2013). “Aqueous two-phase system for citrinin extraction from fermentation broth.” *Sep. Purif. Technol.*, *110*, 158-163.

Pinto-Júnior, V.R., de Santiago, M.Q., Osterne, V.J.D.S., Correia, J.L.A., Pereira-Júnior, F.N., Cajazeiras, J.B., de Vasconcelos, M.A., Teixeira, E.H., do Nascimento, A.S., Miguel, T.B., Miguel Ede, C., Sampaio, A.H., do Nascimento, K.S., Nagano, C.S. and Cavada, B.S. (2013). “Purification, partial characterization and immobilization of a mannose-specific lectin from seeds of Dioclea lasiophylla mart.” *Molecules*, *18*(9), 10857-10869.

Platis, D. and Labrou, N. E. (2009). “Application of a PEG/salt aqueous two- phase partition system for the recovery of monoclonal antibodies from unclarified transgenic tobacco extract.” *Biotechnol. J.*, 4(9), 1320-1327.

Pommier, Y., Johnson, A.A. and Marchand, C. (2005). “Integrase inhibitors to treat HIV/AIDS.” *Nat. Rev. Drug Discovery*, 4(3), 236.

Porto, A.L.F., Sarubbo, L.A., Lima-Filho, J.L., Aires-Barros, M.R., Cabral, J.M.S. and

Tambourgi, E.B. (2000). “Hydrodynamics and mass transfer in aqueous two-phase protein extraction using a continuous perforated rotating disc contactor.” *Bioprocess Eng.*, 22(3), 215-218.

Porto, A.L.F., Sarubbo, L.A., Lima-Filho, J.L., Aires-Barros, M.R., Cabral, J.M.S. and Tambourgi, E.B. (2000). "Hydrodynamics and mass transfer in aqueous two-phase protein extraction using a continuous perforated rotating disc contactor." *Bioprocess Eng.*, 22(3), 215-218.

Porto, A.L.F., Sarubbo, L.A., Lima-Filho, J.L., Aires-Barros, M.R., Cabral, J.M.S. and Tambourgi, E.B. (2000). "Hydrodynamics and mass transfer in aqueous two-phase protein extraction using a continuous perforated rotating disc contactor." *Bioprocess Eng.*, 22(3), 215-218.

Porto, A.L.F., Sarubbo, L.A., Moreira, K.A., Melo, H.J.F.D., Lima-Filho, J.L., Campos-Takaki, G.M. and Tambourgi, E.B. (2004). "Recovery of ascorbic oxidoreductase from crude extract with an aqueous two-phase system in a perforated rotating disc contactor." *Braz. Arch. Biol. Technol.*, 47(5), 821-826.

Porto, C.S., Porto, T.S., Nascimento, K.S., Teixeira, E.H., Cavada, B.S., Lima-Filho, J.L. and Porto, A.L. (2011). "Partition of lectin from Canavalia grandiflora Benth in aqueous two-phase systems using factorial design." *Biochem. Eng. J.*, 53(2), 165-171.

Porto, T.S., Marques, P.P., Porto, C.S., Moreira, K.A., Lima-Filho, J.L., Converti, A., Pessoa, A. and Porto, A.L.F. (2010). "Extraction of ascorbate oxidase from Cucurbita maxima by continuous process in perforated rotating disc contactor using aqueous two-phase systems." *Appl. Biochem. Biotechnol.*, *160*(4), 1057-1064.

Priyanka, B.S., Rastogi, N.K., Raghavarao, K.S.M.S. and Thakur, M.S. (2013). "Optimization of extraction of luciferase from fireflies (Photinus pyralis) using aqueous two-phase extraction." *Sep. Purif. Technol.*, 118, 40-48.

Qureshi, I.A., Dash, P., Srivastava, P.S. and Koundal, K.R. (2006). "Purification and characterization of an N- acetyl- d- galactosamine- specific lectin from seeds of chickpea (Cicer arietinum L.)." *Phytochem. Anal.*, *17*(5), 350-356.

Raghavarao, K.S.M.S., Rastogi, N.K., Gowthaman, M.K. and Karanth, N.G. (1995). "Aqueous two-phase extraction for downstream processing of enzymes/proteins." *Adv. Appl. Microbiol.*, 41, 97-171.

Rahaie, M. and Kazemi, S.S. (2010). "Lectin-based Biosensors: As Powerful Tools in Bioanalytical Applications." *Biotechnology*, 9(4), 428-443.

Rahimpour, F. and Baharvand, A.R. (2009). "Phase Equilibrium in Aqueous Two-phase Systems containing Poly (propylene glycol) and Sodium Citrate at different pH." *Int. J. Chem. Biomol. Eng.*, 59.

Rahimpour, F., Hatti-Kaul, R., Mamo, G. (2016). "Response surface methodology and artificial neural network modelling of an aqueous two-phase system for purification of a recombinant alkaline active xylanase." *Process Biochem*., 51(3), 452-462.

Rahimpour, F., Mamo, G., Feyzi, F., Maghsoudi, S. and Hatti-Kaul, R. (2007). "Optimizing refolding and recovery of active recombinant Bacillus halodurans xylanase in polymer–salt aqueous two-phase system using surface response analysis." *J. Chromatogr. A*, 1141(1), 32-40.

Raja, S. and Murty, V.R. (2012). "Development and evaluation of environmentally benign aqueous two phase systems for the recovery of proteins from tannery waste water." *ISRN Chem. Eng*.

Raja, S. and Murty, V.R. (2013). “Optimization of aqueous two-phase systems for the recovery of soluble proteins from tannery wastewater using response surface methodology.” *J. Eng*., 2013.

Raja, S., Murty, V.R., Thivaharan, V., Rajasekar, V. and Ramesh, V. (2011). “Aqueous two phase systems for the recovery of biomolecules–a review.” *Sci. Technol.,* 1(1), 7-16.

Rao, J.R., and Nair, B.U. (2011). “Novel approach towards recovery of glycosaminoglycans from tannery wastewater”. *Bioresour. Technol., 102*(2), 872-878.

Rathnasamy, S., Vedhahari, B.N. and Kumaresan, R. (2014). “Partition and purification of lysozyme from quail egg white using aqueous two phase system.” *Biotechnol.: Indian J.,* 9(4), 166-170.

Regupathi, I., Murugesan, S., Govindarajan, R., Amaresh, S.P. and Thanapalan, M. (2009). “Liquid− liquid equilibrium of poly (ethylene glycol) 6000+ triammonium citrate+ water systems at different temperatures.” *J. Chem. Eng. Data*, 54(3), 1094-1097.

Renkonen, K.O. (1948). “Studies on hemagglutinins present in seeds of some representatives of the family of Leguminosae.” *Ann. Med. Exp. Biol. Fenn.*, 26, 66-72.

Rini, J.M., Hardman, K.D., Einspahr, H., Suddath, F.L. and Carver, J.P. (1993). “X-ray crystal structure of a pea lectin-trimannoside complex at 2.6 A resolution.” *J. Biol. Chem., 268*(14), 10126-10132.

Rito-Palomares, M. (2004). “Practical application of aqueous two-phase partition to process development for the recovery of biological products.” *J. Chromatogr. B*, 807(1), 3-11.

Rito-Palomares, M. and Hernandez, M. (1998). "Influence of system and process parameters on partitioning of cheese whey proteins in aqueous two-phase systems." *J. Chromatogr. B: Biomed. Sci. Appl.*, *711*(1-2), 81-90.

Roberts, D.D. and Goldstein, I.J. (1983). "Binding of hydrophobic ligands to plant lectins: titration with arylaminonaphthalene sulfonates." *Arch. Biochem. Biophys*., 224(2), 479-484.

Rodríguez, O., Silverio, S.C., Madeira, P.P., Teixeira, J.A. and Macedo, E.A. (2007). "Physicochemical characterization of the PEG8000-Na2SO4 aqueous two-phase system." *Ind. Eng. Chem. Res.*, 46(24), 8199-8204.

Rosa, P.A., Azevedo, A.M. and Aires-Barros, M.R. (2007). "Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies." *J. Chromatogr. A*, 1141(1), 50-60.

Rosa, P.A., Azevedo, A.M., Sommerfeld, S., Mutter, M., Bäcker, W. and Aires- Barros, M.R. (2013). "Continuous purification of antibodies from cell culture supernatant with aqueous two- phase systems: from concept to process." *Biotechnol. J.*, *8*(3), 352-362.

Rosa, P.A.J., Azevedo, A.M., Sommerfeld, S., Bäcker, W. and Aires-Barros, M.R. (2012). "Continuous aqueous two-phase extraction of human antibodies using a packed column." *J. Chromatogr. B*, 880, 148-156.

Rosa, P.A.J., Azevedo, A.M., Sommerfeld, S., Mutter, M., Aires-Barros, M.R. and Bäcker, W. (2009). "Application of aqueous two-phase systems to antibody purification: a multi-stage approach." *J. Biotechnol*., 139(4), 306-313.

Ruckenstein, E. and Guo, W. (2001). “Crosslinked mercerized cellulose membranes and their application to membrane affinity chromatography.” *J. Membr. Sci.*, *187*(1-2), 277-286.

Ruiz-Ruiz, F., Benavides, J., Aguilar, O. and Rito-Palomares, M. (2012). “Aqueous two-phase affinity partitioning systems: current applications and trends.” *J. Chromatogr. A,* 1244, 1-13.

Sadeghi, A., Smagghe, G., Broeders, S., Hernalsteens, J.P., De Greve, H., Peumans, W.J. and Van Damme, E.J. (2008). “Ectopically expressed leaf and bulb lectins from garlic (Allium sativum L.) protect transgenic tobacco plants against cotton leafworm (Spodoptera littoralis).” *Transgenic Res.*, 17(1), 9.

Sadeghi, R., Golabiazar, R. and Shekaari, H. (2010). “The salting-out effect and phase separation in aqueous solutions of tri-sodium citrate and 1-butyl-3-methylimidazolium bromide.” *J. Chem. Thermodyn.*, *42*(4), 441-453.

Salmon, D. N. X., Walter, A., Porto, T. S., Moreira, K. A., Vandenberghe, L. P. D. S., Soccol, C. R., Spier, M. R. (2014). Aqueous two-phase extraction for partial purification of Schizophyllum commune phytase produced under solid-state fermentation. *Biocatalysis and Biotransformation*, *32*(1), 45-52.

Sanchez, E., Shibata, T. and Zadeh, L.A. (1997). “Genetic Algorithms and Fuzzy Logic Systems.” *World Scientific*.

Sano, K. and Ogawa, H. (2014). “Hemagglutination (inhibition) Assay.” *Lectins*, 47-52. Humana Press, New York.

Santos, A.F., da Silva, M.D.C., Napoleão, T.H., Paiva, P.M.G., Correia, M.T.S. and Coelho, L.C.B.B. (2014). "Lectins: Function, structure, biological properties and potential applications." *Curr. Top. Pept. Protein Res.*, 15, 41-62.

Santos, A.F., Napoleão, T.H., Bezerra, R.F., Carvalho, E.V.M.M., Correia, M.T., Paiva, P.M. and Coelho, L.C.B.B. (2013). "Strategies to obtain lectins from distinct sources." *Adv. Med. Biol.*, *63*, 33-60.

