CLONING, EXPRESSION, PRODUCTION, PURIFICATION AND CHARACTERIZATION OF NOVEL GLUTAMINASE FREE RECOMBINANT L-ASPARAGINASE II OF *PECTOBACTERIUM CAROTOVORUM* MTCC 1428 AND *ERWINIA CAROTOVORA* SUBSP. *ATROSEPTICA* SCRI 1043 IN *ESCHERICHIA COLI*

A THESIS

submitted by

RACHNA GOSWAMI

for the award of the degree

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Dedicated to my Parents

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Venkata Dasu Veeranki.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: 26-12-2011 **Rachna Goswami**

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Cloning, Expression, Production, Purification and Characterization of Recombinant L-asparaginase II of *Pectobacterium carotovorum* MTCC 1428 and *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *Escherichia coli***" by Ms. Rachna Goswami for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision at the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

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ABSTRACT

Bacterial L-asparaginase is used as a therapeutic agent for the treatment of acute lymphoblastic leukemia, and a processing aid in the manufacture of starchy food products such as potato chips and biscuits to reduce the formation of acrylamide. L-asparaginase is also used as a model enzyme for the development of new drug delivery system and L-asparagine biosensor to diagnose leukemia. The different side effects of this drug are attributed due to the presence of partial glutaminase activity of L-asparaginase. Therefore, glutaminase-free L-asparaginase is highly enviable for its successful clinical applications.

Among the tested microorganisms, *Pectobacterium carotovorum* MTCC 1428 and *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 have produced novel glutaminase-free periplasmic L-asparaginase. Hence, further studies were carried out with *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043. The genes encoding L-asparaginase (L-asparaginase, L-asparaginase I and L-asparaginase II) in *P. carotovaram* MTCC were identified. These three genes of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were cloned and expressed in *Escherichia coli* BL21 (DE3), with and without 6x histidine at C-terminus. The histidine tag has proved to be facilitated the purification and also promoted expression level of the cytoplasmic L-asparaginase I and L-asparaginase II. But, in the case of L-asparaginase, lower expression of recombinant L-asparaginase was observed with histidine tag as compared to without tag. As L-asparaginase II is used as anticancer agent, the production of glutaminase-free recombinant L-asparaginase II of both the strains have been carried out both in shake flask and bioreactor (batch and fed-batch).

Response surface methodology (RSM) was applied to optimize the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043. Central composite design (CCD) was employed to optimize the medium components (tryptone, yeast extract and NaCl) and physical parameters (pH, agitation and inoculum size). For recombinant L-asparaginase II of *P. carotovorum* MTCC 1428, the optimal levels of tryptone, yeast extract and NaCl were found to be 13.30 g l^{-1}, 6.38 g l^{-1} and 7.12 g l^{-1}, respectively, and most favorable combination of pH, agitation and inoculum size were determined to be 7.1, 212 rpm and 2.50 %, respectively. At the optimal levels of medium components and physical parameters, the maximum production and productivity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 was found to be 70.25 U mg^{-1} of protein (16.41 U ml^{-1}) and 2735.00 U l^{-1} h^{-1}, respectively. The production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 was increased by 1.94 fold as compared to the un-optimized production conditions (36.15 U mg^{-1}). For the production of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043, the optimal levels of tryptone, yeast extract and NaCl were found to be 14.73 g l^{-1}, 5.30 g l^{-1} and 4.03 g l^{-1}, respectively, and the best levels of pH, agitation and inoculum levels were observed to be 7.4, 216 rpm and 2.34 %, respectively. After optimization of chemical and physical parameters, the production of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 67.26 U mg^{-1} of protein (16.05 U ml^{-1}) with productivity of 2675.00 U l^{-1} h^{-1}, resulting an overall 1.95 fold increase in the production as compared to the un-optimized production conditions (34.49 U mg^{-1}).

The effect of pH, dissolved oxygen (DO) and initial glucose concentration on the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp.

atroseptica SCRI 1043 in *E. coli* were studied in a batch bioreactor. The optimum level of pH (controlled), dissolved oxygen (DO) and glucose (g l^{-1}) were found to be 7.0, 40% and 1.5, respectively, and under these optimal levels, the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was increased from 17.83 Uml^{-1} to 23.88 U ml^{-1}, and 17.58 Uml^{-1} to 24.57 U ml^{-1}, respectively.

In fed-batch culture, the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 95.85 U ml^{-1} and 96.78 U ml^{-1}, respectively. The overall production of novel glutaminase free recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was increased by 10.2 and 9.4 fold, respectively. The productivity was increased by 7.66 and 7.05 fold for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively as compared to un-optimized production conditions in shake flask culture.

Glutaminase-free recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* were purified to apparent homogeneity. The molecular mass of the subunit and native purified recombinant L-asparaginase II of both the strains were determined to be approximately 37.5 kDa and 150 kDa by SDS-PAGE and native PAGE, respectively. Among the substrates tested, the recombinant L-asparaginase II of both the strains was found to be very specific for its natural substrate, L-asparagine and no glutaminase activity was observed. So, the purified recombinant glutaminase-free L-asparaginase II of *P. carotovorum* MTCC 1428 and

E. carotovora subsp. *atroseptica* SCRI 1043 may reduce the possibility of side effects during the course of anti-cancer therapy.

Among the salts tested, loss of activity was observed with Hg^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+} Cu^{2+}, Fe^{3+}, Mg^{2+} and Zn^{2+}, whereas Na^{+} and K^{+} acted as an enhancer for recombinant L-asparaginases II of both the strains. Inhibition of enzyme activity in the presence of different metal ions and no effect with the metal chelator (EDTA) inferred that L-asparaginases do not belong to a metalloproteins. Inhibition of enzymes activity in the presence of Hg^{2+} Cd^{2+}, and Zn^{2+} confirmed that the presence of essential vicinal sulfhydryl group(s) of the enzymes for catalysis. Furthermore, the role of sulfhydryl groups in the catalytic activity of the enzymes were also confirmed by stimulation of activity by the reducing source, *viz.,* 2-mercaptoethanol and glutathione, and inhibition by thiol group blocking reagents (iodoactamide). However, L-cysteine and histidine were stimulated activity of enzymes. The K_m of purified recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 0.666 mM and 0.656 mM, respectively. The V_{max} of purified recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was determined to be 303.03 IU mg-1 and 312.50 IU mg^{-1}, respectively. Turnover number (k_{cat}) of recombinant L-asparaginase II of both the strains was found to be 1.3×10^2 s^{-1}. Specificity constants (k_{cat}/K_m) of recombinant L-asparagine II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were determined to be 2.01×10^5 $M^{-1}s^{-1}$ and 2.11×10^5 $M^{-1}s^{-1}$, respectively. Optimum pH of both the purified recombinant L-asparaginase II for hydrolysis of L-asparagine was in the range of 7.5–8.5, and their optimum range of temperature was found to be 47-52 °C (with 50 mM Tris-HCl buffer). The combined effect of pH and temperature on the performance of the

purified recombinant L-asparaginase II was studied under assay conditions using response surface methodology. The optimal levels of pH and temperature for maximum activity of recombinant L-asparaginase II of both the strains were found to be 7.8 and 47.0 °C, respectively. After optimization of process parameters, specific activity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was increased by 1.8 and 1.9 fold, respectively.

The minimum value of k_d of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and for *E. carotovora* subsp. *atroseptica* SCRI 1043 was found be 0.02 h^{-1}. The combination of pH and temperature at which the above mentioned minimum deactivation rate was observed at 8.6 and 35 ºC, respectively. The deactivation process was found to be faster at lower pH of 6.5 and higher pH of 9.5. Thermodynamic parameters (ΔG^*, ΔH^*, ΔS^*, and activation energy) were also evaluated for purified recombinant L-asparaginase II of both the strains.

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ABBREVIATIONS AND NOTATIONS

Abbreviations

ALL	Acute lymphoblast leukemia
Amp	Ampicillin
ANOVA	Analysis of variance
ARS	Agricultural research service
bp	Base pair
CBB	Coomassie brilliant blue
CCD	Central composite design
DCW	Dry cell weight (g l^{-1})
DEAE	Diethylaminoethyl
DF	Degree of freedom
DNA	Deoxyribonucleic acid
DNS	3,5-Dinitrosalicylic acid
DO	Dissolved oxygen (%)
EC	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria broth
MS	Mean square
MTCC	Microbial type culture collection
NRRL	Northern regional research laboratories
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis

Abbreviations

PB	Plackett-Burman
PCR	Polymerase chain reaction
PEG	Polyethylene
RNA	Ribonucleic acid
RSM	Response surface methodology
SB	Super broth
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SF-ASNase	silk fibroin L-asparaginase
sp.	Species
SS	Sum of square
SS-ASNase	Silk sericin peptides-L-asparaginase
SSD	Sum of squares of the differences
SSF	Solid state fermentation
TB	Terrific broth
TCA	Tri-chloro acetic acid
TEA	Triethylamine
Tris	Tris hydroxylmethyl amino methane buffer
UV	Ultraviolet

Notations

%	Percentage
°C	Degree centigrade
C	Integration constant
E	Activation energy
F	Feeding rate
g	Gram
ΔG*	Change in free energy
g	Gravitational acceleration
h	Hour
ΔH*	Change in enthalpy
kb	Kilobase
k_d	Deactivation constant (h^{-1})
kDa	Kilo Dalton
k_{cat}	Turnover numbers (s^{-1})
K_m	Michaelis constant (mM)
k_{cat}/K_m	Specificity constant ($M^{-1}s^{-1}$)
L	Litre
M	Molar (mol l^{-1})
min	Minute
ml min^{-1}	Milliliter per minute
M_r	Molecular weight of enzyme (kDa)
nm	Nanometer
pI	Isoelectric point
R^2	regression coefficient
rpm	Rotational per minute
s	Second
ΔS*	Change in entropy
t	Time

Notations

U	Unit of enzyme activity
U l^{-1} h^{-1}	Unit of enzyme activity per liter per hour
U mg^{-1}	Unit of enzyme activity per milligram
U ml^{-1}	Unit of enzyme activity per milliliter
vvm	Volume of air per volume of medium per minute
v/v	Volume/volume
V_0	The initial (batch culture) volume
V_{max}	Maximal reaction velocity (U mg^{-1})
Y	Yield coefficient
Y_{SA}	response (enzyme production)

Greek letter

β_i	Linear effect
β_{ii}	Square effect
β_{ij}	Interaction effect
β_o	Offset term
Δ	Delta
μ	Specific growth rate (h^{-1})
µl	Microlitre
κ	Boltzmann constant
h	Planck's constant
µM	Micromolar (µmol l^{-1})
µmol	Micromoles

CHAPTER 1
INTRODUCTION

1.1. Generalities

L-Asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) is a clinically acceptable antitumor drug for the effective treatment of acute lymphoblastic leukemia and lymphosarcoma. L-asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. Acute lymphocytic leukemia (ALL) cells are unable to synthesize sufficient asparagine for their growth and are dependent on plasma L-asparagine for their protein synthesis. This amino acid deficiency would leads to inhibition of RNA and DNA synthesis and causes apoptotic cell death of leukemic cells (Douer 2008; Kotzia *et al.,* 2007). It is also used in food processing to reduce the formation of acrylamide (a suspected carcinogen) in starchy food products such as potato chips and biscuits (Pedreschi *et al.,* 2008). As a food processing aid, L-asparaginase can effectively reduce the level of acrylamide up to 90% in a range of starchy foods without changing the taste and appearance of the end product (Hanne *et al*., 2009). Moreover, L-asparaginase is used as model enzyme for the development of novel drug delivery system (Teodor *et al.,* 2009) and L-asparagine biosensor for diagnosis of leukemia (Verma *et al.,* 2007). The functional form of L-asparaginase exists as a tetramer of identical subunits with molecular mass in the range of 140–160 kDa (Aung *et al*., 2000; Aghaiypour *et al*., 2001a; Aghaiypour *et al*., 2001b; Kozak *et al*., 2002). Each of the four active sites is located between the N-terminal and C-terminal domains of two adjacent monomers. Thus, the L-asparaginase tetramer is considered as a dimer of dimmers (Khushoo *et al*., 2004; Swain *et al*., 1993). There are two types of L-asparaginase are found in *E. coli* B

(EC1 and EC2) and *E. coli* K12 (Asn1 and Asn2). Type II L-asparaginase has higher affinity towards asparagine than Type I L-asparaginase. Hence, it has been studied extensively for chemotherapy (Cambell *et al*., 1967; Sanches *et al*., 2007).

A wide range of microorganisms such as bacteria, fungi, yeast, actinomycetes, algae and plants have been proven to be proficient sources of this enzyme. But enzyme properties vary from organism to organism (Verma *et al.,* 2007). The production of L-asparaginase have been studied using *Serratia marcescens* (Heinemann and Howard, 1969; Khan *et al.,* 1970), *Erwinia carotovora* (Howard and Carpenter, 1972; Maladkar *et al.,* 1993), *E. coli* (Barnes *et al.,* 1977; Wei and Liu, 1998), *Enterobacter aerogenes* (Mukherjee *et al.,* 2000), *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2002), *Bacillus subtilis* (Fisher and Wray, 2002) and *Aspergillus* species (Mishra, 2006). Due to severe side effects and immunological reactions in the patients, most of the bacterial L-asparaginases are not suitable for the treatments. To find out the L-asparaginase, which has fewer side effects, several members of a larger family of homologous L-asparaginases have been investigated over many years (Boyse *et al.,* 1967; Cammack *et al.,* 1972; Ehrman *et al.,* 1971; Kotzia and Labrou 2005; Krasotkina *et al.,* 2004; Wriston and Yellin, 1973). The allergic response is due to long term use of a foreign protein and owing to partial glutaminase activity of most of the L-asparaginases (Distasio *et al.,* 1982; Gallagher *et al.,* 1989; Manna *et al.,* 1995). In most of the bacterial L-asparaginases, the presence of partial glutaminase activity (up to 9%) of L-asparaginase was reported (Manna *et al.,* 1995; Muller and Boos, 1998). Therefore, isolation of a glutaminase-free L-asparaginase from a novel source is extremely enviable for successful remedial studies. Clinically available L-asparaginase is derived from *E. coli* and *E. chrysanthemi*. The enzymes from these two microorganisms have been found to have lower

toxicity among the large variety of similar enzymes with known antitumor activity (Duval *et al.,* 2002; Narta *et al.,* 2007).

1.2. Subcellular localization of L-asparaginase

To develop any bioprocess, subcellular localization of enzyme need to be explored. The presence of periplasmic, cytoplasmic and membrane bound enzyme was reported in L-asparaginase producing gram negative strains (Geckil and Gencer, 2004; Geckil *et al.,* 2005; Mukherjee *et al.,* 2000; Schwartz *et al.,* 1966; Triantafillou *et al.,* 1988). Several gram-negative bacteria possess two L-asparaginases, a high-affinity periplasmic enzyme and a low-affinity cytoplasmic enzyme. In *E. coli* and various other bacteria, synthesis of the cytoplasmic L-asparaginase I is constitutive. But, the expression of periplasmic L-asparaginase II is activated during anaerobiosis. It has been proposed that L-asparaginase II perhaps has a particular role in anaerobic fumarate respiration by providing aspartate, which is followed by conversion to fumarate. Moreover, only the type II L-asparaginase has significant antitumor activity (Fisher and Wray, 2002). The intracellular localization of microbial enzymes have been studied for the production of alkaline phosphatase, deoxy ribonuclease (Neu and Heppel, 1965), cyclic phosphodiesterase, 5'-nucleotidase, acid phosphatase (Nossal and Heppel, 1966), carboxymethyl cellulase (Srinivas and Panda, 1998) and 17β-hydroxysteroid dehydrogenase (Egorova *et al.,* 2005). Enzyme localization in bacteria has been studied by using various methods (Alexander, 1956; Marr, 1960; Neu and Heppel, 1965).

1.3. Cloning and expression of L-asparaginase

The production of large amount of L-asparaginase using wild strain is limited due to the extremely low efficiency of the system. Therefore, it is essential to search for systems that display high expression levels of L-asparaginase and construct suitable genes for L-asparaginase through recombinant DNA techniques (Guo *et al*, 2002). It was observed that the genes encoding L-asparaginase I and L-asparaginase II were not sequence related (Verma *et al*., 2007). The recombinant L-asparaginase has great potential as an antitumor agent as compared to the wild type L-asparaginase (Guo *et al*, 2002; Zhao *et al*., 2002). Recombinant *Erwinia carotovora* L-asparaginase has been expressed in *E. coli* (Borisova *et al*. 2003; Krasotkina *et al*. 2004). L-asparaginases from *E. coli, E. carotovora, E. crysanthemi, Helicobacter pylori* and *Saccharomyces cerevisiae* have been cloned and successfully expressed in bacterial, and yeast expression systems (Cappelletti *et al*., 2008; Kotzia and Labrou 2005 and 2007; Khushoo *et al*., 2004; Khushoo *et al*., 2005; Maria *et al*., 2006). The recombinant L-asparaginase was fused to pelB leader sequence under the inducible T7 lac promoter and expressed in to *E. coli* BL21 (DE3) (Khushoo *et al*., 2005).

1.4. Optimization of process parameters for enhance production of L-asparaginase

Process optimization plays a significant role in industrial production processes in which even small improvements is very important for commercial achievement. The production of L-asparaginase by gram-negative bacteria is influenced significantly by process parameters *viz.,* pH, temperature, dissolved oxygen, incubation time, inoculum volume, aeration, agitation and medium constituents (Khan *et al.,* 1970; Mukherjee *et al.,* 2000). In any

bioprocess, the improvement in productivity of any metabolite could be achieved through manipulation of nutritional and physical parameters. Screening and evaluation of nutritional requirements for microorganism is an important step for bioprocess development. Optimization studies involving one-factor-at-a-time approach is tedious, tends to overlook the effects of interaction among the factors and might guide to misinterpretation of results. In contrast, statistical methodologies are generally preferred due to their advantages (Abdel-Fattah and Olama, 2002; Dasu and Panda, 2000; Prakasham *et al.,* 2007; Reddy *et al.,* 2008) and statistically designed experiments minimize the error in determining the effect of parameters in an economical manner (Sharma and Satyanarayana, 2006). Response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system would be determined. Abdel-Fattah and Olama, (2002) have applied Plackett-Burman and Box-Behnken experimental designs for screening and optimization of process parameters, respectively to improve the production of L-asparaginase from *P. aeruginosa.* Kumar *et al*., (2009) have applied Plackett-Burman and central composite design (CCD) for screening and optimization of medium components, respectively, for higher production of L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. Neto *et al.,* (2006) and Prakasham *et al.,* (2007) also reported the enhanced production of L-asparaginase in submerged fermentation from *Zymomonas mobilis* and *Staphylococcus* species using Box-Behnken experimental and Taguchi experiments design, respectively. Hymavathi *et al.,* (2009) reported that the enhancement of L-asparaginase production by *Bacillus circulans* MTCC 8574 using RSM in solid state fermentation. Recently, Kenari *et al*., (2010) have optimized the L-asparaginase production from *E. coli* ATCC 11303 using response surface methodology.

1.5. Enhanced production of recombinant L-asparaginase II in bioreactors

Bioreactors have an edge over shake flasks as it allows better control on process parameters *viz*., aeration, agitation and pH maintenance. A large number of investigations have been performed to clarify the molecular structure (Aung *et al.*, 2000; Borek and Jaskolski, 2002; Kozak and Jurga, 2002), catalysis (Kelo *et al.*, 2002), clinical aspects (Ettinger *et al.*, 1997; Narta *et al.*, 2007), genetic determinants involved in regulation (Huser *et al.*, 2002; Ortuno-Olea and Duran-Vargas, 2000) and stabilization to enhance biological half-life (Baran *et al.*, 2003; O'Fagain, 2003; Soares *et al.*, 2002) of L-asparaginase. But very few reports are available for improving the enzyme production (Geckil *et al.*, 2004; Mukherjee *et al.*, 2000). Further studies on the production of L-asparaginase in bioreactor level have not been attempted so far.

1.6. Purification and characterization of L-asparaginase

Downstream processing of biomolecules obtained from fermentation broths is a fundamental step in process biotechnology as it often represents the major manufacturing cost. The economic viability of a biochemical process depends not only on improvements achieved in the production but also on innovations and optimization of downstream processes (Haki and Rakshit, 2003; Wheelwright 1987). The final quality of biotechnological products is determined at the purification level, which may be regarded as the most important stage in the whole production process. This requirement is even more critical for therapeutic products such as therapeutic enzymes, vaccines and antibiotics that require an extremely high purity level (Alvarez *et al*., 2001). Chromatographic techniques are widely used as high performance purification steps in biotechnology (Martın *et al.*, 2002). As each enzyme requires specific strategy for purification, it is necessary to develop a strategy for the purification of individual

enzyme. The purification and characterization of L-asparaginase have been studied from different bacterial sources *viz.*, *E. coli, E. chrysanthemi, E. carotovora, P. stutzeri, B. circulans, Enterobacter aerogenes,* and *P. aeruginosa* (El-Bessoumy *et al.,* 2004; Khushoo *et al.,* 2005; Kotzia and Labrou, 2005; Kotzia and Labrou, 2007; Manna *et al.,* 1995; Mukherjee, *et al.,* 2000; Prakasham *et al.,* 2010). The hydrolysis of L-asparagine by L-asparaginase depends on several physicochemical factors. The temperature of incubation and pH influence the rate of reaction to a large extent (El-Bessoumy *et al.,* 2004). Thermal stability studies would explore the mechanism of relation between structure and function of a particular enzyme. Very few studies have been carried out on thermodynamic aspects of this enzyme (Kotzia and Labrou, 2005; Krasotkina *et al.,* 2004). Thermal stability studies would help to understand the relation between structure and function of a particular enzyme (Sadana, 1995). L-asparaginase deactivation plays a crucial role in cancer therapy as rapid inactivation may reduce its efficiency. The deactivation studies would provide valuable physical insights into the structure and function of the enzyme. Deactivation is defined as a process where the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds (Joly, 1965). The ability of enzyme is a measure to catalyze a reaction and stability of the enzyme is judged by its residual activity. Both of these properties are modified to a large extend by temperature, pH and modifiers such as activators, inhibitors etc. The examination of relationships between enzyme properties and environmental conditions plays a critical role to predict, manipulate and engineer the protein structure and function.

1.7. Objectives and scope

Based on an extensive literature survey on the production of recombinant L-asparaginase, the present study focused on bioprocess development for the production of glutaminase-free

recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3). In order to achieve the objective, a suitable bioprocess was developed from shake flask to bioreactor level and purified recombinant enzymes of both the strains, and characterized. The following objectives have been envisaged in the present investigation:

- Selection of glutaminase-free L-asparaginase producing strains.
- Subcellular localization of L-asparaginase.
- Cloning and expression of L-asparaginase encoding genes of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3).
- Development of medium to maximize the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3).
- Optimization of physical process parameters for enhanced production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3).
- Production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* BL21 (DE3) in a batch and fed- batch bioreactor.
- Purification and characterization of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* BL21 (DE3).

1.8. Organization of the thesis

In this thesis, the presentation of the work has been done in five chapters. The current **Chapter 1** presents a general introduction, objective and scope of the work. The literature that supports the present work is presented in **Chapter 2. Chapter 3** includes the details of the materials and methods adopted in the current study. Details of subcellular localization in wild strains, cloning and expression of recombinant L-asparaginase of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* and optimization of process parameters in shake flask and bioreactors, purification and characterization of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* are presented in this chapter. It also provides technical information about the analytical methods adopted in this work. **Chapter 4** comprises the results and discussions, where the results of bioprocess development of recombinant L-asparaginase production in shake flask and bioreactor are presented and thoroughly discussed. This chapter addresses subcellular localization in wild strains, cloning, expression, purification and characterization, and production of glutaminase-free recombinant L-asparaginase II in shake flask and bioreactor. **Chapter 5** draws summary and appropriate conclusion based on the previous results and discussions. This chapter also provides some useful recommendations to carry out further work in this field.

CHAPTER 2
REVIEW OF LITERATURE

2.1. L-asparaginase

L-asparaginase (L-asparaginase amidohydrolase EC 3.5.1.1) hydrolyzes L-asparagine to L-aspartic acid and ammonia. It has been used in leukemia treatment from last four decades. It is widely used for treatment of Hodgkin disease, acute lymphocytic leukemia (mainly in children), acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma (Cunningham *et al.,* 1979; Capizzi *et al.,* 1984; Klumper *et al.,* 1995; Larson *et al.,* 1995; Mitchell *et al.,* 1994; Ravindranath *et al.,* 1992). Recently, L-asparaginase have been used for the production of acrylamide free food (Pedreschi *et al.,* 2008), new drug delivery development system (Teodor *et al.,* 2009) and L-asparaginase based biosensor (Verma *et al.,* 2007).

The antileukemic effect of L-asparaginase is postulated to result from the rapid and complete depletion of the circulating pool of L-asparagine as most of the cancer cells are dependent on an exogenous source of this amino acid for survival. However, normal cells are able to synthesize L-asparagine and less affected by its rapid depletion due to treatment with this enzyme. The asparagine deficiency rapidly impairs the protein synthesis and leads to delayed inhibition in DNA and RNA synthesis and due to an impairment of cellular function, resulting in cell death (Muller and Boos, 1998; Narta *et al.,* 2007). Schematic diagram of reaction mechanism of L-asparaginase to hydrolyze the L-asparagine into L-aspartic acid and ammonia is given in Fig. 2.1.

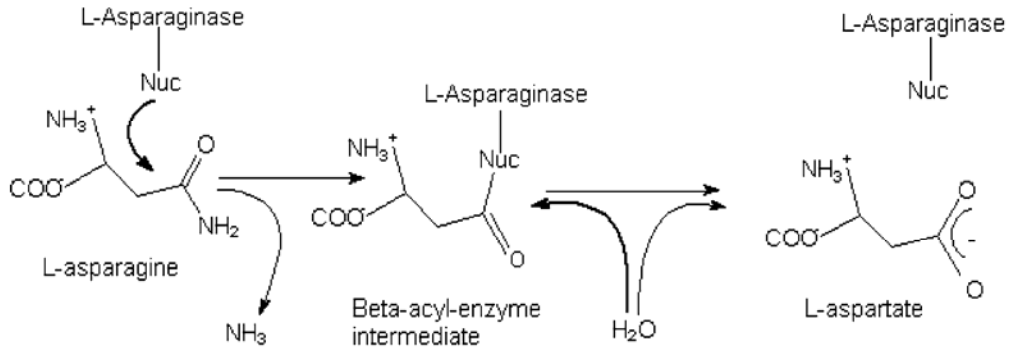

Fig. 2.1. Schematic illustration of the reaction mechanism of L-asparaginase. The proposed covalent intermediate is formed through nucleophilic attack by the enzyme. Bold arrows indicate nucleophilic attack (Sanson and Jaskolski, 2004).

L-asparaginase is produced from a variety of biological sources *viz*., plants, animals and microorganisms (fungus, yeast, bacteria etc.) (Adamson *et al.,* 1968; Broome, 1961). Although L-asparaginase is produced by many microorganisms (Verma *et al.,* 2007), L-asparaginase from *E. chrysanthemi* and *E. coli* are currently in clinical use as effective drugs for the leukemia diseases (Kozak *et al.,* 2000). Unfortunately, despite the wide use of L-asparaginase, most of the treatments have been interrupted due to severe side effects and immunological reactions in the patients (Narta *et al.,* 2007). The allergic response is due to long-term use of a foreign protein, and this can be circumvented by using serologically unrelated L-asparaginase. With a view to characterize enzymes with less toxic side effects, several members of a larger family of homologous L-asparaginases have been thoroughly studied over many years (Ehrman *et al.,* 1971; Krasotkina *et al.,* 2004; Kotzia and Labrou 2005). L-asparaginases with high specificity towards L-asparagine and low-to-negligible activity towards L-glutamine are reported to be less troublesome during the course of anti-cancer therapy (Hawkins *et al.,* 2004; Gallagher *et al.,* 1989; Manna *et al.,* 1995). Therefore, for successful clinical studies, glutaminase free L-asparaginase isolated from a novel source will be more advantageous.

2.2. Historical development

Initially, Clementi (1922) have observed the potential antineoplastic property of L-asparaginase that isolated from the serum of guinea pig. High L-asparaginase activity was observed only in guinea pig serum, whereas other mammals were found devoid of this enzyme. Kidd (1953a, 1953b) described the regression of transplanted lymphomas in mice and rats by the administration of guinea pig serum. This cytotoxic activity was not present in horse or rabbit serum. Further studies by Neumann and McCoy, (1956) proved that asparagine is required to grow the Walker carcinosarcoma 256 *in vitro*. Haley and co-workers, (1961) found that the murine L5178Y leukemia cells also require asparagine for *in vitro* growth. Looking at the biochemical processes involved in these observations, it was concluded that leukemic blast cells are sometimes unable to synthesize enough asparagine for their own metabolism, so that the asparaginase-induced deficiency in asparagine will impair cellular function and eventually cause cellular death. Broome (1963a, 1963b) confirmed that the tumor regression is due to hydrolysis of asparagine to aspartic acid and ammonia by L-asparaginase.

The first clinical trials in patients with acute lymphoblastic leukemia were carried out using L-asparaginase preparations from guinea pig serum and *E. coli*. Both these enzymes showed clinical efficacy (Boyse *et al.,* 1967; Brome *et al*, 1967; Campbell and Mashburn, 1969; Roberts *et al.,* 1966). Yellin and Wriston, (1966a) succeeded in partial purification of two isoforms of L-asparaginase from the serum of guinea pig and it was observed that only one isoform exhibited anti lymphoma activity *in vivo* (Yellin and Wriston, 1966b).

Systematic studies on identification of other potential sources of L-asparaginase were performed in phytopathogenic microorganisms that hydrolyse asparagine for energy production. These studies were identified some bacterial species other than *E. coli*, which also showed relevant asparaginase activity. Among the species studied, *E. chrysanthemi* showed the highest asparaginase activity (Ohnuma *et al.,* 1972). These findings provided a practical base for large-scale production of enzyme for pre-clinical and clinical studies (Boyse *et al.,* 1967; Roberts *et al.,* 1966; Whelan and Wriston, 1969). Initially, Oettgen *et al.,* (1967) confirmed the efficacy of L-asparaginase in humans suffering from leukemia. Old *et al.,* (1967) were demonstrated *in vivo* antitumor activity in a dog model with spontaneous lymphosarcoma. While other microorganisms such as *Serratia marcescens* and *Vibrio succinogenes* (later classified to the species *Wolinella)* also possess L- asparaginases that act on lymphomas (Distasio *et al.,* 1982). However, only native asparaginases from various *E. coli* and from *E. chrysanthemi* were used for clinical use. Hypersensitivity reactions of L-asparaginase from *E. coli* were recognized. An L-asparaginase from *E. carotovora* was found to lack cross reactivity with *E. coli* enzyme (Ohnuma *et al.,* 1972). Both these enzymes have high rates of immunogenicity. Conjugation to poly ethylene glycol (PEG) was observed as a process by which the activity of the drug could be preserved while reducing its potential immunogenicity. This modification was shown in animal models to reduce antibody formation compared to native L-asparaginase and to markedly extend the duration of action of the drug (Ashihara *et al.,* 1978; Park *et al.,* 1981). Peg-aspargase have not administrated frequently as compared to native L-asparaginases. Recently, the use of L-asparaginase in leukemia and lymphoma treatment has been explored. While earlier use focused on its use in remission induction for patients with acute lymphoblastic leukemia

(Haskell *et al.,* 1969; Jones *et al.,* 1977; Oettgen *et al.,* 1967; Ortega *et al.,* 1977). More recent studies have explored the administration of L-asparaginase for 20–30 weeks as consolidation treatment for lymphoid malignancies (Amylon *et al.,* 1999; Clavell *et al.,* 1980). Till 2000, most of the production studies from various microorganisms were performed in shake flasks, and emphasis was given on *in vitro* and clinical studies for novel source of microorganisms for maximizing the production of L-asparaginase. Last few years, many researchers worked on improvement of L-asparaginase production from a variety of microorganisms. However, literature of various process parameters still lacks of knowledge, which influence the L-asparaginase fermentation.

2.3. Subcellular distribution of L-asparaginase

First time, Yellin and Wriston, (1966a) reported the two isoforms of L-asparaginase from the serum of guinea pig. There are many reports for the existence of periplasmic, cytoplasmic and membrane bound L-asparaginase (Geckil *et al.,* 2004; Geckil *et al.,* 2005; Kumar *et al.,* 2010; Mukherjee *et al.,* 2000; Schwartz *et al.,* 1966; Triantafillou *et al.,* 1988). Many gram-negative bacteria contain two L-asparaginases, a high-affinity periplasmic enzyme and a low-affinity cytoplasmic enzyme. Campbell and Mashburn, (1969) have improved the production of asparaginase and confirmed the two asparaginases (EC-I and EC-II) in *E. coli*. In *E. coli* and many other bacteria, synthesis of the cytoplasmic asparaginase I is constitutive, while expression of the periplasmic asparaginase II is activated during anaerobiosis. It has been suggested that the asparaginase II probably has a special function in anaerobic fumarate respiration by providing aspartate, which is then converted to fumarate. Furthermore, only the type II enzyme has substantial antitumor activity (Fisher and Wray, 2002). Ireland and Joy, (1983) have reported that the subcellular distribution of L-asparaginase in pea's leaves by

sucrose density gradient. Based on the alignments of asparaginases available in databases, biochemical and crystallographic data, the known asparaginase sequences can be divided into three families' *viz*., bacterial, plant and the enzymes similar to *Rhizobium etli* asparaginase (Fig. 2.2).

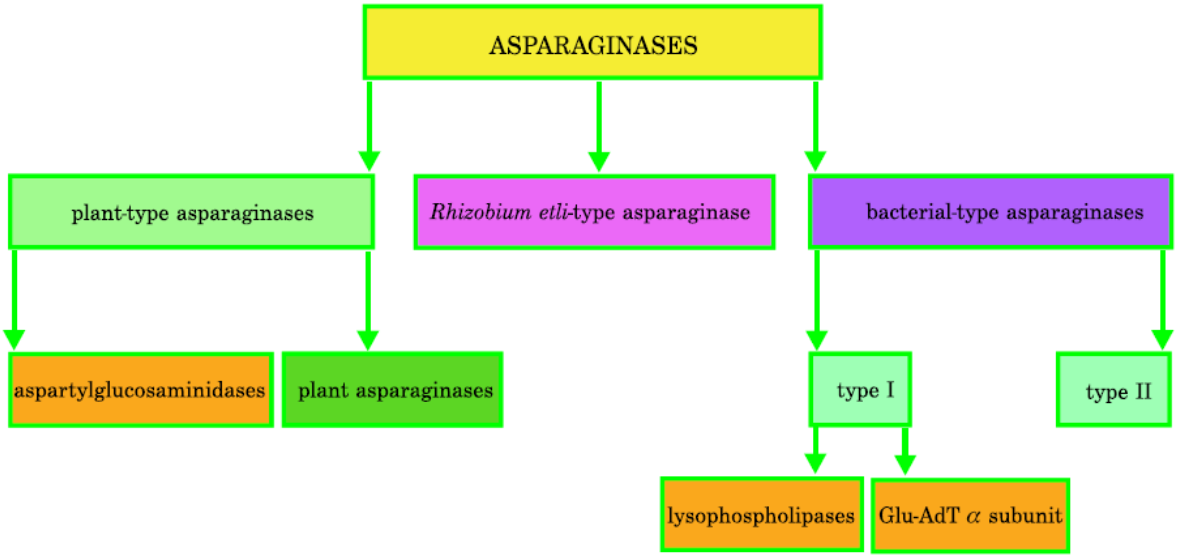

Fig. 2.2. General classification of asparaginases (Borek and Jaskólski, 2001).

2.4. Sources of L-asparaginase

A wide range of microorganisms such as bacteria, fungi, yeast, Actinomycetes and plants have proven to be proficient sources of this enzyme.

2.4.1. Bacterial sources

Bacterial asparaginases have shown considerable medical significance and are being employed in the therapy of acute lymphoblastic leukemia. L-asparaginase from *E. carotovora* is serologically and biochemically distinct from the L-asparaginase of *E. coli*, though its antineoplastic activity and toxicity is comparable. L-asparaginase production has been reported from *E. coli* (Cedar And Schwartz 1968), *Pseudomonas*

flourescens AG (Mardashev *et al.,* 1975), *Mycobacterium phlei* (Pastuszak *et al.,* (1976), *Staphylococci* (Mickucki *et al.,* (1977), *Tetrahymena pyriformi* (Triantafillou *et al.,* 1988) and *Thermus aquaticus* (Curran *et al.,* 1985). Production of L-asparaginase by marine luminous bacteria had been reported by Ramaiah *et al.,* (1992). Mohapatra *et al.,* (1995) have characterized the partially purified L-asparaginase of a *Bacillus* strain isolated from the intertidal marine alga, *Surgassuni*. Nawaz *et al.,* (1998) have isolated and characterized L-asparaginase from *Enterobacter cloacae* that have utilized L-asparagine as a sole carbon source. Glutaminase-free L-asparaginase from *Thermus thermophilus* and from a new *Erwinia sp.* was reported by Pritsa and Kyriakidis (2001) and by Borkotaky and Bezbaruah (2002), respectively. New L-asparaginase from *Pseudomonas aeruginosa* 50071 was reported by El-Bessoumy *et al.,* (2004) that is serologically different from the previously reported L-asparaginases. Neto *et al.,* (2006) have optimized the culture conditions for the production of L-asparaginase from *Zymomonas mobilis* CP4. Production of L-asparaginase from *Staphylococcus* sp. – 6A and *Bacillus circulans* MTCC 8574 has been reported by Prakasham *et al.,* (2007) and Hymavathi *et al.,* (2009), respectively. A novel glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428 was reported by Kumar *et al.,* (2009). Recently, Moharam *et al.,* (2010) and Sunitha *et al.,* (2010) have reported the production of L-asparaginase from *Bacillus* sp R36 and *Bacillus cereus* MNTG-7, respectively. The summarized sources of bacterial L-asparaginase are presented in the Table 2.1. The dendrogram for the bacterial-type asparaginases (Fig. 2.3) is in good agreement with the tree of life (Woese, 2000) and is clearly divided into two main parts. One of them comprises mainly sequences from archaea and eukaryota, while the other includes bacterial asparaginases.

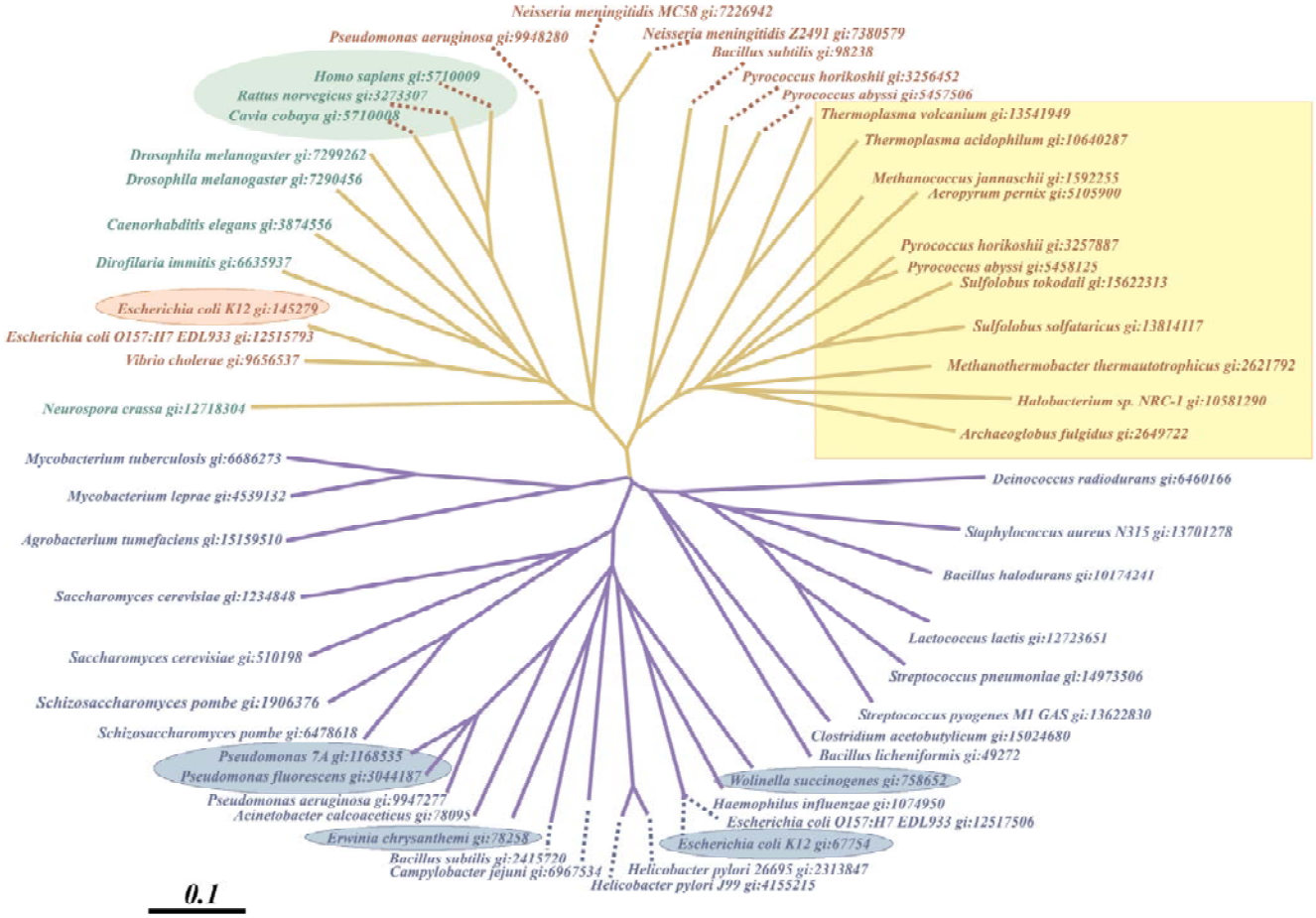

Fig. 2.3. A dendrogram of bacterial asparaginases. (Borek and Jaskólski, 2001). *Orange branch:* type I bacterial asparaginases, *blue branch:* type II bacterial asparaginases. Lettering colors: *blue:* sequences with high homology to EcA II, *brown:* sequences with high homology to EcAI, *green:* sequences with high homology to lysophospholipases. *Yellow box:* subunit α of Glu-AdT amidotransferases, *green oval:* lysophospholipases, *pink oval*: EcAI, reported to be active in dimeric form (Jerlstrom *et al.*, 1989), *blue ovals:* tetrameric bacterial type II asparaginases with antitumor activity. *gi* is a unique sequence identifier assigned by the NCBI (Ortuno-Olea and Duran-Vargas, 2000). The scale bar represents 10% divergence.

Table 2.1. Sources of L-asparaginases from various bacteria

Organism	Mol. weight kDa	L-asparaginase production	References
Pseudomonas aeruginosa	138	11.18 U mg^{-1}	Abdel-Fattah and Olama, (2002)
Escherichia coli	140	27.5 U ml^{-1}	Barnes *et al*., (1977)
Serratia marcescens	180	0.7 U mg^{-1}	Boyd and Phillips, (1971); Stern *et al.,* (1976)
Thermus aquaticus.	80	30.35 U ml^{-1}	Curran *et al.,* (1985)
Pseudomonas acidovorans	156	0.039 U mg^{-1}	Davidson *et al.,* (1977)
Azotobacter vinelandii	84	4 U mg^{-1}	Gaffar and Shethna, (1977)
Helicobacter pylori	30	5.1 U mg^{-1}	Gladilina *et al*., (2009)
Bacillus subtilis	--	0.11 U ml^{-1}	Gulati *et al.,* (1997)
Baciliius lichiniformis	--	0.15 U ml^{-1}	
Bacillus circulans	140	2322 U gds^{-1} (SSF)	Hymavathi *et al.,* (2009)
Escherichia coli ATCC 11303	--	1.03 Uml^{-1}	Kenari *et al*., (2011)
P. carotovorum MTCC 1428	144.42	27.99 U mg^{-1}	Kumar *et al*., (2009)
Pseudomonas stutzeri	34	1.1 U mg^{-1}	Manna *et al.,* (1995)
Enterobacter aerogenes	--	1.20 U ml^{-1}	Mukherjee *et al.,* (2000)
Enterobacter cloacae	--	35.0 U mg^{-1}	Nawaz *et al.,* (1998)
Zymomonas mobilis	--	0.17 U ml^{-1}	Neto *et al.,* (2006)
Bacillus cereus	--	56.5 U ml^{-1}(SSF)	Sunitha *et al.,* (2010)
Bacillus cereus MAB5	--	51.54 U ml^{-1}	Thenmozhi *et al.,* (2011)
Tetrahymena pyriformis	200	0.54 U ml^{-1}	Triantafillou *et al.,* (1988)
Staphylococcus sp.	--	55.6 U ml^{-1} (SSF)	Prakasham *et al.,* (2007)
Thermus thermophilus	200	0.09 U mg^{-1}	Pritsa and Kyriakidis, (2001)
Mycobacterium phlei	126	32.6 U mg^{-1}	Pastuszak *et al.,* (1976)

Organism	Mol. weight kDa	L-asparaginase production	References
Erwinia aroideae	--	1.1 U mg^{-1}	Peterson and Ciegler, (1969a)
Pseudomonas flourescens	--	140 U g^{-1} of dry cells	Peterson and Ciegler, (1969b)
Pseudomonas putida	--	100 U g^{-1} of dry cells	Peterson and Ciegler, (1969b)
Xanthomonas campestris	--	50 U g^{-1} of dry cells	
Erwinia carotovora	150	1.36 U mg^{-1}	Warangkar and Khobragade, (2010)

SSF- solid state fermentation; gds – gram per dry substrate; submerged fermentation production was mentioned in U ml^{-1} or U mg^{-1} or U g^{-1} of dry cells

2.4.2. Fungi and yeast sources

Many reports are available on the production of L-asparaginase from yeast and filamentous fungi (Pinheiro *et al.,* 2001; Wade *et al.,* 1971, Wiame *et al*., 1985). The mitosporic fungi genera such as *Aspergillus*, *Penicillium* and *Fusarium* are commonly used for the production of asparaginase (Angeli *et al.,* 1970; Arima *et al.,* 1972; Curran *et al.,* 1985; Imada *et al.,* 1973; Nakahama *et al.,* 1973). Dunlop and Roon, (1975) observed that the extracellular production of L-asparaginase from *Saccharomyces cerevisiae*. The production of a new asparaginase from a *Rhodotorula* sp. (red imperfect yeast) was reported by Foda *et al.,* (1980). A homodimer of L-asparaginase from *Rhodotorula toruloides* has been found by Ramakrishnan *et al.,* (1996). Mishra, (2006) and Sarquis *et al.,* (2004) reported that the L-asparaginase production by *Aspergiluus niger* and *A. terreus,* respectively. The summarized sources of fungi and yeast L-asparaginase are given in Table 2.2.

Table 2.2. Sources of L-asparaginase from fungi and yeast

Organism	Molecular weight kDa	L-asparaginase production	References
Saccharomyces cerevisiae	--	0.125 U mg^{-1}	Dunlop and Roon, (1975)
Rhodosporidium toruloides	--	4 U ml^{-1}	Foda *et al.,* (1980)
Aspergillus niger	--	40.9 U gds^{-1} (SSF)	Mishra, (2006)
Fusarium oxysporum		8.14 U ml^{-1} (SSF)	Pallem *et al.,* (2011)
Aspergillus terrus	--	0.058 U ml^{-1}	Sarquis *et al.,* (2004)
Aspergillus oryzae	--	0.038 U ml^{-1}	
Fusarium tricinctum	161	5 U mg^{-1}	Scheetz *et al.,* (1971)

SSF- solid state fermentation; gds – gram per dry substrate; submerged fermentation production was mentioned either U ml-1 and U mg -1

2.4.3. Actinomycetes sources

L-asparaginases were also reported in *Streptomyces plicatus* (Balakrish *et al.,* 1977; Maya 1992; Mathew 1995; Selvakumar, 1979). Gunasekaran *et al.,* (1995) reported that the production of L-asparaginase by *Nocardia sp*. The production of intracellular and extracellular asparaginase from *S. longsporusflavus* (F-15) has been reported (Abdel-Fatah and Olama, 1998). *Streptomyces sp.* isolated from the gut of fish, *Therampon jarbua* and *Villorita cyprinoids* have shown L-asparaginase activity (Dhevendaran and Anithakumari, 2002). Dhevagi and Poorani, (2006) have studied production and characterization of L-asparaginase from marine actinomycetes and assessment of its antitumor activity using animal cell lines. Sahu *et al.,* (2007) studied the L-asparaginase activity of actinomycetes associated with different parts of three species of estuarine fish *viz*., *Mugil cephalus*, *Chanos chanos* and *Etroplus suratensis* under various experimental culture conditions and identified the potential L-asparaginase producing actinomycetes through chemotaxonomical and conventional methods of identification. Among the actinomycetes, several *Streptomyces* species such as *S. karnatakensis*,

S. venezualae, *S. longsporusflavus* and a marine *Streptomyces* sp. PDK2 have been explored for L-asparaginase production (Narayana *et al.,* 2008). Amena *et al.,* (2009) reported that the production of an extra-cellular L-asparaginase by a novel isolate, *S. gulbargensis* under submerged culture using groundnut cake extract. The summarized sources of L-asparaginase from actinomycetes are presented in Table 2.3.

