A PROCESS DEVELOPMENT ON PURIFICATION STRATEGIES FOR L-ASPARAGINASE FROM *Capsicum annum* L.

PROJECT REPORT

Submitted by

JAYASHREE C

Register No.: 1020203006

in partial fulfilment for the award of the degree

of

MASTER OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

(An Autonomous Institution Affiliated to Anna University of Technology, Coimbatore)

COIMBATORE - 641 049

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DEPARTMENT OF BIOTECHNOLOGY

PROJECT WORK

PHASE II

APRIL 2012

This is to certify that the project entitled

A PROCESS DEVELOPMENT ON PURIFICATION STRATEGIES FOR L-ASPARAGINASE FROM

***Capsicum annum*.L.**

is the bonafide record of project work done by

JAYASHREE C
Register No.: 1020203006

of M.Tech (Biotechnology) during the year 2011-2012

……………..	.........………………..
Project Guide	Head of the Department

Submitted for the Project Viva-Voce examination held on....................

……………….....	... ...…………………
Internal Examiner	External Examiner

DECLARATION

I affirm that the project work titled "**A PROCESS DEVELOPMENT ON PURIFICATION STRATEGIES FOR L-ASPARAGINASE FROM *Capsicum annum* L.**" being submitted in partial fulfilment for the award of M.Tech (Biotechnology) is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other university.

JAYASHREE C

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I certify that the declaration made above by the candidate is true.

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JAYASHREE C

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ABSTRACT

A process was developed for purification of L-asparaginase from *Capsicum annum* in a single step using TPP by optimizing the system parameters. The optimized conditions were 50% ammonium sulphate saturation, crude:t-butanol ratio of 1:0.75, temperature 30 ºC and pH 9.0. L-asparaginase partitioned predominantly in the interfacial region between the upper solvent and lower aqueous phases. With the process parameters optimized the enzyme was purified to 6.83 fold with 567.4% recovery of activity. The enzyme obtained from TPP showed considerable purification on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as a single band with molecular weight around 35-37 KDa. The optimum temperature was determined as 40 ºC and the enzyme was found to stable at a range of 25-45 ºC. The stability decreased rapidly with zero activity after 2 h at 50 and 55 ºC. The enzyme showed maximum activity at pH 8.5 and was stable around neutral to alkaline pH. V_{max} and K_m were calculated from Lineweaver-Burk plot as 1111.11 U and 0.04 M respectively. The influence of various effectors ($CaCl_2$, $CuSO_4$, EDTA, $FeCl_3$, $HgCl_2$, KOH, $MgSO_4.7H_2O$, $NaNO_3$, SDS, urea, $ZnSO_4.H_2O$, β-mercaptoethanol) at various concentrations (1, 5, 10 mM) on enzyme activity was investigated and Magnesium sulphate was known to be an activator. EDTA showed less inhibiton and all other effectors inhibited L-asparaginase activity strongly with increasing concentration, SDS being the strongest. The influence of ionic strength on enzyme activity was also investigated and a maximum activity was observed at 50 mM. The enzyme showed good scavenging activity and the total antioxidant capacity was found to be 88.58 µM ascorbic acid equivalent, indicating that L-asparaginase from green chillies will be effective drug against cancer.

Key words: *Capsicum annum*, L-asparaginase, TPP, enzyme activity, antioxidant capacity

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LIST OF ABBREVATIONS

ALL	Anti-lymphoblastic luekemia
BSA	Bovine Serum Albumin
ºC	Degree Celsius
h	Hour
IU	International Unit
KDa	Kilo Dalton
µl	Micro Liter
µg	Micro gram
mA	Milli ampere
mg	Milli gram
mg/L	Milli gram/Liter
mM	Milli molar
mL	Milli Liter
min	Minutes
nm	Nano meter
pH	Potential of Hydrogen
rpm	Rotaions Per Minute
SDS-PAGE	Sodium Dodecyl Sulphate - Poly Acrlamide Gel Electophoresis
TPP	Three-Phase Partitioning
TCA	Trichloro acetic acid
v/v	Volume / volume
w/v	Weight / volume

CHAPTER 1

INTRODUCTION

1.1 L-ASPARAGINASE

1.1.1 General

L-Asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) is an enzyme that catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. L-asparaginase is produced by a large number of various organisms including *Saccharomyces cerevisiae, Chlamydomonas sp, Serratia marcescens, Proteus vulgaris, Klebsiella aerogenes, Cavia porcellus, Fusarium tricinctum, Escherichia coli, Lupinus arboreus, Serratia marcescens, Azotobacter vinelandii, Pectobacterium carotovorum, Pseudomonas geniculata* etc. (Wriston, 1985).

The presence of L-asparaginase was first reported by Clementi (1922) in guinea pig serum. This serum was found to have inhibitory effect on tumour cells which was attributed to the L-asparaginase activity (Kidd, 1953). In general L-asparaginases fall under two categories-bacterial type and plant type. Bacterial-type was further subdivided into type I and type II. Type I is intracellular enzyme whereas type II is extracelluar in origin and found to have anticancer property (Cedar and Schwartz, 1967).

Plant type L-asparaginases falls under two sub-classes namely potassium dependent and potassium independent (Sodek *et al.,* 1980). L-asparagine serves as the major nitrogen source for plants for building up of new proteins. It accounts to about 50 to 70% nitrogen supplied to seeds in case of lupin (Atkins *et* al., 1975). However, more than 80% of asparagines that enters the fruit gets metabolized. L-asparaginase is the key enzyme that breaks down asparagines into aspartic acid and ammonia, making asparagine available for utilization by the plants (Chang and Farnden, 1981; Siециечowicz, 1988).

The mechanism of L-asparaginases activity has been compared to that of classic serine proteases, whose activity depends on a set of amino-acid residues, typically Ser-His-Asp, known as the “catalytic triad” (Carter and Wells, 1988).

1.1.2 Importance of L-asparaginase

L-Asparaginase has received its importance in recent years owing to its anti-neoplastic effect. The anti-tumour activity of this enzyme was successfully detected for the first time when the guinea pig was used against leukoses (Schwartz *et al*., 1966). Neoplastic cells lack asparagine synthetase, hence require a continuous supply of exogenous asparagine for protein build up, (Swain *et al*., 1993). This aspect is taken into account and when L-asparaginase is administered it will make the tumor cells starve by excluding the exogenous asparagine supply. However, normal cells are not affected, since they produce their own L-asparagine in suffiently high levels.

Due to this potential L-asparaginase is used in combination with other drugs for the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosarcoma (Stecher *et al.,* 1999; Verma *et al*., 2007).

The commercially available drugs for cancer are obtained only from *E.coli* and *Erwinia chrysanthemi* have been used in humans eventhough L-asparaginase can be obtained from various microbial sources. Erwinase, and Elspar are two of the various formulations containing L-asparaginase. These drugs are mostly administered through intravenous or intramuscular pathways.

However, bacterial originated drugs results in a number of series side effects including anaphylaxis, nuerological seizures, pancreatitis, diabetes etc. (Vishal *et al*., 2009). Other common side effects include headache, nausea, mild stomach cramps, loss of apetite and weight loss. These side effects may be due to the fact that L-asparaginase is an exogeneous macromolecule which may lead to immunological responses against the drug.

L-Asparaginase has another important application in food industries. While baking starchy food materials at high temperatures the asparagine present is known to get converted

into acrylamide, a potential carcinogen. Hence, L-asparaginase is added to food stuffs prior to baking. This could inhibit about 80% formation of acrylamide.

1.2 ENZYME PURIFICATION

1.2.1 Conventional techniques

All the enzymes used in foods or medical treatments must be of high grade purity. These enzymes should be free from toxic materials, pathogens and should not cause allergic reactions. A number of techniques are available for purification of enzymes. After preliminary extraction, enzymes can be separated from other biomolecules by precipitation. One method to achieve precipitation is by adjusting the pH close to the isoeletric point of the desired protein. However, isoelectric precipitation makes it difficult to recover the biological activity of proteins/enzymes. Precipitation using organic solvents like acetone also poses such a problem.

The most commonly employed method to precipitate proteins is to increase salt concentration. Ammonium sulphate is mostly employed for precipitation owing to its high solubility. As the concentration of salt increases the ions of the salt compete with proteins for the ions in the solution. This is called salting out of proteins. The protein precipitates obtained can be re-dissolved in appropriate buffers. A disadvantage associated with salt mediated precipitation is the requirement for additional steps like daialysis or size-exclusion chromatography, to remove the excess salts (Trevor and Philip, 2008).

Other techniques like ultrafiltration, precipitation using polymers can also be employed for protein purification. But these techniques cannot provide complete purification, since other unwanted proteins may also be present due to the overlapping solubility ranges.

Chromatographic techniques are popular for purification of enzymes, since these techniques make use of slight differences in the enzyme characteristics. The various chromatographic techniques are ion exchange chromatography based on the surface charge of proteins, size-exclusion chromatography/gel filtration chromatography based on the size of proteins, hydrophobic interaction/reversed phase chromatography based on the hyrophobicity of proteins, affinity chromatography based on the biospecificity of proteins. The foretold properties vary greatly between different enzymes and this fact is exploited for their fine purification. A general problem in case of chromatographic techniques is associated with the

elution of purified proteins. When mixtures containing widely disparate components are to be separated, using conditions acceptable to components with short retention time would affect those components with longer retention time and vice versa. An alternative method for this is to use gradient elution (Sivasankar, 2005). Disadvantages of these techniques include high cost and dilution of the protein end product.