Sarangi, B.K., Pattanaik, D.P., Rathinaraj, K., Sachindra, N.M., Madhusudan, M.C. and Mahendrakar, N.S. (2011). "Purification of alkaline protease from chicken intestine by aqueous two phase system of polyethylene glycol and sodium citrate." *J. Food Sci. Technol.*, *48*(1), 36-44.

Saravanan, S., Rao, J.R., Murugesan, T., Nair, B.U. and Ramasami, T. (2007). "Partition of tannery wastewater proteins in aqueous two-phase poly (ethylene glycol)-magnesium sulfate systems: effects of molecular weights and pH." *Chem. Eng. Sci.*, *62*(4), 969-978.

Sarubbo, L.A., Oliveira, L.A., Porto, A.L.F., Lima-Filho, J.L., Campos-takaki, G.M. and Tambourgi, E.B. (2003). "Performance of a perforated rotating disc contactor in the continuous extraction of a protein using the PEG–cashew-nut tree gum aqueous two-phase system." *Biochem. Eng. J.*, *16*(3), 221-227.

Sattsangi, P.D. and Sattsangi, S. (1984). "Acetone Precipitation - An Improved Procedure for the Isolation of Soybean Agglutinin." *Prep. Biochem.*, 14(5), 471-483.

Sawant, S.B., Sikdar, S. K. and Joshi, J. B. (1990). "Hydrodynamics and mass transfer in two- phase aqueous extraction using spray columns." *Biotechnol. Bioeng.*, *36*(2), 109-115.

Sedlak, M. (2005). “Recent advances in chemistry and applications of substituted poly (ethylene glycol) s.” *Collect. Czech. Chem. Commun.*, 70(3), 269-291.

Settu, S., Velmurugan, P., Jonnalagadda, R.R. and Nair, B.U. (2015). “Extraction of bovine serum albumin using aqueous two-phase poly (ethylene glycol)–poly (acrylic acid) system.” *J. Sci. Ind. Res., Sect. A*, 74, 348-353.

Shad, Z., Mirhosseini, H., Hussin, A.S.M., Forghani, B., Motshakeri, M. and Manap, M.Y.A. (2018). “Aqueous two-phase purification of α-Amylase from white pitaya (Hylocereus undatus) peel in polyethylene glycol/citrate system: Optimization by response surface methodology.” *Biocatal. Agric. Biotechnol.*, 14, 305-313.

Shang, Q.K., Li, W., Jia, Q. and Li, D.Q. (2004). “Partitioning behavior of amino acids in aqueous two-phase systems containing polyethylene glycol and phosphate buffer.” *Fluid Phase Equilib.*, *219*(2), 195-203.

Shao, B., Wang, S., Zhou, J., Ke, L. and Rao, P. (2011). “A novel lectin from fresh rhizome of Alisma orientale (Sam.) Juzep.” *Process Biochem.*, 46(8), 1554-1559.

Shao, M., Zhang, X., Li, N., Shi, J., Zhang, H., Wang, Z., Zhang, H., Yu, A. and Yu, Y. (2014). “Ionic liquid-based aqueous two-phase system extraction of sulfonamides in milk.” *J. Chromatogr. B*, *961*, 5-12.

Sharma, A., Ng, T.B., Wong, J.H. and Lin, P. (2009). “Purification and characterization of a lectin from Phaseolus vulgaris cv. (Anasazi beans).” *BioMed Res. Int.*

Sharon, N. and Lis, H. (1990). “Legume lectins - a large family of homologous proteins.” *FASEB J.*, 4(14), 3198-3208.

Sharon, N. and Lis, H. (1990). “Legume lectins--a large family of homologous proteins.” *FASEB J.*, *4*(14), 3198-3208.

Shimura, K. and Kasai, K.I. (1987). “Affinophoresis of pea lectin and fava bean lectin with an anionic affinophore, bearing p-aminophenyl-α-D-mannoside as an affinity ligand.” *J. Chromatogr. A*, 400, 353-359.

Shu, Y., Gao, M., Wang, X., Song, R., Lu, J. and Chen, X. (2016). “Separation of curcuminoids using ionic liquid based aqueous two-phase system coupled with in situ dispersive liquid–liquid microextraction.” *Talanta*, *149*, 6-12.

Silva, M.C.C., Santana, L.A., Mentele, R., Ferreira, R.S., de Miranda, A., Silva-Lucca, R. A., Sampaio, M.U., Correia, M.T.S. and Oliva, M.L. (2012). “Purification, primary structure and potential functions of a novel lectin from Bauhinia forficata seeds.” *Process Biochem.*, 47(7), 1049-1059.

Sindhu, N., Kalaivani, S. and Regupathi, I. (2013). “Bovine Serum Albumin Partitioning in Aqueous Two-Phase Systems: Effects of Variables and Optimization.” *BioProcess. J.*, 2013; 12(1): 29-41.

Sindra Virtuoso, L., Ramos Paes Lima, L., Silva Figueiredo Vello, K.A., Costa Couto, H.H., Medeiros Alves, M., Simone Garcia, J. and Schneedorf Ferreira da Silva, J.M. (2010). “Bovine serum albumin partitioning in aqueous two-phase systems.” *Rev. CENIC, Cienc. Quim.*, 41

Singh, R.S., Bhari, R. and Kaur, H.P. (2010). “Mushroom lectins: current status and future perspectives.” *Crit. Rev. Biotechnol.*, *30*(2), 99-126.

Sitohy, M., Doheim, M. and Badr, H. (2007). “Isolation and characterization of a lectin with antifungal activity from Egyptian Pisum sativum seeds.” *Food Chem.*, 104(3), 971-979.

Smith, P.E., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985). “Measurement of protein using bicinchoninic acid.” *Anal. Biochem.* 150, 76-85.

Soares, P.A., Nascimento, C.O., Porto, T.S., Correia, M.T., Porto, A.L. and Carneiro-da-Cunha, M.G. (2011). “Purification of a lectin from Canavalia ensiformis using PEG–citrate aqueous two-phase system.” *J. Chromatogr. B, 879*(5-6), 457-460.

Srinivas, N.D., Barhate, R.S. and Raghavarao, K.S.M.S. (2002). “Aqueous two-phase extraction coupled with ultrafiltration for downstream processing of a plant peroxidase.” *J. Food Eng.*, 54, 1–6.

Su, C.K. and Chiang, B.H. (2006). “Partitioning and purification of lysozyme from chicken egg white using aqueous two-phase system.” *Process Biochem.*, 41(2), 257-263.

Sumner, J.B. (1919). “The globulins of the Jack bean, *Canavalia Ensiformis* - preliminary paper.” *J. Biol. Chem.*, 37(1), 137-142.

Sun, J., Wang, L., Wang, B., Guo, Z., Liu, M., Jiang, K. and Luo, Z. (2007). “Purification and characterisation of a natural lectin from the serum of the shrimp Litopenaeus vannamei.” *Fish Shellfish Immunol.*, 23(2), 292-299.

Sung, L.A., Kabat, E.A. and Chien, S. (1985). “Interaction of lectins with membrane receptors on erythrocyte surfaces.” *J. Cell Biol.*, 101(2), 646-651.

Suseelan, K.N., Bhagwath, A., Pandey, R. and Gopalakrishna, T. (2007). “Characterization of Con C, a lectin from Canavalia cathartica Thouars seeds.” *Food Chem.* 104, 528–535.

Suzuki, T., Amano, Y., Fujita, M., Kobayashi, Y., Dohra, H., Hirai, H., Muruta, T., Usui, T., Morita, T. and Kawagishi, H. (2009). "Purification, characterization, and cDNA cloning of a lectin from the mushroom Pleurocybella porrigens." *Biosci., Biotechnol., Biochem., 73*(3), 702-709.

Swanson, M.D., Winter, H.C., Goldstein, I.J. and Markovitz, D.M. (2010). "A lectin isolated from bananas is a potent inhibitor of HIV replication." *J. Biol. Chem.*, 285(12).

Teotia, S., Mondal, K. and Gupta, M.N. (2006). "Integration of affinity precipitation with partitioning methods for bioseparation of chitin binding lectins." *Food Bioprod. Process.*, 84(1), 37-43.

Thakur, A., Rana, M., Lakhanpal, T.N., Ahmad, A. and Khan, M.I. (2007). "Purification and characterization of lectin from fruiting body of Ganoderma lucidum: Lectin from Ganoderma lucidum." *Biochim. Biophys. Acta, Gen. Subj.*, 1770, 1404–1412.

Trowbridge, I.S. (1973). "Mitogenic properties of pea lectin and its chemical derivatives." *Proc. Natl. Acad. Sci.*, 70(12), 3650-3654.

Trowbridge, I.S. (1974). "Isolation and chemical characterization of a mitogenic lectin from Pisum sativum." *J. Biol. Chem.*, 249(18), 6004-6012.

Tubío, G., Pellegrini, L., Nerli, B.B. and Picó, G.A. (2006). "Liquid− liquid equilibria of aqueous two-phase systems containing poly (ethylene glycols) of different molecular weight and sodium citrate." *J. Chem. Eng. Data*, 51(1), 209-212.

Tyagi, R., Agarwal, R. and Gupta, M.N. (1996). "Purification of peanut lectin using guar gum as an affinity ligand." *J. Biotechnol., 46*(2), 79-83.

Valadez-Vega, C., Guzmán-Partida, A.M., Soto-Cordova, F.J., Álvarez-Manilla, G., Morales-González, J.A., Madrigal-Santillán, E., Villagómez-Ibarra, J.R., Zúñiga-Pérez, C., Gutiérrez-Salinas, J. and Becerril-Flores, M.A. (2011). "Purification, biochemical characterization, and bioactive properties of a lectin purified from the seeds of white tepary bean (Phaseolus acutifolius variety latifolius)." *Molecules*, *16*(3), 2561-2582.

Van Damme, E.J., Briké, F., Winter, H.C., Leuven, F., Goldstein, I.J. and Peumans, W.J. (1996). "Molecular cloning of two different mannose- binding lectins from tulip bulbs." *Eur. J. Biochem.*, 236(2), 419-427.

Van Damme, E.J.M., Lannoo, N. and Peumans, W.J. (2008). "Plant lectins." *Adv. Bot. Res.*, 48, 107-209.

Van Damme, E.J.M., Peumans, W.J., Barre, A. and Rougé, P. (1998). "Plant Lectins: A Composite of Several Distinct Families of Structurally and Evolutionary Related Proteins with Diverse Biological Roles." *Crit. Rev. Plant Sci.*, 17, 575–692.

Van Parijs, J., Broekaert, W.F., Goldstein, I.J. and Peumans, W. J. (1991). "Hevein: an antifungal protein from rubber-tree (Hevea brasiliensis) latex." *Planta*, 183(2), 258-264.

Vázquez-Villegas, P., Aguilar, O. and Rito-Palomares, M. (2011). "Study of biomolecules partition coefficients on a novel continuous separator using polymer-salt aqueous two-phase systems." *Sep. Purif. Technol.*, 78(1), 69-75.

Vernau, J. and Kula, M.R. (1990). "Extraction of proteins from biological raw material using aqueous polyethylene glycol-citrate phase systems." *Biotechnol. Appl. Biochem.*, 12(4), 397-404.

