PRODUCTION, PURIFICATION AND CHARACTERIZATION OF GLUTAMINASE-FREE L-ASPARAGINASE FROM *PECTOBACTERIUM CAROTOVORUM* MTCC 1428

A THESIS

submitted by

SANJAY KUMAR

for the award of the degree

of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

JUNE 2010

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Dedicated to my Parents

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DEPARTMENT OF BIOTECHNOLOGY

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. Veeranki Venkata Dasu and Dr. Kannan Pakshirajan.

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: ……. **Sanjay Kumar**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

It is certified that the work described in this thesis entitled "**Production, Purification and Characterization of Glutaminase-free L-Asparaginase from *Pectobacterium Carotovorum* MTCC 1428**" by Mr. Sanjay Kumar for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in 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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Date: …… **Sanjay Kumar**

ABSTRACT

Bacterial L-asparaginase has been widely used as a therapeutic agent in the treatment of certain kinds of cancer, mainly in treatment of acute lymphoblastic leukemia. Moreover, L-asparaginase is used in food industry for the production of acrylamide free food, as model enzyme for development of new drug delivery system and L-asparagine biosensor for diagnosis of leukemia. The various side effects of this drug are mainly due to the presence of partial glutaminase activity of L-asparaginase. Hence, glutaminase-free L-asparaginase is highly desirable for its successful application.

Among the tested microorganism, *Pectobacterium carotovorum* MTCC 1428 has the ability to produce novel glutaminase-free L-asparaginase. Hence, further studies were carried out using *P. carotovorum* MTCC 1428. Localization, production, purification and characterization of glutaminase-free L-asparaginase have been performed by *P. carotovorum* MTCC 1428. Production of glutaminase-free L-asparaginase was performed both in the shake flask and bioreactor (batch and fed-batch). Furthermore, Kinetic models were developed for dual substrate growth, substrates utilization and L-asparaginase production by *P. carotovorum* MTCC 1428 in batch bioreactor.

The localization of L-asparaginase was carried out using cell fractionation techniques. The specific activity of L-asparaginase was found to be 85 and 77% in the cytoplasm of *Pectobacterium carotovorum* MTCC 1428 that was grown on medium containing L-asparagine, and a combination of both L-asparagine and glucose, respectively. Among the carbon sources tested, L-asparagine and a combination of both L-asparagine and glucose were

found to be the most suitable carbon sources for maximum production of L-asparaginase. Yeast extract, L-asparagine and peptone showed significant effect on the production of L-asparaginase. L-asparagine was used as an essential carbon source for the maximum production of L-asparaginase by *P. carotovorum* MTCC 1428.

Statistically based experimental designs were applied to maximize the production of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428 under shake flask conditions. Nine components of the medium were examined for their significance on the production of L-asparaginase using Plackett-Burman experimental design. The medium components, *viz.*, glucose, L-asparagine, KH_2PO_4 and $MgSO_4.7H_2O$ were screened based on their high confidence levels ($P<0.04$). The optimum levels of glucose, L-asparagine, KH_2PO_4 and $MgSO_4.7H_2O$ were found to be 2.08, 5.20, 1.77 and 0.37 g l^{-1}, respectively using central composite experimental design. The maximum L-asparaginase activity in the optimized medium was found to be 14.7 U ml^{-1} (27.88 U mg^{-1} of protein) which corresponded to a productivity of 1225.8 U l^{-1} h^{-1} of L-asparaginase, resulting 19.4 fold increase in the L-asparaginase activity as compared to the un-optimized medium. The effect of process parameters (initial pH of the medium, temperature, agitation (rpm of the shaking incubator) and inoculum size) on the production of L-asparaginase from *Pectobacterium carotovorum* MTCC 1428 was studied using central composite design (CCD) technique. The individual optimum levels of initial pH of the medium, temperature, agitation and inoculum size were found to be 6.90, 29.8ºC, 157 rpm and 2.61% (v/v), respectively for the production of L-asparaginase. Under optimal levels of process parameters optimization, the production of L-asparaginase was enhanced by 26.39% (specific activity 35.24 U mg^{-1} of protein). Maximum L-asparaginase production obtained at 12 h under the process optimized conditions (shake

flask level), which also resulted in 10.19% increase in the enzyme productivity (1350.83 U l^{-1} h^{-1}).

The purified L-asparaginase showed no glutaminase activity, which may reduce the possibility of side effects of the enzyme during the course of anti-cancer therapy. An intracellular glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428 was purified to apparent homogeneity. The homotetramer enzyme has a molecular mass of 144.4 kDa (MALDI-TOF MS) and an isoelectric point of approximately 8.4. The enzyme is 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-marceptoethanol whereas it is moderately inhibited by various divalent cations and thiol group blocking reagents. Kinetic parameters, K_m, V_{max} and k_{cat} of the purified L-asparaginase from *P. carotovorum* MTCC 1428 were found to be 0.657 mM, 4.45 IU μg^{-1} and 2.751×10^3 s^{-1}, respectively. Optimum pH of the purified enzyme 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 combined effect of pH and temperature on the performance of the purified L-asparaginase was studied under assay conditions using response surface methodology, and were found to be 8.49 and 39.3ºC, respectively. The minimum deactivation rate constant (k_d) and maximum half life ($t_{1/2}$) were found to be 0.041 min^{-1} and 16.9 h, respectively at pH of 8.6 and 40ºC. Thermodynamic parameters (ΔG, ΔH, ΔS, and activation energ y) were also evaluated for purified L-asparaginase. The probable mechanism of deactivation of purified L-asparaginase is described in this study. Further, *in vitro* anti-cancer activity assays using three different human cancer cell lines (HepG2, HeLa and MCF 7) were done to evaluate the therapeutic potential of L-asparaginase.

The effect of important physical parameters such as pH and Dissolved oxygen (DO) was studied to maximize the production of L-asparaginase by *P. carotovorum* MTCC 1428 in a batch bioreactor. The optimum level of dissolved oxygen (DO) was found to be 20% and not much decline in enzyme activity was observed when pH maintained at 8.5 after 12 h of fermentation. Under these conditions, *P. carotovorum* produced 17.97 U ml^{-1} of L-asparaginase corresponding to a productivity of 1497.50 U l^{-1} h^{-1}. Fed-batch production was studied by dual substrate(s) feeding strategies, L-asparagine and glucose were fed individually or together at 12 h of growth for enhancing the production of L-asparaginase. Best strategy for enhanced production of L-asparaginase under fed-batch mode was found to be initial growth medium containing both the substrates (L-asparagine and glucose) and feeding stream containing L-asparagine. Under these conditions, the production of L-asparaginase was 38.78 U ml^{-1}, which corresponded to a productivity of 1615.83 U l^{-1} $h^{-1}$of L-asparaginase. The production and productivity was increased by 115.8% and 7.9%, respectively when compared to the batch culture. The overall enhancement of L-asparaginase production (enzyme activity) and productivity was observed to be 51.0 fold and 25.5 fold, respectively as compared to the un-optimized medium.

Bacterial growth, substrates utilization and L-asparaginase production were studied in batch bioreactor for modeling of fermentation kinetics by *P. carotovorum* MTCC 1428. The kinetic study revealed that the maximum growth of *P. carotovorum* MTCC 1428 was observed at 2 g l^{-1} and 5 g l^{-1} of glucose and L-asparagine, respectively. Various substrate inhibition models were fitted to the growth kinetic data and the additive form of double Luong model was the best to explain the growth kinetics of *P. carotovorum* MTCC 1428. The kinetic parameters of growth studies showed that the predicated maximum inhibition concentration of

glucose (S_{mg}) and L-asparagine (S_{ma}) were close to the experimental (observed) values of 15.0 and 10 g l^{-1}, respectively. Modified form of the Luedeking and Piret model was used to describe the kinetics of L-asparaginase production and system found to be mixed growth associated. Kinetic models of dual substrate growth, L-asparaginase production and substrate(s) utilization by *P. carotovorum* MTCC 1428 were well fitted with experimental data with regression coefficients (R^2) of 0.97, 0.96 and 0.93, respectively.

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ABBREVIATIONS AND NOTATIONS

Abbreviations

ALL	acute lymphoblast leukemia
AML	acute myeloid leukemia
ANOVA	analysis of variance
CBB	coomassie brilliant blue
CCD	central composite design
DCW	dry cell weight (g l^{-1})
DEAE	diethylaminoethyl
DF	degree of freedom
DNS	3,5-dinitrosalicylic acid
DO	dissolved oxygen (%)
EC	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FMOC-Cl	9-fluorenylmethyl chloroformate chloride
GdnCl	guanidine hydrochloride
HPLC	high performance liquid chromatography
IEF–PAGE	isoelectric focusing-polyacrylamide gel electrophoresis
ISE	ammonium selective electrode
MALDI-TOF MS	matrix assisted laser desorption ionization time-of-flight mass spectrometry
mRNA	messenger ribose nucleic acid
MS	mean square
MTCC	microbial type culture collection
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Abbreviations

NCIM	National collection of industrial microorganisms
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PB	Plackett-Burman
p-CMBA	*p*-chloromercuribenzoic acid
PCR	polymerase chain reaction
PEG	polyethylene
PHB	polyhydroxybutyrate
PVDF	polyvinylidene fluoride
RDP	ribosomal Database Project
RQ-PCR	real-time quantitative-polymerase chain reaction
rRNA	ribosomal ribonucleic acid
RSM	response surface methodology
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
TCA	tri-chloro acetic acid
TEA	triethylamine
TFA	trifluoroacetic acid

Notations

°C	degree centigrade
C	integration constant
$C_{L\text{-}asp}$	L-asparagine concentration (µM)
D	dilution factor
E	activation energy
g	gram
g	gravitational acceleration
h	hour
K_{1a}	inhibition constant for L-asparagine (g l^{-1})
K_{1g}	inhibition constant for glucose (g l^{-1})
K_{1i}	inhibition constant for substrate *i* (g l^{-1})
K_{2a}	inhibition constant in Yano and Kago 2 model for L-asparagine (g l^{-1})
K_{2g}	inhibition constants in Yano and Kago 2 model for glucose (g l^{-1})
K_{2i}	inhibition constant in Yano and Kago 2 model for substrate *i* (g l^{-1})
kb	kilobase
k_{cat}	turnover numbers (s^{-1})
k_{cat}/K_m	specificity constant ($M^{-1}s^{-1}$)
kDa	kilo Dalton
K_m	Michaelis constant (mM)
K_{sa}	Monod half saturation constant for L-asparagine (g l^{-1})
K_{sg}	Monod half saturation constant for glucose (g l^{-1})
K_{si}	Monod half saturation constant for substrate *i* (g l^{-1})
M	molar (mol l^{-1})
m_a	L-asparagine used for maintenance of biomass (g of L-asparagine g^{-1} DCW h^{-1})
m_g	glucose used for maintenance of biomass (g of glucose g^{-1} DCW h^{-1})
min	minute
ml min^{-1}	milliliter per minute
$MW_{L\text{-}asp}$	L-asparagine molecular weight

Notations

M_r	molecular weight of enzyme (kDa)
n_a	constant in Loung model accounts the relationship between μ and L-asparagine
n_g	constant in Loung model accounts the relationship between μ and glucose
n_i	constant in Loung model accounts the relationship between μ and substrate *i*
nm	nanometer
P_{asp}	L-asparaginase concentration (U mL^{-1})
pH_{op}	optimum final pH for maximum L-asparaginase production
pH_t	pH value in each sampling time
R^2	regression coefficient
rpm	rotational per minute
s	second
S_a	L-asparagine concentration (g l^{-1})
S_g	glucose concentration (g l^{-1})
S_i	concentration of substrate *i* (g l^{-1})
S_{ma}	maximum inhibitory concentration of L-asparagine (g l^{-1})
S_{mg}	maximum inhibitory concentration of glucose (g l^{-1})
S_{mi}	maximum inhibitory concentration of substrate *i* (g l^{-1})
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
v/v	volume/volume
Ve	elution volume
V_{max}	Maximal reaction velocity (U μg^{-1})
V_o	void volume
vvm	volume of air per volume of medium per minute
w/v	weight/volume
X	biomass concentration (g of DCW l^{-1})

Notations

X_1	biomass concentration in batch bioreactor at time t_1 (g of DCW l^{-1})
X_2	biomass concentration in batch bioreactor at time t_1 (g of DCW l^{-1})
Y	predicated response
$Y_{P/Sa}$	yield of product (L-asparaginase) on L-asparagine (U g^{-1} of L-asparagine)
$Y_{P/Sg}$	yield of product (L-asparaginase) on glucose (U g^{-1} of glucose)
Y_{SA}	response (enzyme production)
$Y_{Sa/X}$	specific L-asparagine uptake rate (g of L-asparagine g^{-1} of cells h^{-1})
$Y_{Sg/X}$	specific glucose uptake rate (g of glucose g^{-1} of cells h^{-1})
$Y_{X/Sa}$	yield coefficient for L-asparagine (g microorganism g^{-1} L-asparagine)
$Y_{X/Sg}$	yield coefficient for glucose (g microorganism g^{-1}glucose)

Greek letter

μ	specific growth rate (h^{-1})
μ_{Expt}	experimental specific growth rate (h^{-1})
µl	microlitre
μ_m	maximum specific growth rate (h^{-1})
µM	Micromolar (µmol l^{-1})
µmol	Micromoles
μ_{Pred}	theoretical specific growth rate (h^{-1})
α	growth-associated constant for L-asparaginase production (U mg^{-1})
β	non growth-associated constant (U mg^{-1} h^{-1})
β_i	Linear effect
β_{ii}	Square effect
β_{ij}	Interaction effect
β_o	Offset term
κ	constant of pH proportionality

CHAPTER 1
INTRODUCTION

1.1. Generalities

L-Asparaginase (L-asparagine amido hydrolase E.C. 3.5.1.1) is an enzyme of high therapeutic value due to its use in certain kinds of cancer therapy, mainly in acute lymphoblastic leukemia (ALL) (Athale and Chan, 2003; Narta *et al.,* 2007). Moreover, it is also used in food industry for the production of acrylamide free food (Pedreschi *et al.,* 2008), as model enzyme for development of new drug delivery system (Teodor *et al.,* 2009) and L-asparagine biosensor for leukemia detection (Verma *et al.,* 2007). The antileukemic effect of 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 thus are less affected by its rapid depletion due to treatment with this enzyme. The asparagine deficiency rapidly impairs the protein synthesis and leads to delayed in inhibition of DNA and RNA synthesis and hence impairs the cell functioning finally resulting in cell death (Muller and Boos, 1998; Narta *et al.,* 2007).

Although L-asparaginase is produced by various microorganisms (Verma *et al.,* 2007), this enzyme from *Erwinia chrysanthemi* and *Escherichia coli* are currently in clinical use as effective drug for various leukemia diseases (Duval *et al.,* 2002; Kozak and Jurga, 2002) 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. The

therapeutic response of patients rarely occurs without some evidence of toxicity (Duval *et al.*, 2002; Narta *et al.*, 2007). With a view to characterize enzymes with less toxic side effects, several members of a larger family of homologous L-asparaginases have been thoroughly investigated over many years (Boyse *et al.*, 1967; Ehrman *et al.*, 1971; Cammack *et al.*, 1972; Wriston and Yellin, 1973; Krasotkina *et al.*, 2004; Kotzia and Labrou 2005). L-Asparaginases with high specificity for L-asparagine and low-to-negligible activity against L-glutamine are reported to be less troublesome during the course of anti-cancer therapy (Hawkins *et al.*, 2004). The allergic response is due to long-term use of a foreign protein, and this can be circumvented by using serologically unrelated L-asparaginase. The other side effects are mainly due to partial glutaminase activity of most of the L-asparaginases (Distasio *et al.*, 1982; Gallagher *et al.*, 1989; Manna *et al.*, 1995). Hence, for successful clinical studies, a glutaminase-free L-asparaginase isolated from a new source will be more beneficial.

In most of the bacterial L-asparaginases, the presence of partial glutaminase activity up to 9% of L-asparaginase activity was reported (Manna *et al.*, 1995; Muller and Boos, 1998). The various side effects of this drug are mainly due to the presence of partial glutaminase activity (Distasio *et al.*, 1982; Muller and Boos, 1998). Hence, for successful clinical use glutaminase-free L-asparaginase is highly desirable. 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), *Escherichia 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), *Aspergillus* species (Mishra, 2007) with various carbon and nitrogen sources. The synthesis of L-asparaginase by gram-negative bacteria is influenced significantly by

process parameters such as pH, temperature, dissolved oxygen, incubation time, inoculum volume, aeration, agitation and medium constituents (Khan *et al.*, 1970; Mukherjee *et al.*, 2000). The results were contradictory in terms of the effect of glucose (Khan *et al.*, 1970; Barnes *et al.*, 1977) and oxygen (Mukherjee *et al.*, 2000) on the production of this enzyme.

1.2. Subcellular localization of L-asparaginase

A study on localization of any enzyme plays an important role in the development of bioprocess. In L-asparaginase producing gram negative strains, the existence of periplasmic, cytoplasmic and membrane bound enzyme was reported (Schwartz *et al.*, 1966; Triantafillou *et al.*, 1988; Mukherjee *et al.*, 2000; Geckil and Gencer, 2004; Geckil *et al.*, 2005). Many gram-negative bacteria contain two L-asparaginases, a high-affinity periplasmic enzyme and a low-affinity cytoplasmic enzyme. In *Escherichia 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 latter one 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). 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 carried out using various methods (Alexander, 1956; Marr, 1960; Neu and Heppel, 1965).

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

Process optimization plays a vital role in industrial production processes in which even small improvements would be decisive for commercial success. In any bioprocess, the improvement in productivity of any metabolite could be achieved through manipulation of nutritional and physical parameters. The Plackett–Burman factorial designs allow for the screening of main factors from a large number of process variables, and these designs are thus quite useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated for further optimization processes. In addition, response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system would be determined. 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 lead to misinterpretation of results. In contrast, statistical methodologies are generally preferred, due to their advantages (Dasu and Panda, 2000; Abdel-Fattah and Olama, 2002; 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). Abdel-Fattah and Olama, (2002) was successfully used Plackett-Burman and Box-Behnken experimental designs for screening and optimization of process parameters to improve production of L-asparaginase from *Pseudomonas 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* species using process parameters optimization by Box-Behnken

experimental and Taguchi methodology design, respectively in submerged fermentation. Recently, Hymavathi *et al.,* (2009) reported the enhancement of L-asparaginase production by isolated *Bacillus circulans* MTCC 8574 using RSM in solid state fermentation.

1.4. Purification and characterization of L-asparaginase

Downstream processing of biomolecules obtained from fermentation broths is a fundamental step in process biotechnology since 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 (Wheelwright 1987; Haki and Rakshit, 2003). Chromatographic techniques are widely used as high performance purification steps in biotechnology (Martın *et al.,* 2002). Since each enzyme requires specific strategy for purification, therefore it becomes necessary to develop strategies for the purification of individual enzyme. The purification and characterization of L-asparaginase has been studied from different bacterial sources *viz., Escherichia coli, Erwinia chrysanthemi, Erwinia carotovora, Pseudomonas stutzeri, Bacillus circulans, Enterobacter aerogenes, Pseudomonas aeruginosa* etc. with various purification strategies (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 proportion of substrate-to-enzyme, 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. An improved knowledge of enzyme deactivation kinetics is needed to enhance the feasibility of its therapeutic use. 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 of its ability to catalyze a reaction while the 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 vital role to predict, manipulate and engineer the protein structure and function. Various human cancerous cell lines for *in vitro* cytotoxicity have been investigated to establish the potential of L-asparaginase from different sources (Cappelletti *et al.*, 2008; Abakumova *et al.*, 2009; Oza *et al.*, 2010; Prakasham *et al.*, 2010).

1.5. Bioreactor studies to improve the L-asparaginase production

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; Kozak and Jurga, 2002; Borek and Jaskólski, 2002), catalysis (Kelo *et al.*, 2002), clinical aspects (Ettinger *et al.*, 1997; Narta *et al.*, 2007), genetic determinants involved in regulation (Ortuño-Olea and Durán-Vargas, 2000; Hüser *et al.*, 2002) and stabilization to enhance biological half-life (Soares *et al.*, 2002;

Baran *et al.*, 2003; O'Fágáin, 2003) of L-asparaginase. But very few reports are available for improving the enzyme production (Mukherjee *et al.*, 2000; Geckil *et al.*, 2004). Further studies on the fermentation kinetics and production in bioreactor level have also not been attempted so far.

1.6. Kinetic modeling of L-asparaginase

Mathematical modeling is necessary to predict the results of industrial fermentations and to choose optimized conditions. Additionally, it helps in understanding the complex mechanisms of growth and product formation of this microorganism (Zinn *et al.*, 2004). Kinetic studies would allow the prediction of fermentation rate, product yield and the control of the fermentation process. The production of L-asparaginase with a batch system from Gram negative bacteria is dependent on many factors as the L-asparaginase production system is of a complex nature. Complete quantitative description of its kinetic behavior requires mathematical expressions describing the time course of each important variable (substrate utilization, growth kinetics and enzyme production). Although the production of L-asparaginase in bioreactor has been studied by few authors (Mukherjee *et al.*, 2000; Geckil *et al.*, 2004), the development of kinetic models for dual substrate growth, L-asparaginase production and substrate utilization have not been considered previously.

1.7. Objectives and scope

Based on an extensive literature survey on the production of L-asparaginase and characterization, the present study focused on bioprocess development for the production of glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428. In order to achieve the

said objective, a suitable bioprocess was developed from shake flask to bioreactor level and the purified enzyme was characterized. The following objectives have been envisaged in the present investigation:

- Isolation, screening and selection of potential glutaminase-free L-asparaginase producing bacterial strains and localization of L-asparaginase from various microorganisms.
- Medium development to maximize the production of L-asparaginase from *P. carotovorum* MTCC 1428.
- Optimization of physical process parameters for enhanced L-asparaginase production by *P. carotovorum* MTCC 1428.
- Purification and characterization of L-asparaginase from *P. carotovorum* MTCC 1428.
- Production of L-asparaginase in a bioreactor operated under batch and fed-batch modes of operation.
- Development of unstructured models for dual substrate utilization kinetics for growth and L-asparaginase production by *P. carotovorum* MTCC 1428.