1.2.2 Three-phase partitioning (TPP)

Three-phase partitioning is an emerging downstream technique employed for protein purification. When a water-miscible cosolvent like t-butanol is added to an aqueous solution containing salt at concentrations above 0.5 M, it separates into two layers. If biomolecules like proteins are present in the soltution then the proteins will be partitioned into a middle layer between the upper solvent phase and lower aqueous phase (Dennison and Lovrien, 1997).

The partitioning of the proteins in the interface is due to the relative buoyancy of the protein compared to the other phases and also the pulling/pushing effects of the solvent or the salt. The other contaminants like lipids, pigments etc. will be removed off in the solvent phase.

Ammonium sulphate is the most preferred salt owing to it being a strong kosmotrope, its high solubility and low cost. As in conventional salting out ammonium sulphate is known to precipitate proteins due to the combined effect of various principles such as ionic strength effects, kosmotropy, cavity surface tension enhancement, osmotic stress and exclusion crowding. Sulphate anion tends to bind to cationic sites on the protein when the proteins have a net positive charge. Because of this protein precipitation is highly pH dependent, since pH of the solution influences the net charge on the protein.

t-Butanol is the most commonly employed cosolvent in TPP techniques. According to Franks and Reid (1973), t-Butanol in water is a powerful structure promoter. t-Butanol does not require cold temperatures as in case of C_2 solvents like ethanol. TPP can be operated efficiently around room temperatures and if needed the process can be carried out at higher temperatures. t-Buatnol acts as a protecting agent. Owing to its large structure t-butanol cannot enter into protein, even though some of t-butanol binds to the protein, t-butanol does not denature the proteins (Dennison and Lovrien, 1997).

1.2.3 Advantages of TPP over conventional purification techniques

Ammonium sulphate amounts required for TPP is lower than that of conventional salting out. Both ammonium sulphate and t-butanol are of low costs. The protein precipitates from TPP contain very little salt, in contrast to the conventional salting-out, desalting may be excluded in most of the cases. This considerably reduces the process time.

TPP also results in rapid precipitation of proteins, hence the chances of protein denaturing due to long time exposures to organic solvents gets eliminated. Both ammonium sulphate and t-butanol are known to be good protecting agents. TPP is comparatively simple, cheaper and rapid technique. An advantage of TPP over chromatographic techniques is that the proteins from TPP are obtained in a concentrated form, whereas chromatography leads to dilution of the proteins.

TPP is simple, rapid, easily scaleable, and known to enhance the enzyme activities in several cases (Tan and Lovrien, 1972). In few cases enzyme activity decreases, but this could be useful if unwanted proteins are shunted aside.

1.3 SCOPE OF THE CURRENT PROJECT

L-Asparaginase currently has its application in medical field for the treatment of anti lymphoblastic leukemia (ALL) and in food industries to eliminate the formation of the carcinogen, acrylamide in baked foods. The conventional L-asparaginase drugs obtained from bacteria are known to cause a number of side effects, some of them even fatal. Hence, researchers started exploring other novel sources like plants and fungal sources. A number of plants have been explored for the purification of L-asparaginase, *Capsicum annum* was reported to be a potential source for L-asparaginase showing good anticancer activity, by Bano and Sivaramakrishnan (1980). In their work L-asparaginase was purified employing salt precipitations followed by Sephadex G-75 filtration and affinity chromatography and obtained a 400 fold purification and 15 % activity recovery. Vishal *et al*., 2009 also explored 34 plant species including *Capsicum annum* for L-asparaginase and reported the purification of the same enzyme from *Withania somnifera* using a combination of ammonium sulphate precipitation and Sephadex-gel filtration.

In the work reported here, *Capsicum annum* was chosen as a source for L-asparaginase and three-phase partitioning (TPP) was employed to purify L-asparaginase, owing to the foretold advantages, as a single step purification process. In general, lower the number of steps in downstream processing, higher the product recovery yield. The critical parameters of TPP process such as ammonium sulphate saturation %, crude enzyme:t-butanol ratio, temperature and pH were optimized to obtain better yield and purification. The L-asparaginase obtained from *Capsicum annum* purified using TPP was characterized.

1.4 OBJECTIVES

- Extraction of L-asparaginase from *Capsicum annum* and purification by Three-phase partitioning (TPP).
- Optimization of system parameters such as ammonium sulphate saturation%, crude enzyme:t-butanol ratio, temperature and pH of TPP.
- Charaterization of the purified L-asparaginase enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 L-ASPARAGINASE (E.C. 3.5.1.1)

L-Asparaginase is an enzyme produced by a number of organisms belonging to different microbial genera, plants and few animals that hydrolyses L-asparagine. In general L-asparaginase can be put under two classes, bacterial and plant-type L-asparaginses. The bacterial L-asparaginases are further subdivided into type I and type II based on their localisation and antitumour activity. Only type II of bacterial L-asparaginase is known to have inhibitory effect on cancer cells (Cedar and Schwartz, 1967). Plant type enzymes differ greatly from that of bacterial type on evolutionary and structural aspects.

2.1.1 Bacterial-type L-asparaginases

EcAII from *Escherchia coli* was the first antileukemic L-asparaginase to be used clinically. The structure of EcAII (PDB code 3ECA) (Figure2.1) was composed of four identical subunits each consisting of 326 residues. Only one subunit involving two Thr residues namely Thr12 and Thr89, was known to form active site where enzyme-substrate interactions take place. Of these two residues Thr12 was reported to supply the –OH nucleophile to attack the C atom of scissile amide bond (Palm *et al*., 1996).

The crystal structure of L-asparaginase was solved incase of *Pyrococcus horikoshii* by Yao *et al.* (2005). This enzyme was also found to be a dimmer. Though type I and type II have relatively high homology these two can be distinguished by phylogenetic analyses. While sequence differences can be correlated with the oligomeric structure, they are not able to explain why the two types have different quaternary structures (Bonthron and Jaskolski, 1997).

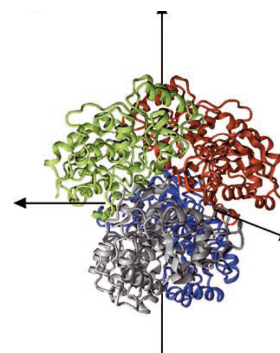

Figure 2.1 Subunit structure of bacterial-type L-asparaginases. The quaternary structure of the periplasmic *E. coli* enzyme EcAII (PDB code 3ECA). The green and red (or gray and blue) monomers form the intimate dimer. The arrows correspond to the two-fold axes defining the 222 symmetry of this homotetramer (Karolina and Mariusz, 2006).

2.1.2 Plant-type L-asparaginases

Plant-type L-asparaginases are not in homology with bacterial type L-asparaginases. The L-asparaginase's role in protein build up was recognized in plants by Grover and Chibnall (1927). Sodek *et al.* (1980) classified plant-type L-asparaginases as potassium-dependent or potassium- independent. These two groups were known to have a high sequence similarity of 66%, but the molecular basis of the K^+-(in) dependence is not clear. The plant-type enzymes belong to the family of Ntn-hydrolases. The nucleophilic (Thr) residue of these enzymes was liberated in an autocatalytic event and is activated by its own free α-amino group. This view is questioned because stereochemical considerations do not favor a strained intramolecular –O–H…NH_2 hydrogen bond and cannot explain how a threonine residue can cleave the α-amide bond in front of it on autocatalytic generation of the mature $(\alpha\beta)_2$ form. A sodium cation coordinated near the active site was known to play a structural role in the positioning of the N-terminal nucleophile. Such a sodium binding has been detected in a subclass of plant-type L-asparaginases, the activity of which was not related to potassium concentration (Karolina and Mariusz, 2006).

Borek (2001) was first to determine the crystal structure of EcAIII (PDB code 1K2X) at 1.65 Å. Prahl *et al.* (2004) reported a similar structure at the same resolution. From these structures an $(\alpha\beta)_2$ oligomeric protein, arranged as a dimer of two (αβ) heterodimers generated in autocatalytic cleavage of the precursor protein was revealed (Figure 2.2). Each of the (αβ) units has the αββα fold typical for Ntn-hydrolases, i.e. consists of two almost exclusively antiparallel β-sheets arranged in a face-to-face manner and flanked on both outer faces by layers of α-helices. The smaller, four-stranded β-sheet is folded exclusively within the β-subunit, while the larger one has eight β-strands contributed by both subunits. The large β-sheet is known to be an important element for the cohesion of the $(\alpha\beta)_2$ oligomer. It includes the N-terminal β-strand of subunit β, which harbors the catalytic Thr residue (179 in EcAIII sequence) is present in the middle.