Table 2.3. Sources of L-asparaginase from actinomycetes

Organism	Molecular Weight (kDa)	L-asparaginase production	References
Streptomyces longsporusflavus	--	22.0 U mg^{-1}	Abdel-Fatah and Olama, (1998)
Streptomyces sp.	--	384.0 U mg^{-1}	Basha *et al.,* (2009)
Streptomyces noursei MTCC 10469.	102	0.803 U mg^{-1}	Dharmaraj (2011)
Streptomyces sp.	140	0.8 U mg	Dhevagi and Poorani (2006)
Nocardia sp.	--	0.5 U ml^{-1}	Gunasekaran *et al.,* (1995)
Streptomyces albidoflavus	112	25.2 U mg^{-1}	Narayana *et al.,* (2008)
Streptomyces griseus ATCC 10137	--	0.1 U ml^{-1}	Peter (1972)
Streptomyces sp. (TA22)	--	8.87 U mg^{-1}	Priya *et al*., (2011)

2.4.4. Plant sources

Heeshen *et al.,* (1996) have observed the presence of two isoforms of L-asparaginase from *Sphagnum fallax*. Among plants, *Lupin araboreus* and *Lupin angustiplius* have produced L-asparaginase (Borek *et al.,* 1999). Borek *et al.,* (2004) reported that the expression, purification and catalytic activity of *Lupinus luteus* asparagine beta-amidohydrolase and its homologue in *E. coli*. L-asparaginase activity has also been found in roots of *Pinus pinaster* and *Pinus radiata* due to ectomycorrhizal fungi in the wheat belt of Western Australia (Bell

and Adams, (2004). A K^+ dependent L-asparaginase from *Arabidopsis*, At3g16150, has been characterized by Bruneau *et al.,* (2006). Recently, anticancer properties of highly purified L-asparaginase from *Withania somnifera* L. was reported (Oza *et al.,* 2010). The summarized sources of plant L-asparaginase are listed in Table 2.4.

Table 2.4. Sources of L-asparaginase from plants

Organism	L-asparaginase production	References
Capsicum annum L. (Green chilies)	9.43 U gm^{-1} of fresh tissue	Bano and Sivaramakrishnan, (1983)
Tamarindus indica (Tamarind)	4.57 U gm^{-1} of fresh tissue	
Amorphophallus (Yam)	4.57 U gm^{-1} of fresh tissue	
Lvcopersicum esculentus (Tomato)	3.72 U gm^{-1} of fresh tissue	
Raphanus sativus (Radish)	3.43 U gm^{-1} of fresh tissue	
Solanum melongna(Brinjal)	3.14 U gm^{-1} of fresh tissue	
Cucurbita maxima (Cucurbita)	2.86 U gm^{-1} of fresh tissue	
Hibiscus esculentus (Ladies finger)	1.71 U gm^{-1} of fresh tissue	
Mangifera indica (Mango)	1.14 U gm^{-1} of fresh tissue	
Lupinus luteus (Lupin)	57 U ml^{-1} of protein	Borek *et al.,* (2004)
Vigna unguiculata	104.50 U mg^{-1} of protein	Ehab (2009)
Pisum sativum (Pea leaves)	100 U ml^{-1} of supernatant	Ireland and Joy, (1983)
Withania somnifera (Ashavgandh)	117 U mg^{-1} of protein	Oza *et al.,* (2010)

The dendrogram for plant-type asparaginases (Fig. 2.4) is clearly divided into four branches (Borek and Jaskolski, 2001). The aspartylglucosaminidase sequences form one of the branches and the enzymes from plants and their close homologs form another branch. The product of *E. coli ybiK* gene is also placed on this branch supporting the hypothesis based on biochemical data that it is very close to plant asparaginases.

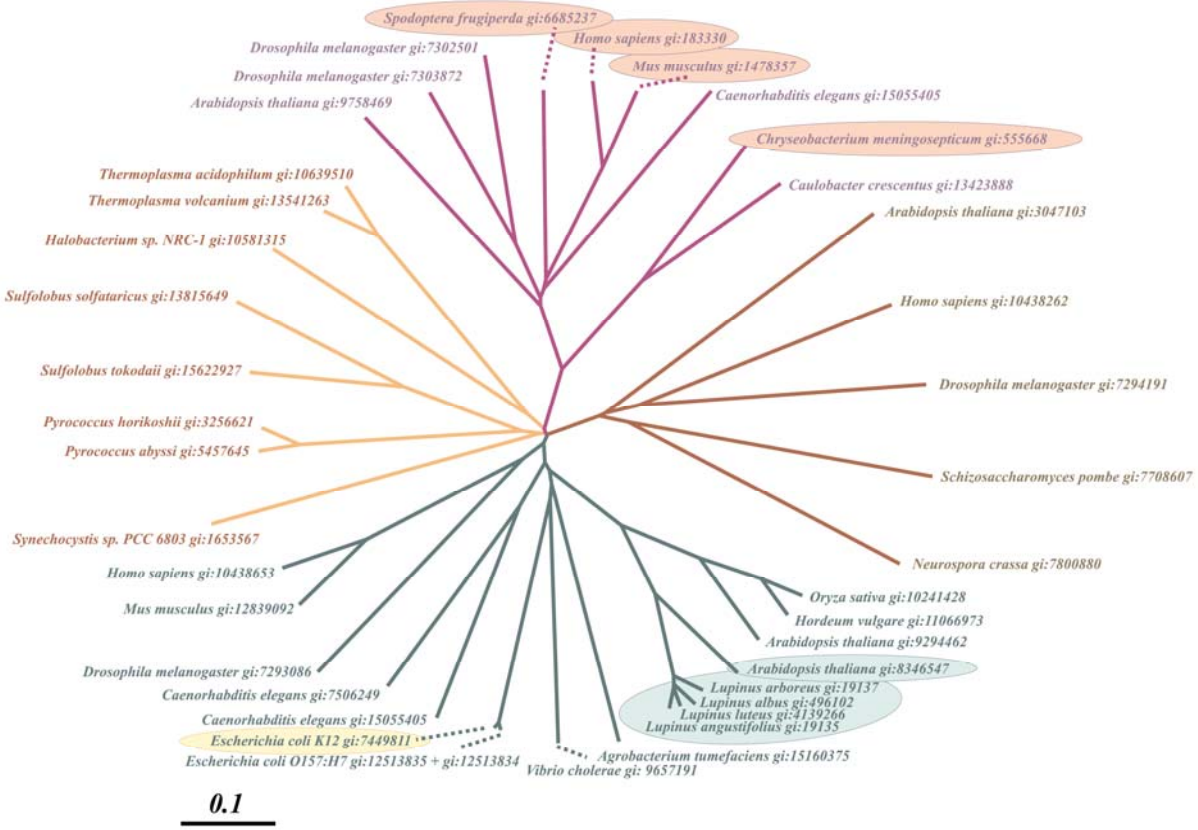

Fig. 2.4. A dendrogram of plant asparaginases (Borek and Jaskolski, 2001). *Violet branch*: aspartylglucosaminidases. *Orange branch*: predominantly archaeal plant-type asparaginases. *Brown branch:* eukaryotic sequences with homology to plant asparaginases but of unknown biochemical characteristics. *Green branch:* biochemically characterized (*green ovals*) asparaginases from plants and their homologs in other organisms. *Pink ovals:* biochemically characterized aspartylglucosaminidases, *yellow oval:* the product of the *E. coli* ybiK gene with asparaginase activity. The scale bar represents 10% divergence.

2.5. Production of L-asparaginase from various microbial sources

Bilimoria (1969) has reported that the production of L-asparaginase II caliform bacteria. Boeck *et al.,* (1970) evaluated the cultural and nutritional requirements for the production of L-asparaginase from *E. coli* K12 and concluded that the conventional aerobic fermentation yielded higher biomass with lower amounts of L-asparaginase. Enzyme synthesis was not induced by terminating aeration-agitation in the absence or presence of glucose. But 0.05% of

L-asparagine was favorable for biosynthesis of L-asparaginase and maximum enzyme production was obtained at pH of 7.5. The production of enzyme was inhibited by the addition of sugars. Minimum inhibition was observed when galactose was used as carbon source, whereas glucose was found to be the most inhibitory carbon source (Cedar, 1967). The synthesis of L-asparaginase from *E. coli*-W and *E. coli* K-12 was suppressed in the presence of glucose due to catabolite repression and catabolite inhibition of the components involved in lactate transport. Lactate was found to be stimulator for L-asparaginase synthesis (Garaev and Golub, 1977). Barnes *et al.*, (1977) have studied the effect of culture conditions on the synthesis of L-asparaginase from *E. coli* A-1 and the production of L-asparaginase was increased by the addition of L-glutamic acid, L-glutamine and mono sodium glutamate. However, the decrease in production was observed in the presence of glucose and chloramphenicol at a pH less than 7.5. Organic acids and amino acids (L-leucine and L-methionine) were observed to enhance the production of L-asparaginase in *E. coli* (Netrval *et al.,* 1977). Heinemann *et al.* (1969 and 1970) have observed the production of L-asparaginase from *S. marcescens* ATCC 60 at a pH range of 8.0-8.6 and level of dissolved oxygen in the culture medium is near to zero. In case of *E. aroideae* NRRL B 138, the production increased at 350 rpm, 28 ^{o}C in TGY medium containing yeast extract (Peterson *et al.,* 1972). Liu *et al*., (1973) used 1% lactose as carbon source, and 1.5 % yeast extract for L-asparaginase production at 28 ^{o}C. Effect of oxygen absorption rates on L-asparaginase II production by *E. coli* 3003C cells was studied by Zubanovic and Johanides, (1975) and it was observed that a definite O_2 absorption rate of 0.4 mm l^{-1} min^{-1} resulted in good growth and maximum enzyme production. In *B. mesentericus* 43A, the biosynthesis of L-asparaginase was inhibited by the addition of L-aspartic acid to the growing cultures (Eremenko *et al.,* 1976). L-asparaginase production was

reported from *Pseudomonas ovalis* (Badr-El-Din and Foda, 1976). There are two forms of asparaginase, L-asparaginase I and L-asparaginase II were observed in *S. cerevisiae*. The two enzymes are genetically and biochemically different. The interactions between these enzymes were studied by Jones, (1977). L-asparaginase I was found to be constitutive, whereas L-asparaginase II was induced in response to nitrogen starvation (Dunlop *et al.,* 1978). In case of *V. succigenes,* L-asparaginase synthesis occurs through out the exponential phase and in early stationary phase. The synthesis of L-asparaginase was induced by ammonium ion. Higher cell growth and enzyme production were observed in the presence of an ammonium formate-sodium fumarate medium (Albanese, 1978). In the presence of nitrogen, high L-asparaginase activity was observed in bacterial cultures (Paul and Cooksey, 1981). A novel L-asparaginase has been characterized by the red imperfect yeast, *Rhodotorula rubra* in the media supplemented with L-asparagine, L-glutamine or L-alanine as carbon and nitrogen sources (Foda *et al*., 1980). The enzyme production was highest during the exponential phase of growth. In *Lupin arboreus*, root-tips, flower buds and developing seeds have been found to be the sources of L-asparaginase (Chang and Farnden, 1981). L-asparaginase of *Aspergillus nidulans* showed the evidence of O_2 repression under N_2 metabolite depressed conditions (Shaffer *et al.,* 1988). The use of keiselguhr composite for large-scale production of asparaginase from *E. chrysanthemi* were described by Goward *et al.,* (1989). The cell growth and L-asparaginase production were studied by Alegre and Minim, (1992) in batch fermentation from *E. aroidae*. They observed that yeast extract plays a vital role for biomass and L-asparaginase synthesis. Strains of luminous bacteria (43 strains) belonging to four species (*Vibrio hawveyi, V. fisheri, Photobacterium leiognathi* and *P. phoshoreum*) isolated from different marine samples were examined for L-asparaginase production. The production of L-asparaginase from luminous

prokaryotes was found to be higher than the reported bacterial sources (Ramaiah and Chandramohan, 1992). Regulation mechanism of production of L-asparaginase from *Staphylococcal* showed that the carbon sources such as sucrose, maltose, galactose, lactose, mannitol and mannose inhibited the production, while exogenous c-AMP improved L-asparaginase production (Rozalska and Mickucki, 1992). The supplementation of cheese whey with tryptone (0.3%) and asparagine (0.5%) improved the production of L-asparaginase from *E. aroideae* NRRL B 138 in pilot scale (Alegre and Minim, 1993). *Nocardia asterodies*, an aerobic actinomycete, was grown on three different media, *viz*., sabourand dextrose broth (SD), tryptic soy broth and synthetic medium in shake flask culture at 37 °C. The production of L-asparaginase was found to be maximum in the SD broth (Gunasekaran *et al.,* 1995). A pH and dye based fast procedure for screening of L-asparaginase producing microorganisms have been reported (Gulati *et al.,* 1997). In the presence of 6% of n-dodecane, the biomass and production of L-asparaginase were increased by 12.7% and 21%, respectively from *E. coli* (Wei and Liu, 1998). An intracellularly expressed L-asparaginase was observed from *Enterobacter cloacae* by Nawaz *et al.,* (1998). These gram negative and rod-shaped bacteria utilized L-asparagine as the sole source of carbon and nitrogen. High L-asparaginase activity was found when cells cultured on L-fructose, D-galactose, sucrose or maltose and L-asparagine. The optimal pH and temperature for L-asparaginase production was found to be 8.5 and 37–42°C, respectively. Production of L-asparaginase from a new *Erwinia sp*. has reported by Borkotaky and Bezbaruah, (2002). The maximum production was observed at 40 ^{o}C and pH of 8.5. Among the various carbon sources, mannitol was found to be the best carbon source for production of L-asparaginase. The combination of L-arginine, L-glutamine and L-asparagine synergistically stimulated the L-asparaginase up to 5.8 fold. At a pH of 7.9, casein hydrolysate

(3.11%) and corn-steep liquor (3.68%) were the most significant factors for improving the enzyme production process. Optimization of solid state fermentation for the production of L-asparaginase by *P. aeruginosa* 50071 has been reported by Abdel-Fattah and Olama, (2002). Furthermore, production and expression of *Pseudomonas* proteins and growth of *Pseudomonas* by utilizing asparagine has been reported (Sonawane *et al.,* 2003). The production of L-asparaginase from filamentous fungi (*Aspergillus tamari* and *A. terreus*) under nitrogen regulation was reported by Sarquis *et al.,* (2004). *A. tamari* have shown the highest L-asparaginase production in 2% proline medium, while the lowest L-asparaginase production levels in both the fungi were found with glutamine and urea as nitrogen sources. L-asparaginase production in *Erwinia aerogenes* expressing *Vitreoscilla* hemoglobin for efficient oxygen uptake has been described by Geckil and Gencer, (2004). Geckil *et al.,* (2006) have performed the experiments to understand the effect of different carbon sources (glucose, lactose, mannitol and glycerol) and *Vitreoscilla* hemoglobin (VHb) on the production of L-asparaginase from *P. aeruginosa* and its VHb-expressing recombinant strain (PaJC). Glucose has shown repression effect on the production of L-asparaginase by the host strain as compared to without carbohydrate in the medium. The production of high levels of L-asparaginase from a new isolate of *Aspergillus niger* in solid state fermentation (SSF), using agro-wastes from three leguminous crops (bran of *Cajanus cajan*, *Phaseolus mungo*, and *Glycine max*) have been reported by Mishra, (2006). Prakasham *et al.,* (2007) evaluated the interactions and effect of nutritional (carbon and nitrogen sources), physiological (incubation temperature, medium pH, aeration and agitation) and microbial (inoculums level) parameters on the production of L-asparaginase by *Staphylococcus* sp.–6A. Among the variables, the incubation temperature, inoculum level and medium pH were influencing significantly at individual level and

contributed to more than 60% of total L-asparaginase production. The maximum production of L-asparaginase by *Streptomyces albidoflavus* under submerged fermentations was observed in the medium containing maltose (1%) and yeast extract (2%) (Narayana *et al.,* 2008). The maximum production was reported to be 5.39 U g^{-1} dry cell weights. Hymavathi *et al.,* (2009) optimized a wide range of SSF parameters to maximize the production of L-asparaginase from *Bacillus circulans* (MTCC 8574) using agricultural waste as carbon source. Kumar *et al*., (2009) have optimized the medium components and physical process parameters for higher production of L-asparaginase from *P. carotovorum* MTCC 1428. Sunitha *et al.,* (2010) have studied the effect of various carbon sources, organic and inorganic nitrogen sources, amino acids and mineral salts on L-asparaginase production by *Bacillus cereus* MNTG-7 by Plackett-Burman experimental Design. Among the 67 nutrients, tapioca starch, L-asparagine, ammonium sulphate, gelatin and $CaCO_3$ were found to be most influencing parameters on the L-asparaginase production. Dharmaraj, (2011) have studied the L-asparaginase production by *Streptomyces noursei* MTCC 10469, isolated from marine sponge, *Callyspongia diffusa*. L-asparaginase production from *E. coli* ATCC 11303 was optimized using response surface methodology (Kenari *et al*., 2010). Pallem *et al*., (2011) have studied the production of L-asparaginase through solid state fermentation using *Fusarium oxysporum.* Priya *et al*., (2011) have optimized the production of L–asparaginase from *Streptomyces* sp (TA22) isolated from Western Ghats region.

Recently, recombinant L-asparaginase has been developed to increase the level of expression of L-asparaginase, besides the novel source of L-asparaginase production. Recombinant L-asparaginase was developed by cloning of gene encoding L-asparaginase from *E. carotovora* NCYC 1526 and expressing in *E. coli* (Kotzia and Labrou, 2005). Expression of recombinant

L-asparaginase fused to pub leader sequence under the inducible T7 lac promoter in BLR (DE) resulted in optimum extracellular production in shake flasks (Khushoo *et al.,* 2005). L-asparaginase from *E. chrysanthemi* 3937 has been expressed in *E. coli* BL21 (DE3) pLysS (Kotzia and Labrou, 2007). Cloning and expression of asparaginase of the *S. cerevisiae* ASP3 gene in *Pichia pastoris* and the production of asparaginase by the recombinant strain in shake flasks and in a 2 l bioreactor were studied by Ferrara *et al.,* (2006). Cappelletti *et al.,* (2008) have investigated the pathological role and therapeutic potential of L-asparaginase II of *Helicobacter pylori* CCUG 17874 expressed in *E. coli* BL21 (DE3). Gladilina *et al.,* (2009) have cloned *H. pylori* asparaginase, which is characterized by extremely low glutaminase activity. Ferrara *et al*., (2010) have produced periplasmic asparaginase by recombinant *Pichia pastoris* harboring the *S. cerevisiae* ASP3. Recently, Vidya *et al*., (2011) have cloned and expressed L-asparaginase II of *E. coli* MTCC 739 and optimized the conditions for higher expression of recombinant L-asparaginase II.

2.6. Purification and characterization of L-asparaginase

The purification of L-asparaginase from various sources have involved separation of cells from the fermentation broth, concentration by precipitation using ammonium sulfate or organic solvents (acetone) and then subjected to chromatography (affinity, ion exchange and/or gel filtration chromatography). L-asparaginase from *S. marcescens* was purified by ammonium sulfate precipitation, DEAE–cellulose column, hydroxy appetite column and polyacrylamide gel electrophoresis (Boyd, 1971). Two type of L-asparaginases, L-asparaginase I and L-asparaginase II were purified from guinea pig serum. L-asparaginase I was purified by 272 folds by ammonium sulphate precipitation and cellulose acetate strip, polyacrylamide disc gel electrophoresis. Whereas, the purification of L-asparaginase II was

achieved 63.5 fold by precipitation with ammonium sulphate, Sephadex G100 gel filtration column and preparative electrophoresis on cellulose bloc. Molecular weight of L-asparaginase I and L-asparaginase II were found to be ~150.00 kDa ~21.50 kDa, respectively by gel filtration (Rogez *et al.,* (1975). L-asparaginase from *Mycobacterium phlei* was purified by fractionation with ammonium sulphate, absorption on calcium phosphate gel and chromatography on Sephadex G-150 and DEAE cellulose. The activation energy and apparent K_m for L-asparagine were observed as 9800 cal mol^{-1} and 0.7 mM, respectively (Pastuszak and Szymona, 1976). Extracellular L-asparaginase from *Candida utilis* was partially purified using acetone and DEAE cellulose column, Sephadex A-50 and followed by purification from Sephadex G-200. The optimum pH for purified enzyme was found to be 6.0 and the enzyme was stable for 10 min. at 50°C. Metal ions, SH inhibitor and chelating agents did not have any inhibitory effect on the enzyme activity (Sakamoto *et al.,* 1977). Whereas, an extracellular asparaginase from *Rhodosporidium rubra* exhibited maximum enzyme activity at optimum pH of 7.0 and it was activated by Mg^{2+} and inhibited by Fe^{2+} and Pb^{2+} (Foda *et al.,* 1980). However, an extracellular asparaginase from *Rhodosporidium toruloides* has been reported to be a homodimer having pH and temperature optima of 6.35 and of 37 °C, respectively (Ramakrishnan and Chandramohan, 1996). Maximum activity of purified *Staphylococcal* L-asparaginase was found to be between pH 8.6 and 8.8 and 30-32 °C (Sobis and Mickucki, 1991). Asparagine catabolism has been studied in bryophytes. The purification and characterization of two forms of L-asparaginase, L-asparaginase I and L-asparaginase II, from *Sphagnum fallax* was carried out by anion-exchange chromatography. Optimum pH and molecular weight of the enzymes were 8.2 and 126 kDa, respectively. They were intermediate between those from higher plants and those from microorganisms (Heeshen *et al.,* 1996). KSCN, $NaClO_4$ and Triton X- 100 have

been used for the solubilization of purified enzyme from *Tetrahymena pyriformis* (Triantafillou *et al.,* 1988). Four forms of L-asparaginase were isolated from *T. pyriformis* and regulation of enzyme activity was studied (Tsirka and Kyriakidis, 1989). L-asparaginase from *T. pyriformis* was found in microsomal membranes. It was activated by lipids while phospholipase-C inactivated the enzyme. The enzyme exhibited an intrinsic phosphorylation activity with a K_m value of 0.5 mM for ATP (Tsrika and Kyriakidis, 1990). Two forms of L-asparaginase, L-asparaginase I and L-asparaginase II were extracted and purified from *Thermus thermophilus* (Tsavdaridis *et al.,* 1994). L-asparaginase from *T. thermophilus* was purified and its apparent molecular mass by SDS-PAGE was found to be ~33.0 kDa. It was hexamer of 200 kDa with pI = 6.0. The optimum pH and K_m was found to be 9.2 and 2.8 mM, respectively. It is a thermostable enzyme and follows linear kinetics even at 77 °C. Chemical modification experiments showed that the existence of histidyl, arginyl and carboxylic residues located at or near active site, while serine and mainly cysteine seems to be necessary for active form (Pritsa and Kyriakidis, 2001). L-asparaginase activity from *Erwinia* strain RLB-1 was not inhibited by 10 mM metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}), whereas the activity was strongly inhibited by the addition of EDTA (Borkotaky and Bezbaruai, 2002). Purification of the enzyme from *P. aeruginosa* 50071 by Sephadex G-100 and SDS-PAGE analysis of the protein was performed by El-Bessoumy *et al.,* (2004). L-asparaginase from *P. aeruginosa* 50071 showed a K_m value of 0.147 mM and V_{max} of 35.7 IU with molecular weight of 160 kDa. The enzyme showed maximum activity at a pH of 9 at 37 ºC for 30 min. The recombinant L-asparaginase II of *E. coli* was 3.3 folds purified with 86% yield purified using Ni–NTA affinity chromatography (Khushoo *et al.,* 2004). L-asparaginase from *E. carotovora* NCYC 1526 (ErA) was cloned and expressed in *E. coli* by Kotzia and Labrou, (2005). The enzyme was

purified to homogeneity by a two-step procedure comprising cation-exchange chromatography and affinity chromatography Kotzia and Labrou, 2005). Dhevagi and Poorani, (2006) have obtained 83 fold purification of L-asparaginase from marine actinomycetes by ammonium sulphate and two step gel exclusion chromatography (Sephadex G-50 and G-200). The enzyme activity was found maximum between in the pH range of 8 to 9 and stable at pH 8. The L-asparaginase showed maximum activity at 60°C and stable up to 50°C. The enzyme has a M_r of 140.0 kDa and cytotoxic effects on lymphoblast cells. Comparative experimental evaluation of immuno-depressive and toxic effects of L-asparaginase from *E. coli* and *E. carotovora* showed that the L-asparaginase from *E. coli* is more immuno-depressive and immune toxic than *E. carotovora* (Cavana *et al.,* 1976). Cell disruption by high pressure homogenizer and the extraction by *in situ* aqueous two-phase extraction (ATPE) system have been demonstrated to isolate intracellular L-asparaginase from *E. coli* by Zhu *et al.,* (2007). This novel *in situ* ATPE process yielded a product of L-asparaginase with a higher specific activity of 94.8 U mg^{-1} and a higher yield of 73.3%. Kotzia and Labrou (2007) cloned and expressed L-asparaginase from *E. chrysanthemi* 3937 in *E. coli* BL21 (DE3) pLysS. The enzyme was purified to homogeneity in a single-step procedure involving cation exchange chromatography on an S-Sepharose FF column. The k_{cat}, K_m and k_{cat}/K_m of the recombinant L-asparaginase were found to be 0.058 mM, 23.8×10^3 s^{-1} and 411.8×10^3 $mM^{-1}s^{-1}$, respectively. L-asparaginase from *Yersinia pesudotuberculosis* and *E. carotovora* were purified to homogeneity by 60% ammonium sulphate followed by sephadex G25 and anionnite Q sepharose FF column and investigated antitumor activity with various cell lines (Abakumova *et al.,* 2009). Basha *et al.,* (2009) observed that the extracellular L-asparaginase from marine actinomycetes with maximum activity at pH 7.5 and 50 ºC. The apparent K_m value for the substrate was 25 µM. Mg^{2+} ion

slightly stimulated activity while Cu^{2+}, Zn^{2+} and EDTA inhibited the enzyme activity. The apparent M_r of L-asparaginase from *E. carotovora* under non denaturing and denaturing conditions was 150 kDa and 37 ± 0.5 kDa, respectively. L-asparaginase activity was studied in the presence of thiols, namely, L-cystine (Cys), L-methionine (Met), N-acetyl cysteine (NAC), and reduced glutathione (GSH). Kinetic parameters in the presence of thiols (10–400 μM) showed an increase in V_{max} values and a decrease in K_m values (Warangkar and Khobragade, 2010). The apparent purified molecular weight of extra-cellular L-asparaginase from *Streptomyces gulbargensis* have reported to be 85 kDa. The optimum pH and temperature for the maximum enzyme activity were 9.0 and 40°C, respectively (Amena *et al.,* 2010). A novel L-asparaginase from *Withania somnifera* L. was purified with ammonium precipitation followed by gel exclusion (Sephadex G-75) and ion exchange (CM-Sephadex C-50) chromatography and characterized (Oza *et al.,* 2010). The purified enzyme was a homodimer with a molecular mass of 72 kDa, and the *pI* value of the enzyme was around 5.1. The data obtained from the MTT assay showed a LD_{50} value of 1.45±0.05 U ml^{-1}. The molecular weight of L-asparaginase is approximately half of that of prokaryotic asparaginase and similar to plant L-asparaginase. L-asparaginase from a soil isolate of *Bacillus circulans* was purified by Prakasham *et al.,* (2010). The M_r of L-asparaginase was found to be 140 kDa and reported the antineoplastic activity with IC_{50} ~100 U per millions cells. Lappa *et al*., (2010) evaluated the thermodynamic properties of recombinant L-asparaginase of *E. carotovora.* They concluded that the low thermal stability of the enzyme is of entropic origin and is most likely due to structural determinants that cause a higher degree of local disorders at specific locations. Wink *et al*., (2010) purified the recombinant L-asparaginase II of *E. carotovora* with signal peptide (AspSP) and without the signal peptide (AspMP). AspMP was purified to homogeneity by a SP

XL cation exchange chromatography column with 91% yield, and AspSP by SP XL column and Sephacryl S-200 HR column with 28% yield. In addition, both enzymes have shown specific activities of 208.1 and 237.6 U mg^{-1}, respectively. K_m , k_{cat} and k_{cat}/K_m values for AspMP and AspSP were found to be 1.1 mM, 160.0 s^{-1}, 1.45 x 10^5 M^{-1} s^{-1} and 0.16 mM, 160.0 s^{-1} and 9.98 M^{-1} s^{-1}, respectively. Recently, Kumar *et al*., (2011b) isolated an intracellular glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428 to apparent homogeneity. The homotetramer enzyme has a molecular mass of 144.4 kDa (MALDI-TOF MS) and an isoelectric point of 8.4 (approximately). The enzyme was found to be very specific for its natural substrate, L-asparagine. The activity of L-asparaginase is activated by mono cations and various effectors including Na+, K+, L-cystine, L-histidine, glutathione and 2-mercaptoethanol, whereas it is moderately inhibited by various divalent cations and thiol group blocking reagents. Kinetic parameters, K_m, V_{max} and k_{cat} of purified L-asparaginase from *P. carotovorum* MTCC 1428 were found to be 0.657 mM, 4.45 U μg^{-1} and 2.751x 10^3 s^{-1}, respectively. Optimum pH of the purified L-asparaginase for the hydrolysis of L-asparagine was in the range of 8.0–10.0, and its optimum temperature was found to be 40 °C. The purified L-asparaginase did not shown glutaminase activity. L-asparaginase of chicken liver was purified using different purification steps (including ammonium sulphate fractionation followed by separation on Sephadex G-100 gel filtration and Sephadex G-200 gel filtration). The enzyme was purified to 128.5 ± 0.5 fold and showed a final specific activity of 158.11 ± 5.0 U mg^{-1} with a 17.1 ± 8.6 % yield. SDS-PAGE and gel filtration of the purified enzyme revealed that one peptide chain with Mr of 33 kDa and 36 kDa, respectively. K_m and V_{max} of L-asparaginase were found to be 1.66 mM and 34.47 U mg^{-1}, respectively. The enzyme showed maximum activity at a pH of 9.5 when incubated at 60 °C for 20 min. The enzyme inhibited the growth of the two human cell lines

including hepatocellular carcinoma (Hep-G2) and colon carcinoma (Hct-116) with IC50 value of 8.38 μg ml^{-1} and 4.67 μg ml^{-1}, respectively. While IC50 was greater than 10 μg $well^{-1}$ for MCF7 (breast carcinoma) cell line (Sayed *et al*., 2011). Eisele *et al*., (2011) purifed L-aspraginase from a basidiomycete, *Flammulina velutipes*. Native PAGE and SDS–PAGE revealed a protein of about 85 kDa with 13 kDa subunits, respectively. They expressed gene of *Flammulina velutipes* asparaginase (FvNase) in *E. coli* and achieved an L-asparagine-hydrolyzing activity of 16 U ml^{-1} in crude extract, which was five times higher than its L-glutamine-hydrolyzing ability. The enzyme showed a pH-optimum at pH 7, remarkable tolerance towards elevated temperature and sodium chloride concentration in both the native and recombinant form. Vidya *et al*., (2011) purified the recombinant L-asparaginase of *E. coli* MTCC 739 fused with hexahistidine tag at C-terminus using nickel-nitrilotriacetic acid chromatography and the optimum pH and temperature were found to be 37 °C and pH 6.0, respectively. The summarized properties of microbial purified L-asparaginase are shown in Table 2.5.

2.7. L-asparaginase preparation

Although L-asparaginase has been reported in a variety of plant and animal species, microorganisms were explored. Microorganisms have proved to be very proficient and inexpensive sources of this enzyme. A broad range of microbes comprising bacteria, fungi, yeast, actinomycetes and algae are very efficient producers of this enzyme. However, the enzyme properties vary from organism to organism. It was produced in large quantities from *E. coli* and *E. carotovora*. The enzyme from these two sources has been found to have lowest toxicity among the large variety of similar enzymes with known antitumor activity (Duval *et al*., 2002).

Table 2.5. Properties of some of the microbial purified L-asparaginases

Microbial source	pH optima	Temp. optima (°C)	K_m (M)	Activity (U mg^{-1})	Antitumor activity	pI	Glutaminase activity (%)	References
Serratia marcescens	6.8	--	1.00×10^{-4}	255	+	5.2	5	Boyd and Phillips, (1971); Stern *et al.*, (1976)
Streptomyces noursei MTCC 10469	8.0	50	--	78.88	NA	--	--	Dharmaraj (2011)
Pseudomonas acidovorans	9.5	--	1.50×10^{-5}	86	+	--	147	Davidson *et al.*, (1977)
Pseudomonas aeruginosa	9.0	37	1.47×10^{-4}	1900	NA	--	--	El-Bessoumy *et al.*,(2004)
Azotobacter vinelandii	8.6	48	1.10×10^{-4}	622	+	--	--	Gaffer and Shethna, (1977)
Pectobacterium carotovorum MTCC 1428.	8.5	40	6.57×10^{-4}	2020.91	+	8.4	0	Kumar *et al.*, (2011)
Pseudomonas stutzeri	9.0	37	1.45×10^{-4}	732	+	6.38	0	Manna *et al.*, (1995)
Erwinia chrysanthemi	8.0	45	1.20×10^{-5}	650	+	8.5	2	Muller and Boos, (1998)
E. coli	5.5	37	1.00×10^{-5}	280	+	5.0	6	
Erwinia carotovora	8.0	50	1.80×10^{-5}	167	+	8.7	9	Narta *et al.*, (2007)
Pseudomonas 7A	7.2	37	4.40×10^{-6}	162	+	--	--	
Corynebacterium glutamicum	7.0	40	2.50×10^{-3}	2020	NA	--	--	
Vibrio succinogenes	8.5	37	1.70×10^{-5}	5.6	+	--	>1	
Bacillus sp.	8.0	37	2.40×10^{-4}	--	+	--	--	
Aspergillus terrus	5-7	40-45	5.80×10^{-4}	--	+	--	--	
Thermus thermophilus	9.2	77	2.80×10^{-3}	840	+	6.0	--	Pritsa and Kyriakidis, (2001)
Fusarium tricinctum	8.0	--	5.2×10^{-4}	1000	NA	5.18	0	Scheetz *et al.*, (1971)
Tetrahymena pyriformis	8.6	--	2.20×10^{-3}	382	NA	--	0	Triantafillou *et al.*, (1988)

NA- data not available

Currently, L-asparaginase is available in three preparations in the dug market *viz.*, two native forms (purified from bacterial sources) and modified form, PEG-L-asparaginase. The native preparations are derived from *E. coli* (marketed commercially by Merck & Co. as Elsper), and *E. carotovora* (available as *Erwinia* L-asparaginase from Ogden Bioservices Pharmaceutical Repository in the United States). The *Erwinia* product is commercially offered in Canada and Europe as Erwinase, marketed by Porton. In Europe, two different preparations of L-asparaginase (L-asparaginase Medac and Crasnitin) from of *E. coli* are available with different pharmacokinetic properties (Boos *et al*., 1996). PEG-L-asparaginase (non-proprietary name pegasparaginase) is a chemically modified form of the enzyme, which is a native L-asparaginase from *E. coli*, covalently conjugated to PEG. PEG- L-asparaginase, now referred to as pegasparaginase or pegasparagase, was developed in 1970s and subjected to clinical trials in 1980s. Pegaparaginase (available commercially from Rhone- Poulenc Rorer as Oncaspar) was approved by the Food and Drug Administration for the treatment of patients with acute lymphoblastic leukemia, who are hypersensitive to native form of *E. coli* L-asparaginase (Table 2.6).

Table 2.6. Asparaginase preparations in clinical use

Commercial product	Pharmaceutical company	Biological source
Asparaginase medac©	Medac, Kyowa Hakko	*E. coli*
Crasnitin©	Bayer AG[a]	*E. coli*
Ciderolase©	Rhoˆne-Poulenc Rorer	*E. coli*
Elspar©	MSD, Rhoˆne-Poulenc Rorer	*E. coli*
Erwinase©	Speywood	*Erwinia chrysanthemi*
Oncaspar©	Enzon, Rhoˆne-Poulenc Rorer, Medac	*E. coli*

[a] Production of this preparation is terminated.

2.8. Mechanism of action

A detailed understanding of the catalytic mechanism of L-asparaginase would explore the modifications of the wild type proteins that could reduce the severe side effects of their application in cancer therapy. Iiboshi *et al.,* (1999) have suggested that the amino acid deficiency selectively regulates p70 S6 kinase ($p70^{s6k}$) and eukaryotic I initiation factor 4E-binding protein 1 (4E-BP1) both of which are targeted by the anti-proliferative drug rapamycin. It was demonstrated that addition of L-asparaginase to human leukemic cells inhibited activity of $p70^{s6k}$ and phosphorylation of 4E-BP1. The rate and kinetics of $p70^{s6k}$ inhibition by L-asparaginase were comparable to those seen by deprivation of Asn from cell culture media, suggesting that the effect of L-asparaginase on $p70^{s6k}$ is explained by depletion of Asn. Moreover, L-asparaginase as well as rapamycin selectively suppressed synthesis of ribosomal proteins at the level of mRNA translation. These findings indicated that L-asparaginase and rapamycin target a common signaling pathway in leukemic cells. Han *et al.,* (1974) suggested that L-asparaginsase acts on the cell membrane causing impairment of antigen or mitogen binding and this is responsible for its inhibitory effect. Story *et al.,* (1993) examined the cellular morphology of a lymphoma in a dog and LY-TH cells (derived from a mouse B- cell lymphoma), before and 4h after L-asparaginase therapy. They determined the mode of cell death caused by L-asparaginase exposure to be apoptosis. Ueno *et al.* studied that the apoptotic cell death of murine leukemia cells induced by *E. coli* L-asparaginase. They observed that deprivation of asparagine from the culture of L5178Y cells by L-asparaginase caused the fragmentation of chromosomal DNA of the leukemia cells within 24 h. Prior to the degradation of DNA, cell cycles were found to be arrested in G1 phase, and evidence of the DNA strand breaks was initially observed in G1 phase cells as early as 8 h after the L-asparaginase treatment. Thus, apoptosis of leukemia cells induced by L-asparaginase is an

event that is associated with the cell cycle arrest in G1 phase (Ueno *et al*. 1997). Kessels (1970) observed that L-asparaginase inhibits the incorporation of fucose and glucosamine like cyclohexamide. Presently, the accepted mechanism for alteration of L-asparagine into L-aspartate and ammonia by L-asparaginase proceeds *via* a covalently bound intermediate involving a β -aspartyl enzyme. This is followed by the removal of ammonia. This intermediate is then attacked by a second nucleophile, normally water, resulting in the hydrolysis of the acyl-enzyme intermediate yielding the acidic product and free enzyme (Sanches *et al.,* 2007), which may be possible due to the proximity of their side chains to the aspartate ligand, Thr-12, Tyr-25, Ser-58, and Thr-89 and these could be potential nucleophiles (Swain 1993).

2.9. Structure of L-asparaginase

The preliminary crystallographic studies on L-asparaginase from *E. coli* (Epp *et al.,* 1971), *Proteus vulgaris* (Lee, 1973), *Acinetobacter glutaminasficans* (Wlodawer, 1975), *Pseudomonas*7A (Wlodawer, 1977), *Wolinella succinogenes* (previously called *Vibrio succinogenes)* (Ammon 1985) and *E. carotovora* (Wikman *et al*., 2005) have been carried out. In 1976, Murthy and Knox performed small angle X-ray scattering studies on *E. coli* L-asparaginase, which inferred that the enzyme had a radius of gyration of 34.0±0.5 Å at pH 7.0. Further, the structure of *E. coli* L-asparaginase was studied by Swain *et al.,* (1992) and this enzyme is active as a homotetramer with 222 symmetry, belongs to the class of α/β proteins. Each subunit has two domains with unique topological features. They proposed locations for the active sites between the N- and C-terminal domains belonging to different subunits and postulate a catalytic role for Thr-89. Structure of the enzyme with bound L-aspartate indicated a threonine residue as a catalytic nucleophile (Miller *et al.,* 1993). The

enzyme was found to be a tetramer with each monomer having a general shape of a prolate ellipsoid. Tryphtophan and cystein are both present in *E. coli,* but completely absent in *Erwinia* (Shifrin *et al.,* 1973). The *Erwinia* enzyme contained a relatively large portion of arginine (Stern, 1976). Homer and Allsopp (1976) discovered a new histidyl residue of L-asparaginase in *E. coli* that affected its properties. Amino acid sequence of tryptic peptides was determined by fragmentation with various enzymes and by Edman degradation (Maita *et al.,* 1979). Secondary structure of L-asparaginase from *E. coli* in a broad pH region has been described by Illarionova *et al.,* (1980). Hellman *et al.,* (1983) showed that freeze-drying altered the tetramer structure of the enzyme into monomer. L-glutaminase-L-asparaginase purified from *Acinetobacter. glutaminasificans* was studied for its binding sites, catalytic activity, substrate specificity, K_m and V_{max} by Steckel *et al.,* (1983). Ionization/deionization of the functional groups of the active center of L-asparaginase was found to be responsible for enzyme activity (Frankenberger and Page, 1983). Enzyme activity was decreased in high acidic/alkaline conditions. Danileichenko *et al.,* (1990) studied that the electron microscopic structure of L-asparaginase from *E. carotovora* 268. *Erwinia* L-asparaginase (ErA) monomer consists of about 330 amino acid residues that form 14β strands and 8α helices, arranged into two easily individual domains (N-terminal domain is larger and C terminal domain is smaller). These both two domains are attached by a linker consisting of ~20 residues. Each of the four active sites is located between the N and C-terminal domains of two adjacent monomers. Therefore, the L-asparaginase tetramer can be treated as a dimer of dimmers. Part of the active site formed by strictly conserved residues is a flexible loop (between residues 10 and 40) that contains two important residues, Thr-12 and Tyr-25 (Aghaiypour *et al.,* 2001). Thr-12 is the nucleophile involved in the acylation reaction (Aung *et al.,* 2000). Access to the active site cavity is controlled by this flexible loop that opens and closes in a ligand dependent

manner (Aung *et al.,* 2000, Kotzia *et al.,* 2005). The tertiary structure and amino acid sequence of L-asparaginase from *Wolinella. succinogenes* has been compared with bacterial L-asparaginase by Lubkowski *et al.,* (1996) and a difference in structures was found and specificity was affected due to distinction between amino acid sequences. All bacterial type II L-asparaginases are active as homotetramers containing around 330 amino acids per monomer and four identical non-cooperative active sites. The four subunits are labeled A, B, C and D. Two active sites are formed at the dimerization interface between the A and C subunits (as well as between B and D), and so-called intimate dimer, and two such intimate dimers join to form a tetramer with 222 symmetry. The assembly of the close dimers is mostly hydrophobic in nature. The tetramer keeps the mainly hydrophobic part of the protein concealed inside its core, away from contact with solvent, providing stabilization to the structure and rendering a globular shape to the molecule, with the polarizable surface oriented towards the exterior medium (Jones, 1996). A new L-asparaginase from *E. coli* belonging to the class of Ntn aminohydrolases has been crystallized by the vapor diffusion method by using PEG-4000 as a precipitant by Borek and Jasloski, (2001). Kozak *et al.,* (2000) made crystallographic studies on a Y25 F mutant of periplasmic *E. coli* L-asparaginase and also studied its kinetic properties. Structural analysis of L-asparaginase by *E. coli* has been studied by HPLC and mass spectroscopy by Han *et al.,* (2001). The enzyme from *E. coli* has been crystallized in a novel form and its asymmetric unit has been found to be a dimer instead of tetramer, and also other structural differences have been found (Sanches *et al.,* 2003). The crystal structure of L-asparaginase for a complex obtained by co-crystallization with L-aspartate has been determined at 1.9 Å resolutions by Michalska *et al.,* (2005). The architecture of L-asparaginase by *E. coli* (EcA III) active site sheds light on the subject of substrate specificity. The crystal structure of type I L-asparaginase from *Pyrococcus harikoshii* has

been described by Yao *et al.,* (2005) by diffraction. It has considerable difference with the structure of type II L-asparaginase. Most catalytic residues are conserved except two that recognize the amino group of the substrate. Mezentsev *et al.,* (2006) carried out a comparative molecular-graphic analysis of subunits interfaces and developed an experimental approach for an enzyme oligomerization study along with homology modeling of the special structure of L-asparaginase from *E. carotovora*. In this study, L-asparaginase was immobilized on a CM5 chip surface of optical biosensor Biacore 3000 based on surface plasmon resonance technology. Study for determination of the crystal structure of potassium-independent L-asparaginase from yellow lupine (LlA) and the corroboration of classification of this group of enzymes in the family of Ntn-hydrolases has been done by Michalska *et al.,* (2006). The (αβ) heterodimer has a sandwich-like fold with two beta-sheets flanked by two layers of alpha-helices ($\alpha_2\beta_2$) in common with other Ntn-hydrolases. The nucleophilic Thr193 residue, which is liberated in the autocatalytic event at the N terminus of subunit β, is part of an active site that is similar to that observed in a homologous bacterial enzyme. *Lupinus luteus* L-asparaginase is a dimer of heterodimer formed by subunits α (1-192 residue) and β (residue193-325) and thr 193 acts as nucleophile. An unusual sodium-binding loop of the bacterial protein, necessary for proper positioning of all components of the active site, shows strictly conserved conformation and metal coordination in the plant enzyme. A chloride anion complexed in the LlA structure marks the position of the α-carboxylate group of the L-aspartyl substrate/product moiety. Analytical ultracentifugation and X-ray crystallography studies on cytoplasmic asparaginase from *E. coli* by Yun *et al.,* (2007) revealed that the L-asparaginase I forms a tetrameric structure as a dimer of two intimate dimers. Kinetic analysis of the enzyme reveals that L-asparaginase I (AnsA) is positively cooperative, displaying a sigmoidal substrate dependence curve with an $(S)_{0.5}$ of 1mM L-asparagine and a

Hill coefficient (nH) of 2.6. Binding of L-asparagine to an allosteric site was observed in the crystal structure concomitant with a reorganization of the quaternary structure, relative to the apo enzyme. Li *et al.,* (2007) replaced an amino acid by proline at a certain hydrogen-bonded turn (β-turn). From the viewpoint of stearic structure and stability of entropy, proline should be a more constant structure, and hydrogen-bonded turns are very important positions for the stability of protein at which proteins modify their overall chain direction. The results showed that the thermostability of the point mutant L-asparaginases II from two thermophiles (*Streptococcus thermophilus* and *Corynebacterium efficiens*) is significantly enhanced by substitution with proline in a predicted β-turn structure while the enzyme's activity is not affected. Bansal *et al.,* (2010) have studied the structural stability of recombinant L-asparaginase of *Pyrococcus furiosus* (*PfA*). Taking coordinates from a recently published structure of a homologous L-asparaginase of *Pyrococcus horikoshii* (PhA) (Yao *et al.,* 2005), a model of *PhA* has been developed. In contrast, crystal structure of the type I L-asparaginase PhA forms an intimate dimer and not associates as a homotetramer. A clear hydrophobic area is absent from the region of the PhA surface, which corresponds to the tetramerization edge of the type II L-asparaginase (Yao *et al.,* 2005, Sanches *et al.,* 2007).

2.10. Methods of assay

Some common methods were used for measuring L-asparaginase activity such as the Nesslerization reaction and Indooxine method (Lanvers *et al.,* 2002), a coupled enzyme assay with excess glutamic-oxaloacetate transaminase and malic dehydrogenase (Cooney and Handschumacher, 1970), and fluorometric assay using L-aspartic acid β-(7-amido-4-methlcoumarin) as a substrate (Ylikangas and Mononen, 2000). The drawbacks are multistep operation, requirement for the highly toxic reagent and inapplicability for real time. Therefore

many attempts have been made by researchers for the assay of L-asparaginase. Furthermore, proficient methods for the assay of L-asparagine have been developed. Methods showing the substrate–enzyme relationship have been developed as well. An online gas analyzer for automated enzymatic analysis with potentiometer ammonia detection has been described by Fraticelli and Meyerhoff, (1983). Where, an ammonia electrode was incorporated in combination with predialysis unit. Drainas and Drainas, (1985) developed a method to measure the L-asparaginase activity by conductimetrically. The method is based on the increase of conductivity, which is due to the production of ammonia and/or aspartate in a reaction mixture containing cell-free extract and asparagine or aspartate hydroxamate. This conductivity is linear with time and enzyme concentration and follows Michaelis-Menton kinetics. The direct measurement of L-asparagine in human plasma samples through the use of L-asparaginase from *E. coli* in the soluble form is a major clinical application of this system. A multi-analyte miniature conductance biosensor using urease and L-asparaginase and a three–enzyme system consisting of urease, creatinase and creatininase for determining urea, L-asparagine and creatinine, respectively, have been described by Cullen *et al.,* (1990). The device responded to changes in electrode double layer capacitance, as the ionic strength is increased by the enzyme-catalyzed production of changed reaction products.

An enzymatic method has been developed for the kinetic measurement of L-asparaginase activity and L-asparagine with an ammonia gas-sensing electrode. This method is based upon the deamination of L-asparagine by L-asparaginase from *E. coli* resulting in the formation of ammonia (Tagami and Mastsuda, 1990). A specific quantitative colorimetric assay for L-asparagine by mixing it with dilute ethanolic ninhydrin solution and measuring its absorption at 340–350 nm has been reported by Sheng *et al.,* (1993). Both L-asparaginase and asparagine

synthetase activities were estimated by this procedure and followed by HPLC amino acid analysis.

Determination of L-asparagine using a garlic tissue electrode has been reported by Kim *et al.,* (1995). Garlic tissue cells were used for conversion of L-asparagine into ammonia. An ammonium gas electrode (ISE) was employed as the detector. The combination of L-asparaginase in garlic tissue cells and gas electrode responds linearly to L-asparagine concentration. L-asparaginase from *E. chrysanthemi* was assayed by incubating it with beta L-aspartic acid and the formation of 7-amino-4-methylcoumarin was measured fluorometrically (Ylikangas and Mononen, 2000). This is also using to monitor L-asparaginase activity in the serum of ALL patients during L-asparaginase therapy. Lanvers *et al.,* (2002) developed a sensitive plate reader-based method by means of L-aspartic β-hydroxamate as substrate for the quantification of L-asparaginase derived from *E. coli, E. chrysanthemi* and pegylated L-asparaginase from *E. coli* in human serum. A thermostable recombinant asparaginase from *Archaeoglobus fulgidus* was cloned and expressed in *E. coli* as a fusion protein. It was purified by an immobilized metal ion affinity chromatography and its activity was determined by monitoring the change in ammonia concentration in the solution. The enzyme was immobilized and used with an ammonium selective electrode (ISE) to develop a biosensor for L-asparaginase (Wang and Bachas, 2002). An automated kinetic enzymatic method for monitoring plasma L-asparaginase activity during therapy of acute lymphoblastic leukemia has been developed by Orsonneau *et al.,* (2004). The method has precision as compared to Nessler's method. Tsurusawa *et al.,* (2004) demonstrated that the highly sensitive enzyme coupling method to determine the minimum levels of L-asparaginase necessary for maintaining asparagine depletion under L-asparaginase treatment in acute lymphoblastic leukemia. It was revealed that

asparagine levels are strongly correlated with plasma L-asparaginase even at low enzyme activities (50 IU ml^{-1}). Using pyrrole/polyimide as a probe, a new PPY-MSPQC system was developed by Ren *et al.,* (2010) for detection of NH_3 at high humidity and room temperature and this was used for L-asparaginase assay. The PPY-MSPQC method is very simple, direct, real time, and non-toxic.