1.8. Organization of the thesis

The presentation of the work has been divided into five chapters. The current **Chapter 1** presents a general introduction, objective and scope of the present work. While 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 present study. Details of the subcellular localization, optimization of process parameters in shake flask and bioreactors, purification and characterization, and kinetic modeling for L-asparaginase fermentation by *P. carotovorum* MTCC 1428 are presented in this chapter. It also provides technical information about the

analytical methods adopted in this present work. **Chapter 4** contains the results and discussions, where the results of bioprocess development of L-asparaginase production in shake flask and bioreactor are presented and thoroughly discussed. This chapter addresses subcellular localization, purification and characterization of novel glutaminase-free L-asparaginase. Finally, the chapter emphasizes the development of kinetic models of dual substrate growth, L-asparaginase production and substrate(s) utilization by *P. carotovorum* MTCC 1428. **Chapter 5** draws summary and appropriate conclusions 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) is the enzyme having antitumor activity and obtained from various biological sources *viz*., plants, animals and many other microorganisms (fungus, yeast, bacteria etc.) (Broome, 1961; Adamson *et al.*, 1968). The enzyme acquired clinical importance in 1961 when the antitumor effect of Guinea pig serum originally discovered by Kidd, (1953) was traced by Broome, (1961). It has been used in leukemia treatment last four decades. The most common therapeutic indications are treatment of Hodgkin disease, acute lymphocytic leukemia (mainly in children), acute myelocytic leukemia, acute myelomonocytic leukemia, and chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosarcoma (Cunningham *et al.*, 1979; Capizzi *et al.*, 1984; Ravindranath *et al.*, 1992; Mitchell *et al.*, 1994; Klumper *et al.*, 1995; Larson *et al.*, 1995). Recently, some more applications of L-asparaginase have been reported *viz*., acrylamide free food production (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 thus are less affected by its rapid depletion due to treatment with

this enzyme. The asparagine deficiency rapidly impairs the protein synthesis and leads to a delayed inhibition in DNA and RNA synthesis and hence to an impairment of cellular function, resulting in cell death (Fig. 2.1) (Muller and Boos, 1998; Narta *et al.*, 2007). Schematic diagram of reaction mechanism of L-asparaginases to hydrolyzed the L-asparagine into L-aspartic acid and ammonia is given in Fig. 2.2.

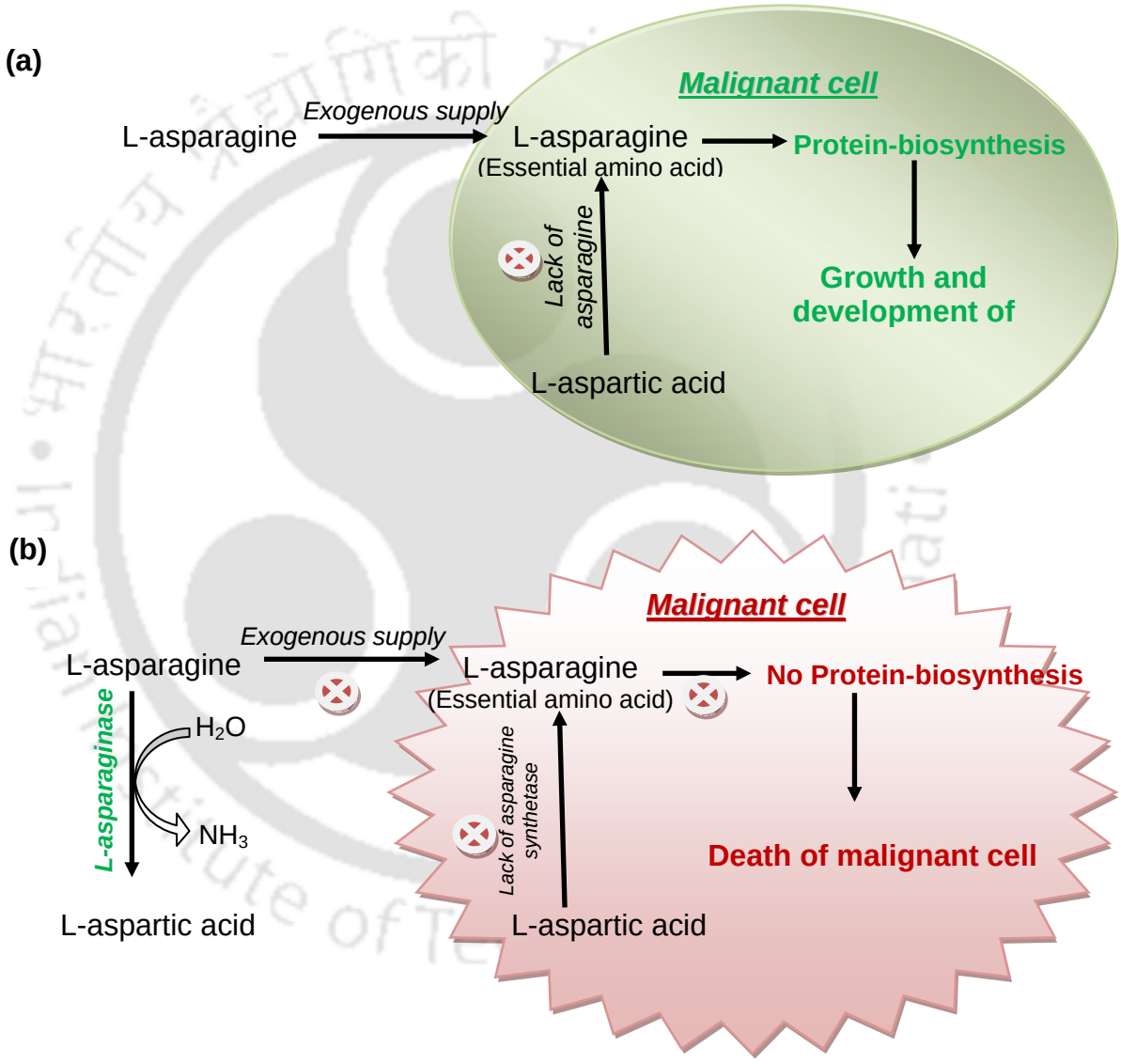


Fig. 2.1. Mechanism of action of L-asparaginase (a) Proliferation of malignant cell (b) Death of malignant cell in presence of L-asparaginase

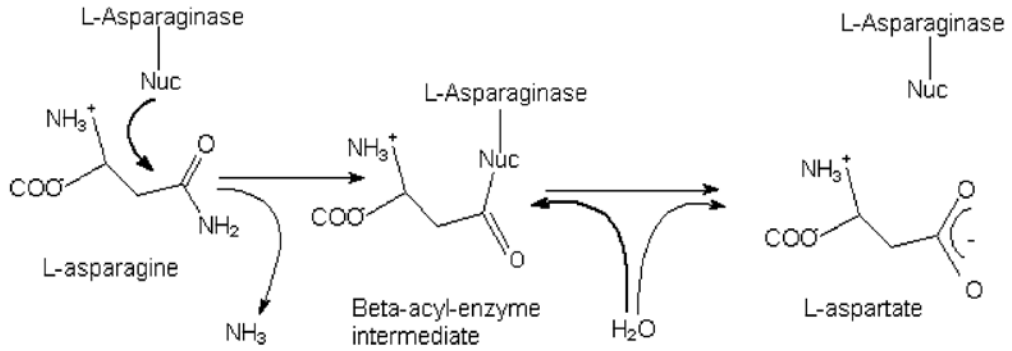


Fig. 2.2. Schematic illustration of the reaction mechanism of L-asparaginases. The proposed covalent intermediate is formed through nucleophilic attack by the enzyme. Bold arrows indicate nucleophilic attack (Sanson and Jaskolski, 2004).

Although L-asparaginase is produced by various microorganisms (Verma *et al.,* 2007), L-asparaginase from *Erwinia chrysanthemi* and *Escherichia coli* are currently in clinical use as effective drugs for the various 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. The therapeutic response of patients rarely occurs without some evidence of toxicity (Narta *et al.,* 2007). With a view to characterize enzymes with less toxic side effects, several members of a larger family of homologous L-asparaginases have been thoroughly investigated 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 against L-glutamine are reported to be less troublesome during the course of anti-cancer therapy (Hawkins *et al.,* 2004). The allergic response is due to long-term use of a foreign protein, and this can be circumvented by using serologically unrelated L-asparaginase. The other side effects are mainly due to glutaminase contamination of most of L-asparaginases (Gallagher *et al.,* 1989; Manna *et al.,* 1995). Hence, for successful clinical studies, glutaminase free L-asparaginase isolated from a new source will be more advantageous.

2.2. Historical development

The pioneer observation that turned out to be important for the development of L-asparaginase as a potential antineoplastic agent was made by Clementi, (1922): revealing the presence of high activity of L-asparaginase in 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,b) 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) served to prove that the basically non-essential amino acid asparagine is needed to grow the Walker carcinosarcoma 256 *in vitro*. Haley and co-workers, (1961) found that murine L5178Y leukemia cells also require asparagines 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 asparagines will impair cellular function and eventually cause cellular death. Setting out from Kidd's publications, Broome (1963ab) later showed that it was not the complement in the serum, which provoked the tumor regression, but rather the L-asparaginase. This observed effect is due to the relative substrate specificity and hydrolyses the amino acid asparagine to aspartic acid and ammonia.

In 1964, Mashburn and Wriston in search of other L-asparaginase sources for clinical use established that the activity of the enzyme isolated from *Escherichia coli* compares with the guinea pig asparaginase. Publications by other researchers in 1966 and 1967 confirmed these results (Roberts *et al.,* 1966; Boyse *et al.,* 1967; Brome *et al,* 1967). At the same time it

became possible to produce and utilize larger quantities of the enzyme, and a series of preclinical and clinical studies was initiated **(**Boyse *et al.,* 1967; Whelan and Wriston, 1969). The first clinical trials in patients with acute lymphoblastic leukemia were carried out with asparaginase preparations both from guinea pig serum and *E. coli*. Both enzymes showed clinical efficacy, which was confirmed in further studies (Roberts *et al.,* 1966; Boyse *et al.,* 1967). Yellin and Wriston, (1966a) succeeded in partial purification of two isoforms of L-asparaginase from the serum of guinea pig. Interestingly, only one isoform exhibited anti lymphoma activity *in vivo* (Yellin and Wriston, 1966b).

Systematic studies to identify other potential sources of L-asparaginase were performed in phytopathogenic microorganisms that hydrolyse asparagine for energy production. These studies identified bacterial species other than *E. coli* which also showed relevant asparaginase activity. Among the species studied *E. carotovora*, later re-named *Erwinia chrysanthemi*, showed the highest asparaginase activity and was hence utilized for larger scale enzyme production (Philips *et al.,* 1971). Mashburn and Wriston, (1964) and Campbell and Mashburn, (1969) reported that the purification of *E. coli* L-asparaginase, and demonstrated its tumoricidal activity similar to that of guinea pig sera. These findings provided a practical base for large-scale production of enzyme for pre-clinical and clinical studies (Roberts *et al.,* 1966; Boyse *et al.,* 1967). Oettgen *et al.,* (1967) were first to show the efficacy of L-asparaginase in humans suffering from leukemia. Another important contemporary observation was that of Old *et al.,* (1967), demonstrating *in vivo* antitumor activity in a dog model with spontaneous lymphosarcom. While other microorganisms such as *Serratia marcescens* and *Vibrio succinogenes*, later classified to the species *Wolinella*, also possess asparaginases that act on lymphomas (Distasio *et al.,* 1982), only native asparaginase from various *E. coli* strains and

from *Erwinia chrysanthemi* went on to gain importance for clinical use. Hypersensitivity reactions to the *E. coli* protein were recognized to be fairly common. An L-asparaginase from *Erwinia caratovora* was found to lack cross reactivity with *E. coli* enzyme (Ohnuma *et al.,* 1972). Both enzymes, however, have high rates of immunogenicity, and conjugation to poly ethylene glycol (PEG) was recognized 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). Clinical studies described below have borne out a reduced rate of hypersensitivity reactions and have allowed for much less frequent administration of the peg-aspargase compared to native L-asparaginases.

The use of L-asparaginase in leukemia and lymphoma treatment has expanded in recent years. While earlier use focused on its use in remission induction for patients with acute lymphoblastic leukemia (Oettgen *et al.,* 1967; Haskell *et al.,* 1969; Ortega *et al.,* 1977; Jones *et al.,* 1977), more recent studies have explored the administration of L-asparaginase for 20–30 weeks as consolidation treatment for lymphoid malignancies (Clavell *et al.,* 1980; Amylon *et al.,* 1999). For these reasons, L-asparaginase has established itself to be an indispensable component in modern procedures of combination chemotherapy.

Till 2000, most of the production studies from various microorganisms were performed under shake flasks and emphasis was given on *in vitro*, *in vivo* and clinical studies for novel source of microorganism rather for maximizing the production of L-asparaginase. Last few years, many researchers worked on improvement of L-asparaginase production from a variety of

microorganism. However, literature still lack of knowledge of various process parameters, which influence the L-asparaginase fermentation.

2.3. Subcellular distribution of L-asparaginase

Yellin and Wriston, (1966a) reported first time that the two isoforms of L-asparaginase from the serum of guinea pig. Various workers have been reported for the existence of periplasmic, cytoplasmic and membrane bound L-asparaginase was reported (Schwartz *et al.,* 1966; Triantafillou *et al.,* 1988; Mukherjee *et al.,* 2000; Geckil *et al.,* 2004; Geckil *et al.,* 2005; Kumar *et al.,* 2010). Campbell and Mashburn, (1969) had attempted to improve the production of asparaginase and shown that the two asparaginases detectable in *Escherichia coli*, named EC-I and EC-II, where EC-II enzyme has relevant antitumour activity. Many gram-negative bacteria contain two L-asparaginases, a high-affinity periplasmic enzyme and a low-affinity cytoplasmic enzyme. 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 latter one 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) studied that the subcellular distribution of L-asparaginase in pea's leaves by sucrose density gradient. Cytosolic L-asparaginase was observed in pea leaves. Based on the alignments of asparaginases available in databases, as well as on biochemical and crystallographic data, the known asparaginase sequences can be divided into three families (Fig. 2.3) *viz*., bacterial, plant and the enzymes similar to *Rhizobium etli* asparaginase.

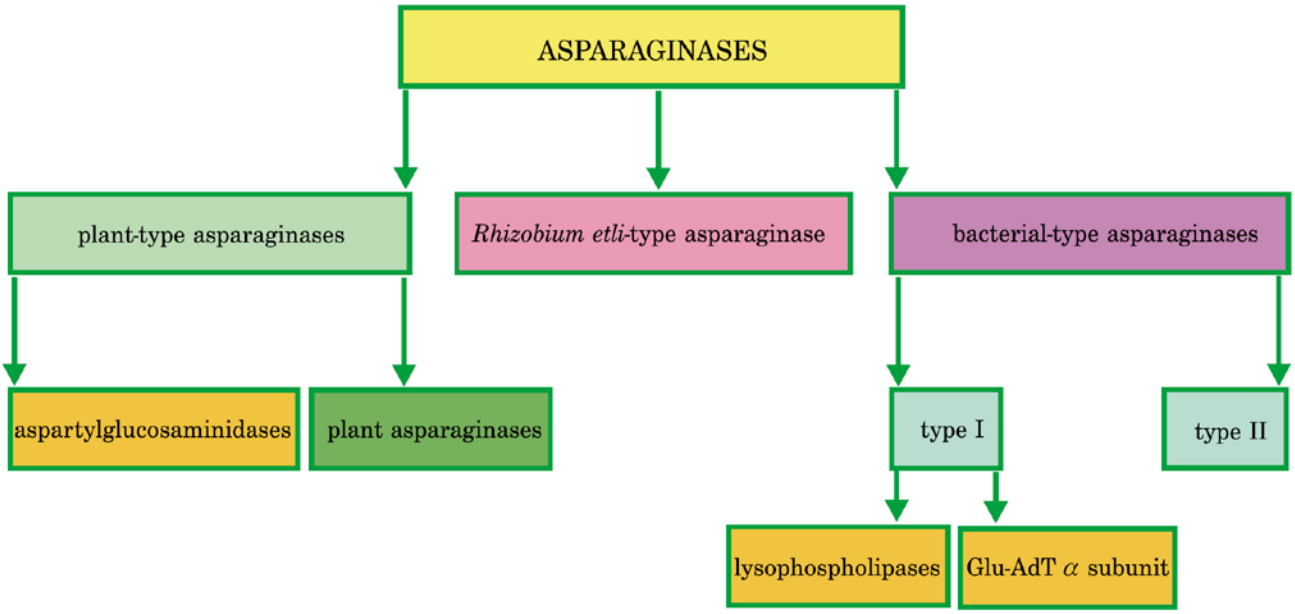


Fig. 2.3. General classification of asparaginases (Borek and Jaskólski, 2001).

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 been the subject of considerable medical interest and are being employed in the therapy of acute lymphoblastic leukemia. L-asparaginase from *Erwinia carotovora* is serologically and biochemically distinct from the L-asparaginase of *E. coli*, although its antineoplastic activity and toxicity is similar. L-asparaginase production from *Pseudomonas flourescens* AG has been reported by Mardashev *et al.*, (1975). Pastuszak *et al.*, (1976) reported that the *Mycobacterium phlei* has also been found to be a good L-asparaginase source. L-asparaginase production in *Staphylococci* has been

described by Mickucki *et al.,* (1977). In *Tetrahymena pyriformis*, maximum activity of the enzyme has been found at the stationary phase of growth and most of the activity has been associated with the endoplasmic reticulum (Triantafillou *et al.,* 1988). Curran *et al.,* (1985) have obtained a specific L-asparaginase that has been further characterized from the thermophilic bacteria, *Thermus aquaticus*. 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 the *Enterobacter cloacae* that can utilize L-asparagine as the sole growth substrate and produce L-asparaginase. Glutaminase-free L-asparaginase from *Thermus thermophilus* has been reported by Pritsa and Kyriakidis, (2001). L-asparaginase from a new *Erwinia sp.* has been reported by Borkotaky and Bezbaruah, (2002). El-Bessoumy *et al.,* (2004) have obtained a new L-asparaginase producer from *Pseudomonas aeruginosa* 50071 that is serologically different from the previously reported L-asparaginases. Neto *et al.,* (2006) has been obtained L-asparaginase from *Zymomonas mobilis* CP4 and optimized culture conditions. Production of L-asparaginase from *Staphylococcus* sp. – 6A has been reported by Prakasham *et al.,* (2007). Hymavathi *et al.,* (2009) has been reported the production of L-asparaginase using *Bacillus circulans* MTCC 8574. Recently, Sunitha *et al.,* (2010) has been obtained L-asparaginase from *Bacillus cereus* MNTG-7. The summarized sources of bacterial L-asparaginase are presented in Table 2.1. The dendrogram for the bacterial-type asparaginases (Fig. 2.4) 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 one includes eubacterial asparaginases.

Table 2.1. Sources of L-asparaginases from various bacteria

Organism	Mol. weight kDa	L-asparaginase production	References
Escherichia coli	140	27.5 U ml^{-1}	Barnes *et al*., 1977
Erwinia carotovora	150	1.36 U mg^{-1}	Warangkar and Khobragade (2010)
Azotobacter vinelandii	84	4 U mg^{-1}	Gaffar and Shethna, (1977)
Pseudomonas aeruginosa	138	11.18 U mg^{-1}	Abdel-Fattah and Olama, (2002)
Pseudomonas stutzeri	34	1.1 U mg^{-1}	Manna *et al.,* (1995)
Staphylococcus sp.	--	55.6 U ml^{-1} (SSF)	Prakasham *et al.,* (2007)
Bacillus cereus	--	56.5 U ml^{-1}(SSF)	Sunitha *et al.,* (2010)
Bacillus circulans	140	2322 U gds^{-1} (SSF)	Hymavathi *et al.,* (2009)
Bacillus subtilis	--	0.11 U ml^{-1}	Gulati *et al.,* (1997)
Baciliius lichiniformis	--	0.15 U ml^{-1}	
Thermus thermophilus	200	0.09 U mg^{-1}	Pritsa and Kyriakidis, (2001)
Tetrahymena pyriformis	200	0.54 U ml^{-1}	Triantafillou *et al.,* (1988)
Pseudomonas acidovorans	156	0.039 U mg^{-1}	Davidson *et al.,* (1977)
Enterobacter cloacae	--	35.0 U mg^{-1}	Nawaz *et al.,* (1998)
Helicobacter pylori	30	5.1 U mg^{-1}	Gladilina *et al.,* (2009)
Serratia marcescens	180	0.7 U mg^{-1}	Boyd and Phillips, (1971); Stern *et al.,* (1976)
Erwinia aroideae	--	1.1 U mg^{-1}	Peterson and Ciegler, (1969a)
Zymomonas mobilis	--	0.17 U ml^{-1}	Neto *et al.,* (2006)
Enterobacter aerogenes	--	1.20 U ml^{-1}	Mukherjee *et al.,* (2000)
Mycobacterium phlei	126	32.6 U mg^{-1}	Pastuszak *et al.,* (1976)
Thermus aquaticus.	80	30.35 U ml^{-1}	Curran *et al.,* (1985)
Pseudomonas flourescens	--	140 U g^{-1} of dry cells	Peterson and Ciegler, (1969b)
Pseudomonas putida	--	100 U g^{-1} of dry cells	
Xanthomonas campestris	--	50 U g^{-1} of dry cells	

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

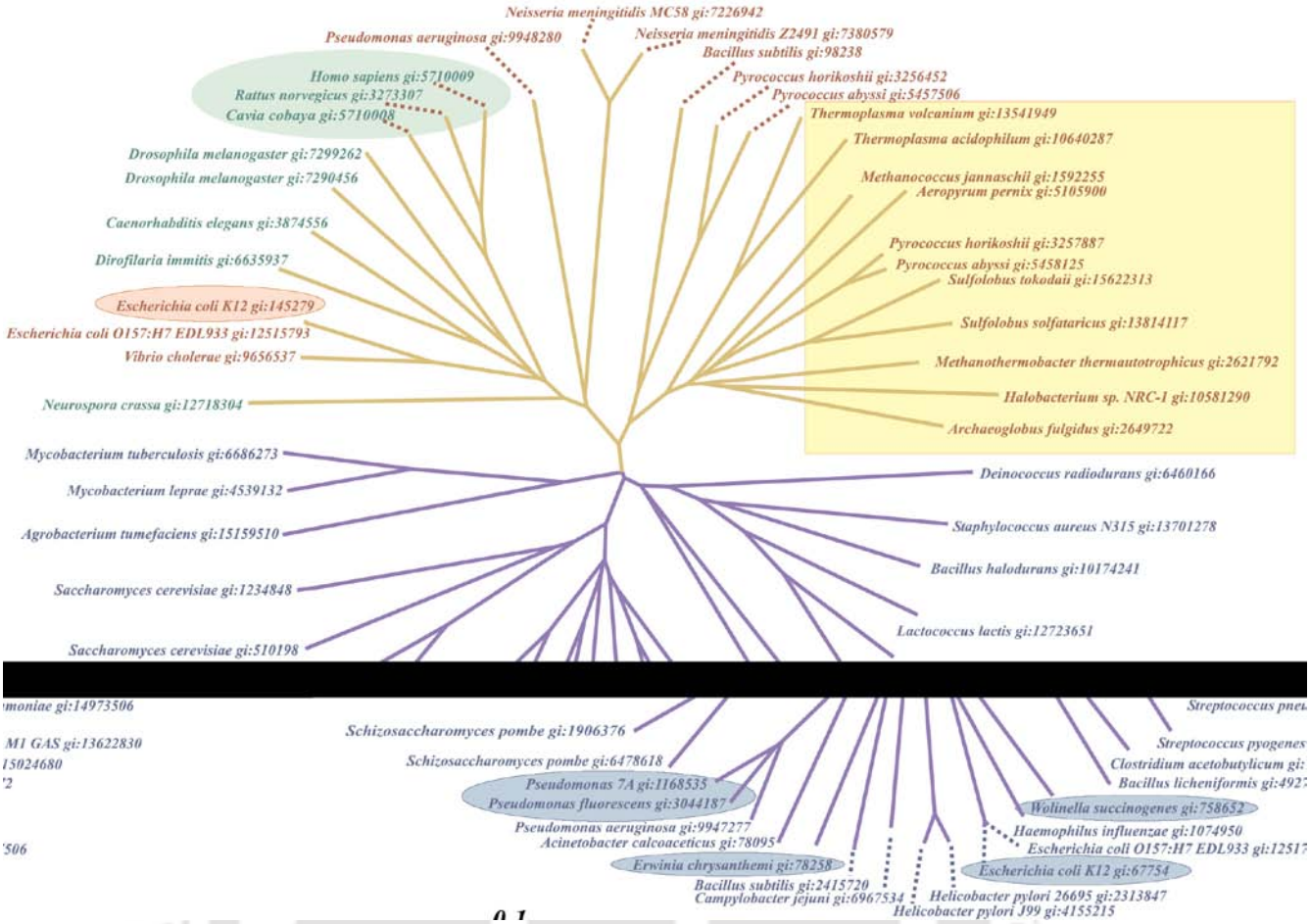


Fig. 2.4. A dendrogram of bacterial asparaginases. (Borek and Jaskólski, 2001). *Orange branch* - type I bacterial asparaginases, *blue branch* - type II bacterial asparaginases. Lettering colors: *blue* - sequences with high homology to EcAII, *brown* - sequences with high homology to EcAI, *green* - 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.