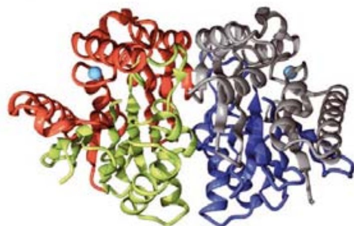

Figure 2.2 Subunit structure of plant-type L-asparaginases. Quaternary structure of the EcAIII (αβ)2 heterotetramer. The red and gray chains correspond to subunit α, the green and blue ones to subunit β. The blue spheres mark the position of the sodium cation coordinated by α subunit of each (αβ) unit (Karolina and Mariusz, 2006).

2.1.3 Mechanism of action

L-Asparaginase (E.C. 3.5.1.1) is an amidohydrolase that catalyses the conversion of L-asparagine into L-aspartic acid and ammonia. The mechanism of L-asparaginases has been compared to that of classic serine proteases, whose activity depends on a set of amino-acid residues, typically Ser-His-Asp, known as the "catalytic triad" (Carter and Wells, 1988). In the first step, the enzyme's nucleophile which is activated *via* a strong O-H…B hydrogen

bond to an adjacent basic residue. This then attacks the C atom of the amide substrate, leading through a tetrahedral transition state to an acyL-enzyme intermediate product. The negative charge developed on the O atom of the amide group in the transition state is stabilized by interactions with adjacent hydrogen-bond donors. The second step involves the the attack on the ester C atom launched by an activated water nucleophile (Karolina and Mariusz, 2006).

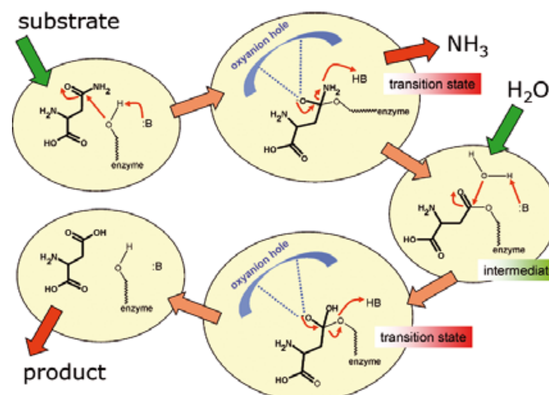

Figure 2.3 General mechanism of L-asparaginase catalysed reaction (Karolina and Mariusz, 2006).

2.2 APPLICATIONS OF L-ASPARAGINASE

The enzyme has been isolated from a variety of sources: animals and plant cells, yeast, fungi, and bacteria. However only asparaginase obtained from *Escherichia coli* and *Erwinia corotovora* are commercially available for the treatment of anti-lymphoblastic leukaemia (ALL) and certain other types of cancer. Erwinase, Kidrolase, Crisantipase, Oncospar and Elspar are some of the commercially available formulations obtained from

these two bacteria. However bacterial L-asparaginase is associated with a number of side effects including anaphylaxis, pancreatitis, diabetes, etc.

All cells require L-asparagine for build up of proteins. Normal cells are capable of producing their own L-asparagine whereas neoplastic cells cannot, since they lack asparagine synthetase. They depend on the continuous supply of L-asparagine from the circulating pool (Swain *et al*., 1993). This aspect has been taken into account for treatment of cancer by administering L-asparaginase which will eliminate the supply of L-asparagine to tumour cells without affecting normal healthy cells (Maria *et al.,* 2004).

Clementi (1922) was the first to report the presence of L-asparaginase in guinea pig serum. The antitumor property of guinea pig serum was discovered by Broome (1961) which was attributed to the activity of L-asparaginase by Kidd (1953).

Another application of L-asparaginase finds its way in the food industry where the enzyme is used to reduce the formation of acrylamide, a carcinogen which is produced when starchy food materials are baked at higher temperatures. The enzyme is added to food materials before baking which inhibits the formation of acrylamide by breaking down L-asparagine into L-aspartic acid (Franco *et al*., 2002).

2.3 L-ASPARAGINASE SOURCES

2.3.1 Animal sources

Certain animals are also exploited for the production of L-asparaginase to reduce immunological risk when used for chemotherapy. This enzyme is found in serum of only few mammals. Production of L-asparaginase from liver and serum of *Cavia porcellus* and its immunological properties were studied by Zhang *et.al.,* 1995.

L-Asparaginase from liver of *Gallus gallus* has been reported by Wriston and Yellin (1973). They have also reported that L-asparaginase is found in human serum in little amounts.

2.3.2 Bacterial sources

A number of potential sources for L-sparaginase including bacteria like *Pectobacterium corotovorum, Proteus vulgaris, Citrobacter freundii, Wolinella succinogenes, Serratia marcescens, Klebsilla aerogenes, Pseudomonas geniculata, Clostridium cadaveris, Mycobacterium avis, Mycobacterium bovis, Mycobacterium phlei, Mycobacterium smegmatis, Acinetobacter caleoaceticus, Acinetobacter glutaminasificans* were reported by Wriston and Yellin (1973) and Wriston (1985). Gaffar and Shethna (1977) reported asparaginase from soluble fraction of *Azotobacter vinelandii* which was found to retain the activity even when the organism was grown in a nitrogen free medium.

Thermus thermophillus was exploited for production of L-asparaginase and the enzyme was characterized by Prista and Kyriakidis (2001) and the antiproliferative activity of L-asparaginase from the same species against different human cancer cell lines was investigated.

Verma *et al.* (2007) reported L-asparaginase production from different bacterial species including *Nocardia asteroides, Photobacterium leiognathi, Thermus thermophilus, Tetrahymena pyriformis, Pseudomonas putida, Pseudomonas aeruginosa and Vibrio harveyi etc.* Production of L-asparaginase by certain actinomycetes was also reported by some researchers. Sahu *et.al.*(2007) reported L-asparaginase with anti-luekernic activity from actinomycete strain LA-29, isolated from estuaries fish (*Mugil cephalus* Linn).

The attempt of Hymavathi *et al.* (2009) was an evidence that L-Asparaginase production was possible by *Bacillus circulans* (MTCC 8574) using agricultural waste and they optimized the production using response-surface methodology. Saleem *et al.* (2009) reported the production of extracellular L-asparaginase with antileukaemic activity by marine actinomycetes using solid-state and submerged fermentations.

The potential of *Streptomyces tendae* TK-VL 333 to produce L-asparaginase was reported by Kavitha and Vijayalakshmi, 2010. Another species of *Streptomyces*, *S. gulbargensis* was reported to produce the same enzyme by Amena *et al.,* 2010. Narta *et al.* (2011) reported the improved production of the enzyme by *Bacillus brevis* at 4% (v/v) concentration of three different oxygen-vectors namely liquid paraffin, silicone oil and n-dodecane which were found to a stimulatory effect.

2.3.3 Fungal source

Dox reported the presence of L-asparaginase in the fungi, *Penicillium camemberti* early as 1909. Later Bach (1928) and Schmalfuss and Mothes (1930) studied the production of L-asparaginase by *Aspergillus niger*. *A. terreus* was reported to produce L-asparaginase by De-Angeli *et al.*,1970, Gulati *et al.*,1997. Maria *et al.* (2004) screened 26 strains of filamentous fungi belonging to *Aspergillus, Penicillium* and *Fusarium* genera and found that only *Aspergillus* strains showed L-asparaginase production. Further studies on *A. terreus* (IOC 217), *A.tamarii* (IOC 186) using different nitrogen sources like urea, glutamine and proline revealed that L-asparaginase production is nitrogen regulated in these organisms.

Production of L-asparaginase by a number of species belonging to *Fusarium* genera such as *F. oxysporum, F. solani, F. roseum, F. culmorum, F. caucasium* and *Fusarium* related species of ascomycetes genera, Hypomcetes, Nectria having a *Fusarium* imperfect state *Gibberella* were reported by Nakahama *et al.,* 1973. A fungal strain *Cylindrocarpon obtusisporum* MB-10 was isolated from soil purified and characterized by Raha *et al.,* 1990. Yeast like *Saccharomyces cerevisiae, Rhodotrula mucilaginosa, Rhodosporidum toruloides* were also reported to produce L-asparaginase by Verma *et al.,* 2007.

2.3.4 Plant sources

Though L-asparaginase from bacterial source is used as chemotherapeutic agent it is known to have many side effects including anaphylaxis, pancreatitis, neurological seizures (Vishal *et al.,* 2009). Attempts have been made by researchers to produce L-asparaginase from novel sources like plants. The amino acid sequence of which though did not have any homology with microbial asparaginase, it was known to have 23% identity and 66% similarity to human glycosyL-asparaginase (Lough *et al.,* 1992). They reported the distribution of L-asparaginase in *Lupinus cuteus* and *Dolichos lab lab* seedlings. L-Asparaginase production was investigated only in a few higher plants including legume like *Lupinus anqustifolius, L. arboreus, L. polyphyllus* (Wriston, 1985; Chang and Farnden,1981).

The occurrence of L-asparaginase in various plant sources like Chillies, Tamarind, Plaintain, Brinjal, Curcurbita etc have been investigated by Bano and Sivaramakrishnan (1980). They found that Chillies (*Capsicum annum* L.) and Tamarind (*Tamarind indica*)

contained appreciable amount of L-asparaginase with activity of 9.43 µmol of ammonia liberated by 1 g of fresh tissue in 1 L. No trace of the enzyme was found in drumstick and ginger. While only trace amounts were present in lemons, onions and potatoes.