Vidal- Valverde, C., Frias, J., Hernandez, A., Martín- Alvarez, P.J., Sierra, I., Rodríguez, C., Blazquez, I. and Vicente, G. (2003). "Assessment of nutritional compounds and antinutritional factors in pea (Pisum sativum) seeds." *J. Sci. Food Agric.*, *83*(4), 298-306.

Voros, N., Proust, P. and Fredenslund, A. (1993). "Liquid-liquid phase equilibria of aqueous two-phase systems containing salts and polyethylene glycol." *Fluid Phase Equilib.*, *90*(2), 333-353.

Watkins, W.M. and Morgan, W.T.J. (1952). "Neutralization of the anti-H agglutinin in eel serum by simple sugars." *Nature*, *169*(4307), 825.

Wenzel, M. and Rüdiger, H. (1995). "Interaction of Pea (Pisum sativum L.) Lectin with Pea Storage Proteins." *J. Plant Physiol.*, 145, 191-194.

Wongmongkol, N. and Prichanont, S. (2006). "Partition of alkaline protease in aqueous two-phase systems of polyethylene glycol 1000 and potassium phosphate." Korean J. Chem. Eng., 23(1), 71-76.

Wu, W.C., Ng, H.S., Sun, I.M. and Lan, J.C.W. (2017). "Single step purification of bromelain from Ananas comosus pulp using a polymer/salt aqueous biphasic system." *J. Taiwan Inst. Chem. Eng.*, 79, 158-162.

Wu, Z., Hu, G., Wang, K., Zaslavsky, B.Y., Kurgan, L. and Uversky, V.N. (2017). "What are the structural features that drive partitioning of proteins in aqueous two-phase systems?" *Biochim. Biophys. Acta, Proteins Proteomics*, 1865(1), 113-120.

Xiao, J.X., Sivars, U. and Tjerneld, F. (2000). “Phase behavior and protein partitioning in aqueous two-phase systems of cationic–anionic surfactant mixtures.” *J. Chromatogr. B: Biomed. Sci. Appl.*, 743(1-2), 327-338.

Yan, Q., Jiang, Z., Yang, S., Deng, W. and Han, L. (2005). “A novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity.” *Arch. Biochem. Biophys.*, 442, 72–81.

Yanagi, K., Ohyama, K., Yamakawa, T., Hashimoto, K. and Ohkuma, S. (1990). “Purification and characterization of anti-N lectin from Vicia unijuga leaves.” *Int. J. Biochem.*, 22(1), 43-52.

Yang, L., Huo, D., Hou, C., He, K., Lv, F., Fa, H. and Luo, X. (2010). “Purification of plant-esterase in PEG1000/NaH2PO4 aqueous two-phase system by a two-step extraction.” *Process Biochem.*, *45*(10), 1664-1671.

Yang, N., Li, D.F., Feng, L., Xiang, Y., Liu, W., Sun, H. and Wang, D.C. (2009). “Structural basis for the tumor cell apoptosis-inducing activity of an antitumor lectin from the edible mushroom Agrocybe aegerita.” *J. Mol. Biol.*, 387(3), 694-705.

Yucekan, İ. and Önal, S. (2011). “Partitioning of invertase from tomato in poly (ethylene glycol)/sodium sulfate aqueous two-phase systems.” *Process Biochem.*, 46(1), 226-232.

Zafarani-Moattar, M.T. and Hamidi, A.A. (2003). “Liquid− liquid equilibria of aqueous two-phase poly (ethylene glycol) − potassium citrate system.” *J. Chem. Eng. Data*, *48*(2), 262-265.

Zem, G.C., Badali, O., Gaytan, M., Hekmatjou, H., Alvarez, M., Nnoli, J., Katus, E. and Oppenheimer, S.B. (2006). “Microbead analysis of cell binding to immobilized lectin: an

alternative to microarrays in the development of carbohydrate drugs and diagnostic tests." *Acta Histochem.*, 108(4), 311-317.

Zeng, X. and Ruckenstein, E. (1998). "Cross-linked macroporous chitosan anion-exchange membranes for protein separations." *J. Membr. Sci.*, *148*(2), 195-205.

Zhang, C., Medina-Bolivar, F., Buswell, S. and Cramer, C.L. (2005). "Purification and stabilization of ricin B from tobacco hairy root culture medium by aqueous two-phase extraction." *J. Biotechnol.*, *117*(1), 39-48.

Zhao, S., Zhao, Y., Li, S., Zhao, J., Zhang, G., Wang, H. and Ng, T. B. (2010). "A novel lectin with highly potent antiproliferative and HIV-1 reverse transcriptase inhibitory activities from the edible wild mushroom Russula delica." *Glycoconjugate J.*, *27*(2), 259-265.

Zhi, W., Song, J., Bi, J. and Ouyang, F. (2004). "Partial purification of α-amylase from culture supernatant of Bacillus subtilis in aqueous two-phase systems." *Bioprocess Biosyst.* Eng., *27*(1), 3-7.

Zhi, W., Song, J., Ouyang, F. and Bi, J. (2005). Application of response surface methodology to the modeling of α-amylase purification by aqueous two-phase systems. *J. Biotechnol.*, *118*(2), 157-165.

Zijlstra, G.M., de Gooijer, C.D. and Tramper, J. (1998). "Extractive bioconversions in aqueous two-phase systems." *Curr. Opin. Biotechnol.*, 9(2), 171-176.

APPENDIX 1

Table A1.1 Binodal data developed in the present study for PEG-6000 (Sodium citrate (SC)), PEG 8000-Sodium citrate/potassium citrate (PC) and ammonium citrate (AC)

SC	PEG 6000	SC	PEG 8000	PC	PEG 8000	AC	PEG 8000
3.6	45.10	32.03	0.1	32	0.1	32.1	0.14
3.9	40.02	29.56	0.21	30.4	0.21	30.9	0.23
4.2	38.14	27.12	0.42	27.3	0.44	27.5	0.45
4.4	36.06	25.08	0.58	25.8	0.59	25.9	0.61
4.8	34.08	24.10	0.63	24.3	0.6	24.45	0.65
5.0	30.20	23.06	0.68	23.3	0.69	23.4	0.71
5.4	28.54	20.04	0.764	20.4	0.764	20.5	0.9
6.0	24.65	19.02	0.79	19.3	0.8	19.5	0.9
7.06	20.42	18.04	0.84	18.4	0.85	18.7	1.5
8.4	17.21	15.81	0.9	17.6	0.94	18.1	1.8
9.12	13.85	15.02	1.03	16.6	1.03	17.4	2.1
10.14	11.53	14.13	2.21	15.9	2.3	16.5	3.2
11.09	9.54	12.64	4.3	14.4	4.4	15.9	4.5
12.03	7.38	11.81	5.84	13.7	5.9	15.1	6.1
13.5	5.5	11.22	6.8	12.9	6.8	14.3	7.5
14.3	4.3	10.20	9.02	12	9.02	13.4	8.9
15.6	2.9	9.7	10.2	11.3	10.2	12.4	10.6
17.8	1.5	8.9	11.98	10.5	12.1	11.1	12.9
18.7	1.4	7.8	14	9.2	14	9.8	15.2
23.6	1.3	7.2	15.9	8.2	15.9	8.6	16.9
30.5	1.2	6.6	17.62	7.5	17.62	7.9	18.64
35	1.0	5.8	19.97	6.8	20.3	7.2	21.2
37	0.9	5.2	22.12	5.9	22.7	6.2	24.34
42	0.8	4.8	25.03	5.5	25.03	5.8	26.98
		4.2	27.68	5.2	28.05	5.4	29.6
		3.9	30.12	4.8	30.12	4.95	32.2
		3.4	33.75	4.5	34.23	4.7	34.8
		3.3	36.38	4.2	36.45	4.4	37.3
		3.1	41.32	3.6	41.76	3.3	42.7
		3.02	47.02	3.1	47.85	3.13	48.2
		2.9	48.63	2.9	49.3	3.02	50

