CHAPTER 2

Literature Review

2.1. Sources of production of L-glutaminase

Glutaminase is ubiquitous in bacteria, yeast, fungi and mammalian tissues (Wade et al., 1971; Imada et al., 1973; Spiers and wade, 1979; Sabu et al., 2000). Microbial glutaminases are preferred as flavor enhancing agent in food industry (Yano et al., 1988; Moriguchi et al., 1994; Tachiki et al., 1996; Sabu et al., 2000; Kumar et al., 2012) and therapeutic agent in pharmaceutical industry (El-Asmar and Greenberg, 1996; Roberts et al., 1970). Among bacteria, the production of glutaminases from *Escherichia coli* (Prusiner et al., 1976), *Rhizobium etli* (Tomita et al., 1988), *Vibrio costicola* (Renu and Chandrasekaran, 1992), *Micrococcus luteus* (Moriguchi et al., 1994), *Pseudomonas aeruginosa* (Ohshima et al., 1976), and *Pseudomonas fluorescens* (Eremenko et al., 1975) were well studied. Among yeast strains, *Hansenula, Rhodotorula, Cryptococcus albidus, Candida scottii*, *Candida utilis, Debaryomyces* sp., and *Zygosaccharomyces rouxii* (Imada et al., 1973; Fukushima and Motai, 1990; Iyer and Singhal, 2008; Dura et al., 2002) were reported to produce significant level of glutaminase under submerged fermentation. Among fungi, *Beauveria bassiana* (Keerthi et al., 1999), *Tilachlidium humicola* (Imada et al., 1973), and *Aspergillus oryzae* (Yano et al., 1988) were reported to produce glutaminase.

2.2. Classification of glutaminase

L-glutaminase hydrolyzes L-glutamine into L-glutamic acid and ammonia as shown in Fig.2.1. Glutaminase in mammalian tissue is generally categorized as the kidney type and liver type glutaminases (Heini et al., 1987). Microbial glutaminases belong to family of amidohydrolase that catalyzes the deamination of glutamine and are classified in two classes (Nandakumar et al., 2003):

(i) **Glutaminase**: It is highly specific for glutamine and catalyzes the hydrolysis of glutamine to glutamic acid.

(ii) **Glutaminase-asparaginase:** It catalyzes the hydrolysis of glutamine to glutamic acid and asparagine to aspartic acid with similar efficiency.

The homology in amino acid sequences of glutaminase and glutaminase-asparaginase indicates common mechanism of deamination.

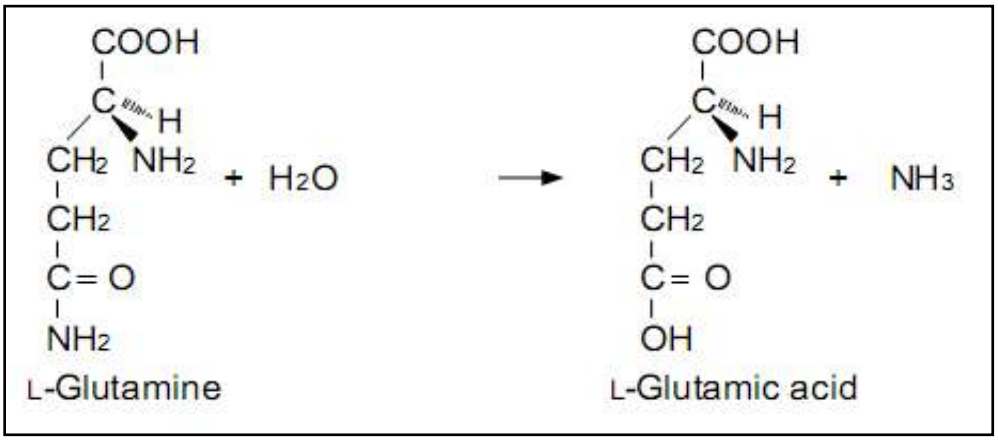

Fig.2.1. Catalytic activity of L-glutaminase to hydrolyze L-glutamine into L-glutamic acid and ammonia