2.11. Modification of the enzyme

In order to overcome frequent-injection and short half life of L-asparaginase, physical entrapment and conjugation of the enzyme with water-soluble polymers have been developed (Capizzi *et al.,* 1971). L-asparaginase was successfully conjugated with natural and artificial polymers such as albumin, dextran, polyethylene glycol (PEG) and polyvinyl alcohol (Ashihara *et al.,* 1978; Nambu, 1986; Poznansky *et al.,* 1982; Wileman, 1991). Uren and Ragin (1979) utilized poly-dl-alanyl peptides to block immunogenic epitopes of *E. coli* and *Erwinia* L-asparaginase, but the clinical studies have not been performed to date. Nerker and Gangadharan (1989) conjugated L-asparaginase from *Erwinia* to human serum albumin. L-asparaginase entrapped in red blood cells was quite stable and had markedly prolonged *in vivo* half-life (Deloach *et al*., 1990; Nagi *et al*., 1998). Attempts were made for the chemical modification of the enzyme by Bluma *et al.,* (1975). They modified the enzyme with N-bromosuccinimide, which inhibited enzyme activity. To study the sub-unit interactions, Parrott and Shifrin, (1976) modified the L-asparaginase of *E. coli* with 2, 4, 6–trinitrotoluene. Covalent attachment to the fibers of a hollow fiber hemodialyzer resulted in ready access of the substrate to the enzyme (Jackson *et al.,* 1979). Reductive alkylation of L-asparaginase by *E. coli* affected the pH optimum and catalytic activity without any effect on thermal stability and *pI* (Shprunka *et al.,* 1980). But *pI* of the L-asparaginase from *E. coli* increased by

succinylation and acetylation (Nickle *et al.,* 1982). Immobilization of the enzyme by polyacrylamide increased its stability to denaturation and proteolysis (Galaev *et al.,* 1981). Modification of L-asparaginase by albumin polymers led to augment in resistance towards proteolysis (Poznansky *et al.,* 1982). Covalent attachment of poly-D-alanine peptides to lysyl residues on the surface of L-asparaginase from *E. carotovora* helped to decrease the immunogenicity of the enzyme (Uren *et al.,* 1982). Spherical microparticles of polyacrylamide were used for immobilization of L-asparaginase by Edman *et al*., (1983). These led to an increase in efficiency of the action of enzyme and acted as adjuvant for the production of antibodies. However, a decrease in enzyme activity was observed when modification of tyrosyl residues and carboxyl groups was carried out as reported by Qian *et al.,* (1984). Immobilization on polyglucin led to better thermal stability and storage stability as well as decreased antigenic affinity (Karsakevich *et al.,* 1986a). The therapeutic potential of L-asparaginase was greatly improved by complexing with dextran sulphate. The enzyme had good substrate specificity, increased thermal stability, and better storage stability. Additionally, it was resistant to proteolysis (Karsakevich *et al.,* 1986b). Immobilization of the enzyme on soluble CM cellulose by using azide binding was carried out by Karsakevich *et al.,* (1987). Electrophoretic, enzymatic and immunologic properties of CM cellulose asparaginase depend on the quantity of CM cellulose in the polymer conjugate of the enzyme. Chemical modification by acylation increased the catalytic activity of the enzyme, but the limitation of this approach is that enzyme becomes hydrophobic after modification (Martins *et al*., 1990). Yoshimoto *et al.,* (1987) have modified the enzyme by coupling a magnetic modifier to amino groups of L-asparaginase. The anti-leukemic activity of the enzyme was further enhanced by immobilizing the enzyme on a water-soluble vinylpyrrolidone and acrolein copolymer and the enzymatic activity depended on the amount of acrolein radicals in the copolymer

(Karsakevich *et al.,* 1988). Modification of L-asparaginase by acetic anhydride, dextran and monomethoxy PEG has been reported by Cao *et al.,* (1990). A noticeable increase in half-life of the enzyme was observed by Qian *et al.,* (1996) when the enzyme was chemically modified by N,O-carboxy methyl chitosan in the presence of L-aspartic acid. PEGylation, the conjugation of L-asparaginase to PEG turned out to be the most successful process of chemical modification, which is usually employed in the event of hypersensitivity reactions to the native forms. Immobilization of L-asparaginase into a biocompatible poly (ethylene glycol) albumin hydrogel has been reported by Jean-Francois and Fortier, (1997). Monopolyethylene glycol (mPEG) is acknowledged to reduce immunogenicity of enzymes and to stabilize the catalytic activity of various enzymes. Abuchowski *et al*. (1979) have successfully coupled the L-asparaginase with PEG. Biochemical properties of PEG-L-asparaginase, commercially known as pegasparagase (Oncaspar®, ENZON, Piscataway, NJ, and Rhone-Poulenc Rorer Pharmaceuticals, Collegeville, PA) that have been commercially available since 1994. Its apparent molecular weight is higher and its reactivity with specific antibodies is very low. But it increases if the drug is subjected to freeze-thawing cycles (Korholz *et al*., 1989). Clinical studies proved that the modified enzyme have antitumor activity both in animal models (Abuchowski *et al*., 1979; Yashimoto *et al*.,1986) and humans (Jurgens *et al*., 1988; Yashimoto *et al*.,1986). A BSA-PEG matrix has been observed to be very proficient for L-asparaginase immobilization by Jean-Francois and Fortier, (1996) and the K_m value for the substrate increased by 200 fold due to immobilization. Soares *et al.,* (2002) have reported that the modification with PEG changed physico-chemical and biological properties of the enzyme, resulting in less immunogenicity and a longer half time of plasmatic life. The stability of the enzyme improved and abolished immunogenicity (Zhang *et al.,* 2004b). PEG-L-asparaginase activity resulted in effective asparagine depletion in serum

and cerebrospinal fluid (Hawkins *et al.,* 2004). Moreover, the modified enzyme had more activity than native L-asparaginase. PEG-modified enzyme has shown promising therapeutic potential (Veira *et al.,* 2005). Rizzari *et al.,* (2006) reported that the adequate plasma enzymatic activity and asparagine depletion was achieved by PEG- asparaginase.

The K_m, optimum pH range and thermo stability were increased by coupling the levan from *Z. mobilis* with L-asparaginase from *E. carotovora*. But the electrophoretic mobility was reduced (Vina *et al.,* 2001). Encapsulation of L-asparaginase into PHB nanocapsules and study of adverse effects and anaphylaxis in mice was performed by Baran *et al.,* (2003). In polynanospheres, biological activity of L-asparaginase improved but the release profile was changed considerably by co-encapsulation of the stabilizers (Wolf *et al.,* 2003). A chemically modified derivative of the enzyme (Palmitoyl L-asparaginase) encapsulated in liposome has also been studied in animals (Jorge *et al*., 1994) and it exhibited about 10-fold prolongation in half-life without acute toxicity. Removal of immunodominant epitopes in the enzyme by site directed mutagenesis was studied in *E. chrysanthemi* using synthetic hexapeptide and polyclonal antisera from rabbits and mice. It resulted in substantial dwindle in binding of the antibodies leads to decrease in immunogenicity without altering enzyme activity (Moola *et al*., 1994). Fernandes and Gregoriadis (1997) reported that the chemical modification by polysialic acid improved therapeutic use by enhancing beneficial anti-tumor activities of L-asparaginase. The modified enzyme had improved functionality. Zhang *et al.,* (2004a) studied the immobilization of L-asparaginase on silk particles (Sericin) from *Bombyx mori* (silkworm) by covalent binding of the enzyme. Silk fibroin L-asparaginase (SF-ASNase) bioconjugates have been developed by covalent attachment of L-asparaginase with silk fibroin (Zhang *et al.,* 2005). Bioconjugation of silk fibroin significantly helps to reduce the

immunogenicity and antigenicity of the enzyme. The covalent attachment of the silk sericin peptides to L-asparaginase producing silk sericin peptides-L-asparaginase (SS-ASNase) are active, stable and have a lower immune response with extended half-lives in human serum under *in vitro* conditions (Zhang *et al.,* 2006). The modified L-asparaginase by conjugation to the oxidized inulin polymer increased the thermostability, *in vitro* half life, resistance to protease digestion, reusability after repeated freezing–thawing and widened the range of optimum pH (Tabandeh and Aminlari, 2009). The immunogenicity of the modified L-asparaginase was significantly decreased as compared to the native enzyme. Kwon *et al.,* (2009) have developed an innovative method for encapsulation of therapeutically active L-asparaginase into functionally intact erythrocytes for enhanced L-asparaginase therapy for ALL. Teodor *et al.,* (2009) have obtained nanosize biocompatible materials /tissues, which are used for entrapment of L-asparaginase. Recently, Bandgar *et al.,* (2010) have synthesized antioxidant thiomorpholides in the presence of a catalytic amount of solid-supported fluoroboric acid (HBF_4–SiO_2) and act as activators of therapeutically important L-asparaginase from *E. carotovora*.

2.12. Recombinant L-asparaginase

Due to short half life of this enzyme, repeated use of the drug is needed, which is a major drawback of this therapy. So, improvement in thermo stability, half-life, specific activity and reduction in glutaminase activity of L-asparaginase plays a vital role in the anticancer therapy. In plants, the asparaginase from *Lupinus angustifolius* seeds was cloned and characterized. The gene consisted of four exons and three introns (Dickson *et al*., 1992). Also, a cDNA corresponding to the gene that code for the asparaginase from *Lupinus arboreus* was isolated (Lough *et al*., 1992). Southern blot experiments have shown that the gene is a single-copy and

expressed only in seeds (Lough *et al*., 1992). Casado *et al*. (1995) showed that both *Arabidopsis* and *Lupinus* genes have a similar molecular organization and consist of three introns and four exons. A high homology between genes encoding L-asparaginase from *A. thaliana* and *L. angustifolius* was found (>71% and 75% for nucleotides and amino acids, respectively). Cloning gene (*ansB*) encoding L-asparaginase II of *E. coli* have cloned and expressed (Bonthron 1990). Jennings and Beecham, (1993) have observed that the expression of L-asparaginase II (encoded by *ansB* in *Salmonella enteric*) was positively regulated by a cAMP receptor protein (cRP) and anaerobiosis. Newsted *et al.*, (1995) reported that the preparation of trypsin sensitive L-asparaginase by genetically fusing its gene with a single chain antibody derived from preselected antibody, which is capable of providing protection against trypsin. The formation of the fusion protein, L-asparaginase Sc Fv expressed in *E. coli* in the form of inclusion bodies. The fusion protein conferred that the stearic hindrance, blocked cleavage sites and changed the electrostatic potential surface of the enzyme (Guo *et al.* (2000). Wang *et al.* (2002) have cloned and expressed thermostable recombinant asparaginase from *Archeoglobus fulgidus* in *E. coli.* Recombinant L-asparaginase of *E. carotovora* was expressed in *E. coli* and characterized by Borisova *et al.* (2003). Recombinant L-asparaginase was developed by cloning of L-asparaginase of *E. carotovora* NCYC 1526 (Er A) and *E. chrysanthemi* 3937 (Erl-ASNase), and expressed in *E. coli.* The enzymatic and structural properties of the recombinant enzymes were investigated and the kinetic parameters (K_m, k_{cat}) were determined. The enzyme was later immobilized on epoxy-activatedSepharose CL-6B. The immobilized enzyme retained most of its activity (60%) and showed high stability at 4^{o}C (Kotzia and Labrou, 2005; Kotzia and Labrou, 2007). Studies have been carried out for the development of a novel growth hormone to release hormone (hGHRH) analog by using 127 amino acid residues of the C-terminus from L-asparaginase.

The peptide formed has been found to have good function-selectivity and species specificity. Ferrara *et al*., (2006) have cloned asp3 gene that encodes the periplasmic, nitrogen regulated, asparaginase II from *S. cerevisiae*, and expressed in the methylotrophic yeast, *Pichia pastoris*, under the control of the aox1 gene promoter. Huser *et al*., (2006) have cloned *ansB* gene encoding a class II glutaminase/asparaginase from *Pseudomonas fluorescens* and characterized by DNA sequencing, promoter analysis and heterologous expression in *E. coli*. They confirmed that *ansB* is monocistronic and depends on the alternate sigma factor σ54 for expression. A second open reading frame located downstream of *ansB* is highly homologous to a number of bacterial genes that encode secreted endonucleases of unknown function. Liang-Zhu *et al*., (2007) have developed a higher thermostable mutant that produce L-asparaginase II by replacing Asp178 with proline in a hydrogen-bonded turn (178–180 DGR), which contributed for thermostability of the enzyme. Cloning, biochemical characterization and *in vitro* cytotoxicity of a novel L-asparaginase from *Helicobacter pylori* CCUG 17874 was reported by Cappelletti *et al*., (2008). *In vitro* cytotoxicity assays have shown that different cell lines displayed a variable sensitivity towards the enzyme. Wink *et al*., (2010) have cloned L-asparaginase II of *E. carotovora* with and without signal peptide (AspSP), expressed, purified and determined steady-state kinetic parameters. Recently, Vidya *et al*., 2011 have cloned L-asparaginase II encoding gene, *ansB* of *E. coli* MTCC 739 by excluding the native signal, in the frame with pelB leader sequence of prokaryotic expression vector pET20b and expressed in *E. coli* (DE3) cells.

2.13. Treatment with L-asparaginase

Information regarding the clinical trials of native L-asparaginases of *Erwinia* and *E. coli* have widely discussed in the last 40 years. Current treatment protocols of ALL and lymphosarcoma

do not employ L-asparaginase as a single agent. In fact, it is always a part of multiple agent regimens and combined with drugs having definitive immunosuppressive effects. The PEGylated enzyme is more preferred as L-asparaginase from *E. coli* and *Erwinia*, which is allergic to most of the patients. It is also known to have delayed a plasma clearance property that evades the need of frequent medication (Kurtzberg *et al*., 1990, Cavana *et al.,* 1976). Strappinni *et al.,* (1984) have observed that the enhanced polymerization rate of fibrin monomers or fibrin clottability by L-asparaginase treatment. The binding of L-asparaginase from *E. coli* to the plasma membrane of normal human mononuclear cells was demonstrated by Mercado-Vianco and Arenas-Diaz, *(*1999a). L-asparaginase in amalgamation with methotrexate has shown synergistic anti-leukemic activity in a schedule dependent manner (Aguayo *et al.,* 1999). Cell cycle arrest in G1 phases, which consequences in apoptosis of leukemia cells is induced by L-asparaginase (Ueno *et al.,* 1997). L-asparaginase by *E. coli* has been found to phosphorylate endogenous polypeptides in immune cells. Products of L-asparaginase specially NH^{4+} ions diffuse into the cytosol and modify the pH, which activates signal transduction pathways associated with phosphorylation of substrates (Mercado-Vianco and Arenas-Diaz, 1999b). Kelo *et al.,* (2002) have observed that the L-asparaginase action on peptides and their effect on metabolism in the human body. L-asparaginase in lidocaine decreases the pain intensity of an intramuscular injection in children without changes in bioavailability and absorptive rates of the enzyme. L-asparaginase has been found to be efficient in nasal type leukemia treatment (Yong *et al.,* 2003). Hyakuna *et al.,* (2004) have reported that the successful asparaginase treatment followed by bone marrow transplantation in leukemia patients. Deamination of glutamine may enhance the anti-leukemic effect of L-asparaginase (Panosyan *et al.,* 2004). Asparagine levels have been found to be strongly correlated with plasma L-asparaginase activity even at

low enzyme activities of <50 U/l (Tsurusawa *et al.,* 2004). Relationship between the presence of anti-asparaginase antibodies and L-asparaginase activity has been observed by Zalewska *et al.,* (2004). Immunologic cross-reaction between antibodies against various formulations of native L-asparaginase from *E. coli* and PEG L-asparaginase have been reported, but no such reaction has been found against L-asparaginase from *Erwinia* (Avramis and Panosyan, 2005). A significant decrease in α 2-antiplasmin and plasminogen levels was measured when the L-asparaginase from *E. coli* was used as drug but not in the Erwinase-treated patients. Dana-Farber Cancer Institute has shown that L-asparaginase from *Erwinia* is less toxic and less efficacious than L-asparaginase from *E. coli* (Moghrabi *et al.,* 2006). Steiner *et al.,* 2006) have reported that the undulating course of ammonia concentrations during anticancerous therapy by using L-asparaginase as drug. Patients with ALL and prothrombin gene mutation may have an advanced risk of clotting complications in contrast to patients with factor V Leiden mutation. A randomized trial of low molecular weight heparin should be performed during L-asparaginase therapy to assess its safety and efficacy in preventing venous thrombotic events (Harlev *et al.,* 2010).

2.14. Side effects of L-asparaginase

L-asparaginase has a discrete toxicity profile, which ranges from acute hypersensitivity and hyperglycemia to hepatocellular dysfunction and pancreatitis (Oettgen *et al.*, 1970). Toxicity of asparaginase fall under two major categories, those pertaining to immunological sensitization (hypersensitivity) to a foreign protein and the adverse events related to the inhibition of protein synthesis. There is a resemblance in the frequency of toxicity with all commercially available asparaginase preparations. However, in the case of pegaspargase suppression in allergic reactions is observed. Unlike the other chemotherapeutic agents of the multi-agent treatment, L-asparaginase causes modest bone marrow depression and usually

does not affect the gastrointestinal or oral mucosa or hair follicles. The most common clinical manifestation of hypersensitivity is urticaria. However, the spectrum of allergic reactions ranges from localized erythema to systemic anaphylaxis. Other reported hypersensitivity reactions consist of indurations, edema, swelling, chill, fever, tenderness and skin rashes (Evoy 1993). To avoid allergic reactions, combination chemotherapy is considered. In case of toxicities arising out of inhibited protein synthesis, normal tissues with high rates of protein synthesis (e.g. liver, pancreas and coagulation systems) are most frequently affected by L-asparaginase therapy. Although an elevation in transaminase and bilirubin levels are the most common phenomena and abnormal alkaline phosphatase levels have been observed. Reduction in serum albumin, fibrinogen and serum lipoprotein levels is also a manifestation of liver dysfunction. Reduced synthesis of protein in the liver can also affect the endocrinium. Thyroxinebinding globulin (TBG), the major thyroid hormone transport protein, has been found to be transiently but distinctly decreased during L-asparaginase therapy (Garnick and Larsen 1979; Ferster *et al*., 1992). Pathologically, fatty acid infiltration of the liver is commonly observed after L-asparaginase therapy (Oettgen, 1970). A study on the consequences of L-asparaginase on antithrombin levels in plasma from acute lymphoblastic leukemia patients, HepG2 cells, plasma and livers from mice treated with this drug has been carried out by Hernandez-Espinosa *et al*., (2006). Imbalances in the formation of clotting factors are common side effects associated with L-asparaginase therapy (Anderson *et al*., 1979; Elliott *et al*. 2004; Gugliotta *et al.,* 1990; Homans *et al.,* 1987; Priest *et al.,* 1982). Pancreatitis is a well-documented complication of L-asparaginase therapy (Weetman and Bachner 1974). Elevated serum amylase and lipase were additional manifestation of pancreatitis. A number of patients develop signs and symptoms of diabetes due to damage in islet cells, and succeeding decrease in synthesis of insulin. Hyperglycemia may be more

severe when L-asparaginase is administered in combination with prednisone, but the risk can be reduced if L-asparaginase is administered after prednisone (Ortega *et al*., 1970; Sutow *et al*., 1976). Toxicity profile of L-asparaginase is given in the Table 2.7.

Table 2.7. Toxicity profile of L-asparaginase therapy (Narta *et al.*, 2007)

System	**complications**	
Immune	Hypersensitivity	
	Reaction	*Grading (CTC)*
	No reaction	0
	Mild reaction	1
	Urticaria	2
	Bronchospasm, serum sickness, sever local reaction (>10cm, >24 h)	3
	Hypertension, anaphylaxis	4
liver	Hypoalbumenia; elevations in transaminase; bilirubin and alkaline phosphatase; lipoprotein abnormalities; decrease in serum cholesterol	
Pancreas	Acute hemorrhagic pancreatitis; pancreatitis; decreased serum insulin; diabetic insulin.	
Coagulation	Increase in prothrombin time; hypofibrinogenemia; decrease in plasminogen; factor V, VII, VIII, IX and others; thromboembolism; hemorrhagic events.	
CNS	Mild depression and personality changes; confusion and hallucination	
Others	Parotitis; lethargy; comma; seizure.	

Neurotoxicity (depression, lethargy, fatigue, somnolence, confusion, irritability, agitation, dizziness) occurs up to 25% of adult patients treated with L-asparaginase (Ashihara *et al*., 1978), but rarely found with children. Neurotoxity may also consequence from lack of L-asparagine and serum L-glutamine in the brain. While many patients had elevation ammonia levels in blood, but a correlation between ammonia levels and degree of toxicity has not been firmly established. Meyer *et al*., (2003) have observed that L-asparaginase associated

hyperlipidemia with hyperviscosity syndrome in a patient with T-cell lymphoblast lymphoma. Neutropenic enterocolitis has been observed as an unusual acute complication of neutropenia, associated with leukaemia and lymphoma (Radulovic *et al*., 2004). Myocardial ischemia has been observed in a patient with acute lymphoblastic leukaemia (Saviola *et al*., 2004) due to L-asparaginase therapy (Table 2.8). A comparative induction toxicity of native and PEG-L-asparaginase has given in the Table 2.8.

Table 2.8. A comparative study on induction toxicity of native and PEG-L-asparaginase (Narta *et al.*, 2007).

Toxicity type		**L-asparaginase preparation**	
		Native multi-agents (%)	**PEG-L-asparaginase single/multi-agents (%)**
Hepatic		59	71
Pancreatic		15	0
Coagulopathic	↑ PT	18	15
	↓FN	44	29
Hyperglycemic		5	0
Neurological dysfunction		18	5
Hypersenstivity (grade3-4)		3	0

(↑ PT: increased prothrombin time, ↓ FN: decreased fibrinogen).

2.15. Resistance to L-asparaginase

ALL patients have been found resistant to chemotherapeutic agents such as anthracyclines, vincristine and asparaginase. Holleman *et al.*, (2003) reported that the drug resistance in ALL is related with impaired ability of cells to induce apoptosis, and procaspase-2 expression is related to drug resistance in childhood ALL. Resistance to L-asparaginase results in a decreased capability of tumor cells to undergo apoptosis (Savitsky *et al.*, 2003). The differential expression of genes has been studied in drug sensitive and drug resistant ALL and

it is linked with drug-resistance and response to treatment (Holleman *et al.,* 2004). Resistance to L-asparaginase has been associated with the over expression of asparagine synthetase. An *in vitro* chemosensitivity assay is an excellent indicator of cellular response to chemotherapy (Arriffin *et al.,* 2005). In rat serum and ARJ cells, L-asparaginase treatment depletes cellular asparagines. The cellular glutamine levels have been found to be severely reduced along with a marked decrease in the activity of glutamine synthetase (Rotoli *et al.,* 2005). Krejci *et al.,* (2005) reported that the up regulation of asparagine synthetase does not prevent cell-cycle arrest induced by L-asparaginase in leukemic cells. The 45 genes that are differentially expressed in ALL and exhibiting cross-resistance to prednisolene, vincristine, asparaginase and duanorubicity have been acknowledged. The 139 genes responsible for distinct phenotype of discordant resistance to L-asparaginase have been identified (Lugthart *et al.,* 2005). Targeting particular genes involved in response to amino acid starvation in ALL cells may offer a novel way to overcome L-asparaginase resistance (Fine *et al.,* 2005). Epigenetic changes (changes involved in the expression of genes) in the repression and induction of asparagine synthesis in human leukemic cell lines have been studied and might be targeted for studying L-asparaginase resistance (Ding and Broome, 2005; Fine *et al.,* 2005). Li *et al.,* (2006) have reported that the down regulation of asparagine synthetase expression can augment the sensitivity of cells resistant to L-asparaginase.

2.16. L-asparaginase-a subject of considerable medical interest and persistent research

Currently, the therapeutic potential of L-asparaginse is well established as it has remarkably induced remission in most of the patients suffering from ALL. A comparative assessment of preparations of L-asparaginase from *E. coli* produced in the USSR, Germany and Japan was

made by Kondrat (1984) and it was observed that the clinical properties of these preparations were identical. The antileukemic action of the preparation made in the USSR was superior to the preparation (leumase) made in Japan. L-asparaginase made in USSR and Germany was recommended for clinical use. A novel asparaginase, GLIAP localized to rat brain astrocytes. It is involved in astroglial production of the neuroactive amino acid, L-aspartate, has been identified and characterized by Dieterich *et al.,* (2003). A novel way of eliminating cancer-causing acrylamide from bread has been developed by researchers from Dutch chemical firm DSM, using gene technology to degrade L-asparagine, the free amino acid that is a precursor to acrylamide, prior to baking. This involved cloning of gene encoding L-asparaginase from *Aspergillus niger* (Dunn, 2004). Taeymans *et al.,* (2005) have demonstrated the application of L-asparaginase in the food industry to determine acrylamide and acrylamide formation in food products. Dutch state mines and Novozymes have obtained patents for utilizing L-asparaginase in the production of acrylamide free food from *A. niger* and *A. oryzae* recombinant strains. In Australia and New Zealand, Novozyme has applied patent to utilize the asparaginase as a processing aid with the objective to supply the food industry. A chimeric enzyme, AnsB-TTP-CETPC, comprising asparaginase, tetanus toxin helper T cell epitope and human CETP B cell epitope have been expressed as a soluble protein in *E. coli* (Gaofu *et al.,* 2006). The purified chimeric enzyme exhibited about 83% activity of the native asparaginase. High titers of anti-CETP antibodies were induced in mice after immunization with three doses of the chimeric enzyme. The chimeric enzyme may have potential use in the vaccine against atherosclerosis. Verma *et al.,* (2007) reported that the use of L-asparaginase for the development of a novel diagnostic biosensor for the detection of levels of asparagine in leukemia cells.

CHAPTER 3
MATERIALS AND METHODS

3.1. Chemicals and reagents

All restriction enzymes, *Pfu* DNA polymerase and T4 DNA ligase were purchased from NEB, USA. Most of the chemicals used in the study (Isopropyl- β-d-thiogalactopyranoside (IPTG), ampicillin, agarose, succinamic acid, L-asparagine-t-butyl ester HCl, BOC-L-asparagine, N-α-acetyl-L-asparagine, EDTA, iodoacetamide, 2-Marceptoethanol, Ni-NTA agarose gel) were procured from Sigma-Aldrich (Bangalore, India). D-asparagine, DL-asparagine, L-glutamine, D-glutamine, D-aspartic acid, DL-aspartic acid, L-glutamic acid, L-aspartic acid amide, L-cystine, L-histidine, glutathione and reagents used in the medium development study were of analytical grade and obtained from HiMedia (India). Chemicals and markers used in native PAGE and SDS PAGE were obtained from Bangalore Genei, India. All other chemicals used in protein and glucose analysis were of analytical grade and obtained from Merck (India).

3.2. Microorganisms and vector

Different strains used for this study are *Erwinia aroideae* NRRL B 134, *Erwinia aroideae* NRRL B 136, *Erwinia aroideae* NRRL B 138 (procured from Agriculture Research Service (ARS) culture collection, USDA , Peoria, USA), *Pectobacterium carotovorum* MTCC 1428, *Escherichia* coli DH5α (obtained from Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh, India) and *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 [kindly provided by Dr. Paul Birch, Scotland Crop Research Institute

(SCRI), Scotland]. *Escherichia coli* BL21 (DE3) and plasmid pET 22b(+) obtained from Novagen, USA.

3.3. Growth and maintenance of microorganisms

All strains were maintained on LB-agar medium containing (g l^{-1}): Yeast extract 5.0, Tryptone 10.0, NaCl 10.0 and agar 20.0 (initial pH 7.0). For active culture, freeze dried microorganism (stored at –80 °C) was streaked on LB-agar plate and incubated at 28 °C (*E. aroideae* NRRL B 136, *E. aroideae* NRRL B 138, *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043) or 37 °C [*E. coli* BL21 (DE3) and *E. coli* DH5α]. A single colony was inoculated in to 8.0 ml of LB medium and incubated in shaking incubator at 180 rpm at 28 °C (*E. aroideae* NRRL B 136, *E. aroideae* NRRL B 138, *P. carotovorum* MTCC 1428 and *E. carotovora* sub sp. *atroseptica* SCRI 1043) or 37 °C [*E. coli* BL21 (DE3) and *E. coli* DH5α] for 10-12 h. An aliquot of this culture was used to inoculate the main seed medium for production study or for preparation of competent cells [*E. coli* DH5α or *E. coli* BL 21 (DE3)]. The organisms were sub-cultured on LB-agar plate every month and stored at 4±1 °C and glycerol stock of organisms was stored at -80 °C.

3.4. Production of L-asparaginase by wild strains

The production of L-asparaginase by wild strains was performed in the modified basal semisynthetic medium containing (g l^{-1}): carbon source 3.0, $Na_2HPO_4.2H_2O$ 6.0; KH_2PO_4 3.0; NaCl 0.5; $MgSO_4.7H_2O$ 0.5; $CaCl_2.2H_2O$ 0.015; yeast extract 1.0; peptone 1.0 and initial pH was maintained at 6.5 (Gulati *et al.,* 1997). The inoculum was prepared by adding a loop full of freshly prepared pure culture on a slant into 50 ml of autoclaved above mentioned medium containing glucose as the sole source of carbon in a 250 ml Erlenmeyer flask. The culture

flask was incubated at 28 ºC and 180 rpm in an orbital shaking incubator for 10-12 h [Optical density (OD) at 600nm ~0.8]. A 2% of inoculum from the above seed culture was added to 50 ml of the medium containing 1.5 g l^{-1} L-asparagine in 250 ml Erlenmeyer flasks and incubated in an orbital shaking incubator at 28 °C and 180 rpm. Samples were withdrawn at regular intervals to determine cell growth and L-asparaginase activity.

3.5. Subcellular localization of L-asparaginase in wild strains

Samples were collected at regular intervals of time and cells were separated by centrifugation at 10,000*g* for 10 min at 4±1 ºC. The cells pellet was washed with 0.05 M Tris-HCl buffer (pH 8.5) and resuspended in the same volume of buffer. The cell-free supernatant was used to determine the extracellular activity of L-asparaginase. The suspended cells were divided into two equal portions. The first portion was ultrasonicated on ice (Model VC 505 Microprocessor based cell Ultrasonic processor, Sonics & Materials Inc, CT, USA) at 20 MHz, 30% amplitude, 4 cycles (2 min per cycles with 2 s and 1 s off) and the contents were centrifuged at 20,000*g* for 15 min (4±1 °C). The supernatant was analyzed for intracellular cytoplasmic L-asparaginase. The cell debris was resuspended in the same volume of Tris-HCl buffer (pH 8.5) and divided in to three equal parts. SDS, Triton X100 and EDTA were added to each part separately. The final concentration of SDS, Triton X100 and EDTA were 0.001% (w v^{-1}), 1.0% (v v^{-1}), and 5 mM, respectively. The cell debris were ultrasonicated on ice and incubated in a shaking incubator at 180 rpm and 25 °C for 30 min. The samples were ultracentrifuged at 100,000*g* for 30 min (4±1 °C) and the activity of membrane bound L-asparaginase was analyzed in the supernatant (Triantafillou *et al.* 1988; Geckil *et al.,* 2005). The second half of the pallet was used for the extraction of periplasmic fluid using cold

osmotic technique (Nossal and Heppel 1966). In this method, 1 g of the pellet was suspended in 40 ml of the periplasmic fluid extraction medium [0.033 M Tris-HCl (pH 8.0) with 30% sucrose and 1mM Na_2EDTA]. The mixture was stirred on a rotary shaker for 15 min at 30 °C and 180 rpm. The mixture was centrifuged at 10,000*g* at 4±1 ºC for 10 min. The supernatant fluid was removed and the pellet was rapidly dispersed in distilled water (1 g of wet cell per 40 ml of distilled water) and incubated in ice-cold for 15 min with vigorous agitation to minimize destabilization of the extracted enzyme. The osmotic shock treated cells were removed from the fluid by centrifugation at 20,000*g* for 15 min at 4±1 °C and resulting supernatant was analyzed for periplasmic L-asparaginase.

3.6. Analytical methods

3.6.1. Assay of L-asparaginase and L-glutaminase

Samples were centrifuged at 10,000*g* for 10 min at 4±1 ºC and washed twice with 0.05 M Tris-HCl buffer (pH 8.5) and ultrasonicated on ice (Model VC 505 Microprocessor based cell Ultrasonic processor, Sonics & Materials Inc, CT, USA) at 20 MHz, 30% amplitude, 4 cycles (2 min per cycles with 2 s on and 1 s off). The contents were centrifuged at 20,000*g* for 15 min (4±1 °C) and the supernatant was analyzed for intracellular L-asparaginase activity by modified method of Wriston (1970). L-asparaginase catalyzes the L-asparagine to L-aspartic acid and ammonia. Ammonia would react with the Nessler's reagent to produce an orange product. The enzyme assay mixture consisted of 900 µl of L-asparagine (10 mM) in Tris HCl buffer (pH 8.5) and 100 µl of crude extract of the enzyme. The reaction mixture was incubated at 37 °C for 30 min and 100 µl of 15% trichloroacetic acid (TCA) was added to stop the reaction. The reaction mixture was centrifuged at 10,000*g* for 5 min at room temperature to remove the precipitates and ammonia released in the supernatant was

determined colorimetrically by adding 100 µl Nessler reagent into sample containing 100 µl of supernatant of above reaction mixture and 800 µl distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min and OD at 425 nm was measured against the blank that received TCA before the addition of enzyme. The ammonia produced in the reaction was determined based on a standard curve obtained with ammonium sulfate as standard (1 unit OD at 425 nm = 14.12 U ml^{-1}). L-glutaminase activity was determined as described above for L-asparaginase activity using modified method of Mashburn and Wriston (1964). L-asparaginase activity (IU ml^{-1}) was defined as the micromoles of ammonia released in one minute by one ml of enzyme and specific activity is expressed as units per milligram of protein.

3.6.2. Protein determination

The total protein content of the samples was determined according to the method described by Lowry *et al*. (1951). The protein assay mixture consisted of 200 µl of diluted crude extract of enzyme (5 times) and 1 ml of freshly prepared complex forming reagent (2% Na_2CO_3 in 0.1 N NaOH : 1.0% $CuSO_4.5H_2O$: 2% potassium sodium tartrate ≡ 100:1:1). The contents in the sample were vortexed and incubated at room temperature for 10 min and 100 µl of freshly prepared diluted Folin reagent with distilled water (1:1) was added and vortexed. This reaction mixture was incubated in the dark condition at room temperature for 30 min. After 30 min. of incubation, developed blue color was measured as OD at 660 nm against the blank. The protein concentration in the reaction was determined based on a standard curve obtained with bovine serum albumin (Sigma) as standard (1 unit OD at 660 nm = 0.4829 mg ml^{-1} of protein).

3.6.3. Dry cell weight (DCW)

For preparation of standard curve of DCW, cells were centrifuged at 10,000*g* for 10 min at 4±1 ºC and the supernatant was discarded. The pellet was resuspended with same volume of Tris-HCl buffer (pH 8.5) and centrifuged at 10,000*g* for 10 min at 4±1 ºC. Cells were washed twice with the same buffer and supernatant free cells were used to determine the DCW. Different dilution of cell samples were used for measuring the cell OD (~0.1-1.0) at 600 nm and corresponding dry cell weight (DCW) was determined by centrifugation of culture in a pre-weighted microcentrifuge tube, followed by washing the cells twice with the buffer and drying to a constant weight at 80 ºC in an oven. DCW of the unknown sample was determined by measuring the cell OD at 600 nm using UV-visible spectrophotometer and compared with standard curve between OD at 600 nm vs. DCW (1 unit OD at 600 nm = 0.4358 g l^{-1} DCW).

3.6.4. Estimation of glucose

Glucose concentration in the samples was estimated by 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). DNS reagent was prepared by mixing 10 g of DNS and 0.5 g of sodium sulphite in 1 L of 0.25 N NaOH. The glucose estimation mixture consists of suitably diluted 500 µl of sample in the range of 0.1-1.0 g l^{-1} of glucose and 500 µl of DNS reagent in a tightly capped test tube to avoid evaporation. The reaction mixture was incubated in water bath at 95 ºC for 10 min to develop the red-brown color. After that 1 ml of 40% (w v^{-1}) potassium sodium tartrate solution was added to stabilize the color. Samples were allowed to cool down to room temperature and the absorbance was measured at 540 nm using UV-visible spectrophotometer against blank. The glucose concentration in the sample

was determined based on a standard curve obtained with glucose (0-1.0 mg ml^{-1}) as standard (1 unit OD at 540 nm = 0.6860 mg ml^{-1} of glucose).

3.6.5. Measurement of plasmid stability

Samples were taken at regular interval of time and diluted appropriately under aseptic conditions. A diluted sample was spread on a non-selective (amp^-) and selective (amp^+) LB agar plate. The plate was incubated at 37 °C for 16 h. The ratio of the number of colony forming units (CFU) on the selective agar plate to that on the non-selective agar was used to determine the percentage of plasmid-carrying cells, which was used as an index of plasmid stability (Goyal *et al*., 2009).

3.7. Cloning of genes encoding the L-asparaginase (*ans*), L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043

3.7.1. Isolation of genomic DNA

Genomic DNA was isolated from *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* subsp. SCRI 1043 using genomic DNA isolation kit (Sigma) as per protocol provided by manufacture. The purified genomic DNA was analyzed in agarose gel (0.6 %) and the quantity of genomic DNA was analyzed using spectrophotometer by measuring absorbance at 260 nm (1 OD= 50 µg ml^{-1}).

3.7.2. Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* using plasmid DNA isolation kit (Himedia, India) by following the manufacture's instruction provided with kits. The purified plasmid DNA was

analyzed in agarose gel (0.8 %) and the quantity of plasmid DNA was analyzed using spectrophotometer by measuring absorbance at 260 nm (1 OD= 50 µg ml^{-1}).

3.7.3. Preparation of competent cells of E. coli DH5α and E. coli BL21 (DE3)

A single isolated colony of *E. coli* DH5α or *E. coli* BL21 (DE3) was transferred in to LB broth and incubated at 37 °C at 180 rpm for 10-12h. The above culture (2% v v^{-1}) was transferred in to 50 ml of LB medium and incubated in a shaking incubator at 37 °C at 200 rpm. The culture was grown till its OD at 600nm reached ~0.5-0.6. The cells were harvested in sterile 50 ml oakridge tubes by centrifugation at 5,000*g* for 10 minutes at 4.0 °C and supernatant was discarded. The cells were gently resuspended in 1/10th of initial volume in TSS buffer [LB medium 85% (vv $^{-1}$), Polyethylene glycol (PEG) 10% (w v^{-1}), Dimethyl sulfoxide (DMSO) 5% (v v $^{-1}$), Magnesium chloride (MgCl2) 50 mM final]. Cells were incubated on ice for 30 minutes. After 30 minutes, 100 µl of aliquot of cells were dispensed in to sterile pre-chilled cryovials and stored at –80 ºC until further use (Chung *et al*., 1987).

3.7.4. Transformation of pET 22b (+) in to E. coli DH5α and E. coli BL21 (DE3)

Frozen aliquots of competent cells of *E. coli* DH5α and *E. coli* BL21 (DE3) were taken from –80 ºC and allowed to thaw on ice. The vector, pET 22b(+) (25 ng) was added to competent cells (in aseptic conditions) and mixed gently by tapping and incubated in ice for 45 min. After incubation, heat shock was given to the cells for 1.5 minutes at 42 ºC and tubes were kept on ice for 5 min. 900 µl of LB medium and 100 µl of 200 mM glucose was added to tubes and cells were allowed to grow at 37 ºC and 200 rpm for 1 h. The transformed culture was spread over LB-agar plates containing ampicillin (100 µg ml^{-1}). The plates were incubated at 37 °C for 14-16 h. Single isolated colony was inoculated in 8 ml of LB broth

supplemented with ampicillin (100 µg ml^{-1}) and incubated at 37 ºC. 1% of inoculum was transferred in 20 ml of LB medium supplemented with ampicillin (100 µg ml^{-1}). Plasmid DNA was isolated from the above culture and analyzed by agarose gel (0.8%) electrophoresis and quantified by measuring absorption at 260 nm.

3.7.5. Cloning of genes encoding the L-asparaginase (ans), L-asparaginase I (ansA) and L-asparaginase II (ansB2) of P. carotovorum MTCC 1428 and E. carotovora subsp. atroseptica SCRI 1043

There is no L-asparaginase gene sequence available in gene bank for *P. carotovorum* MTCC 1428. Therefore, primers were designated as per respective gene sequence available in the NCBI genome database of *E. carotovora* subsp. *atroseptica* SCRI 1043 (NCBI genome database: accession code BX950851). The segments encoding for the L-asparaginase (*ans*), L-asparaginase I (*ansA*), L-asparaginase II (*ansB2*) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were amplified by PCR using gene specific primers (Table 3.1). Conditions for amplification of genes are provided in Table 3.2. Restrictions sites were incorporated in forward and reverse primers for cloning the gene sequence in between specific restriction sites. To study the effect of 6x histidine tag at C-terminus of protein, each gene was cloned with and without stop codon to express the recombinant protein without and with 6xHis-tag, respectively. The PCR amplified products and pET22b(+) were double digested with *Bam*HI and *Xho*I. After ensuring the complete digestion by running small aliquots on agarose gel, double digested pET22b(+) was eluted from gel by gel extraction kit (Bangalore Genei, India) and double digested PCR product was purified using PCR purification kit (Bangalore Genie, India).

Table-3.1. Primers for the amplification of the genes encoding L-asparaginases of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043

Name	primer	Sequence	Restriction sites
L-asparaginase	Forward primer	5'- ggaattcggatccaatgacgaaacccgtgattgtg -3'	*Eco*RI and *Bam*HI
	Reverse Primer[a]	5'- gaagcttctcgagttaacgatagatatcggcgacg 3'	*Hind*III and *Xho*I
	Reverse Primer[b]	5'- gaagcttctcgagacgatagatatcggcgacggg - 3'	*Hind*III and *Xho*I
L-asparaginase I	Forward primer	5'- ggagctcggatccaatgcaaaagaaatccat -3'	*Sac*I and *Bam*HI
	Reverse Primer[a]	5'- ggaagcttctcgagtcaatctttatcgctcaa -3'	*Hind*III and *Xho*I
	Reverse Primer[b]	5'- ggaagcttctcgagatctttatcgctcaattc-3'	*Hind* III and *Xho*I
L-asparaginase II	Forward primer	5'- ggaattcggatccaatgcaactctcatttatcgcc -3'	*Eco*RI and *Bam*HI
	Reverse Primer[a]	5'- gaagcttctcgagttactgctcgaaataggtacg -3'	*Hind*III and *Xho*I
	Reverse Primer[b]	5'- gaagcttctcgagctgctcgaaataggtacggatt-3'	*Hind*III and *Xho*I

Reverse Primer[a]: primer for amplification of genes with stop codon.

Reverse Primer[b]: primer for amplification of genes without stop codon

Table 3.2. Conditions for the amplification of the genes encoding L-asparaginases of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043

Name	L-Asparaginase	L-Asparaginase I	L-Asparaginase II
Forward Primer	0.5µM	0.5µM	0.5µM
Backward Primer[a/b]	0.5µM	0.5µM	0.5µM
Template DNA	1.0 ng	1.5 ng	2.0 ng
dH_2O	10 µl	5 µl	-
dNTPs	0.2mM	0.2mM	0.2mM
Pfu DNA polymerase	1U	1U	1U
Pfu DNA polymerase buffer (5x)	10 µl	10 µl	10 µl
Denaturation temperature	94 °C for 30 Sec	94 °C for 30 Sec	94 °C for 30 Sec
Annealing temperature	55 °C for 45 Sec	55 °C for 45 Sec	55 °C for 45 Sec
Amplification temperature	72 °C for 60 sec	72 °C for 60 sec	72 °C for 60 sec
Last cycle extension	72 °C for 10 min	72 °C for 10 min	72 °C for 10 min
Total Cycles	30	30	30

Double digested PCR product and linearized double digested pET22b(+) were ligated using instant ligation kit from NEB (Cat No. M0202L). The reaction was set up in the molar ratio of 1:3 (vector: insert) in final volume of 20 µl as per the manufacturer's instruction

(http://www.neb.com/nebecomm/products/protocol2.asp). From this ligation mixture, 2.5 µl to 5 µl of ligation mix (~10 ng of DNA) was transformed aseptically in to *E. coli* DH5α as described earlier in the section 3.7.4. The transformed culture was spread over LB-agar plates containing ampicillin (100 µg ml^{-1}) and plates were incubated at 37 ºC for 14-16 h. Single colony was streaked on LB-agar plates containing ampicillin (100 µg ml^{-1}) and incubated at 37 °C for 14-16 h. A loop full culture grown from single colony was inoculated in to 8 ml of LB broth supplemented with ampicillin (100 µg ml^{-1}) and incubated at 37 °C. Plasmid DNA was isolated from the above culture and analyzed. Screening of the positive clones carrying gene of interest was done by colony PCR, by restriction digestion with *Bam*HI and *Xho*I, and analyzed by electrophoresis (1.0 % agarose gel). In addition, sequences were confirmed by sequencing of inserts using T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5'-CTAGTTATTGCTCAGCGGT-3') specific primers.

3.8. Expression of recombinant L-asparaginase (*ans*), L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in to *E. coli* BL21 (DE3)

3.8.1. Transformation of recombinant construct in to E. coli BL 21 (DE3)

The recombinant constructs with desired genes were transformed in to *E. coli* BL 21 (DE3) as described in the section 3.7.4 and plated on LB agar plates containing ampicillin (100 µg ml^{-1})

3.8.2. Expression of recombinant proteins

The active culture was prepared by inoculating frozen glycerol stock of recombinant strain (kept at -80 ºC) on a LB agar plate (yeast extract 5 g l^{-1}, tryptone 10 g l^{-1}, NaCl 10 g l^{-1}, 2% agar, pH 7.0), supplemented with ampicillin (100 µg ml^{-1}) and incubated at 37 ºC for 12 h. A single isolated colony was then transferred in to 20 ml of LB medium containing ampicillin, (100 µg ml^{-1}) and incubated on a rotary shaking incubator at 37 ºC and 200 rpm for 10 h. This pre-inoculum was transferred (2% vv^{-1}) in to 50 ml of LB medium supplemented with ampicillin (100 µg ml^{-1}) in 250 ml conical flasks and incubated in a rotatory shaking incubator at 37 °C and 200 rpm. The production of recombinant protein was induced by the addition of 1mM isopropyl β-D-thiogalactopyranoside (IPTG) (culture concentration= OD at 600 nm ~ 0.8) at different temperatures (25 °C, 30 °C and 37 °C). Samples were collected at different time intervals of post-induction and determined the profile of recombinant proteins expression. Cells were separated from fermentation broth by centrifugation at 10,000*g* at 4 ºC for 10 minutes. The harvested cells were ultrasonicated on ice (Model VC 505 Microprocessor-based cell ultrasonic processor, Sonics & Materials, CT, USA) at 20 MHz, 30% amplitude, two cycles (2 min per cycles with 2 sec. on and 1 sec. off) and centrifuged at 20,000g for 15 minutes at 4 ºC. The supernatant was analyzed for intracellular L-asparaginase as described in the section 3.6.1. Experiments were conducted in duplicates and enzyme assay was performed in duplicates for each sample. For checking the expression of recombinant proteins in soluble and insoluble fractions, after 6 or 8 h of post-induction, *E. coli* BL21 (DE3) cells were harvested by centrifugation. 100 mg of cell pellet was resuspended in 1.0 ml of lysis buffer [100 mM Tris HCl (pH 8.5), 100 mM NaCl, 5 mM EDTA (pH 8.0), and 1mM PMSF] and cells were sonicated on ice (Model VC

505 Microprocessor-based cell ultrasonic processor, Sonics & Materials, CT, USA) at 20 MHz, 30% amplitude, two cycles (2 min per cycles with 2 sec. on and 1 sec. off). After sonication, supernatant was separated by centrifugation at 20,000g for 15 minutes at 4 ^{o}C. The supernatant was analyzed for soluble recombinant proteins and sonicated cell pellet was used to check the presence of insoluble protein (inclusion body) according to the method described by Sambrook *et al.,* (1989). SDS–PAGE of recombinant proteins was performed in a 12.5% polyacrylamide gel under reducing conditions according to the method of Laemmli (1970). Proteins were reduced by treatment with 10% of 2-mercaptoethanol at 95 °C for 10 min and stained with coomassie brilliant blue R-250.

3.9. Subcellular localization of recombinant L-asparaginase II of *P. carototovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3)

The subcellular localization of recombinant L-asparaginase II of *P. carototovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3) cells was carried out as described in the section 3.5.

3.10. Selection of medium for higher expression of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora subsp. atroseptica* SCRI 1043

Experiments were performed to screen the best suitable medium for maximum production of recombinant L-asparaginase II (with His. tag) of both strains using different media [Luria–Bertani (LB), 2x YT, Terrific broth (TB), Super broth (SB), M9 minimal and

Reisenberg). The optimum temperature for expression of recombinant L-asparaginase II of both strains was found to be at 30 °C. Hence, further production studies were carried out at 30 °C. Samples were collected at different interval of time of post-induction to determine the profile of recombinant L-asparaginase II production.

3.11. Optimization of chemical and physical process parameters for the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora subsp. atroseptica* SCRI 1043 from *E. coli* BL21 (DE3)

3.11.1. Optimization of the chemical parameters by central composite design for the production of recombinant L-asparaginase II of P. carotovorum MTCC 1428 and E. carotovora subsp. atroseptica SCRI 1043

Maximum expression of recombinant L-asparaginase II of both the strains from *E. coli* BL21 (DE3) was observed in the LB medium. Therefore, LB medium was selected for optimization study. The central composite design (CCD) (Khuri and Cornell 1987) has been applied to optimize the levels and explain the combined effect of medium constituents (tryptone, yeast extract and NaCl) on the production of recombinant L-asparaginase II. Each variable (medium component) was assessed at five coded levels (-2, - 1, 0, +1, and +2) with 20 ($=2^k+2k+6$) combinations. Where, k is the number of independent variables (Araujo and Brereton 1996). The minimum and maximum ranges of the variables and the experimental plan with regard to their values in actual and coded form are presented in Table 3.3 and Table 3.4, respectively. The specific activity of recombinant L-asparaginase II was taken as the response.