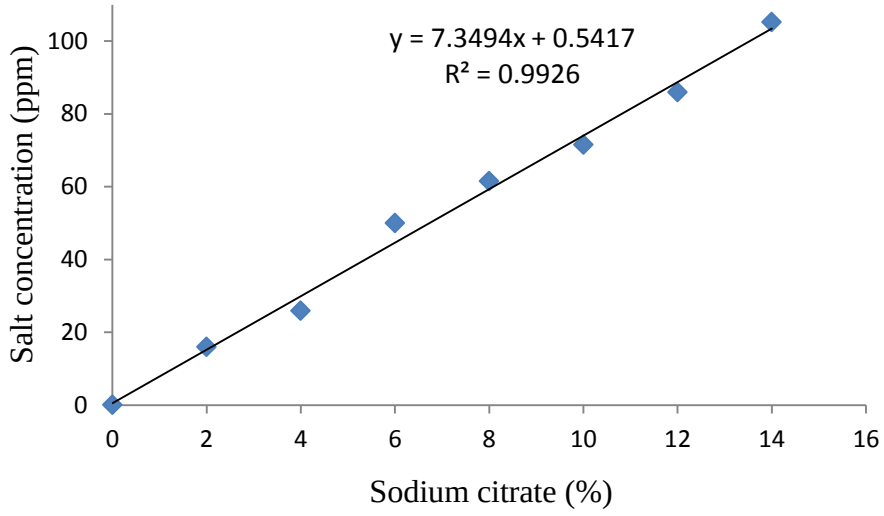


Fig. A1.1 Calibration curve developed for the estimation of trisodium citrate

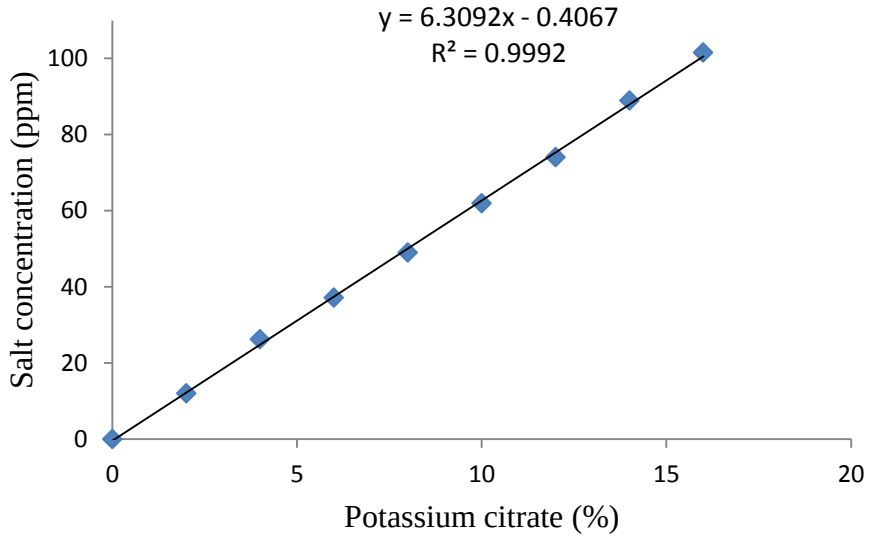


Fig. A1.2 Calibration curve developed for the estimation of potassium citrate

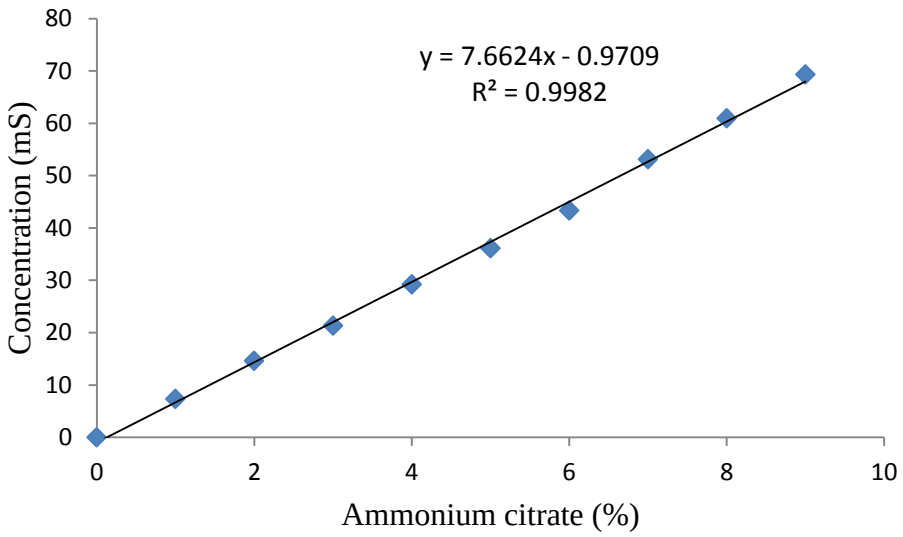


Fig. A1.3 Calibration curve developed for the estimation of ammonium citrate

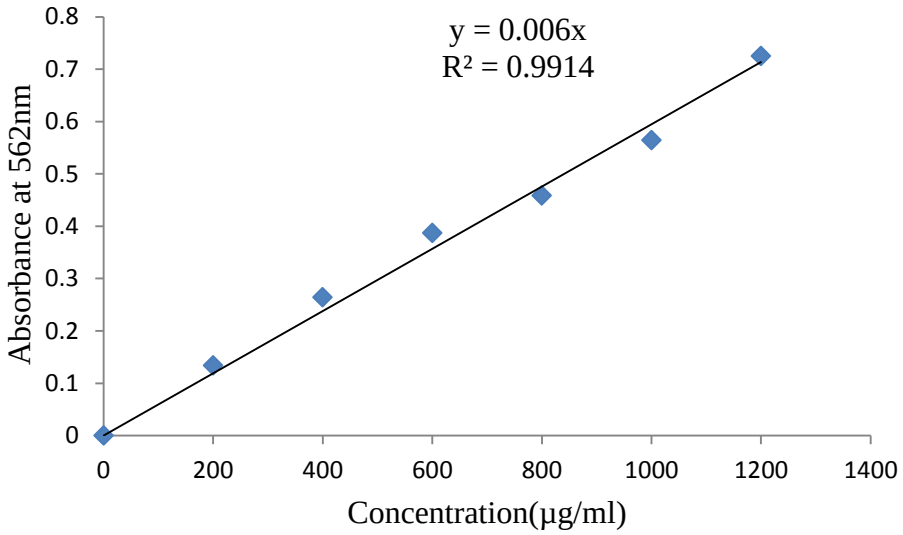


Fig. A1.4 Calibration curve of pure *Pisum sativum* lectin.

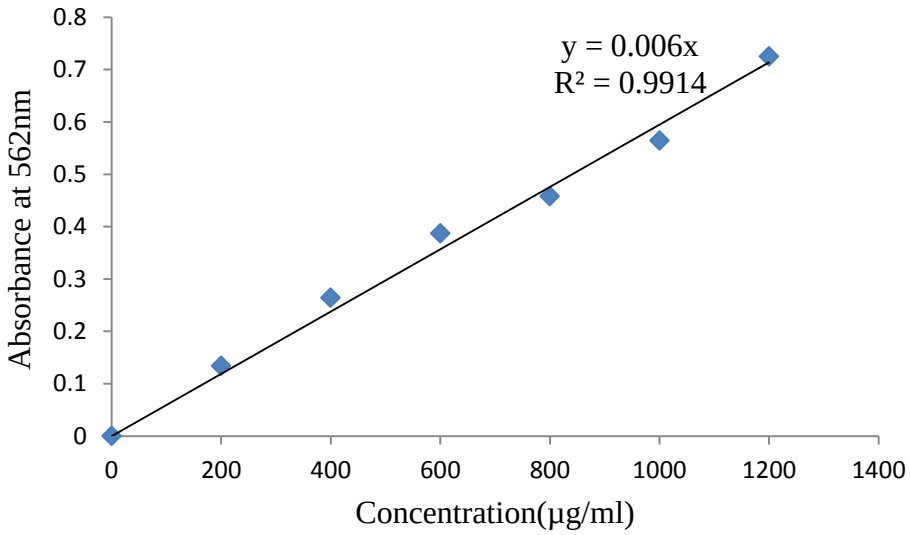


Fig. A1.5 Calibration plot for protein estimation

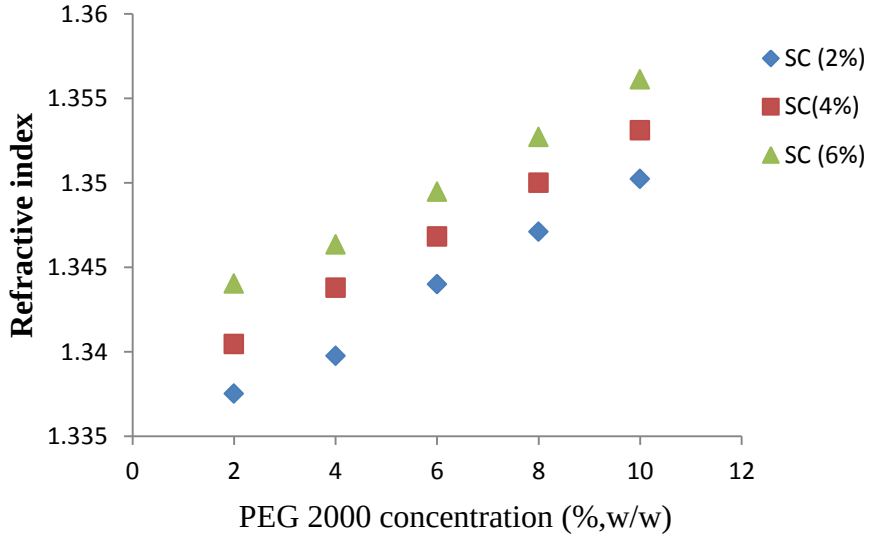


Fig. A1.6 Calibration curve for different PEG 2000 and sodium citrate (SC) concentrations

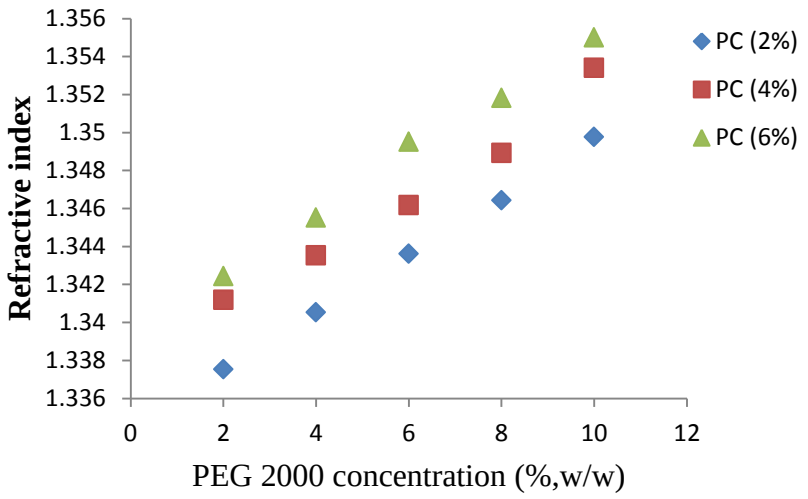


Fig. A1.7 Calibration curve for different PEG 2000 and potassium citrate (PC) concentrations

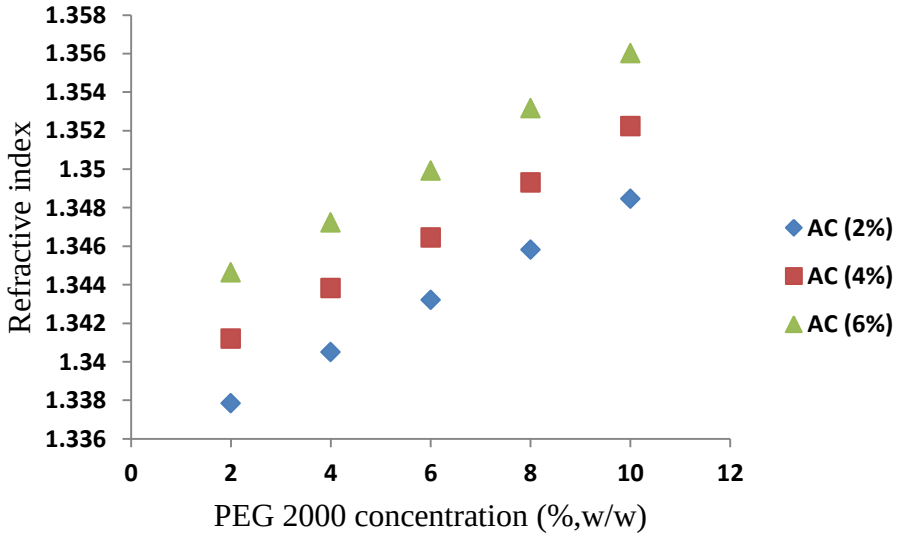


Fig. A1.8 Calibration curve for different PEG 2000 and ammonium citrate (AC) concentration.