The homology in amino acid sequences of glutaminase and glutaminase-asparaginase indicates common mechanism of deamination. Many of glutaminases catalyze γ-glutamyl transfer from glutamine and categorized as γ-glutamyltransferase (EC 2.3.2.2). Hartman (1968) classified microbial glutaminases into four groups based on the catalysis of cleavage of γ-acyl bond in glutamine:

i. Only catalyzing glutamine hydrolysis like glutaminases from *Pseudomonas putrefaciens* (Holcenberg et al., 1973; Nandakumar et al., 2003), and *Micrococcus luteus* K-3 (Moriguchi et al., 1994).

ii. Glutaminase using water as a preferential acceptor and hydrolyze glutamine prior to the transfer reaction like glutaminase from *Escherichia coli* (Prusiner et al., 1976) and *Pseudomonas aeruginosa* (Soda et al., 1972). Glutaminase from *Pseudomonas nitroreducens* hydrolyzed glutathione prior to γ-glutamyl transfer to glycylglycine (Tachiki et al., 1996).

iii. Transfer reaction prior to glutamine hydrolysis like glutaminase from *Pseudomonas nitroreducens* reported to transfer γ-glutamyl to ethylamine or methylamine with repressed hydrolysis of glutamine (Tachiki et al., 1996).

iv. Only γ-glutamyl transfer reaction like glutaminase from *Lactobacillus rhamnosus* (Ziade et al., 2003)

These reactions are proposed to proceed through the formation of an acyl enzyme derivative through acylation and deacylation steps (Ohshima et al., 1976) as shown in Fig.2.2.

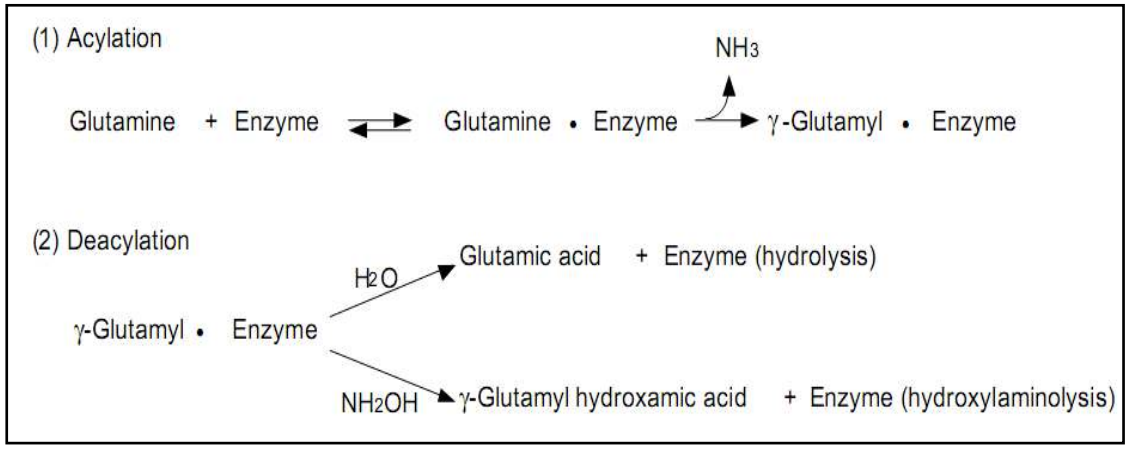

Fig.2.2. Reaction mechanism proposed for hydrolytic deamination of glutamine by glutaminase

2.3. Biochemical properties of microbial glutaminase

The production, purification and biochemical characterization of glutaminase from diverse group of microorganisms were well studied. Table2.1 shows that glutaminases from different microorganisms exhibit different biochemical properties like molecular weight, optimal pH, optimal temperature and substrate specificity. The range of optimum and stable temperature for many glutaminase were reported as 40-50˚C. Glutaminase from *Aspergillus oryzae* was optimally active in a temperature range of 37-45˚C (Koibuchi, et al., 2000), whereas glutaminase A and B from *Escherichia coli* showed optimal activity at 37˚C (Prusiner et al., 1976). Glutaminase from *Aspergillus oryzae* was markedly inhibited by the high-salt concentration during soy-sauce fermentation process (Yano et al., 1988). Therefore, salt-tolerant glutaminase plays potentially significant roles in soy sauce fermentation process. Glutaminase from *Micrococcus luteus* K-3 (Moriguchi et al., 1994; Kennedy et al., 2001), and *Bacillus subtilis* (Satomura et al., 2005; Ebel et al., 2000) were reported to be salt tolerant up to 16 and 25% of NaCl, respectively. Glutaminases A and B from *Saccharomyces cerevisiae* had pH optima at 7.5 and 8.1, respectively (Soberon and Gonzalez, 1987). The optimum activity of glutaminases from *Escherichia coli, Proteus morganii* and *Clostridium welchii* was reported at about pH-5 (Hughes and Williamson, 1952) and found to be unsuitable for clinical use. Glutaminases from *Saccharomyces cerevisiae* (Soberon and Gonzalez, 1987), *Bacillus pasteurii* (Marcus et al., 2002) and *Pseudomonas acidovorans* (Lois et al., 1977) were reported to have optimum pH above neutrality and hence preferred for clinical purpose.