For statistical calculations, the relation between the coded values and real values were described as given in the following Eq. 3.1:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \qquad i=1,2,3.....k \tag{3.1}$$

Where, x_i is the independent variable coded value, X_i is the real value of the independent variable, X_o is the real value of the independent variable on the center point, ΔX_i is the step change and the central point was set with a α of 1.682 for optimization of medium components. For predicting the optimal levels, a second-order polynomial equation was fitted to correlate the relationship between variables and response (Sp. activity). The quadratic model for predicting the optimal levels was expressed according to the Eq. 3.2:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \tag{3.2}$$

Where, Y is the predicted response (sp. activity), k is the number of variables. X_i *and* X_j are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the i^{th} quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient. Statistical analysis of the data was performed by statistical software package MINITAB® Release 15.1, PA, USA to evaluate the analysis of variance (ANOVA) for determining the significance of each term in the equations and to estimate the goodness of fit in each case. *P*-value (Probability > *F*) less than 0.05 indicated that the model terms are significant and adequacy of the method developed was further analyzed. Response surface plots were drawn for the experimental results obtained from the effect of different variables on recombinant L-asparaginase II production to determine the individual and cumulative effects of variables and the mutual interactions among them.

Table 3.3. Ranges and levels of the independent variables used in the optimization study

Variables	Symbol coded	Range and levels				
		-2	-1	0	+1	+2
Tryptone (g l^{-1})	X_1	0.23	5.00	12.00	19.00	23.78
Yeast Extract (g l^{-1})	X_2	0.27	3.00	7.00	11.00	13.73
NaCl (g l^{-1})	X_3	0.23	5.00	12.00	19.00	23.78

Table 3.4. A 2^3 full-factorial central composite design matrix in coded units and real values (parenthesis)

Run Order	Uncoded and Coded levels		
	Tryptone (X_1) (g l^{-1})	Yeast Extract ($X2$) (g l^{-1})	NaCl ($X3$) (g l^{-1})
1	5.00 (-1)	3.00 (-1)	5.00 (-1)
2	19.00 (+1)	3.00 (-1)	5.00 (-1)
3	5.00 (-1)	11.00 (+1)	5.00 (-1)
4	19.00 (+1)	11.00 (+1)	5.00 (-1)
5	5.00 (-1)	3.00 (-1)	19.00 (+1)
6	19.00 (+1)	3.00 (-1)	19.00 (+1)
7	5.00 (-1)	11.00 (+1)	19.00 (+1)
8	19.00 (+1)	11.00 (+1)	19.00 (+1)
9	0.23 (-2)	7.00 (0)	12.00 (0)
10	23.78 (+2)	7.00 (0)	12.00 (0)
11	12.00 (0)	0.27 (-2)	12.00 (0)
12	12.00 (0)	13.73 (+2)	12.00 (0)
13	12.00 (0)	7.00 (0)	0.23 (-2)
14	12.00 (0)	7.00 (0)	23.77 (+2)
15	12.00 (0)	7.00 (0)	12.00 (0)
16	12.00 (0)	7.00 (0)	12.00 (0)
17	12.00 (0)	7.00 (0)	12.00 (0)
18	12.00 (0)	7.00 (0)	12.00 (0)
19	12.00 (0)	7.00 (0)	12.00 (0)
20	12.00 (0)	7.00 (0)	12.00 (0)

3.11.2. Optimization of the process parameters by CCD for the production of recombinant L-asparaginase II of P. carotovorum MTCC 1428 and E. carotovora subsp. atroseptica SCRI 1043

Initial pH of the medium, inoculum size and rpm of the shaking incubator were considered to enhance the production of recombinant L-asparaginase II. The optimization of process parameters was performed using 2^3 full factorial central composite designs (CCD) with 6 axial points, resulting in a total number of twenty experiments, which covers the entire range of combinations of variables. The number of treatment combinations were 20 ($=2^k+2k+6$), where, k is the number of independent variables (Araujo and Brereton 1996). The range, level of variables and full experimental plan with regard to their values in actual and coded form are shown in Table 3.5 and Table 3.6, respectively.

Table 3.5. Ranges and levels of the independent variables used in the optimization of process parameters by CCD.

Variables	Symbol used	Range and levels				
		-2	-1	0	+1	+2
Initial pH	X_1	6.16	6.5	7	7.5	7.84
Inoculums size (%, v/v)	X_2	1.32	2	3	4	4.68
Agitation (rpm)	X_3	132.37	160	200	240	267.27

The relationship among the variables, *i.e.* initial pH of medium, inoculum size and rpm of the shaking incubator were expressed mathematically in the form of a quadratic model (Eq. 3.2), which gave the response as a function of the independent variables. Where, Y is the response (specific activity), β_0 the constant coefficient, X_i (i = 1–3) are non-coded variables (initial pH of medium X_1, inoculum size (%) X_2 and agitation speed X_3), β_i are the linear, β_{ii} are the quadratic, and β_{ij} (i and j = 1–3) are the second-order interaction coefficients.

The statistical software package, MINITAB® Release 15.1, PA, USA was used for the regression analysis of the experimental data and also to plot the response surface graphs. The *F*-test was used to evaluate the significance of the model.

Table 3.6. A 2^3 full-factorial CCD matrix in real values and coded units (in parenthesis)

Run Order	Uncoded and Coded levels		
	pH (X_1)	Inoculum size (X_2) (%)	Agitation (X_3) (rpm)
1	6.50 (-1)	2.00 (-1)	160.00 (-1)
2	7.50 (+1)	2.00 (-1)	160.00 (-1)
3	6.50 (-1)	4.00 (+1)	160.00 (-1)
4	7.50 (+1)	4.00 (+1)	160.00 (-1)
5	6.50 (-1)	2.00 (-1)	240.00 (+1)
6	7.50 (+1)	2.00 (-1)	240.00 (+1)
7	6.50 (-1)	4.00 (+1)	240.00 (+1)
8	7.50 (+1)	4.00 (+1)	240.00 (+1)
9	6.16 (-2)	3.00 (0)	200.00 (0)
10	7.80 (+2)	3.00 (0)	200.00 (0)
11	7.00 (0)	1.30 (-2)	200.00 (0)
12	7.00 (0)	4.70 (+2)	200.00 (0)
13	7.00 (0)	3.00 (0)	133.00 (-2)
14	7.00 (0)	3.00 (0)	267.00 (+2)
15	7.00 (0)	3.00 (0)	200.00 (0)
16	7.00 (0)	3.00 (0)	200.00 (0)
17	7.00 (0)	3.00 (0)	200.00 (0)
18	7.00 (0)	3.00 (0)	200.00 (0)
19	7.00 (0)	3.00 (0)	200.00 (0)
20	7.00 (0)	3.00 (0)	200.00 (0)

3.11.3. Validation of the model at predicted optimum levels

In order to validate the model, experiments were performed at optimal levels of initial pH of the medium, inoculum size and rpm of the shaking incubator. All experiments were conducted in duplicates and averages of the results were taken as response.

3.12. Comparison between IPTG and lactose induction

To compare the efficiency of induction by IPTG or lactose, a comparative study on the expression of recombinant L-asparaginase II of both the strains was carried out. Expression of recombinant L-asparaginase II of both the strains was induced by the addition of either IPTG or lactose at different concentrations (0.1 mM to 2.5 mM).

3.13. Effect of glucose and glycerol on the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043

In order to study the effect of initial concentration of glucose and glycerol on the production of recombinant L-asparaginase II of both the strains, experiments were performed at different concentrations of glucose and glycerol under previously optimized production conditions.

3.14. Production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in batch and fed batch bioreactor

3.14.1. Inoculum development

A loop full of frozen glycerol culture stock (kept at -80 °C) was streaked on a LB-agar plate containing ampicillin (100 μg ml^{-1}) and incubated at 37 °C for 14–16 h. A single isolated

colony was transferred in to 20.0 ml of sterile LB medium supplemented with 100 mg ml^{-1}ampicillin in a 100 ml Erlenmeyer flask on a rotary shaker at 37 °C and 200 rpm for 6–8 h (when cell OD at 600 nm was ~0.8). This pre-inoculum was transferred (2% v v^{-1}) in to the main inoculum medium and incubated for 8-10 h. The seed culture (2.5% v v^{-1}) was transferred to the production medium (1.0 L) in 3.0 L bioreactor.

3.14.2. Production of recombinant L-asparaginase II of P. carotovorum MTCC 1428 and E. carotovora subsp. atroseptica SCRI 1043 in a batch bioreactor

The production of any metabolite varies from shake flask to bioreactor system due to agitation pattern, uncontrolled pH and aeration in shake flask fermentation. It was reported that the addition of glucose less than 0.05%, improves the plasmid stability and protein yields (Zhang *et al*., 2003; Studier *et al*., 2005). Production of any metabolite in a bioreactor is significantly influenced by agitation, uncontrolled/controlled pH, agitation and aeration. Initially, the effect controlled and uncontrolled pH on the production of L-asparaginase II was studied. It was observed that the production of recombinant protein was higher in controlled pH as compared to un-controlled pH conditions. Therefore, production of recombinant L-asparaginase II was carried out at different levels of controlled pH. Taguchi's method was applied to evaluate and optimize significantly influencing parameters for the production of recombinant L-asparaginase II of both the strains in a batch bioreactor. According to Taguchi's orthogonal array of three variables (glucose, controlled pH and DO), nine experiments were performed to evaluate the influence of various parameters on recombinant L-asparaginase II production of both the strains. The variables and their levels employed in Taguchi's robust experimental design are given in Table 3.7. The levels of (glucose, pH and DO) were varied according to the experimental plan given in Table 3.8.

Table 3.7. Variables and their levels employed in the Taguchi's robust design method for optimal recombinant L-asparaginase II production in batch bioreactor

Variable	Constituents	Levels		
		1	2	3
A	Glucose (g l^{-1})	0.5	1.0	1.5
B	pH	6.5	7.0	7.5
C	DO (%)	10	25	40

Table 3.8. Optimization of recombinant L-asparaginase II production of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in a batch bioreactor by Taguchi's method

Run No.	Glucose (g l^{-1})	pH	DO (%)
1	0.5	6.5	10
2	0.5	7.0	25
3	0.5	7.5	40
4	1.0	6.5	25
5	1.0	7.0	40
6	1.0	7.5	10
7	1.5	6.5	40
8	1.5	7.0	10
9	1.5	7.5	25

In each experimental run, the response was recorded as the recombinant L-asparaginase II production (U ml^{-1}) and corresponding signal-to-noise (S/N) ratio was calculated using Eq. 3.3 with an overall objective of estimating the effects of various parameters on recombinant L-asparaginase II production, where a large S/N ratio is preferred.

$$\frac{S}{N} = -10\log\left(\frac{\frac{1}{y^2}}{n}\right) \tag{3.3}$$

Where, y is the response [enzyme activity (U ml^{-1})] and *n* is the number of experimental runs. Statistical analysis of the results in the form of analysis of variance (ANOVA) was performed.

To verify the validity of the model, experiment was conducted at optimal levels of parameters in a batch bioreactor. All batch experiments were conducted in a 3.0 L Bioreactor (Applikon, Holland) with 1.0 L working volume. The bioreactor was fitted with necessary controllers. The pH of the medium was adjusted with 2 M NaOH and/or 2 M HCl. The levels of pH (controlled), the DO was maintained according to experimental plan by varying airflow and impeller speed by cascade mode of operation. The production of recombinant L-asparaginase II was induced with 1mM IPTG when cell density at Ab_{600nm} reached to 1.50 -1.8 (after 3 h) and further cultivated for 12 h at 30 °C. The samples were withdrawn at regular intervals of time and enzyme activity was measured in duplicates.

3.14.3. Fed-batch cultivation

To maximize the cell density, production and productivity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 from *E. coli*, experiments were performed in fed-batch culture (3.0 L bioreactor (Applikon, Holland)). Fed-batch cultivations were started as batch cultures (1.0 L) in the previously optimized production conditions. To find out the best strategy, different feeding approaches were applied *viz.*, exponential feeding, constant feeding and intermittent pulse feeding.

3.14.3.1. Exponential feeding

Exponential feeding method allows cells to grow at a constant specific growth rate and also provides the advantage that acetate production can be minimized by controlling the specific growth rate below the critical value of acetate formation (Nayak and Vyas, 1999). After the exhaustion of initial glucose, exponential feeding was started with the solution containing 200 g l^{-1} of glucose, 200 g l^{-1} of yeast extract and 100 mg l^{-1} ampicillin in growth phase. When the

cell concentration reached to ~10.00 (OD at 600 nm), the recombinant L-asparaginase II expression was induced by adding 1mM IPTG and temperature was adjusted to 30 °C. In production phase, feeding was carried out with the solution containing 100 g l^{-1} of glucose, 100 g l^{-1} of yeast extract and 100 mg l^{-1} ampicillin. Feeding was performed by assuming quasi-steady-state with the following substrate balance equation.

$$F = \frac{\mu XoVo\exp(\mu t)}{YSo} \quad (3.4)$$

Where, F is the feeding rate (l h^{-1}), μ the specific growth rate (0.20 h^{-1}), X_o the cell concentration at the time of starting the fed-batch (g l^{-1}), V_o the initial (batch culture) volume (l), t the cultivation time after initiation of the fed-batch culture (h), S_o is the substrate concentration in feeding solution and Y is the yield coefficient (0.60 g DCW g^{-1} glucose) pre-determined from batch experiments. After induction, feeding was continued for another 12 h.

3.14.3.2. Constant feeding

Fed-batch cultivation was started after the exhaustion of glucose in batch culture, feeding was started at a constant rate of feeding. The feed medium used for constant feeding contained 100 g l^{-1} of glucose, 100 g l^{-1} of yeast extract and 100 mg l^{-1} ampicillin. Feeding of the glucose was carried out at constant rate of 2.0 g glucose l^{-1} h^{-1} (Goyal *et al*., 2009; Ramalingam and Gautam, 2007). When the cell concentration reached to ~8.00 (OD at 600 nm), the recombinant L-asparagianse II expression was induced by adding 1mM IPTG at 30 ^{o}C and feeding was continued for another 12 h.

3.14.3.3. Intermittent pulse feeding

In intermittent pulse feeding, concentration of glucose in the medium was monitored at regular interval of time and fed in a stepwise-increasing manner in proportional to the

biomass (Ramalingam and Gautam, 2007; Nayak and Vyas, 1999; Giridhar and Srivastava 2000). The feed medium used for intermittent pulse feeding contained 100 g l^{-1} of glucose, 100 g l^{-1} of yeast extract and 100 mg l^{-1} ampicillin. In first experiment, residual glucose was maintained below 2.0 g l^{-1} and feeding of glucose with specific feed rate of 0.50 g glucose (g DCW) $^{-1}$ h^{-1} was carried out. In the second experiment, feeding of glucose with specific feed rate of 0.5 g glucose (g DCW)$^{-1}$ h^{-1} and 0.25 g glucose (g DCW)$^{-1}$ h^{-1} was carried out in the growth phase and production phase, respectively, and the residual glucose was maintained below 1.0 g l^{-1}. The biomass concentration in the bioreactor (X) was calculated throughout the fermentation by measuring the optical density (OD) as well as the dry cell weight. When the cell concentration was reached to ~10.00 (OD at 600nm), the recombinant L-asparaginase II expression was induced by adding 1mM IPTG at 30 °C and feeding was changed according to experiment and continued at the similar mode for another 12 h.

In all the fed batch experiments, DO was controlled between 35-40% of saturation by cascade mode of operation (350-1200 rpm) and aeration (2.0-3.0 vvm) and the pH was controlled at 7.0 by the addition of 2N HCl and/or 2N NaOH.

After finding out the best strategy for production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 from *E. coli*, same feeding strategy was employed for the production of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli.*

3.15. Purification of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* BL21 (DE3)

3.15.1. Enzyme production for purification

A loop full of frozen culture in glycerol stock (kept at -80 °C) was streaked on a LB-agar plate containing ampicillin (100 μg ml^{-1}) and incubated at 37 °C for 14–16 h. A single isolated colony was transferred in to 10.0 ml of sterile LB medium supplemented with 100 mg ml^{-1}ampicillin in a test tube and incubated in an orbital shaking incubator at 37°C and 200 rpm for 6–8 h. This pre-inoculum was transferred (2% v v^{-1}) to the main inoculum medium and incubated for 8-10 h. The seed culture (2.5% v v^{-1}) was transferred to the 50 ml of the optimized production medium in 250 ml Erlenmeyer flask and incubated in an orbital shaking incubator under optimized conditions. The recombinant protein was induced (at cell OD at 600 nm ~0.8) by the addition of 1.0 mM IPTG to the culture medium.

3.15.2. Isolation and purification

After 6 h of post induction, cells were harvested from 50 ml of culture broth by centrifugation and washed twice with 50 mM Tris-HCl (pH 8.5) and the pellet was suspended in 5.0 ml of 50 mM sodium phosphate buffer (pH 7.0, 500 mM NaCl, 10 mM imidazole), and ultrasonicated on ice (Model VC 505 Microprocessor based cell Ultrasonic processor, Sonics & Materials Inc, CT, USA) at 20 MHz, 30% amplitude, 4 cycles (2 min per cycles with 2 s and 1 s off). After ultrasonication, the contents were centrifuged at 20,000*g* for 15 min (4±1 °C). The supernatant was loaded onto a 2.0 ml Ni affinity column equilibrated with 50 mM sodium phosphate buffer (pH 8.0, 500 mM NaCl, 10 mM imidazole). After

30 min, the column was washed with 20 ml of the buffer containing 20 mM imidazole. The protein was eluted with 200 mM imidazole and dialyzed in 50 mM Tris–HCl buffer for 24 h. After dialysis, the enzyme activity and protein concentration of the purified L-asparaginase was determined.

3.16. Characterization of purified recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* BL21 (DE3)

3.16.1. Effect of pH on activity and stability of purified enzyme

The effect of pH on the activity of purified recombinant L-asparaginase II of both the strains was determined under assay conditions over a pH range of 5.5-10.5. For pH stability determination, enzyme preparations were incubated at different pH (5.5, 6.5, 7.5, 8.5, 9.5 and 10.5) for 24 h at 4±1 °C in the absence of substrate and residual activity was determined.

3.16.2. Effect of incubation temperature and time on activity of purified enzyme

To study the effect of incubation temperature on the activity of recombinant L-asparaginase II of both the strains, enzyme assay was performed at different temperatures ranging from 27 to 57 °C. To study the effect of the incubation time, enzyme was incubated separately with substrate for 15, 30, 45, 60, 75 and 90 minutes under the standard conditions and then enzyme activity was measured.

3.16.3. Effect of ionic strength

To study the effect of ionic strength on the activity of recombinant L-asparaginase II of both the strains, the activity of the enzyme was determined at different levels of ionic strength of buffer (5 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM).

3.16.4. Effect of various effectors on L-asparaginase activity

The activity of recombinant L-asparaginase II of both the strain was determined in the presence of different modifiers (metal ions, Iodoacetamide, L-histidine, gltutathione, 2-marceptoethanol, EDTA, L-cystine, SDS and urea). The purified enzyme was incubated with various modifiers for 30 min, and the residual activities were determined. The relative activity was expressed as the percentage ratio of the activity of the enzyme incubated with metal ions to that of the untreated enzyme. All of the metal ions (Hg^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}+, K^{2+}, Fe^{3+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+}) were in the form of chloride.

3.16.5. Substrate specificity

The activity of recombinant L-asparaginase II of both strains was monitored using various substrates (at a final concentration of 10 mM) *viz*., D-asparagine, DL-asparagine, L-glutamine, D-glutamine, D-aspartic acid, DL-aspartic acid, L-glutamic acid, succinamic acid, L-aspartic acid amide, L-asparagine-t-butyl ester HCl, BOC-L-asparagine and N-α-acetyl-L-asparagine. The enzymatic activity was carried out according to the method described in the section 3.16.4.

3.16.6. Determination of kinetic parameters

The Michaelis constant (K_m), maximal velocity (V_{max}) and Turnover numbers (k_{cat}) of the purified recombinant L-asparaginase II of both the strains were determined using L-asparagine as

substrate in the range of 0.05 to 2.5 mM. The reported reaction velocity is the mean of at least four measurements, which were normalized relative to a blank. The kinetic parameters K_m, V_{max} and k_{cat} were calculated by non-linear regression analysis of experimental steady-state data to the Michaelis–Menten equation using the computer program GraFit, Erithacus Software (Leatherbarrow 1998). Turnover numbers were calculated on the basis of one active site per 37.5 kDa subunit by SDS-PAGE.

3.16.7. Electrophoresis

Native PAGE of the purified recombinant L-asparaginase II of both strains was performed using 7.5 % polyacrylamide gel in glycine buffer (pH 10.0) at 5±1 ºC as described by Gallagher (1999). SDS-PAGE was performed by following the modified method of Laemmli (1970) with a 12.5 % separating acrylamide gel (pH 8.8) with a 5% stacking gel (pH 6.8) containing 0.1% SDS. After electrophoresis in a Tris-glycine buffer (pH 8.3) at 120 V for 1.5 h at room temperature, the proteins in the gel were stained with coomassie brilliant blue R-250. Subunit molecular weight and intact molecular weight of L-asparaginase were determined using standard molecular weight markers in SDS–PAGE and Native PAGE, respectively.

3.16.8. Effect of pH and temperature on the performance of purified recombinant L-asparaginase II of P. carotovorum MTCC 1428 and E. carotovora subsp. atroseptica SCRI 1043 under assay condition

The central composite design (CCD) was applied to optimize the levels and explain the combined effect of pH and temperature on the performance of purified recombinant L-asparaginase II of both the strains under assay conditions. Each variable (pH and

temperature) was assessed at five coded levels (-α, - 1, 0, +1, and +α). The minimum and maximum ranges of the variables are used and the full experimental plans with regard to their values (actual and coded) are provided in Table 3.9 and Table 3.10, respectively.

Table 3.9. Experimental ranges and levels of pH and temperature for optimization of recombinant L-asparaginase II activity

Variables	Range and levels				
	-α	**-1**	**0**	**+1**	**+α**
pH	6.1	6.5	7.0	7.5	8.9
Temperature	37.9	40	45	50	52.1

Table 3.10. Experimental design at various combinations of pH and temperature

Run order	Uncoded and coded levels	
	pH	**Temperature (°C)**
1	6.5 (-1)	40 (-1)
2	8.5 (+1)	40 (-1)
3	6.5 (-1)	50 (+1)
4	8.5 (+1)	50 (+1)
5	7.5 (0)	45 (0)
6	7.5 (0)	45 (0)
7	7.5 (0)	45 (0)
8	6.1 (-2)	45 (0)
9	8.9 (+2)	45 (0)
10	7.5 (0)	37.9 (-2)
11	7.5 (0)	52.1 (+2)
12	7.5 (0)	45 (0)
13	7.5 (0)	45 (0)
14	7.5 (0)	45 (0)

According to this design, the total number of treatment combinations were 14 ($=2^k + 2k + 6$). Where, k is the number of independent variables (Araujo and Brereton 1996). CCD with four axial points ($\alpha = 1.414$) and six replicates at the center point with a total number of 14 experiments were employed to evaluate the error. The quadratic model for predicting the

optimal levels was expressed according to the Eq. (3.2). Where, Y is the predicted response, k is the number of variables. X is the coded levels of the independent variables, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the i^{th} quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficients of pH and temperature. The statistical software package, MINITAB® Release 15.1, PA, USA was used for the regression analysis of the experimental data, and also to plot the response surface graphs. All the experiments were performed in duplicates and averages of the results were taken as the response.

3.16.9. Deactivation studies

To study the thermal stability of purified recombinant L-asparaginase II of both strains, the enzyme was incubated at different combinations of pH and temperature. The levels of pH and range of temperature selected to study the deactivation of recombinant L-asparaginase II enzymes were 6.5, 7.5, 8.5, 9.5 and 35-55°C, respectively. The enzyme samples were deactivated at various combinations of pH and temperature as given in the Table 3.11 and aliquots of samples were collected at different intervals of time, and the residual activity of recombinant L-asparaginase II was measured.

Table 3.11. Experimental condition adopted to study the deactivation of purified recombinant L-asparaginase II at different pH and temperature combinations

pH	**Temperature (^oC)**				
6.5	35	40	45	50	55
7.5	35	40	45	50	55
8.5	35	40	45	50	55
9.5	35	40	45	50	55

3.16.10. Estimation of deactivation rate constant (k_d) and half-life time ($t_{1/2}$)

Since the deactivation of L-asparaginase is one of the major constraints in the efficiency of chemotherapy, a better understanding of the mechanism of deactivation is very important. The deactivation of L-asparaginase is assumed to follow first-order kinetics. This is called single step two-stage theory (Naidu and Panda 2003; Sadana 1995). The two-state mechanism is as follows:

$$E \rightarrow E_d \tag{3.5}$$

The assumption in the mechanism is that the active enzyme state E directly converts to inactive state E_d without providing any significant amount of intermediates. The first-order deactivation is represented as:

$$\frac{dE}{dt} = -k_d[E] \tag{3.6}$$

Integration of Eq. (3.6) leads to:

$$\alpha = \exp(-k_d t) \tag{3.7}$$

Where, $\alpha = \frac{E_d}{E}$

From the plot of $\ln(\alpha)$ *vs.* t, the slope gives the value of deactivation rate constant k_d. The half-life of an enzyme is defined as the time required by the enzyme to loose half of its initial activity, which is given by:

$$t_{1/2} = \frac{\ln(2)}{k_d} \tag{3.8}$$

3.16.11. Estimation of thermodynamic parameters

The energies and entropies of deactivation were estimated by making use of absolute reaction rates (Eyring 1935; Naidu and Panda 2003). The temperature dependency of deactivation rate constant can be expressed as:

$$k_d = \frac{\kappa T}{h} \exp\frac{\Delta S^*}{R} \exp\frac{-\Delta H^*}{RT} \tag{3.9}$$

or,

$$\ln\frac{k_d}{T} = \ln\frac{\kappa}{h} + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT} \tag{3.10}$$

The values of ΔH^* and ΔS^* were calculated from the slope and intercept of the plot of $\ln(k_d/T)$ versus $1/T$ respectively. ΔG^* was estimated by the following relationship

$$\Delta G^* = \Delta H^* - T\Delta S^* \tag{3.11}$$

The activation energy is calculated from the following Arrhenius equation

$$k_d = k_0 \exp\frac{-E}{RT} \tag{3.12}$$

or,

$$\ln(k_d) = \ln(k_0) - \frac{E}{RT} \tag{3.13}$$

The values of E and k_o were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus $1/T$, respectively.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Selection of microorganism

Among the tested strains, *E. aroideae* NRRL B 136 (9.27 U mg^{-1}), *E. carotovora* subsp. *atroseptica* SCRI 1043 (9.84 U mg^{-1}) and *P. carotovorum* MTCC 1428 (10.64 U mg^{-1}) were selected for the maximum production of glutaminase free-L-asparaginase (Fig. 4.1). L-asparaginases with high specificity towards L-asparagine and low-to-negligible activity against L-glutamine were reported to be less troublesome during the course of anti-cancer therapy (Hawkins *et al.,* 2004). Most of the L-asparaginases from various microorganisms possess partial glutaminase activity, which causes various side effects during therapy (Distasio *et al.,* 1982; Gallagher *et al.,* 1989; Muller and Boos, 1998; Willis and Woolfolk, 1974). The absence of glutaminase activity would minimize the risk factor for successful clinical studies. Hence, the existence of glutaminase activity was tested from selected microorganisms. The substrate specificity of the L-asparaginase from *E. aroideae* NRRL B 136, *E. carotovora* subsp. *atroseptica* SCRI 1043 and *P. carotovorum* MTCC 1428 was observed to be very specific for its natural substrate, L-asparagine and no glutaminase activity was observed with L-glutamine as substrate. Therefore, the glutaminase free L-asparaginase reported in this study will be an advantageous and value-added product. Further, subcellular localization studies were carried out.

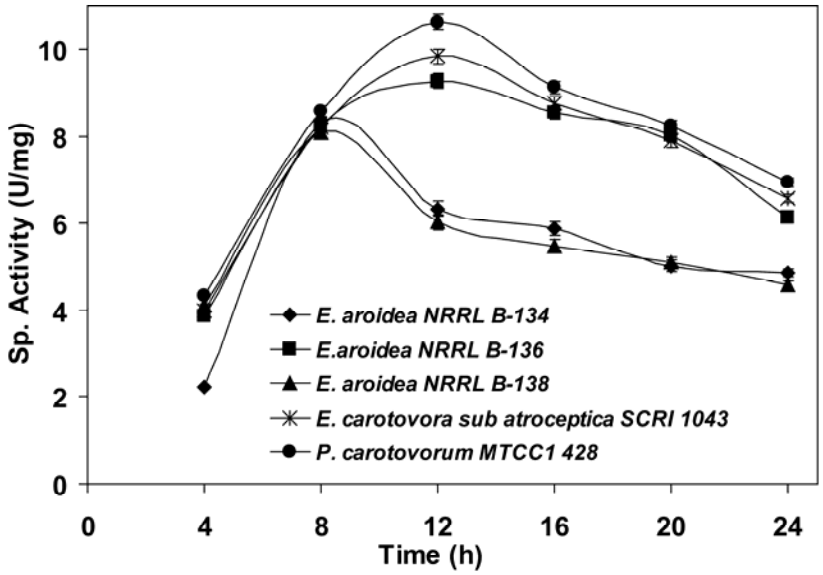

Fig. 4.1. Production of L-asparaginase by various strains (*E. aroidea* NRRL B 134, *E. aroidea* NRRL B 136, *E. aroidea* NRRL B 138, *E. carotovora* subsp. *atroseptica* SCRI 1043, *P. carotovorum* MTCC 1428).

4.2. Subcellular localization of L-asparaginase

Localization of L-asparaginase from *E. aroideae* NRRL B 136 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was carried according to the method described in the section 3.5. Localization of L-asparaginase from *P. carotovorum* MTCC 1428 has been performed by Kumar *et al*., 2010. There was no trace of extracellular L-asparaginase activity observed in the culture filtrates, which implied that the enzyme is an intracellular product in all these microorganisms. The solubilization of membrane bound enzyme was carried out using SDS, Triton X-100 and EDTA as described in the section 3.5. The maximum enzyme activity of L-asparaginase was observed in the membrane bound fraction using 0.001% SDS in all the microorganisms. The specific activity of L-asparaginase was varied from 1.46 to 7.89 U mg^{-1} of protein in various fractions (cytoplasm, periplasm and membrane bound) of microorganisms (Fig. 4.2). The existence of L-asparaginase in the membrane fraction of *Tetrahymena pyriformis,* and periplasmic space of *Enterobacter aerogenes* and

P. aeruginosa have been reported (Geckil *et al.,* 2005; Triantafillou *et al.,* 1988). There are reports on the production of L-asparaginase from *E. coli* (Cedar and Schwartz, 1967), filamentous fungi of genera, *Aspergillus, Penicillium* and *Fusarium* (Sarquis *et al.,* 2004*), Vibrio succinogenes* (Albanese and Kafkewitz, 1978; Kafkewitz and Goodman, 1974), *E. aroideae* (Peterson and Ciegler, 1969a)*, Serratia marcescens* (Heinemann and Howard 1969; Sukumaran *et al.,* 1979)*, E. aerogenes* (Geckil and Gencer, 2004; Mukherjee *et al.,* 2000) and *P. aeruginosa* (Abdel-Fattah and Olama, 2000; El-Bessoumy *et al.,* 2004; Geckil *et al.,* 2004). However, there is no literature available on the localization of L-asparaginase from *E. aroideae* NRRL B 136 and *E. carotovora* subsp. *atroseptica* SCRI 1043.

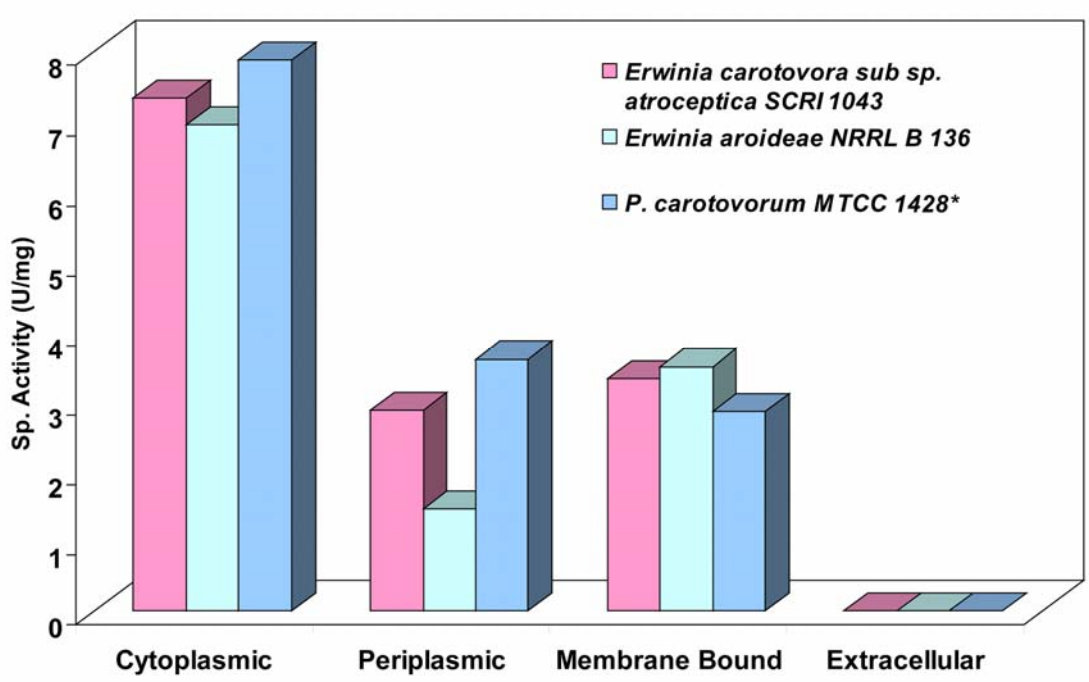

Fig. 4.2. Localization of L-asparaginase in *E. aroideae* NRRL B 136, *E. carotovora* subsp. *atroseptica* SCRI 1043 and *P. carotovorum* MTCC 1428 (* Kumar *et al*., 2010).

The production of L-asparaginase from *E. coli* (Cedar and Schwartz, 1967), *V. succinogenes* (Kafkewitz and Goodman, 1974) and *S. cerevisiae* (Jones, 1977) was found to be

constitutive both in periplasmic and cytoplasmic fractions. Among these strains, *E. carotovora* subsp. *atroseptica* SCRI 1043 and *P. carotovorum* MTCC 1428 have produced higher levels of periplasmic glutaminase-free L-asparaginase, which is considered to be type II L-asparaginase (L-asparaginase II) and used as anticancer agent. Therefore, *E. carotovora* subsp. *atroseptica* SCRI 1043 and *P. carotovorum* MTCC 1428 were considered for further studies.

4.3. Cloning of genes encoding L-asparaginase (*ans*), L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043

4.3.1. Amplification of genes by PCR

Amplification of L-asparaginase (*ans*), cytoplasmic L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) were carried out with gene specific primers. To study the effect of 6xHis-tag on expression of recombinant proteins, amplification of each gene was performed with and without stop codon for expressing the recombinant proteins without and with 6xHis-tag, respectively. After amplification of the genes by PCR, products of expected sizes of *ans* (960 bp), *ansA* (1020 bp) and *ansB2* (1050 bp) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were observed in agarose gel (1.0%) electrophoresis as shown in Fig. 4.3 A-D.

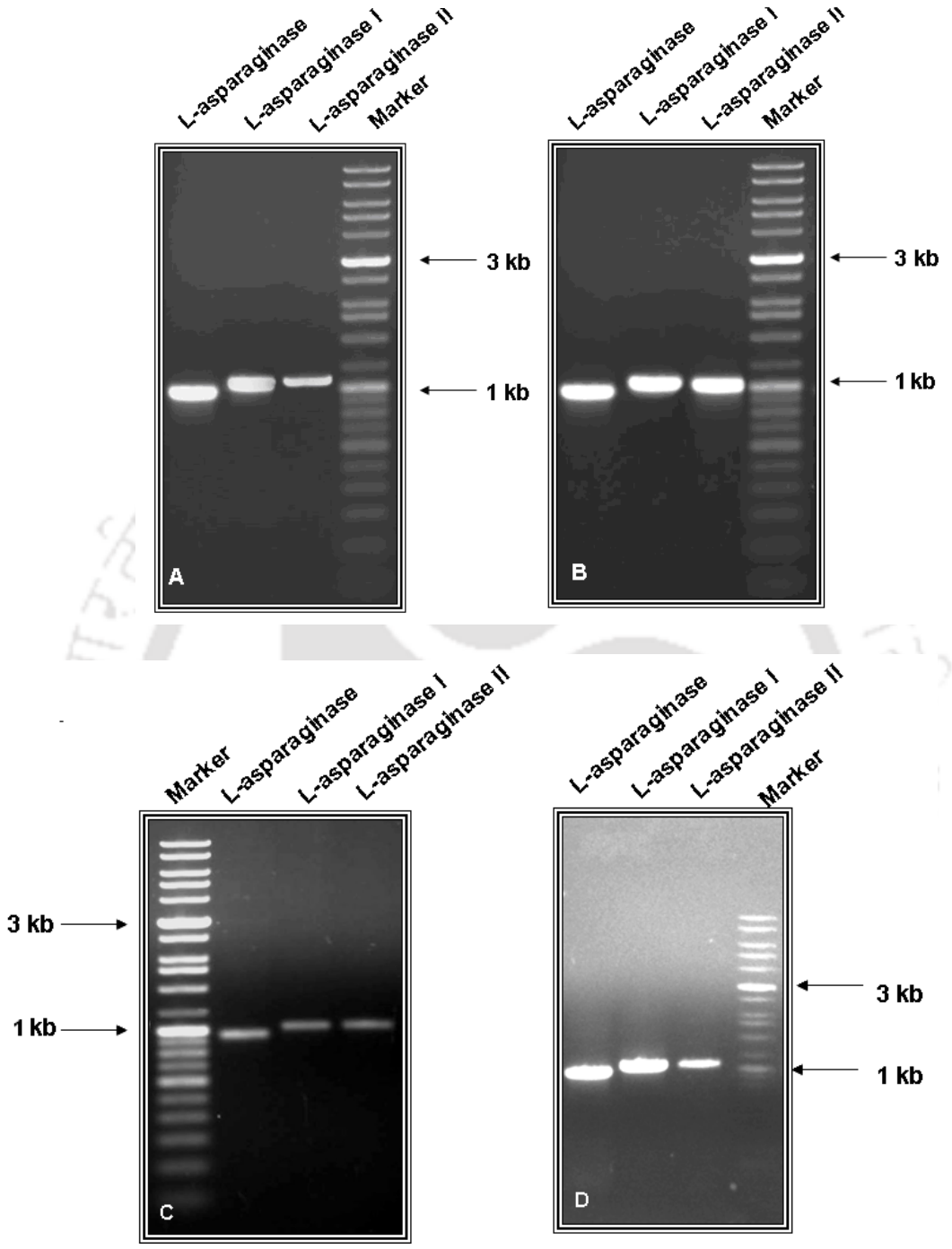

Fig. 4.3. PCR amplification of genes encoding L-asparaginase (**A** and **B**: PCR amplification of L-asparaginase, L-asparaginase I and L-asparaginase II of *P. carotovorum* MTCC 1428 with stop codon and without stop codon, respectively. **C** and **D**: PCR amplification of L-asparaginase, L-asparaginase I and L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 with stop codon and without stop codon, respectively).

4.3.2. Cloning of L-asparaginase encoding genes (ans , ansA and ansB2)

The amplified PCR products and pET 22b(+) were double digested with *Bam*HI and *Xho*I restriction enzyme. DNA fragments were cloned into the T7 expression vector, pET22b(+) in-frame with pel B leader sequence between *Bam*HI and *Xho*I restriction sites. Each gene (*ans, ansA, ansB2*) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was cloned in to pET22b(+) with and without 6xHis-tag (C-terminus) to observe the effect of 6xHis-tag on the expression level of protein. Conformation of positive clones was carried out by colony PCR, restriction digestion with *Bam*HI and *Xho*I (Fig.4.4 and 4.5) and followed by sequencing. The over-expression of cloned gene was under the regulation of T7 polymerase responsive promoter and a Lac operator in pET22b(+). The recombinant plasmid has the ampicillin resistance gene for selection of plasmid containing bacterial clones. Positive clones were maintained in 20% sterile glycerol at -80 ^{o}C.

4.3.3. Nucleotide sequencing and sequence analysis

For confirmation of sequence of cloned genes, sequencing was performed using T7 promoter and T7 terminator specific primers. BLAST-n result of the sequence of the genes (*ans, ansA and ansB2*) of *E. carotovora* subsp. *atroseptica* SCRI 1043 have shown 100% similarity with published gene sequences in gene bank and there was no PCR introduced mutation was detected. BLAST-n result of gene sequence of L-asparaginase (*ans*) of *P. carotovorum* MTCC 1428 has shown 90% similarity with L-asparaginase gene sequence (*ans*) of *E. carotovora* subsp. *atroseptica* SCRI 1043 and *P. carotovorum* subsp. carotovorum PC1.

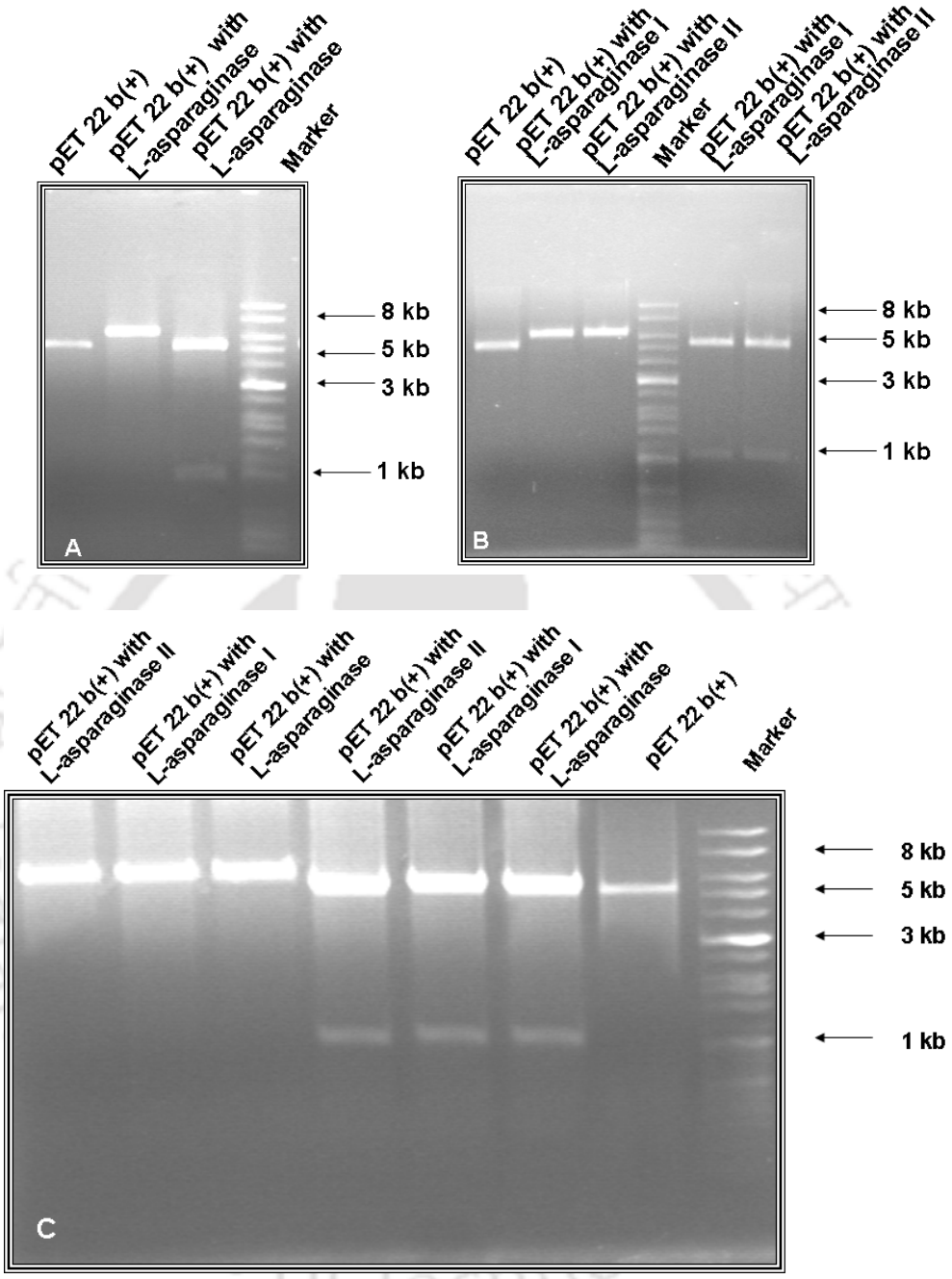

Fig. 4.4. Restriction digestion of recombinant construct carrying L-asparaginase genes of *P. carotovorum* MTCC 1428 (**A**: Single digestion (with *Xho*I) of pET 22b (+) and single and double digestion (with *Bam*HI and *Xho*I) of recombinant construct with L-asparaginase gene of *P. carotovorum* MTCC 1428 with His. tag, **B**: Single digestion (with *Xho* I) of pET 22b (+) and single digestion (with *Xho*I) and double digestion (with *Bam*HI and *Xho*I) of recombinant clone with L-asparaginase I and L-asparaginase II of *P. carotovorum* MTCC 1428 with His. tag and **C**: Single digestion (with *Xho* I) of pET 22b (+) and single and double digestion (with *Bam*HI and *Xho*I) of recombinant clone with L-asparaginase, L-asparaginase I and L-asparaginase II gene of *P. carotovorum* MTCC 1428 without histidine tag).

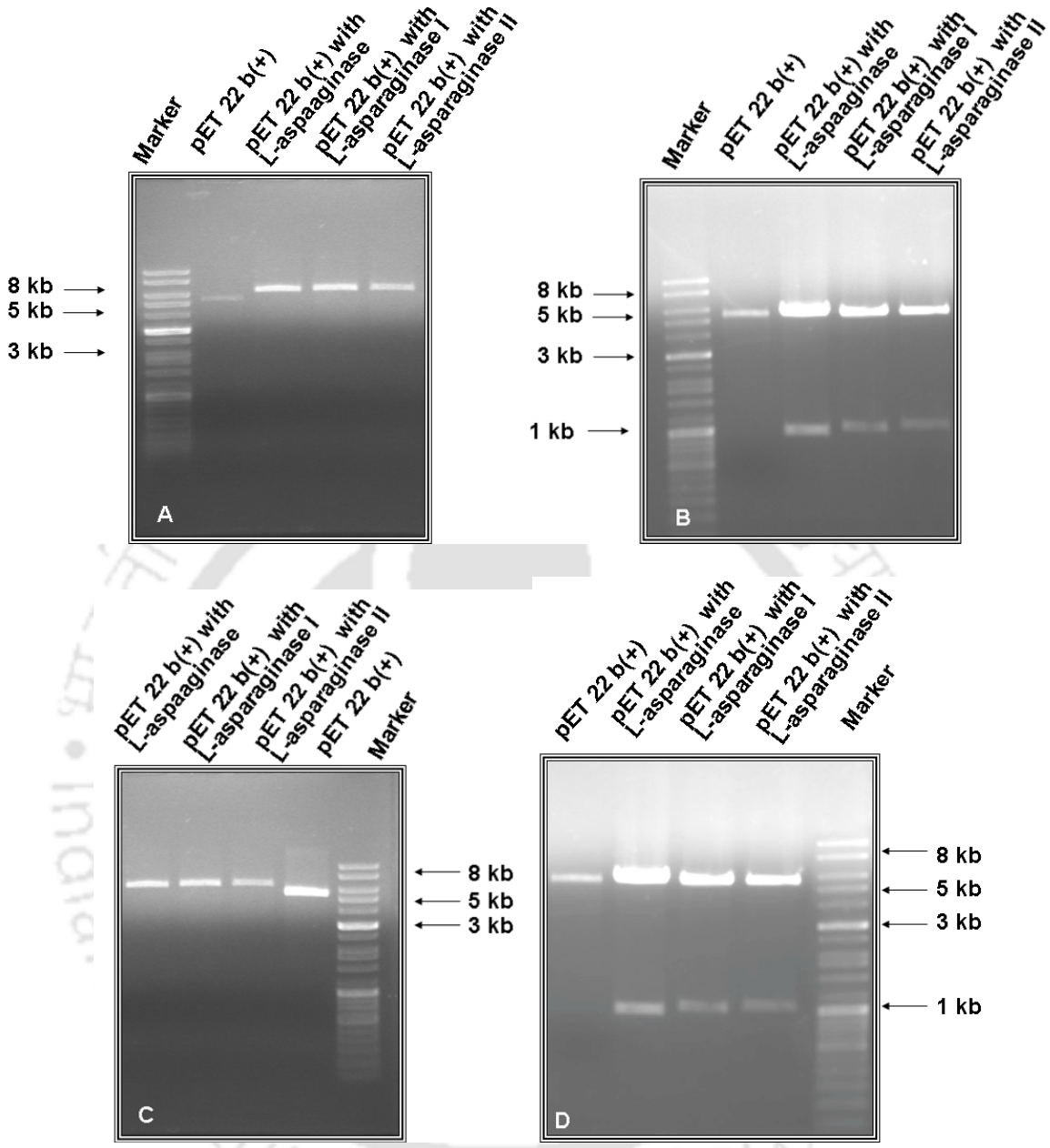

Fig. 4.5. Restriction digestion of recombinant construct carrying L-asparaginase genes of *E. carotovora* subsp. *atroseptica* SCRI 1043 (**A**: Single digestion (with *Xho*I) of pET 22b (+) and recombinant clone with gene of *E. carotovora* subsp. *atroseptica* SCRI 1043 with His. Tag, **B:** Double digestion (with *Bam*HI and *Xho*I) of pET 22b (+) and recombinant clone with gene of *E. carotovora* subsp. *atroseptica* SCRI 1043 with His. Tag, **C:** Single digestion (with *Xho* I) of pET 22b (+) and recombinant clone with gene of *E. carotovora* subsp. *atroseptica* SCRI 1043 without His. Tag and **D**: Double digestion (with *Bam*HI and *Xho* I) of pET 22b (+) and recombinant clone with gene of *E. carotovora* subsp. *atroseptica* SCRI 1043 without histidine tag).