2.4.2. Fungi and yeast sources

Many reports are available, which showed that eukaryote microorganisms like yeast and filamentous fungi have a potential for asparaginase production (Wade *et al.*, 1971, Wiame *et*

al., 1985, Pinheiro *et al.,* 2001). For example, the mitosporic fungi genera such as *Aspergillus*, *Penicillium* and *Fusarium* are commonly reported in the literature to produce asparaginase (De-Angeli *et al.*, 1970, Arima *et al.,* 1972, Imada *et al.,* 1973, Nakahama *et al.,* 1973, Curran *et al.,* 1985). Dunlop and Roon, (1975) reported 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 *Rhodosporidium toruloides* has been reported by Ramakrishnan *et al.,* (1996). Sarquis *et al.,* (2004) and Mishra, (2006) reported that the L-asparaginase production by *A. terreus* and *A. niger,* 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 terrus	--	0.058 U ml^{-1}	Sarquis *et al.*, (2004)
Aspergillus oryzae	--	0.038 U ml^{-1}	
Aspergillus niger	--	40.9 U gds^{-1} (SSF)	Mishra, (2006)
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 are also found in *Streptomyces plicatus* isolated from the alimentary canal of the fish, *Gerres filamentous* (Balakrish *et al.*, 1977; Selvakumar, 1979; Maya 1992; Mathew 1995). Gunasekaran *et al.,* (1995) reported that the production of L-asparaginase by *Nocardia*

sp. strain. Production of intracellular and extracellular asparaginase from *Streptomyces longsporusflavus* (F-15) has been described by Abdel-Fatah and Olama (1998). *Streptomyces sp.* Isolated from the gut of the fish, *Therampon jarbua* and *Villorita cyprinoids* have shown L-asparaginase activity (Dhevendaran and Anithakumari, 2002). Dhevagi and Poorani, (2006) have been investigated the isolation, 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 also to identify 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, *Streptomyces 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 albidoflavus	112	25.2 U mg^{-1}	Narayana *et al.,* (2008)
Streptomyces sp.	140	0.8 U mg	Dhevagi and Poorani (2006)
Streptomyces sp.	--	384.0 U mg^{-1}	Basha *et al.*, (2009)
Nocardia sp.	--	0.5 U ml^{-1}	Gunasekaran *et al.*, (1995)
Streptomyces longsporusflavus		22.0 U mg^{-1}	Abdel-Fatah and Olama, (1998)
Streptomyces gulbargensis	85	12.1 U mg^{-1}	Amena *et al.,* (2010)

2.4.4. Plant sources

Two isoforms of the enzyme have been reported by Heeshen *et al.,* (1996) from the Bryophyte *Sphagnum fallax.* Among plants, L-asparaginase has been reported to be produced from *Lupin araboreus* and *Lupin angustiplius* (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 *Escherichia coli*. L-asparaginase activity has also been reportedly found in the soil of roots of *Pinus pinaster* and *Pinus radiata* due to ectomycorrhizal fungi in the wheat belt of Western Australia by 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. against ALL was observed by Oza *et al.,* 2010. The summarized sources of plant L-asparaginase are listed in Table 2.4.

Table 2.4. Sources of the L-asparaginase from plants

Organism	L-asparaginase production	References
Capsicum annum L. (Green chillies)	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	
Withania somnifera (Ashavgandh)	117 U mg^{-1} of protein	Oza *et al.,* (2010)
Lupinus luteus (Lupin)	57 U ml^{-1} of protein	Borek *et al.,* (2004)
Pisum sativum (Pea leaves)	100 U ml^{-1} of supernatant	Ireland and Joy, (1983)

The dendrogram for plant-type asparaginases (Fig. 2.5) is clearly divided into four branches (Borek and Jaskólski, 2001). The aspartylglucosaminidase sequences form one of the branches. The enzymes from plants and their close homologs form another branch. The product of the *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.

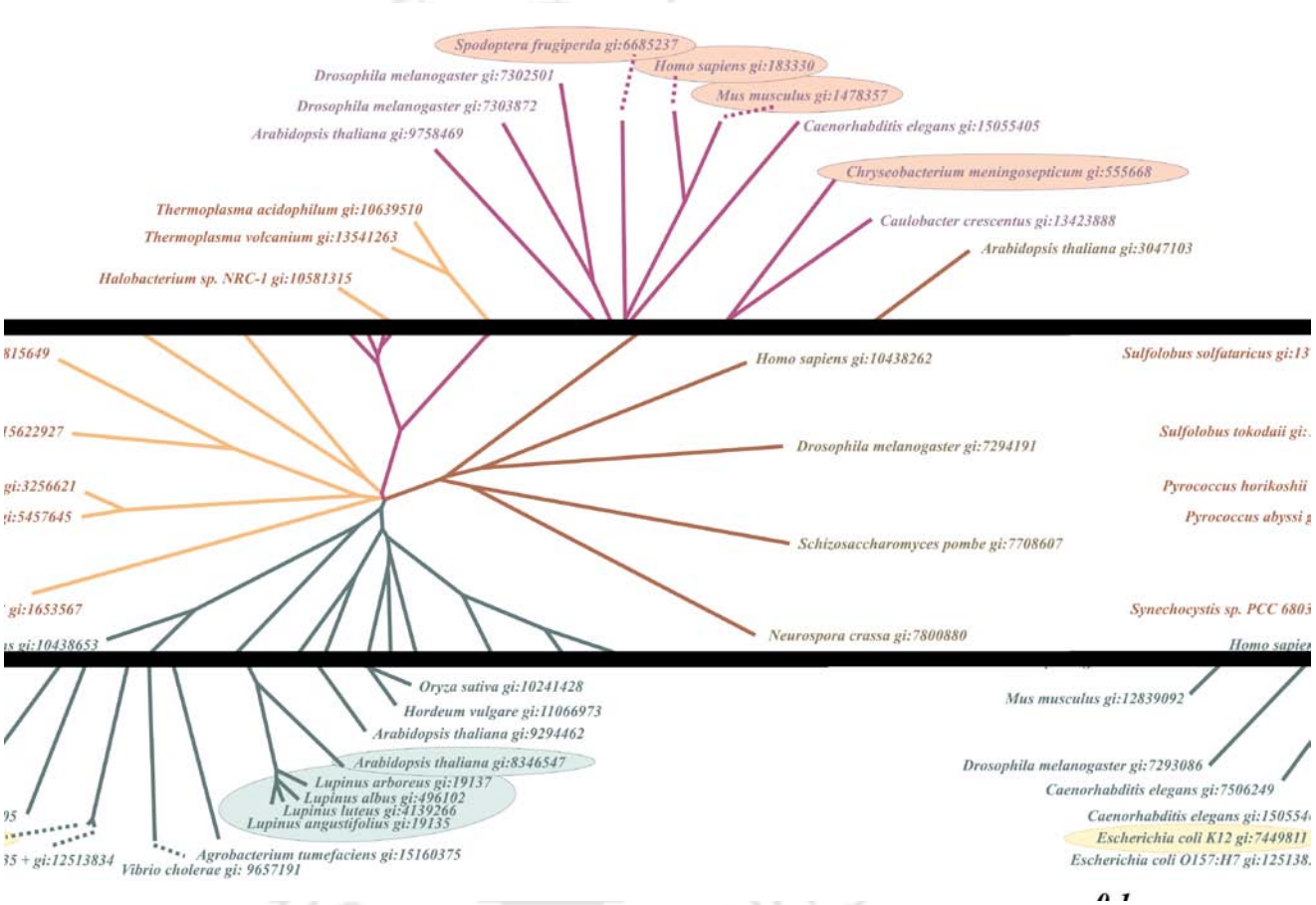


Fig. 2.5. A dendrogram of plant asparaginases (Borek and Jaskólski, 2001). *Orange branch* - predominantly archaeal plant-type asparaginases. *Violet branch* - aspartylglucosaminidases. *Brown branch* - eukaryotic sequences with homology to plant asparaginases but of unknown biochemical characteristics. *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

There have been many reports about the production of L-asparaginase under various conditions by various microorganisms. L-asparaginase from *Pseudomonas ovalis* was reported by Badr-El-Din and Foda, (1976). L-asparaginase from *Serratia marcescens* (Stern *et al.,* 1976) has also been reported. The synthesis of L-asparaginase in *E. coli* -W and *E. coli* K-12 was almost completely suppressed in the presence of glucose at a concentration of 0.5%. This is due to catabolite repression and catabolite inhibition of the components involved in lactate transport (Garaev and Golub, 1977) and lactate stimulated L-asparaginase synthesis. Organic acids and amino acids such as L-leucine and L-methionine were found to enhance production of L-asparaginase in *E. coli* (Netrval *et al.,* 1977). *S. cerevisiae* synthesizes two forms of asparaginase, L-asparaginase-I and L-asparaginase-II. The two enzymes are genetically and biochemically distinct. The interactions between both were studied by Jones, (1977). L-asparaginase-I is constitutive and L-asparaginase-II is secreted in response to N starvation (Dunlop *et al.,* 1978). High L-asparaginase activity has been observed in bacterial cultures in the presence of ample nitrogen (Paul and Cooksey, 1981). In *Lupin arboreus*, parts such as leaves, root-tips, flower buds and developing seeds have been found to be sources of L-asparaginase (Chang and Farnden, 1981). Use of keiselguhr composite and CM sepharose for large-scale production of asparaginase from *Erwinia chrysanthemi* has been described by Goward *et al.,* (1989). Cell growth and enzyme formation were studied by Alegre and Minim, (1992) in batch fermentation for the production of therapeutic L-asparaginase from *Erwinia aroidae*. Yeast extract was important for the cell mass formation and L-asparaginase synthesis, but L-asparaginase production was inhibited at high concentration. 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. Amounts of enzyme secreted by luminous prokaryotes were found to be higher than those reported from other bacterial species (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, while in presence exogenous c-AMP stimulated carbon sources and ultimately L-asparaginase enzyme production (Rozalska and Mickucki, 1992). The supplementation of cheese whey with tryptone (0.3%) and asparagine (0.5%) was used (Alegre and Minim, 1993). The operator conditions of a bioreactor have shown significant effect on the production of L-asparaginase by *Erwinia arodiae* NRRL B-138. *Nocardia asterodies*, an aerobic actinomycete, was grown on three different media, namely sabourand dextrose broth (SD), tryptic soy broth and synthetic medium, as a shake culture at 37°C for six days. The SD broth yielded maximum growth and maximum L-asparaginase production (Gunasekaran *et al.,* 1995). A pH and dye based fast procedure for screening of L-asparaginase producing microorganisms has been reported by Gulati *et al.,* (1997). The compound n-dodecane at 6% increased cell concentration by 12.7% and production of L-asparaginase by 21% to achieve 60.8 U/ml from *E. coli* (Wei and Liu, 1998). An intracellularly expressed L-asparaginase was detected from *Enterobacter cloacae* by Nawaz *et al.,* (1998). These gram negative, 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 pH and temperature optimum of L-asparaginase was found to be 8.5 and 37–42°C, respectively. Production of L-asparaginase from a new *Erwinia sp*. has been reported by Borkotaky and Bezbaruah, (2002). Optimum pH and K_m were found to be 9.2 and 2.8 mM for L-asparagine, respectively. L-

asparaginase from new *Erwinia sp*. was found to be glutaminase-free, thermostable, hexamer and followed linear kinetics upto 77°C.

Optimization of solid state fermentation for the production of L-asparaginase by *Pseudomonas aeruginosa* 50071 has been reported by Abdel-Fattah and Olama, (2002). 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. Production and expression of *Pseudomonas* proteins and growth of *Pseudomonads* by utilizing asparagine has also been reported (Sonawane *et al.*, 2003). Release of L-asparaginase from *E. coli* cells with aqueous two-phase micellar systems (Qin *et al.*, 2003) by using K_2HPO_4 and Triton X-100 has also been reported. The production of L-asparaginase from filamentous fungi (*Aspergillus tamari* and *Aspergillus terreus*) under nitrogen regulation was reported by Sarquis *et al.*, (2004). *Aspergillus 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 in the presence of 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). *Vitreoscilla* hemoglobin in *Erwinia aerogene* and *P. aeruginosa* respond differently to catabolic and oxygen repression for L-asparaginase production (Geckil *et al.*, 2004). Also, the recovery of L-asparaginase from *P. aeruginosa* and *Erwinia aerogenes* by membrane permeabilization by 50 mM potassium phosphate with 1% hexane has been carried out (Geckil *et al.*, 2005). 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 in *Pseudomonas aeruginosa* and its VHb-expressing recombinant strain

(PaJC). Glucose has shown a slight repression 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) fermentation process parameter on the production of L-asparaginase by *Staphylococcus* sp.–6A. They reported that among all fermentation factors the incubation temperature, inoculum level and medium pH were the major influential parameters at the 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 when maltose (1%) and yeast extract (2%) present in the medium (Narayana *et al.,* 2008). The maximum production was reported by 5.93 U $g^{-1}$of dry cell weight. Hymavathi *et al.,* (2009) optimized wide range of SSF parameters to maximize the production of L-asparaginase from isolated *Bacillus circulans* (MTCC 8574) in agricultural waste materials and yield improvement was observed more than 300%. Recently, Sunitha *et al.,* (2010) was investigated 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 in 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 (product yield and cost).

Recently, recombinant L-asparaginase has been also 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 L-asparaginase from *Erwinia 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) host cells resulted in optimum extracellular production in shake flasks (Khushoo *et al.*, 2005). L-asparaginase from *Erwinia chrysanthemi* 3937 has been expressed in *Escherichia coli* BL21 (DE3) pLysS (Kotzia and Labrou, 2005; Kotzia and Labrou, 2007). Cloning and asparaginase expression of the *S. cerevisiae* ASP3 gene in *P. 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 from *H. pylori* CCUG 17874 expressed in *E. coli* BL21 (DE3) and undertaken a functional study of this enzyme. Gladilina *et al.*, (2009) have cloned *Helicobacter pylori* asparaginase, which is characterized by extremely low glutaminase activity. The recombinant enzyme was expressed in *E. coli* cells.

2.6. Properties of L-asparaginase obtained from various microbial sources

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 shown that a definite O_2 absorption rate of 0.4 mm l^{-1} min^{-1} resulted in good growth and high enzyme production. In *Bacillus mesentericus* 43A, the biosynthesis of L-asparaginase was inhibited by the addition of L-aspartic acid in the growing cultures (Eremenko *et al.*, 1976). Comparative experimental evaluation of immuno-depressive and toxic effects of L-asparaginase from *E. coli* and from *Erwinia carotovora* showed

that the L-asparaginase from *E. coli* is more immuno-depressive and immune toxic than that from *E. carotovora* (Cavana *et al.*, 1976). Sulphydryl groups of L-asparaginase from *P. flourescens* were reported to be essential for enzyme activity (Sokolov and Nikolaev, 1976). *E. coli* L-asparaginase has been shown to inhibit the growth of cultured pancreatic cells by Wu *et al.*, (1978). A novel L-asparaginase has been characterized by the red imperfect yeast *Rhodotorula rubra* by growth on media supplemented with L-asparagine, L-glutamine or L-alanine as carbon and nitrogen sources. Enzyme activity was highest during the exponential phase of growth. Optimum pH of the enzyme was 7 and it was activated by Mg^{2+} and inhibited by Fe^{2+} and Pb^{2+} (Foda *et al.*, 1980). L-asparaginase of *Aspergillus nidulans* showed the clearest evidence of O_2 repression under N_2 metabolite derepressed conditions (Shaffer *et al.*, 1988). Four forms of L-asparaginase were isolated from *Tetrahymena pyriformis* (Tsirka and Kyriakidis, 1989) and regulation of enzyme activity was studied. Lipids activated the enzyme and phospholipase-C inactivated the enzyme. L-asparaginase from *Tetrahymena pyriformis* was found in microsomal membranes. The enzyme exhibited an intrinsic phosphorylation activity with a K_m value of 0.5 mM for ATP (Tsrika and Kyriakidis, 1990). The pH optima of purified *Staphylococcal* L-asparaginase were found to be between 8.6 and 8.8 while the temperature optima were 30-32°C. The K_m of the enzyme was found from the Lineweaver Burk plot and was 3-71 times 10^{-2} M of L-asparagine (Sobis and Mickucki, 1991). Two forms of L-asparaginase, L-asparaginase-I and L-asparaginase-II, were extracted and purified from *Thermus thermophilus* by Tsavdaridis *et al.*, (1994). The two forms acted optimally at pH 8.6. An extracellular asparaginase from *Rhodosporidium toruloides* has been reported to be a homodimer having pH optima of 6.35 and temperature optima of 37°C (Ramakrishnan and Chandramohan, 1996). The summarized properties of microbial purified L-asparaginase are shown in Table 2.5.

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

Microbial source	pH optima	Temperature optima (°C)	K_m (M)	Enzyme activity (U/mg)	Antitumor activity	pI	Glutaminase activity (%)	References
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}	--	+	--	--	
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	
Serratia marcescens	6.8	--	1.00×10^{-4}	255	+	5.2	5	Boyd and Phillips, (1971); Stern *et al.*, (1976)
Azotobacter vinelandii	8.6	48	1.10×10^{-4}	622	+	--	--	Gaffer and Shethna, (1977)
Pseudomonas acidovorans	9.5	--	1.50×10^{-5}	86	+	--	147	Davidson *et al.*, (1977)
Tetrahymena pyriformis	8.6	--	2.20×10^{-3}	382	NA	--	0	Triantafillou *et al.*, (1988)
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)
Pseudomonas aeruginosa	9.0	37	1.47×10^{-4}	1900	NA	--	--	El-Bessoumy *et al.*, (2004)
Pseudomonas stutzeri	9.0	37	1.45×10^{-4}	732	+	6.38	0	Manna *et al.*, 1995

NA- data not available

2.7. Purification and characterization of L-asparaginase

L-asparaginase from *Mycobacterium phlei* was purified by fractionation with ammonium sulphate, absorption of contaminating proteins on calcium phosphate gel and chromatography on Sephadex G-150 and DEAE cellulose. The energy of activation 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 by acetone and by column chromatography on DEAE, Sephadex A-50 and Sephadex G-200. Optimum pH was 6 and the enzyme was stable for 10 min. at 50°C. Metal ions, –SH inhibitor, and chelating agents did not show any inhibition or activation of the enzyme (Sakamoto *et al.,* 1977). KSCN, $NaClO_4$ and Triton X- 100 have been used for the solubilization of enzyme purified from *T. pyriformis* (Triantafillou *et al.,* 1988). Asparagine catabolism has been studied in Bryophytes. The purification and characterization of two forms of L-asparaginase, L-asparaginase-I and L-asparaginase-II, obtained from *Sphagnum fallax* was carried out by anion-exchange chromatography (Heeshen *et al.,* 1996). They observed that the pH optimum and molecular weight of the enzyme were 8.2 and 126 kDa, respectively. It had characteristics that were intermediate between those from higher plants and those from microorganisms. L-Asparaginase from *Thermus thermophilus* has a dual L-asparaginase/kinase activity. It was purified and its apparent molecular mass by SDS-PAGE was found to be 33 KDa by Pritsa and Kyriakidis, (2001). The native molecular mass of L-asparaginase from *Thermus thermophilus* was reported hexamer of 200 kDa with pI = 6.0. The optimum pH was found as 9.2 and the K_m for L-asparagine was observed to be 2.8 mM. It is a thermostable enzyme and it 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).