2.4. Biotechnology applications of microbial glutaminase

2.4.1. Use of microbial glutaminase in food industry

Microbial glutaminases are commonly used as flavor enhancing agent in food industry. The hydrolytic product "glutamic acid" contributes sharp sour taste during soy sauce fermentation (Nakadai and Nasuno, 1989).

The soy products are generally preferred in Western country due to its purported health benefits of cancer protective, estrogenic effect. Yokotsuka and Sasaki (1986) first used glutaminase from *Aspergillus oryzae* for soy sauce fermentation process. Glutaminase from *Aspergillus oryzae* was found to be inhibited by 17–18% salt concentration of soy sauce fermentation process (Yano et al., 1988). Glutaminase from marine isolated strain like *Micrrococcus leuteus* K3 was reported as salt tolerant with 1.3 fold higher activity in the presence of 8–16% NaCl (Moriguchi et al., 1994) and thus preferred for soy sauce fermentation process (Moriguchi et al., 1994). Salt-tolerant glutaminase from yeasts such as *Candida*, *Cryptococcus*, and *Torulopsis* were also used to increase the glutamate content of soy sauce (Nakadai and Nasuno, 1989). In recent years, strain improvements of *koji* molds (*Aspergillus oryza*) for overproduction of glutaminase enzyme are also attempted (Ushijima and Nakadai, 1987). Immobilization methods are currently used to improve the taste of soy sauce by immobilizing glutaminase enzyme or glutaminase-producing microorganisms (Fukushima and Motai, 1990).

The other major application of microbial glutaminase in food industry is the production of theanine (γ-L-glutamylethylamide) component. Theanine is used as unique taste-enhancing amino acid for Japanese green tea and preferred for clinical purpose because of their ability to (a) suppress stimulation by caffeine (b) improve the

Table2.1. Biochemical properties of glutaminase produced from different bacteria, yeast and fungi

Microorganism	Molecular mass (KD)		Optimum pH	Optimum temperature (°C)	K_m value	References
Bacteria	**Native**	**Subunit**				
Actinetobacter glutaminisificans	132	33	7.0	35	5.8±1.5µm	Roberts et al., 1972
Bacillus pasteurii	100	55	9.0	37	9.5 mM	Yano et al., 1988
Bacillus subtilis	55		6.0	50	0.64mM	Kozlov et al., 1972
Escherichia coli A	110	28	5.0	34	0.42mM	Hartman and Stochaj, 1973; Hartman and McGrath, 1973
Escherichia coli B	90	35	7.1-7.9	34	0.39mM	Prusiner et al., 1976
Micrococcus luteus I	86	43	8	50	4.4mM	Moriguchi et al., 1994
Micrococcus luteus II	86		8.5	50	6.5mM	Moriguchi et al., 1994
Pseudomonas aeruginosus A	137	35	7.5-9.0	35	0.12mM	Soda et al., 1972
Pseudomonas aeruginosus B	67		8.5	35	0.18mM	Ohshima et al., 1976
Pseudomonas nitroreducens	40		9.0	35	6.5 mM	Tachiki et al., 1996
Pseudomonas aurantica	148	37	6.8-8.0	35	0.53mM	Imada et al., 1973.
Yeast						
Saccharomyces cerevisiae	148		7.5-8.1	38-40	4.0 mM	Soberon and Gonzalez, 1987
Debaryomyces	115	50-65	8.5	40	4.5 mM	Dura et al., 2002
Fungi						
Aspergillus oryzae MA-27-1M	113		9.0	45	0.096mM	Yano et al., 1988; Yokotsuka and Sasaki, 1986
Aspergillus oryzae AJ11728	82		9.0	37-45	1.2mM	Klein et al., 2002; Koibuchi et al., 2000