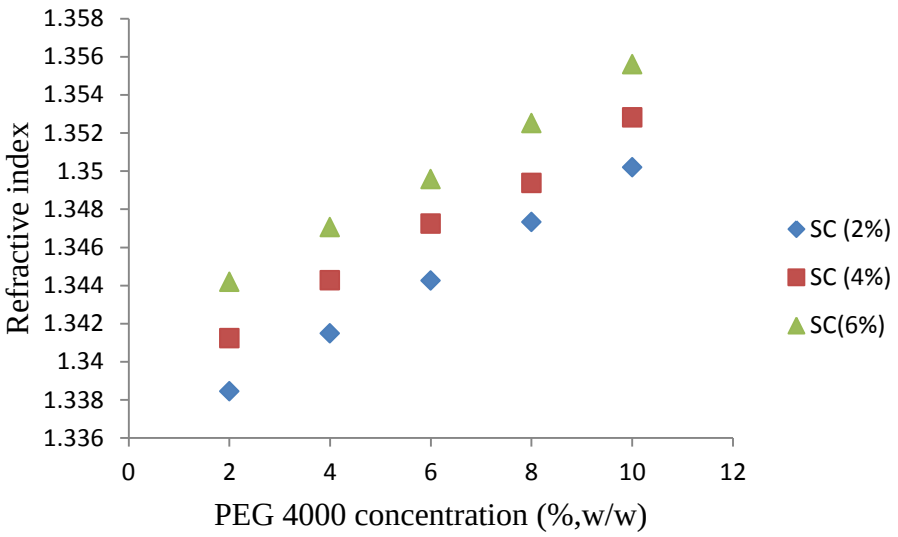


Fig. A1.9 Calibration curve for different PEG 4000 and sodium citrate (SC) Concentrations

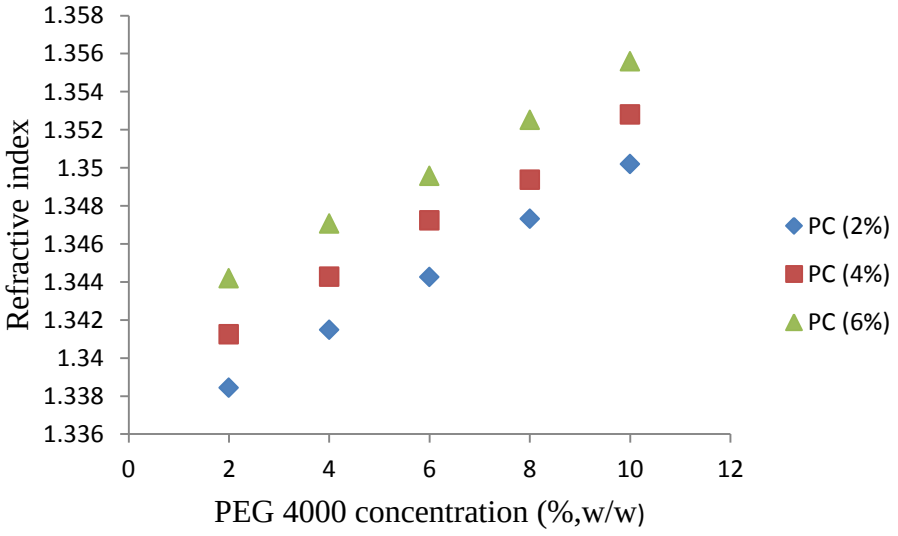


Fig. A1.10 Calibration curve for different PEG 4000 and potassium citrate (PC) concentrations.

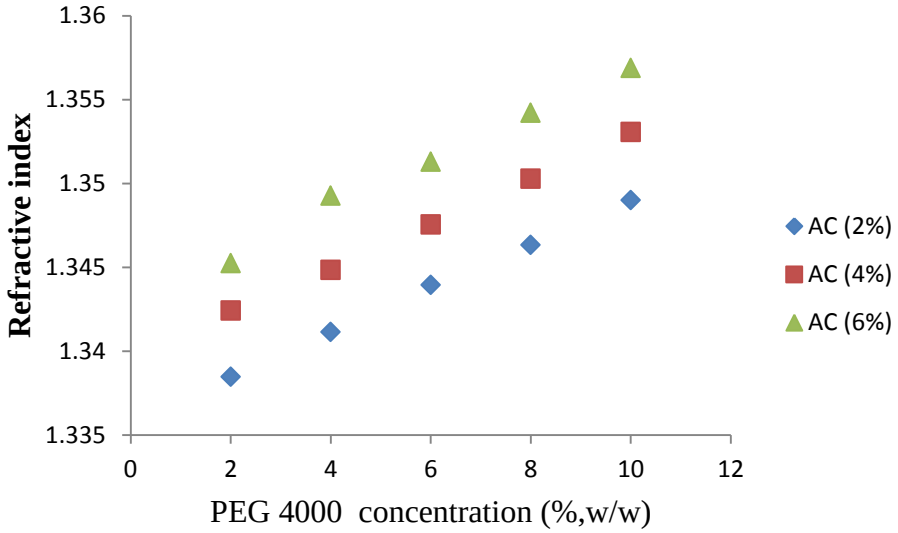


Fig. A1.11 Calibration curve for different PEG 4000 and ammonium citrate (AC) concentrations

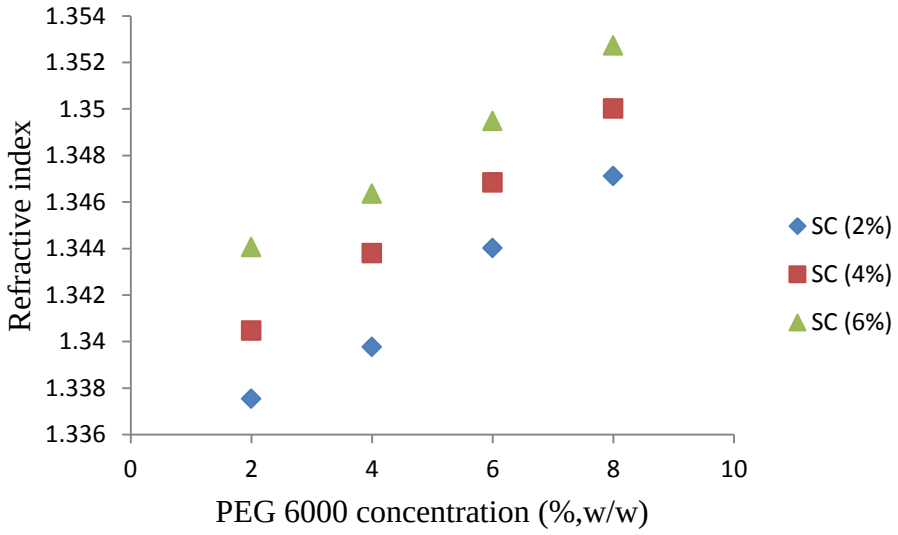


Fig. A1.12 Calibration curve for different PEG 6000 and sodium citrate (SC) concentrations

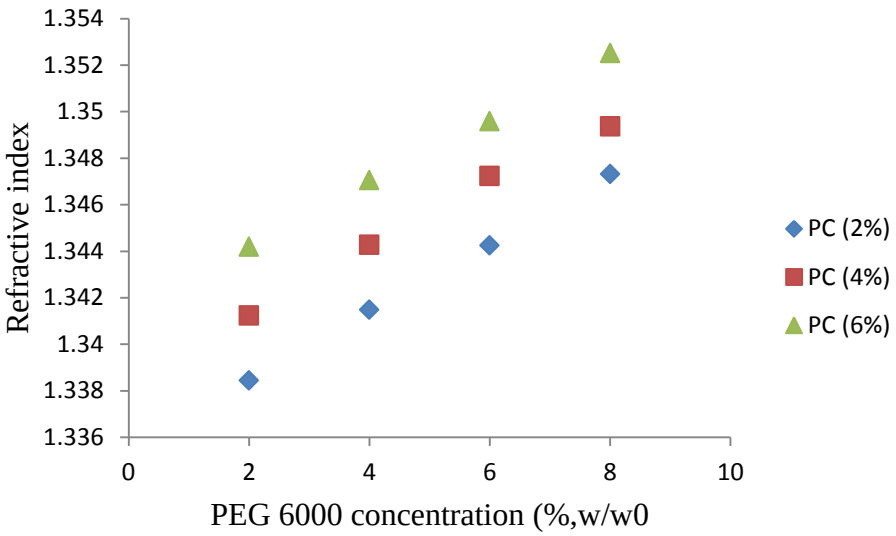


Fig. A1.13 Calibration curve for different PEG 6000 and potassium citrate (PC) concentrations

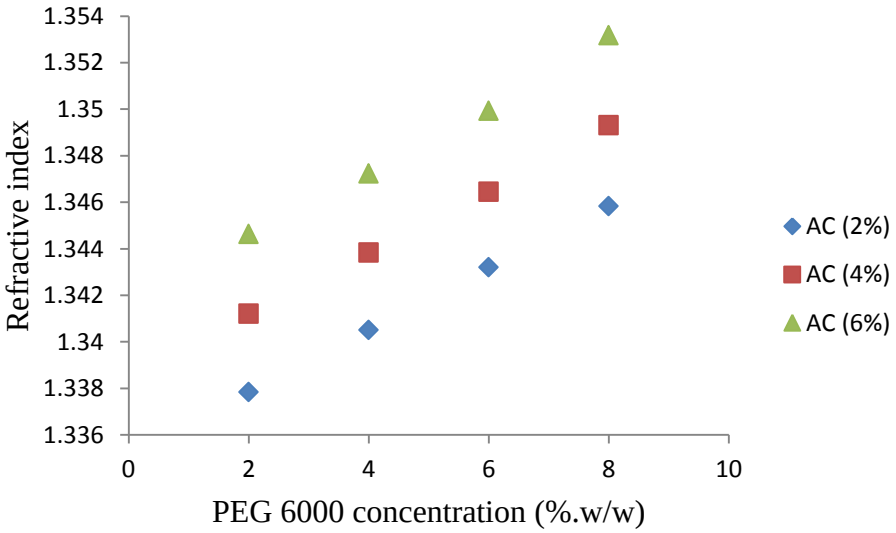


Fig. A1.14 Calibration curve for different PEG 6000 and ammonium citrate (AC) concentrations

APPENDIX 2

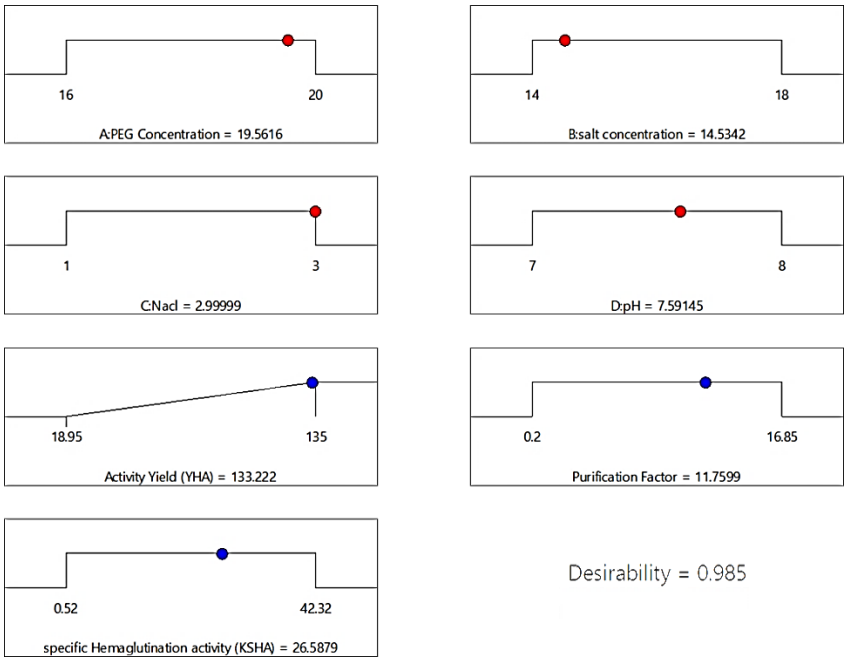


Fig. A2.1 Ramp graph for the maximization of hemagglutination activity yield % (goal I)