Borkotaky and Bezbaruai, (2002) showed that the L-asparaginase activity from *Erwinia* sp. 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 addition of EDTA. Purification of the enzyme from *Pseudomonas 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 pH 9 when incubated at 37ºC for 30 min. Khushoo *et al.,* 2004 was purified the recombinant L-asparaginase from *E. coli* from the culture supernatant in a single step using Ni–NTA affinity chromatography, which gave an overall yield of 95mg/L of purified protein, with a recovery of 86%. L-asparaginase from *Erwinia 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. The pH-dependence of V_{max} shows that one transition in the acidic pH range with pK_a = 5.4, and the pH-dependence of V_{max}/K_m exhibits two transitions with pK_a =5.4 and 8.5 (Kotzia and Labrou, 2005).

Dhevagi and Poorani, (2006) showed that the 83 fold purification of crude 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 range of 8 to 9 and stable at pH 8. The L-asparaginase showed maximum activity at 60°C and stable upto 50°C. The enzyme has a M_r of 140kDa and cytotoxic effects on lymphoblast cells. The

feasibility and generic applicability of directly integrating conventional discrete operations of cell disruption by high pressure homogenizer and the product capture by *in situ* aqueous two-phase extraction (ATPE) system have been demonstrated for the extraction of 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) reported that the cloning and expression of L-asparaginase from *Erwinia 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 for L-asparagine 0.058 mM, 23.8×10^3 s^{-1} and 411.8×10^3 mM^{-1} s^{-1}, respectively. Abakumova *et al.,* (2009) has been purified L-asparaginase to homogeneity from *Yersinia pesudotuberculosis* and *Erwinia carotovora* by 60% ammonium sulphate followed by sephadex G25 and then anionnite Q sepharoseFF and investigated antitumor activity with various cell lines. Basha *et al.,* (2009) reported that the extracellular L-asparaginase from marine actinomycetes optimum activity at pH 7.5 and 50ºC. The apparent K_m value for the substrate was 25 µM. Mg^{2+} ion slightly stimulated activity while Cu^{2+}, Zn^{2+} and EDTA were inhibitory. The apparent M_r of L-asparaginase from *Erwinia carotovora* under nondenaturing 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 presence of thiols (10–400 µM) showed an increase in V_{max} values (2000, 2223, 2380, 2500, and control 1666.7 µmoles $mg^{-1}min^{-1}$) and a decrease in K_m values (0.086, 0.076, 0.062, 0.055 and control

0.098mM) (Warangkar and Khobragade, 2010). The apparent purified molecular weight of extra-cellular L-asparaginase from *Streptomyces gulbargensis* has been reported 85 kDa and optima pH and temperature for the enzyme were 9.0 and 40°C, respectively (Amena *et al.*, 2010). Recently, 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. This was the first report of the plant containing L-asparaginase with antitumor activity. 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 was soil isolated from *Bacillus circulans* and purified by Prakasham *et al.,* (2010). The M_r of L-asparaginase was found 140 kDa and reported the antineoplastic activity with IC_{50} ~100 U per millions cells.

2.8. L-asparaginase preparations

The search for biological sources to be utilized in large-scale enzyme production, which began after the antitumor activity of L-asparaginase from different microbial and plant species had been discovered, led to the establishment of drug preparations almost exclusively from *E. coli* or *Erwinia chrysanthemi* derived asparaginase. All of the asparaginase preparations, which have been approved and are currently commercially available are derived from various *E. coli* strains or *Erwinia chrysanthemi*. Apart from the native enzymes, PEG-conjugated *E. coli* asparaginases (PEG asparaginases) are used which may exhibit lower immunogenicity than the native forms (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.

2.9. Structure of L-asparaginase

In 1976, Murthy and Knox performed small angle X-ray scattering studies on *E. coli* L-asparaginase solutions, which showed that the enzyme had a radius of gyration of 34.0±0.5 ANG at pH 7. The enzyme was found to be a tetramer with each monomer having a general shape of a prolate ellipsoid. In the same year, Homer and Allsopp 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 wide pH region has been described by Illarionova *et al.,* (1980). Hellman *et al.,* (1983) showed that freeze-drying changed the tetramer structure of the enzyme into monomer. Enzyme L-glutaminase-L-asparaginase purified from *A. 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 reduced in high acidic/alkaline conditions. Danileichenko *et al.,* (1990) studied that the

electron microscopic structure of L-asparaginase from *Erwinia carotovora* 268. The structure of *E. coli* L-asparaginase was studied by Swain *et al.,* (1993) and two domains were observed. Location of the active site was found to be between the N and C terminals. Structure of the enzyme with bound L-aspartate indicated a threonine residue as a catalytic nucleophile (Miller *et al.,* 1993). The tertiary structure and amino acid sequence of L-asparaginase from *Wollinella succinogenes* has been compared with bacterial L-asparaginase by Lubkowski *et al.,* (1996) and a difference in structures was found and sub-specificity was affected due to differences between amino acid sequences. 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. The catalytic role of both Thr15 and Try29 residues of L-asparaginase in bacteria has been confirmed by Aghaiypour, *et al.,* (2001).

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 new 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 A° resolution 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 significant 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 spatial structure of L-asparaginase from *Erwinia 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 confirmation of classification of this group of enzymes in the family of Ntn-hydrolases has been done by Michalska *et al.,* (2006). The (alphabeta) heterodimer has a sandwich-like fold with two beta-sheets flanked by two layers of alpha-helices (alphabetabetaalpha) in common with other Ntn-hydrolases. 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 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 quarternary structure, relative to the apo enzyme. Li *et al.,* (2007) replaced an amino acid by proline (Pro) at a certain hydrogen-bonded turn (β-turn). From the viewpoint of steric structure and stability of entropy, Pro should be a more stable structure, and hydrogen-bonded turns are very important sites for the stability of protein at which proteins change 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 Pro in a predicted β-turn structure while the

enzyme's activity is not affected. Bansal *et al.,* (2010) has been investigated the structural stability of recombinant L-asparaginase of *Pyrococcus furiosus* (*PfA*). *In vitro* unfolding/refolding was studied in the presence of guanidine hydrochloride (GdnCl) and urea. The protein displayed a two-state unfolding transition in the presence of GdnCl and refolded back, regaining 100% activity. Taking coordinates from a recently published structure of a homologous L-asparaginase of *Pyrococcus horikoshii* (PhA) (Yao *et al.,* 2005), a model of *PfA* has been developed.

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). Some drawbacks in analysis are present methods, *e.g*., multistep operation, requirement for the highly toxic reagent and inapplicability for real time, continuous monitoring.

Therefore many attempts have been made by researchers for the assay of L-asparaginase and for monitoring its activity. Also, efficient methods for the assay of L-asparagine have been developed. Methods showing the substrate–enzyme relationship have been devised 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 conjunction with a predialysis unit. Drainas and Drainas, (1985) developed a method to measure the L-asparaginase activity *via* 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 it follows Michaelis kinetics. The direct measurement of L-asparagine in human plasma samples through the use of L-asparaginase by *E. coli* in the soluble form has been a major clinical application of this system. A multi-analyte miniature conductance biosensor using enzymes such as 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 generation 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 (max) at 340–350 nm has been reported by Sheng *et al.,* (1993). Both L-asparaginase and asparagine synthetase activities were measured by this procedure, 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 employed for conversion of L-asparagine into ammonia. An ammonium gas electrode (ISE) was used as the detector. The combination of L-asparaginase in garlic tissue cells and gas electrode responds linearly to L-asparagine concentration. L-

asparaginase from *Erwinia chrysanthemi* was assayed fluorometrically by incubating it with beta L-aspartic acid and measuring the release of 7-amino-4-methylcoumarin (Ylikangas and Mononen, 2000). Rapid analysis of L-asparaginase activity was made by this assay and it can be used for monitoring L-asparaginase activity in the serum of ALL patients during L-asparaginase therapy. Lanvers *et al.,* (2002) developed a sensitive plate reader-based method using 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 later purified by an immobilized metal ion affinity chromatography and its activity was determined by monitoring the change in the ammonia concentration in 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 plasmatic L-asparaginase activity during therapy of acute lymphoblastic leukemia has been described by Orsonneau *et al.,* (2004). The method is easy and requires less time to perform along with having better specificity and precision than the Nessler method. Tsurusawa *et al.,* (2004) demonstrated that the highly sensitive enzyme coupling method to determine the minimum levels of L-asparaginase activity necessary for maintaining asparagine depletion under L-asparaginase treatment in acute lymphoblastic leukemia. It was shown that asparagine levels are strongly correlated with plasma L-asparaginase activity even at low enzyme activities (50 IU/ml). 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. The system was used for L-asparaginase activity assay. In comparison with Nesslerization method, the PPY-MSPQC method is simple, direct, real time, and non-toxic.

2.11. Modification of the enzyme

Finally, polyethylene glycol (PEG) conjugated enzymes have become available which are usually employed in the event of hypersensitivity reactions to the native forms. In order to overcome its usual shortcomings and maintain a therapeutic level of L-asparaginase in the blood of a patient, such methods as physical entrapment and conjugation of the enzyme with water-soluble polymers have been developed to replace frequent-injection therapy (Capizzi *et al.,* 1971). L-asparaginase was successfully conjugated with natural and artificial polymers such as albumin, dextran, polyethylene glycol (PEG) and polyvinyl alcohol (Poznansky *et al.,* 1982; Wileman, 1991; Ashihara *et al.,* 1978 and Nambu, 1986). A very less half-life and high immunogenicity of the native enzyme coerced researchers to modify the enzyme, resulting in its increased half-life and better storage stability. 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. Parrott and Shifrin, (1976) modified the L-asparaginase of *E. coli* with 2, 4, 6–trinitrotoluene for studying sub-unit interactions. 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 had no effect on thermal stability and *pI* of L-asparaginase by *E. coli*. However, pH optimum and catalytic activity were affected (Shprunka *et al.,* 1980). 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 increase in resistance towards proteolysis (Poznansky *et al.,* 1982). The *pI* of the L-asparaginase by *E. coli* increased by succinylation and acetylation (Nickle *et al.,* 1982). Covalent attachment of poly-D-alanine peptides to lysyl residues on the surface of *Erwinia carotovora* L-asparaginase decreased 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 adjuvants 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 greater 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, better storage stability and was resistant to proteolysis (Karsakevich *et al.,* 1986b). Immobilization of the enzyme on soluble LM 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 amount of CM cellulose in the polymer conjugate of the enzyme.

Yoshimoto *et al.,* (1987) chemically modified the enzyme by coupling a magnetic modifier to amino groups of L-asparaginase. The anti-leukemic activity of the enzyme was further improved 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). The anti-leukemic activity of L-asparaginase was studied when it was covalently bound with water-soluble CM-cellulose (Karsakevich *et al.,* 1989) and the activity depended on the amount of polymer bound to the enzyme. Modification of L-asparaginase by acetic anhydride, dextran and monomethoxy PEG has been reported by Cao *et al.,* (1990). Chemical modification by acylation increased the catalytic activity of the enzyme (Martins *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. A BSA-PEG matrix has been found to be very efficient 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. Holle, (1997) reported that PEG modified L-asparaginase was extremely efficient, when given intramuscularly or intravenously it proved to be a good alternative to native L-asparaginase in patients allergic to ALL treatment. PEG and dextran, being macromolecular modifiers, are better than the small molecular modifier acetic anhydride. Fernandes and Gregoriadis (1997) reported that chemical modification by polysialic acid improved therapeutic use by enhancing potentially beneficial anti-tumor activities of L-asparaginase. 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 known to reduce immunogenicity of bacteria enzymes and their blood clearance and to stabilize the catalytic activity of various enzymes. PEG-L-asparaginase or pegaspargase (Oncaspar®, ENZON, Piscataway, NJ, and Rhone-Poulenc Rorer Pharmaceuticals, Collegeville, PA) is a bioconjugation form of L-asparaginase that has been commercially available since 1994.

The modified enzyme had improved functionality. The biologically active fructose polymer levan from *Z. mobilis* was covalently coupled to L-asparaginase from *Erwinia carotovora* and the K_m of the native enzyme and the pH-optima range widened, thermostability increased 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.,* (2002). 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 as well. In polynanospheres, biological activity of L-asparaginase improved but the release profile was altered significantly (Wolf *et al.,* 2003) by co-encapsulation of the stabilizers. Improved biological activity and storage stability was also observed. Zhang *et al.,* (2004a) studied that the immobilization of L-asparaginase on silk particles (Sericin) from *Bombyx mori* silkworm, resulting in covalent binding of the enzyme. It was also reported that the modification by PEG resulted in increased half-life 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). Also, the modified enzyme had more activity than native L-asparaginase. Studies on the effect of PEG-asparaginase on asparagine concentration and serum asparagine activities in cerebrospinal fluid have been observed by Veira *et al.,* (2005), showing that the PEG-modified enzyme had promising therapeutic potential. Rizzari *et al.,* (2006) reported that the adequate plasma enzymatic activity and asparagine depletion was achieved during both exposures to PEG-ASP given as a first-line ASP product during an intensive chemotherapy protocol for pediatric ALL. 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. Zhang *et al.,* (2006) also reported that the covalent attachment of the silk sericin peptides to L-asparaginase producing silk sericin peptides-L-asparaginase (SS-ASNase) bioconjugates that are active, stable, have a lower immune response, and extended half-lives in vitro in human serum. 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 K_m of the modified enzyme was more than five times lower than that of the native L-asparaginase, showing affinity between L-asparaginase and its substrate has increased after bioconjugation. The immunogenicity of the modified L-asparaginase was significantly decreased compared with the native enzyme. Kwon *et al.,* (2009) have been developed an innovative method for encapsulation of therapeutically active L-asparaginase into functionally intact erythrocytes toward enhanced L-asparaginase therapy for ALL. Teodor *et al.,* (2009) obtained nanosize biocompatible materials, with L-asparaginase entrapped, capable to penetrate cells/tissues and deliver L-asparaginase. Recently, Bandgar *et al.,* (2010) synthesized of 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* and increased the thermostability.

2.12. Treatment with L-asparaginase

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. Comparison of L-asparaginase from *Erwinia carotovora* and *E. coli* has been made (Cavana *et al.,* 1976). Enhanced polymerization rate of fibrin monomers or fibrin clottability by L-asparaginase treatment has been reported by Strappinni *et al.,* (1984). The binding of L-asparaginase by *E. coli* to the plasma membrane of normal human mononuclear cells was demonstrated by Mercado-Vianco and Arenas-Diaz, (1999a). There has been a modification of antileukemic treatment by means of injecting doses of *E. coli* L-asparaginase bound to a patient's own isolated immune cells. L-asparaginase in

combination with methotrexate has shown synergistic anti-leukemic activity in a schedule dependent fashion (Aguayo *et al.,* 1999).

In humans, acute lymphoblastic leukemia cell lines have been markedly inhibited by asparaginases. Cell cycle arrest in G1 phases, which results 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 reported 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 effective in nasal type leukemia treatment as well (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). Asparagine levels have an inverse relation with L-asparaginase activity and chemotherapy. L-asparaginase results in decreased asparagine, glutamine and 5 other amino acid levels in pediatric patients with ALL (Grigoryan *et al.,* 2004).

Correlation 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 has been reported, but no such reaction has been found against *Erwinia* L-asparaginase (Avramis and Panosyan, 2005). The view that selectivity to treatment with L-asparaginase is due, at least in part, to lower asparagine synthetase (AS) expression has been supported by studies carried out by Leslie *et al.,* (2006), where they reported that the median asparagine synthetase (AS) mRNA levels were higher in acute myeloid leukaemia (AML) than ALL blasts in both children and adults, with intermediate levels in normal peripheral blood mononuclear cells. The effect of two different L-asparaginase preparations, native *Escherichia coli* L-asparaginase (Crasnitin; Bayer AG, Leverkusen, Germany; n = 10) and L-asparaginase derived from *Erwinia chrysanthemi* (Erwinase; Porton Products, London, UK; n = 10), on the changes in parameters concerning hypercoagulability was evaluated in ALL patients by Appel *et al.,* (2006). A significant decrease in α 2-antiplasmin and plasminogen levels was measured in the L-asparaginase from *E. coli* but not in the Erwinase-treated patients. Results of the Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 for children with acute lymphoblastic leukemia have shown that *Erwinia* is less toxic than *E. coli* asparaginase, but also less efficacious (Moghrabi *et al.,* 2006). Steiner *et al.,* (2006) have reported that the undulating course of ammonia concentrations during L-asparaginase containing induction treatment. It was concluded that ammonia levels may represent a suitable surrogate parameter of L-asparaginase enzyme activity and may enable the monitoring of silent inactivation of L-asparaginase due to the characteristic fluctuation profile. Patients with ALL and prothrombin gene mutation may have a higher risk of clotting complications in comparison 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).

2.13. Side effects

Besides minor side effects such as an allergic reaction and vomiting, L-asparaginase therapy of ALL has some serious side effects. Onset of venous thrombosis in children undergoing histopathologic disease due to ALL therapy has been reported by Sahoo and Hart (2003). L-asparaginase associated hyperlipidemia with hyperviscosity syndrome in a patient with T-cell lymphoblast lymphoma has been reported by Meyer *et al.,* (2003). *Neutropenic enterocolitis* has been observed as an unusual acute complication of neutropenia, associated with leukaemia and lymphoma (Radulovic *et al.,* 2004). Hypersensitivity reactions to chemotherapeutic actineoplastic agents such as L-asparaginase (Rossi *et al.,* 2004) have been reported. Ikarashi *et al.,* (2004) reported that the tubular and glomerular dysfunction due to ALL chemotherapy. Chen *et al.,* (2004) observed that the urethral obstruction was due to L-asparaginase induced pancreatitis during treatment of ALL. Myocardial ischemia has been observed in a patient with acute lymphoblastic leukaemia (Saviola *et al.,* 2004) due to L-asparaginase therapy.

An outburst of acute pancreatitis, called “drug induced pancreatitis”, has been reported by Trivedi and Pitchumoni, (2005). A cerebral thrombotic complication in adolescent leukaemia patients (Imamura *et al.,* 2005) has been attributed to L-asparaginase treatment. Acute hepatic dysfunction (Aoki *et al.,* 2005) and immunodeficiency in children with ALL (Brodtman *et al.,* 2005) have been other major side effects. Ocular complications arise due to L-asparaginase treatment (Foroozan, 2005), but symptoms ease out by discontinuing treatment and carrying

on treatment with heparin. Growth hormone deficiency in children receiving chemotherapy for acute lymphoblastic leukaemia has been reported by Haddy *et al.*, (2006). Growth impairment after cranial radiation can result in diminished adult height. A study on the consequences of L-asparaginase on antithrombin levels in plasma from acute lymphoblastic leukemia patients, HepG2 cells, and plasma and livers from mice treated with this drug has been carried out by Hernandez-Espinosa *et al.*, (2006). They reported that asparaginase treatment induced severe, acquired, and transient type I deficiency of antithrombin (and 1-antitrypsin) with intracellular accumulation of the nascent molecule leading to increase in the risk of thrombosis.

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
	Immunoseparation	
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.	

2.14. Resistance to L-asparaginase

A cell line resistant to L-asparaginase expressed high levels of asparagine synthetase activity as reported by Andrulis *et al.,* (1990). This was due to increased expression but without amplification of the genes encoding asparagine synthetase. Holleman *et al.,* (2003) reported that the drug resistance in ALL is associated with impaired ability of cells to induce apoptosis. Also, PARP and Procaspase-2 expression is related to drug resistance in childhood ALL. Resistance to L-asparaginase results in a decreased ability 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 associated 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. ALL patients have been found resistant to chemotherapeutic agents such as anthracyclines, vincristine and asparagine.

An *in vitro* chemosensitivity assay is a good indicator of cellular response to chemotherapy (Arriffin *et al.,* 2005). In rat serum cells and in ARJ cells, L-asparaginase treatment depletes cellular asparagine; also, cellular glutamine levels have been found to be severely reduced along with a marked decrease in the activity of glutamine synthetase (GS). The inhibition of GS in the presence of L-asparaginase triggers apoptosis (Rotoli *et al.,* 2005). GS may thus form a target for the suppression of a L-asparaginase resistant phenotype. Krejci *et al.,* (2005) reported that the up regulation of asparagine synthetase does not avert 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 identified. 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 provide 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 may be targeted for studying L-asparaginase resistance (Ding and Broome, 2005). Fine *et al.*, (2005) also described that L-asparaginase resistance can be studied by targeting epigenetic changes. Li *et al.*, (2006) have reported that the down regulation of asparagine synthetase expression can increase the sensitivity of cells resistant to L-asparaginase.

2.15. L-Asparaginase - a subject of considerable medical interest and persistent research

L-asparaginase has been a major research subject for many researchers worldwide. Its chemotherapeutic potential in treating acute lymphoblastic leukemia and lymphosarcoma has been one of the most eminent discoveries of modern times. Its therapeutic potential is now well established, as it has remarkably induced remission in most of the patients suffering with ALL. A comparative examination of preparations of *E. coli* L-asparaginase produced in the USSR, Germany and Japan was made by Kondrat Eva, (1984) and it revealed that the clinical characteristics of the preparation made in the USSR and the preparation made in Germany (crasnitin) 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 and involved in astroglial production of the neuroactive amino acid L-aspartate, has been identified and characterized by Dieterich *et al.,* (2003). An innovative way of eliminating cancer-causing acrylamide from bread has been developed by researchers of the 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 a strain of asparagine busting L-asparaginase from food-grade *Aspergillus niger*. The resulting strain was fermented and purified before being added to the dough. This resulted in a considerable decrease in acrylamide levels (Dunn, 2004). A study by Taeymans *et al.,* (2005) has shown that the application of L-asparaginase in the food industry, where it is being used to determine acrylamide presence 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 stuffs from *Aspergillus niger* and *Aspergillus oryzae* recombinant strains. In Australia and New Zealand, Novozyme has applied to use the asparaginase enzyme as a processing aid with the intention to supply the food industry, according to Food Standards Australia and New Zealand. Asparaginase is produced using DNA techniques from a strain of the host micro-organism *Aspergillus oryzae*, and converts asparagine to aspartic acid to reduce acrylamide formation in baked or fried wheat dough based products.

A chimeric enzyme, AnsB-TTP-CETPC, comprising asparaginase, tetanus toxin helper T cell epitope and human CETP B cell epitope has been expressed as a soluble protein in *Escherichia coli* (Gaofu *et al.,* 2006). The purified chimeric enzyme exhibited approximate 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 future 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. The research being carried out on L-asparaginase may only be the tip of the iceberg. Although, much has been unraveled, it appears that there is still a long way to go in exploring this amazing enzyme.