effects of antitumor agents (c) their role as antihypertensive agents. Theanine has been produced from glutamate and ethylamine using a combination reaction of bacterial glutamine synthetase with a sugar fermentation reaction of baker's yeast (Tachiki et al., 1996). Glutaminase from *Pseudomonas nitroreducens* generally catalyzes γ-glutamyl transfer reaction and hence used as a new enzymatic method for producing theanine (Tachiki et al., 1996).

2.4.2. Use of microbial glutaminase in pharamaceutical industry

Some bacterial glutaminases have antitumor activity and hence used for therapeutic purpose. Glutaminase from *Achromobacter* sp. has been used as preliminary clinical trial for patients suffered with acute lymphoblastic leukemia (Spiers and Wade, 1979). The antitumoric activity of glutaminase from *Pseudomonas* 7A has been also studied in mice (Roberts et al., 1979). The chemical modification method of succinylation has been used to enhance antitumor activity of glutaminase from *Actinetobacter glutaminasificans* (Warrell et al., 1982).

The growing cancerous cells generally synthesize nitrogenous compounds in the form of nucleotides and non essential amino acids (NEAAs). Glutamine is the obligate nitrogen donor for enzymatic steps during synthesis of purine and pyrimidine nucleotides (Voet and Voet, 1995; Ahluwalia et al., 1990; Cowan et al., 2012). In these reactions, glutamine donates its amide (γ nitrogen) group for the synthesis of the NEAAs (Voet and Voet, 1995; Cowan et al., 2012) as shown in Fig2.3. Transaminase enzyme transfers the amine group from glutamic acid to α-ketoacids which is used to synthesize alanine, serine, aspartate, and ornithine. Serine is a precursor for glycine and cysteine biosynthesis, ornithine is a precursor for arginine biosynthesis, and

aspartate is a precursor for asparagine biosynthesis. Glutamic acid contributes its carbon skeleton and nitrogen to the synthesis of proline.

A wide variety of human cancer cell lines (pancreatic cancer, acute myelogenous leukemia and small cell lung cancer) has shown sensitivity to glutamine starvation (Wu et al., 1978). Azaserine, 6diazo-5-oxo-L-norleucine (L-DON) and acivicin have significant activity to inhibit the glutamine dependent enzymatic steps in nucleotide biosynthesis and used as glutamine analogue (Ovejera et al., 1979; Griffiths et al., 1993). The growth of cancer cells has been inhibited by targeting to block the glutaminase metabolic pathway using some of inhibitor as shown in Fig2.4. L-γ-glutamyl-p-nitroanilide (GPNA), L-asparaginase and phenyl-butyrate inhibited cancer cell growth by inhibiting uptake of L-glutamine through ASCT2 importer from extracellular space (Thibault et al., 1994; Esslinger et al., 2005; Nicklin et al., 2009). L-asparaginase possesses significant glutaminase activity which hydrolyzes glutamine to glutamic acid and ammonia and used for treatment of leukemia patient (Wu et al., 1978; Avramis and Panosyan, 2005). In human, phenylbutyrate spontaneously breaks down to form phenylacetate, which is conjugated with glutamine by the hepatic enzyme phenylacetyl coenzyme A: glutamine acyltransferase to yield phenylacetylglutamine (Thibault et al., 1994). Glutamine is metabolized through glutaminolysis within cells to provide NADPH or exported to facilitate TOR kinase activation. Myc enables conversion of glutamine into glutamic acid via upregulation of glutamine synthetase, an enzyme whose activity can be inhibited by treatment with L-DON and azaserine (David et al., 2008). Transamination of glutamic acid to α-ketoglutarate can be inhibited with amino-oxyacetic acid (AOA). The electron transport chain to regenerate of NAD^+ in mitochondrial metabolism is targeted to

inhibit using inhibitors like biguanides, phenformin and metformin (Dykens et al., 2008; Hirsch et al., 2009).