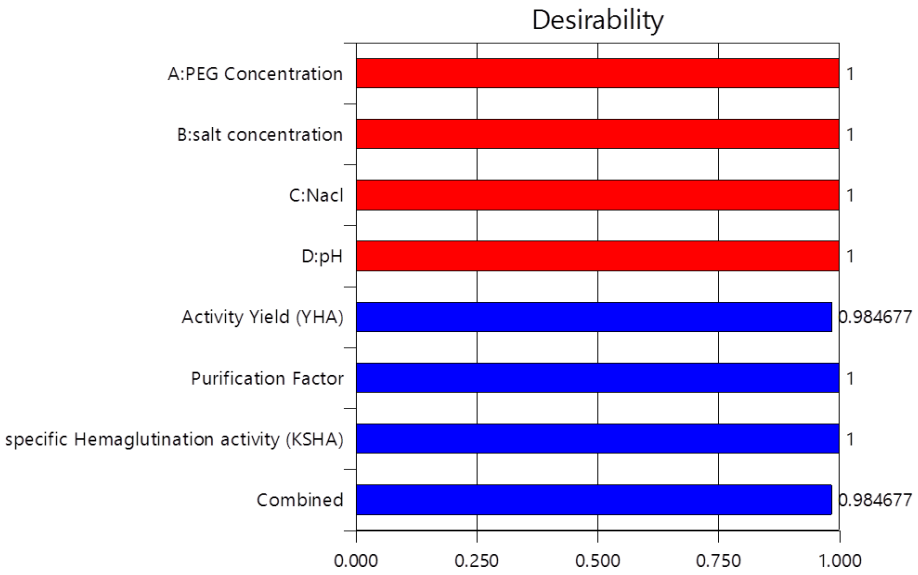


Fig. A2.2 The desirability of individual target achieved for the maximization of hemagglutination activity yield % (goal I)

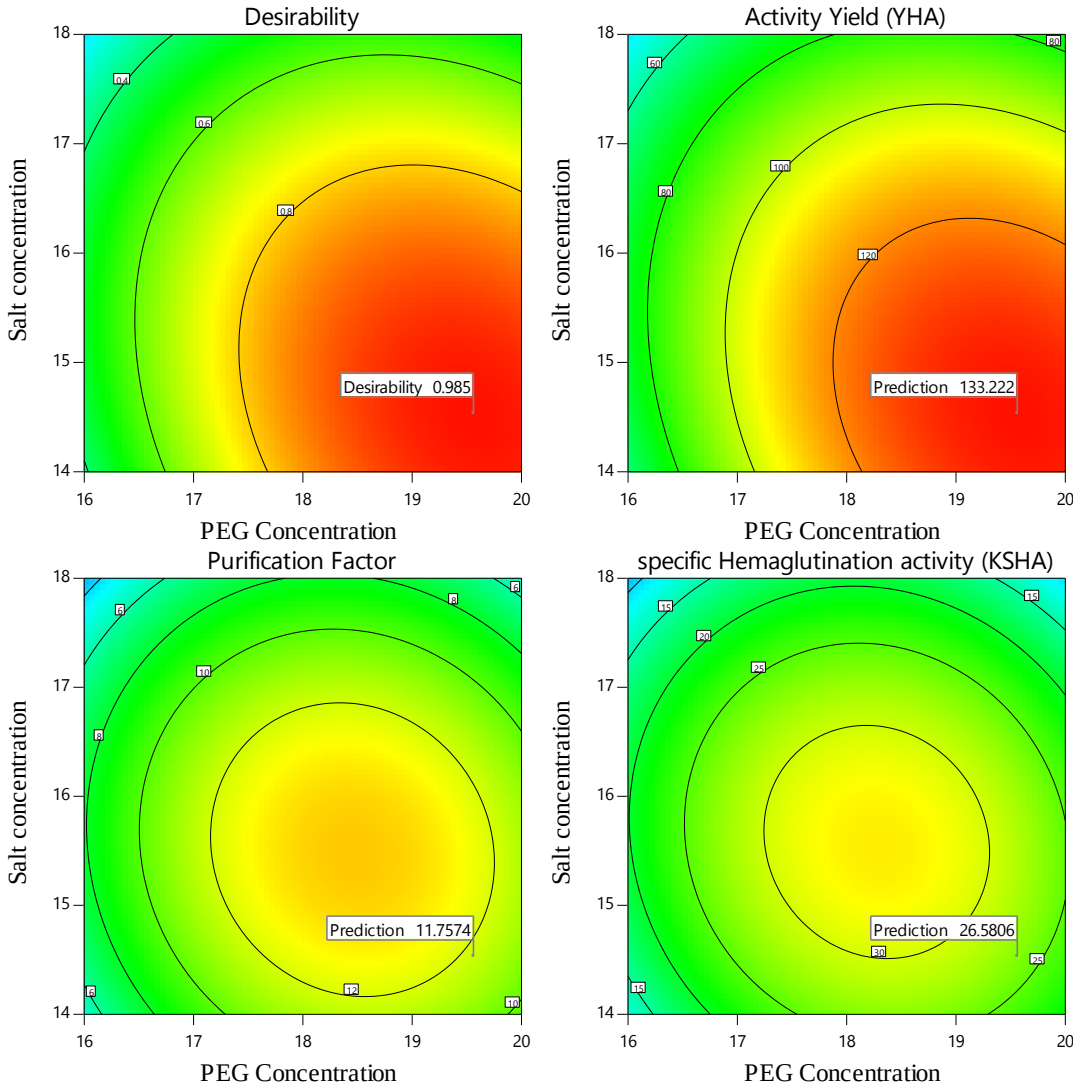


Fig. A2.3 Contour plots for the responses for maximization of hemagglutination activity yield % (goal I)

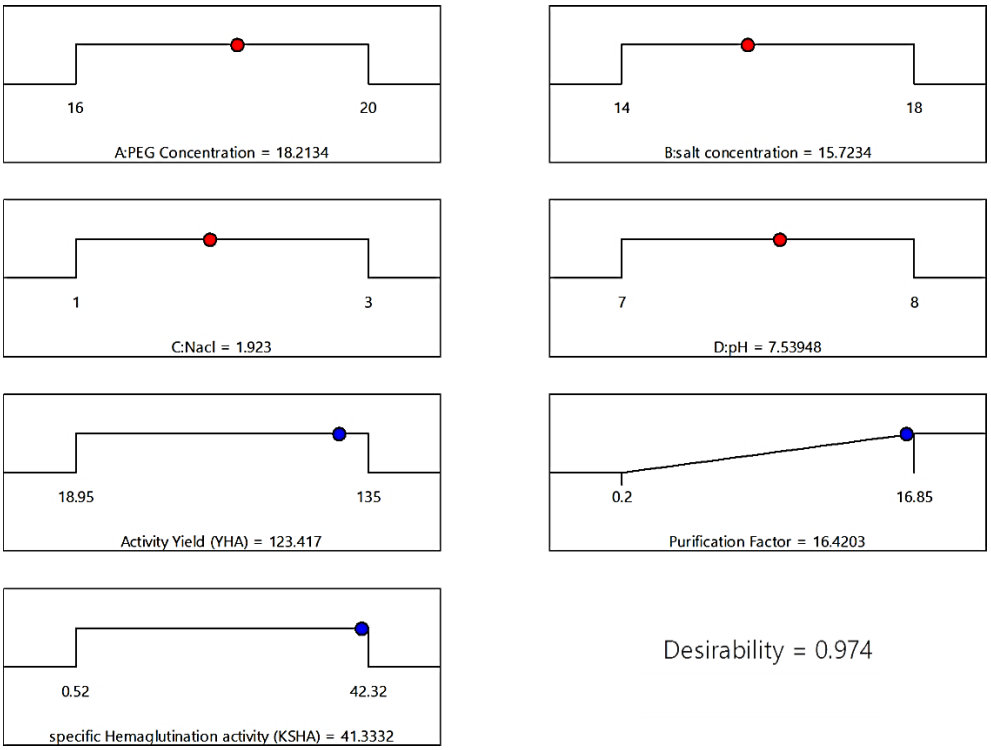


Fig. A2.4 Ramp graph for the maximization of purification factor (goal II)

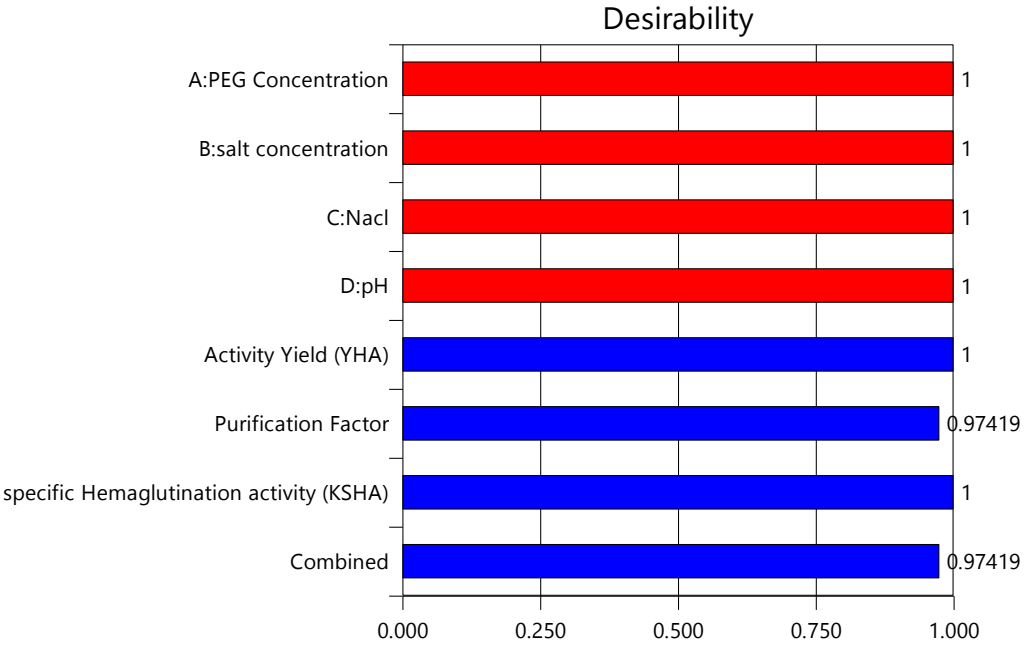


Fig. A2.5 The desirability of individual target achieved for the maximization of purification factor (goal II)