CHAPTER 3
MATERIALS AND METHODS

3.1. Chemicals and reagents

Chemicals and reagents used in the medium development study were of analytical grade and obtained from HiMedia Company, India. Most of the chemicals used in the study of specificity (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, N-α-acetyl-L-asparagine), effectors (EDTA, Iodoacetamide, L-Cystine, L-Histidine, Glutathione, 2-Marceptoethanol, *p*-Chloromercuribenzoic acid (*p*CMBA), purification (chromatographic matrixes) and antitumor activity (Cell culture plates, culture media, and other related chemicals) were procured from Sigma-Aldrich (Bangalore, India). Chemicals and markers used in native PAGE and SDS PAGE were obtained from Bangalore Genei, India. HPLC analysis of L-asparagine was performed with Merck HPLC grade chemicals and solvents. All other chemicals used in protein and glucose analysis were of analytical grade and of the highest purity available in the local market.

3.2. Microorganisms

Serratia marcescens NCIM 2919 and *Wautersia eutropha* NRRL B 2804 were obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India and Agricultural Research Service Culture Collection, Illinois, USA, respectively. *Serratia marcescens* MTCC 97 and *P. carotovorum* MTCC 1428 were obtained from Microbial Type

Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh, India. Nine bacterial strains were isolated from soil samples collected from the premises of an amino acid packaging industry, Himalaya Herbs, Saharanpur, Uttar Pradesh, India for the production of L-asparaginase. All strains were maintained on a medium containing (g l^{-1}): beef extract 1.0, yeast extract 2.0, NaCl 5.0, peptone 5.0 and agar 15.0 (pH 7.0) at 30°C. The organisms were sub-cultured every month and stored at 4±1°C.

3.3. Isolation of microorganisms from soil and identification

Soil samples were diluted appropriately in sterile saline solution (0.9% w/v) and plated onto L-asparagine containing pH indicator phenol red plate (Gulati *et al.*, 1997) and incubated at 30ºC. After 36 h, the isolates grown on plates were streaked on another phenol red plates to get single colony of isolate and screened for potential L-asparaginase producers. Among all the isolated strains, highest level of L-asparaginase producer (designated as SK-07) was selected for further studies. SK-07 was identified based on 1.5 kb of 16S rRNA fragment amplification using consensus primers (Bangalore Genei, India). Forward and reverse sequences thus obtained were aligned to produce a high quality consensus sequence. Related sequences were retrieved from Ribosomal Database Project (RDP II release 9) using blast programme. A phylogenetic tree was constructed using MEGA 3.1 software based on the 16S rRNA sequence of 10 strains close to isolated strain.

3.4. Cultivation medium and culture conditions

The production of L-asparaginase 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 30ºC and 180 rpm in an orbital shaking incubator for 10-12 h (Optical density (OD) at 600nm = 0.6~0.8). A 2% of inoculum from the above seed culture was added to 50 ml of the medium in 250 ml Erlenmeyer flasks and incubated in an orbital shaking incubator at 30°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

Samples were collected at different intervals of time and centrifuged at 10000*g* for 10 min at 4±1ºC. The cells were separated, washed with 0.05 M Tris-HCl buffer (pH 8.6) 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 (Model VC 505 Microprocessor based cell Ultrasonic processor, Sonics & Materials Inc, CT, USA) at 20 MHz, 35% amplitude, 4 cycles (2 min per cycles with 1.5 s and 0.5 s off) and the contents were centrifuged at 20000*g* for 10 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, made in to three equal parts and SDS, Triton X100 and EDTA were added to each part individually. The final concentration of SDS, Triton X100 and EDTA were 0.001% (w/v), 1.0% (v/v), and 5 mM, respectively in the samples of cell debris. The cell debris were ultrasonicated and incubated in a shaking incubator

at 180 rpm and 25°C for 30 min. The samples were ultracentrifuged at 100000*g* for 30 min (4±1°C) and the activity of membrane bound L-asparaginase was measured in the supernatant (Triantafillou *et al.*, 1988; Geckil *et al.*, 2005). The second half of the pellet 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 10000*g* and 4±1ºC for 10 min. The supernatant fluid was removed and the well drained 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 shock treated cells were removed from the fluid by centrifugation at 20000*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 10000*g* for 10 min at 4±1ºC and washed twice with 0.05 M Tris-HCl buffer (pH 8.6) and ultrasonicated (Model VC 505 Microprocessor based cell Ultrasonic processor, Sonics & Materials Inc, CT, USA) at 20 MHz, 35% amplitude, 4 cycles (2 min per cycles with 1.5 s on and 0.5 s off). The contents were centrifuged at 20000*g* for 10 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 and the latter can react with the Nessler's reagent to produce an orange product. The enzyme assay mixture consisted of 900 µl of L-asparagine (100 mM) in Tris

HCl buffer (pH 8.6) 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 10000*g* for 5 min to remove the precipitates and the ammonia released in the supernatant was determined colorimetrically by adding 100 µl Nessler reagent into sample containing 100 µl supernatant 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 blanks that received TCA before the addition of cell suspension. 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 = 14.036 U ml^{-1}). L-glutaminase activity was determined as described above for L-asparaginase activity using modified method of Mashburn and Wriston, (1964). One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 µmol of ammonia per min at 37°C. Specific activity is expressed as units per milligram of protein. In some cases, particularly during studies with modifiers, the L-asparaginase activity was measured as a function of hydroxamate formation (hydroxylaminolysis reaction) through a colorimetric couple reaction with $FeC1_3$ (Drainas *et al.*, 1977).

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 1ml 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 15 min. Then 100 µl of freshly prepared diluted Folin reagent with distilled water (1:1) was added

and vortexed and kept in the dark at room temperature for 30 min. Blue color was developed and OD at 660 nm was measured against the blank (no crude enzyme, 200 µl distilled water). 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 = 0.370 mg ml^{-1} of protein).

3.6.3. Dry cell weight (DCW)

For standard curve of DCW, cells were centrifuged at 10000*g* for 10 min at 4±1ºC and discarded the supernatant. The pellet was resuspended with same volume of distilled water and centrifuged at 10000*g* for 10 min at 4±1ºC. Twice washed with distilled water and supernatant free cells were used to determine the DCW. Different dilution of cell samples were used for measuring cell OD (~0.1-1.0) at 600 nm and corresponding DCW determined at 105°C for 24 h. DCW of the unknown sample was determined by measuring the OD of the culture broth at 600 nm using UV-visible spectrophotometer and compared with standard curve between OD at 600 nm vs. DCW (1 unit OD at 600 = 0.272 g l^{-1} DCW).

3.6.4. Estimation of glucose

Glucose concentration in the media 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 0.2-1.0 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 95ºC for 10 min to develop the red-brown color. Then 1 ml of 40% (w/v) potassium sodium tartrate solution was added to stabilize the color. Absorbance was measured at 540 nm using UV-visible spectrophotometer after cooling to room temperature

against blank (no glucose, 500 µl distilled water). The glucose concentration in the reaction was determined based on a standard curve obtained with glucose (0-1.0 mg/ml) as standard (1 unit OD at 540 = 0.636 mg ml^{-1} of glucose).

3.6.5. Estimation of L-asparagine

L-asparagine concentration was estimated using HPLC (Varian, USA). Reversed phase C-18 HPLC column (PTH amino acid column, Hypersil gold, 5µl particle size, 250 × 4.6 mm, Thermo Ltd. USA) was used with UV (263nm) and Fluorescence (exc. 263 nm, emm. 313 nm) detection system using 9-fluorenylmethyl chloroformate chloride (FMOC-Cl) derivatisation method (Haynes *et al.*, 1991). The L-asparagine concentration in the sample was determined based on a standard curve obtained with L-asparagine concentration (10-500 µM) *vs*. peak area as standard. L-asparagine sample was diluted in range of 10-500 µM with boric acid buffer (pH 8.5, 200 mM). 5µl of amino acid solution was mixed with 5µl derivatization reagent FMOC-Cl (16 M) in acetonitrile and allowed to react for 2 min. Then 5µl cleavage reagent (0.85 M NaOH: 0.5 M Hydoxylamine HCl: 2-methylthioethanol ≡ 36:15:1) was added and allowed to react for 4 min. Then 5 µl of quenching reagent (Acetonitrile: HPLC water: acetic acid ≡ 20:3:2) was added and mixed well. Injected the sample into the column and L-asparagine peak was detected using UV (263nm) and Fluorescence (exc. 263nm, emm. 313nm) detection system at ~6.3 min in chromatogram. Mobile phase was contained HPLC water (A) and acetonitrile (B) with 0.1% trifluoroacetic acid (TFA) and 0.015% triethylamine (TEA) at flow rate of 1 ml min^{-1}. A linear gradient was applied for 10 min from mobile phase A to B as follows: 0-2 min for 7% of B; 2-7 min for 17% of B; 7-10 min for 35% of B.

3.7. Effect of various carbon sources with or without L-asparagine on L-asparaginase production from *P. carotovorum* MTCC 1428

In order to select the most suitable substrate(s) for the maximum production of L-asparaginase from *P. carotovorum* MTCC 1428, experiments were conducted with various carbon sources (glucose, sucrose, sorbitol, starch, lactose) with or without L-asparagine. The medium contained basal semisynthetic medium (carbon source (2.0 g l^{-1}), with or without L-asparagine (3.0 g l^{-1})).

3.8. Effect of initial concentration of glucose and L-asparagine on L-asparaginase production from *P. carotovorum* MTCC 1428

The effect of initial concentration(s) of glucose and/or L-asparagine on the production of L-asparaginase was studied. The effect of initial glucose concentration was investigated by keeping L-asparagine concentration at a predetermined level (1.5 g l^{-1}) and by varying the concentration of glucose from 1 to 10 g l^{-1}. Similarly, experiments were performed at optimal level of glucose concentration (2 g l^{-1}) obtained from the above experiment and by varying L-asparagine concentration from 1 to 10 g l^{-1}.

3.9. Role of nitrogen sources on the production of L-asparaginase from *P. carotovorum* MTCC 1428

A 2^3 full factorial design was applied to evaluate the significance of three different nitrogen sources (L-asparagine, yeast extract and peptone) on the production of L-asparaginase. The range and the levels of the variables investigated in this study are given in Table 3.1. A total of 8

set of experiments were employed in this study (in duplicates) to evaluate the significance of nitrogen sources on the production of L-asparaginase (Table 3.2).

Table 3.1. Experimental range and levels of the independent variables

Variables (Symbol coded)	Coded low level	Corresponding low level value (g l^{-1})	Coded high level	Corresponding high level value (g l^{-1})
L-asparagine (A)	-1	0.00	+1	3.00
Yeast extract (Y)	-1	0.00	+1	1.00
Peptone (P)	-1	0.00	+1	1.00

Table 3.2. Experimental design matrix of 2^3 full factorial design

Run	Code level		
	L-asparagine (A)	Yeast extract (Y)	Peptone (P)
1	+1	+1	+1
2	-1	-1	-1
3	+1	-1	+1
4	-1	+1	-1
5	-1	-1	+1
6	-1	+1	+1
7	+1	+1	-1
8	+1	-1	-1

3.10. Optimization of chemical and physical process parameter for the production of L-asparaginase from *P. carotovorum* MTCC 1428

3.10.1. Screening of medium components by Plackett-Burman experimental design

The Plackett-Burman experimental design was applied to screen the significantly influencing medium components to maximize the production of L-asparaginase from *P. carotovorum* MTCC 1428 (Plackett and Burman, 1946). Total of nine parameters *viz.*, L-asparagine,

glucose, $Na_2HPO_4.2H_2O$, KH_2PO_4, NaCl, $MgSO_4.7H_2O$, $CaCl_2$, yeast extract and peptone have been considered for screening experiment. Each variable is represented at two levels, *i.e*., a high (+) and low (-). According to Plackett–Burman experimental design a total of 12 experiments were performed. The levels of variables and design matrix in the coded levels and real values are shown in the Table 3.3 and Table 3.4, respectively. Plackett–Burman (PB) experimental design is based on the first order polynomial model.

$$Y = \beta_0 + \sum \beta_i X_i \quad (3.1)$$

Where, Y is the response (enzyme activity), β_o is the model intercept and β_i is the linear coefficient, and X_i is the level of the independent variable. The significance of each variable was determined using Student's t-test by statistical software package, MINITAB® Release 15.1, PA, USA. All experiments were conducted in duplicates and averages of the results were taken as the response.

Table 3.3. Experimental variables at different levels used for the production of L-asparaginase by *P. carotovorum* MTCC 1428 using PB design

Variables	Symbol code	Experimental values (g l^{-1})		
		Lower (-1)	Medium (0)	Higher (+1)
Glucose	X_1	1.00	3.00	5.00
L- Asparagine	X_2	3.00	5.00	7.00
$Na_2HPO_4.2H_2O$	X_3	1.00	6.00	11.00
KH_2PO_4	X_4	0.50	3.00	5.50
NaCl	X_5	0.00	0.50	1.00
$MgSO_4.7H_2O$	X_6	0.00	0.50	1.00
$CaCl_2.2H_2O$	X_7	0.00	0.015	0.03
Yeast extract	X_8	0.25	1.00	1.75
Peptone	X_9	0.25	1.00	1.75

Table 3.4. PB design matrix in coded units and real values in parenthesis

Run Order	Coded and uncoded values								
	Glucose ***(X_1)***	**L-asparagine** ***(X_2)***	**$Na_2HPO_4.2H_2O$** ***(X_3)***	**KH_2PO_4** ***(X_4)***	**NaCl** ***(X_5)***	**$MgSO_4.7H_2O$** ***(X_6)***	**$CaCl_2.2H_2O$** ***(X_7)***	**Yeast extract** ***(X_8)***	**Peptone** ***(X_9)***
1	1(5.0)	-1(3.0)	1(11.0)	-1(0.5)	-1(0)	-1(0)	1(0.03)	1(1.75)	1(1.75)
2	1(5.0)	1(7.0)	-1(1.0)	1(5.5)	-1(0)	-1(0)	-1(0)	1(1.75)	1(1.75)
3	-1(1.0)	1(7.0)	1(11.0)	-1(0.5)	1(1.0)	-1(0)	-1(0.0)	-1(0.25)	1(1.75)
4	1(5.0)	-1(3.0)	1(11.0)	1(5.5)	-1(0)	1(1.0)	-1(0)	-1(0.25)	-1(0.25)
5	1(5.0)	1(7.0)	-1(1.0)	1(5.5)	1(1.0)	-1(0)	1(0.03)	-1(0.25)	-1(0.25)
6	1(5.0)	1(7.0)	1(11.0)	-1(0.5)	1(1.0)	1(1.0)	-1(0)	1(1.75)	-1(0.25)
7	-1(1.0)	1(7.0)	1(11.0)	1(5.5)	-1(0)	1(1.0)	1(0.03)	-1(0.25)	1(1.75)
8	-1(1.0)	-1(3.0)	1(11.0)	1(5.5)	1(1.0)	-1(0)	1(0.03)	1(1.75)	-1(0.25)
9	-1(1.0)	-1(3.0)	-1(1.0)	1(5.5)	1(1.0)	1(1.0)	-1(0)	1(1.75)	1(1.75)
10	1(5.0)	-1(3.0)	-1(1.0)	-1(0.5)	1(1.0)	1(1.0)	1(0.03)	-1(0.25)	1(1.75)
11	-1(1.0)	1(7.0)	-1(1.0)	-1(0.5)	-1(0)	1(1.0)	1(0.03)	1(1.75)	-1(0.25)
12	-1(1.0)	-1(3.0)	-1(1.0)	-1(0.5)	-1(0)	-1(0)	-1(0)	-1(0.25)	-1(0.25)

X_1-X_9 are mentioned in g l^{-1}

3.10.2. Optimization of the screened chemical parameters by Central Composite Design (CCD)

The CCD was applied to optimize the levels and explain the combined effect of the screened medium constituents, *viz.* L-asparagine, glucose, KH_2PO_4 and $MgSO_4.7H_2O$ on the production of L-asparaginase from *P. carotovorum* MTCC 1428 (Khuri and Cornell, 1987). Each variable (medium component) was assessed at five coded levels (-2, - 1, 0, +1, and +2) with 30 ($=2^k + 2k + 6$) treatment combinations, where k is the number of independent variables (Araujo and Brereton, 1996). The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded form is presented in Table 3.5 and Table 3.6, respectively. A total number of 30 experiments were conducted. Twenty four experiments were augmented with six replications at the center points to evaluate the error in the experiment.

Table 3.5. Ranges and levels of the independent variables used in the optimization study

Variables	**Symbol**	**Range and levels**				
	coded	**-2**	**-1**	**0**	**+1**	**+2**
Glucose	X_1	0.50	2.00	3.50	5.00	6.50
L-Asparagine	X_2	1.00	3.00	5.00	7.00	9.00
KH_2PO_4	X_4	0.50	2.00	3.50	5.00	6.50
$MgSO_4.7H_2O$	X_6	0.05	0.30	0.55	0.80	1.05

Data was fitted to a nonlinear Eq 3.2 using regression method for predicting the optimal levels.

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

Table 3.6. A 2^4 full-factorial central composite design matrix in coded units and real values (parenthesis).

Run Order	Coded and Uncoded levels			
	Glucose ***(X_1)***	**L-asparagine** ***(X_2)***	**KH_2PO_4** ***(X_4)***	**$MgSO_4.7H_2O$** ***(X_6)***
1	-1 (2.0)	-1 (3.0)	-1 (2.0)	-1 (0.30)
2	-1 (2.0)	1 (7.0)	-1 (2.0)	-1 (0.30)
3	1 (5.0)	-1 (3.0)	-1 (2.0)	-1 (0.30)
4	1 (5.0)	1 (7.0)	-1 (2.0)	-1 (0.30)
5	-1 (2.0)	-1 (3.0)	1 (5.0)	-1 (0.30)
6	-1 (2.0)	1 (7.0)	1 (5.0)	-1 (0.30)
7	1 (5.0)	-1 (3.0)	1 (5.0)	-1 (0.30)
8	1 (5.0)	1 (7.0)	1 (5.0)	-1 (0.30)
9	-1 (2.0)	-1 (3.0)	-1 (2.0)	1 (0.80)
10	-1 (2.0)	1(7.0)	-1 (2.0)	1 (0.80)
11	1 (5.0)	-1 (3.0)	-1 (2.0)	1 (0.80)
12	1 (5.0)	1 (7.0)	-1 (2.0)	1 (0.80)
13	-1 (2.0)	-1 (3.0)	1 (5.0)	1 (0.80)
14	-1 (2.0)	1 (7.0)	1 (5.0)	1 (0.80)
15	1 (5.0)	-1 (3.0)	1 (5.0)	1 (0.80)
16	1 (5.0)	1 (7.0)	1 (5.0)	1 (0.80)
17	0 (3.5)	0 (5.0)	0 (3.5)	0 (0.55)
18	0 (3.5)	0 (5.0)	0 (3.5)	0 (0.55)
19	0 (3.5)	0 (5.0)	0 (3.5)	0 (0.55)
20	0 (3.5)	0 (5.0)	0 (3.5)	0 (0.55)
21	0 (3.5)	-2 (1.0)	0 (3.5)	0 (0.55)
22	0 (3.5)	2 (9.0)	0 (3.5)	0 (0.55)
23	-2 (0.5)	0 (5.0)	0 (3.5)	0 (0.55)
24	2 (6.5)	0 (5.0)	0 (3.5)	0 (0.55)
25	0 (3.5)	0 (5.0)	-2 (0.5)	0 (0.55)
26	0 (3.5)	0 (5.0)	2 (6.5)	0 (0.55)
27	0 (3.5)	0 (5.0)	0 (3.5)	-2 (0.05)
28	0 (3.5)	0 (5.0)	0 (3.5)	2 (1.05)
29	0 (3.5)	0 (5.0)	0 (3.5)	0 (0.55)
30	0 (3.5)	0 (5.0)	0 (3.5)	0 (0.55)

X_1, X_2, X_4 and X_6 are mentioned in g l^{-1}

Where, Y is the predicted response, k is the number of factor variables. X is the coded levels of the independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the ii^{th} quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient. 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.

3.10.3. Optimization of the process parameters by CCD

Initial pH of medium, temperature, rpm of the shaking incubator and inoculums size were considered in the present optimization process by considering production of L-asparaginase (specific activity) by *P. carotovorum* MTCC 1428 as the response. According to CCD, the total number of treatment combinations was 30 ($=2^k + 2k + 6$), where, k is the number of independent variables (Araujo and Brereton, 1996). The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded form is shown in Table 3.7 and Table 3.8, respectively. Twenty four experiments were augmented with six replications at the center points to evaluate the pure error.

Table 3.7. Experimental codes, ranges and levels of the independent variables for response surface methodological experiment

Variables	**Symbol used**	**Range and levels**				
		-2	**-1**	**0**	**+1**	**+2**
Initial pH	*A*	6.0	6.5	7	7.5	8.0
Temperature (°C)	*B*	24	28	32	36	40
Agitation (rpm)	*C*	100	140	180	220	260
Inoculums size (%, v/v)	*D*	1	2	3	4	5

Table 3.8. A 2^4 full-factorial CCD matrix in real values and coded units (in parenthesis)

Run Order	Uncoded and Coded levels			
	A	***B***	***C***	***D***
1	6.5 (-1)	28 (-1)	140 (-1)	2 (-1)
2	7.5 (+1)	28 (-1)	140 (-1)	2 (-1)
3	6.5 (-1)	36 (+1)	140 (-1)	2 (-1)
4	7.5 (+1)	36 (+1)	140 (-1)	2 (-1)
5	6.5 (-1)	28 (-1)	220 (+1)	2 (-1)
6	7.5 (+1)	28 (-1)	220 (+1)	2 (-1)
7	6.5 (-1)	36 (+1)	220 (+1)	2 (-1)
8	7.5 (+1)	36 (+1)	220 (+1)	2 (-1)
9	6.5 (-1)	28 (-1)	140 (-1)	4 (+1)
10	7.5 (+1)	28 (-1)	140 (-1)	4 (+1)
11	6.5 (-1)	36 (+1)	140 (-1)	4 (+1)
12	7.5 (+1)	36 (+1)	140 (-1)	4 (+1)
13	6.5 (-1)	28 (-1)	220 (+1)	4 (+1)
14	7.5 (+1)	28(-1)	220 (+1)	4 (+1)
15	6.5 (-1)	36 (+1)	220 (+1)	4 (+1)
16	7.5 (+1)	36 (+1)	220 (+1)	4 (+1)
17	7.0 (0)	32 (0)	180 (0)	3 (0)
18	7.0 (0)	32 (0)	180 (0)	3 (0)
19	7.0 (0)	32 (0)	180 (0)	3 (0)
20	7.0 (0)	32 (0)	180 (0)	3 (0)
21	6.0 (-2)	32 (0)	180 (0)	3 (0)
22	8.0 (+2)	32 (0)	180 (0)	3 (0)
23	7.0 (0)	24 (-2)	180 (0)	3 (0)
24	7.0 (0)	40 (+2)	180 (0)	3 (0)
25	7.0 (0)	32 (0)	100 (-2)	3 (0)
26	7.0 (0)	32 (0)	260 (+2)	3 (0)
27	7.0 (0)	32 (0)	180 (0)	1 (-2)
28	7.0 (0)	32 (0)	180 (0)	5 (+2)
29	7.0 (0)	32 (0)	180 (0)	3 (0)
30	7.0 (0)	32 (0)	180 (0)	3 (0)

The relationship among the variables, *i.e.* initial pH of medium, temperature, rpm of the shaking incubator and incoulum size 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 (enzyme production), β_0 the constant coefficient, X_i (i = 1–4) are non-coded variables (initial pH of medium - A, temperature - B, agitation speed - C and inoculums size - D), β_i are the linear, β_{ii} are the quadratic, and β_{ij} (i and j = 1–4) 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.