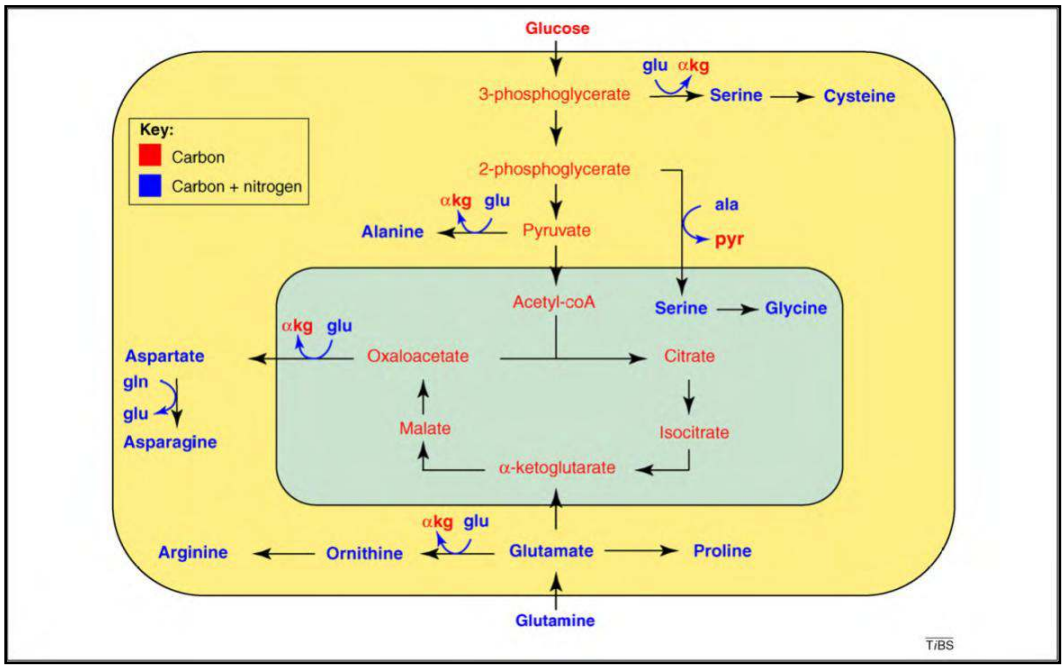

Fig.2.3. Biosynthetic pathway to synthesize non essential amino acids using glucose and glutamine as carbon catabolites (Brown et al., 2008)