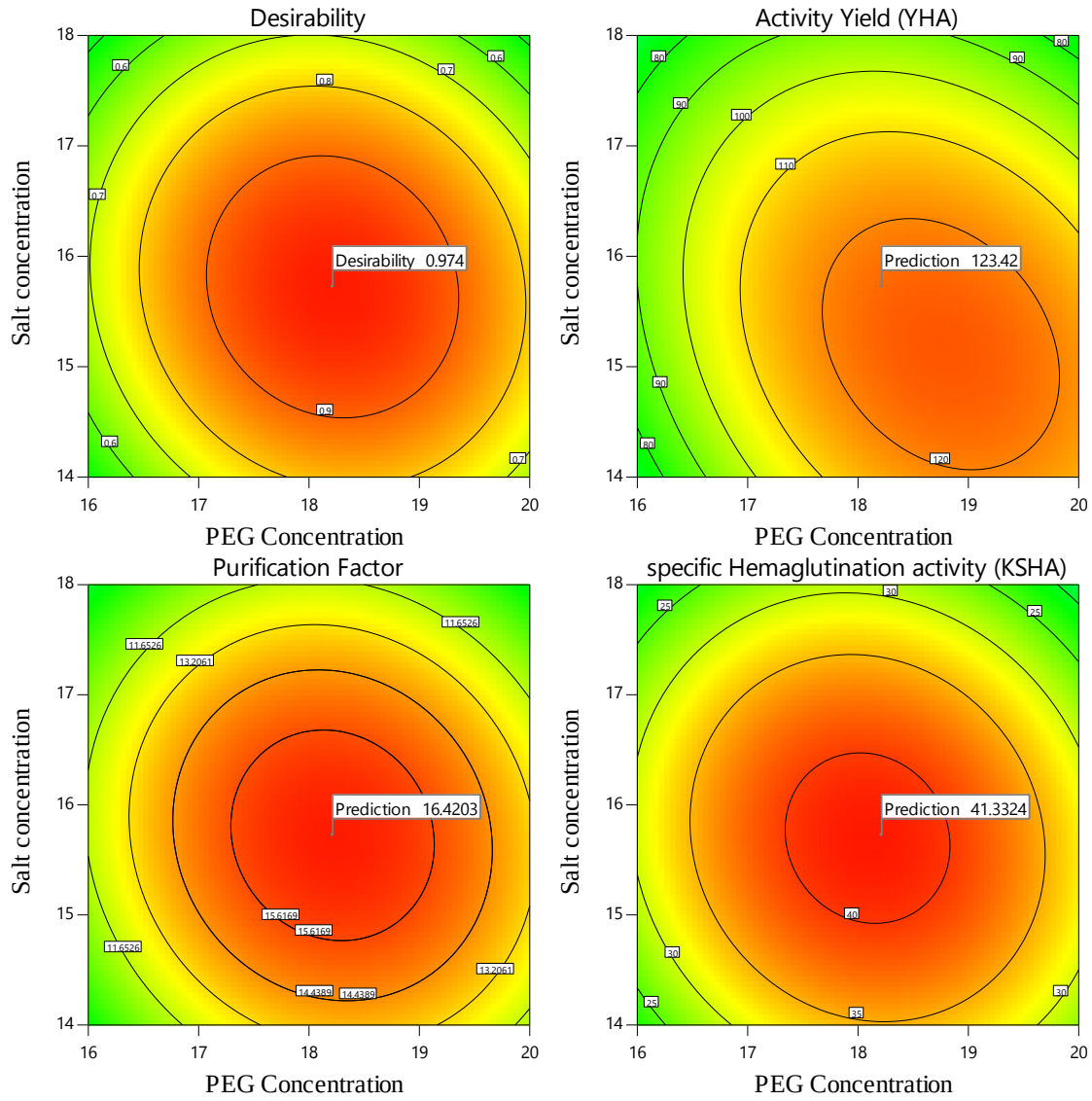


Fig. A2.6 Contour plots for the responses for maximization of Purification factor (goal II)

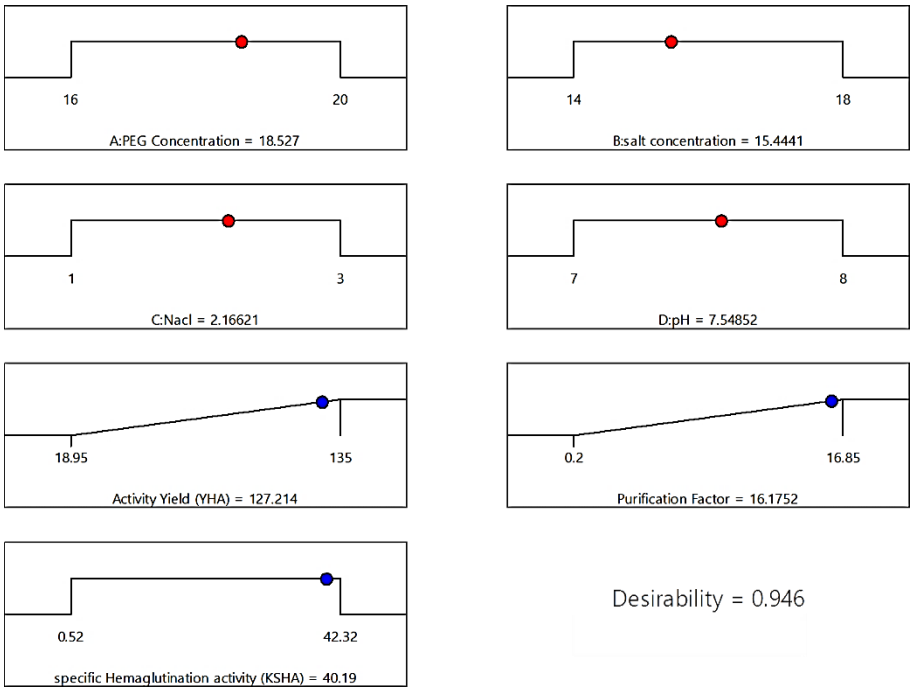


Fig. A2.7 Ramp graph for the maximization of of hemagglutination activity yield %. purification factor (goal III)

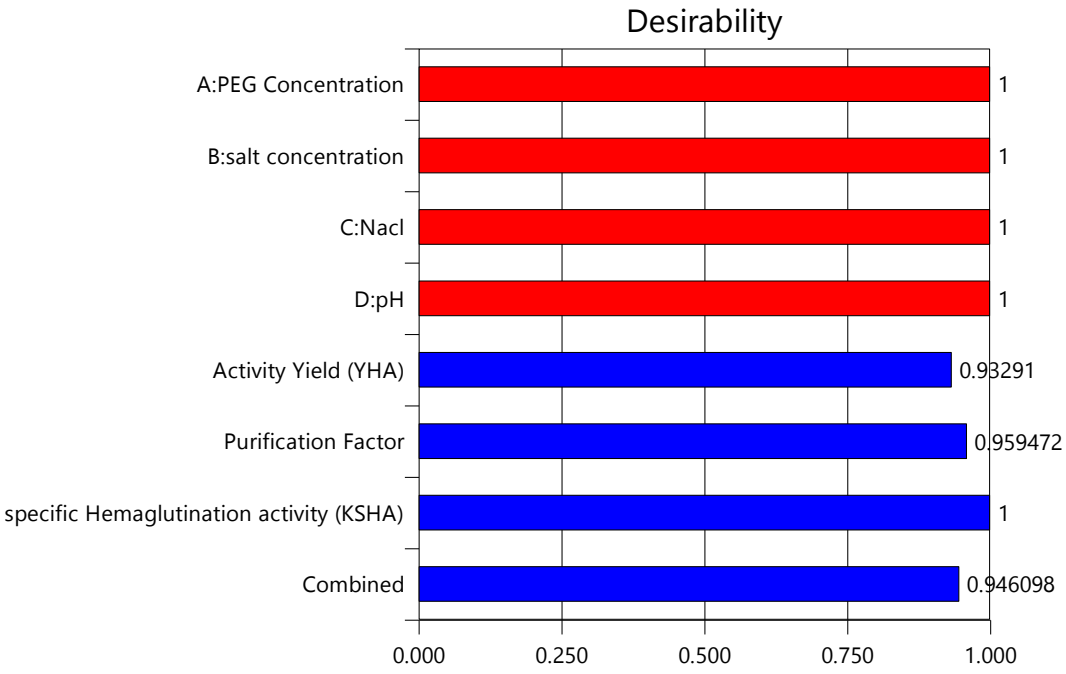


Fig. A2.8 The desirability of individual target achieved for the maximization of of hemagglutination activity yield % purification factor (goal III).

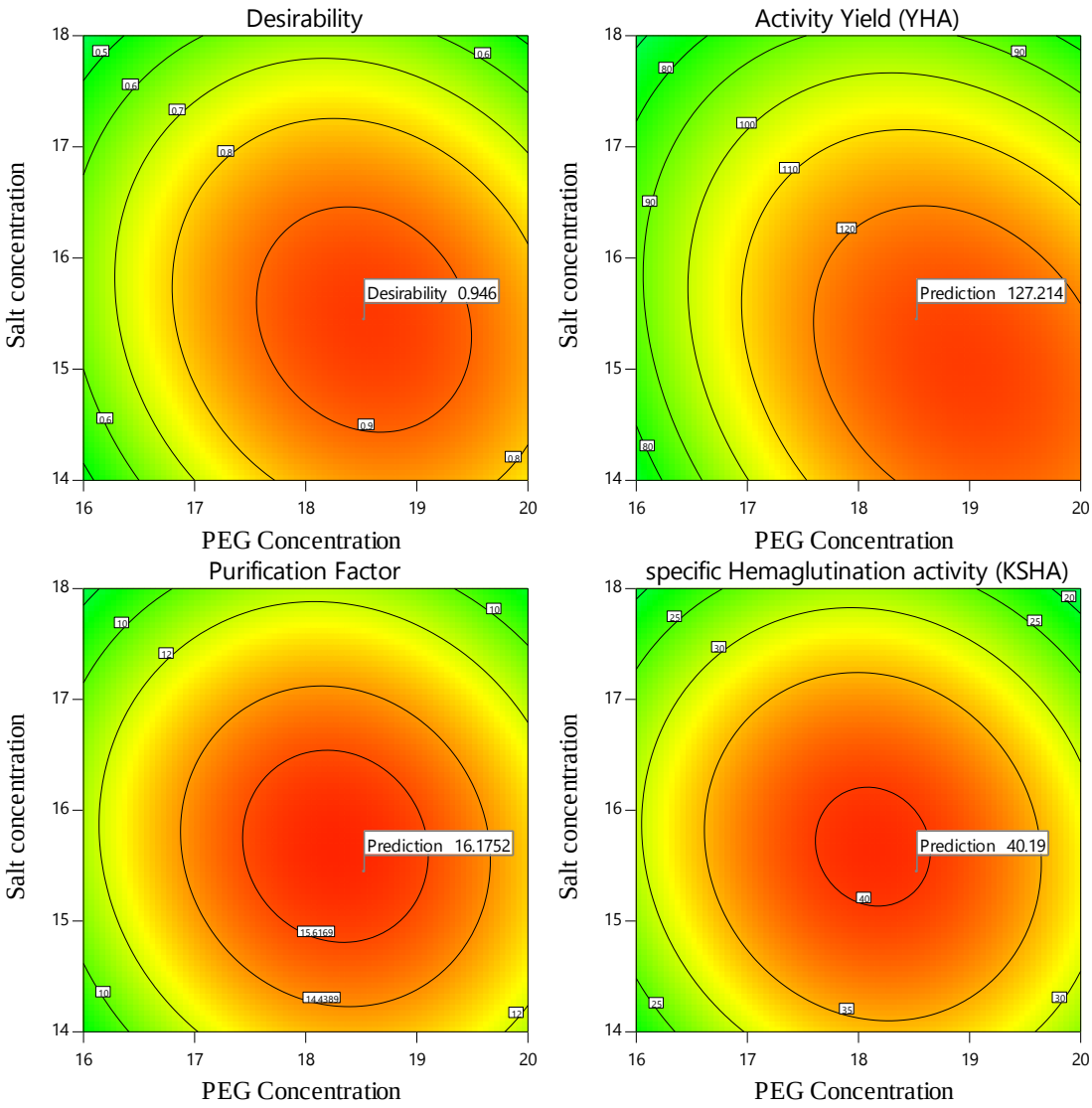


Fig A2.9 Contour plots for the responses for maximization of Purification factor (goal III)