3.10.4. Validation of the model at predicted optimum levels of chemical and physical process parameters

In order to validate the model, experiments were performed at optimal levels of the most significant variables and at middle level of other medium components both in shake flask and a 7 L bioreactor (Biostat B plus, Sartorius, Germany). The medium was inoculated with 2% inoculum and the bioreactor was operated at 30°C, 200 rpm and 1.5 vvm with uncontrolled pH. Similarly, experiment for model validation was performed at optimal levels of physical process parameters in pre-optimized medium in shake flask and bioreactor. The bioreactor was operated at optimal levels of physical process parameters and at aeration rate of 1.5 vvm with uncontrolled pH. Samples were collected at regular interval of time and specific activity was measured (in duplicates). All experiments in shake flask were conducted in duplicates and averages of the results were taken as response.

3.11. Isolation and purification of L-asparaginase produced from *P. carotovorum* MTCC 1428

3.11.1. Enzyme production for purification

The production of L-asparaginase was performed in the optimized semisynthetic medium containing (g l^{-1}): Glucose 5.202; L-asparagine 2.076; $Na_2HPO_4.2H_2O$ 6.0; KH_2PO_4 1.772; NaCl 0.5; $MgSO_4.7H_2O$ 0.373; $CaCl_2.2H_2O$ 0.015; yeast extract 1.0, peptone 1.0 with initial pH of 6.5 (Kumar *et al.,* 2009). The inoculum was prepared into 80 ml of sterile medium mentioned above containing glucose as the sole carbon source in a 500 ml Erlenmeyer flask as described in Section 3.4. The inoculated culture was transferred (2 % v/v) in a stirred tank fermenter – Sartorius Biostat B plus, Germany with a working volume of 4 l. The reactor was operated under uncontrolled pH, at a stirrer speed of 200 rpm, aeration rate of 1.5 vvm and 30°C. After 12 h fermentation, cells were centrifuged and washed at 10,000*g* for 10 min at 4±1ºC with 0.05 M Tris-HCl buffer (pH 8.6) and resuspended in the same buffer. The washed cells suspension was used as the crude enzyme preparation as described method in section 3.6.1 and clear supernatant obtained was used as the enzyme source.

3.11.2. Isolation and purification

All isolation and purification steps were carried out at 0-4°C unless otherwise indicated and all chromatographic runs in the study were monitored for protein at 280 nm. Isolation and purification steps are summarized in the following flowchart (Fig. 3.1).

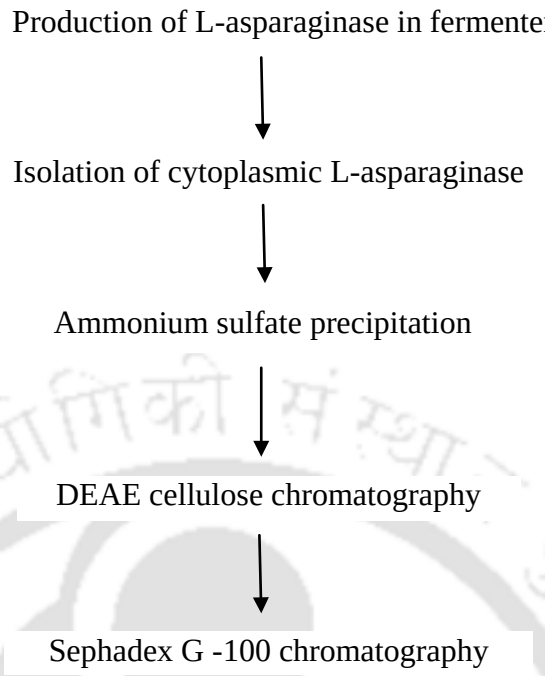


Fig. 3.1. Flow chart of the isolation and purification steps involved in downstream of intracellular L-asparaginase from *P. carotovorum* MTCC 1428.

a) Ammonium sulfate precipitation

Finely powdered ammonium sulfate was added to the clear supernatant obtained after ultrasonication. L-asparaginase activity was associated with the fraction precipitated at 60-80% saturation, with constant stirring and left overnight at refrigerated conditions. The precipitate was collected by centrifugation at 10,000*g* for 30 min, dissolved in minimal amount of 50mM Tris HCl buffer (pH 8.6) and dialyzed against the same buffer for 24 hr.

b) DEAE cellulose chromatography

Dialyzed ammonium sulphate fraction was loaded on diethylaminoethyl (DEAE) cellulose column pre-equilibrated with Tris HCl buffer (50mM, pH 9.6) at a flow rate of 1 ml min^{-1}. The column was washed with 2 column volume of the above buffer and the adsorbed protein was eluted using linear gradient of KCl (0-200 mM) in Tris HCl buffer (50mM, pH 8.6).

Active fractions obtained after the ion exchange chromatography were pooled separately, dialyzed against the same buffer and concentrated using lyophilizer.

c) Sephadex G-100 chromatography.

The DEAE concentrated fractions were further purified on gel filtration chromatography using Sephadex G -100. Sample obtained from above step was loaded into pre-equilibrated column with Tris HCl buffer (50mM, pH 8.6). The protein elution was done with the same buffer containing 100 mM NaCl at a flow rate of 0.2 ml min^{-1}. The active fractions were pooled, concentrated and dialyzed against the Tris HCl buffer (50mM, pH 8.6). This concentrated fraction was stored at -20°C till further usage for characterization.

3.12. Characterization of purified L-asparaginase from *P. carotovorum* MTCC 1428

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

The optimum pH for L-asparaginase activity was determined under the assay condition over a pH range of 5.5-10.5. For pH stability determination, enzyme preparations were incubated at pH values in the range of 5.5-10.5 for 24 h at 4 °C in the absence of L-asparagine and residual activity was determined.

3.12.2. Effect of temperature on activity of purified enzyme

The optimum temperature range for enzyme activity was determined by incubating the assay mixture at different temperatures ranging from 30 to 60°C. Enzyme preparations were incubated at temperatures in the range of 30-60°C at different pH 7.6, 8.6 and 9.6, for 5 h at optimum pH, and residual activity was determined by Nessler's method at pH 8.6 and 37°C.

3.12.3. Effect of various effectors on L-asparaginase activity

The enzyme activity was determined in the presence of different modifiers (metal ions, Iodoacetamide, *p*CMBA, 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 then measured. 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+}, Sn^{2+}, Pb^{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.

3.12.4. Substrate specificity

The L-asparaginase activity 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 methodology described in section 3.12.3.

3.12.5. Kinetic parameters

The Michaelis constant (K_m), maximal velocity (V_{max}) and Turnover numbers (k_{cat}) of the purified enzyme were determined using L-asparagine as substrate in the range of 0.05 to 2.0 mM. Each 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 36.105 kDa subunit by MALDI-TOF MS.

3.12.6. Electrophoresis

Native PAGE of the purified L-asparaginase was performed using 7.5 % polyacrylamide gel in glycine buffer at 5±1ºC as described by Gallagher, (1999). SDS-PAGE was performed 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 3 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 was determined using standard molecular weight markers in SDS–PAGE and Native PAGE respectively.

3.12.7. N-terminal sequencing of the L-asparaginase

Semi dry electro-blotting of the purified L-asparaginase from unstained SDS–PAGE gel on to poly-vinyl difluoride (PVDF) membrane was carried out at constant voltage of 20 V (5.5 mA $(cm^2)^{-1}$ of gel) for 30 min at room temperature. The membrane was removed from the semidry trans-blotting sandwich and saturated with 100% methanol followed by staining with Coomassie Brilliant Blue R-250. The blotted membrane was de-stained with 50% methanol and the band of L-asparaginase was excised. N-terminal sequencing was determined by Edman degradation sequencing of the protein using an Applied Biosystems Procise Sequencer (Biochemistry Protein Sequencing facility, IISC, Bangalore).

3.12.8. Molecular mass determination

The native molecular weight of purified enzyme was determined by gel filtration chromatography through a column (1 cm × 50 cm) of sephadex G-100 by the method of Andrew, (1964). The column was calibrated with blue dextran and standard moleculaer marker proteins. The elution volume (*Ve*) of each marker protein and void volume (V_o) of the

column were estimated. A plot of (Ve/V_o) against log M_r was used to find the native weight (M_r) of L-asparaginase. The intact molecular mass was also determined by matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF), using Bruker Ultraflex MALDI-TOF/TOF mass spectrometer equipped with a 337 nm nitrogen laser (The Centre for Genomic Application, New Delhi). The matrix was prepared in deionized water containing sinapinic acid (10 mg ml^{-1}), 50% acetonitrile and 0.1% TFA. L-asparaginase was mixed with matrix (1:1) and 2 µl of the sample was spotted on plate, dried at room temperature and analyzed.

3.12.9. Estimation of pI

The isoelectric focusing polyacrylamide gel electrophoresis (IEF–PAGE) was performed in a vertical gel apparatus using ampholytes (Bio-Rad) of the pH range 3-10. Approximately 20 µg of purified protein was applied to the gel and focused at 200-400 V for a period of 4-5 h. Protein band was visualized with silver stain (Deutscher, 1990).

3.12.10. Experimental design to evaluate the combined effect of pH and temperature for purified L-asparaginase under assay condition

The central composite design was applied to optimize the levels and explain the combined effect of pH and temperature for purified L-asparaginase under assay condition. 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 were used and the full experimental plan with regard to their values in actual and coded form is provided in Table 3.9 and Table 3.10, respectively. According to this design, the total number of treatment combinations was 14 ($=2^k + 2k + 6$). Where, k is the number of independent variables (Araujo and Brereton, 1996). CCD with four axial points (α = 2) and six replicates at the center point with a total number of 14

experiments were employed to evaluate the error. All variables were taken at a central coded value, which was considered as zero.

Table 3.9. Experimental ranges and levels of the independent variables for response surface methodological experiment

Variables	Range and levels				
	-α	**-1**	**0**	**+1**	**+α**
pH	7.4	8.0	8.6	9.2	9.8
Temperature	34	37	40	43	46

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

Run order	Uncoded and coded levels	
	pH	**Temperature (ºC)**
1	8.0(-1)	37(-1)
2	9.6(+1)	37(-1)
3	8.0(-1)	43(+1)
4	9.6(+1)	43(+1)
5	8.6(0)	40(0)
6	8.6(0)	40(0)
7	8.6(0)	40(0)
8	7.4(-2)	40(0)
9	9.8(+2)	40(0)
10	8.6(0)	34(-α)
11	8.6(0)	46(+α)
12	8.6(0)	40(0)
13	8.6(0)	40(0)
14	8.6(0)	40(0)

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 triplicates and averages of the results were taken as the response.

3.12.11. Deactivation studies

Experiments were conducted to study the thermal stability of purified L-asparaginase. L-asparaginase was incubated at different combinations of pH and temperature. The levels of pH and range of temperature selected to study the deactivation of L-asparaginase were 7.6, 8.6, 9.6 and 30-50°C, 40-60°C, 30-50°C, respectively. The enzyme samples were deactivated at various combinations of pH and temperature as mentioned in the Table 3.11 and aliquots of samples were collected at different intervals of time. The pH of the samples was adjusted to standard assay conditions and the activity of L-asparaginase was measured.

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

pH	Temperature (ºC)
7.6	30
	35
	40
	45
	50
8.6	40
	45
	47
	50
	60
9.6	30
	35
	40
	45
	50

3.12.12. 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 (Sadana, 1991; Naidu and Panda, 2003). The two-state mechanism is as follows:

$$E \rightarrow E_d \tag{3.3}$$

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:

$$\frac{dE}{dt} = -k_d[E] \tag{3.4}$$

Integration of Eq. (3.4) leads to:

$$\alpha = \exp(-k_d t) \tag{3.5}$$

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

From the plot of ln(α) *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:

$$t_{1/2} = \frac{\ln(2)}{k_d} \tag{3.6}$$

3.12.13. Estimation of thermodynamic parameters

The energies and entropies of deactivation can be estimated by making use of absolute reaction rates (Eyring, 1991; Naidu and Panda, 2003). The temperature dependency of deactivation rate constant can be expressed as:

$$k_d = \frac{\kappa T}{h} \exp\frac{\Delta S^*}{R} \exp\frac{-\Delta H^*}{RT} \quad (3.7)$$

or,

$$\ln\frac{k_d}{T} = \ln\frac{\kappa}{h} + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT} \quad (3.8)$$

The values of ΔH^* and ΔS^* values 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:

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (3.9)$$

The activation energy is calculated from the Arrhenius equation as:

$$k_d = k_0 \exp\frac{-E}{RT} \quad (3.10)$$

or,

$$\ln(k_d) = \ln(k_0) - \frac{E}{RT} \quad (3.11)$$

The values of E and k_0 were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus $1/T$ respectively.

3.12.14. Cytotoxicity assay

Human liver hepatocarcinoma cell line (HepG2), human breast adenocarcinoma cancer cell line (MCF 7) and Human cervical adenocarcinoma cell line (HeLa) were obtained from National Centre for Cell Science (Pune, India), and were cultured in Dulbecco's modified

eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, heat inactivated), 1.5 g l^{-1} sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate and 1% antibiotic – antimycotic solution (1,000 U ml^{-1} penicillin G, 10 mg ml^{-1} streptomycin sulfate, 5 mg ml^{-1} gentamycin, and 25 µg ml^{-1} amphotericin B). Cells were grown in T-25 culture flasks (Corning®) under a humidified atmosphere with 5% CO_2 at 37°C (Healforce).

At sub-confluent stage, cells were harvested by trypsinization, seeded in a 96 well culture plate (Corning®) approximately at a rate of 1×10^4 cells per well and cultured for 24 h in a CO_2 incubator. The medium from each well was removed and the cells were washed twice with Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+}. The cells were then exposed to different concentrations (ranging from 20 to 200 U ml^{-1} in serum-free DMEM) of purified L-asparaginase to study the dose dependent action and to calculate the IC_{50} values. After 24 h of exposure, the contents of each well were replaced with equal volume of MTT (3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide) solution (0.5 mg ml^{-1} in serum-free DMEM without phenol red). The cells were further incubated for a period of 4 h, after which the contents of each well were replaced with an equal volume of dimethyl sulfoxide (DMSO). Finally, the absorbance was read at 570 nm in a multi-mode microplate reader (infinite m200, Tecan) and the cell viability (%) was calculated by the Equation 3.12 (Kasoju *et al.*, 2009).

$$Viability(\%) = Abs_{Test} / Abs_{Control} \times 100 \qquad (3.12)$$

Where, Abs_{Test} and $Abs_{Control}$ are the absorbance values of the treated and untreated cells, respectively. The IC_{50} value was calculated as the concentration of the compound at which 50% cell death was achieved.

The changes in cell morphology induced by L-asparaginase were tracked by inverted light microscope (Proscitech) equipped with a digital camera (Proscitech). For this purpose, cells were cultured in a 35 mm culture dish (Corning®) and were then exposed to L-asparaginase, followed by incubation for 24 h in a CO_2 incubator.

3.13. Cultivation conditions for Batch and fed batch production of glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428

The production of L-asparaginase was performed in the optimized semisynthetic medium as mentioned in section 3.11.1. The inoculum was prepared using 40 ml of sterile medium containing glucose as a sole carbon source in a 250 ml Erlenmeyer flask as described by the method in section 3.4 and then 2.61% of the seed culture was transferred to the production medium (1.5 l) in a bioreactor. Physical process parameters for the production of L-asparaginase *viz*., pH (7.0), temperature (30°C), agitation (~160 rpm) and inoculum size (2.61%) were maintained at previously optimized levels under shake flask conditions.

Batch and fed-batch experiments were carried out for the production of L-asparaginase in 7.0 l Biostat B plus (Sartorious, Germany) containing 1.5 l of the optimized medium. The fermenter is equipped with a DO, cell OD analyzer and an automatic pH controller (proportional integral derivative mode). The bioreactor was operated at a temperature of 30°C, at a fixed agitation (160 rpm) or cascade mode (160 - 600 rpm) at an aeration rate of 1.5 vvm. Experiments were performed according to the experimental plan given in Table 3.12. In order to determine the optimum DO level in the culture, experiments were performed at uncontrolled, 10, 20 and 30% of DO.

Table 3.12. Strategies used to evaluate the effect of controlled pH[a] (8.5) after 12 h and DO level on L-asparaginase production[b] from *P. carotovorum* MTCC 1428 in batch bioreactor.

Batch run	DO level (%)	pH	Agitation (rpm)
Batch 1	Uncontrolled	Uncontrolled	160
Batch 2	Uncontrolled	Controlled[a]	160
Batch 3	10	Controlled[a]	160-600 (Cascading mode)
Batch 4	20	Controlled[a]	160-600 (Cascading mode)
Batch 5	30	Controlled[a]	160-600 (Cascading mode)

[b]Production conditions: Initial pH 7.0; aeration -1.5 vvm; inoculum size – 2.61% (v/v); volume of broth 1.5 L; optimized medium under shake flask conditions.

Fed-batch experiments were performed by cultivating the culture in fermenter under batch mode until 12th h of fermentation using optimized medium and by feeding glucose (30 g l^{-1}) and/or L-asparagine (30 g l^{-1}). Four experiments were carried out to evaluate the effect of feeding substrate(s) (L-asparagine and/or glucose) on the production of L-asparaginase as shown in Table 3.13. The organism was grown at a temperature of 30°C, DO level of 20% (agitation in cascading mode: 160 - 600 rpm), controlled pH (8.5±0.1) after 12 h and aeration of 1.5-2 vvm. The concentrations of glucose and L-asparagine in the culture were maintained in the range of ~0.3-2.0 and ~0.5-4.0 g l^{-1}, respectively by monitoring and regulating feeding accordingly. Samples were withdrawn at regular intervals to analyze for cell growth, substrate(s) consumption and L-asparaginase production. All the experimental results reported are average of two experimental runs. The activity values reported are mean values with < ±5% standard deviation.

Table 3.13. Feeding strategies adopted for the production of L-asparaginase[a] by *P. carotovorum* MTCC 1428 in the bioreactor operated under fed-batch mode.

Fed-batch run	Batch (12 hr)		Feeding in Fed-Batch	
	Initial glucose	Initial L-asparagine	glucose	L-asparagine
Fed-batch 1	N	Y	N	Y
Fed-batch 2	Y	N	Y	Y
Fed-batch 3	Y	Y	Y	Y
Fed-batch 4	Y	Y	N	Y

[a]Production conditions: Initial pH - 7.0; DO level - 20%; aeration - 1.5-2.0 vvm; inoculum size - 2.61% (v/v); agitation - cascading mode 160-600 rpm; broth volume - 1.5 L; feeding substrate(s) concentrations – 30 g l^{-1}each; pH maintained 8.5±0.1 after 12 h. Y - present; N – absent.

3.14. Cultivation conditions for models of dual substrate growth kinetics, glutaminase-free L-asparaginase production and its substrates utilization by *P. carotovorum* MTCC 1428 in batch bioreactor

The growth assessment, substrate utilization and production of L-asparaginase were studied in a batch bioreactor with working volume of 1.5 l (Biostat B plus, Sartorius, Germany) using optimized semisynthetic basal medium at 8 different combinations of influent substrate(s) concentration of glucose and/or L-asparagine as shown in Table 3.14. Medium containing (g l^{-1}): Glucose 0-7; L-asparagine 0-10; $Na_2HPO_4.2H_2O$ 6.0; KH_2PO_4 1.773; NaCl 0.5; $MgSO_4.7H_2O$ 0.373; $CaCl_2.2H_2O$ 0.015; yeast extract 1.0; peptone 1.0 and initial pH of 7.0 used in this study (Kumar *et al.*, 2009). The inoculum was prepared similarly as described in section 3.13. A 2.61% of inoculum from the seed culture was added to 1.5 l of the medium in a bioreactor and operated at 20% dissolved oxygen (previously optimized level) under cascading mode (agitation - 160-600 rpm), aeration rate of 1.5 vvm and at 30°C.

Samples were withdrawn at regular interval of time and measured for L-asparaginase production, cell growth and substrate utilization. Experiments were conducted in duplicates and enzymatic assay was performed in duplicates for each sample.

Table 3.14. Different concentration of glucose and/or L-asparagine in optimized semisynthetic basal medium used in batch bioreactor for unstructured modeling of L-asparaginase fermentation by *P. carotovorum* MTCC 1428.

Batch bioreactor run	Initial substrate concentration	
	Glucose (S_g) ($g\ l^{-1}$)	L-asparagine (S_a) ($g\ l^{-1}$)
1	0	7
2	7	0
3	2	5
4	1	2.5
5	4	7.5
6	5	10
7	2	0
8	0	5

Process conditions: T_m, 30ºC; pH controlled when reaches < 8.5; aeration, 1.5 vvm; agitation, 160 – 600 rpm (cascading mode); DO level, 20%.

3.15. Kinetic model development in batch bioreactor

3.15.1 Microbial growth

Three types of multiple-substrate growth models are considered when growth is limited by more than one substrate (Bailey and Ollis, 1986).