Whereas gene sequence of L-asparaginase I *(ansA)* of *P. carotovorum* MTCC 1428 has revealed 91 % and 92 % identity with gene sequence of L-asparaginase I of *E. carotovora* subsp. *atroseptica* SCRI1043 and *P. carotovorum* subsp. carotovorum PC1, respectively. Blast-n results of gene sequence of L-asparaginase II (*ansB2)* of *P. carotovorum* MTCC 1428 has revealed 100% and 90% similarity with *E. carotovora* subsp. *atroseptica* SCRI 1043 and *P. carotovorum* subsp. carotovorum PC1, respectively. The nucleotide sequences of L-asparaginase *(ans),* L-asparaginase I *(ansA)* and L-asparaginase II *(ansB2)* of *P. carotovorum* have been submitted to NCBI and assigned the accession number of JN585953, JN631810 and JN638885, respectively.

4.3.4. Expression of recombinant proteins

E. coli is one of the most attractive systems available for heterologous protein production, and has been widely used in basic research studies as a host strain for the overproduction of recombinant proteins (Baneyx 1999; Bhandari and Gowrishankar, 1997). The present work was undertaken to develop novel recombinant glutaminase-free L-asparaginase of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043. For expression of recombinant proteins, *E. coli* BL21 (DE3) cells were transformed with recombinant constructs. To find out the optimum cell density for expression of recombinant proteins (L-asparaginase, L-asparaginase I and L-asparaginase II) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, cultures were induced at different cell OD at 600 nm (0.4, 0.6, 0.8, 1.0, 1.2). Optimum cell OD at 600nm for expression of recombinant proteins (L-asparaginase, L-asparaginase I and L-asparaginase II) of both the strains was found to be 0.8 (data not shown). Similarly, to observed the effect of IPTG concentration on expression of recombinant proteins, cultures were induced with different concentration of IPTG (0.1 mM,

0.25 mM, 0.5 mM, 0.75 mM, 1.0 mM, 1.25 mM, 1.5 mM). The optimum concentration of IPTG for induction of recombinant proteins was observed to be 1 mM (data not shown). To find out the optimum temperature for expression of recombinant proteins of both the strains, cells were initially grown at 37 °C and when cell OD at 600nm was ~0.8, cultures were induced with 1.0 mM IPTG. After IPTG addition, cells were incubated at different temperatures (25 °C, 30 °C and 37 °C) and samples were collected at different time points of post-induction to determine the profile of recombinant protein expression.

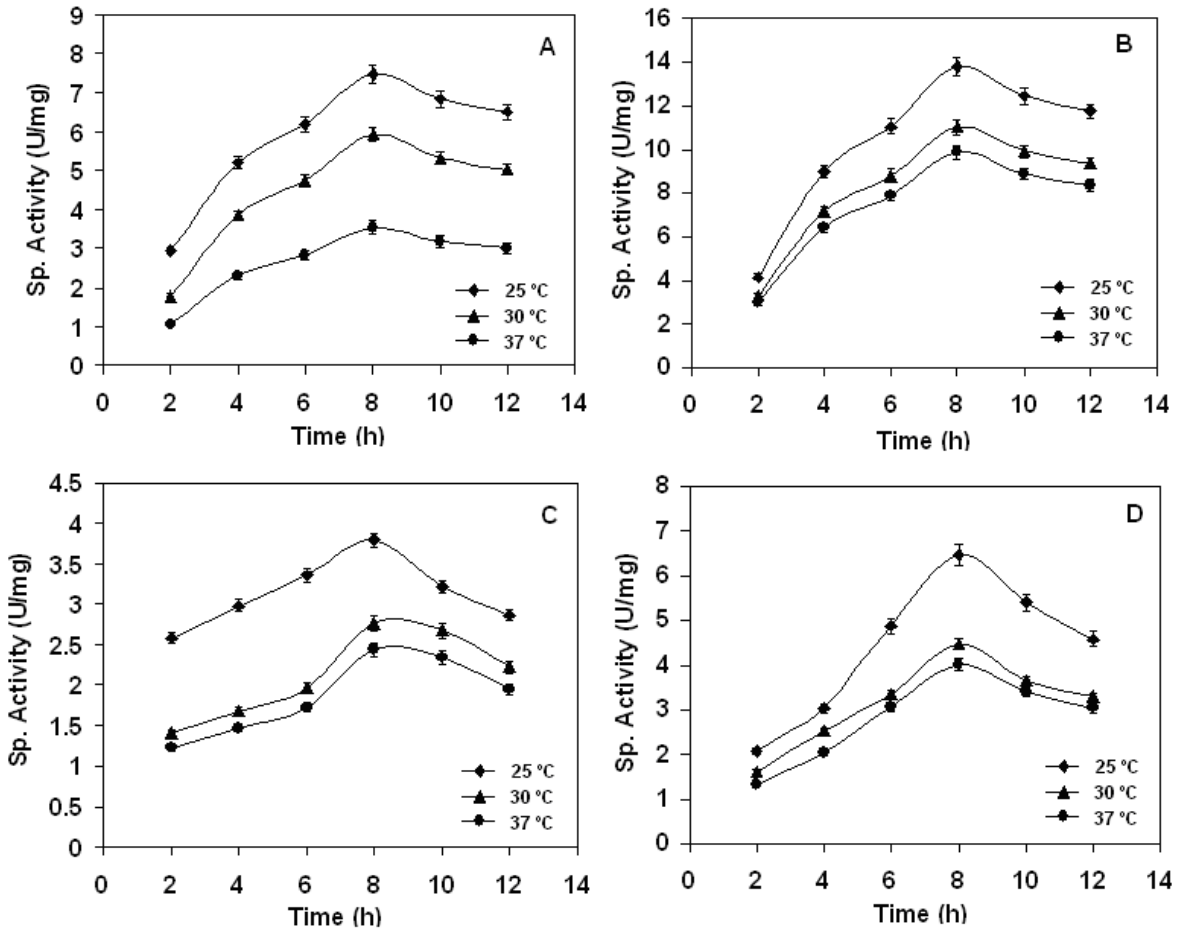

Fig. 4.6. Expression of recombinant L-asparaginase in *E. coli* (**A** and **B**: recombinant L-asparaginase of *P. carotovorum* MTCC 1428 with His. tag and without His. tag, respectively. **C** and **D**: recombinant L-asparaginase of *E. carotovora* subsp. *atroseptica* SCRI 1043 with and without His. tag, respectively).

The optimum temperature for recombinant L-asparaginase (with and without 6xHis-tag) and L-asparaginase I (with and without 6 xHis-tag) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 25 °C (Fig. 4.6 and 4.7).

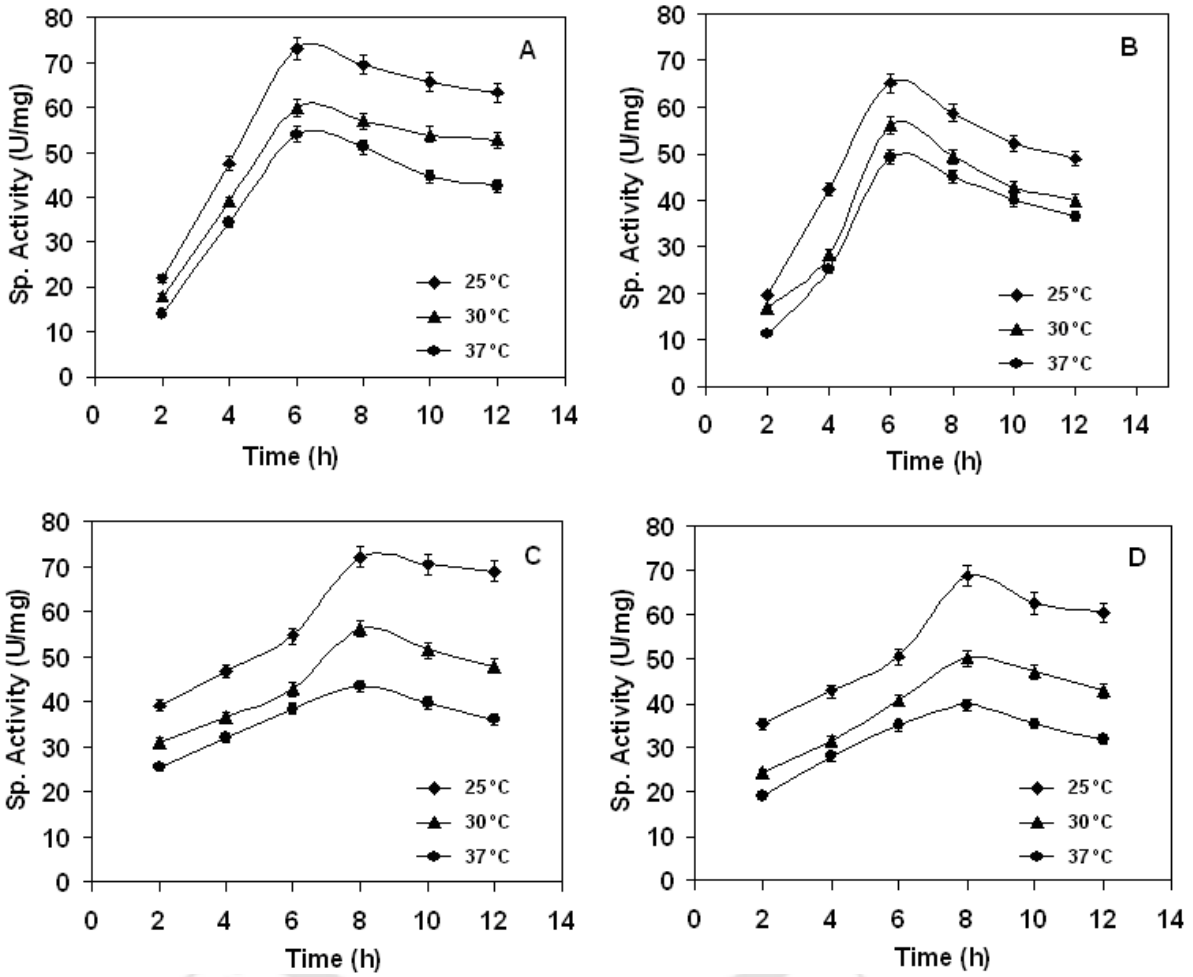

Fig. 4.7. Expression of recombinant L-asparaginase I in *E. coli* (**A** and **B**: recombinant L-asparaginase I of *P. carotovorum* MTCC 1428 with His. tag and without His. tag, respectively. **C** and **D**: recombinant L-asparaginase I of *E. carotovora* subsp. *atroseptica* SCRI 1043 with and without His. tag, respectively).

But the optimum temperature for expression of recombinant L-asparaginase II (with and without 6xHis-tag) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 30 °C (Fig 4.8). The expression of recombinant proteins

(L-asparaginase, L-asparaginase I and L-asparaginase II) of both the strains was checked in the soluble and insoluble fractions as described by Sambrook *et al.,* (1989).

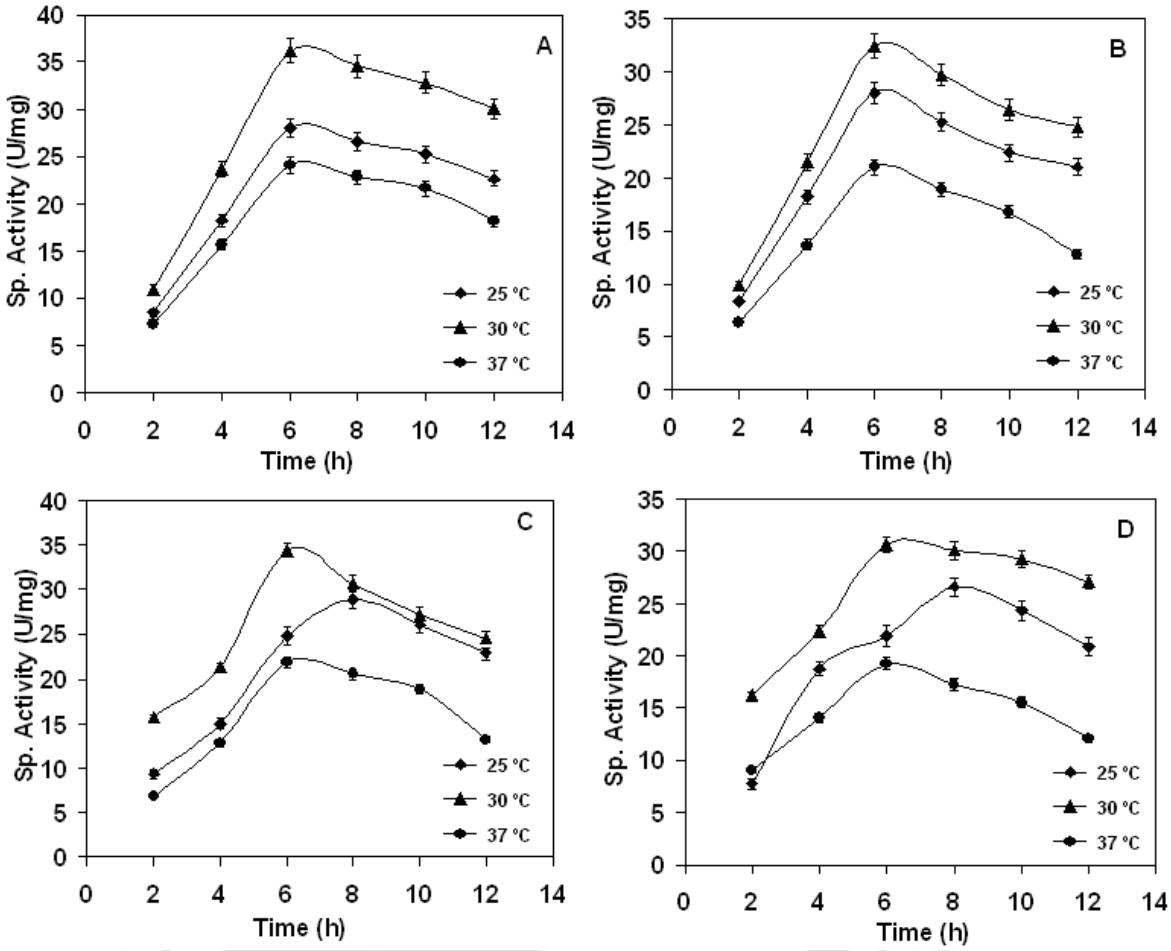

Fig. 4.8. Expression of recombinant L-asparaginase II in *E. coli* (**A** and **B**: recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 with His. tag and without His. tag, respectively. **C** and **D**: recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 with and without His. tag, respectively).

It was observed that all the recombinant proteins (L-asparaginase, L-asparaginase I and L-asparaginase II) of both the strains were expressed as soluble proteins (Fig. 9 and 10) and no expression of recombinant proteins was observed as insoluble protein (inclusion body) (data not shown).

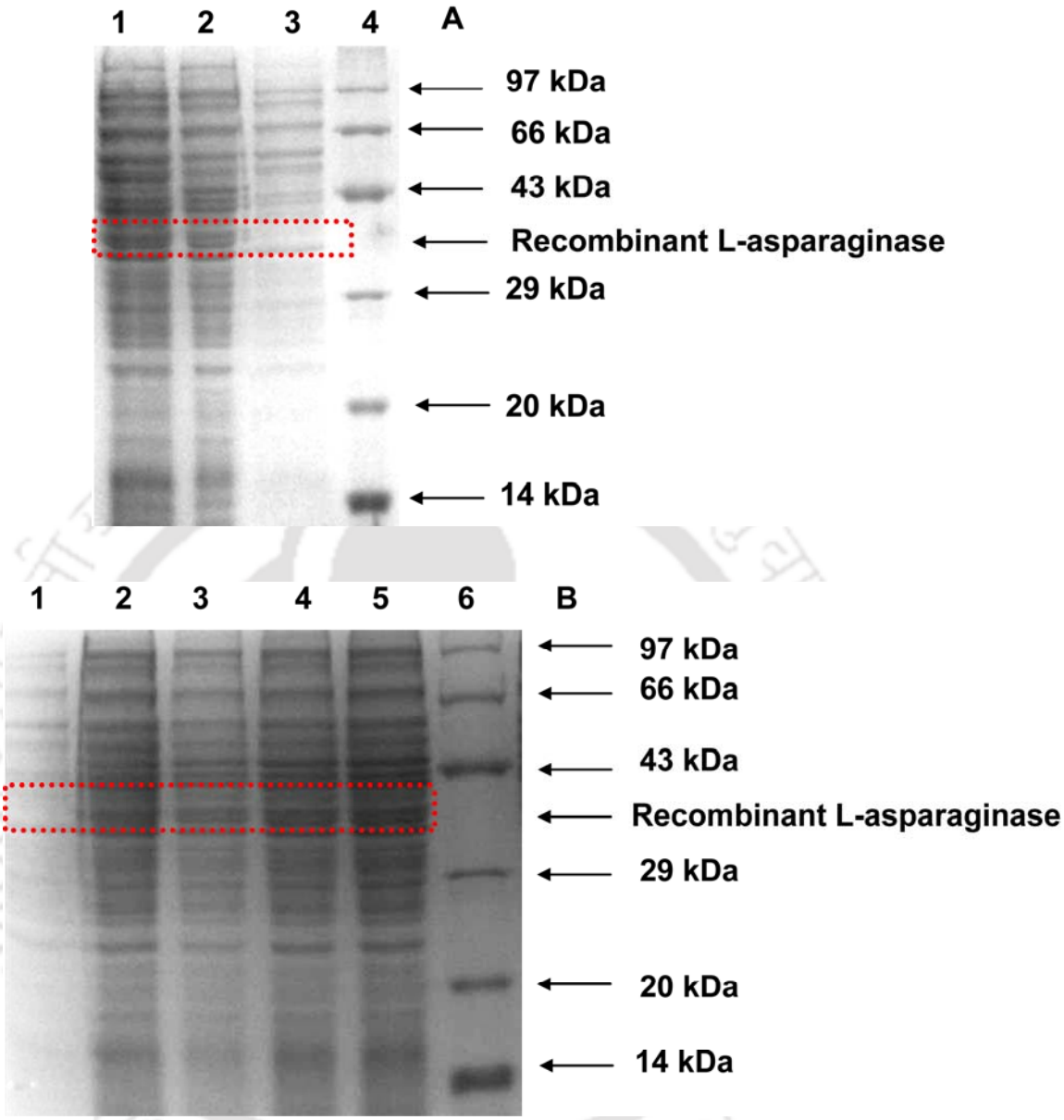

Fig. 4.9. SDS PAGE of recombinant protein of *P. carotovorum* MTCC 1428 (**A:** Lane 1 and 2: recombinant L-asparaginase with and without His. Tag, respectively; Lane 3: pET 22b(+) (without any insert) in *E. coli* BL21 (DE3) and Lane 4: Marker). **B:** SDS PAGE of recombinant protein of *P. carotovorum* MTCC 1428 (Lane 1: pET 22b(+) (without any insert) in *E. coli* BL21 (DE3); Lane 2 and 3: recombinant L-asparaginase I with and without His. Tag, respectively; Lane 4 and 5: recombinant L-asparaginase II with and without His. Tag, respectively and Lane 6: Marker).

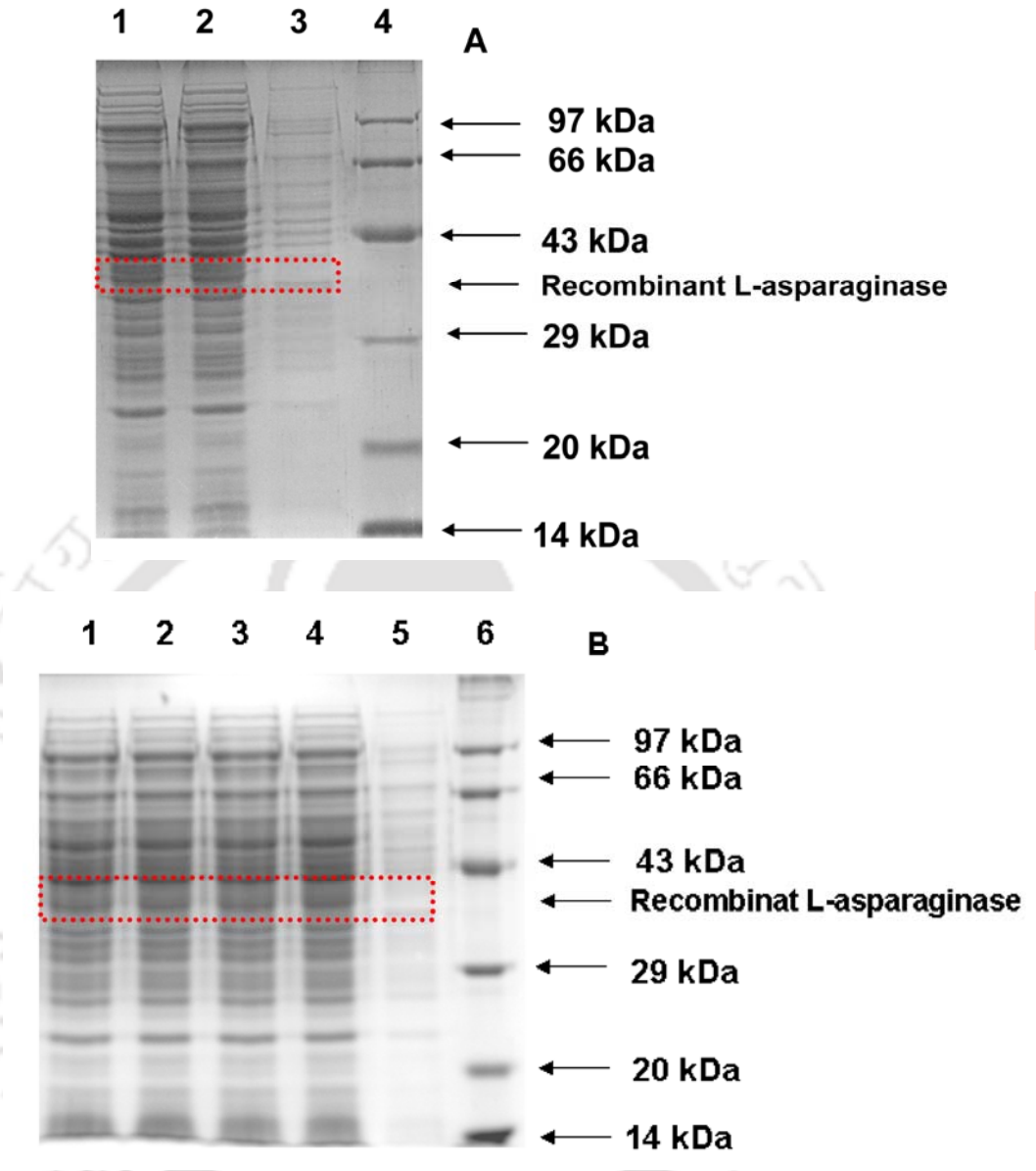

Fig. 4.10. SDS PAGE of recombinant protein of *E. carotovora* subsp. *atroseptica* SCRI 1043 (**A:** Lane 1 and 2: recombinant L-asparaginase with and without His. Tag, respectivel;, Lane 3: pET 22b(+) (without any insert) in *E. coli* BL21 (DE3) and Lane 4: Marker); **B:** SDS PAGE of recombinant protein of *E. carotovora* subsp. *atroseptica* SCRI 1043 (Lane 1 and 2: recombinant L-asparaginase I with and without His. Tag, respectively; Lane 3 and 4: recombinant L-asparaginase II with and without His. Tag, respectively; Lane 5: pET 22b(+) (without any insert) in *E. coli* BL21 (DE3) and Lane 6: Marker).

4.4. Effect of histidine tag on expression of recombinant L-asparaginase (*ans*), L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3)

E. coli is widely used as a host strain for the overproduction of recombinant proteins, especially for the T7-based gene expression system and several proteins of pharmaceutical value are also produced on a commercial scale (Makrides 1996; Nikerel *et al*., 2006). The presence of His-tag helps in the purification of the proteins in a single step due to the affinity towards Ni^{+} metal ions. As expected on the basis of available literature, it was observed that the effect of His-tag varies from protein to protein (Amor-Mahjoub *et al.,* 2006; Horchani *et al.,* 2009). The presence of histidine tag does not shown similar effect with all three expressed recombinant proteins of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3) (Fig. 4.6-4.8). In case of recombinant L-asparaginase of both the strains, lower expression was observed due to the presence of tag. However, in case of recombinant L-asparaginase I and L-asparaginase II of both the strains, higher expression was achieved with His-tag as compared to without tag, which might be due to improved protein yield of tagged protein (Arnau *et al*., 2006). The positive effect of the His-tag on the expression of recombinant protein may be due to the hydrophilic nature of the His-tag as generating a more hydrophilic protein, which is more compatible with the host cell (Sun *et al*., 2005; Svensson *et al*., 2006). According to Hammestrom *et al*. (2002) and Chen *et al.,* (2005), the presence of affinity tag is helpful in improving the solubility of the protein. Khoshoo *et al*. (2005) also cloned and expressed *E. coli* L-asparaginase II with and without His-tag at C-terminus and they observed that His-tag at the C-terminus had no effect on total secretary

expression. It is confirm that His-tag at C-terminus of protein has an effect on the expression level of protein. The glutaminase-free L-asparaginase II have shown less allergic reactions. Hence, glutaminase-free L-asparaginase will always be a useful product (Manna *et al*., 1995). L-glutaminase activity in all recombinant proteins (L-asparaginase, L-asparaginase I and L-asparaginase II with/without His-tag) of both the strains was checked and none of them possess L-glutaminase activity.

Among the L-asparaginases , L-asparaginase I and L-asparaginase II, only L-asparaginase II is used as anticancer agent (Cambell *et al*., 1967; Sanches *et al*., 2007). The higher expression of recombinant L-asparaginase II of both the strains was observed with 6x His. tag as compared to without 6x His. Tag (Fig 4.8). Therefore, further studies have been carried out with recombinant L-asparaginase II fused with histidine tag of both the strains.

4.5. Subcellular localization of recombinant L-asparaginase II of *P. carototovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3)

Subcellular localization of the recombinant L-asparaginase II of both the strains was carried out in *E. coli* BL21 (DE3) as described in the materials and methods section 3.5. Recombinant L-asparaginase II of both the strains was expressed as intracellular protein. It was observed that 59% and 63% of recombinant L-asparaginase II of *P. carototovorum* MTCC 1428 (21.83 U mg^{-1}) and *E. carotovora* subsp. *atroseptica* SCRI 1043 (20.79 U mg^{-1}) in *E. coli* BL21 (DE3) was localized in periplasm, respectively. But 41% and 37% of recombinant recombinant L-asparaginase II of *P. carototovorum* MTCC 1428 (15.17 U mg^{-1}) and *E. carotovora* subsp. *atroseptica* SCRI 1043 (12.21 U mg^{-1}) in *E. coli* BL21 (DE3) was

localized in the cytoplasm, respectively. There was no extracellular and membrane bound recombinant L-asparaginase II of both the strains was observed. pET-22b(+) carries an N-terminal pelB signal sequence for potential periplasmic localization. However, localization of the recombinant protein, cloned in pET 22 b(+), varied from protein to protein (Cao *et al.,* 2009; Khushoo *et al.,* 2004; Verheyden *et al.,* 2009). Cao *et al.,* (2009) have cloned B subunit of the *E. coli* heat-labile enterotoxin (LTB) in pET 22b(+) and expressed in *E. coli* BL21 (DE3) and it was expressed in the periplasmic space. Verheyden *et al.,* (2009) have cloned recombinant chymotrypsin(ogen) in pET 22b(+) and expressed in *E. coli* BL21 (DE3), and found that recombinant chymotrypsin(ogen) was localized in the cytoplasm. However, Khushoo *et al.,* (2004) have cloned *E. coli* L-asparaginase II in pET 22b(+) and observed extracellular expression in *E. coli* BL21 (DE3).

4.6. Selection of medium for production of recombinant L-asparaginase II

To find out the best medium for expression of recombinant L-asparaginase II of both the strains, six media (LB, 2x YT, TB, SB, M9, and Reseinberg) were studied. The maximum expression of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 in *E. coli* was observed in LB medium (36.15 U mg^{-1}) as compared to other media (2x YT: 28.18 Umg^{-1}, TB: 25.37U mg^{-1}, SB: 20.11 U mg^{-1}, M9: 11.46 U mg^{-1} and Reseinberg: 18.45 U mg^{-1}). Similarly, highest expression of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* was observed in LB medium (34.49 U mg^{-1}) than other media (2x YT: 26.01 U mg^{-1}, TB: 22.82 U mg^{-1}, SB: 21.00 U mg^{-1}, M9: 13.94 U mg^{-1} and Reseinberg: 16.08 U mg^{-1}). The lower expression of recombinant cytochrome P450 monooxygenase CYP102A1 in TB medium than LB medium was also reported by Pfug *et al*. (2007). Zhang *et al.,* (2009) have observed that accumulation of excessive amount of

ammonium due to the higher concentration of nitrogen source in the culture medium was not favorable for production of recombinant TATm-survivin (T34A) in *E. coli*. Probably, at higher concentration of undefined nitrogen sources, the concentration of some unknown factor crosses the favorable level and reduces the recombinant protein expression (Pfug *et al*. 2007). Ramalingama *et al*., (2007) have observed that the excessive carbon and nitrogen substrates in the medium would lead to decrease in recombinant protein productivity. So, the lower expression of recombinant L-asparaginase II was observed in 2x YT, TB and SB media. It was observed that lower cell growth and recombinant L-asparaginase II expression in M9 and Reisenberg media as synthetic media are not composed of undefined nitrogen sources (yeast extract/peptone/tryptone), which supports higher growth of organism. In addition, due to the presence of higher concentration of glucose (catabolite repressor) in M9 and Reisenberg media, the lower expression of recombinant L-asparaginase II was observed. Hence, the optimization of medium components was performed using LB medium for both the strains.

4.7. Optimization of chemical and physical process parameters for the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora subsp. atroseptica* SCRI 1043 in *E. coli* using response surface methodology (RSM)

4.7.1. Optimization of the medium constituents for maximizing the recombinant L-asparaginase II production

The experiments were performed to optimize the levels of medium components of selected LB medium (tryptone, yeast extract and NaCl) by central composite experimental design. The experimental design matrix and the corresponding observed and predicted responses

(production of L-asparaginase) are shown in Table 4.1. By applying the multiple regression analysis on the experimental data, the following second order polynomial equations 4. 1 and 4.2 were found to explain the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively.

$$Y_{\text{specific activity}} = 19.7934 + 4.2029\,X_1 + 3.0391\,X_2 + 0.7708\,X_3 - 0.1580\,X_1^2 - 0.2460\,X_2^2 - 0.0778\,X_3^2 + 0.0094\,X_1X_2 - 0.0069\,X_1X_3 + 0.0367\,X_2X_3 \quad (4.1)$$

$$Y_{\text{specific activity}} = 24.9880 + 3.7113\,X_1 + 0.7158\,X_2 + 0.5537\,X_3 - 0.1296\,X_1^2 - 0.0833\,X_2^2 - 0.0527\,X_3^2 + 0.0047\,X_1X_2 - 0.0178\,X_1X_3 + 0.0237\,X_2X_3 \quad (4.2)$$

Where, X_1 is tryptone, X_2 is yeast extract and X_3 is NaCl.

The results were analyzed using the analysis of variance (ANOVA) and are shown in Table 4.2 and 4.3 for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively. According to the ANOVA of the quadratic regression model, the model was highly significant, as is evident from the Fisher, *F*-test (The *F* value is the ratio of the mean square due to regression to the mean square due to error). The *F* values for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 3877.67 and 589.49, respectively (the confidence interval is 0.05), indicating that the model was adequate (Table 4.2 and 4.3). For recombinant L-asparaginase II production, *P*-value for 'lack of fit' was greater than 0.05. *P* values for *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 0.242 and 0.739, respectively.

Table 4.1. A 2^3 full-factorial central composite circumscribed design matrix in real values and coded values (in parenthesis) with experimental and predicted values for recombinant L-asparaginase II production of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL 21 (DE3)

Run Order	Tryptone (X_1) (g l^{-1})	Y. Extract (X_2) (g l^{-1})	NaCl (X_3) (g l^{-1})	Recombinant L-asparaginase II of *P. carotovorum* MTCC 1428			Recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043		
				Sp. Activity (U mg^{-1})		Activity (U ml^{-1})	Sp. Activity (U mg^{-1})		Activity (U ml^{-1})
				Observed	Predicted		Observed	Predicted	
1	5.00 (-1)	3.00 (-1)	5.00 (-1)	46.230±0.119	46.250	7.892±0.411	43.198±0.023	43.203	8.844±0.038
2	19.00 (+1)	3.00 (-1)	5.00 (-1)	51.964±0.217	52.098	11.532±0.151	51.129±0.103	51.468	10.051±0.033
3	5.00 (-1)	11.00 (+1)	5.00 (-1)	44.252±0.146	44.098	7.985±0.425	40.671±0.009	40.736	8.282±0.012
4	19.00 (+1)	11.00 (+1)	5.00 (-1)	48.893±0.320	48.891	12.523±0.257	49.877±0.210	49.528	11.189±0.026
5	5.00 (-1)	3.00 (-1)	19.00 (+1)	33.011±0.250	32.906	3.958±0.321	32.686±0.018	33.011	4.858±0.014
6	19.00 (+1)	3.00 (-1)	19.00 (+1)	40.053±0.288	40.099	10.025±0.302	37.882±0.023	37.794	11.258±0.312
7	5.00 (-1)	11.00 (+1)	19.00 (+1)	35.101±0.137	34.860	4.122±0.595	33.556±.0.053	33.193	4.098±0.078
8	19.00 (+1)	11.00 (+1)	19.00 (+1)	41.122±0.348	40.998	10.001±0.754	38.531±0.320	38.503	9.783±0.052
9	0.23 (-2)	7.00 (0)	12.00 (0)	30.854±0.242	31.083	3.896±0.297	27.793±0.051	27.763	4.002±0.192
10	23.78 (+2)	7.00 (0)	12.00 (0)	41.253±0.271	41.162	9.526±0.256	39.115±0.056	39.179	9.400±0.231
11	12.00 (0)	0.27 (-2)	12.00 (0)	47.521±0.400	47.409	9.852±0.619	48.388±0.036	48.031	9.406±0.065
12	12.00 (0)	13.73 (+2)	12.00 (0)	46.101±0.379	46.356	7.065±0.108	46.163±0.019	46.553	7.743±0.123
13	12.00 (0)	7.00 (0)	0.23 (-2)	56.210±0.254	56.157	0.023±0.360	52.731±0.011	52.684	9.758±0.232
14	12.00 (0)	7.00 (0)	23.77 (+2)	38.103±0.279	38.299	8.123±0.423	34.762±0.002	34.843	8.070±0.212
15	12.00 (0)	7.00 (0)	12.00 (0)	57.950±0.152	58.016	12.012±0.419	50.752±0.235	51.061	11.25±0.0309
16	12.00 (0)	7.00 (0)	12.00 (0)	58.122±0.191	58.016	10.986±0.343	51.639±0.163	51.061	10.101±0.052
17	12.00 (0)	7.00 (0)	12.00 (0)	57.712±0.240	58.016	11.524±0.538	50.516±0.142	51.061	11.253±0.068
18	12.00 (0)	7.00 (0)	12.00 (0)	58.001±0.331	58.016	12.012±0.161	50.839±0.020	51.061	12.244±0.045
19	12.00 (0)	7.00 (0)	12.00 (0)	58.134±0.281	58.016	11.023±0.554	51.623±0.015	51.061	10.492±0.087
20	12.00 (0)	7.00 (0)	12.00 (0)	58.212±0.322	58.016	13.210±0.380	50.805±0231	51.061	12.520±0.099

Table 4.2. Analysis of variance (ANOVA) for quadratic model for production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	1640.03	1640.03	182.226	3877.67	0.000
Linear	3	508.91	546.74	182.248	3878.14	0.000
Square	3	1121.23	1121.23	373.745	7953.09	0.000
Interaction	3	9.89	9.89	3.296	70.13	0.000
Residual error	10	0.47	0.47	0.047		
Lack of fit	5	0.31	0.31	0.062	1.94	0.242
Pure Error	5	0.16	0.16	0.032		
Total	19	1640.50				

R-Sq = 99.97% R-Sq(pred) = 99.84% R-Sq(adj) = 99.95%
DF degrees of freedom, SS sum of squares, MS mean square

Table 4.3. Analysis of variance (ANOVA) for quadratic model for production of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043

Source	DF	SS	SS	MS	*F*	*P*
Regression	9	1171.62	1171.617	130.180	589.49	0.000
Linear	3	544.18	427.949	142.650	645.96	0.000
Square	3	617.72	617.719	205.906	932.40	0.000
Interaction	3	9.71	93713	3.238	14.66	0.001
Residual error	10	2.21	2.208	0.221		
Lack of fit	5	0.78	0.780	0.156	0.55	0.739
Pure Error	5	1.43	1.428	0.286		
Total	19	1173.82				

R-Sq = 99.81% R-Sq(pred) = 99.28% R-Sq(adj) = 99.64%

This was higher than 0.05, indicating that the 'lack of fit' of model was insignificant. In other words, the model was fit with the responses data collected. R^2 values for *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 99.97 % and 99.81%, respectively. This implies that more than 99.5% for recombinant L-asparaginase II

production is attributed to the chemical parameters and ~0.5 % of the total variation is not explained by the model.

Table 4.4. Student *t* test of the model coefficients estimated by multiple linear regression for recombinant L-asparaginase II production of *P. carotovorum* MTCC 1428

Source	Coef.	SE Coef.	*t* value	*P*
Constant	19.7934	0.501483	51.434	0.000
X_1	4.2029	0.039648	106.005	0.000
X_2	3.0391	0.069782	43.551	0.000
X_3	0.7708	0.039648	19.442	0.000
X_1^2	-0.1580	0.001165	-135.550	0.000
X_2^2	-0.2460	0.003569	-68.930	0.000
X_3^2	-0.0778	0.001165	-66.794	0.000
$X_1 * X_2$	-0.0094	0.002737	-3.441	0.006
$X_1 * X_3$	0.0069	0.001564	4.387	0.001
$X_2 * X_3$	0.0367	0.002737	13.390	0.000

Table 4.5. Student *t* test of the model coefficients estimated by multiple linear regression for recombinant L-asparaginase II production of *E. carotovora* subsp. *atroseptica* SCRI 1043

Source	Coef.	SE Coef.	*t* value	*P*
Constant	24.9880	1.08710	22.986	0.000
X_1	3.7113	0.08595	43.181	0.000
X_2	0.7158	0.15127	4.732	0.001
X_3	0.5537	0.08595	6.443	0.000
X_2^2	-0.1296	0.00253	-50.240	0.000
X_3^2	-0.0833	0.00774	-10.765	0.000
X_1^2	-0.0527	0.00253	-20.844	0.000
$X_1 * X_2$	0.0047	0.00593	0.793	0.446
$X_1 * X_3$	-0.0178	0.00339	-5.241	0.001
$X_2 * X_3$	0.0237	0.00593	3.986	0.003

This indicates that the combined effects of all the independent variables significantly contributed to maximizing the production of recombinant L-asparaginase II of both the strains (Table 4.1). Model coefficients estimated by regression analysis for each variable are shown in Table 4.4 and Table 4.5. The significance of each coefficient was determined by *t*-values and *P*-values. The larger the magnitude of *t*-test value and smaller the *P*-value indicates that the high significance of the corresponding coefficient (Karthikeyan *et al*., 1996; Tanyildizi *et al*., 2005). The results inferred that tryptone (X_1) have shown significant effect ($P < 0.05$) on recombinant L-asparaginase II production as it had largest coefficient for *P. carotovorum* MTCC 1428 and for *E. carotovora* subsp. *atroseptica* SCRI 1043, followed by yeast extract (X_2) and NaCl (X_3). Positive coefficients of X_1, X_2 and X_3 indicated a linear effect for the increase in recombinant L-asparaginase II production. Negative coefficients were observed for quadratic terms of all the three variables. Low *P*-value of X_1, X_2 and X_3 variables for quadratic terms indicated that they are significant. A positive sign indicates that a higher-level variable setting consequences in a higher response than the lower-level variable setting, while a negative sign indicates that the lower-level variable setting results in a higher response than the high-level variable setting. The 3D response surface plots and the 2D contour plots are the graphical representation of the regression equation, used to visualize the relationship between the response and experimental levels of each variable and the type of interaction among the variables to deduce the optimum conditions (Tanyildizi *et al*., 2005). Response surface plots described by the Eq. 4.1 and 4.2 are represented in Fig. 4.11 and 4.12, respectively. The response surface plots were constructed by plotting the response (specific activity) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels.

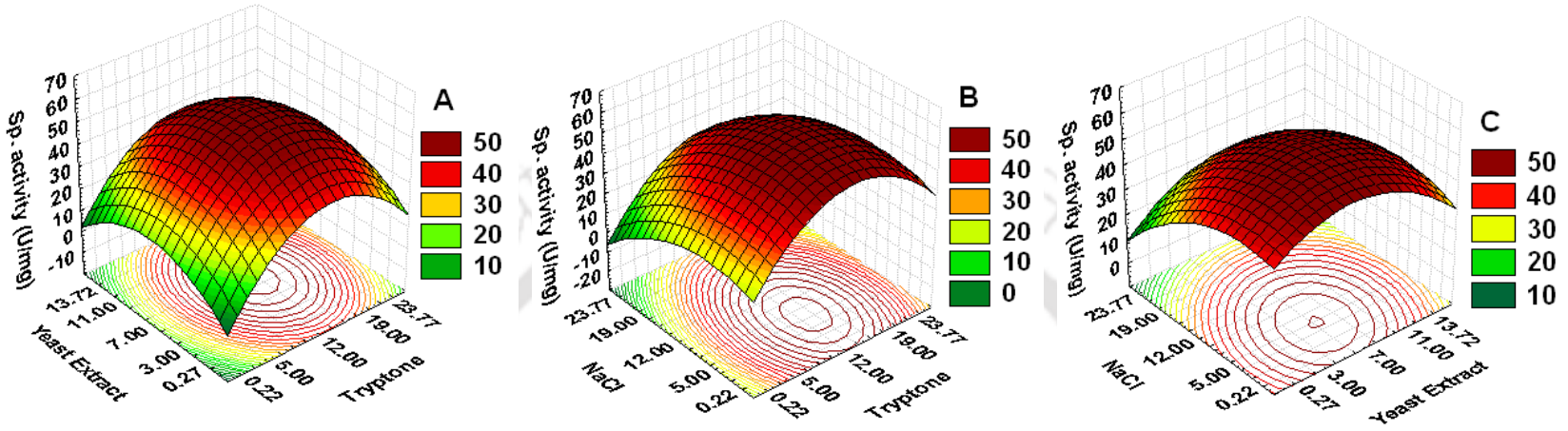

Fig. 4.11. Three-dimensional response surface plots and contour plots for recombinant L-asparaginase II production of *P. carotovorum* MTCC 1428, showing the interactive effects of **A**: Yeast Extract and Tryptone, **B**: NaCl and Tryptone and **C**: NaCl and Yeast Extract.

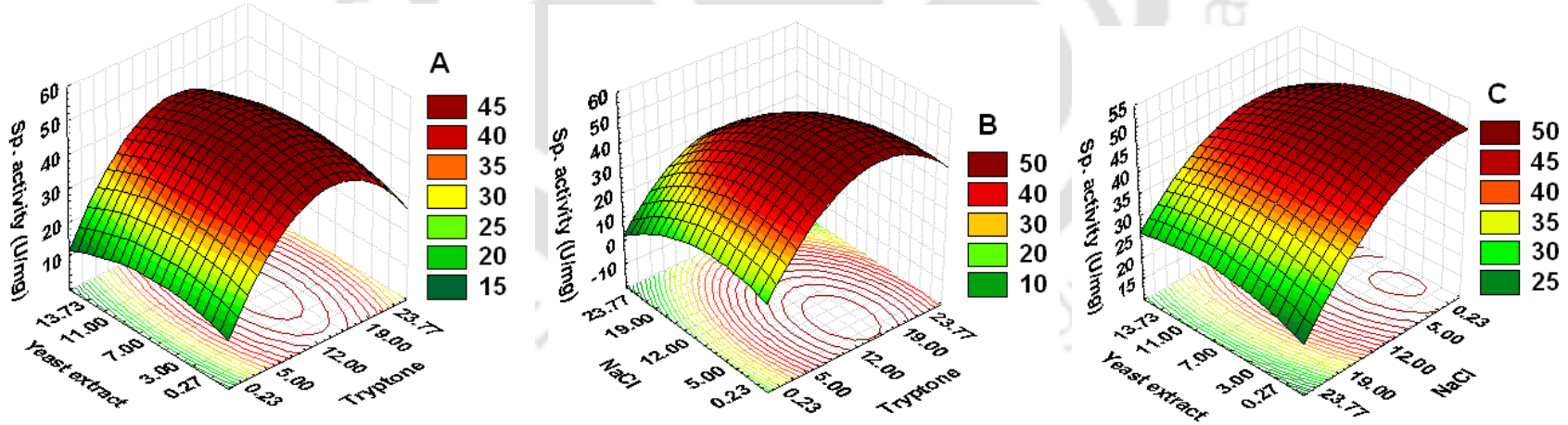

Fig. 4.12. Three-dimensional response surface plots and contour plots for recombinant L-asparaginase II production of *E. carotovora* subsp. *atroseptica* SCRI 1043 showing the interactive effects of **A**: Yeast Extract and Tryptone, **B**: NaCl and Tryptone **C**: Yeast Extract and NaCl.

Response surface plots, which are the graphical description of regression equation for visualizing the relationship between the response and variable and the interaction between the variables for assuming the optimum conditions and depict the interaction between two variables by keeping the other variables at their middle levels for recombinant L-asparaginase II production. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram (Tanyildizi *et al.*, 2005). As shown in the Fig. 4.11 and 4.12, there was a steep increase in the production of recombinant L-asparaginase II with an increase in tryptone concentration and a maximum level of recombinant L-asparaginase II production was reached when tryptone was 5-19 g l^{-1}. Maximization of the regression equation (Eq. 4.1 and 4.2) was carried out using an iterative technique to obtain optimum levels of chemical parameters by substituting the corresponding coded concentration levels of the factors into the regression equation. The maximum predictable response for production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were calculated by applying the regression analysis to the Eq.-4.1 and 4.2 using 'response optimizer' in Minitab software. For production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428, the optimum levels of tryptone, yeast extract and NaCl were found to be 13.30 g l^{-1}, 6.38 g l^{-1} and 7.12 g l^{-1}, respectively. The optimal values of tryptone, yeast extract and NaCl for recombinant L-asparaginase II production of *E. carotovora* subsp. *atroseptica* SCRI 1043 were determined to be 14.50 g l^{-1}, 5.30 g l^{-1} and 4.03 g l^{-1}, respectively. The production of recombinant L-asparaginase II of both the strains was enhanced in the presence of high concentration of tryptone. It is well known that nitrogen source is mainly utilized to synthesize protein, nucleic acid and metabolites of nitrogen and nitrogen sources as a key factor and supports enzyme production (Mctigue *et al*.,

1994). Relatively lower concentration of nutriment is the limited factor for cell growth and protein expression. The existing reports are also in the favor of high nitrogen source requirement for cell growth and protein synthesis (Ibrahim *et. al*., 2005). This significant interaction effects simplified the scale-up process for the enzyme production and desirable for most of the large-scale production of protein. The concentration of NaCl is lower in the optimized medium than un-optimized medium. Probably, higher salt concentration is not favorable for growth and enzyme production (Chan *et al.*, 1999). It was observed that the combined concentration of nitrogen sources (tryptone and yeast extract) was lower than other undefined medium (2xTY, TB and SB media) for the production.

4.7.2. Experimental validation of model predictions of optimized chemical parameter levels

To verify the validity of the model, experiments were performed at optimal levels of medium composition. The observed production (specific activity) of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 61.38 U mg^{-1} and 57.06 U mg^{-1} and this is very good agreement with the value predicted by the model, 60.18 U mg^{-1} and 54.77 U mg^{-1}, respectively. After optimization of medium components, 1.69 and 1.65 fold higher production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively was achieved as compared to un-optimized medium.

Response surface methodology has been applied in several similar studies to optimize the medium components for the production of pectinase from *Bacillus pumilus* (Sharma and Satyanarayana, 2006), alkaline protease from *Bacillus sp.* RKY3 (Reddy *et al.,* 2008), chitosanase by *Microbacterium sp.* OU01 (Sun *et al.,* 2007), griseofulvin production by

Penicillium griseofulvum (Dasu *et al.,* 2002), cis-epoxysuccinate hydrolase from recombinant *E. coli* (Pan *et al*., 2010), L-aspraginase production by *P. carotovorum* MTCC 1428 (Kumar *et al*., 2009), L-asparaginase production by *E. coli* ATCC 11303 (Kenari *et al*., 2010), L-asparaginase by mangrove derived *Bacillus cereus* MAB5 (*Thenmozhi et al.,* 2011), etc. There is no report available in the literature on the optimization of medium components for the production of glutaminase-free recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli*. The range of specific activity of L-asparaginases from different microorganisms was reported in between 0.1–35.0 U mg^{-1} of protein (Abdel-Fattah and Olama 2002; El-Bessoumy *et al.,* 2004; Kumar *et al.*, 2009; Maladkar *et al.,* 1993; Manna *et al.,* 1995; Nawaz *et al.,* 1998; Prakasham *et al.,* 2007; Raha *et al.,* 1990). L-asparaginase from *E. carotovora, E. crysanthemi, E. coli* and *S. cerevisiae* have been cloned and successfully expressed in bacterial and yeast expression systems (Khushoo *et al.,* 2004; Khushoo *et al.,* 2005; Kotzia and Labrou 2005; Kotzia and Labrou 2007; Maria *et al.,* 2006;). However, the L-asparaginase obtained from *E. coli* and *Erwinia* species have been employed for many years as effective anticancer drugs but their therapeutic response rarely occurs without some evidence of toxicity. After optimization of medium components, experiments were performed to optimize physical process parameters to maximize the production of recombinant L-asparaginase II.

