CHAPTER 1

General Introduction

L-glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) belongs to superfamily of β-lactamase that catalyzes hydrolytic deamination of L-glutamine into L-glutamic acid and ammonia (Scheer et al., 2011). This enzyme is strictly specific to glutamine and differ from glutaminase-asparaginase (EC 3.5.1.1), which hydrolyzes both asparagine and glutamine. This enzyme plays a major role in the cellular nitrogen metabolism by supplying nitrogen required for biosynthesis of a variety of metabolic intermediates (Sinsuwan et al., 2012). In recent years, this enzyme is commonly used as an excellent antitumor agent for glutamine-dependent tumor cells as it catalyzes the hydrolysis of L-glutamine into glutamic acid and ammonia, which causes depriving of tumor cells with the essential nutrient, glutamine (Pal and Maity, 1992). It is also used for treatment of acute lymphocytic leukemia and HIV (Roberts et al., 2001). In food industry, it is used as flavor enhancer for producing sharp taste in fermented Chinese foods such as soy sauce (Sabu et al., 2000; Iyer and Singhal, 2008) and production of L-theanine in Japanese green tea which has antihypertensive property and also improves the effects of antitumor agents by suppressing stimulation by caffeine (Tachiki et al., 1996). L-glutaminase based biosensors are used to monitor glutamine level in mammalian and hybridoma cell cultures (Sabu et al., 2000).

Although L-glutaminase is produced by almost all living cells for catabolism of glutamine, microbial L-glutaminase has received greater attention for its potential biotechnological applications and easiness in large scale production. Glutaminase is produced by a wide variety of microorganisms mainly bacteria, fungi and yeast (Wade et al., 1971; Imada et al., 1973; Spiers and Wade, 1979; Sabu et al., 2000) as listed in Table1.1.

Table1.1.Production of L-glutaminase from different bacteria, fungi and yeast

Microorganism	References
Bacteria	
Actinetobacter glutaminisificans	Holcenberg, 1978
Achromobacter sp.	Spiers and Wade, 1979
Aerobacter aerogenes	Donald et al., 1965
Aeromonas hydrophila	Wade et al., 1971
Alcaligenes faecalis	Imada et al., 1973
Bacillus licheniformis	Cook et al., 1981
Bacillus subtilis	Harayama and Yasuhira, 1991
Bacillus megaterium	Imada et al., 1973
Bacillus pasteurii	Klein et al., 2002
Erwinia cartowora	Wade et al., 1971
Micrococcus luteus	Moriguchi et al., 1994
Pseudomonas 7A	Sabu et al., 2000
Pseudomonas aurantica	Lebedeva and Berezova, 1997
Pseudomonas fluorescens	Eremenko et al., 1975
Pseudomonas nitroreducens	Tachiki et al., 1996
Pseudomonas sp.	Kumar and Chandrasekaran, 2003
Vibrio costicola	Prabhu and Chandrasekaran,1997
Fungi	
Aspergillus oryzae	Yano et al., 1988; Sabu, 2000
Aspergillus sojae	Ushijima and Nakadai, 1987
Aspergillus sp.	Kumar et al., 2009
Beauveria sp.	Sabu et al., 2000
Trichoderma koningii	EI-Sayed, 2009
Yeasts	
Candida scottii	Imada et al., 1973
Cryptococcus albidus	Fukushima and Motai, 1990
Cryptococcus nodaensis	Sato et al., 1999
Zygosaccharomyces rouxii	Iyer and Singhal, 2008; 2010
Debaryomyces sp.	Dura et al., 2002
Saccharomyces cerevisiae	Soberon et al., 1987

Its commercial importance demands not only search for new and better yielding microbial strains, but also economically viable bioprocesses for its large scale production. Several reports are available for the production of L-glutaminase from bacteria, yeast, and fungi (Wade et al., 1971; Imada et al., 1973; Ramkrishna and Prakasham, 1999; Renu and Chandrashekharan, 1992; Nandakumar et al., 2003) in shake flask studies. The metabolic processes of the microorganisms during shake flask fermentation are very much influenced by the environmental conditions like media components and cultural conditions. Therefore, it is essential to optimize the environmental conditions to reach maximum enzyme production. The classical method of "one-at-a-time" optimization strategy is generally used for optimization of conditions by varying one factor at a time (Rathi et al., 2001), but this needs to perform large number of experiments and is very time consuming. Nowadays, optimization strategies based on statistical tools (Response surface methodology and artificial neural network) are preferred because of quick screening of a large experimental domain and considering interactive effect of each variable (Sim and Kamaruddin, 2008; Bas and Boyaci, 2007; Dutta et al., 2004; Lou and Nakai, 2001). Moreover, statistical optimization methods reflect the role of each component with screening and prediction of large experimental domain. In response surface methodology (RSM), parameters are optimized using factorial design, regression analysis and analysis of variance (ANOVA). A quadratic linear correlation is assumed for optimizing the parameters which is the main limitation of RSM. Artificial neural network (ANN) is more accurate modeling technique as compare to RSM with better flexibility, data fitting ability, prediction and modeling of nonlinear relationships (Bas and Boyaci, 2007; Haykin, 2009).