Interactive or multiplicative form:

$$\frac{\mu}{\mu_m} = [\mu(S_1)][\mu(S_2)]...[\mu(S_i)] \qquad (3.13)$$

Additive form:

$$\frac{\mu}{\mu_m} = \frac{\mu(S_1) + \mu(S_2) + ... + \mu(S_i)}{i} \quad (3.14)$$

Non-interactive form:

$$\frac{\mu}{\mu_m} = \mu(S_1) \text{ or } \mu(S_2) \text{ or } ... \text{ or } \mu(S_i) \quad (3.15)$$

Many mathematical models are reported in the literature to correlate the single substrate concentration with microbial growth rate, μ versus S_i (Luong, 1980; Bailey and Ollis 1986; Shuler and Kargi, 1992). To develop multiple-substrate growth kinetics, these individual models can be combined as given in Eqs. (3.13) - (3.15), to obtain equations consistent with the experimental data (Shuler and Kargi, 1992). The specific growth rate in exponential phase is calculated by the following Eq. 3.16.

$$\frac{dx}{dt} = \mu X \quad => \ln(X_2 - X_1) = \mu(t_2 - t_1) + C \quad (3.16)$$

Where, X_1 and X_2 are the dry cell weight (DCW) obtained at time t_1 and t_2, respectively. C is integration constant. In our study, we have used the following growth kinetics models considered to explain the cell growth kinetics for a single substrate, S_i (Yano and Koga, 1969; Luong, 1980; Bailey and Ollis, 1986, Shuler and Kargi, 1992; Gokulakrishnan *et al.*, 2006):

Double exponential model: $\frac{\mu}{\mu_m} = \mu_m[\exp(-\frac{S_i}{K_{1i}}) - \exp(-\frac{S_i}{K_{Si}})]$ (3.17)

Luong model: $\mu = \mu_m \frac{S_i}{K_{Si} + S_i} (1 - \frac{S_i}{S_{mi}})^{ni}$ (3.18)

Yano and Kago 2 model: $\mu = \mu_m \frac{S_i}{K_{Si} + S_i + \frac{S_i^3}{K_{2i}^2}}$ (3.19)

Where, μ_m is the maximum specific growth rate (h^{-1}), μ is the specific growth rate (h^{-1}), S_i is the limiting substrate concentration (glucose and L-asparagine in this study), K_{si} is the Monod half saturation constant for substrates (glucose or L-asparagine) g l^{-1}, S_{mi} is the maximum inhibitory substrate concentration g l^{-1}, n_i is the constant which accounts the relationship between μ and S_i, and K_{1i}, K_{2i} are inhibition constants, subscript i represents either g or a as glucose and L-asparagine, respectively.

To develop growth models for multiple-substrates, for each combination of L-asparagine and glucose, specific growth rate (μ) was calculated in exponential phase. Based on single substrate, various growth models (Eqs. (3.17) - (3.19)) were inserted into Eq. (3.13) or (3.14), or (3.15) to find the best additive-substrate model.

3.15.2 L-asparaginase production

L-asparaginase production in batch bioreactor was modelled using a modified form of Luedeking and Piret model (Luedeking and Piret, 1959, Nelson *et al.*, 2007) (Eq. 3.20), which includes a term for the effect of the optimum final pH value on L-asparaginase synthesis:

$$\frac{dP_{asp}}{dt} = (\alpha.\mu.X + \beta.X)(1-\kappa|\frac{pH_{op} - pH_t}{pH_{op}}|) \qquad (3.20)$$

Where, P_{asp} is the L-asparaginase concentration (U ml^{-1}), α is a growth-associated constant for L-asparaginase production (U mg^{-1}), which corresponds to the yield product on biomass formed ($Y_{P/X}$) for growth-associated metabolites, and β is the non growth-associated constant (U mg^{-1} h^{-1}), X is the biomass concentration (g of DCW l^{-1}), κ is a constant of pH proportionality, pH_{op} is the optimum final pH for maximum L-asparaginase production and pH_t is the pH value in each sampling time.

3.15.3 Substrate utilization

The substrates utilization kinetics was described by the following Eqs. 3.21 and 3.22, which considers substrate conversion to cell mass, product formation and substrate consumption for maintenance.

Glucose utilization kinetics: $$\frac{dS_g}{dt} = -\frac{1}{Y_{X/Sg}}\frac{dX}{dt} - \frac{1}{Y_{P/Sg}}\frac{dP_{asp}}{dt} - m_g X \quad (3.21)$$

L-asparagine utilization kinetics: $$\frac{dS_a}{dt} = -\frac{1}{Y_{X/Sa}}\frac{dX}{dt} - \frac{1}{Y_{P/Sa}}\frac{dP_{asp}}{dt} - m_a X \quad (3.22)$$

Where, S_g, S_a are glucose and L-asparagine concentration (g l^{-1}), $Y_{X/Sg}$, $Y_{X/Sa}$ are yield of biomass on glucose and L-asparagine (g of DCW g^{-1} of substrate), $Y_{P/Sg}$, $Y_{P/Sa}$ are yield of product (L-asparaginase) on glucose and L-asparagine (U of L-asparaginase g^{-1} of substrate), and m_g, m_a are substrates used for maintenance of biomass (g of substrate g^{-1} DCW h^{-1}) respectively.

3.16. Estimation of kinetic parameters from various models by non-linear regression analysis

The kinetic parameters of various models (dual substrate growth, product formation and substrate utilization kinetics) considered were estimated using Microsoft Excel 2007 Solver®, which solves non-linear regression problems using Newton's method (Larson *et al.,* 1978). The following constraint was given to kinetic constants in all models while estimating the parameters are shown in the Table 3.15. The equations were solved to find values of the kinetic parameters that

minimize the objective function, the sum of squares of the differences (SSD) between experimental and theoretical data for specific growth rates, as given by Eq. (3.23).

$$SSD=\sum_{i=1}^{N}(\mu_{Expt}-\mu_{Pred})^2 \quad (3.23)$$

To calculate the maintenance and yield factors (Eqs. 3.21 and 3.22), described the objective function as the SSD between the substrate consumption rates, which were experimentally measured and theoretically estimated, separately for glucose and L-asparagine. The selected growth model equation for growth kinetics was fitted by nonlinear surface fit using Origin® Pro 8.0.

Table 3.15. The search range for kinetic constants of *P. carotovorum* MTCC 1428 in the non-linear regression analysis.

Constant	Allowable range
μ_m (h^{-1})	μ_{max} – $3\mu_{max}$
K_{sg} (g l^{-1})	0 – 20
K_{sa} (g l^{-1})	0 – 20
K_{1g} (g l^{-1})	0 – 30
K_{1a} (g l^{-1})	0 – 30
pH_{op}	8.5 – 8.8

3.17. Best model for dual substrate growth kinetics

Selected the additive substrate growth kinetics using non-linear regression and calculated the kinetic parameters as described before. Among various combinations of models (Eqs. (3.17) - (3.19)), the best additive-substrate growth model was selected to achieve the minimum sum of

squares of differences (SSD) and maximum R^2 between the experimental data and model solutions.

CHAPTER 4
RESULTS AND DECUSSION

4.1. Isolation and screening of microorganism

A total of 27 colonies were isolated from the soil and nine formed pink zone in L-asparagine pH indicator phenol medium plates indicative of L-asparaginase producing strains (Gulati *et al.*, 1997). All nine strains grown well at 30°C and produced L-asparaginase ranging from 0.57 to 20.79 U mg^{-1} of protein in the medium containing L-asparagine as sole source of carbon. The isolate that produced the highest level of L-asparaginase (designated as SK-07) was considered for further localization studies with other strains procured from different culture collection centers. The presence of glutaminase activity in all isolates was observed to 3.2 to 6.7% of relative activity of L-asparaginase. L-asparaginases with high specificity for L-asparagine and low-to-negligible activity against L-glutamine are reported to be less troublesome during the course of anti-cancer therapy (Hawkins *et al.*, 2004).

The partial 16S rRNA sequence of SK-07 (comprising of 1.5 kb nucleotides) was determined (GenBank accession no. FJ612597). A phylogenetic tree was constructed on 16S rRNA sequences (Fig. 4.1). From the phylogenetic tree, it was observed that the isolate was related to the *Serratia* lineage, and closely clustered with *S. marcescens* (GenBank accession no. FJ462701) and nearest homolog was found to be *Serratia nematodiphila* (Accession no. EU914257). Therefore, the isolate was tentatively identified to be *S. marcescens* (designated as *S. marcescens* SK-07). Initially the basal medium containing glucose was used for the production of L-asparaginase from *S. marcescens* SK-07.

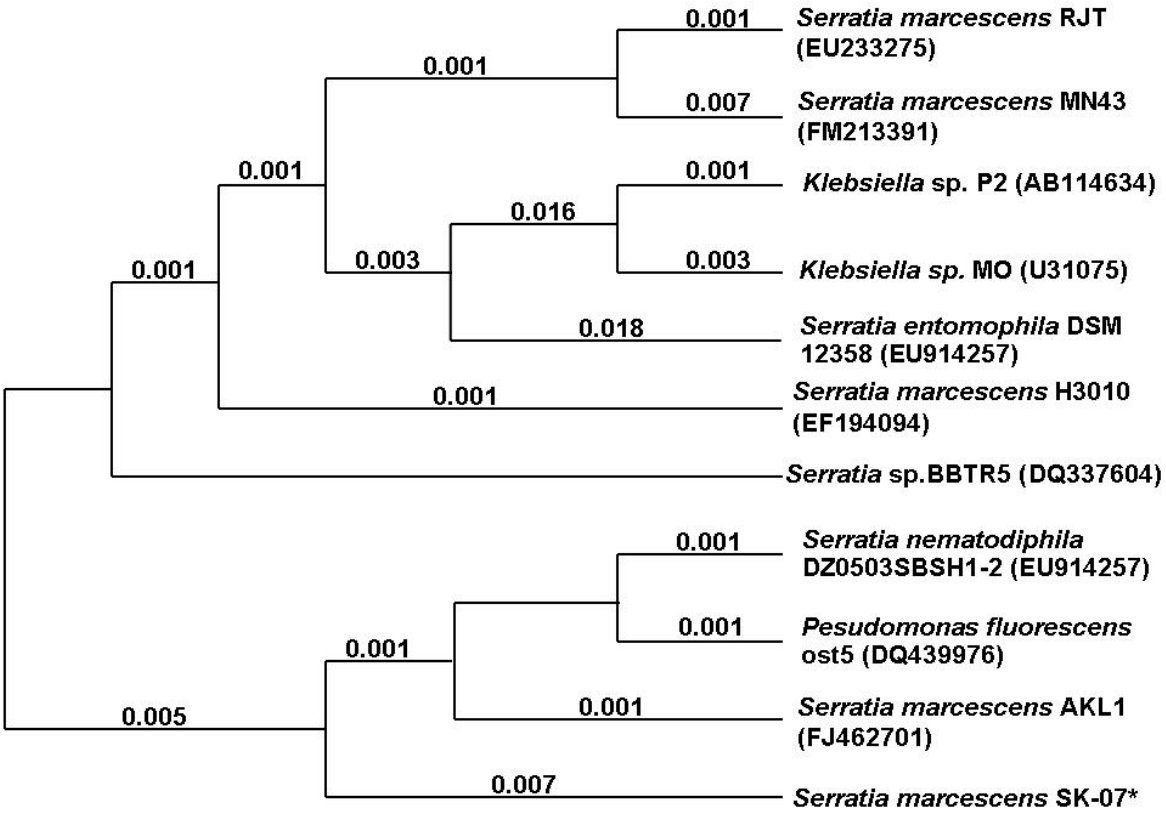


Fig. 4.1. Phylogenetic tree diagram based on 16S rRNA analysis using MEGA 3.1 software. Number in parenthesis indicates various gene database accession number. *indicates the isolated strain in this study.

4.2. Subcellular localization of L-asparaginase

In order to develop an economically feasible bioprocess for the production of L-asparaginase, subcellular localization of this enzyme was carried out using cell fractionation techniques in various organisms *viz.*, *S. marcescens* SK 07, *S. marcescens* NCIM 2919, *S. marcescens* MTCC 97, *W. eutropha* NRRL B 2804 and *P. carotovorum* MTCC 1428 using glucose or L-asparagine or combination of glucose and L-asparagine. There was no trace of extracellular activity observed in the culture filtrates, which implied that the enzyme was present locally in intracellular regions in all these microorganisms. The maximum cell

growth was observed in the medium containing L-asparagine (in comparison with glucose or combination of glucose and L-asparagine) from all microorganisms (Table 4.1). 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 varied from 2.66 to 8.36 U mg^{-1} of protein in the various fractions (cytoplasm, periplasm and membrane bound) of microorganisms grown on glucose medium. Whereas, the specific activity of the enzyme was higher, about 8 fold, in the cytoplasm when the microorganisms were cultivated on the medium containing L-asparagine or combination of L-asparagine and glucose. The existence of L-asparaginase in the membrane fraction of *Tetrahymena pyriformis* and periplasmic space of *Enterobacter aerogenes* and *Pseudomonas aeruginosa* have been reported (Triantafillou *et al.*, 1988; Geckil *et al.*, 2005). There are reports on the production of L-asparaginase from *Escherichia coli* (Cedar and Schwartz, 1968), filamentous fungi of genera *Aspergillus*, *Penicillium*, and *Fusarium* (Sarquis *et al.*, 2004*), Vibrio succinogenes* (Kafkewitz and Goodman, 1974; Albanese and Kafkewitz, 1978), *Erwinia aroideae* (Peterson and Ciegler, 1969a), *Serratia marcescens* (Heinemann and Howard 1969; Sukumaran *et al.*, 1979), *Enterobacter aerogenes* (Mukherjee *et al.*, 2000; Geckil and Gencer, 2004) and *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2000; El-Bessoumy *et al.*, 2004; Geckil *et al.*, 2004). However, there is no literature report available on the localization of L-asparaginase from *P. carotovorum* MTCC 1428. 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.

Most of the L-asparaginases from various microorganisms possess partial glutaminase activity, which causes various side effects during therapy (Muller and Boos, 1998; Distasio *et al.*, 1982; Gallagher *et al.*, 1989; 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 all microorganisms. The substrate specificity of the L-asparaginase from *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 novel glutaminase free L-asparaginase reported in this study will be an advantageous and a value-added product. The presence of glutaminase activity in all other microorganisms was observed (relative activity from 1.67 to 8.9% of L-asparaginase) and results are shown in Table 4.1. Therefore, L-asparaginase produced from *P. carotovorum* MTCC 1428 seems to be a novel glutaminase free enzyme. Hence, further studies were carried out using *P. carotovorum* MTCC 1428.

4.3. Effect of various carbon sources with or without L-asparagine

In order to select the most favorable carbon source(s) for maximization of L-asparaginase production, experiments were performed using various carbon sources and their combination with L-asparagine (Table 4.2). The maximum L-asparaginase activity was found to be 10.69 U ml^{-1} (22.45 U mg^{-1} of protein) using L-asparagine as sole source of carbon from *P. carotovorum* MTCC 1428. The most favorable substrate for the production of L-asparaginase from *P. carotovorum* MTCC 1428 was found to be L-asparagine or the combination of L-asparagine and glucose. The combination of both L-asparagine and glucose seems to be a better choice to develop an economically feasible process, because

Table 4.1. Subcellular distribution of L-asparaginase in various strains

Strains	Substrate	Cell mass (g l^{-1})	L-asparaginase activity (U ml^{-1})			Specific activity of L-asparaginase (U mg^{-1})			L-glutaminase (% cytoplasmic relative activity)
			cytoplasm	Periplasm	Membrane bound with SDS	Cytoplasm	Periplasm	Membrane bound with SDS†	
***Serratia marcescens* NCIM 2919**	Glucose	0.52±0.030	2.83±0.24	1.23±0.22	0.87±0.13	7.25±0.35	3.81±0.59	5.96±0.37	7.68±1.72
	Glucose + L-asparagine	0.50±0.017	12.05±0.76	1.14±0.17	1.48±0.34	31.45±1.34	3.64±0.34	10.20±0.28	7.89±2.10
	L-asparagine	0.58±0.037	14.07±1.23	0.74±0.36	0.58±0.11	33.73±2.44	2.11±0.40	3.64±0.34	8.90±1.37
***Serratia marcescens* MTCC 97**	Glucose	0.46±0.006	2.87±0.43	0.92±0.27	0.74±0.16	8.36±1.93	2.94±0.77	5.62±0.32	8.12±0.98
	Glucose + L-asparagine	0.42±0.024	10.09±0.96	0.76±0.08	1.19±0.23	30.57±2.22	2.52±0.17	8.35±0.07	6.45±1.32
	L-asparagine	0.60±0.028	11.81±1.23	1.23±0.15	1.04±0.21	28.13±0.89	2.99±0.83	4.81±0.58	7.70±1.94
***Pectobacterium carotovorum* MTCC 1428**	Glucose	0.53±0.013	1.25±0.23	1.34±0.21	0.41±0.09	3.35±0.49	4.30±0.42	2.66±0.36	0
	Glucose + L-asparagine	0.78±0.014	9.63±0.73	1.32±0.24	0.49±0.06	21.60±1.55	3.61±0.30	2.86±0.65	0
	L-asparagine	0.76±0.020	10.69±0.62	0.71±0.13	0.38±0.08	22.45±0.65	1.65±0.36	2.29±0.16	0
Wautersia eutropha* NRRL B-2804	Glucose	0.36±0.007	2.40±0.22	1.59±0.11	0.88±0.14	7.48±1.14	5.41±0.16	5.97±0.66	2.16±1.37
	Glucose + L-asparagine	0.44±0.021	6.24±0.47	1.24±0.26	1.39±0.11	18.29±0.55	3.63±0.32	8.42±0.10	2.42±1.45
	L-asparagine	0.55±0.015	5.02±0.32	0.62±0.03	0.48±0.06	15.36±0.93	1.99±0.42	4.30±0.15	1.67±0.93
***Serratia marcescens* SK-07 (soil isolate)**	Glucose	0.51±0.007	2.05±0.33	1.04±0.16	0.68±0.07	5.38±0.12	3.33±0.32	4.94±0.77	4.11±1.32
	Glucose + L-asparagine	0.46±0.060	6.03±0.79	1.09±0.12	0.75±0.18	16.20±0.99	3.81±0.30	5.67±0.39	3.59±0.45
	L-asparagine	0.62±0.029	8.46±0.65	0.95±0.10	0.59±0.14	20.79±0.84	2.76±0.37	4.50±0.42	3.78±0.87

Each value = mean value ± S.D.

*16th hour (rest 12th hour)

†Membrane bound with Triton X 100 not able to detect enzyme activity and protein concentration due to turbidity of solution.

Table 4.2. Effect of various carbon sources on pH of the broth and L-asparaginase production from *P. carotovorum* MTCC 1428 at 12th hour

Different carbon source with SM	pH	Specific activity (U mg^{-1})
SM	7.25±0.028	2.46±0.513
L-asparagine	8.40±0.042	22.45±0.65
Glucose	6.96±0.042	3.35±0.493
Glucose + L-asparagine	7.61±0.014	21.60±0.986
Sucrose	6.69±0.014	9.50±0.704
Sucrose + L-asparagine	7.30±0.014	11.41±0.576
Sorbitol	6.70±0.014	7.63±0.607
Sorbitol + L-asparagine	7.24±0.000	11.90±0.560
Starch	7.15±0.014	4.60±0.559
Starch + L-asparagine	8.19±0.028	13.17±0.897
Lactose	7.17±0.028	2.44±0.627
Lactose + L-asparagine	8.24±0.028	12.45±0.640

SM - Basal semisynthetic medium; Each value = mean value ± S.D.

glucose is a much low cost substrate than L-asparagine. The production of L-asparaginase was marginally favorable with low cost substrates *viz.*, glucose, sucrose and sorbitol, whereas starch and lactose did not show significant effect on the production. In general, glucose was regarded as a repressor for L-asparaginase production in bacteria (Heinemann and Howard, 1969; Barnes *et al.*, 1977; Jones, 1977; Cedar and Schwartz, 1967; Mukherjee *et al.*, 2000). The existing reports are contradictory regarding the effect of glucose and nitrogen sources on L-asparaginase production in different and sometimes in the same bacterium (Khan *et al.*, 1970; Barnes *et al.*, 1977). It has been reported that the production of L-asparaginase in *Enterobacter cloacae* is down-regulated due to lowering of pH of the medium. There was no activity of L-asparaginase detected at acidic pH (<6.0) and optimal activity was observed at neutral and alkaline pH (6.5 - 9.5) (Geckil *et al.*, 2004; Tanyildizi *et al.*, 2005). In order to

evaluate the role of L-asparagine on the production of L-asparaginase, time-dependent induction or depression experiments were carried out. During the fermentation, L-asparagine (final concentration of 1.5 g l^{-1}) was added to the each culture flask containing 1.5 g l^{-1} glucose at different time interval (0, 2, 4, 8, 10 h of the cultivation). Then the culture was grown until 12 h. The maximum production of L-asparaginase (21.55 U mg^{-1} of protein) was obtained when L-asparagine was added to the culture medium at the beginning of fermentation (0 h). The decrease in the production of L-asparaginase was observed with late addition of L-asparagine to the culture medium, which revealed that *P. carotovorum* MTCC 1428 utilized L-asparagine as an essential carbon source to maximize the production of L-asparaginase.

4.4. Effect of initial concentrations of glucose and/or L-asparagine

In order to select the most appropriate and optimal concentration(s) of substrate(s) (L-asparagine or combination of L-asparagine and glucose) for maximum production of L-asparaginase by *P. carotovorum* MTCC 1428, experiments were performed according to the method described in the section 3.8 and results are shown in Fig. 4.2. The results obtained at different initial concentrations of glucose or L-asparagine were considered as control experiments. The effect of initial concentrations of glucose (2.0 to 10.0 g l^{-1}) on the production of L-asparaginase from *P. carotovorum* MTCC 1428 was found to be insignificant. The decrease in pH of the culture broth was observed in the glucose medium, which might influence the enzyme production. Similar repression of L-asparaginase synthesis in the glucose medium has been observed with *S. marscences* (Heinemann and Howard, 1969) and *E. coli* (Barnes *et al.*, 1977). However, observations have been made on the enhancement of L-asparaginase

synthesis by glucose from *S. marcescens* by Khan *et al.*, (1970). The maximum L-asparaginase activity was found to be 11.62 U ml^{-1} (24.23 U mg^{-1} of protein) at 4.0 g l^{-1} of L-asparagine without glucose in the medium. Similarly, by varying glucose concentration from 1 to 10 g l^{-1}, the maximum L-asparaginase activity, 9.52 U ml^{-1} (21.03 U mg^{-1} of protein), was achieved at 2 and 1.5 g l^{-1} of glucose and L-asparagine (fixed) respectively. Effect of initial concentration of L-asparagine (1.0 to 10.0 g l^{-1}) was also studied by keeping glucose concentration at predetermined level obtained from previous experiment (2.0 g l^{-1}) and the L-asparaginase activity was found to be 14.56 U ml^{-1} (26.98 U mg^{-1} of protein), at 4.0 g l^{-1} of L-asparagine. Hence, an enhancement of 2.75 U mg^{-1} of protein was achieved by the addition of 2 g l^{-1} of glucose to the medium containing 4 g l^{-1} of L-asparagine.