4.7.3. Optimization of physical process parameters for higher production of recombinant L-asparaginase II of P. carotovorum MTCC 1428 and E. carotovora subsp. atroseptica SCRI 1043

In order to optimize the physical process parameters (initial pH of the medium, rpm of the shaking incubator and inoculum size) for enhanced production of recombinant L-asparaginase

II, experiments were performed according to the central composite experimental design (as described in the section 3.10.2). The design matrix and the corresponding results of observed and predicted responses (production of recombinant L-asparaginase II) are shown in Table 4.6. The production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 varied from 42.56 U mg^{-1} to 67.91 U mg^{-1} and 36.12 U mg^{-1} to 63.29 U mg^{-1}, respectively. By applying the multiple regression analysis on the experimental data, the following second-order polynomial equations, 4.3 and 4.4 were found to explain the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively.

$$Y_{\text{specific activity}} = -1037.520 + 240.183\ X_1 + 1.906\ X_2 + 40.583\ X_3 - 15.033\ X_1^2 - 0.002\ X_2^2 - 4.020\ X_3^2 - 0.097\ X_1X_2 - 2.390\ X_1X_3 - 0.016\ X_2X_3 \quad (4.3)$$

$$Y_{\text{specific activity}} = -917.896 + 207.171\ X_1 + 1.915\ X_2 + 33.074\ X_3 - 12.792\ X_1^2 - 0.003\ X_2^2 - 3.030\ X_3^2 - 0.096\ X_1X_2 - 1.875\ X_1X_3 - 0.026\ X_2X_3 \quad (4.4)$$

Where X_1 is pH, X_2 is inoculum size in % and X_3 is agitation. As shown in Table 4.7 and 4.8, all the variables were found to be significant for recombinant L-asparaginase II production of both the strains. All the variables have shown positive effect on the production of recombinant L-asparaginase II of both the strains. The Student's *t* distribution and the corresponding *P* values, along with the parameter estimate are shown in Table 4.7 and Table 4.8, respectively for *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043. The *P* values of all linear and quadratic relationships between physical process variables suggest that they are highly significant. Furthermore, the effect of interactions of variables is very significant on the production. The results were also analyzed using the ANOVA (Table 4.9 and 4.10).

Table 4.6. A 2^3 full-factorial CCD matrix in real values and coded values (in parenthesis) with experimental and predicted values for recombinant L-asparaginase II production of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043

Run Order	pH (X_1)	Inoculum size (%) (X_2)	Agitation (rpm) (X_3)	Recombinant L-asparaginase II of *P. carotovorum* MTCC 1428			Recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043		
				Sp. Activity (U mg^{-1})		Activity (U ml^{-1})	Sp. Activity (U mg^{-1})		Activity (U ml^{-1})
				Observed	Predicted		Observed	Predicted	
1	6.50 (-1)	2.00 (-1)	160.00 (-1)	45.718±0.230	46.488	11.221± 0.125	43.030±0.230	42.735	10.840± 0.186
2	7.50 (+1)	2.00 (-1)	160.00 (-1)	56.377±0.120	56.852	10.157± 0.114	52.930±0.120	52.639	10.220± 0.162
3	6.50 (-1)	4.00 (+1)	160.00 (-1)	59.410±0.009	59.803	10.971± 0.425	58.490±0.009	58.358	11.370± 0.236
4	7.50 (+1)	4.00 (+1)	160.00 (-1)	60.243±0.015	60.457	9.586± 0.142	58.806±0.015	58.644	9.619± 0.012
5	6.50 (-1)	2.00 (-1)	240.00 (+1)	43.077±0.320	43.485	9.823± 0.352	40.701±0.320	40.383	9.523± 0.288
6	7.50 (+1)	2.00 (-1)	240.00 (+1)	48.840±0.119	49.069	10.012± 0.159	46.884±0.119	46.538	10.175± 0.071
7	6.50 (-1)	4.00 (+1)	240.00 (+1)	53.420±0.089	53.566	11.121± 0.411	51.013±0.089	50.830	11.094± 0.043
8	7.50 (+1)	4.00 (+1)	240.00 (+1)	49.587±0.032	49.439	10.998± 0.152	47.551±0.032	47.367	11.258± 0.092
9	6.16 (-2)	3.00 (0)	200.00 (0)	53.576±0.045	53.854	9.750± 0.195	52.601±0.045	50.922	11.620± 0.076
10	7.84 (+2)	3.00 (0)	200.00 (0)	59.257±0.026	59.099	11.168± 0.254	55.981±0.026	56.338	11.168± 0.117
11	7.00 (0)	1.32 (-2)	200.00 (0)	42.560±0.302	41.741	7.520± 0.242	36.122±0.302	36.637	8.047± 0.215
12	7.00 (0)	4.68 (+2)	200.00 (0)	53.311±0.212	53.250	13.897± 0.623	50.309±0.212	50.471	14.591± 0.022
13	7.00 (0)	3.00 (0)	132.73(-2)	62.432±0.142	61.631	14.952±0.120	59.540±0.142	59.835	15.521±0.271
14	7.00 (0)	3.00 (0)	267.27(+2)	49.920±0.120	49.841	15.001± 0.041	47.992±0.120	48.374	15.250± 0.159
15	7.00 (0)	3.00 (0)	200.00 (0)	66.726±0.087	67.107	14.252± 0.102	63.288±0.087	62.675	13.697± 0.088
16	7.00 (0)	3.00 (0)	200.00 (0)	67.980±0.240	67.107	13.988± 0.454	62.542±0.240	62.675	14.364± 0.234
17	7.00 (0)	3.00 (0)	200.00 (0)	66.426±0.055	67.107	14.012± 0.232	62.488±0.055	62.675	14.364± 0.334
18	7.00 (0)	3.00 (0)	200.00 (0)	67.124±0.263	67.107	14.355± 0.120	62.686±0.263	62.675	14.364± 0.034
19	7.00 (0)	3.00 (0)	200.00 (0)	66.325±0.201	67.107	13.587± 0.141	62.888±0.201	62.675	14.362± 0.120
20	7.00 (0)	3.00 (0)	200.00 (0)	67.912±0.012	67.107	14.852±0.012	62.275±0.012	62.675	15.340± 0.012

Table 4.7. Student *t* test of the model coefficients estimated by multiple linear regressions for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428

Source	Coef	SE Coef	*t* value	*P*
Constant	-1037.520	44.2016	-23.472	0.000
X_1	240.183	11.5399	20.813	0.000
X_2	1.906	0.0835	22.834	0.000
X_3	40.583	4.0991	9.900	0.000
X_1^2	-15.033	0.8011	-18.767	0.000
X_2^2	-0.002	0.0001	-34.622	0.000
X_3^2	-4.020	0.2003	-20.074	0.000
$X_1 * X_2$	-0.097	0.0108	-9.032	0.000
$X_1 * X_3$	-2.390	0.5376	-4.446	0.001
$X_2 * X_3$	-0.016	0.0054	-3.008	0.013

Table 4.8. Student *t* test of the model coefficients estimated by multiple linear regressions for recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043

Source	Coef	SE Coef	*t* value	*P*
Constant	-917.896	25.3097	-36.267	0.000
X_1	207.171	6.6077	31.353	0.000
X_2	1.915	0.0478	40.060	0.006
X_3	33.074	2.3471	-27.889	0.000
X_1^2	-12.792	0.4587	14.091	0.000
X_2^2	-0.003	0.0000	-58.953	0.000
X_3^2	-3.030	0.1147	-26.425	0.000
$X_1 * X_2$	-0.096	0.0062	-15.622	0.000
$X_1 * X_3$	-1.875	0.3078	-6.091	0.000
$X_2 * X_3$	-0.026	0.0031	-8.408	0.000

According to the ANOVA of the quadratic regression model, the model is highly significant, as is evident from the Fisher *F* test. *F* value for *P. carotovorum* MTCC 1428 is 267.52 and for *E. carotovora* subsp. *atroseptica* SCRI 1043 is 767.99, corresponding to very low probability value ($P < 0.05$).

Table 4.9. Analysis of variance (ANOVA) for quadratic model for the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	1391.61	1391.609	154.623	267.52	0.000
Linear	3	360.88	480.188	160.063	276.93	0.000
squar	3	966.92	966.918	322.306	557.64	0.000
Interaction	3	63.81	63.809	21.270	36.80	0.000
Residual error	10	5.78	5.780	0.578		
Lack of fit	5	3.15	3.152	0.630	1.20	0.423
Pure Error	5	2.63	2.628	0.526		
Total	19	1397.39				

R-Sq = 99.59% R-Sq(pred) = 98.02% R-Sq(adj) = 99.21%

SS sum of squares, DF degrees of freedom, MS mean square

Table 4.10. Analysis of variance (ANOVA) for quadratic model for production of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043

Source	DF	SS	SS	MS	*F*	*P*
Regression	9	1309.81	1309.815	145.535	767.99	0.000
Linear	3	424.98	422.906	140.969	743.89	0.000
squar	3	818.16	818.155	272.718	1439.13	0.000
Interaction	3	66.98	66.675	22.225	117.28	0.000
Residual error	10	1.90	1.895	0.190		
Lack of fit	5	1.26	1.263	0.253	2.00	0.232
Pure Error	5	0.63	0.632	0.126		
Total	19	1311.71				

R-Sq = 99.86% R-Sq(pred) = 99.20% R-Sq(adj) = 99.73%

This signifies that the combined effects of all the independent variables significantly contributed to maximize the production of recombinant L-asparaginase II of both the strains. The goodness of the model was checked by coefficient of determination, R^2. R^2 values for *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be

99.59 % and 99.86%, respectively. This implies that more than 99.5% for recombinant L-asparaginase II production is attributed to the physical parameters and ~0.5 % of the total variation is not explained by the model. The three-dimensional response surface plots were constructed to predict the recombinant L-asparaginase II production of both the strains (Fig. 4.13 and 4.14). Maximization of the regression equations (Eq. 4.3 and 4.4) were carried out by substituting the corresponding coded concentration levels of the factors into the regression equation. The maximum predictable response for recombinant L-asparaginase II production for both the strains were calculated. The optimul levels of physical process parameters for the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 were determined to be as follows: pH 7.09, agitation 212 rpm and inoculum concentration 2.50 %, respectively. The best combination of pH, agitation, inoculum size for the production of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 7.11, 216 rpm and 2.34 %, respectively.

4.7.4. Verification of the model

The model was validated by performing the experiments under the optimal levels of pH, agitation and inoculum size. The maximum production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 70.25 U mg^{-1} and 67.26 U mg^{-1}, respectively. After optimization of chemical and physical parameters, an overall 1.94 and 1.95 fold increase in recombinant L-asparaginase II production of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively was achieved.

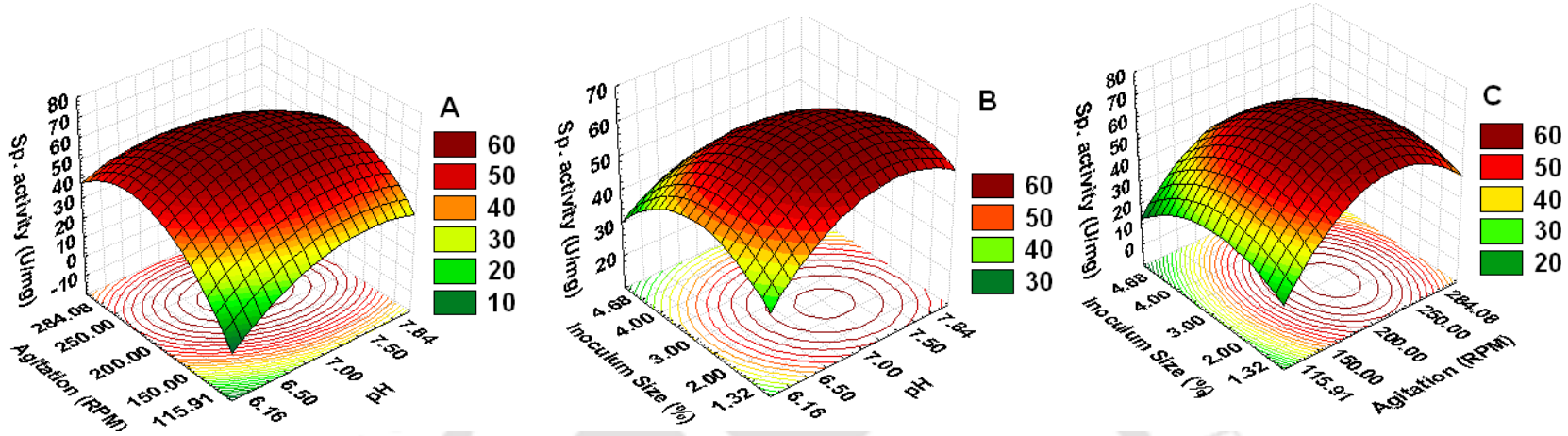

Fig. 4.13. Three-dimensional response surface plots and contour plots for the recombinant L-asparaginase II production of *P. carotovorum* MTCC 1428, showing the interactive effects of **A**: Agitation and pH, **B**: Inoculum size (%) and pH, **C**: Inoculum size (%) and Agitation.

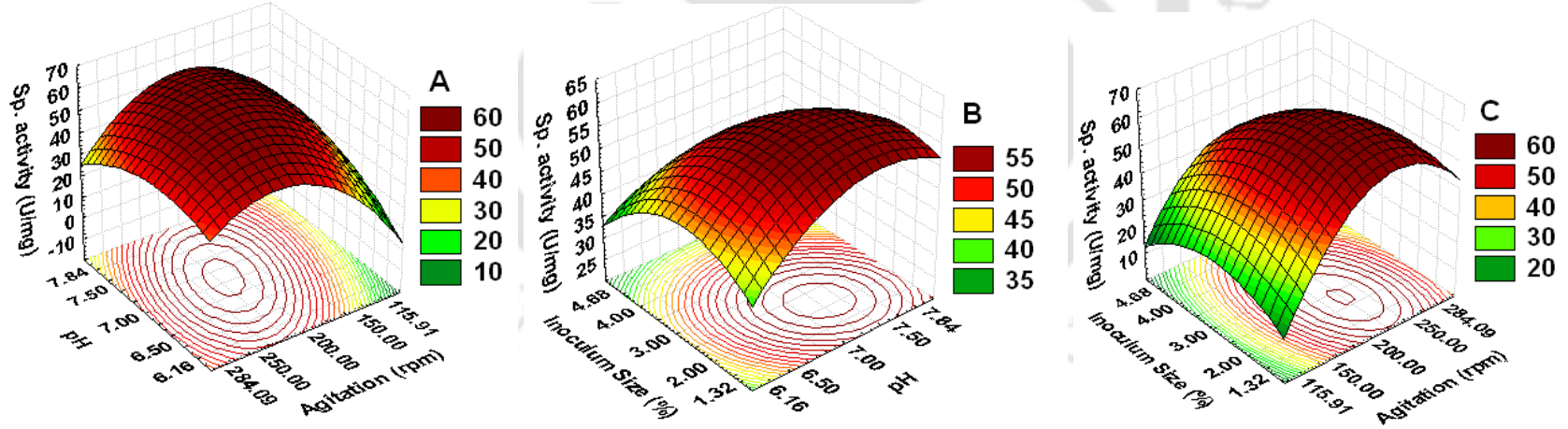

Fig. 4.14. Three-dimensional response surface plot and contour plot for the recombinant L-asparaginase II production of *E. carotovora* subsp. *atroseptica* SCRI 1043, showing the interactive effects of **A**: pH and Agitation **B**: Inoculum size (%) and pH and **C**: Inoculum size (%) and Agitation.

Table 4.11. Comparison of production of recombinant L-asparaginase II

Name	Condition	Recombinant L-asparaginase II Production			
		Sp. Activity (U mg^{-1})		Activity (U ml^{-1})	Productivity (U h^{-1} l^{-1})
		Observed	Predicted		
P. carotovorum MTCC 1428	Unoptimized Medium	36.15	-	9.39	1565.00
	Optimized Medium	61.38	60.18	12.86	2143.33
	Optimized physical process conditions	70.25	68.53	16.41	2735.00
E. carotovora subsp. *atroseptica* SCRI 1043	Unoptimized Medium	34.49	-	10.30	1716.67
	Optimized Medium	57.06	54.77	13.23	2205.00
	Optimized physical process conditions	67.26	64.66	15.53	2588.33

CCD has been applied in several similar studies to optimize the physical process parameters for the production of glucansucrase (Ellaiah *et al.,* 2002), citric acid (Lotfy *et al.,* 2006), cephalosporin C (Adinarayana *et al.,* 2003), bacteriocin (Motta and Brandelli, 2008), L-asparaginase (Kumar *et al*., 2011a) etc. There is no report available in the literature on the optimization of pH, agitation and inoculum size on the production of recombinant glutaminase-free L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043. However, a few reports are available on the production of L-asparaginase using statistical methods. Abdel-Fattah and Olama (2002) have applied Plackett-Burman and Box-Behnken experimental designs for screening and optimization of process parameters to improve the production of L-asparaginase from *P. aeruginosa* in solid state fermentation. Neto *et al.,* (2006) and Prakasham *et al.,* (2007) also reported the enhanced production of L-asparaginase from *Zymomonas mobilis* and *Staphylococcus* at optimal level

of process parameters by Box-Behnken experimental and Taguchi methodology design, respectively in submerged fermentation.

4.8. Optimization of IPTG and lactose levels

The lac promoter-derived expression systems are generally used for the production of heterologous proteins in *E. coli* (Viitanen *et al*., 2003) and one of the most commonly used strategies is its induction with the nonmetabolizable analog of lactose, isopropyl-β-D-thiogalactopyranoside (IPTG) (Bhandari and Gowrishankar, 1997; Woyski and Cupp-Vickery 2001). In spite of its higher cost and toxicity of IPTG, few reports are available in the literature on the lactose as inducer for foreign gene expression. It is very difficult to establish the ideal induction conditions with lactose as it serves simultaneously as an inducer, a carbon and energy source (Gombert and Kilikian, 1998; Kilikian *et al*., 2000). However, lactose has been chosen in large-scale production of recombinant proteins due to its lower cost as compared to IPTG. However, the expression of recombinant protein was found to be lower with lactose than IPTG (Pan *et al*., 2008). To study the effect of lactose or IPTG as inducer, expression of recombinant L-asparaginase II of both the strains was carried out using different concentration of lactose/IPTG. Expression of recombinant L-asparaginase II of both the strains increased when, 0.1 mM to 1.0 mM of IPTG was used as inducer and beyond that it decreased (Manderson *et al*., 2006; Yee and Blanch 1993). Similar trend was also observed with lactose. Expression of recombinant L-asparaginase II was increased when lactose concentration was increased from 0.1 to 1.0 mM and beyond that higher concentration was not found to be favorable for expression. Probably, due to conversion of lactose in to glucose and galactose and it is well acknowledged that glucose have shown catabolite repression (Zhang *et al*., 2003). Maximum expression of recombinant L-asparaginase II of *P.*

carotovorum MTCC 1428 was observed to be 70.62 Umg^{-1} and 64.03 Umg^{-1} with 1mM IPTG and with 1mM lactose, respectively (Fig. 4.15 A).

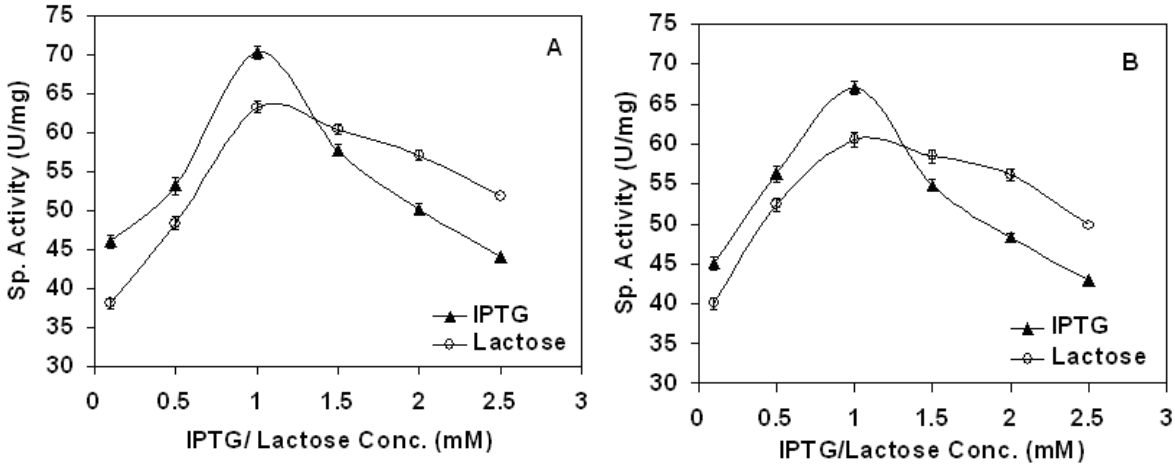

Fig. 4.15. Expression of recombinant L-aspraginase II with IPTG/lactose (**A**: *P. carotovorum* MTCC 1428 and **B**: *E. carotovora* subsp. *atroseptica* SCRI 1043).

But highest expression of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 was observed (67.26 U mg^{-1} of protein) with 1mM IPTG as compared to induction with 1mM lactose (60.53 $Umg^{-1}$of protein) (Fig. 4.15 B). Kweon *et al*., (2001) and Kilikian *et al*., (2000) have also reported that the higher expression of recombinant protein with IPTG than lactose as an inducer.

4.9. Effect of glucose and glycerol on the production of recombinant L-asparaginase II

Glucose and glycerol are the most commonly used carbon sources for the production of recombinant proteins (Losen *et al*., 2004). It was reported that addition of 0.05% glucose, improves not only plasmid stability but also protein yields (Studier *et al*., 2005; Zhang *et al*., 2003). In order to determine the optimum levels of glucose/glycerol for enhance production of recombinant L-asparaginase II of both the strains, experiments were carried out at different

concentration of glucose/glycerol. Optimal concentrations of glucose and glycerol were found to be 0.1% and 0.2 %, respectively and beyond that decrease in expression of recombinant L-asparaginase II was observed (Fig. 4.16 A and B).

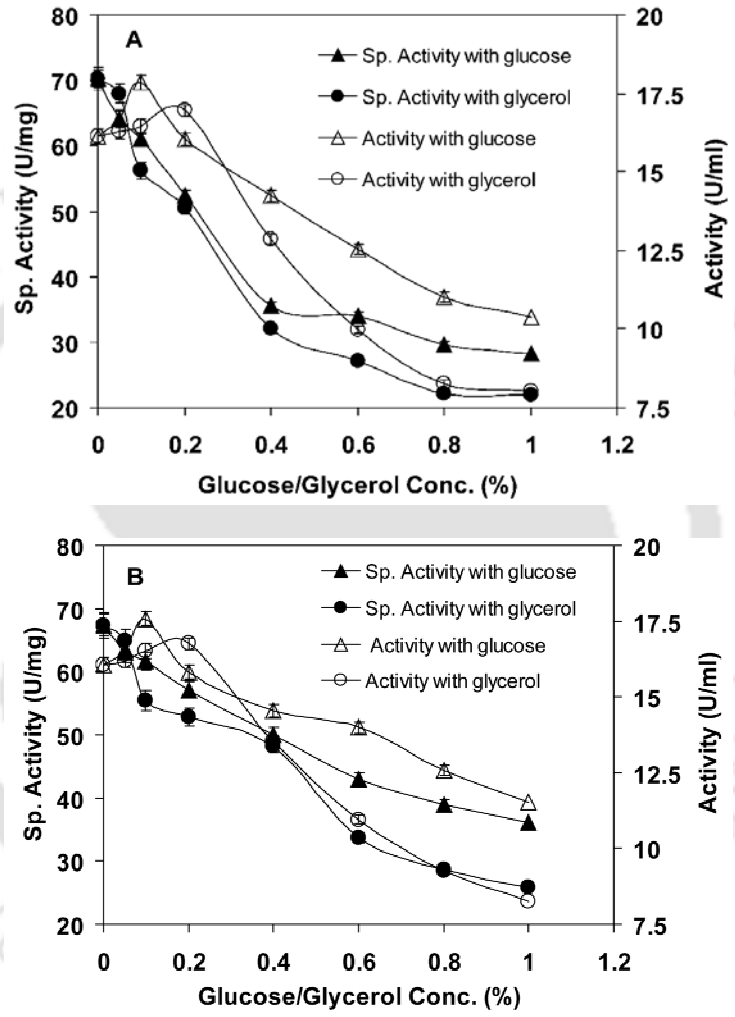

Fig. 4.16. Effect of glucose/glycerol on recombinant L-aspraginase II production (**A**: *P. carotovorum* MTCC 1428 and **B**: *E. carotovora* subsp. *atroseptica* SCRI 1043).

Due to higher cell growth in the medium containing glucose, higher productivity was observed as compared to glycerol. Higher concentrations of glucose, causes catabolite repression and also triggers the acetic acid production, which is not favorable for growth and

expression of recombinant proteins (Mark and Elliot, 2006). Similarly, in the present study, catabolite repression was observed above 0.1% of glucose in the medium. It is reported that the production of acetic acid and oxygen limitation at higher concentration of glycerol in the medium. The specific production rate of acetic acid from glycerol was reported to be approximately 70% of the rate from glucose (Strandberg and Enfors, 1991). Due to accumulation of acetate, decrease in cell growth and expression of recombinant protein was observed under oxygen limited condition (Losen *et al*., 2004). Therefore, higher concentration of glucose (>0.1%) and glycerol (>0.2%) were not found to be favorable for the production of recombinant L-asparaginase II of both the strains.

4.10. Production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 *and E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3) in a batch and fed batch bioreactor

After optimization of production conditions at shake flask level, final efforts were employed to optimize the production of recombinant L-asparaginase II of both the strains in a lab scale bioreactor. Initially, the effect of controlled and un-controlled pH on the production of recombinant L-asparaginase II was studied in a batch bioreactor. It was observed that controlled pH (pH 7.0) was favorable for higher production of enzymes. After 7 h of post-induction, reduction in production was observed under uncontrolled pH as compared to control pH conditions (Fig. 4.17 and 4.18). After 8 h of post induction, pH tends to increase beyond 7.0, which might not be favorable for recombinant L-asparaginase II production. Therefore, production of recombinant L-asparaginase II of both the strains was studied under control pH conditions. There are many reports available in the

literature on the production of recombinant proteins under control pH condition (Lau *et al.*, 2004; Panda *et al*., 1999; Ramalingam *et al*., 2007).

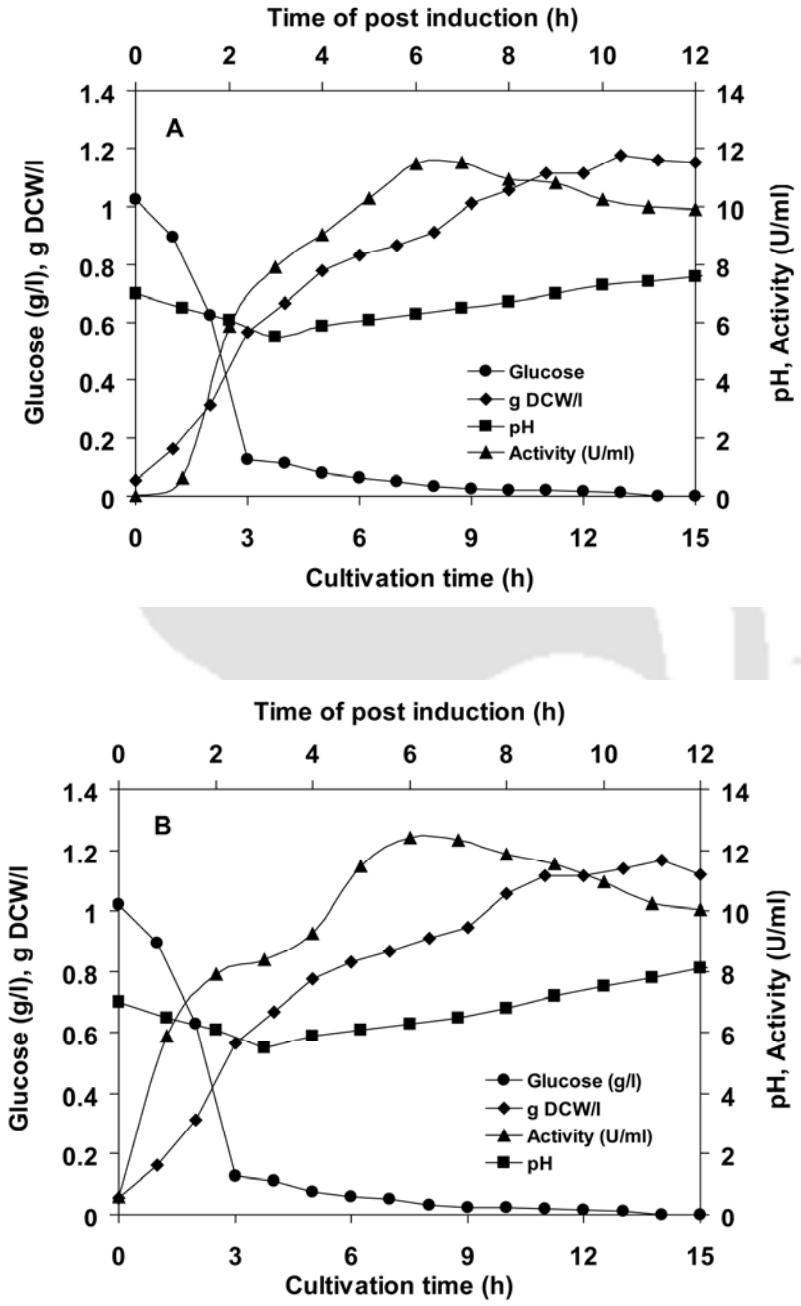

Fig. 4.17. Production of recombinant L-asparaginase II under un-controlled pH conditions (**A**: *P. carotovorum* MTCC 1428 and **B**: *E. carotovora* subsp. *atroseptica* SCRI 1043).

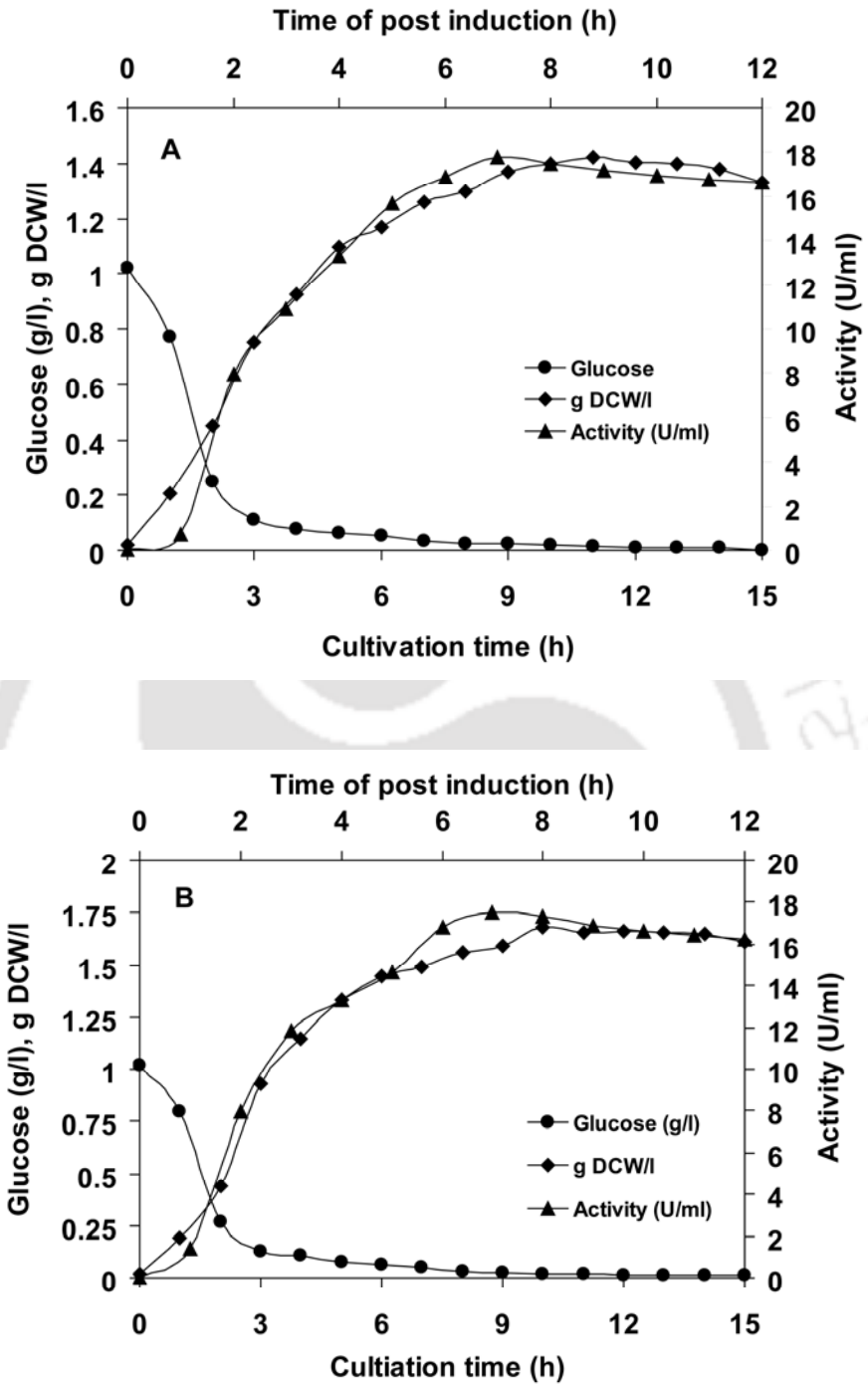

Fig. 4.18. Production of recombinant L-asparaginase II under controlled pH conditions (**A**: *P. carotovorum* MTCC 1428 and **B**: *E. carotovora* subsp. *atroseptica* SCRI 1043).

4.10.1. Optimization of production of recombinant L-asparaginase II of P. carotovorum MTCC 1428 and E. carotovora subsp. atroseptica SCRI 1043 in E. coli BL21 (DE3) using Taguchi's method in a batch bioreactor

According to Taguchi's orthogonal array of L9, 9, experiments were performed as described in the section 3.14.2 and results are presented in the Table 4.12.

Table 4.12. Taguchi's robust experimental design matrix and corresponding recombinant L-asparaginase II production in a batch bioreactor

Run No.	Glucose ($g\ l^{-1}$)	pH	DO (%)	*P. carotovorum* MTCC 1428			*E. carotovora* subsp. *atroseptica* SCRI 1043		
				Activity (Uml^{-1})	Sp. activity (Umg^{-1})	S/N	Activity (Uml^{-1})	Sp. activity (Umg^{-1})	S/N
1	0.5	6.5	10	5.50	28.65	14.80	3.81	27.41	11.61
2	0.5	7.0	25	16.14	43.87	24.15	14.98	49.84	23.51
3	0.5	7.5	40	12.94	39.32	22.23	12.35	42.62	21.83
4	1.0	6.5	25	9.83	49.46	19.85	6.02	41.06	15.59
5	1.0	7.0	40	20.69	60.75	26.13	16.98	57.95	24.59
6	1.0	7.5	10	14.14	45.30	23.00	11.12	35.63	20.92
7	1.5	6.5	40	12.06	50.82	21.63	13.13	49.02	22.36
8	1.5	7.0	10	20.12	53.68	26.07	19.24	52.21	25.68
9	1.5	7.5	25	17.19	52.96	24.70	16.99	51.25	24.60

Depending upon the combination of parameters (concentration of glucose, controlled pH and DO level) and their levels, production of recombinant L-asparaginase II of both the strains varied in each run significantly, as indicating the strong influence of the variables and their levels on the response (production of L-asparaginase). Further, to understand the effect of these variables on the production of recombinant L-asparaginase II, their ranking was performed based on the calculated delta S/N ratio (Table 4.13). In general, delta value for a factor, calculated by measuring the difference between the highest and lowest characteristic average S/N ratio of the factor, specify its relative significance over others on a given response. Higher value of delta for a factor indicates a

larger significant effect than others, while S/N ratio indicates effect of factors on a response. Thus delta S/N ratio have used as a decisive factor for ranking the variables for their effects on the response (Dasu *et al.,* 2003; Daverey *et al.,* 2010). In this investigation, based on the delta S/N ratio obtained for each factor, three parameters were ranked accordingly, which observed that pH had the maximum effect and followed by glucose concentration and DO level (Table 4.13).

Table 4.13. S/N ratio and ranking of the process variables at various levels of variables

Level	*P. carotovorum* MTCC 1428			*E. carotovora* subsp. *atroseptica* SCRI 1043		
	Glucose	pH	DO	Glucose	pH	DO
1	20.40	18.76	21.29	18.99	16.53	19.41
2	23.06	25.52	22.91	20.37	24.60	21.24
3	24.14	23.32	23.40	24.22	22.45	22.93
Delta	3.74	6.75	2.10	5.23	8.07	3.52
rank	2	1	3	2	1	3

To validate these findings on the significance of the individual parameters and their contribution on L-asparaginase II production, ANOVA of the results was performed. Table 4.14 and 4.15 present the ANOVA of production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3), respectively that shows a term for error (the value of mean sum of squares: MS). In general, low *P* value of a term in ANOVA indicates that high significance of the term. Hence, in this study, all the parameters *viz.*, glucose conc., pH and DO level were found to have a significant effect on the production of recombinant L-asparaginase II. The effect of parameters on the production of recombinant L-asparaginase II by ANOVA is in good agreement with those observed earlier from the factors ranking based on their delta S/N ratio.

Table 4.14. Analysis of variance for the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428

Source	DF	Seq SS	Ads SS	Ads MS	*F*	*P*
Glucose	2	38.118	38.118	19.0588	215.13	0.005
pH	2	146.525	146.525	73.2623	826.94	0.001
DO	2	5.929	5.929	2.9646	33.46	0.029
Residual Error	2	0.177	0.177	0.0886		
Total	8	190.749				

R-Sq = 99.9% , R-Sq(adj) = 99.6%

Table 4.15. Analysis of variance for the production of recombinant L-asparaginase II *E. carotovora* subsp. *atroseptica* SCRI 1043

Source	DF	Seq SS	Ads SS	Ads MS	*F*	*P*
Glucose	2	63.678	63.678	31.8392	120.51	0.008
pH	2	135.455	135.455	67.7275	256.34	0.004
DO	2	11.477	11.477	5.7387	21.72	0.044
Residual Error	2	0.528	0.528	0.2642		
Total	8	211.139				

R-Sq = 99.7%, R-Sq(adj) = 99.0%

4.10.2. Selection of optimum levels of parameters for enhanced recombinant L-asparaginase II production

The three parameters (concentration of glucose, pH and DO level) were analyzed at different levels. Particular levels of the variables caused a significant increase in the mean response as compared to other levels of the variables. For example level 3 of glucose, DO and level 2 of pH were found to exhibit a noteworthy positive consequence on the response (Fig. 4.19 A and B). These levels of the variables were therefore selected as optimum for the maximum production of recombinant L-asparaginase II of both the strains.

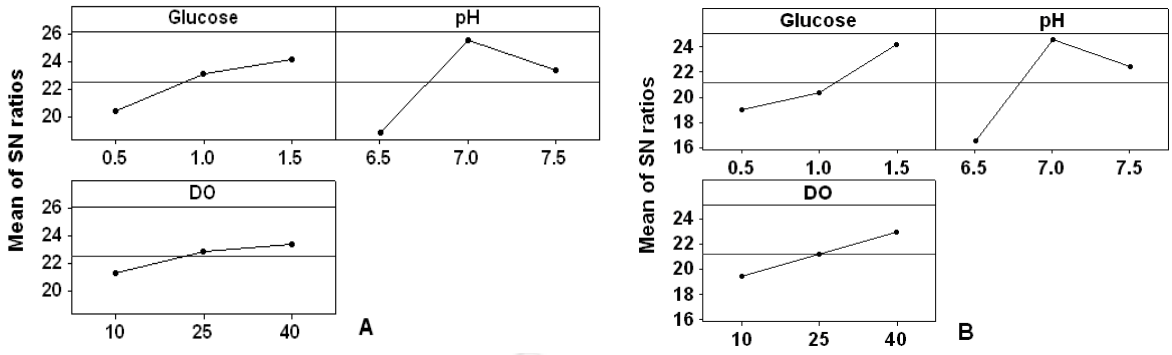

Fig. 4.19. Main effect plot for SN ratios for recombinant L-asparaginase II production (**A**: *P. carotovorum* MTCC 1428 and **B**: *E. carotovora* subsp. *atroseptica* SCRI 1043

At the optimum levels of parameters, the production of recombinant L-asparaginase II of both the strains was performed in a batch 3 L bioreactor (Applikon, Holland) with 1.0 L medium. The maximum production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 23.88 U ml^{-1} and 24.57 U ml^{-1}, respectively. The production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 1.34 and 1.40 fold higher under optimal levels of parameters, respectively as compared to optimized conditions in shake flask culture. Maximum production of recombinant L-asparaginase II of both the strains was achieved at 7 h of post induction in a batch bioreactor and the overall productivity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 increased from 2971.66 U l^{-1} h^{-1} to 3411.43 U l^{-1} h^{-1} and 2930.00 U l^{-1} h^{-1} to 3510.00 U l^{-1} h^{-1}, respectively.

In the literature, reports are available on the optimization of production conditions by Taguchi's method. Dasu *et al*., (2003) and Niccolai *et al*., (2003) have applied Taguchi's method to enhance the production of griseofulvin from *Penicillium griseofulvum* MTCC 1898 and recombinant neutrophila activating (NAP) protein of *Helicobacter pylori* from *E. coli*, respectively in a batch

bioreactor. Hao *et al*., (2006) have also exploited Taguchi's method to screen the significant medium components and optimization of the medium components for higher production of cytochrome P450 2C9 from *E. coli* DH5α. Ghane *et al.*, (2008) have achieved 28% higher production of interferon beta from *E. coli* using Taguchi's method. Recently Daverey *et al*., (2010) has optimized the sophorolipid (SL) production by the yeast *Candida bombicola* in a batch bioreactor using same method. Experiments were performed to enhance the production and productivity of recombinant L-asparaginse II in Fed-batch bioreactor using different strategies.

4.10.3. Enhanced production of recombinant L-asparaginase II in fed-batch culture

Fed-batch cultures were performed to achieve maximum cell density, production and productivity of recombinant L-asparaginase II in a 3 L bioreactor under previously optimized batch conditions. Various feeding strategies were applied to enhance the cell density, production and productivity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428.

4.10.3.1. Exponential feeding

To achieve high cell density, bioreactor was operated in fed-batch mode with commonly used host organism, *E. coli*, for the production of recombinant proteins (Lee, 1996). Maintaining the specific growth rate at an appropriate range would provide a desirable metabolic condition and results in maximum productivity (Babaeipour *et al*., 2007). Therefore, exponential feeding is adopted as a convenient method to avoid by-product formation and to obtain maximum attainable cell density (Shiloach and Fass, 2005). But, the details of such feeding are still a matter of debate and new techniques are aimed at optimizing the feeding method (Babaeipour *et al*., 2008; Bahrami *et al*., 2008; Ting *et al*., 2008). In this study, after exhaustion of initial glucose, exponential feeding was started after 3 h of the onset of batch

cultivation with the solution containing 200 g l^{-1} of glucose, 200 g l^{-1} of yeast extract and 100 mg l^{-1} ampicillin in growth phase. Exponential feeding was adopted starting from an initial glucose feeding rate of 0.5 g l^{-1} h^{-1} to a final rate of 15.20 g l^{-1} h^{-1} to achieve the maximum cell density at a controlled growth rate (μ = 0.2 h^{-1}). Maximum biomass of *E. coli* BL21 (DE3) was found to be 5.18 g l^{-1} and stability of the recombinant plasmid dropped to 52%. Maximum production of recombinant L-asparaginase II (40.34 U ml^{-1}) of *P. carotovorum* MTCC 1428 was observed after 8h of post induction (Fig. 4.20). The constant decrease in growth rate of plasmid bearing cells may be due to the metabolic stress caused by an increased production of foreign gene product. Exponential feeding methods have been well characterized and have been utilized to produce numerous heterologous proteins in *E. coli* (Choi *et al.*, 200; Frings *et a*l., 1995; Harrison *et al*., 1997; Hoffman *et al*., 1995; Shin *et a*l., 1997). During exponentially feeding, slower growth of plasmid bearing cells and nutrient accumulation was observed in the form of high residual glucose after post induction and therefore, further feeding was stopped. Since, glucose was accumulated in the later stages of feeding during post induction, the decline in growth rate of plasmid bearing cells may not be due to carbon limitation, but might be due to acetate accumulation or due to cell lysis (Fu *et al*., 2005). Chung *et al*., (2000) have also observed the lower production of recombinant human insulin-like growth factor-I in *E. coli* by exponential feeding. Similarly, Ramalingam *et al*. (2007) have also observed lower production of recombinant streptokinase in *E. coli* by exponential feeding due to accumulation of carbon and nitrogen sources. But, Li *et al.*, (1998) have observed higher production of glutathione production from recombinant *E. coli* by exponential feeding. Furthermore, Khushoo *et al*., (2005) have also observed that the production of recombinant L-asparaginase of 8.7×10^5 units l^{-1} from *E. coli.*

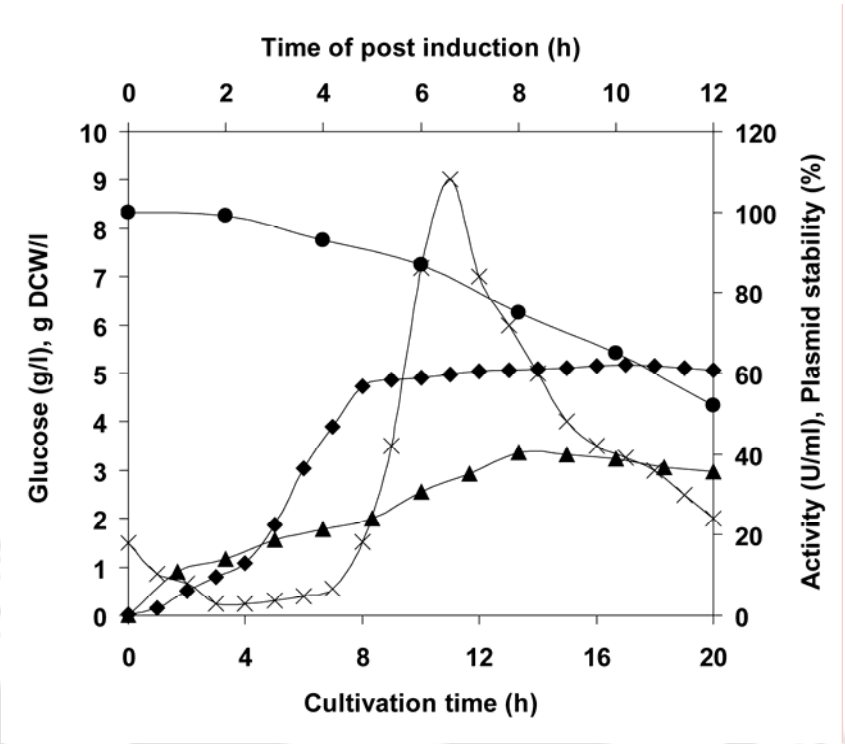

Fig. 4.20. Production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 in *E.coli* using exponential feeding (g DCW/l, Glucose (gl^{-1}), Activity (U ml^{-1}) and Plasmid stability).

4.10.3.2. Constant feeding

The simplest predetermined feeding profile is constant rate feeding. In constant- rate feeding, feeding solution (100 g l^{-1} of glucose, 100 g l^{-1} of yeast extract and 100 mg l^{-1} ampicillin) was fed into the bioreactor at predetermined fixed rates (Goyal *et al*., 2009). Feeding of glucose was started after 3 h of batch cultivation at a predetermined rate of 2.0 g l^{-1} h^{-1}. Owing to the increase in culture volume and cell concentration in the bioreactor, the increase in cell concentration slows down over time. A maximum cell mass and enzyme activity of 3.75 g l^{-1} and 22.15 U ml^{-1} was achieved, respectively. The stability of plasmid was dropped to 43% (Fig. 4.21). During the constant feeding, the feed rate was limiting throughout the entire phase and therefore the residual glucose levels were found to be consistently low. The residual glucose was found to be ~0.35 g l^{-1}. Probably, the feed rate in this case was lower than the

substrate uptake rate and therefore, may not be able to supply the nutrient levels required for maintaining the growth rate and recombinant protein production.

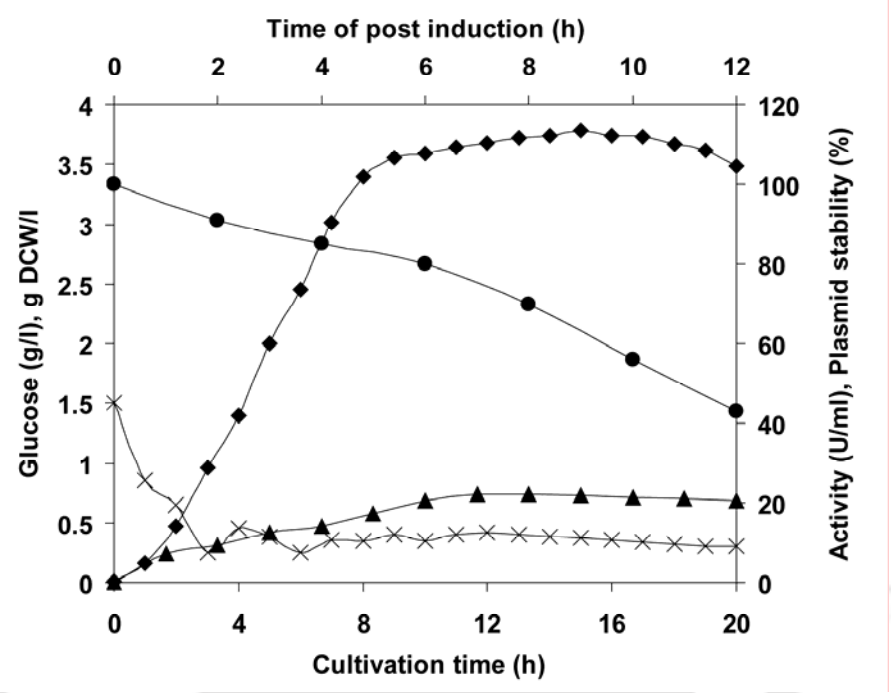

Fig. 4.21. Production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 in *E. coli* using constant feeding (→ g DCW/l, → Glucose (gl^{-1}), → Activity (U ml^{-1}) and →Plasmid stability).