Some bryophytes belonging to the genus *Sphagnum* such as *S. fallax, S. cuspidatum, S. fimbricatum, S. imbricatum, S. magellanicum, S. papillosum, S. russowii, S. squarrosum, S. subnitens, S. subsecundum* was reported by Heeschen *et al.*, 1996.

Vishal *et al.* (2009) investigated the presence of L-asparaginase in 34 plant species belonging the Solanaceae and Fabaceae families. *Withania somnifera,* an Indian medicinal plant was reported to be a novel source of this enzyme. Some plants and their particular part known to produce high amounts of L-asparaginase are listed below (Table 2.1).

2.4 PURIFICATION OF L-ASPARAGINASE

L-Asparaginase enzyme from various sources has been purified by application of different techniques/combination of two or more techniques like salt precipitation, gel filtration, ion-exchange chromatography etc. The enzyme produced by *Acinetobacter calcoaceticus* has been purified by precipitation with Streptomycin, followed by chromatography on DEAE-Cellulose and CM-Celluose, subsequently gel filtration on agarose and chromatography on Phosphocellulose in a work done by Joner, 1976.

Recombinant L-asparaginase produced by *E.coli* BL21 was purified to homogeneity by a single step procedure involving cation-exchange chromatography on S-Sepharose FF column in a work done by Kotzia and Labrou, 2007. L-Asparaginase produced by *E. coli* L-asparaginase mutans (mL-ASPs) was purified by native polyacrylamide gel electrophoresis. Wei *et al.* (2008) found this approach to be simple and reproducible and efficient than that column/affinity chromatography. They were able to get a specific activity of approximately 400 U/mg. Purification of native wild-type and recombinant mutant chimeric L-asparaginases produced from *E. coli* BL21 (DE3) by periplasm preparation was purified through osmotic shock and anion-exchange chromatography (Gaofu *et al.,* 2005). Recombinant L-asparaginase produced by *E.coli* BL21 was purified to homogeneity by a single step procedure involving cation-exchange chromatography on S-Sepharose FF columm in a work done by Kotzia and Labrou, 2007. L-Asparaginase produced by *E. coli*

L-asparaginase mutans (mL-ASPs) was purified by native polyacrylamide gel electrophoresis. Wei *et al.* (2008) found this approach to be simple and reproducible and efficient than that column/affinity chromatography. They were able to get a specific activity of approximately 400 U/mg. Purification of native wild-type and recombinant mutant chimeric L-asparaginases produced from *E. coli* BL21 (DE3) by periplasm preparation was purified through osmotic shock and anion-exchange chromatography (Gaofu *et al.,* 2005).

Table 2.1 List of some of the plants reported to produce L-asparaginase.

Sl. No.	Plant	Plant parts	References
1.	*Aradopsis thaliana*	Flower	Bruneau *et al.,* 2006
2.	*Lupius arboreus*	Flower and leaf	Chang and Farnden,1981; Verma *et al.,* 2007
3.	*Capsicum annum*	Fruit	Vishal *et al.,* 2009; Bano and Sivaramakrishnan, 1980
4.	*Datura inoxia*	Fruit	Vishal *et al.,* 2009
5.	*Pisum sativum*	Fruit	Vishal *et al.,* 2009
6.	*Solanum lycopersium, Tamarindus indica, Vigna ungiculata*	Leaf	Vishal *et al.,* 2009
7.	*Lupinus arqulitifolius*	Root and seed	Chang and Farnden, 1981
8.	*Lupinus polyphyllus*	Seed (testa)	Sodek and Lea, 1993

A single step purification of L-asparaginase isoenzyme using Ni-NTA affinity chromatography with a yield of 95 mg/L and 86% recovery has been reported by Khushoo *et al.,* 2005. A two-step procedure involving cation-exchange chromatography and affinity

chromatography on immobilized L-asparaginase was employed by Kotzia and Labrou (2005) to purify the enzyme to near homogenity.

Native enzyme from *P. aeruginosa* 50071 was purified to get 106 fold purity and 43% yield by employing different purification steps including ammonium sulphate fractionation, Sephadex G-100 gel filtration and CM-Sephadex C50 by El-Bessoumy *et al.* (2001). A recombinant L-asparaginase protein produced by *Heliobacter pylori* 99 was purified to get 1-8 fold by sonication followed by soluble fraction of cell free extract at pH 7.2 and 5.8, and chromatography SP-Separose by Gladilina *et al.* (2009) with more than 60% yield.

L-Asparaginase from *Streptomyces tendae TK-VL333* was purified to homogeneity by Kavitha and Vijayalakshmi (2010) by employing ammonium sulphate precipitation, Sephadex G-100 and CM-Sephadex G-50 gel filtration and obtained 7.2 fold purity and 25.7% recovery. A fold purify of 82.12 and 32% yield has been reported from *Streptomyces gulbargensis* by Amena *et al.* (2010). They employed puification steps such as ammonium sulphate fractionation, Gel filtration, Sephacryl and CM-Sephadex C-50 gel filtration. A combination of ammonium sulphate precipitation, dialysis, gel filtration on Sephadex G-100 and SDS-PAGE were employed by Saleem *et al.* (2009) to purify the enzyme to homogeneity from marine actinomycetes.

Crude L-asparaginase obtained from *Aspergillus vinelandii* by grinding with glass powder along with phosphate buffer and was subjected to protamine sulphate precipitation followed by ammonium sulphate precipitation, and then chromatography on Sephadex G-150 column by Gaffar and Shethna (1977) to obtain the enzyme purity near homogeneity.

L-Asparaginase produced by *Bacillus sp.* isolated from soil was purified by employing ammonium sulphate precipitation followed by dialysis, Ion-exchange chromatography using DEAE-Columm by Vidhya *et al.* (2010). Suchita and Chandra (2010) purified L-asparaginase produced by *Erwinia corotovora* by employing ammonium sulphate fractionation (60-70%) followed by chromatography on Sephadex G-100, CM-Cellulose and DEAE-Sephadex columm subsequently. John and Robert (1976) employed different purify steps like ammonium sulphate precipitation, hydroxylapative chromatography followed by CM-Sephadex and DEAE-Sephadex chromatography to purify L-asparaginase produced by *Vibrio succinogenes* and have obtained 40-45% overall yield.

Vigna ungiculata cultivar L-asparaginase was purified by dialysing the crude enzyme precipitation against potassium phosphate buffer followed by DEAE-Sepharose chromatography which yielded 3 peaks, the highest activity being detected for asparaginase II. The asparaginase II was purified using gel filtration which yielded a final purity of 9.27 fold with 36% recovery (Ehab, 2009). Bano and Shivashankar (1980) employed different purifying steps including ammonium sulphate precipitation (60 %), sodium sulphate precipitation (30 %), Sephadex G-75 filtration and affinity chromatography to purify L-asparaginase from *Capsicum annum* and reported a 400 fold purification and final recovery of 15 %. Vishal *et al.,* 2009 purified L-asparaginase from *Wthania somnifera* by ammonium sulphate precipitation (40-60 %) followed by Sephadex G-25 filtration to remove salt. They obtained a final recovery of 66 % and 16-28 fold purity.

2.5 THREE-PHASE PARTITIONING (TPP)

TPP is an up coming bioseparation technique which employs collective operations of principles involved in numerous techniques such as salting out, isoionic precipitation, cosolvent precipitation, osmolytic and kosmotropic precipitation of proteins. TPP was first developed as an upstream technique for precipitation of crude cellulases in litre scale (Odegaard *et al*., 1984). However later TPP was applied for downstream processing of biomolecules. Cosolvents like t-butanol even though completely miscible in water, upon addition of enough salt the solvent becomes immiscible and if any protein is present it will be precipitated in the middle of the upper solvent phase and lower aqueous phase with salt (Dennison and Lovrien, 1997). Tan and Lovrien (1972) reported that TPP retained the activity of most of the enzymes and enhanced the catalytic activity in some cases. In few cases as that of haemoglobin, the activity was lost which becomes useful when those proteins become unwanted and need to be shunted off.

TPP has some origins with conventional salting out. Sulphate is the leading kosmotrope in the Hofmeister series (von and Schleich, 1969) and also a strong water structure promoter (Collins and Washabaugh, 1985). t-Butanol is also described as a powerful water structure promoter by Franks and Reid (1973). The anionic sulphate and neutral t-butanol thus influence the physiochemical property each other in water.

Sulphate ion salts out protein due to combined effect of five principles namely ionic strength effects, kosmotropy, cavity surface tension enhancement, osmotic stress and exclusion crowding. The binding of sulphate anion into few cationic sites of proteins at concentrations above 0.5 M, leading to conformational shrinkage when proteins have appreciable net positive charge Z_H^+ was reported by Fink (1995) and Matulis and Lovrien (1996). This behaviour was reported as the main cause for the strong dependence of the salting out mechanism on pH (Dennison and Lovrien, 1997). Some of t-butanol is also known to bind to the proteins. Thus the proteins subjected to TPP were obtained with little of bound salt, t-butanol and modest amounts of water. The positioning of proteins in the interface is attributed to the bound t-butanol increasing the buoyancy of the protein, pulling effects of t-butanol and the pushing effects of ammonium sulphate.