Optimization of agitation and aeration rate is important aspects for pilot scale production of enzyme in bioreactor. The optimum agitation speed causes proper oxygen transfer and homogeneous mixing of the nutrients in medium, which results better cell growth and higher enzyme production (Felse and Panda, 2000). Supply of oxygen to the growing cell population is the rate-limiting factor in many aerobic fermentation processes due to poor solubility of oxygen in the culture medium, which is determined by the oxygen transfer rate and is governed by the volumetric oxygen transfer coefficient (K_La), one of the most important parameters in scaling-up aerobic fermentation processes (Dick et al., 1994; Roman and Gavrilescu, 1994). Development of suitable mathematical model for production of microbial enzyme is difficult and challenges exist at all stages of model development like, estimation of selected parameters, model formulation, and solution of the equations (Nielsen and Villadsen, 1992). By combining experimental work with kinetic modeling, it is possible to provide meaningful interpretation of the experimental results to analyze new aspects of microbial physiology.

Extractive fermentation in aqueous two-phase systems is a meaningful approach to overcome low product yield in a conventional fermentation process by preferentially partitioning the biomolecule product in one phase and interfering substances into other phase (Albertsson, 1986). Aqueous Two Phase System (ATPS) result from incompatibility between two polymers or between a polymer and a salt in water. ATPS contain about 80 to 90% water which provides favorable environment for cells, cell organelles and biologically active substances like, enzymes. During extractive fermentation, the partitioning of desired product in one phase is possible with careful adjustment of the composition of phase forming components along with other

physicochemical parameters (Mattiasson and Holst, 1991). The biocompatibility of aqueous two phase systems provides a very low interfacial tension between the phases, which results in high mass transfer and ease of scale up. Recently, aqueous two-phase systems has led to their use for extraction and recovery of a variety of intracellular enzymes from disrupted cell broth (Mayerhoff et al., 2004), affinity purification of enzymes and proteins (Xu et al., 2003), purification of interferon from mammalian cell cultures (Guan et al., 1996) etc. The polyethylene glycol (PEG)/salt ATPS are particularly useful because of their low cost and ease of handling (Kula et al., 1982) for purification of many biomolecules like β-glucosidase (Persson et al.,1989), protease (Lee and Chang, 1990), cellulase (Persson et al., 1991), acetone-butanol-ethanol (Kim and Weigand, 1992), surfactin (Drouin and Cooper, 1992), α-amylase (Kim and Yoo, 1991; Stredansky et al., 1993), subtilin (Kuboi et al., 1994), β-galactosidase (Kuboi et al., 1995), lactic acid (Dissing and Mattiasson, 1994; Kwon et al., 1996). Aqueous two-phase system using PEG/dextran provides a relatively high polar environment (Kula et al., 1982) and has been used for production of amphiphilic enzymes, such as pectinase (Antov and Pericin, 2000), chitinase (Chen and Lee, 1995), and endoglucanase (Sinha et al., 2000).

Pure form of glutaminase is required for study of most of its biotechnological applications. The purification of enzymes allows successful determination of their primary amino acid sequence and three dimensional structures. Most of the microbial glutaminase are extracellular in nature. Methods like centrifugation, filtration have been applied to remove cells from the culture broth. The cell-free culture broth is then concentrated by using methods like ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents (ethanol, acetone). Most of the

purification schemes have been attempted with ammonium sulphate precipitation followed by a combination of several chromatographic methods such as ion exchange, gel filtration and affinity chromatography (Moriguchi et al., 1994; Tachiki et al., 1996; Ziade et al., 2003; Chantawannakul et al., 2003).

The study of structure-function relationship of catabolic enzyme by using bioinformatics tools enable scientists to identify the amino acid residues in active site of the enzyme and their mechanism of action which is used to enhance catalytic activity of the enzyme (Wang et al., 2009; Baig and Manickem, 2010). The three dimensional structure of a protein can be determined at high resolution by either experimental methods such as X-ray crystallography, Nuclear magnetic resolution (NMR) or by computational analysis. In absence of crystallographic structures, a variety of advanced homology modeling methods have been developed which can provide reliable models of proteins that share 30% or more sequence identity with a known structure (Bodade et al., 2010; Xiang, 2006; Nayeem et al., 2006; Khobragade et al., 2011; Beedkar et al., 2012).

The current study is focused on developing a process of production of L-glutaminase in shake flask fermentation and in lab scale bioreactor using a potent bacterial strain. Different optimization strategies were used to optimize reaction conditions, cultural condition and nutrient media components to achieve maximum production of L-glutaminase in shake flask. Aeration and agitation parameters were then optimized to scale up the production of glutaminase in 5litre fermenter. Logistic equation, Luedeking-Piret equation and modified Luedeking-Piret equation were applied to develop suitable kinetic models for cell biomass, product formation and substrate consumption respectively. Extractive fermentation for production of glutaminase were

also studied using different aqueous two phase systems composed of PEG X (X= 2000, 4000, and 6000) and salts (magnesium sulphate, sodium sulphate, and sodium citrate) / polymers (dextran 40, and dextran T500). L-glutaminase was purified from the fermented broth by centrifugation, ammonium sulfate precipitation, dialysis, and DEAE-cellulose chromatography. The molecular weight of native enzyme and its subunits was determined by using Native-PAGE and SDS-PAGE electrophoresis method. The peptide sequences of the enzyme were obtained using MALDI-TOF/TOF analyzer. The biochemical characterization of the enzyme includes the study of molecular weight, pH optima, temperature optima, thermo stability, thermal inactivation, substrate specificity, and effect of metal ions/chelators/thiol binding agents. Bioinformatics tools were applied to predict three dimensional structures of the enzyme and docking analysis of the enzyme with L-glutamine as substrate. The purified enzyme was then used to study antitumor activity using human carcinoma cells.