Ramalingam *et al*., (2007) have also observed lower cell growth and production of recombinant streptokinase in *E. coli* due to lower supply of glucose. However, Goyal *et al.,* (2009) have improved recombinant streptokinase production in fed batch culture using constant rate of feeding.

4.10.3.3. Intermittent pulse feeding

In order to avoid the inhibitory effect of glucose concentration on the biomass and production of recombinant L-asparaginase II, intermittent pulse feeding was applied with solution containing 100 g l^{-1} of glucose, 100 g l^{-1} of yeast extract and 100 mg l^{-1} ampicillin. To supply the sufficient amount of glucose to the system, glucose was fed into the bioreactor in a step-

wise increasing rate in proportional to the biomass and feeding rate was adjusted every hour accordingly (Giridhar and Srivastava 2000; Nayak and Vyas, 1999; Ramalingam and Gautam, 2007).

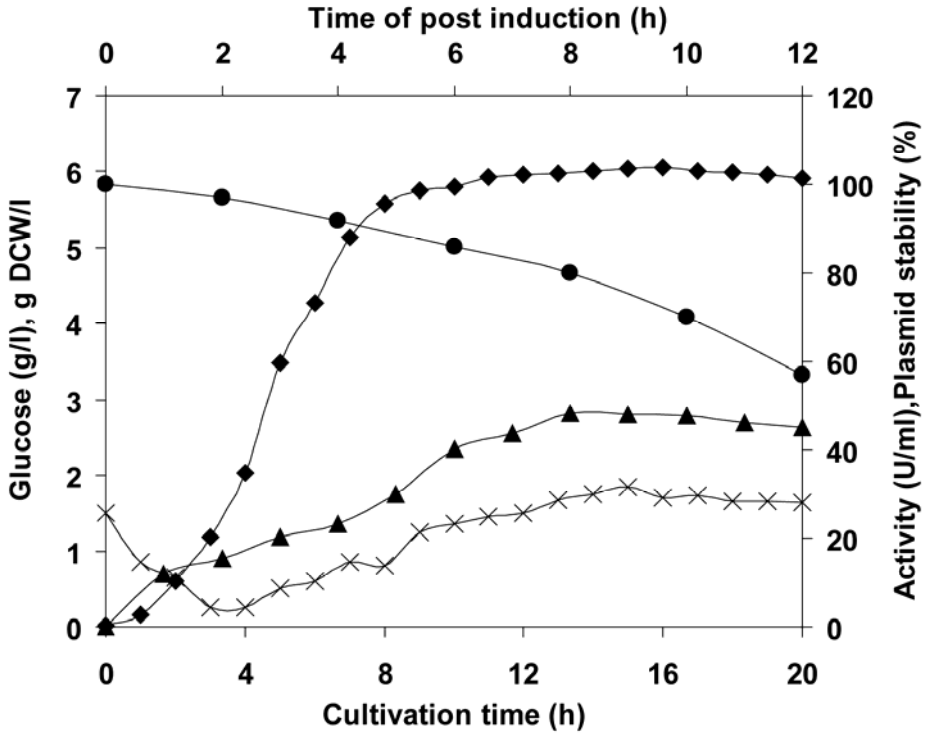

Fig. 4.22. Production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 in *E.coli* using intermittent pulse feeding with specific glucose supply rate of 0.50 g glucose (g DCW)$^{-1}$ h^{-1} (g DCW/l, Glucose (g.l), Activity (U ml^{-1}) and Plasmid stability).

In this strategy, feeding was done based on the residual substrate concentration available in the reactor. As the residual concentration of glucose was ~1.5 g l^{-1}, the feeding was started with specific supply rate of 0.5 g glucose (g DCW)$^{-1}$ h^{-1}. When the cell OD at 600 nm reached ~10.00, the expression of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 was induced by the addition of 1mM IPTG and glucose was fed with specific supply rate of 0.5 g glucose (g DCW)$^{-1}$ h^{-1}. There was an inverse correlation between the glucose feeding rate and the production level was observed. When feeding rate of 0.5 g glucose (g DCW)$^{-1}$ h^{-1} both in

growth and production phase, the maximum biomass, enzyme activity and plasmid stability of 6.05 g l^{-1}, 48.54 U ml^{-1} and 57% were achieved, respectively (Fig. 4.22). Probably, due to lower accumulation of glucose the production was improved in this method (48.45 U ml^{-1}) as compared to exponentially feeding (40.34 U ml^{-1}). Therefore, in the next experiment, the rate of glucose feeding was reduced in the production phase. The glucose was fed at a rate of 0.5 g glucose (g DCW)$^{-1}$ h^{-1} in growth phase. To avoid the glucose accumulation in production phase, glucose was fed with specific feed rate of 0.25 g glucose (g DCW)$^{-1}$ h^{-1} in the production phase. As the concentration of glucose dropped below 1.0 g l^{-1}, the feeding was started. It was observed that the residual glucose was detected to be below 0.75 g l^{-1} with feeding rate of 0.25 g glucose (g DCW)$^{-1}$ h^{-1} in the production phase, and the maximum biomass (7.32 g l^{-1}), plasmid stability (64%) and production (95.85 U ml^{-1}) obtained were much higher than the other feeding regimes (Fig. 4.23). After finding out best strategy for production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 in *E. coli*, the same intermittent pulse feeding strategy with specific feed rate of 0.5 g glucose (DCW)$^{-1}$ h^{-1} and 0.25 g glucose (g DCW)$^{-1}$ h^{-1} in growth phase and production phase, respectively was used for production of recombinant L-aspraginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* in fed-batch culture. In fed-batch mode of fermentation, four fold higher production (96.78 U ml^{-1}) of recombinant L-aspraginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* was achieved as compared to the batch culture (24.57 U ml^{-1}). The maximum biomass and plasmid stability were found to be 7.35 g l^{-1} and 62 %, respectively (Fig. 4.24).

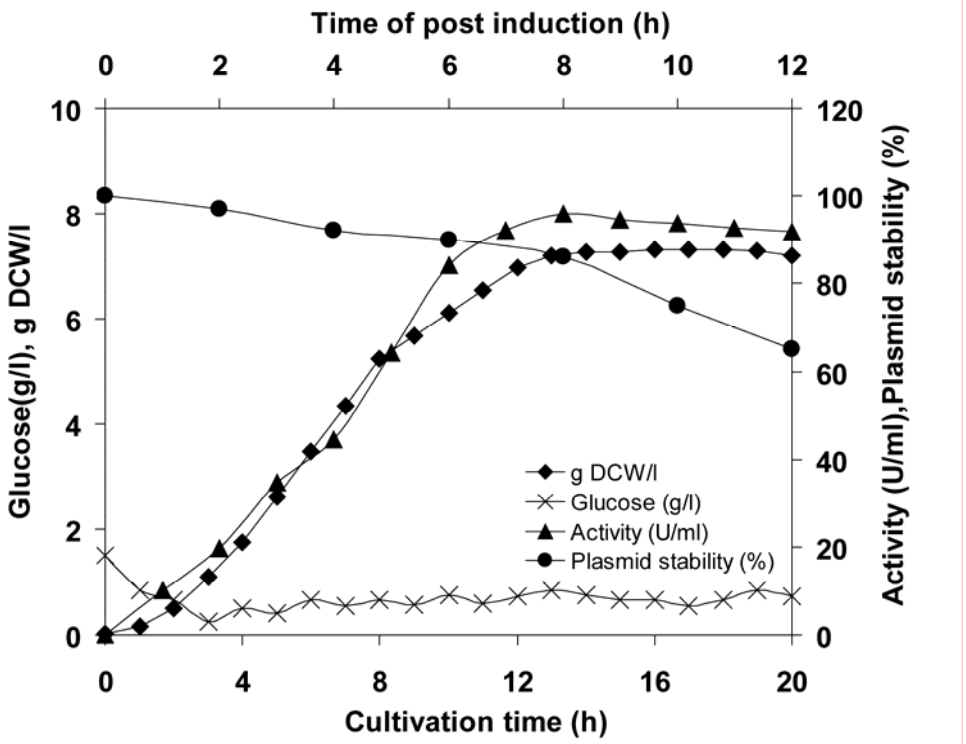

Fig. 4.23. Production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 in *E. coli* using intermittent pulse feeding with specific glucose supply rate of 0.50 g glucose $(gDCW)^{-1}$ h^{-1} and 0.25 g glucose $(gDCW)^{-1}$ h^{-1} in growth phase and production phase, respectively.

The production of recombinant L-aspraginase II of both the strains was increased to a maximum value and then began to decline in all the feeding methods, within 8 h of post-induction. It might be due to significant plasmid loss was observed after 8 h of post induction. However, higher plasmid loss was observed in constant feeding as compared to other feedings, which might be due to stress in nutriment limiting conditions.

Many authors have studied the effect of glucose feeding profile on recombinant protein production in *E. coli* (Hoffmann *et al*., 200; Lin and Neubauer 2000; Madurawe *et al.*, 2000). Chen *et al*., (2008) have studied the production of L-Threonine by recombinant *E. coli* by constant feeding and intermittent pulse feeding in DO stat mode and concluded that pulse fed-

batch could activate the oscillation of microorganism metabolism during the whole culture process and the medium was prone to be utilized completely.

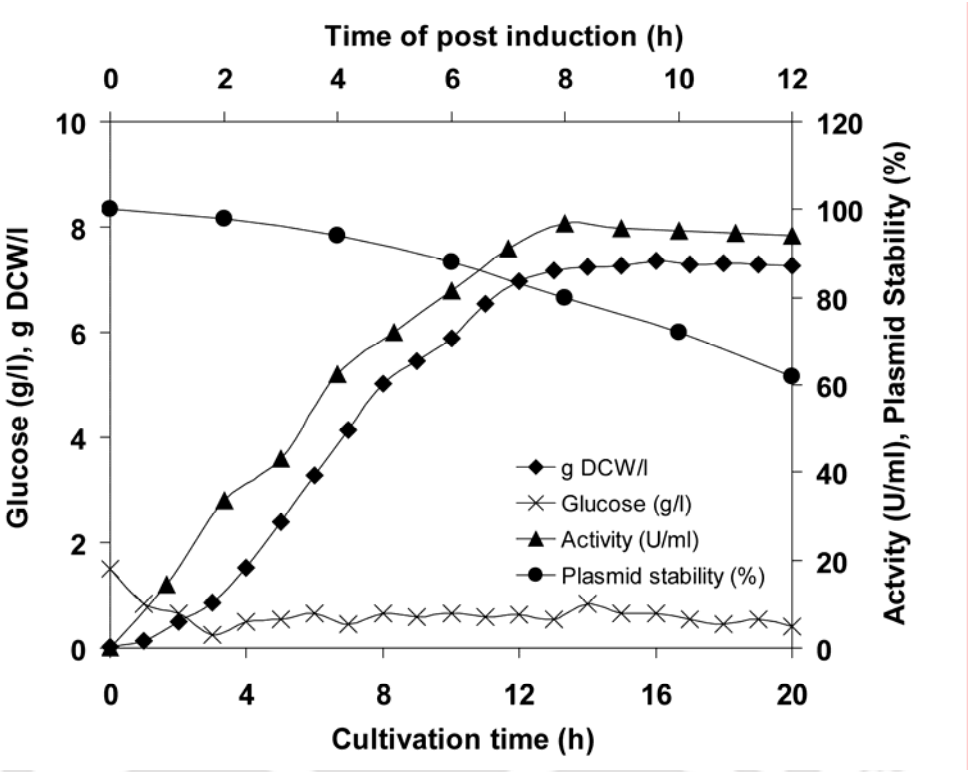

Fig. 4.24. Production of recombinant L-asparagin ase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E.coli* using intermittent pulse feeding with specific glucose supply rate of 0.50 g glucose $(gDCW)^{-1}$ h^{-1} and 0.25 g glucose $(gDCW)^{-1}$ h^{-1} in growth phase and production phase, respectively.

Giridhar and Srivastava (2000) have achieved higher production of L-sorbose by *Acetobacter suboxydans* by pulse feeding. Kweon *et al*., (2001) have also achieved higher production of *Phytolacca insularis* protein in recombinant *E. coli* by stepwise increasing feeding. By using intermittent pulse feeding of substrate, Gummadi and Bhavya (2011) have achieved enhanced production of caffeine demethylase from *E. coli*.

Table 4.16. Comparison of recombinant L-asparaginase II production and productivity at various stages of *P. carotovorum* MTCC 1428 in *E. coli*.

Process conditions	Shake flask/ Bioreactor	L-asparaginase production		Productivity ($U\ L^{-1}\ h^{-1}$)	Enhanced Productivity (fold)	Enhanced production* (fold)
		Activity ($U\ ml^{-1}$)	Specific activity ($U\ mg^{-1}$)			
Before optimization	Shake flask	9.39±0.50	36.15±0.22	1565.00±0.15	--	--
Optimized medium	Shake flask	12.86±0.33	61.38±0.32	2143.33±16.52	1.37	1.37
Optimized process parameters	Shake flask	16.41±0.48	70.25±0.69	2735.00±45.23	1.75	1.75
Optimized Glucose Conc.	Shake flask	17.83±0.56	67.95±0.86	2971.66±21.78	1.90	1.90
Optimization in bioreactor	Batch bioreactor	23.88±0.44	63.19±0.52	3411.43±58.25	2.18	2.54
Fed Batch	Fed-batch bioreactor	95.85±0.43	52.40±0.71	11981.00±48.50	7.66	10.20

*Enhanced production on the basis of enzyme activity ($U\ ml^{-1}$).

Table 4.17. Comparison of recombinant L-asparaginase II production and productivity at various stages of the *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli*.

Process conditions	Shake flask/ Bioreactor	L-asparaginase production		Productivity ($U\ L^{-1}\ h^{-1}$)	Enhanced Productivity (fold)	Enhanced production* (fold)
		Activity ($U\ ml^{-1}$)	Specific activity ($U\ mg^{-1}$)			
Before optimization	Shake flask	10.30±0.50	34.49±0.12	1716.67±0.40	--	--
Optimized medium	Shake flask	13.63±0.31	57.06±0.56	2271.66±35.21	1.32	1.32
Optimized process parameters	Shake flask	16.05±0.63	67.26±0.41	2675.15±50.30	1.56	1.56
Optimized Glucose Conc.	Shake flask	17.58±0.62	64.62±0.78	2930.00±37.71	1.71	1.71
Optimization in bioreactor	Batch bioreactor	24.57±0.39	61.71±0.85	3510.25±38.98	2.04	2.38
Fed Batch	Fed-batch bioreactor	96.78±0.69	54.59±0.75	12097.50±52.23	7.05	9.39

*Enhanced L-asparaginase production calculated from L-asparaginase activity ($U\ ml^{-1}$).

Zhang *et al.,* (2009) have studied three feeding rates of glucose supply [1.06 g (g DCW)$^{-1}$ h^{-1}, 0.64 g (g DCW)$^{-1}$ h^{-1} and 0.25 g (g DCW)$^{-1}$ h^{-1}]. They observed that the inverse correlation between the glucose feeding rate and achieved highest production of TATm-survivin (T34A) with lowest feeding rate of 0.25 g (g DCW)$^{-1}$ h^{-1}.From the various feeding regimes, it was observed that the amount of nutrient fed is critical for the production of the recombinant proteins. Nutrient limitation leads to lowered raw material and energy availability for growth and product formation. It has also been observed that excessive levels of carbon and nitrogen substrates (Deutch 1989; Villaverde 1993; Vila *et al.*, 1994) or high levels of acetate accumulation (Anda *et al.*, 2006; Shiloach *et al*., 1996) lead to reduced recombinant protein productivity, and this might be the reason for lower production in cases of exponential and intermittent feeding with 0.5 g glucose (gDCW)$^{-1}$ h^{-1} in the production phase. Hence, low levels of nutrient feed as well as excessive nutrient feed are both detrimental for the production of recombinant L-asparaginase II. Fed-batch techniques are often employed to achieve a high volumetric yield. Fed-batch cultures have been successfully used to increase the production and productivity of protein for *e.g* recombinant human interferon-γ (Babaeipour *et al*. 2007), recombinant streptokinase from *E. coli* (Goyal *et al*., 2009), recombinant anticancer drug TATm-survivin from *E. coli* (Zhang *et al*. (2009), recombinant human soluble catechol-O-methyltransferase (hSCOMT) from *E. coli* (Passarinha *et al*. 2009), microbial production of acetoacetate by recombinant *E. coli* (Hu *et al*., 2010), monoamine oxidase by recombinant *E. coli* (Voulgaris *et al.*, 2011) and recombinant potato carboxypeptidase inhibitor from *E. coli* (Puertas *et al.*, 2011). The overall increase in the production of novel glutaminase free recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 by 10.2 and 9.4 fold,

respectively. The productivity was increased by 7.66 and 7.05 fold for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively.

4.11. Purification of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* BL21 (DE3)

The purification of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovorum* subsp. *atroseptica* SCRI 1043 was carried out by single step Ni–NTA affinity chromatography. The specific activity of recombinant asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 increased from 70.25 to 287.36 U mg^{-1} and 67.26 to 282.45 U mg^{-1}, respectively. The recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were purified by 4.09 and 4.20 fold with 77% and 82% of yield, respectively (Table 4.18).

Table 4.18. Purification profile of L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. SCRI 1043 from *E. coli* BL21 (DE3)

Protein	Step	Total Units (IU)	Total protein (mg)	Sp. Activity (U mg^{-1})	Purification (fold)	Yield (%)
P. carotovorum MTCC 1428	Crude Extract	2148.36	30.58	70.25	--	100
	Ni- NTA Column	1654.24	5.75	287.36	4.09	77
E. carotovora subsp. atriceptica SCRI 1043	Crude Extract	1896.52	28.20	67.26	--	100
	Ni- NTA Column	1555.15	5.50	282.45	4.20	82

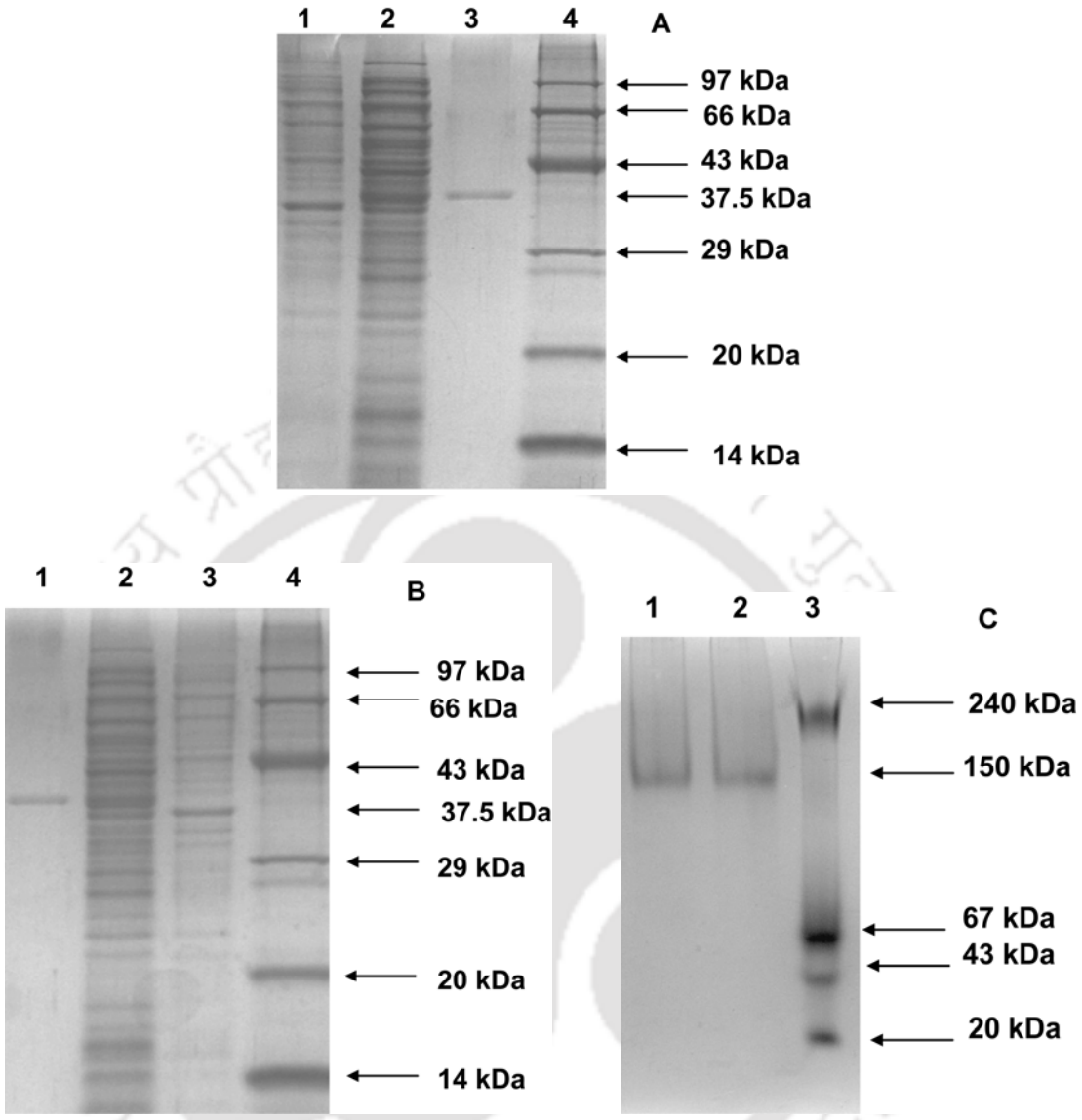

Fig. 4.25. Purification of recombinant protein (**A**: SDS polyacrylamide gel electrophoresis (12.5%) of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 (Lane 1: pET 22b(+) in *E. coli* BL21 (DE3) without any insert Lane 2: Crude extract, Lane 3: Purified recombinant L-asparaginase II, and Lane 4: Marker. **B**: SDS Polyacrylamide gel electrophoresis (12.5%) of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 (Lane 1: Purified recombinant L-asparaginase II, Lane 2: Crude extract, Lane 3: pET 22b(+) in *E. coli* BL21 (DE3) without any insert and Lane 4: Marker. **C**: Native PAGE (7.5%) of L-asparaginase (Lane 1: Purified recombinant L-asparaginase II of *P. carotovorum* MTCC 1428, Lane 2: Purified recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 and Lane 3: Marker).

SDS–PAGE and Native PAGE analysis of the purified recombinant L-asparaginase II of both the strains have confirmed the presence of a single protein band of ~37.5 kDa and ~150.0 kDa, respectively (Fig. 4.25). Khushoo *et al*., (2004) have also cloned L-asparaginase II of *E. coli* in pET22 b(+) and expressed in *E. coli* BL21(DE3). They have achieved 3.3 folds purification of recombinant L-asparaginase II with 86% yields using Ni-NTA column. Kotzia *et al*., (2007) have cloned and expressed L-asparaginase of *E. chrysanthemi* 3937 in T7 expression pCR®T7/CT-TOPO® and expressed in *E. coli* BL21(DE3) pLysS cells. They have achieved 15.4 folds purification with 69.8% yield using S-Sepharose FF column.

4.12. Characterization of purified recombinant L-asparaginase II

4.12.1. Effect of pH on enzyme activity and stability

An enzyme has a characteristic pH optimum for maximum activity and beyond that reduction in activity was observed (Lubkowski *et al*., 1994). Recombinant L-asparaginase II of both the strains at different pHs show that the enzymes have a wide range of activity between 6.5 and 9.5 with optimum pH of 8.5. According to Fig. 4.26 A and B, the profile of enzyme activity shows that it is not constant and there is a small augment in the activity as pH increases. The activity is maximum at alkaline pH due to the equilibrium between L-aspartic acid and L-aspartate. In acidic pH, L-aspartic acid has a greater affinity for the active site of the enzyme. Under such conditions, it becomes a competitive inhibitor (Miller *et al*., 1993). In alkaline pH, the equilibrium was shifted towards the aspartate, which has lower affinity to the active site (Lubkowski *et al*., 1994). At pH below 6.5 and above 9.5, the enzymes have lost approximately 65% and 45% activity, respectively (Fig. 4.26 A and B). Though maximum activity at a physiological pH is one of the prerequisites of L-asparaginase for antitumor activity, by virtue of its broad pH activity profile ~85% of the enzyme activity was retained at

pH 7.5. The enzymes have shown stability at alkaline pH range (pH 7.5–9.5) as they retained ~80% of their original activity after incubation for 24 h.

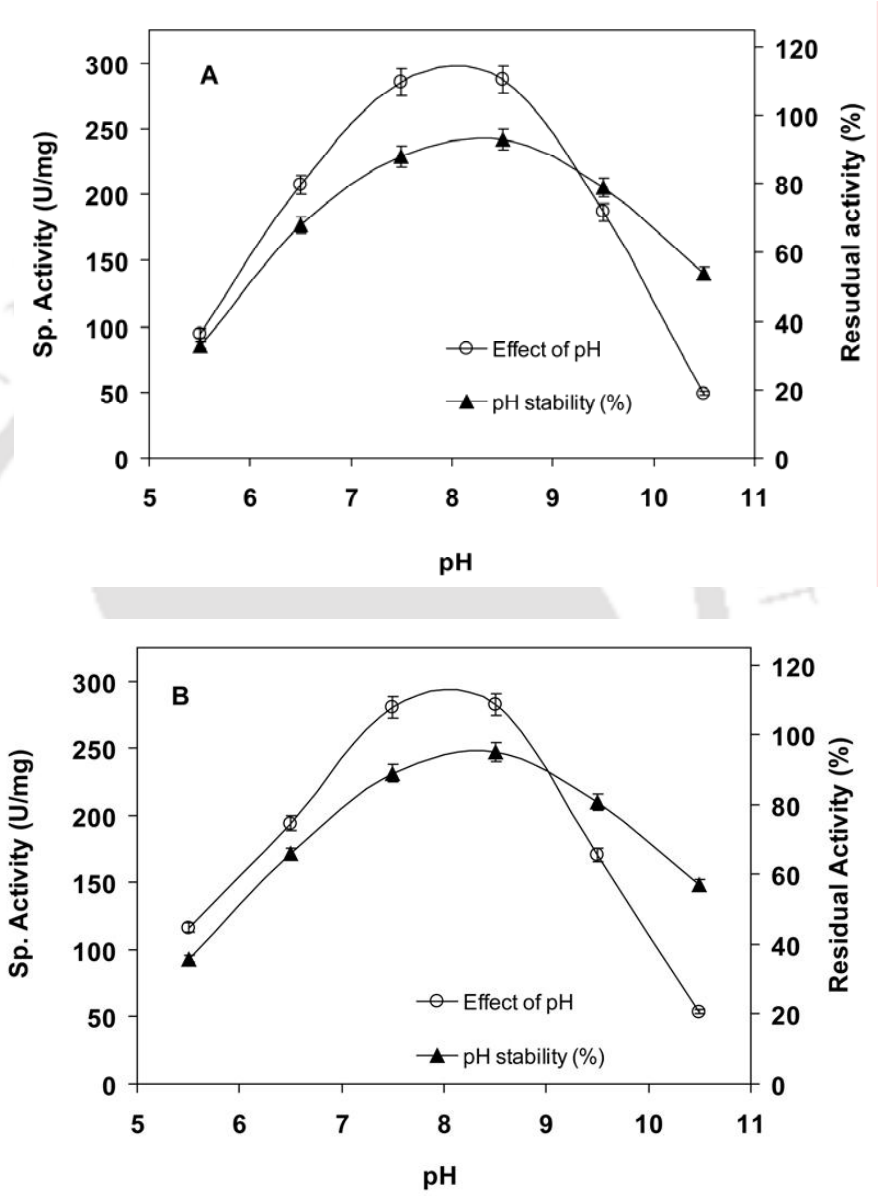

Fig. 4.26. Effect of pH of assay buffer and stability of purified recombinant L-asparaginase II at different pH levels after incubation for 24 h at 4±1 °C (**A**: *P. carotovorum* MTCC 1428 and **B**: *E. carotovora* subsp. *atroseptica* SCRI 1043).

Most of the L-asparaginases from *Erwinia* species showed that the optimal pH in the alkaline range (8.0-9.0). But L-asparaginase from *E. coli* has exhibited optimum pH in the acidic range

from 5.0-6.0 (Muller and Boos, 1998). But L-asparaginase from *E. coli* has exhibited optimum pH in the acidic range from 5.0-6.0 (Muller and Boos, 1998).

4.12.2. Effect of incubation temperature and incubation time on the enzyme activity

Hydrolysis of L-asparagine at different assay temperature was studied. The purified enzymes of both the strains exhibited maximum activity at 47-52 ºC and pH of 8.6. The activity of enzymes was lost by 75% at 57 ºC (Fig. 4.27). Narta *et al*. (2007) have also observed that the optimum temperature for *Erwinia* L-asparaginase at 50 ºC. At lower temperature, the activity of the enzymes was found to be very low due to slow reaction rate.

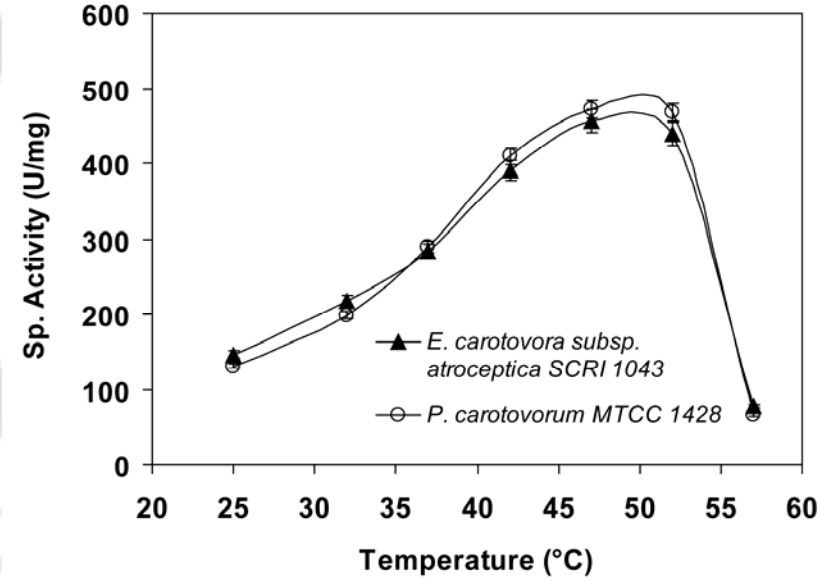

Fig. 4.27. Effect of incubation temperature on purified recombinant L-asparaginase II.

Experiments were performed to observe the effect of incubation time on the activity of recombinant L-asparaginase II of both the strains. Maximum enzymes activity was observed after 30 minutes of incubation. Incubation time shows inverse effect on activity of enzymes (Fig. 4.28). El-Sayed *et al.,* (2011) have also observed that the decrease in L-asparaginase activity after incubating for 90 min. due to product inhibition.

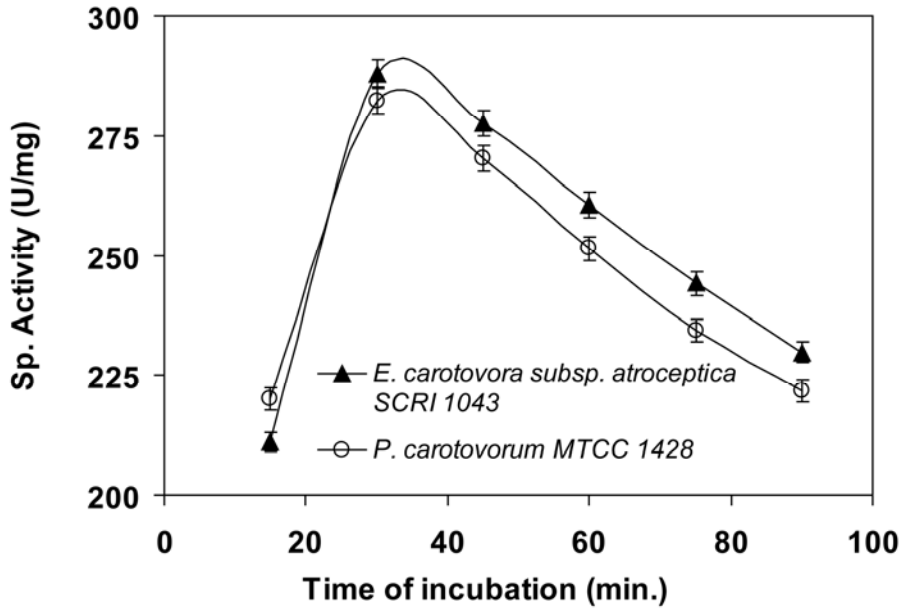

Fig. 4.28. Effect of incubation time on purified recombinant L-asparaginase II

Cunningham *et al*., (2008) reported that the decrease in the activity of phospholipase A2 by lysophosphatidylcholines (product) formation. Rakels *et al*., (1994) have observed modification of enatioselectivity of carboxylesterase NP by product (methanol) inhibition.

4.12.3. Effect of ionic strength of buffer

As the activity of an enzyme would vary with ionic strength of buffer in the assay mixture, the assay of recombinant L-asparaginase II was performed on different ionic strength of buffers. For example, inorganic ions may bind to some of the ionic side chains of a protein. Although this kind of interaction was not affecting the three dimensional shape of the enzyme in a substantial manner but could make it easier for a substrate molecule to locate or bind to the active site of the enzyme (Jimnez *et al*., 1964). It was observed that the decrease in enzyme activity at lower and higher ionic strength of buffers. Minimum and maximum L-asparaginae hydrolysis occurred at buffer concentration of 5 mM and 50 mM, respectively (Fig. 4.29).

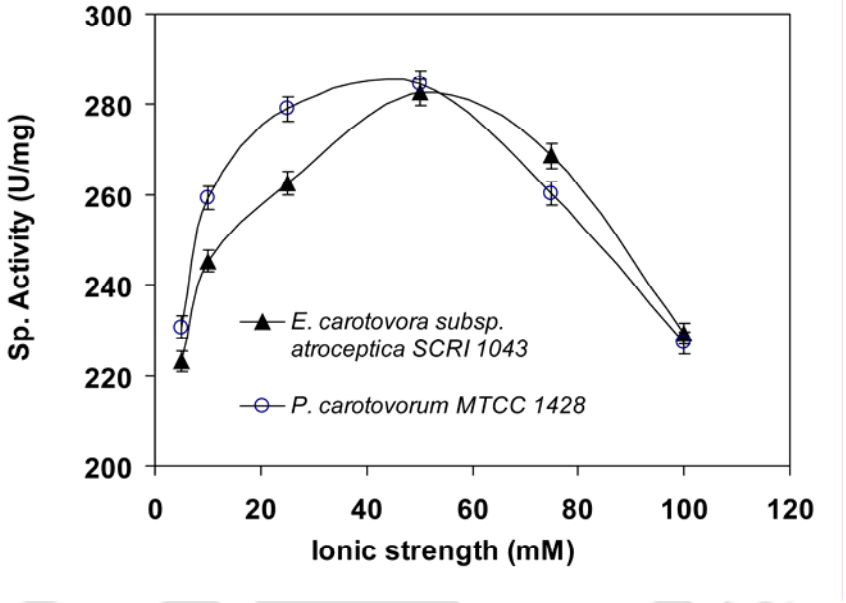

Fig. 4.29. Effect of ionic strength on enzyme activity of recombinant L-asparaginase II.

Reduction in the performance of enzymes at lower and higher ionic strength of buffer is due to the inability of enzymes to form non covalent interaction with substrate (Jimnez *et al*., 1964). Assay of L-asparaginase was performed by various researchers at a constant ionic strength of 0.05M (Heinemann *et al*., 1969; Khushoo *et al*., 2004; Stecher *et al*., 1999; Tosa *et al*., 1971).

4.12.4. Effect of various effectors and substrate specificity

Activity of recombinant L-asparaginase II of both the strains was assayed in the presence of different reagents (Table 4.19). Among the salts tested, significant loss of activity was observed with Hg^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+} Cu^{2+}, Fe^{3+}, Mg^{2+} and Zn^{2+}, whereas Na^{+} and K^{+} acted as an enhancer for recombinant L-asparaginase II of both the strains. Inhibition of enzymes activity in the presence of different metal ions and no effect with the metal chelator (EDTA), inferred that they were not a metalloproteins.

Table 4.19. Influence of different effectors on recombinant L-asparaginase II activity

Addition	Conc. (mM)	Recombinant L-asparaginase II of *P. carotovorum* MTCC 1428	Recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043
		Relative Activity (%)	Relative Activity (%)
No addition	-	100	100
Na^+ (NaCl)	50.0	119	109
K^+ (KCl)	150.0	126	114
Mg^{2+} ($MgCl_2$)	40.0	70	65
Ca^{2+} ($CaCl_2$)	150.0	35	32
Mn^{2+} ($MnCl_2$)	100.0	4	3
Zn^{2+} ($ZnCl_2$)	100.0	11	14
Fe^{3+} ($FeCl_3$)	100.0	5	6
Ni^{2+} ($NiCl_2$)	10.0	58	43
Co($CoCl_2$)	10.0	34	39
Cu^{2+} ($CuCl_2$)	10.0	26	22
Hg^{2+}($HgCl_2$)	10.0	44	43
2-mercaptoethanol	0.5	120	132
SDS	2.5	22	17
Urea	2.5	58	62
EDTA	5.0	106	112
Iodoacetamide	5.0	82	72
L-cysteine	25.0	104	107
L-histidine	25.0	113	116
Glutathione reduced	0.5	115	112

Inhibition of enzyme activity in the presence of Hg^{2+}, Cd^{2+}, and Zn^{2+} inferred that the presence of essential vicinal sulfhydryl group(s) of both enzymes for catalysis. The role of sulfhydryl groups in the catalytic activity of the enzymes is also confirmed by the stimulation of activity by the reducing source *viz.*, 2-mercaptoethanol and glutathione, and inhibition by thiol group blocking reagent, iodoactamide. L-cysteine and histidine found to be stimulator for recombinant L-asparaginase II activity of both the strains. The recombinant L-asparaginase II of both the strains lost ~40 % of their activity at 2.5 M urea and ~20 % of

activity was retained at 2.5 M sodium dodecyl sulfate (SDS) inferred that L-asparaginase possesses the thiol group binding domain with high affinity towards free-SH group containing effectors and these effectors convert the asparaginase from one conformation to another catalytically more active conformation. The L-asparaginase activation induced by GSH and Cys supports the hypothesis that all thiol group containing compounds and amino acids may interact with the same activator site on L-asparaginase. Earlier, Thiol reactivity has been reported with the purified L-asparaginase from *E. carotovora* and *P. carotovorum* MTCC 1428 (Kumar *et al*. 2011b; Warangkar and Khobragade, 2010). This kind of stimulatory action has been studied with the glyoxalase, mitogen activated protein kinase (Ernst *et al*., 1979).

Table 4.20. Substrate specificities of purified recombinant L-asparaginase II

Substrate	Conc. (mM)	Recombinant L-asparaginase II of *P. carotovorum* MTCC 1428	Recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043
		Relative Activity (%)	Relative Activity (%)
L-asparagine	10	100.00	100.0
D-asparagine	10	65.00	68.0
DL-asparagine	10	77.00	80.0
L-glutamine	10	N. D.	N. D.
L-aspartic acid	10	N. D.	N. D.
D-asparatic acid	10	N. D.	N. D.
L-glutamic acid	10	N. D.	N. D.
succinamic acid	10	79.00	84.0
L-asparagine-t-butyl ester HCL	10	77.00	70.0
BOC-L-asparagine	10	88.00	89.0
N-alfa-acetyl L-asparagine	10	16.00	17.0
DL-asparatic acid	10	N. D.	N. D.
L-aspartic acid amide	10	73.00	75.0

The substrate specificity of the recombinant L-asparaginase II is presented in the Table 4.20. No positive hydrolysis was observed when L-glutamine, L-aspartic acid, D-aspartic acid, DL-aspartic acid and L-glutamic acid were used separately as substrates. The absence of glutaminase activity would minimize the risk factor for successful clinical studies (Gallagher *et al.,* 1989; Manna *et al.,* 1995). It was observed that the enzyme was very specific for its natural substrate, L-asparagine (Table 4.20). This characteristic property makes the recombinant L-asparaginase II is very valuable in therapeutic applications. Therefore, the purified recombinant glutaminase-free L-asparaginase reported in this study will be an advantageous and value-added product.

4.12.5. Determination of kinetic parameters

The K_m and V_{max} of purified recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 0.666 mM and 303.03 IU mg^{-1}, and 0.656 mM and 312.50 IU mg^{-1}, respectively. This indicates high affinity of the enzymes towards its substrate (Fig. 4.30). The substrate affinity in terms of K_m is very low, which is 5-9 times lower than the reported cytosolic bacterial L-asparaginase (Kumar *et al*., 2011b). Turnover number (k_{cat}) and specificity constants (k_{cat}/K_m) for recombinant L-asparagine II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were determined to be 1.34×10^2 s^{-1} and 2.01×10^5 $M^{-1}s^{-1}$, and 1.38×10^2 s^{-1} and 2.11×10^5 $M^{-1}s^{-1}$, respectively. L-Asparaginase of different microorganisms has different substrate affinities and different physiological roles in the enzyme activity.

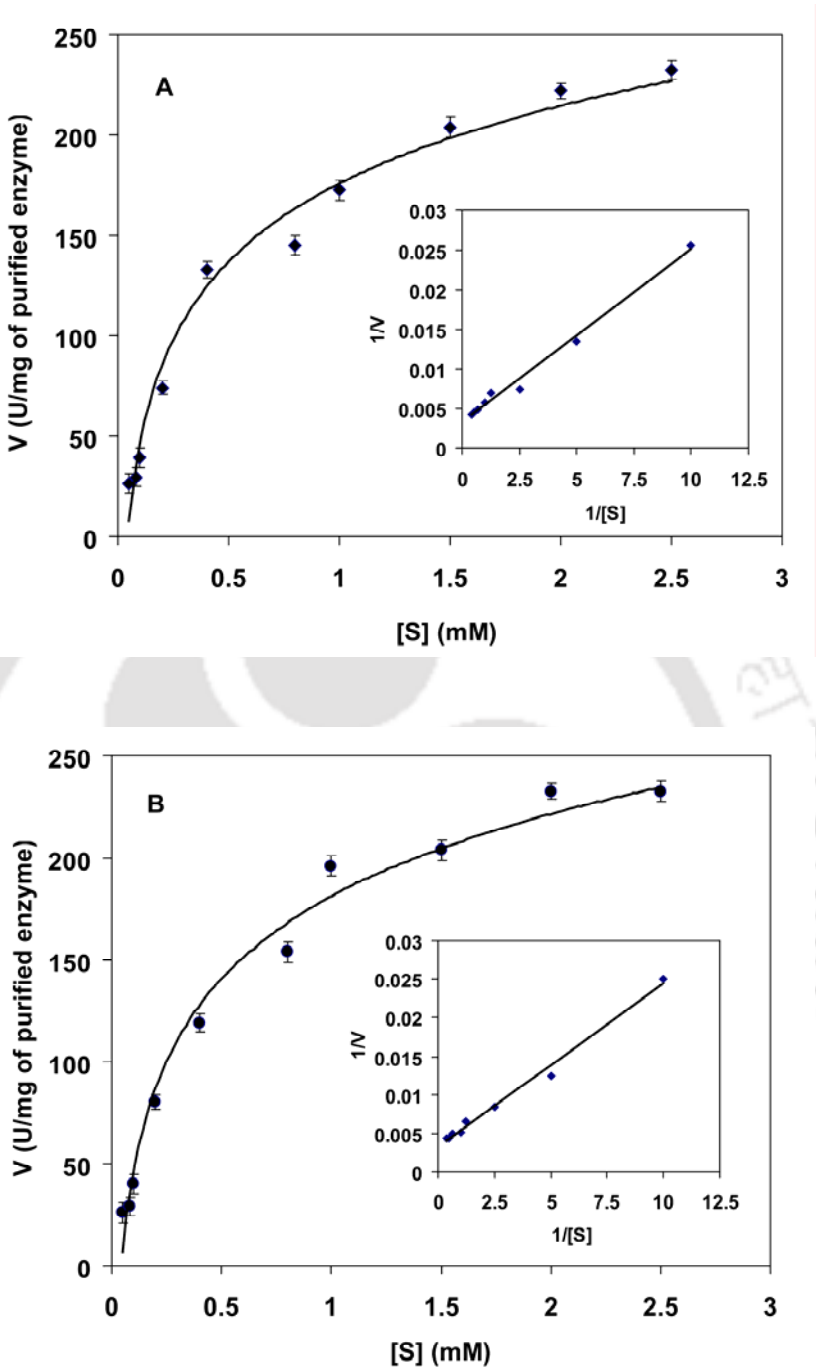

Fig. 4.30. Plot of the reaction velocities (*V*) *vs*. substrate concentration (S: 0.05-2.5 mM) fitted to the Michaelis–Menten equation and determination of K_m and V_{max} of purified recombinant L-asparaginase II using L-asparagine as substrate by non-linear regression analysis of experimental steady-state data. (*Inset*) The corresponding Lineweaver-Burk plot for L-asparaginase catalyzed reaction (**A**: *P. carotovorum* MTCC 1428 and **B**: *E. carotovora* subsp. *atroseptica* SCRI 1043).

Higher K_m values of 2.5 mM and 3.5 mM have been reported for L-asparaginase from *C. glueamicum* and *E. coli*, respectively (Willis and Woolfolk, 1974). The K_m value of L-asparaginase from *Pseudornonas stutzeri* MB-405A was reported to be 0.145 mM (Manna *et al.,* 1995). L-Asparaginase with lower K_m value of 0.074 mM was reported for L-asparaginase from *V. succinogenes* (Willis and Woolfolk, 1974). Warangkar and Khobragade (2010) have reported that L-asparaginase from *E. carotovora* with lower K_m value of 0.09 mM. K_m, V_{max}, turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) of purified L-asparaginase of *P. carotovorum* MTCC 1428 are reported to be 0.657 mM, 4.45 $U\mu g^{-1}$, $12.751 \times 10^3\ s^{-1}$ and $4.187 \times 10^6\ M^{-1}s^{-1}$, respectively (Kumar *et al.,* 2011b). The kinetic parameters determined in this study were comparable with those reported for many bacterial recombinant L-asparaginases (Kotzia and Labrou, 2005; Kotzia and Labrou, 2007).

4.12.6. Molecular mass determination

The molecular weight of subunit of recombinant L-asparaginase II of both the strains were found to be equal and it was approximately 37.5 kDa by SDS-PAGE analysis (Fig. 4.25 A and B). The purified recombinant L-asparaginase II of each strain had an approximate molecular weight of ~150.0 kDa as assessed by Native PAGE (Fig. 4.25 C). The findings of the current study corroborate with the available reports, which showed that the functional form of bacterial L-asparaginase exists as a tetramer of identical subunits with molecular mass in the range of 140–160 kDa (Aghaiypour *et al.,* 2001; Kozak *et al.,* 2000; Prakasham *et al.,* 2010). Each of the four active sites is located between the N and C-terminal domains of two adjacent monomers. Thus, the L-asparaginase tetramer have considered as a dimer of dimmers (Khushoo *et al.,* 2004; Swain *et al.,* 1993).

4.12.7. Optimization of combined effect of pH and temperature on the performance of recombinant L-asparaginase II under assay conditions

The most important physical factors, which influence the enzymatic reaction rate, are pH and temperature of incubation with the substrate. Each enzyme has a characteristic pH and temperature optima beyond which the reduction in activity would observed. The optimal temperature and pH for L-asparagine hydrolysis were determined using statistical experimental design. In order to determine the optimal conditions, preliminary experiments were performed to study the effect of pH on the activity of recombinant L-asparaginase II of both the strains (keeping at constant temperature). The results clearly showed that the maximum activity was observed when the pH was varied from 7.5-8.5 (Fig. 4.26 A and B). Similarly, experiments were performed to study the effect of assay temperature on the activity of recombinant L-asparaginase II of both the strains. The maximum activity was observed when the temperature varied between 47 to 52 ºC (Fig. 4.27). Therefore, experiments were conducted at various combinations of pH and temperature. The design matrix and the corresponding results of observed and predicted responses (L-asparaginase sp. activity) are shown in the Table 4.21. The enzyme activity varies from 330.13 to 512.25 U mg^{-1} and 335.16 to 529.18 U mg^{-1} for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively. By applying the multiple regression analysis on the experimental data, the following second-order polynomial equation 4.5 (for *P. carotovorum* MTCC 1428) and 4.6 (for *E. carotovora* subsp. *atroseptica* SCRI 1043) were found to explain the dependence of L-asparaginase activity on pH and temperature of incubation.

$$Y_{specific\ \text{activity}} = -6210.25 + 885.94\,A + 137.05B - 58.39\,A^2 - 1.49B^2 + 0.61\,AB \qquad (4.5)$$

$$Y_{specific\ activity} = -6917.98 + 936.90\ A + 160.82\ B - 65.48\ A^2 - 1.85\ B^2 + 1.81\ AB \quad (4.6)$$

Where, *A* is pH and *B is* temperature.