Even though Dennison and Lovrien (1997) explained the background of this technique, they failed to explain few other phenomena with their idea and they also ignored that fact of presence of two liquid phases. Reka *et al. (*2003) through their investigation concluded that protein interfacial adsorption and the rheological properties of such films might also be important for separation of protein in TPP. The extraction of protein in the middle layer is actually due to partitioning rather than precipitation (Kiss *et al*., 1998). Borbas *et al.* (2001) quantitatively analyzed the middle phase and suggested that it is a heterogeneous system and this layer is known to have bulk viscoelastic behaviour.

2.5.1 Purification of enzymes and proteins by TPP

Melius and Simmons (1965) were first to apply TPP-like separation for purification of lipases. Their work involved the use of n-butanol instead of t-butanol and they were able to obtain nearly a 10-fold increase in specific activity. Rudd *et al*. (1987) reported the application of TPP involving t-butanol for the isolation of a cholesterol esterase from pancreas with a 2.8-fold increase in specific activity.

The precipitation of the three forms of cellulase enzyme namely exocellulase, endocellulase and β-glucosidase was achieved using TPP from *Trichoderma reesei* with 90-95% recovery of activity, in a work done by Odegaard *et al.,* 1984. Niehaus and Dilts (1982) isolated mannitol dehydrogenase from a crude homogenate of *Aspergillus parasiticus* mycelia. In their work TPP was proved efficient in removing the unwanted pigments and

many other unwanted proteins which gave a four fold increase in specific activity. TPP was also successfully applied for the isolation of cathepsin L in a single chain without being proteolytically snipped which was attributed to the speed of this particular technique by Dennison *et al*. (1992). Pike *et al*. (1992) were able to obtain proteolytically active cathepsin L complex with stefin B using TPP.

Gavin *et al*. (1989) isolated cathepsin D from bovine in two steps involving three-phase partitioning in t-butanol/water/ammonium sulfate followed by affinity chromatography on pepstatin-agarose and observed that there was a great reduction in time when compared to previous methods. The observation by Pol *et al. (*1990) that TPP denatures haemoglobin was exploited by Fortgens (1991) to successfully isolate cathepsin D from human spleen. A similar work was reported earlier by Jacobs *et al*. (1989) incase of isolation of cathepsin from bovine spleen. The same fact was proved useful in case of rapid isolation of soluble proteins such as carbonic anhydrase, catalase and superoxide dismutase from human erythrocytes by Pol *et al*. (1990).

Dennison and Lovrien (1997) in their work summarized the precipitation of about 20 enzymes and protein by the application of t-butanol mediated TPP. Table 2.2 gives the information of which they were aware of. A batch mode operation involving three-phase partitioning and the use phenyl Sepharose-6B was employed to purify alkaline phosphatase from crude extract of chicken liver by Aparna *et al.* (2000). They were able to obtain a fold purification of 80 and 61% recovery of activity.

Aparna and Gupta (2001) were able to obtain 25 fold purification with 85% recovery for optimized conditions of 30% ammonium sulphate saturation (w/v), ratio of t-butanol to crude extract is 1:1 And a temperature of 20°C, in case of wheat grain bifunctional protease / amylase inhibitor. Pectinases from *Aspergillus niger* and tomato were purified by addition of *tert*-butanol in the presence of ammonium sulphate. The yields of 76% (*Aspergillus niger*) and 183% (tomato) and purifications of 10-fold (*Aspergillus niger*) and 9-fold (tomato) were obtained (Aparna and Gupta, 2001).

Sulakshana *et al.* (2004) reported purification of recombinant Green Florescence Protein (GFP) by TPP in two rounds. The first round most GFP retained in lower aqueous phase with 20% ammonium sulphate saturation and crude: t-butanol ratio of 1:1 (v/v). In

second step 60% ammonium sulphate saturation and crude: t-butanol ratio of 1:2 were employed to achieve 78% recovery and 20-fold purification. Ipsita *et al*. (2005) showed that the biological activities of cellulase, cellobiase and β-glucosidase can be recovered from their urea-denatured forms by the application of TPP which resulted in 94%, 98% and 90% regain of activities with 73, 65 and 101 fold purifications for cellulase, cellobiase and β-glucosidase respectively.

Shweta and Gupta (2001) have purified phospholipase D from carrot (*Dacus carota*) by applying TPP and obtained 13-fold purify and 72% recovery of activity with optimum conditions for 50% ammonium sulphate saturation, 1:1 ratio of crude extract to T-butanol at pH 4.5. Pol *et al.* (1990) have report the purification of human carbonic anhydrase I and II, catalse and superoxide dismutase, by TPP was applied for removal of hemoglobin. This partitioning step has resulted in 60-80 fold enrichment of enzyme activities. These enzymes were subjected further purification to which resulted in over all yield of 84 and 29% for carbonic anhydrase I and II respectively, 38% for catalase and 52% for superoxide dismutase. Çalcı *et al.* (2009) were able to purify α- galactosidase from tomato (*Lycopersicon esculeutum*) and obtained 4.3 fold purification and 80% activity yield. Narayan *et al.* (2008) have purified *Ipomea palmata* leaf peroxidase using TPP. They reported a 18-fold purification after second cycle TPP with 81% activity recovery.

Ipsita and Munishwar (2002) reported purification of soyabean trypsin inhibitor with 13-fold and 72% recovery using TPP. Ipsita and Munishwar 2004 found that the activity of α-chymotrysinogen was increased to 119% after TPP. The enzyme obtained through TPP showed a 2.94 and 3.58 fold increase when compared to that of normal α-chymotrysinogen in initial rates for formation of the ester Ac-Phe-OEt (from Ac-Phe and ethanol) in low water containing toluene and n-octane, respectively.

Lalit *et al.* (2007) have carried out TPP in two step for purification of bifunctional amylase/protease inhibitor from *Eleusine coracana* (Ragi). 30% ammonium sulphate saturation with 1:1 ratio of crude to t-butanol were the optimized conditions for step 1 which gave 8.90 and 8.65 fold purification with 83% and 80% yield of amylase inhibitor and trypsin inhibitor respectively and 20.1 and 16 fold purification with 39.50% and 32% yield for the same in case step 2.Where the optimized conditions were to be 60% ammonium sulphate saturation and ratio of aqueous phase to t-butanol of 1:2.

Table 2.2 Enzymes purified by t-butanol-TPP (Dennison and Lovrien, 1997)

Enzyme	Source	Increase in specific activity	Yield %
Cellulase	*T.reesei*		95
Peroxidase	Horse radish	2X-3X	
Pepsin	Porcine stomach		Almost 100
Protease	*B.subtilis*	5X	300
α –Amylase	Barley malt	10X	100
α –Amylase	*A.oryzae*	2-3X	100
α –Amylase	*B.amyloliquifaciens*	6-8X	200-300
α –Amylase	*B.licheniformis*	4X	200-300
Invertase	*S.cerevisiae*	75X	500-1000
β-Galactosidase	*A.orysae*	8-10X	600
β-Galactosidase	Almond	10-20X	80
Amlyoglucosidase	*Rhizopus sp.*	3X	200-300
Lipase	*Candida cylindracea*	8X	900
B/B-protease inhibitor	Soybean	4X	100
Cathepsin D	Bovine spleen	13X	27
Cathepsin L	Sheep liver	3.5X	60
Cathepsin S	Bovine spleen	Considerably improved	Considerably improved
Mannitol dehydrogenase	*A.parasiticus*	4-5X	77
Carbonic anhydrase	Human erythrocytes	68X	96
Catalase	Human erythrocytes	86X	143
Trypanopain-Tb protease	Trypanosomes	6X	25

Smita *et al.* (2008) were first to report the application of TPP to obtain proteins from solubilized inclusion bodies expressed in *E.coli* in a correctly refolded and active form.They were able to obtain higher refolding yield for 12 different proteins including RNase A, Cc Db-FITP, MBP264D,CD4D12, TRX mutant etc, than the earlier reported methods. Their work has indicated that TPP could serve as a useful complement to existing refolding strategies of diverse proteins from inclusion bodies, including for those proteins with/without disulphide bonds for single and multi domain protein and for monomeric and dimeric one.

Dhananjay and Mulimani (2009) have reported the purification of α- galactosidase from fermented media of *Aspergillus oryzae* using TPP in a single step with ammonium sulphate saturation 60%, crude: T-butanol ratio of 1:1, which gave activity recovery of 92% with 12-fold purification. Isolation of trypsin from legume seeds such as navy bean, red kidney bean and adzuki bean with 30% ammonium sulphate saturation (w/v), t-butanol by was done Richa *et al.* (2009). They reported 5, 14, and 7-fold purification with 315%, 441% and 228% recovery for navy bean, red kidney bean and adzuki bean respectively. Purification of Aryl alcohol oxidase (AAO) from *Pleurotus ostreatus* was carried out by TPP by Kumar and Rapheal (2011). The enzyme was purified upto 10.19 fold with 10.95% recovery.