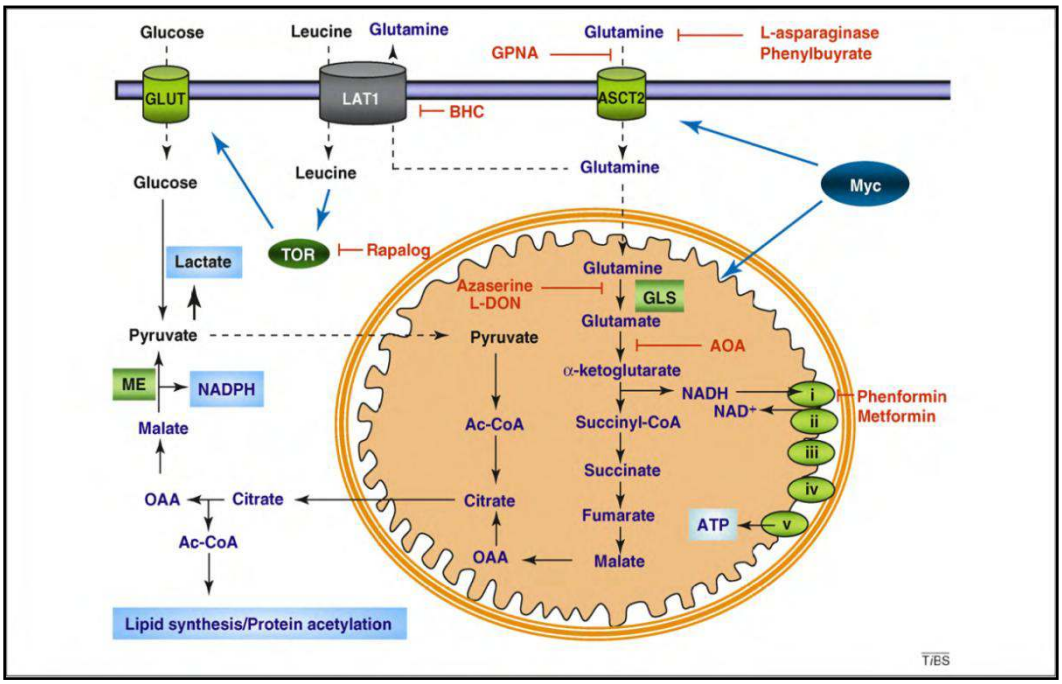

Fig2.4. Pharmacologic targets to block glutamine metabolism in cancer cells.

2.5. Optimization of fermentation process parameters

The optimization of a bioconversion process using classical method (One variable at one time) is laborious, time-consuming, does not depict the complete effects of the parameters in the process and ignores the combined interactions between the physicochemical parameters. Nowadays, response surface methodology (RSM) is used to design the experiments and analyze the results for optimizing the interactive influences of different factors and reducing the number of laborious experiments. This methodology uses statistical experimental design to develop empirical models which relates response to variables. This methodology has one main limitation to deal non linearity behavior of model up to quadratic non- linear correlation (Desai et al., 2008). In recent year, many researchers have investigated artificial neural networks (ANN) as the artificial learning tool in a wide range of biotechnology applications including optimization of bioprocesses and enzyme production from microorganisms. ANN is biologically inspired and mimics human brain. They are consisting of a large number of simple processing elements named neurons. These neurons are connected with connection link. Each link has a weight that multiplied with transmitted signal in network. Each neuron has an activation function to determine the output. There are many kind of activation function. Usually nonlinear activation functions such as sigmoid and step functions are used. ANNs are trained by experience, when applied a new input to the network it can generalize from past experiences and produce a new result (Haykin, 2009). The simple structure of ANN normally consists of an input layer, a hidden layer and an output layer (Desai et al., 2008; Haykin, 2009). By applying algorithms that mimic the processes of real neurons, the network can learn to solve many types of problems. From the perspectives of process modeling, ANN has

been applied to solve complex engineering problems where it is difficult to develop models from the fundamental principles, particularly when dealing with non-linear systems which also exist in bioconversion process. It provides a mathematical alternative to the quadratic polynomial for representing data derived from statistically designed experiments. ANN is also able to handle a large amount of data to approximate functions to any desired degree of accuracy, thus make it attractive as empirical model.

2.6. Partitioning of biomolecules in aqueous two phase system

Extraction using aqueous two-phase systems (ATPS) has been recently used as simple, fast, cost effective and eco-friendly downstream processing method for recovery of biomolecules. The product recovery technique of liquid-liquid extraction using organic-aqueous phase systems is extensively used in the chemical industry. This method has not commonly used for biotechnology application due to the poor solubility of proteins in the organic solvents and the tendency of the organic solvents to denature them (Srinivas, 2000). Aqueous two-phase extraction (ATPE) has been successful to a large extent in overcoming the limitations of conventional organic-aqueous extraction and hence applicable for the extraction and purification of biomolecules (Walter et al., 1991; Albertsson, 1986; Zaslavasky, 1995). The product is obtained with high yield, lower process time and high selectivity. Several reports are available for use of methodology of aqueous two-phase systems in the extraction and purification of biological materials such as enzymes, proteins, nucleic acids and cell organelles (Kula et al., 1982; Walter et al., 1991; Albertsson, 1986; Diamond and Hsu, 1992; Zaslavasky, 1995). The list of extensively studied extractive fermentative

production of different biomolecules in different phase system is summarized in Table2.2.