Table 4.21. Experimental design and results for the activity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 at various combinations of pH and temperature

Run Order	Uncoded and coded levels		Recombinant L-asparaginase II of *P. carotovorum* MTCC 1428		Recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043	
	pH	Temp (°C)	Sp. activity (U mg ^{1})		Sp. activity (U mg ^{1})	
			Observed	Predicted	Observed	Predicted
1	6.5(-1)	40(-1)	330.13±7.86	331.160	335.13±8.56	334.07
2	8.5(+1)	40(-1)	398.95±9.18	399.914	390.25±10.82	387.85
3	6.5(-1)	50(+1)	401.30±9.23	400.294	389.30±10.62	391.53
4	8.5(+1)	50(+1)	482.23±11.03	481.158	480.52±12.14	481.42
5	7.5(0)	45(0)	498.44±11.51	498.759	509.52±12.28	510.54
6	7.5(0)	45(0)	499.96±11.37	498.759	509.96±13.30	510.54
7	7.5(0)	45(0)	497.79±11.13	498.759	511.79±14.21	510.54
8	6.1(-2)	45(0)	341.64±7.74	341.610	345.64±9.36	344.78
9	8.9(+2)	45(0)	447.34±9.35	447.406	445.34±11.23	446.36
10	7.5(0)	37.9(-2)	385.04±10.52	383.630	378.04±12.01	380.46
11	7.5(0)	52.1(+2)	488.50±12.03	489.963	489.50±11.65	487.57
12	7.5(0)	45(0)	512.25±11.47	511.279	527.25±12.98	526.53
13	7.5(0)	45(0)	510.49±12.01	511.279	523.49±11.95	526.53
14	7.5(0)	45(0)	511.18±11.98	511.279	529.18±12.12	526.53

The results were analyzed using the ANOVA as appropriate to the experimental design used (Table 4.22 and 4.23). According to the ANOVA of the quadratic regression model, the model is highly significant as is evident from the Fisher *F* test. This indicates that the combined effects of pH and temperature significantly contributed for maximizing the recombinant L-asparaginase II activity. The goodness of the model was checked by coefficient of determination, R^2, which implies that the sample variation of 99.98 and 99.93%

for the dependence of recombinant L-asparaginase II activity of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively.

Table 4.22. ANOVA for quadratic model for optimization of pH and temperature for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428

Source	DF	Seq SS	Adj. SS	Adj. MS	*F*	*P*
Block	1	548.7	548.7	548.7	311.64	0.000
Regression	5	55677.1	55677.1	11135.4	6324.70	0.000
Linear	2	33141.0	33141.0	8879.4	5043.31	0.000
Square	2	36.7	36.7	16570.5	9411.72	0.000
Interaction	1	12.3	12.3	36.7	20.82	0.003
Residual error	7	8.3	8.3	1.8		
Lack of fit	3	4.0	4.0	2.8	2.73	0.178
Pure Error	4	56238.1		1.0		
Total	13					

R-Sq = 99.98% R-Sq(pred) = 99.83% R-Sq(adj) = 99.96%

Table 4.23. ANOVA for quadratic model for optimization of pH and temperature for recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043

Source	DF	Seq SS	Adj. SS	Adj. MS	*F*	*P*
Block	1	895.6	895.6	895.6	139.23	0.000
Regression	5	66385.3	66385.3	13277.1	2064.08	0.000
Linear	2	21725.6	21725.6	10563.0	1642.14	0.000
Square	2	44333.9	44333.9	22166.9	3446.11	0.000
Interaction	1	325.9	325.9	325.9	50.66	0.000
Residual error	7	45.0	45.0	6.4		
Lack of fit	3	25.4	25.4	8.5	1.73	0.298
Pure Error	4	19.6	19.6	4.9		
Total	13	67325.9				

R-Sq = 99.93% R-Sq(pred) = 99.54% R-Sq(adj) = 99.88%

The Student's *t* distribution and the corresponding *P* values, along with the parameter estimate are shown in Table 4.24 and 4.25 for *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively. The *P* values of all linear and quadratic relationships

between process parameters and L-asparaginase II activity of both the strains under assay conditions, suggested that they are highly significant because the interaction effect among pH and temperature is lower than linear effect (P<0.05). In addition, it was also found that the coefficient for pH is very much larger than coefficient for temperature.

Table 4.24. Model coefficient estimated by multiple linear regressions for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428

Term	Coef	SE Coef	*T*	*P*
Constant	-6210.25	66.9436	-92.768	0.000
Block	-6.26	0.3546	-17.653	0.000
pH	885.94	9.4613	93.638	0.000
Temp.	137.05	2.0221	67.773	0.000
pH*pH	58.39-	0.4883	-119.574	0.000
Temp.*Temp.	-1.49	0.0195	-76.270	0.000
pH*Temp.	0.61	0.1327	4.563	0.003

Table 4.25. Model coefficient estimated by multiple linear regressions for recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043

Term	Coef	SE Coef	*T*	*P*
Constant	-6917.98	127.957	-54.065	0.000
Block	-8.00	0.678	-11.800	0.000
pH	936.90	18.085	51.807	0.000
Temp.	160.82	3.865	41.609	0.000
pH*pH	-65.48	0.933	-70.160	0.000
Temp.*Temp.	-1.85	0.037	-49.648	0.000
pH*Temp.	1.81	0.254	7.118	0.000

This implies that any change in pH has large effect on the activity of recombinant L-asparaginase II than temperature in the range studied. The 3D response surface plots have explained the behavior of the system and enzymatic activity over independent variables (pH and temperature) and shown in Fig. 4.31.

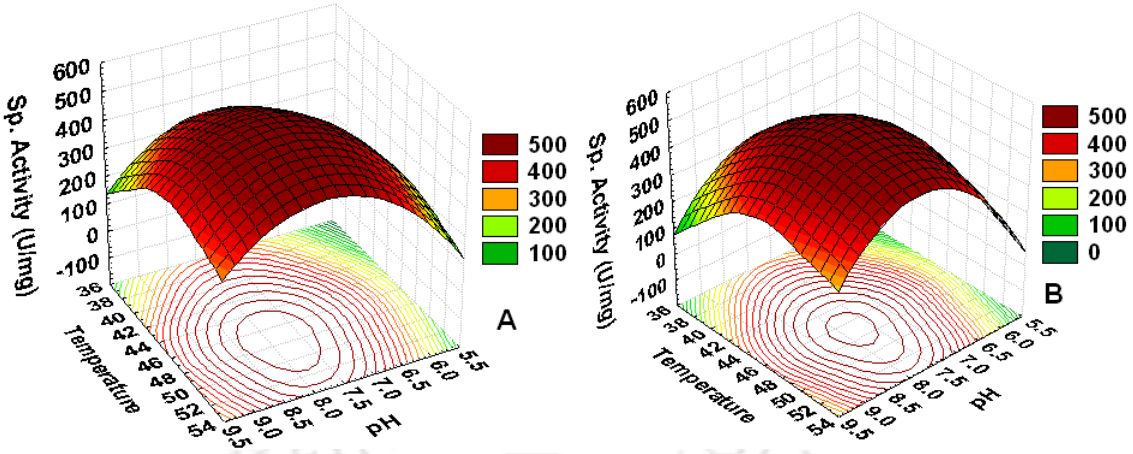

Fig. 4. 31. 3D response surface plots and contour plots, showing the effect of different levels of pH and incubation temperature on activity of recombinant L-asparaginase II (**A**: *P. carotovorum* MTCC 1428 and **B**: *E. carotovora* subsp. *atroseptica* SCRI 1043.

The enzyme activity is higher at a temperature range of 45 to 50 ºC and moderate pH (7.5-8.5). Equations (4.5 and 4.6) were solved by MINITAB optimizer. The optimal levels of pH and temperature were found to be 7.8 and 47.0 °C, respectively for recombinant L-asparagines II of both the strains. Under these optimal levels of pH and temperature the maximum specific activity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were observed to be 523.18 U mg^{-1} and 533.29 U mg^{-1}, respectively. After optimization of pH and temperature, specific activity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were increased by 1.82 and 1.88 fold, respectively in the purified samples.

4.12.8. Enzyme deactivation studies

Thermo stability represents the capability of an enzyme molecule to resist against thermal unfolding in the absence of substrate. In this study, recombinant L-asparaginase II of both the strains were deactivated under various combinations of pH and temperature as described

in the section 3.16.10 and extent of deactivation was measured by deactivation rate at a pH range of 6.5 to 9.5 as discussed in the section 3.16.11. The effect of temperature on half-life time has been studied and the results are shown in the Table 4.26.

Table 4.26. Effect of temperature on deactivation constant (k_d) and half life time ($t_{1/2}$) of the purified recombinant L-aspraginase II

pH	Temp. (°C)	Recombinant L-aspraginase II of *P. carotovorum* MTCC 1428		Recombinant L-aspraginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043	
		K_d (h^{-1})	$t_{1/2}$ (h)	K_d (h^{-1})	$t_{1/2}$ (h)
6.5	35	0.070	9.900	0.071	9.705
	40	0.093	7.451	0.091	7.615
	45	0.290	2.389	0.260	2.658
	50	0.521	1.330	0.524	1.320
	55	0.982	0.705	0.936	0.740
7.5	35	0.039	17.692	0.042	16.500
	40	0.061	11.360	0.065	10.660
	45	0.150	4.620	0.181	3.828
	50	0.250	2.772	0.351	1.974
	55	0.826	0.838	0.801	0.865
8.5	35	0.023	30.130	0.022	31.935
	40	0.049	14.142	0.050	13.887
	45	0.106	6.537	0.126	5.500
	50	0.186	3.725	0.203	3.410
	55	0.650	1.066	0.711	0.974
9.5	35	0.078	8.884	0.076	9.118
	40	0.102	6.794	0.095	7.294
	45	0.258	2.686	0.280	2.475
	50	0.690	1.004	0.620	1.117
	55	1.259	0.550	1.190	0.582

The deactivation rate is proportional to the active enzyme concentration and k_d (deactivation rate constant). The deactivation process was modeled as first-order kinetics and the deactivation rate constant was evaluated. The minimum value of k_d observed for recombinant

L-asparaginase II of *P. carotovorum* MTCC 1428 was 0.023 h^{-1} and for *E. carotovora* subsp. *atroseptica* SCRI 1043 was 0.022 h^{-1}. The combination of pH and temperature at which the above mentioned minimum deactivation rate constant was observed at 8.6 and 35 ºC, respectively.

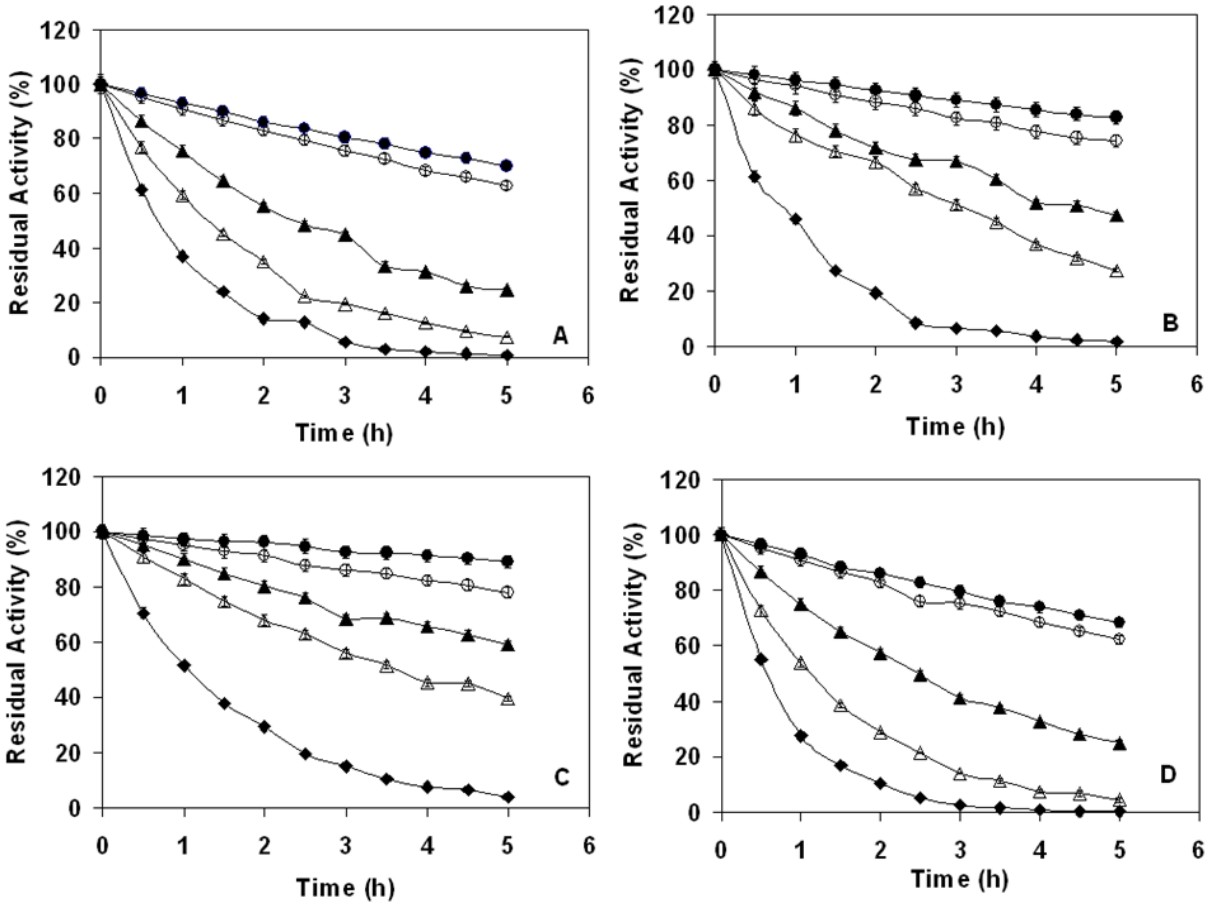

Fig. 4.32. Thermal stability of the recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 at (A) pH 6.5 (B) pH 7.5 and (C) pH 8.5 and (D) pH 9.5 (35 °C, 40 °C, 45 °C, 50 °C 55 °C).

The deactivation process was found to be faster at a pH of 6.5 and 9.5 than pH of 7.5 and 8.5 for recombinant L-asparaginase II of both the strains (Fig. 32 and 33). The maximum value of k_d for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were observed to be 1.259 and 1.190, respectively at a pH of 9.5 and 55 °C. Naidu and Panda, (2003) were also reported that the similar effect

of pH on deactivation rate constant as observed in this study. This is due to disulfide exchange, which usually occurs at near neutral and alkaline conditions (Munch and Tritsch, 1990).

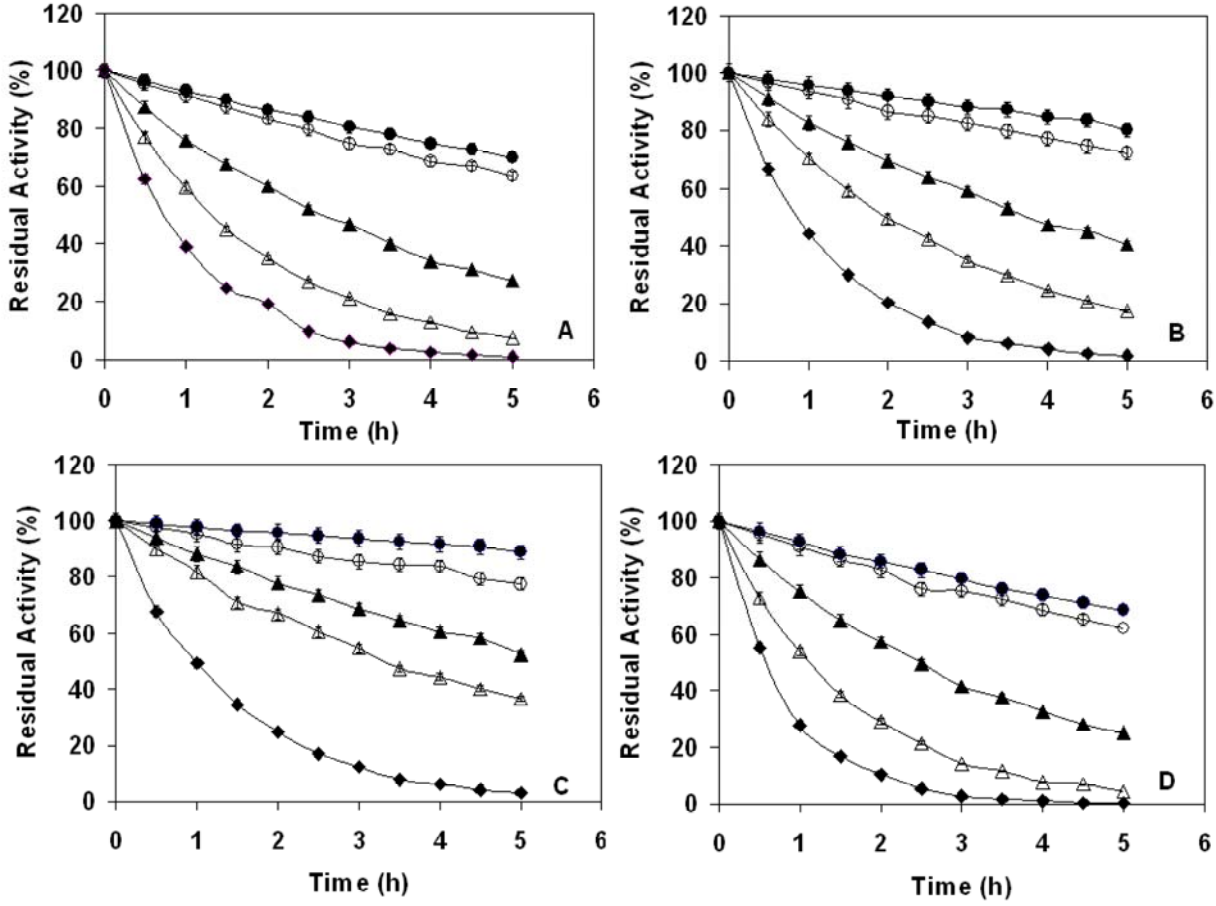

Fig. 4.33. Thermal stability of the recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 at (A) pH 6.5 (B) pH 7.5 and (C) pH 8.5 and (D) pH 9.5 (35 °C, 40 °C, 45 °C, 50 °C, and 55 °C).

Furthermore, the observation of interrelationship between conformational stability and enzyme activity suggested that in naturally occurring enzymes one cannot expect to find stability at temperatures far above than that of growth of an organism (Daniel, 1996). The results obtained in the present study also indicate that the optimum pH and temperature lie near that of the growth conditions. This is in agreement with the result that recombinant L-asparaginase II enzyme are more stable at pH 7.5-8.5 at 35 °C than pH 6.5 and 9.5.

4.12.9. Estimation of thermodynamic parameters

The change in enthalpy and entropy was calculated by transition state theory (Eq. 3.10) and the results are shown in the Table 4.27. The entropy values of purified recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be lower at pH of 6.5 and 9.5 than pH of 7.5 and 9.5. Solvent and structural effects are the two major factors, which influence the numerical values of ΔH^* and ΔS^*. Increase in entropy and enthalpy values were observed when enzymes were incubated at pH of 7.5 and 8.5. The thermal inactivation of enzymes is accompanied by the disruption of lots of non-covalent linkages including hydrophobic interactions with concomitant increase in the enthalpy of activation for recombinant L-asparaginase II. Positive values of ΔH^* would be expected for the breaking of hydrogen bonds as well as for the exposure of hydrophobic groups from the interior of the native protein during the deactivation process and such events would raise the energy of the protein–water solution. Positive value of ΔS^* indicates that the protein has become more disordered as it deactivated by temperature. The probable reason is that the enzymes get unfolded during the deactivation process and the positive sign is due to the increase in the number of protein molecules in a transition activated state, which in turn, gives lower values of ΔG^* or at lower pH (pH 6.5) and higher pH (pH 9.5). The values of ΔG^* (calculated from Eq. (3.11) are given in the Table 4.27 for the enzymes of both the strains. The decrease in entropy and enthalpy values was observed with increase in pH. Probably, at lower and higher pH, the stable three dimensional structure of enzyme gets compressed and the active site of the enzyme gets distorted, as a result decrease in residual activity was observed. The temperature-dependence of the catalytic activity was investigated to expand a deeper insight into the mechanism and specificity of recombinant L-asparaginase II enzymes of both the strains. The temperature dependency of

first-order deactivation rate constant was studied by Arrhenius equation (Eq. 3.13). The activation energy (E) and frequency factor (k_0) were estimated from equation (Eq. 3.13) and they are shown in the Table 4.27. Maximum activation energy was observed at optimum pH and further decrease or increase of pH resulted decrease in activation energy.

Table 4.27. Estimated thermodynamic parameters during the thermal deactivation of recombinant L-asparaginase II[a]

Name	pH	ΔH* (KJ mol^{-1})	ΔS* (J mol^{-1} K^{-1})	ΔG* (KJ mol^{-1})	E (KJ mol^{-1})	k_0 (h^{-1})
Recombinant L-asparaginase II of *P. carotovorum* MTCC 1428	6.5	114.87	104.42	82.71-80.10	117.51	5.13×10^{18}
	7.5	123.30	126.69	84.28-81.11	125.95	7.46×10^{19}
	8.5	131.49	149.78	85.36-81.62	134.13	1.19×10^{21}
	9.5	120.96	124.67	82.56-79.44	123.60	5.86×10^{19}
Recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043	6.5	113.120	98.61	82.74- 80.28	115.756	2.50×10^{18}
	7.5	124.602	131.81	84.00- 80.71	127.237	1.40×10^{20}
	8.5	138.029	170.82	85.41–81.14	135.435	1.50×10^{22}
	9.5	121.110	124.78	82.67- 79.56	123.754	5.90×10^{20}

[a]The temperature range is 35-55°C

Kapat and Panda (1997) and Naidu and Panda (2003) were also observed similar kind of trend on temperature dependency of deactivation rate constant for thermal deactivation of chitinase from *Trichoderma harzianum* and pectolytic enzymes from *Aspergillus niger*, respectively. For recombinant L-asparaginase II, The deactivation energy increased at a pH range of 7.5 to 8.5 at 35 °C, suggesting that recombinant L-asparaginase II enzymes require more amount of energy to deactivate under optimum pH range. For recombinant L-asparaginase II of both the strains, increase in deactivation energy was observed at pH 8.5, suggesting that enzymes require more amount of energy to deactivate. This is in agreement with the result that the recombinant L-asparaginase II is more stable at pH 8.5 with lower temperature rather than pH 6.5, 7.5 and 9.5 at higher temperature.

CHAPTER 5
SUMMARY AND CONCLUSIONS

- *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be potential strains for the production of novel glutaminase free L-asparaginase.
- Cloning and expression of L-asparaginase encoding genes (L-asparaginase (*ans*), L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were carried out with and without histidine tag. The expression of recombinant L-asparaginase (*ans*) of both the strains has decreased when fused with histidine tag. However, the expression of recombinant L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) of both the strains were increased with histidine tag.
- Statistical experimental designs were applied to maximize the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043. The production of recombinant L-asparaginase II of both the strains was enhanced by ~2.0 fold under optimal levels of chemical and physical parameters.
- The optimal levels of glucose, pH (controlled) and DO for the enhanced production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* sub sp.*atroseptica* SCRI 1043 in *E. coli* were found to be 1.5 g l^{-1}, pH 7.0 (controlled) and 40%, respectively. At the optimum levels of parameters, the production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 23.88 U ml^{-1} and 24.57 U ml^{-1}, respectively in a batch bioreactor. Under optimal levels of parameters, the production of recombinant L-asparaginase II of

P. carotovorum MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was increased by 1.34 and 1.40 fold, respectively in a batch bioreactor as compared to shake flask culture.

- The production of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were found to be 95.85 U ml^{-1} and 96.78 U ml^{-1}, respectively in a fed-batch culture. The overall production of novel glutaminase free recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was increased by 10.20 and 9.39 fold, respectively. The productivity was increased by 7.66 and 7.05 fold for recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043, respectively as compared to under un-optimized conditions in shake flask culture.
- A single step purification of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* sub sp.*atroseptica* SCRI 1043 from *E. coli* BL21 (DE3) was carried out. The molecular mass of the subunits and native L-asparaginase of recombinant L-asparaginase II of both the strains were found to be ~37.5 kDa and ~150 kDa by SDS-PAGE and Native PAGE, respectively.
- The purified recombinant L-asparaginase II of both the strains was active over a broad range of pH (6.5-9.5) and temperature (35-50 ºC). The recombinant L-asparaginase II of both the strains showed stability at alkaline range of pH (pH 7.5–9.5) and it retained ~80% of its original activity when incubated at 4±1 ºC for 24 h. Maximum activity of recombinant L-asparaginase II of both the strains was obtained at a pH of 8.5 and 47-52 ºC. Maximum hydrolysis of L-asparagine was observed with 50 mM Tris-HCl buffer. The optimum time of incubation at which maximum activity was observed to be 30 min.

- Studies on effect of different metal ions and reagents on the performance of purified recombinant L-asparaginase II of both the strains revealed that several metal ions are very detrimental for enzymatic activity except Na^+ and K^+. The enzyme is sensitive to thiol group reagent and stimulate with reducing agents. L-cysteine and L-histidine proved to be stimulators of the enzyme activity.
- Specificity studies of purified recombinant L-asparaginase II with various structure analogues of L-asparagine suggested that both the enzymes were very specific for its natural substrate, L-asparagine.
- The substrate affinity of the purified recombinant L-asparaginase II of both the strains, in terms of K_m is very low (~ 0.66 mM), which is 5–9 times lower than the reported cytosolic L-asparaginase. The V_{max} of purified recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 was determined to be 303.03 IU mg-1 and 312.50 IU mg^{-1}, respectively. Turnover number (k_{cat}) of recombinant L-asparaginase II of both the strains was found to be 1.3×10^2 s^{-1}. Specificity constants (k_{cat}/K_m) of recombinant L-asparagine II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 were determined to be 2.01×10^5 $M^{-1}s^{-1}$ and 10^2 s^{-1} and 2.11×10^5 $M^{-1}s^{-1}$, respectively.
- The optimal levels of pH and temperature for maximum activity of recombinant L-asparaginase II of both the strains were found to be 7.8 and 47.0°C, respectively. After optimization of process parameters, specific activity of recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* was increased by 1.8 and 1.9 fold, respectively.

- Thermodynamic parameters (K_d, $t_{1/2}$, ΔH, ΔS, ΔG and E_a) of recombinant L-asparaginase II of both the strains were determined to evaluate the probable mechanism of deactivation of recombinant L-asparaginase II.

Future scope of work

1. *In vivo* studies on purified recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043 to established its full potential as a chemotherapeutic agent.
2. Studies on biophysical properties of these recombinant L-asparaginase II of *P. carotovorum* MTCC 1428 and *E. carotovora* subsp. *atroseptica* SCRI 1043.

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APPENDIX

A.1. Sample calculation for the estimation of L-asparaginase activity

Preparation of standard plot for ammonia

Stock solution of 1 mM (or µmol ml^{-1}) ammonium sulphate ($(NH_4)_2SO_4$) was prepared in 50 mM Tris-HCl buffer of pH 8.5. $(NH_4)_2SO_4$ is a divalent salt of ammonia, hence 1 µmol ml^{-1} of $(NH_4)_2SO_4$ solution equivalent to the 2 µmol ml^{-1} ammonia (NH_4^+) solution. The stock solution was appropriately diluted with miliQ water to get standard solutions of various amount of ammonia (µmol) *viz.*, 0.4 to 4.0 of interval 0.4 as shown in X-axis of Fig A.1. Experiments were performed for standard plot in triplicates and absorbance of the standard samples was measured at 425 nm against the appropriate blank as described for test samples in the section 3.6.1. The data was fitted with a linear regression model using Microsoft Excel® software (Fig. A.1). Activity of L-asparaginase was measured by modified Nessler`s method as described in the section 3.6.1. The concentration of ammonia in the test sample was determined by using standard curve. One unit of L-asparaginase (U) is defined as the amount of enzyme that liberates 1 µmole of ammonia per min at 37°C (1 unit OD at 425 nm = 3.851 µmol of ammonia).

Calculation for L-asparaginase activity

L-asparaginase activity in the test sample was calculated by the following equation.

$$\text{L-asparaginase activity (U ml}^{-1}) = \frac{A_C \times V_T}{V_R \times T \times V_C} \qquad \text{(A.1)}$$

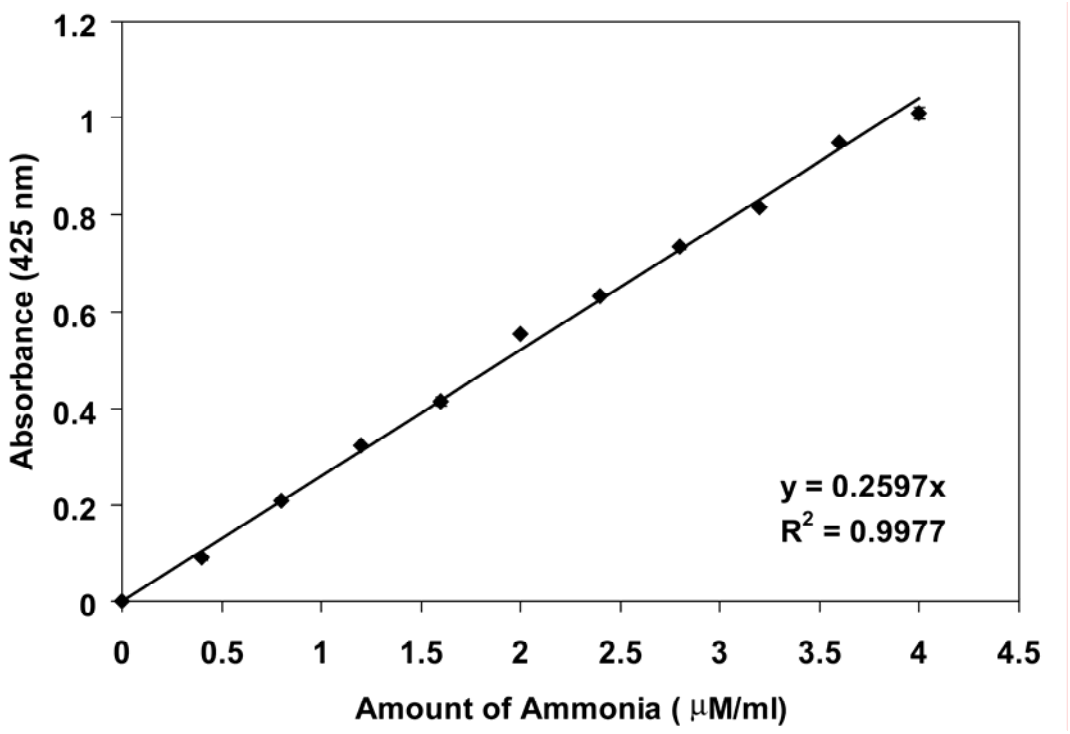

Fig. A.1. Standard curve drawn between known concentration of ammonia and the corresponding OD measured at 425 nm.

Where, A_c = amount of ammonia released during reaction in µmol (test sample absorbance at 425 nm (Abs_{425}) × 3.851 µmol), V_T = total volume of reaction (1.1 ml), V_R = volume of reaction mixture used in step 2 (0.1 ml of reaction mixture), T = time of assay (30 min), V_C = volume of crude enzyme (0.1 ml).

After substituting the all values in Eq. A.1, it simplify into Eq. A.2

L-asparaginase activity (U ml^{-1}) = Abs_{425} × 14.12 (A.2)

Where, Abs_{425} = test sample absorbance at 425 nm against appropriate blank.

A.2. Sample calculation for the estimation of protein

Preparation of standard plot for protein

Stock solution of 0.5 mg ml^{-1} protein (BSA) was prepared in miliQ water. The stock solution was appropriately diluted with same water to get standard solutions of various concentrations of protein (mg ml^{-1}) *viz*., 0.05 to 0.5 as shown in X-axis of Fig A.2. Experiments were performed for standard curve in triplicates and absorbance of the samples was measured at 660 nm against the appropriate blank as described for test samples of protein in the section 3.6.2. The points were fitted with a linear regression model with the help of Microsoft Excel® software (Fig. A.2). Protein concentration in test sample was measured by Lowry method as described in section 3.6.2. Then concentration of protein (mg ml^{-1}) in the test sample was calculated using standard curve (1 unit OD at 660 nm = 0.4829 mg ml^{-1} of protein).

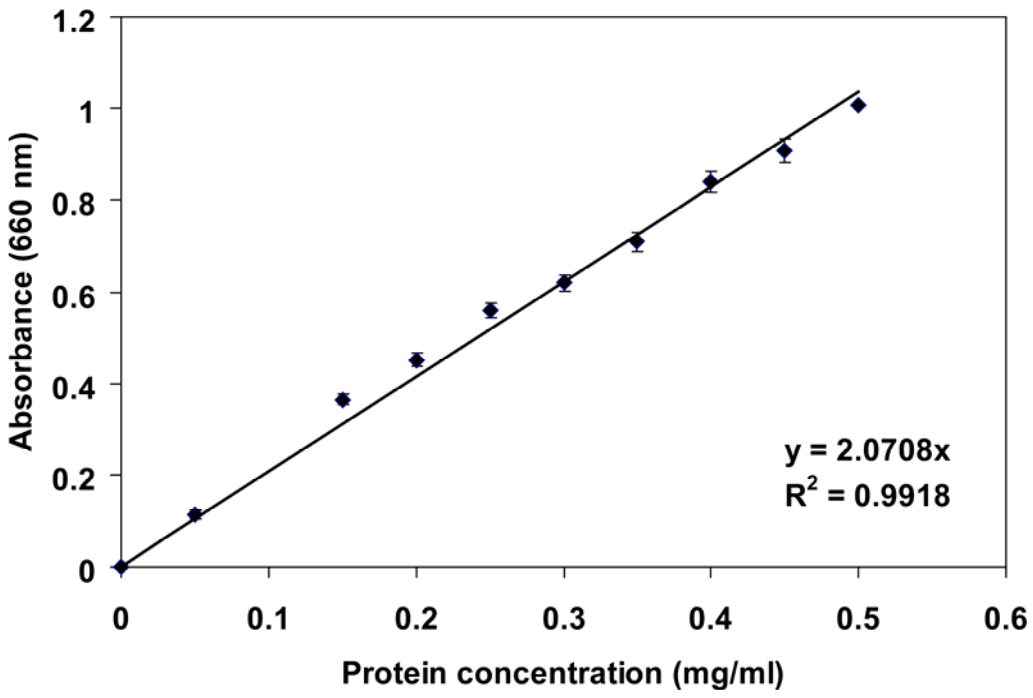

Fig. A.2. Standard curve drawn between known protein concentration and the optical density measured at 660 nm.

Calculation for protein concentration

Protein concentration in test sample was calculated by the following Eq. A.3.

Protein concentration $(mg\ ml^{-1}) = Abs_{660} \times 0.4829$ (A.3)

Where, Abs_{660} = test sample absorbance at 660 nm against appropriate blank.

A.3. Sample calculation for the estimation of dry cell weight (DCW)

Preparation of standard plot for DCW

Experiments were performed for standard curve in triplicates and absorbance of the standard samples was measured at 600 nm against the blank (miliQ water) as described in the section 3.6.3. Different dilutions of cell samples were used for measuring cell OD (0.1-1.0) at 600 nm and corresponding DCW ($g\ l^{-1}$) was determined at 80 °C for 24 h (Fig. A.3).

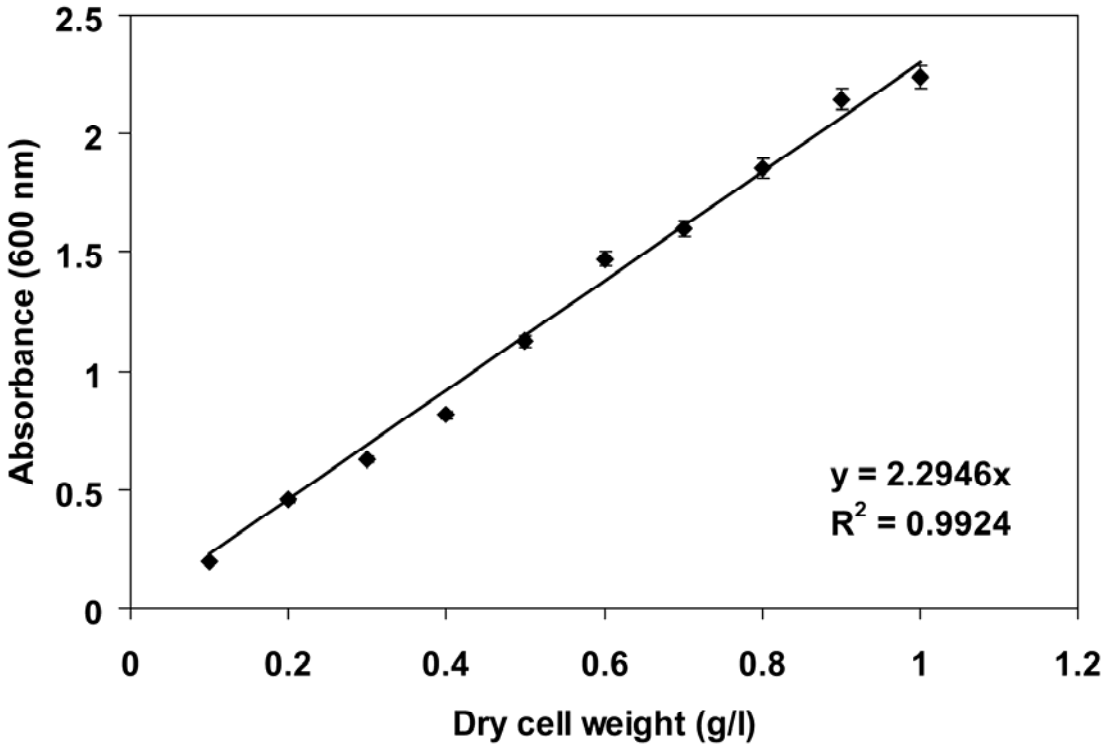

Fig. A.3. Standard curve drawn between dry cell weight of *E. coli* and the optical density measured at 600 nm.

DCW of the unknown sample was determined by measuring the OD of the culture broth at 600 nm using UV-visible spectrophotometer (1 unit OD at 600 nm = 0.4358 g l^{-1} DCW).

Calculation for DCW

Dry cell weight of the test sample was calculated by the following Eq. A.4.

Dry cell weight concentration (g l^{-1}) = Abs_{600} × 0.4358 (A.4)

Where, Abs_{600} = test sample absorbance at 600 nm against appropriate blank.

A.4. Sample calculation for the estimation of glucose

Preparation of standard plot for glucose

Glucose concentration in the test sample was measured by DNS method as described in the section 3.6.4. Stock solution of 1.0 mg ml^{-1} glucose was prepared in miliQ water. The stock solution was appropriately diluted with same water to get standard solutions of various concentrations of glucose (mg ml^{-1}) *viz*., 0.1 to 1.0 as shown in X-axis of Fig A.4. Experiments were performed for standard curve in triplicates and absorbances of the standard samples were measured at 510 nm against the appropriate blank. The data was fitted with a linear regression model by using Microsoft Excel® software (Fig. A.4). The concentration of glucose (mg ml^{-1}) in the test sample was calculated from standard curve (1 unit OD at 510 nm = 0.6860 mg ml^{-1} of glucose).

Calculation for glucose concentration

Glucose concentration in test sample was calculated by the following equation obtained from standard plot.

Glucose concentration (mg ml^{-1}) = Abs_{510} × 0.6860 (A.5)

Where, Abs_{510} = test sample absorbance at 510 nm against appropriate blank.

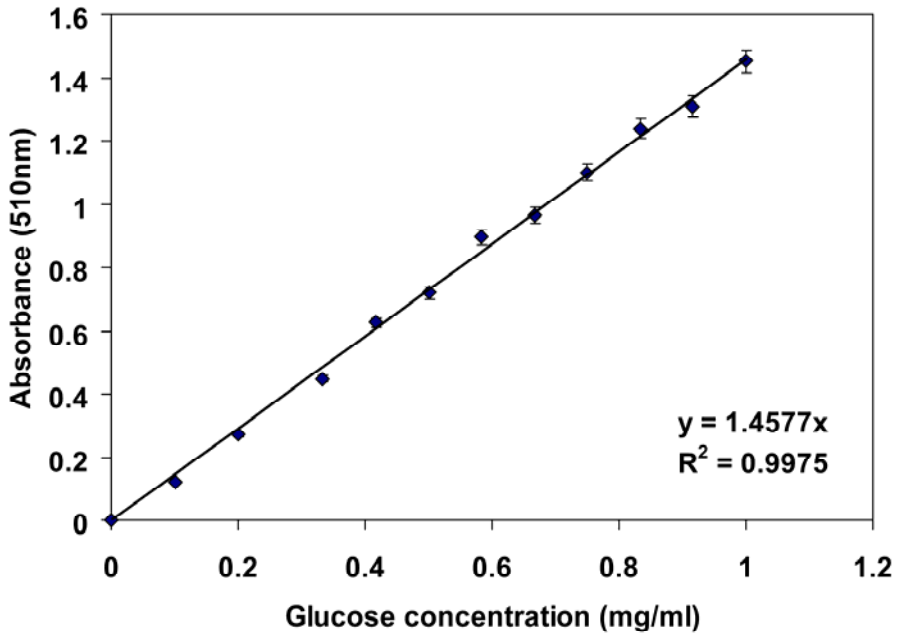

Fig. A.4. Standard curve drawn between known glucose concentration and the optical density measured at 510 nm.

A.5. Gene sequence of L-asparaginase of *P. carotovorum* MTCC 1428 (Gene bank accession no. JN585953)

ATGACGAAACCCGTGATTGTGATCCACGGTGGCGCAGGTGCGCTGACCCGCTCGGCTATGAGCGCT
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GAAAACGGCAGCGCGTTGGATGCCGTAACGGAAGCCGTCCGTCTGTTGGAAGAGTGCCCGCTATTC
AATGCCGGACACGGTTCGGTCTTCACCCACGCAGAAACCCACGAACTGGACGCCAGCATCATGGA
CGGACGGTCGCTGGATGCTGGCGCGGTGAGCTGTGTCAGCCATATCCGTAACCCGATTCTGGCGGC
TCGTACCGTACTCGAAGCCAGCCCGCATGTGATGTTTACCGCCGACGGCGCGGAAGCCTTTGCACA
GCAGCACGGTCTGGAAAGGGTCGAACCTGCGTTTTTCTCCACCGACGAACGCCGCCAGCAGCTTCA
TAACGCACAGGCAGGCTCAGGCCGCGTCATCCTCGATCATGATGGTCAGAACGACCCGATCGATCC
CGACCGCAAATTCGGCACCGTGGGCGCGGTAGCACTGGACAGCGCGGGCAACCTCGCGGCGGCAA
CCTCGACGGGCGGCATGACCAACAAACAGGCCGGACGCGTGGGCGATAGCCCCATCATTGGTGCT
GGCTGTTACGCCAATAACCAGACGGTAGCCGTTTCCTGCACCGGTACTGGCGAGGTCTTTATGCGC
GCCGTCGCGGCCTATGATGTCTCGGCGCTGATGGAATATGCCGGACTCACGCTACAGCAGGCCAGC
GATCGCGTAGTCATGGAGAAACTCGTGCAAATGGACGGTAGCGGCGGGATGATTGCCGTGGACAA
AGCAGGCAACATCGCCCTGCCATTTAACAGCGAAGGCATGTATCGCGGCTATGGCTACGTGGGAG
AAGTTCCCGTCGCCGATATCTATCGTTAA

A.6. Gene sequence of L-asparaginase I of *P. carotovorum* MTCC 1428 (Gene bank accession no. JN631810)

ATGCAAAAGAAATCCATTTATGTCGCCTATACGGGCGGCACCATCGGTATGCAGCGCTCAGCCAAT
GGCTATGTTCCGGTATCCGGTCATTTACAGCAGCAGTTGGCAAAAATGCCGGAATTCCACCGCGAA
GAAATGCCATCGTTCACGATTCATGAATATGACCCGCTAATCGATTCGTCTGACATGACGCCAGCG
GATTGGCAATCCATCGCGGACGATATTCAAAACCACTACGATGATTATGACGGTTTCGTGATTCTG
CATGGTACTGACACGATGGCGTTCACCGCATCTGCGCTCTCATTTATGCTGGAAAATCTTGCCAAGC
CGGTTATCGTGACAGGGTCACAAATTCCGTTGGCGGAATTGCGCTCGGACGGCCAGACCAACCTGC
TAAACGCGCTGTACGTTGCGGCTAATCATCCGATTAATGAAGTCGCCCTCTTCTTCAATAACAAACT
GCTGCGCGGCAACCGCACCACCAAAGCCCACGCGGACGGCTTTGATGCCTTCGCTTCCCCCAACTA
TCCGCCGCTGCTCGAAGCCGGTATCCATATTCGTCGGCTGGCACCGACCGTGGAATGTAGCAACTG
TCCGCCGCTCAAAGTGCATCACATTACGCCACAGCCTATCGGGGTGATTACGATTTATCCTGGTATT
TCTGCCGATGTCATCAGCAATTTCCTCCGCCAGCCAGTAAAAGCGCTCATCCTGCGCTCCTACGGCG
TTGGCAACGCGCCGCAAAGCCCCGGGCTGCTGCACGAGCTACGTGAAGCCTCCGCTCGCGGCATTG
TGGTCGTCAACCTGACACAATGCATTTCCGGACGCGTGAACATGGGCGGCTATGCGACGGGCAATG
CGCTGGCGCATGCGGGCGTCATCAGTGGCTTTGATATGACCGTCGAAGCCGCCTTGACCAAGCTGC
ATTACTTATTGAGCCAGCAAGACTTAAGCGCCGATGAAATCCGCCAGTTGATGCAGCAGAACCTGC
ATGGGGAATTGAGCGATAAAGATTGA

A.7. Gene sequence of L-asparaginase II of *P. carotovorum* MTCC 1428 (Gene bank accession no. JN638885)

ATGCAACTCTCATTTATCGCCCGCACCATCACCGCCGCTTGCCTGATGCTGTCGTCTCATGCGCTGC
TAGCCGATGACGCCAAACCTGGCGTCACTATCTACGCTACCGGCGGCACCATTGCTGGAAAGGCAG
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ATCGATCAAGCGATTCTGTTAAAGCTATCCAAAGCGATTAACAAACAGCTAGGCGATTCAAATACG
CACGGCGTCGTCATTACTCACGGCACCGATACGTTGGAAGAGACCGCGTTCTTTTTGGATCTGACG
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TGGTCCTATGAACCTGCTGGAAGCCGTCACGCTGGCAACCAGTAAGAACGCGGAAAAACGCGGCG
CGCTGGTGTTGCTGAACGATCGCATCGGCTCCGCATTCTACACCACCAAAACCAATGCCACGGCGC
TGGACACGTTCAAAGCCAATGAACAAGGCTATCTGGGCGCATTCTACGGCGGCGTTCCGCGCTTCT
TTTATCAGCCTGCTGCCCCCGAAAACAAACCGTTCTTTGACGTAAGCAATAAGGACACGCTGGCGA
AGGTCGACATTCTCTACGGCTATCAGGATCAAGACAGTGGTCTGCTGAATGCCGCCATTGAACAGG
GCGCAAAAGGCATCATTATCGCGGGTAGCGGTAACGGTGCGACGCCCACGCGTATCAAAGAAGAC
ATCAAGAAAGCGGTAGCCAAAGGCATTCCGGTGGTGATCAGTACGCGCACCGGCAGCGGCTATGT
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TGCTTTCGCTAGCGCTGTCTAACGGCGACGACATCGAGAAAATCCGTACCTATTTCGAGCAGTAA

LIST OF PUBLICATIONS

Communicated/Under Prepration in Referred International Journals

- **Goswami R**, Venkata Dasu V. Optimization of production conditions and effect of process parameters on the performance of novel glutaminase-free L-asparaginase from *Erwinia aroideae* NRRL B-136. ***Process Biochemistry*** (submitted).
- **Goswami R**, Venkata Dasu V. Effect of histidine tag on the expression of recombinant L-asparaginases and optimization of medium components for enhanced production of recombinant L-asparaginase II of *Pectobacterium carotovorum* MTCC 1428 in *Escherichia coli* BL21 (DE3). ***Applied Microbiology and Biotechnology*** (submitted).
- **Goswami R**, Venkata Dasu V. Cloning, expression, purification, characterization and optimization of medium components for maximum production of recombinant L-asparaginase II of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3). ***Bioresource Technology*** (submitted).
- **Goswami R,** Venkata Dasu V. Purification, characterization and enhanced production of recombinant L-asparaginase II of *Pectobacterium carotovorum* MTCC 1428 in *Escherichia coli* BL21 (DE3) in a batch and fed-batch bioreactor (under preparation).
- **Goswami R**, Venkata Dasu V. Production of recombinant L-asparaginase II of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *Escherichia coli* BL21 (DE3) in a batch and fed-batch bioreactor (under preparation).

Published in International Conference Proceedings

- **Rachna Goswami** and Veeranki Venkata Dasu, Purification and characterization of recombinant L-asparaginase II of Erwinia carotovora. International Conference on Perspective and Challenges in Chemical and Biological Sciences; 2012. p. 303.
- **Rachna Goswami** and Veeranki Venkata Dasu, Optimization of chemical and physical Parameters for enhanced production of recombinant L-asparaginase II of *Erwinia carotovora* in *E. coli*. International Conference on Genomic Sciences (ICGS); 2010. p. 86.
- Venkata Dasu Veeranki and **Rachna Goswami**, Cloning and expression of three L-asparaginases of *Pectobacterium carotovorum* in *E. coli*. and Optimization of chemical and Physical parameters for maximum Expression of recombinant L-asparaginase II in *E. coli.* Biochemical and molecular Engineering XVII Emerging Frontiers: 2011 (http://www.engconfintl.org/11amPosters.pdf).
- Venkata Dasu Veeranki and **Rachna Goswami**, Cloning and expression of three L-asparaginases of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *Escherichia coli.* Society for Industrial Microbiology and Biotechnology (SIM), Annual Meeting and Exhibition; 2010 (http://sim.confex.com/sim/2010/web program/Paper16306.html).
- **Rachna Goswami** and Veeranki Venkata Dasu. Localization of glutaminase free L-asparaginase from *Erwinia carotovora.* Emerging Trends in Biotechnology (ETBT); 2009. p. 208-209.

- **Rachna Goswami** and Veeranki Venkata Dasu, Localization and Partial Purification of L-asparaginase isolated from *Erwinia aroideae* NRRL B-136. International Society of Biotechnology (ISBT); 2008. p. 3.