From above literature survey it is clear that TPP is an effective single step process for purification of enzymes, with advantages of obtaining better fold purification and high recovery of enzyme activity. Hence, in the current report TPP has been employed for the purification L-asparaginase from *Capsicum annum* with a view to optimize the TPP process parameters to yield better results.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

All the chemical and reagents were used of analytical grade and were obtained from Hi-media, Bombay. All the reagents were prepared using milli-Q water (Millipore, USA).

3.2 EXTRACTION OF L-ASPARAGINASE

Green chillies were purchased from local market and washed with tap water followed by distilled water prior to use. Extraction was carried out by grinding the chillies using 0.15 M KCl using mortar and pestle. The homogenized sample was centrifuged at 10,000 rpm for 15 min. The supernatant was filtered and named as crude extract. All the steps were carried out at 4º C.

3.3 PURIFICATION BY THREE-PHASE PARTITIONING (TPP)

The crude extract was subjected to TPP for purification of L-asparaginase by addition of ammonium sulphate to attain particular saturation followed by addition of t-butanol at particular pH in separating funnel and allowed to stand for 1 hr at specific temperature. After one hour, the mixture was centrifuged at 5,000 rpm for 10 min to separate the phases. The interfacial layer and lower aqueous layer were collected, the earlier was re- dissolved in 0.15 M KCl, and these two samples were dialysed overnight against 0.015 M KCl to remove excess salt, and assayed for activity and total protein concentration. The parameters such as ammonium sulphate (w/v), crude: t-butanol ratio (v/v), temperatures and pH were optimised as follows.

3.3.1 Optimization of ammonium sulphate saturation

In order to find optimum ammonium sulphate saturation, the salt was added to the crude to attain different saturation (10, 20, 30, 40, 50, 60, 70 and 80 %), while the other

parameters were fixed as ratio of crude: t-butanol-1:1, temperature - room temperature (28±2º C) and pH-7.2.

3.3.2 Optimization of crude: t-butanol

With the optimized ammonium sulphate saturation, ratio of crude: t-butanol was varied from 1:0.25 to 1:2 at fixed temperature and pH conditions as mentioned early.

3.3.3 Optimization of temperature

At optimized condition of ammonium sulphate saturation and crude: t-butanol ratio, the next parameter temperature was optimized by carrying at TPP standing time (1 h) at different temperatures ranging from 30º C to 60 º C at pH 7.0.

3.3.4 Optimization of pH

With the three parameters at optimized conditions, optimum pH was determined by adjusting pH of the crude after saturating with ammonium sulphate ranging from 5 to 9.5 followed by addition of t-butanol at optimized quantity.

3.4 DETERMINATION OF ENZYME ACTIVITY

Enzyme activity was determined by enzymatic assay procedure proposed by Shirfrin *et al* (1974), with a modification in substrate concentration. 0.1 mL of enzyme extract, 0.1 mL of L-asparagine as substrate, 1 mL of 50 mM Tris buffer (pH 8.6) and 0.9 mL of milli-Q water comprised the assay system. The mixture was incubated for 30 min at 37º C. After incubation the reaction was stopped by adding 0.1 mL 1.5 M trichloroacetic acid (TCA). 0.2 mL of this mixture was added to 4.3 mL milli-Q water and the ammonia released due to L-asparagine catalysis by L-asparaginase was estimated by adding 0.5 mL of Nessler's reagent. The intensity of the colour developed was measured at 436 nm using UV-Spectrophotometer (Elico, SL-159, Hydrebad).

The absorbance was compared with ammonium sulphate standard. One international unit (IU) of L-asparaginase is that amount of enzyme which liberate 1 µmol of ammonia in 1 min. at 37º C.

3.5 DETERMINATION OF PROTEIN CONCENTRATION

The total protein was determined by Lowry's method (Lowry *et al.,* 1951), with bovine serum albumin as standard (BSA). Assays were carried out in duplicates and the averages were used for calculations. Specific activity was calculated and expressed as units per milligram of protein.

3.6 PHYSIO-CHEMICAL PROPERTIES OF PURIFIED L-ASPARAGINASE

3.6.1 Effect of temperature on activity and stability of L-asparaginase

In order to determine the effect of temperature on the activity of L-asparaginase, the assays were carried out over the temperature range of 20-55º C. The relative activities were expressed in percentage as the ratio of the purified L-asparaginase activity obtained at a certain temperature to the maximum activity obtained at the given temperature range. To check the thermal stability, the enzyme was incubated at different temperatures (30 - 55º C) for 30 min. After the incubation period the enzyme aliquots were withdrawn, brought to room temperature and then assayed at optimal assay conditions and the residual activity was determined.

3.6.2 Effect of pH on activity and stability of L-asparaginase

In order to determine the effect of pH on the activity of L-asparaginase, the assays were carried out over the pH range of 5.0 to 9.0. The pH stability of the L-asparaginase was studied by incubating the enzyme in buffers of different pH (5.0 to 9.0) at 30º C for 30 min. The buffers (0.05 M) used were acetate (pH 5.0 and 5.5), phosphate (pH 6.0 - 8.0) and glycine (pH 8.5 and 9.0). The residual activities were determined using the standard activity assay procedure.

3.6.3 Effect of substrate concentration (L-asparagine) on activity of L-asparaginase

The effect of substrate concentration on the enzyme activity was determined by incubating the enzyme with initial concentration of L-asparagine ranging from 0.01 to 0.2 M at 37º C. Lineweaver-Burk plot of 1/V against 1/[S] was used to determine the apparent maximum velocity of reaction (V_{max}) and Michaelis-Menten constant (K_m).

3.6.4 Effect of various effectors on L-asparaginase activity

The influence of various effectors ($CaCl_2$, $CuSO_4$, EDTA, $FeCl_3$, $HgCl_2$, KOH, $MgSO_4.7H_2O$, $NaNO_3$, SDS, urea, $ZnSO_4.H_2O$, β-mercaptoethanol) in concentrations of 1, 5, 10 mM on the L-asparaginase activity was investigated by pre-incubating the enzyme with the different effectors for 30 min at room temperature. Residual activity was calculated against control, without any effectors which was taken as 100% activity.

3.6.5 Effect of ionic strength on L-asparaginase activity

To investigate the influence of ionic strength on the enzyme activity, the enzyme assays were carried out at different concentration of Tris buffer (0.01-0.1 M) at pH 8.6 and the relative activities were expressed in percentage as the ratio of the purified L-asparaginase activity obtained at a certain ionic concentration of the buffer to the maximum activity obtained at the given concentration range.

3.6.6 Total antioxidant capacity of L-asparaginase

0.1 mL of the enzyme was mixed with 1 mL of reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The mixture was incubated at 95 º C for 90 min. After incubation the mixture was brought down to room temperature and the absorbance was measured at 695 nm and compared with that of ascorbic acid standard. The reagent blank consisted of 0.1 mL milli-Q water and 1 mL of reagent solution. (Prieto *et al*., 1999)

3.6.7 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SDS–PAGE was performed to analyze the proteins of the samples according to the method of Laemmli (1970) using 3% stacking gel and 10% polyacrylamide separating gels. Electrophoresis was carried out at a constant current of 15 mA per gel using a power-pack electrophoresis unit (Genei, Bangalore). After separation, the gel was stained with Coomassie Brilliant Blue R-250 for 1 hr for the detection of proteins and then destained by diffusion in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid solution.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 TPP OF L-ASPARAGINASE FROM GREEN CHILLIES

In this work TPP has been applied for the purification of L-asparaginase green chillies (*Capsicum annum)* in single step. TPP was carried out using ammonium sulphate and t-butanol, and the four parameters governing the efficacy of TPP were optimized. The influence of different parameters, such as ammonium sulphate saturation percentages (20, 30, 40, 50, 60 and 70 %, w/v), crude extract to t-butanol ratio (1:0.25, 1:0.5, 1:0.75, 1:1 and 1:2, v/v), temperatures (30, 40, 50, 60 and 70ºC) and pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5) were studied. In these experiments L-asparaginase was found to predominately accumulate in the interface, between the upper solvent phase and the lower aqueous phase (Figure 4.1).

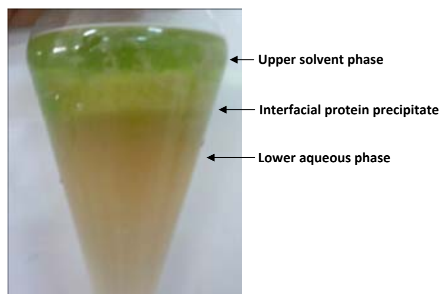

Figure 4.1 Three phases formed in TPP of L-asparaginase. (The three phases, namely the upper solvent phase, interfacial protein precipitate and lower aqueous phase, formed during TPP of L-asparaginase by addition of ammonium sulphate and t-butanol after pH adjustment)

The efficiency of salting out primarily depends upon the sulphate concentration and then the net charge on the protein. Hence, ammonium sulphate saturation is a critical parameter to be optimized (Dennison and Lovrien, 1997). In order to find the most suitable ammonium sulphate concentration, different percent saturations of ammonium sulphate were used for partitioning. The results are shown in Figure 4.2. A saturation of 50% (w/v) was known to give maximum activity hence this percent saturation was taken for further studies.