Table2.2. Extractive fermentative production of different biomolecules using ATPS

Biomolecules	**Microorganism**	**References**
α-amylase	*Bacillus subtilis*	Andersson et al., 1985
	Bacillus amyloliquifaciens	Alam et al., 1989
	Bacillus subtilis	Stredansky et al., 1993
Acetone-butanol	*Clostridium acetobutylicum*	Mattiasson et al., 1982
Alkaline protease	*Bacillus thuringiensis, Bacillus cereus*	Hotha & Banik, 1997; Banik & Prakash, 2006
Amyloglucosidase	*Aspergillus niger*	Ramadas et al., 1996
cellulase	*Trichoderma reesi*	Persson et al., 1984
Ethanol	*Saccharomyces cerevisiae*	Kuhn, 1980
Chitinase	*Serratia marcescens*	Chen and Lee, 1995
Xylanase	*Recombinant Escherichia coli*	Kulkarni et al., 1999
Lactic acid	*Lactobacillus casei*	Katzbauer et al., 1995
	Lactobacillus lactis	Planas et al., 1997; Kwon et al., 1996
L-asparaginase	*Esherichia coli*	Jiang and Zhao, 1999
Pectinase	*Polyporus squamosus*	Antov and Pericin, 2000
6-pentyl-α-pyrone	*Trichoderma harxianum*	Rito-Palomares et al., 2004
Surfactin	*Bacillus subtilis*	Drouin and Cooper, 1992
Subtilin	*Bacillus subtilis*	Kuboi et al., 1994
Lipase	*Bacillus stearothermophilus*	Bradoo et al., 1999
β-galactosidase	*Escherichia. coli*	Kohler et al., 1991; Kuboi et al., 1995
Alkaline phosphatase	*Bacillus licheniformis*	Pandey and Banik, 2011

Aqueous two-phase system has about 80-90% water and provides biocompatible environment for biologically active molecules (Chen and Lee, 1995). Cells are immobilized on one phase of ATPS and essential product is separated towards the other phase. Polymer/salt systems require lesser time to separate and show better selectivity for protein extraction than polymer/polymer systems and hence preferred for industrial application. Simple dialysis method is applied for recovery of biological proteins from the salt phase. Binodal curve (% polymer I vs % salt / polymer II) in ATPS system separates heterogeneous phase from homogeneous phase as shown in Fig2.5. Several reports are available for construction of binodal curve for a number of biomolecules (Bamberger et al., 1984; Walter et al., 1991; Albertsson, 1986; Diamond and Hsu, 1992; Zaslavasky, 1995).

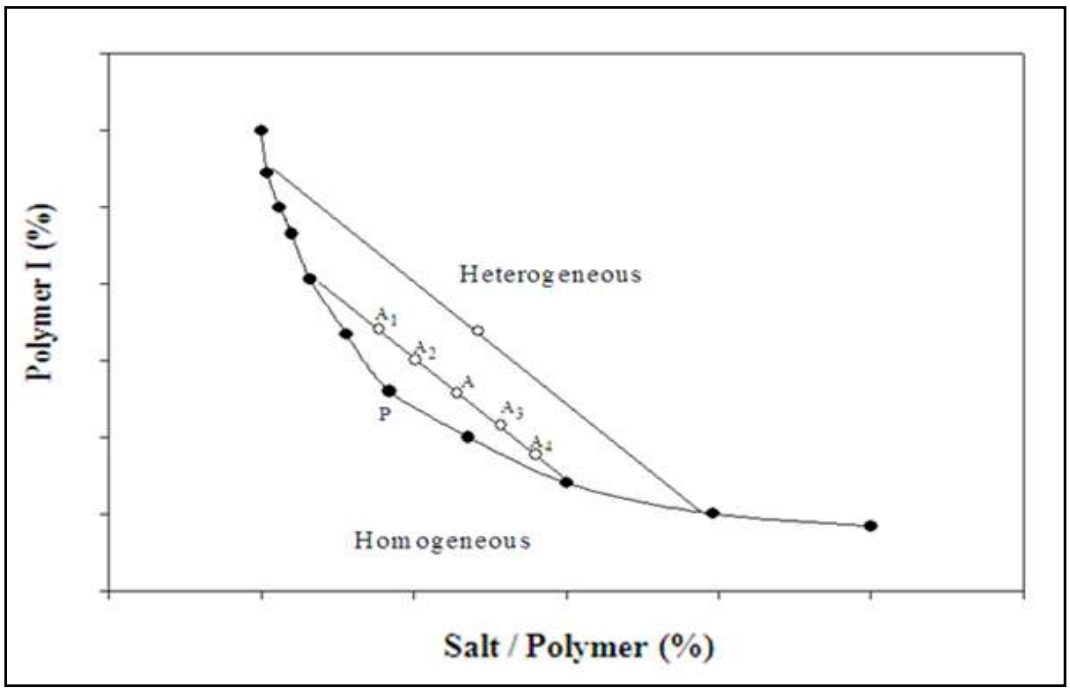

Fig.2.5. Binodal curve for a polymer-polymer or polymer-salt aqueous two-phase system