APPENDIX 3

Table A3 Experimental values considered for ANN modeling of equilibrium characteristics and partitioning of Psl

				Experimental values					**ANN Predicted values**				
PEG (g/mol)	Salt	**PEG (%)**	**Salt (%)**	W^1_B	W^2_B	W^1_T	W^2_T	**K**	W^1_B	W^2_B	W^1_T	W^2_T	**K**
2000	SC	20	12	4.85	20.00	33.11	5.00	0.32	4.90	20.00	32.78	5.00	0.317
		20	14	3.36	21.81	41.55	4.08	0.68	3.39	21.81	42.38	4.16	0.687
		20	16	2.82	24.85	44.70	3.60	0.93	2.82	25.35	45.15	3.56	0.921
		20	18	2.50	27.50	48.23	2.86	0.14	2.45	28.05	48.23	2.89	0.14
2000	PC	20	14	7.59	21.98	31.36	5.42	0.30	7.59	22.42	31.67	5.42	0.297
		20	16	5.61	26.05	37.00	2.20	0.56	5.72	25.53	36.63	2.24	0.554
		20	18	4.38	28.32	44.00	0.92	0.85	4.38	28.89	44.88	0.92	0.85
		20	20	3.75	32.01	48.56	0.41	0.12	3.83	31.69	47.59	0.41	0.122
2000	AC	20	16	5.89	22.35	41.75	4.85	0.10	6.01	22.13	41.75	4.85	0.098
		20	18	4.06	26.53	50.23	2.53	0.20	4.10	26.53	50.73	2.58	0.2
		20	20	3.16	28.63	55.12	2.00	0.42	3.22	28.34	56.22	2.02	0.416
		20	22	2.86	32.00	58.00	0.96	0.68	2.80	32.32	56.84	0.96	0.666
4000	SC	20	12	5.50	25.78	28.00	1.00	0.62	5.56	25.78	27.44	1.00	0.626
		20	14	3.86	27.56	34.10	0.90	1.23	3.94	27.01	34.78	0.89	1.255
		20	16	3.80	28.50	37.50	1.20	3.19	3.72	28.79	37.13	1.18	3.19
		20	18	3.10	30.00	42.10	1.40	1.82	3.13	30.30	42.10	1.39	1.838
4000	PC	20	12	6.40	20.00	33.14	2.50	0.53	6.53	19.80	33.80	2.48	0.541
		20	14	6.00	23.20	36.12	1.20	1.20	6.06	23.43	36.48	1.18	1.212
		20	16	5.50	26.12	41.00	1.03	2.98	5.45	25.86	41.82	1.05	3.04
		20	18	5.03	29.11	43.06	1.30	1.78	4.93	29.11	43.92	1.27	1.798
4000	AC	20	14	9.04	30.10	25.07	1.00	0.26	9.13	29.50	24.82	0.98	0.257
		20	16	8.00	32.00	28.10	1.20	0.98	8.16	32.32	28.38	1.19	0.99
		20	18	6.48	34.06	33.06	1.10	1.23	6.54	33.38	33.39	1.08	1.242

		20	20	5.60	35.21	39.08	1.00	1.96	5.66	35.91	38.69	1.02	1.999
6000	SC	20	10	6.02	30.15	25.52	1.20	0.673	5.96	29.85	25.78	1.21	0.673
		20	12	4.80	34.00	31.00	1.23	1.233	4.75	34.68	31.31	1.24	1.258
		20	14	3.98	36.94	36.94	1.20	1.881	3.98	37.31	36.57	1.19	1.919
		20	16	5.36	39.54	40.23	1.32	3.228	5.31	39.14	40.63	1.35	3.26
6000	PC	20	12	3.99	17.59	44.21	2.35	0.471	3.99	17.41	43.33	2.40	0.48
		20	14	3.50	22.50	55.14	1.11	0.91	3.47	22.73	54.59	1.11	0.901
		20	16	3.35	25.35	57.20	0.40	1.516	3.38	25.86	57.77	0.40	1.486
		20	18	3.10	29.32	60.00	0.35	2.811	3.10	29.32	59.40	0.36	2.755
6000	AC	20	14	4.20	18.50	50.12	3.44	0.489	4.20	18.87	50.12	3.37	0.479
		20	16	3.50	22.50	55.00	1.11	0.869	3.43	22.28	54.45	1.13	0.878
		20	18	3.35	25.35	57.01	0.40	1.374	3.42	25.10	57.01	0.39	1.374
		20	20	3.05	29.32	60.09	0.35	2.225	3.05	29.91	61.29	0.34	2.225
8000	SC	20	12	3.30	21.50	36.38	0.76	1.36	3.30	21.29	37.11	0.78	1.36
		20	14	3.10	26.23	43.32	0.58	2.43	3.16	26.23	42.89	0.59	2.479
		20	16	3.02	32.00	47.02	0.10	2.71	3.08	32.00	46.08	0.10	2.683
		20	18	29.00	3.02	0.21	47.02	1.56	29.29	3.08	0.21	47.96	1.529
8000	PC	20	12	21.70	4.50	0.72	34.23	1.32	22.13	4.55	0.71	34.91	1.307
		20	14	23.30	3.60	0.69	41.76	1.85	23.77	3.67	0.68	42.18	1.832
		20	16	24.90	3.12	0.60	47.80	2.10	25.15	3.18	0.61	48.28	2.121
		20	18	28.00	2.90	0.40	49.30	2.72	28.00	2.90	0.40	50.29	2.774
8000	AC	20	12	22.30	4.80	0.80	34.10	1.36	21.85	4.75	0.82	34.10	1.36
		20	14	24.00	4.20	0.69	38.10	2.32	24.00	4.24	0.70	38.48	2.297
		20	16	27.00	3.30	0.50	42.70	2.42	26.46	3.33	0.50	43.13	2.42
		20	18	29.00	3.05	0.26	48.00	1.56	29.29	3.11	0.26	47.52	1.544

RESEARCH PUBLICATIONS

(A) Research Papers in International Journals

Shashidhara, R.B. and Iyyaswami R. "Aqueous two phase partitioning of Pisum sativum lectin in PEG/citrate salt system". *Prep. Biochem. Biotechnol.,* 48(8), 759-767.

Rashmi, B.S and Regupathi I. (2019). "Aqueous Two Phase based Selective Extraction of Mannose/Glucose Specific lectin from Indian Cultivar of *Pisum sativum* Seed". *J. Chromatogr. B.,* 1114, 13-23

(B) Manuscripts under preparation

Rashmi B.S, Regupathi I. "Statistical optimization and continuous extraction of lectin from *Pisum sativum* seed in PEG/sodium citrate system".

Rashmi B.S, Regupathi I. "Artificial neural network modeling of Phase behavior and partition of *Pisum sativum lectin* in PEG/sodium citrate system".

International conference

Rashmi B.S and Regupathi I (2018). "*Glycine max* lectin partitioning feasibility studies in PEG/Salt aqueous two phase system". *International conference on recent trends in analytical chemistry (ICORTAC-2018),* 15-17th March, 2018, Madras University, Chennai.

CURRICULUM VITAE

ACADEMIC DETAILS

YEAR	EDUCATION DETAILS	CLASS
2014-2019	Ph.D. in Chemical Engineering Department of chemical engineering NITK, Mangalre, Karnataka.	Course work (CGPA-8.2)-Submitted thesis in 2019
2011-2013	M.Tech in Biotechnology RVCE, BANGALORE, VTU.	1st class with distinction (83%)
2007-2011	B.E. in Biotechnology Shridevi Institute of Engineering and Technology, Tumkur. VTU	1st class with distinction (76%)
2007	12th/Pre-University Sarvodhaya PU College, Tumkur; Karnataka Pre-University Board.	1st class (65%)
2005	10th/SSLC ChetanaVidyaMandira, Tumkur; KSEEB.	1st class (85%)

WORKING EXPERIENCE

November 2013-December 2014 Assistant Professor

Department of Biotechnology

Sridevi Institute of Engineering and Technology

Tumkur, Karnataka.

TECHNICAL SKILLS

Downstream processing:

Separation of biomolecules

Biochemistry & Enzyme Technology:

Enzymatic Assay, production, Purification and Characterization.

Software:

Matlab, Minitab, Design expert

PAPERS PRESENTED IN CONFERENCES

a. Presented paper entitled "Enhancement of growth and regeneration efficiency of *Oncidium Gower Ramsey* by the optimization of the media". In First International and Third National Conference on Biotechnology, Bioinformatics and Bioengineering held at Held at Hotel Udayee International, Tirupati,and Andhra Pradesh, South India during June 28-29, 2013.
b. Presented Poster paper entitled "Designing of efficient and low cost medium for the enhanced growth of *Oncidium Gower Ramsey*". in national symposium held at CSIR-CFTRI, MYSORE on 11^{th}-13^{th} march,2013
c. Presented paper entitled "Novel fibrinolytic protease and sulfated polysaccharide from different fungal spp." In International conference held at SIET,Tumkur on 8^{th} and 9^{th}June, 2012.
d. Presented Paper entitled "Production and characterization of Novel Fibrinolytic Enzymes from different Fungal Spp." inNational Conference Held at Acharya College of Engineering, Bangalore, on 4^{th}& 5^{th} April,2012.
e. Presented paper entitiled "*Glycine max* lectin partitioning feasibility studies in PEG/Salt aqueous two phase system". *International conference on recent trends in analytical chemistry (ICORTAC-2018),* 15-17^{th} March, 2018, Madras University, Chennai.

Awarded Best paper Presentation award in National Conference Held at Acharya College of Engineering, Bangalore, on 4^{th}& 5^{th} April, 2012.

PAPER PUBLICATION WORK

Rashmi, B.S and Regupathi I. (2019). "Aqueous Two Phase based Selective Extraction of Mannose/Glucose Specific lectin from Indian Cultivar of *Pisum sativum* Seed". *J. Chromatogr. B.,* 1114, 13-23

Shashidhara, R.B. and Iyyaswami R. "Aqueous two phase partitioning of Pisum sativum lectin in PEG/citrate salt system". *Prep. Biochem. Biotechnol.,* 48(8), 759-767.

Rashmi B.S, Liny P. Production and characterization of novel fibrinolytic enzyme from different soil fungal sp. Int j pharm bio sci 2013 july; 4(3): (b) 454 – 463.

PERSONAL INFORMATION

Name:	Rashmi B.S
Sex:	Female
Date of Birth:	12th July 1989
Nationality:	INDIAN
Father's Name:	Shashidhara B.S
Mother's Name:	Mamatha R.C
Hobbies:	Listening to Music
Languages known:	Kannada,English

ADRESS FOR CORRESPONDENCE

Residence: Rashmi B.S
D/O B.S. Shashidhara
""Shivakrupa", Nittur (H), Gubbi (T), Tumkur (D), Karnataka.
Email : rashmibs.bs@gmail.com
Contact number: M: 9886204405