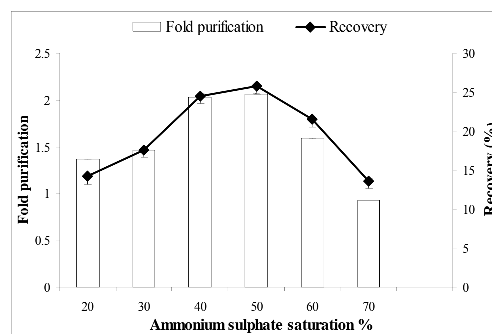

Figure 4.2 Optimization of ammonium sulphate saturation and its effect on the degree of purification and activity recovery of L-asparaginase. (Conditions: crude:t-butanol ratio 1:1, temperature 28±2ºC and pH around 7.2)

After selection of ammonium sulphate saturation the next parameter, relative amount of t-butanol was optimized. t-Butanol is the most preferred cosolvent for TPP. t-butanol owing to its large branched structure does not denature proteins since it cannot penetrate the folded protein structure (Dennison *et al*., 1992). For this study ammonium sulphate saturation was fixed at 50% (w/v) and the crude:t-butanol ratio was varied from 1:0.25 to 1:2.0 (v/v) (Figure 4.3). The best results were observed with 1:0.75 (v/v) ratio and ammonium sulphate saturation of 50% (w/v). t-Butanol is known not to adequately synergize at lower amounts and causes denaturation at higher amounts.

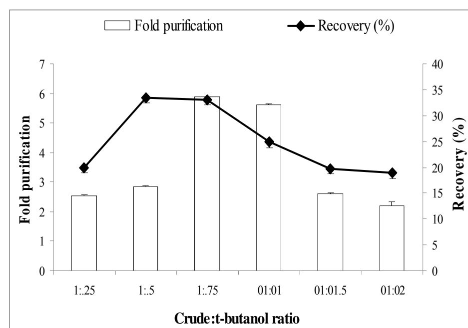

Figure 4.3 Optimization of crude:t-butanol ratio for purification and recovery of L-asparaginase. (Conditions: 50% ammonium sulphate saturation, temperature 28±2ºC and pH around 7.2)

Temperature of the system is also an important parameter in TPP. Most TPPs are carried out around room temperature and there is no need for cold temperatures, as in case of C_2 solvents like ethanol. It can also be carried out at higher temperatures if needed. Hence, an attempt was made to optimize the temperature of the system. The system temperature was varied from 30 to 70ºC during the standing time of 1 h (Figure 4.4). A temperature of 30ºC was known to give best results at 50% ammonium sulphate saturation and 1:0.75 crude enzyme: t-butanol ratio.

Another crucial parameter that governs precipitation of protein is the system pH, since the net charge on the protein is highly pH dependent. Hence, the effect of pH on protein partitioning was investigated. After saturating the crude with 50% (w/v) ammonium sulphate the pH of the system was adjusted to particular values ranging from 5.0 to 9.5, (Figure 4.5) and t-butanol was added at optimized ratio of 1:0.75 and allowed to stand 1 h. After 1 h, of the three phases formed the bottom phase and the interphase were analyzed. L-asparaginase had partitioned selectively at the interphase at pH 9, ammonium sulphate saturation of 50% (w/v), crude to t-butanol ratio of 1:0.75 and a temperature of 30ºC. These optimized

conditions resulted in 6.83 fold purification and 567.4% yield. The overall purification of L-asparaginase from *Capsicum annum* by TPP is summarized in Table 4.1.

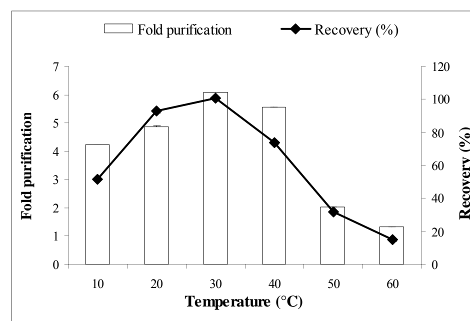

Figure 4.4 Optimization of temperature for purification and recovery of L-asparaginase. (Conditions: 50% ammonium sulphate saturation, crude:t-butanol ratio 1:0.75 and pH around 7.2).

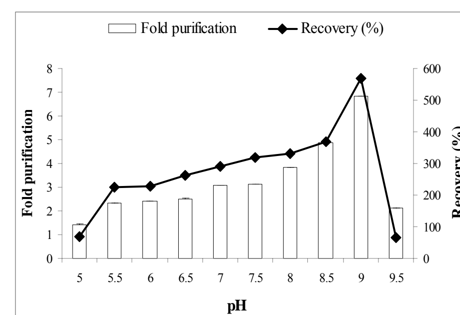

Figure 4.5 Optimization of pH and its effect on purification and recovery of L-asparaginase. (Conditions: 50% ammonium sulphate saturation, crude:t-butanol ratio 1:0.75 and 30ºC).

Table 4.1 Overall purification of L-asparaginase from *Capsicum annum* by TPP[a]

Steps	Total activity (Unit)	Total protein (mg)	Specific activity (Unit/mg)	Purification fold	Activity yield (%)
Crude extract	4992.72	11.33	440.53	1.00	100
TPP-interfacial precipitate	28333.4	9.41	3009.26	6.83	567.4
TPP-aqueous phase	0666.67	5.15	129.45	0.29	13.35

[a] The ammonium sulphate (50%, w/v) was added to the crude extract of L-asparaginse from *Capsicum annum* and then pH was adjusted to pH 9.0, followed by addition of t-butanol in a ratio of 1:0.75 (v/v) (crude extract:t-butanol). Three phases formed were collected separately. The upper phase was removed and then the lower aqueous phase and interfacial precipitate were tested for enzyme activity and protein amount. Each experiment was carried out in duplicates.

4.2 CHARACTERIZATION OF L-ASPARAGINASE

4.2.1 Effect of temperature on L-asparaginase activity and stability

To determine the influence of temperature on L-asparaginase activity, the enzymatic assays were carried out in the temperature range 20-55ºC. The enzyme showed maximum activity at 37ºC. Similar result was observed in cases of *Vigna unguiculata* (Ehab, 2009) and *Streptomyces gulbargensis* (Amena *et al*., 2010). The thermal stability of the enzyme was determined after incubation for 30 min at various temperatures (20-55ºC). The stability was found to decline steeply above 45ºC. The enzyme retained about 99.2% and 86.7% of initial activity at 30ºC and 37ºC respectively after 1 h, however it was reduced to77.5% and 26.6% at the end of 6 h. At higher temperatures of 50ºC and 55ºC the enzyme lost its activity completely after 2 h (Figure 4.7).

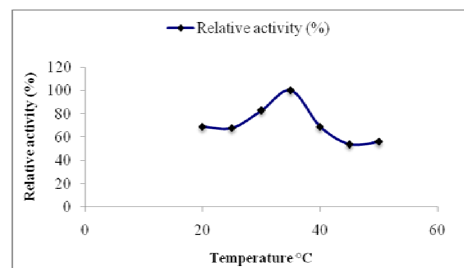

Figure 4.6 Effect of temperature on enzyme activity of L-asparaginase

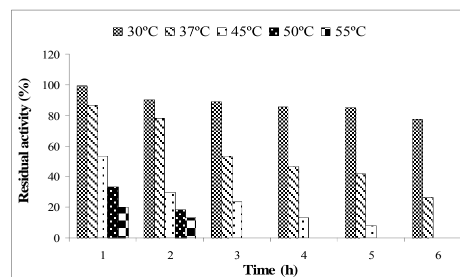

Figure 4.7 Thermal stability of L-asparaginase

4.2.2 Effect of pH on L-asparaginase activity and stability

The effect of pH on L-asparaginase activity was determined by incubating the enzyme in buffers with different pH values (pH 5.0–9.0) and the relative activity was determined. The enzyme showed maximum activity at pH 8.5 (Figure 4.8). L-asparaginase isolated from

different sources including *Lupinus angustifolius, Saccharomyces cerevisiae, Serratia marcescens, Withania somnifera, Bacillus coagulans* (Wriston, 1985) and *Capsicum annum* (Bano and Shivaramakrishnan, 1980). The effect of pH on the stability of L-asparaginase was determined by pre-incubating the enzyme in buffers with varying pH ranging from 5 to 9 for 30 min at room temperature. The enzyme was more stable around neutral to slight alkaline pH of 8.5. Similar results were reported in case of *Pseudomonas chlororaphis subsp. aurantiaca* with maximum stablilty at pH 8 (Lebedeva, 1995) and for *Pseudomonas stutzeri* maximum stability was found in the range of 7.5-9.5 (Manna *et al*., 1995).