The composition of top and bottom phase in binodal curve was determined gravimetrically in term of phase volume ratios (V_t/V_b). The identical composition of top and bottom phase has been obtained at plait point (P) of binodal curve. The distance between the composition of top and bottom phase in binodal curve is termed as tie-line length (TLL), which affects viscosity, density and interfacial tension between the phases. In case of PEG/salt ATPS system, the concentration of PEG in the top phase increases with longer TLL, which results higher the viscosity in the top phase. On the other hand, with longer TLL, the salt concentration increases in the bottom phase which has hardly any effect on the viscosity of the bottom phase. Hence, the viscosity difference between top and bottom phase increases as the TLL increases. In contrast to the effect of TLL on viscosity, the variation in density of the top phase is very small in comparison to density in bottom phase. This is due to the fact that, as the TLL becomes longer, the phases become closer to solutions of pure PEG in the top, and pure salt in the bottom. PEG concentration has a small influence on density, whereas salt concentration affects the density much more strongly. As TLL increases, the difference between the top and bottom phase composition becomes greater and hence the interfacial tension increases (Juan et al., 2012). The phase formation of ATPS is affected with concentration of externally added different salts, different molecular weight, hydrophobicity and concentration of the polymers (Walter et al., 1991; Albertsson, 1986; Diamond and Hsu, 1992; Banik et al., 2003).

There is a critical concentration of phase forming solutes for each ATPS which is termed as plait point. The system exists in heterogeneous form above this plait point and homogenous form below this point. The lower concentration of polymer is

required for higher molecular weight polymer for phase separation and vice-versa for low molecular weight of polymer. The tendency of phase formation decreased with increasing the level of hydrophobicity of the polymers in polymer/polymer systems. System pH and temperature also play crucial parameters for construction of binodal curve. The lower concentration of polymeric phase component is required to separate into two phases at lower temperature in polymer/polymer ATPS system. However, higher concentrations of the phase forming solutes are required to separate into two phases at lower temperature for polymer/salt systems (Zaslavasky, 1995). This methodology is not selective enough to provide the desirable purity and is recognized as a primary purification step in the overall process of protein purification (Abbot et al., 1990).

The crucial parameters of partitioning like partition coefficient of desired protein, volume ratio are required to be optimized in such a way that the cell debris partitioned into one phase and the desired protein partitioned into other phase (Abbot et al., 1990; Hustedt et al., 1985). Partition coefficient of biomolecule is defined as the ratio of the equilibrium concentration of the protein in the top phase to that in the bottom phase. The ionic nature of protein, characteristics of polymer, phase composition, system pH and temperature affects the partition coefficient of biomolecules (Banik et al., 2003; Pandey and Banik, 2011). The removal of polymer/salt with desired biomolecule is essential for further purification of the biomolecule after a successful extraction using ATPS. Addition of new salt separates PEG from the biomolecule by forming a new PEG/salt system and partitioned the desired biomolecule into salt phase with very low concentration of PEG (Hustedt et al., 1985). The residual amount of PEG along with the salt can be removed by ultrafiltration (Hustedt et al., 1985) or using

chromatographic column based on adsorption/ hydroxypatite / ion-exchange/affinity (Albertsson, 1986). ATPS based on affinity partitioning is preferred for purification of most of biomolecules in which affinity ligand will interact with the biomolecule and selectively will be partitioned to one phase. The interaction between affinity ligand and biomolecule may be electrostatic or hydrophobic. Polymer/polymer system is preferred for electrostatic interaction because high salt concentration of polymer/salt system may interfere the electrostatic interaction and lowered the partitioning of desired biomolecules (Walter et al., 1991). Since the interaction of protein with ligand is predominantly of electrostatic, hence polymer/polymer based affinity partitioning is preferred for biomolecules (Diamond and Hsu, 1992).