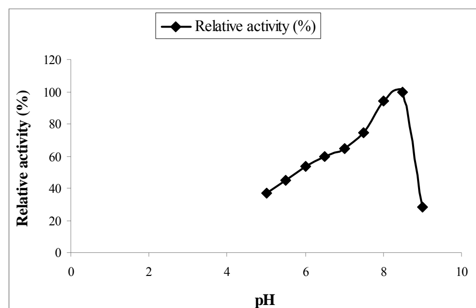

Figure 4.8 Influence of pH on L-asparaginase activity

4.2.3 Effect of substrate (L-asparagine) concentration

To determine the effect of substrate concentration initial concentrations of L-asparagine was varied from 10 to 180 mM and the kinetic parameters V_{max} and K_m were calculated from Lineweaver-Burk plot as 1111.11 U and 0.04 M respectively. Such high K_m values were observed in the cases of *Escherichia coli* mutant enzyme Q59G with a value of 0.05M and in *E.coli* mutant enzyme N248E with a K_m value of 0.07M (Derst *et al.,* 2000).

4.2.4 Effect of various effectors on L-asparaginase activity

The influence of effectors such as $CaCl_2$, $CuSO_4$, EDTA, $FeCl_3$, $HgCl_2$, KOH, $MgSO_4.7H_2O$, $NaNO_3$, SDS, urea, $ZnSO_4.H_2O$, β-mercaptoethanol in concentrations of 1, 5, 10 mM on the L-asparaginase activity were investigated and the residual activities were calculated with respect to control without any effector (Figure 4.9). Magnesium sulphate was known to act as activator at 5 and 10 mM concentrations. EDTA had less inhibitory effect. Other effectors exhibited a inhibitory effect with increasing concentrations, SDS showed strong inhibition at 10 mM concentration. Similar results were observed in a work carried out by Bano and Shivaramakrishnan (1980) in case of *Capsicum annum.*

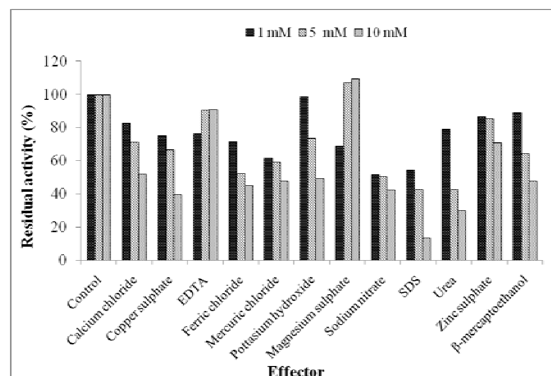

Figure 4.9 Effect of various effectors on L-asparaginase activity

4.2.5 Effect of ionic strength on L-asparaginase activity

The influence of ionic strength on L-asparaginase activity was investigated by varying the initial ionic strength of assay buffer (Tris, pH8.6) from 10 to 100 mM. The relative

activities were determined and L-asparaginase showed maximum activity at a concentration of 50 mM (Figure 4.10).

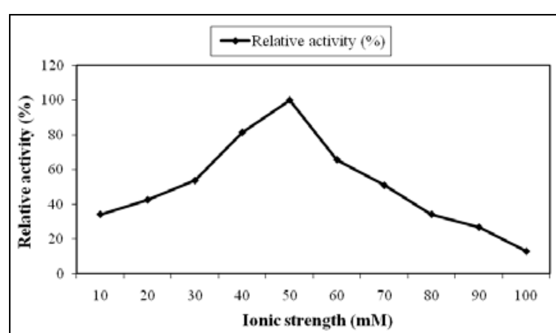

Figure 4.10 Effect of ionic strength on L-asparaginase activity

4.2.6 Total antioxidant capacity of L-asparaginase

The total antioxidant capacity of L-asparaginase was investigated using ammonium molybdate assay. The assay showed that the L-asparaginase purified using TPP has high scavenging activity of 88.58 µM ascorbic acid equivalent when compared to the scavenging activity of the well-known antioxidant, ascorbic acid. The antioxidant capacity of L-asparaginase was also confirmed in *Bacillus sp* R36 by Maysa *et al.*, 2010.

4.2.7 SDS–PAGE analysis

SDS-PAGE analysis showed that, the enzyme obtained from TPP was nearly homogenous with a molecular weight around 37 kDa (Figure 4.9). The result compares well with the previous reports (Cappelletti *et al*., 2008 and Dhavala *et al*., 2008).

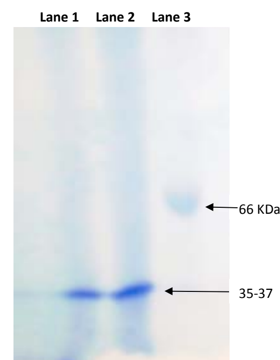

Figure 4.10 SDS-PAGE analysis of L-asparaginase from *Capsicum annum*

(Lane 1, Lane 2: TPP purified L-asparaginase (inter phase), Lane 3: BSA)

CHAPTER 5

CONCLUSION

A simple and rapid process for the purification of L-asparaginase from *Capsicum annum* using TPP was successfully developed. The enzyme was purified to 6.83 fold with a recovery of 567.4%. Three-phase partitioning (TPP) is an elegant way to separate proteins directly from even the large volumes of crude suspensions. The amount of salt required for TPP is lesser when compared to the conventional techniques. t-Butanol is relatively cheaper than that of reagent grade ethanol and is its requirement also small. TPP facilitates the separation of protein of interest from unwanted proteins into separate phases, as in this study L-asparaginase is predominantly partitioned into the interface. TPP can operate practically around room temperatures or even at higher temperatures. A temperature of 30ºC gave best results in this study.

TPP is known to enhance specific activity of certain proteins. These results support the fact that TPP is a simple, scalable, economic and a quick technique which can result in protein purification in single step. This technique will be a good strategy for the production of L-asparaginase from different sources in a more economic and efficient way. The characteristic biochemical properties of the L-asparaginase were also shed light on its suitability for various industrial applications. A high antioxidant value of 88.58 µM ascorbic acid equivalent indicates good scavenging ability of L-asparaginase supporting the fact that it can be applied for the treatment of cancer which is one of the free-radical mediated diseases.

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APPENDICES

APPENDIX 1

A1.1 Acetate Buffer
Stock solution:
A: 0.2 M solution of acetic acid (11. 55 mL in 1000 mL)
B: 0.2 M solution of sodium acetate (16.4 g of C_2H_2Na or 27.2 g of $C_2H_3O_2Na.3H_2O$ in 1000 mL)
x mL of A, y mL of B, diluted to a total of 100 mL.

x	y	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

A1. 2 Phosphate Buffer
Stock solution:
A: 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 mL)
B: 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 mL)
x mL of A, y mL of B, diluted to a total of 200 mL.

x	y	pH	x	y	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	89.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

A1. 3 Glycine NaOH Buffer
A: 0.2 M solution of glycine (15.01g in 1000 mL)
B: 0.2 M NaOH
50 mL of A, + x mL of B, diluted to a total of 200 mL.

x	y
4.0	8.6
6.0	8.8
8.8	9.0
12.0	9.2
16.8	9.4
22.4	9.6
27.2	9.8
32.0	10.0
38.6	10.4
45.5	10.6

APPENDIX 2

SDS-PAGE
Stock Acrylamide (30 %)
The solution contained 28.2 g of acrylamide and 1.8 g of N-N-Methlyene bis-acrylamide in a final volume of 100 mL made in distilled water. The solution was filitered through Whatman number 1 filter paper.

1.857 M Tris buffer (pH 8.8)
22.76 g of Tris was dissolved in distilled water. The pH was adjusted to 8.8 with HCl and fial volume was made up to 100 mL. the solution was stored at refrigerator.

1.25 M Tris-HCl buffer (pH 6.8)
15. 125 g of Tris was dissolved in distilled water. The pH was adjusted to 6.8 with HCl and final volume was made up to 100 mL. The solution was stored at refrigerator.

Sodium Dodecyl Sulphate (SDS) - 10% (W/V)
1 g of APS was dissolved in distilled water and final volume was made up to 100 mL and stored at room temperature.

Ammonium Per sulphate (APS) - 10% (W/V)
1g of SDS was dissolved in distilled water and final volume was made up to 10 mL freshly prepared APS was used every time.

Electrophoresis buffer (10 X)
Tris base 6 g, Glycine 14.4 g and SDS 10 g was dissolved in distilled water and the volume was made up to 1000 mL and stored at room temperature.

Separating gel mixture (10 %)
Stock acrylamide solution - 3.71 mL
Tris-HCl (pH 8.8) - 2 mL
Autoclaved Distilled water - 4.5 mL
APS - 100 µL
SDS - 200 µL
TEMED - 20 µL

Stacking gel mixture (5 %)
Stock acrylamide solution - 340 µL
Tris-HCl (pH 6.8) - 250 µL

Autoclaved Distilled water - 1.87 mL
APS - 50 µL
SDS - 100 µL
TEMED - 10 µL

Coomassie Brilliant Blue Staining
Staining solution
1 g of Coomassie Brilliant Blue (R-250) in a solution containing 40 mL Methanol, 10 mL Glacial acetic acid and 50 mL distilled water.

Destaining solution
Solution containing 40 mL Methanol, 10 mL Glacial acetic acid and 50 mL distilled water.