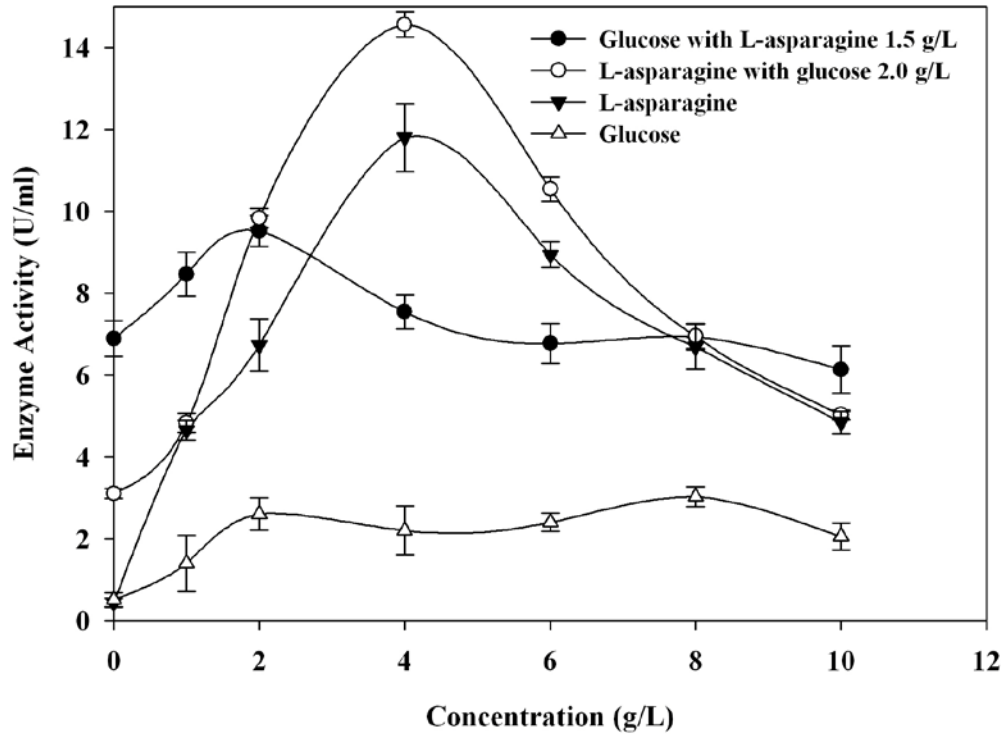


Fig. 4.2. Effect of initial concentration of glucose and L-asparagine on the production of L-asparaginase from *P. carotovorum* MTCC 1428.

4.5. Role of nitrogen source on the production of L-asparaginase

A two level three factor (2^3) full factorial experimental design was applied to observe the influence of parameters (L-asparagine, yeast extract and peptone) on the production of L-asparaginase from *P. carotovorum* MTCC 1428. Experiments were performed according to the design matrix and results are shown in Table 4.3. A linear regression model was fitted for the experimental data by Fisher's statistical test for ANOVA using Minitab Statistical Software release 15.1.1.0, USA. The regression model was applied to evaluate effect of variables (nitrogen sources) on the production of L-asparaginase. The regression model proposed for the production of L-asparaginase is as follows.

$$Y_{\text{Specific activity}} = 7.2072 + 5.5991A + 2.0729Y + 3.6079P + 1.6324AY + 2.2906AP + 0.8353YP + 0.6856AYP \quad (4.1)$$

Where, A is L-asparagine, Y is yeast extract, and P is peptone.

Table 4.3. Results of specific and enzyme activity of L-asparaginase obtained in the factorial design study

Run	Coded and uncoded level			L-asparaginase specific activity (U mg^{-1})		Enzyme activity (U ml^{-1})
	A	Y	P	Experimental	Predicted	
1	+1 (3.0)	+1 (1.0)	+1 (1.0)	23.93±0.48	23.59	11.54±0.54
2	-1 (0.0)	-1 (0.0)	-1 (0.0)	0.00	0.00	0.00
3	+1 (3.0)	-1 (0.0)	+1 (1.0)	13.48±1.04	12.74	4.27±0.37
4	-1 (0.0)	+1 (1.0)	-1(0.0)	0.58±0.09	0.61	0.21±0.02
5	-1 (0.0)	-1 (0.0)	+1(1.0)	2.34±1.02	2.34	0.53±0.27
6	-1 (0.0)	+1 (1.0)	+1(1.0)	3.52±0.36	3.76	1.27±0.14
7	+1 (3.0)	+1 (1.0)	-1(0.0)	9.09±1.29	9.89	3.45±0.58
8	+1 (3.0)	-1 (0.0)	-1(0.0)	4.72±0.80	4.89	1.39±0.21

Each value = mean value ± S.D; *A, Y and P* are mentioned (parenthesis) in g l^{-1}.

In general, if the coefficient of the model was relatively large, it had more significant effect on the

production of L-asparaginase. Furthermore, a variable with positive fitted constant had an enhanced effect towards L-asparaginase production than the one with negative coefficient, which shows inhibitory effect. However the present model had all positive coefficients, thus showing positive effect on the enzyme production. The magnitude and direction of effects of the factors as well as their coefficients of regression and statistical significance of the experimental data for the production of L-asparaginase are shown in Table 4.4. The net effect is a difference between the responses of two levels (high and low level) of factors. The regression coefficients were obtained by dividing the net effect by two. The standardized effects (*t*-values) were calculated by dividing each coefficient by its standard error. The combined effects of parameters were significant for all combinations of them. The significance of the data is judged by its *P*-value being zero or close to zero. The significance of each coefficient in the regression based model was determined by t-values and *P*-values, which are listed in the Table 4.4. Generally, larger the magnitude of standardized effect and smaller the corresponding *P*-value indicates a high significance of the corresponding coefficient (Tanyildizi *et al.,* 2005). It was observed that the variables with largest effect were the linear term in the model for L-asparaginase production. Furthermore, the interactive effect of L-asparagine and peptone was more significant than the other factors. The importance of dehydrolysed protein on the production of L-asparaginase was emphasized in the literature (Heinemann and Howard, 1969; Howard and Carpenter, 1972). This is presumably due to the presence of some essential nutrients or inducers in yeast extract and peptone to enhance L-asparaginase production. However, the effect of yeast extract was observed to be less on the production of L-asparaginase in comparison with peptone. ANOVA was employed for the determination of significant variables (Table 4.5). The *F*-value was the ratio of mean square due to regression to the mean square due to residual and it indicates that the influence (significant or

not) of each controlled factor on tested models. If the model was good predictor of the experimental results, the calculated *F*-value should be several times the tabulated value. In addition, the *P*-value corresponding to the *F*-value indicated the probability that differences between calculated and tabulated value was due to random experimental error. From ANOVA analysis the confidence level was greater than 95% ($P < 0.05$) for L-asparaginase production and *P*-value of the model was zero (Table 4.4). Thus, the model fitted the experimental data very accurately. The coefficient of determination R^2 of the model for L-asparaginase production was calculated to be 0.9948 indicating that the model was able to comprehend more than 99% of the data variability. The enzyme activity varies from 0 to 11.54 U ml^{-1}. The wide variation of L-asparaginase production from 0 to 23.93 U mg^{-1} of protein reflects the importance of the screening of nitrogen source (Table 4.3). These results suggested that all variables (L-asparagine, yeast extract and peptone) significantly influence the production of L-asparaginase. The high level of L-asparagine concentration (3.0 g l^{-1}) favored the increase in the production of L-asparaginase than at lower level (0.00 g l^{-1}). From the ANOVA analysis results (Table 4.5), all three nitrogen sources proved to be important for the production of L-asparaginase.

The Pareto plot displays the absolute value of the effect of variables, which are important in the analysis of results from the design experiment. It draws a reference line to indicate that the variables which extend past this line are potentially important. The interaction effects of the variables on the production have been shown in Fig. 4.3. All the variables (L-asparagine, yeast extract and peptone) and all the combinations of their interactions have shown to influence the production of L-asparaginase significantly (Fig. 4.3). Hence, all the nitrogen sources (L-asparagine, yeast extract and peptone) were considered for the maximum production of L-asparaginase from *P. carotovorum* MTCC 1428 and the data shown within 95% confidence level.

Table 4.4. Regression analysis of the 2^3 full factorial design

Term	Net effect	Coefficients	Standardized effect (*t* value)	*p*-value
Constant		7.2072	37.27	0.000
Asparagine	11.1982	5.5991	28.95	0.000
Yeast Extract	4.1458	2.0729	10.72	0.000
Peptone	7.2158	3.6079	18.66	0.000
Asparagine*Yeast Extract	3.2649	1.6324	8.44	0.000
Asparagine*Peptone	4.5812	2.2906	11.84	0.000
Yeast Extract*Peptone	1.6706	0.8353	4.32	0.003
Asparagine*Yeast extract*Peptone	1.3712	0.6856	3.55	0.008

Table 4.5. Analysis of variance (ANOVA) for specific activity of L-asparaginase

Source	DF	SS	MS	*F*- value	P
Main Effects	3	778.63	259.54	433.77	0.000
2-Way Interactions	3	137.75	45.92	76.74	0.000
3-Way Interactions	1	7.52	7.52	12.57	0.008
Residual Error	8	4.79	0.60		
Pure Error	8	4.79	0.60		
Total	15	928.68			

R^2= 99.48%; Adj R^2= 99.03%;SS, sum of squares; DF, degrees of freedom; MS, mean square.

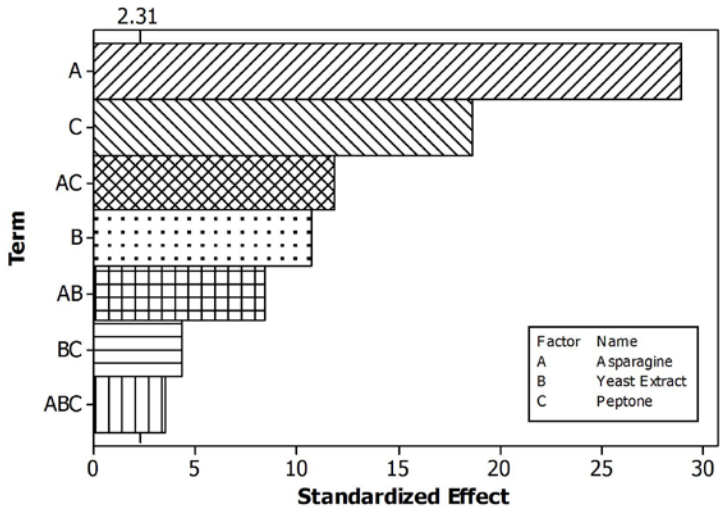


Fig. 4.3. Pareto chart of the standardized effects of nitrogen sources on the L-asparaginase production from *P. carotovorum* MTCC 1428, α = 0.05.

4.6. Screening of significantly influencing medium components

The Plackett-Burman (PB) experimental design was applied to screen the most significantly influencing medium components. Experiments were performed according to the design matrix as shown in Table 4.6. The observed and predicted responses and L-asparaginase production (specific activity) for the different trials are also given in Table 4.6. The cell growth corresponded well with the enzyme activity and the specific activity inferring that the L-asparaginase production to be a growth-associated product formation kinetics. The enzyme activity varied from 4.73 to 12.8 U ml^{-1}, whereas cell growth varied from 0.549 to 0.970 g l^{-1} (Table 4.6). However, wide variation of L-asparaginase production from 16.22 to 25.72 U mg^{-1} of protein reflects the importance of the optimization of medium constituents.

The regression model was applied to screen the significant medium components. The following regression equation was obtained from the analysis of variance (ANOVA) and only significant variables ($P<0.04$) were included.

$$Y_{\text{specific activity}} = 20.854 - 1.888X_1 - 0.655X_2 - 0.727X_4 + 1.156\,X_6 \qquad (4.2)$$

Where, X_1 = L-asparagine, X_2 = glucose, X_4 = KH_2PO_4 and X_6 = $MgSO_4.7H_2O$.

The analysis of the data from the Plackett–Burman experiments involved a first order (main effects) model. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened *via* Student's *t*-test for ANOVA. Table 4.7 represents the effects, values of coefficients, *t* and *P* of each component from the responses. The main effect of each variable upon L-asparaginase production was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (-1) of that variable. On the analysis of the regression coefficients of the medium components,

$Na_2HPO_4.2H_2O$, NaCl and $MgSO_4.7H_2O$ showed positive effect values. Whereas, L-asparagine, glucose, KH_2PO_4, $CaCl_2$, yeast extract and peptone showed negative effect values. The components were screened based on their absolute value of effects (either positive or negative) and *P* value below at 0.04. The absolute values of effect in the Table 4.7 were used to indicate the relative contribution of the variable on the response. A positive sign indicates that at higher level of variables setting results in a higher response than the lower level variable setting. On the other hand, a negative sign indicates that the lower level of variable setting results in a higher response than the high level variable setting. The *P* values of the components $Na_2HPO_4.2H_2O$, NaCl, $CaCl_2$, yeast extract and peptone were above 0.04 for the production of L-asparaginase and hence considered as insignificant ones. The remaining components, L-asparagine, glucose, KH_2PO_4 and $MgSO_4.7H_2O$ have shown *P* value below 0.04 and were considered to be significant ones. The levels of these screened components were optimized using central composite experimental design for enhanced production of L-asparaginase.

Pareto chart (Fig. 4.4) shows that the ranking of important variables used in the design of the experiment for optimization according to the absolute values of standardized effect and it is a convenient way to view the results of a Plackett-Burman experimental design. The reference line (2.14) indicates that the effects were significant with α value of 0.05. The variables effects, which extend past the line, were known to be significant at particular α. The standardized effects were the t statistics shown in Fig. 4.4. The *t* statistics were calculated by dividing each coefficient by its standard error. The variables (L-asparagine, glucose, KH_2PO_4 and $MgSO_4.7H_2O$) were influencing the production of L-asparaginase very significantly (Fig. 4.4). All other insignificant variables were not included in the next optimization experiment, but instead they were used in all experiments at their middle level (centre point).

Table 4.6. Plackett-Burman design matrix in coded units and real values (parenthesis) along with the observed and predicted L-asparaginase production

Run Order	Experimental values									Specific Activity (U mg^{-1})		Enzyme activity (U ml^{-1})	Cell growth (g l^{-1})
	Glucose (X_1)	L-asparagine (X_2)	$Na_2HPO_4.2H_2O$ (X_3)	KH_2PO_4 (X_4)	NaCl (X_5)	$MgSO_4.7H_2O$ (X_6)	$CaCl_2.2H_2O$ (X_7)	Yeast extract (X_8)	Peptone (X_9)	Observed[a]	Predicted		
1	1(5.0)	-1(3.0)	1(11.0)	-1(0.5)	-1(0)	-1(0)	1(0.03)	1(1.75)	1(1.75)	18.19±0.32	18.49	7.22±0.39	0.751±0.05
2	1(5.0)	1(7.0)	-1(1.0)	1(5.5)	-1(0)	-1(0)	-1(0)	1(1.75)	1(1.75)	16.23±0.01	15.92	4.73±0.01	0.549±0.01
3	-1(1.0)	1(7.0)	1(11.0)	-1(0.5)	1(1.0)	-1(0)	-1(0.0)	-1(0.25)	1(1.75)	22.42±0.18	21.96	11.76±0.13	0.968±0.03
4	1(5.0)	-1(3.0)	1(11.0)	1(5.5)	-1(0)	1(1.0)	-1(0)	-1(0.25)	-1(0.25)	20.80±0.16	20.34	10.28±0.25	0.869±0.04
5	1(5.0)	1(7.0)	-1(1.0)	1(5.5)	1(1.0)	-1(0)	1(0.03)	-1(0.25)	-1(0.25)	16.48±0.08	16.77	4.88±0.09	0.573±0.02
6	1(5.0)	1(7.0)	1(11.0)	-1(0.5)	1(1.0)	1(1.0)	-1(0)	1(1.75)	-1(0.25)	20.48±0.44	20.92	9.68±0.37	0.793±0.01
7	-1(1.0)	1(7.0)	1(11.0)	1(5.5)	-1(0)	1(1.0)	1(0.03)	-1(0.25)	1(1.75)	21.48±0.32	21.93	11.43±0.28	0.882±0.03
8	-1(1.0)	-1(3.0)	1(11.0)	1(5.5)	1(1.0)	-1(0)	1(0.03)	1(1.75)	-1(0.25)	22.20±0.48	21.90	11.56±0.36	0.940±0.05
9	-1(1.0)	-1(3.0)	-1(1.0)	1(5.5)	1(1.0)	1(1.0)	-1(0)	1(1.75)	1(1.75)	23.57±0.24	23.87	12.80±0.13	0.965±0.06
10	1(5.0)	-1(3.0)	-1(1.0)	-1(0.5)	1(1.0)	1(1.0)	1(0.03)	-1(0.25)	1(1.75)	21.62±0.55	21.31	11.83±0.38	0.926±0.04
11	-1(1.0)	1(7.0)	-1(1.0)	-1(0.5)	-1(0)	1(1.0)	1(0.03)	1(1.75)	-1(0.25)	24.11± 0.27	23.65	12.82±0.25	0.970±0.02
12	-1(1.0)	-1(3.0)	-1(1.0)	-1(0.5)	-1(0)	-1(0)	-1(0)	-1(0.25)	-1(0.25)	22.66±0.37	23.11	11.63±0.33	0.943±0.01

[a]The observed values of L-asparaginase specific activity were the mean values of duplicates with standard deviation (Mean±S.D).
X_1-X_9 are mentioned in g l^{-1}.

Table 4.7. Statistical analysis of Plackett–Burman design showing coefficient values, t and *P*-value for each variable

Variable	Symbol code	Effect	Coefficient	t-value	*P*-value
Intercept			20.854	168.65	0.0001
Glucose	X_1	-3.776	-1.888	-15.27	0.0004[b]
L-asparagine	X_2	-1.311	-0.655	-5.30	0.0001[b]
$Na_2HPO_4.2H_2O$	X_3	0.149	0.074	0.60	0.5572[a]
KH_2PO_4	X_4	-1.453	-0.727	-5.88	0.0002[b]
NaCl	X_5	0.549	0.274	2.22	0.0446[a]
$MgSO_4.7H_2O$	X_6	2.313	1.156	9.35	<0.0001[b]
$CaCl_2.2H_2O$	X_7	-0.346	-0.173	-1.40	0.1843[a]
Yeast extract	X_8	-0.112	-0.056	-0.45	0.6576[a]
Peptone	X_9	-0.537	-0.268	-2.17	0.0485[a]

[a]Non-significant at P > 0.04; [b]Significant
R^2 = 97.35%; R^2 (adj) = 95.27%

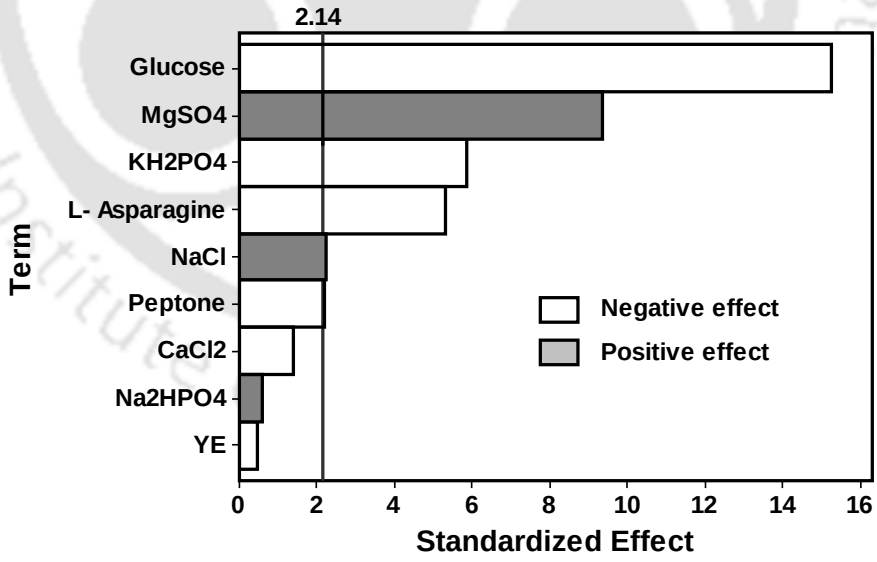


Fig. 4.4. Pareto chart of the standardized effects of the factors on the L-asparaginase production from *P. carotovorum* MTCC 1428, α = 0.05.

In general, glucose was regarded as a repressor for L-asparaginase production in bacteria (Heinemann *et al.,* 1970; Barnes *et al.,* 1977; Mukherjee *et al.,* 2000). In the present study, the production of L-asparaginase was enhanced in the presence of L-asparagine. The existing reports are contradictory regarding the effect of glucose and nitrogen sources on L-asparaginase production in different and sometimes in the same bacterium (Khan *et al.,* 1970; Barnes *et al.,* 1977). The production level of L-asparaginase was low in the basal medium containing glucose. It is very difficult to evaluate the significance of each medium component and interaction among them on the production of L-asparaginase by adopting classical approach (one variable at a time). Hence, experiments were carried out to screen the significant medium components and optimize their levels using Plackett-Burman and central composite experimental design techniques, respectively. According to statistical analysis of Plackett-Burman design, L-asparagine, glucose, KH_2PO_4 and $MgSO_4.7H_2O$ were found to be major factors that influenced the L-asparaginase production. Though there are reports available on the application of Plackett-Burman experimental design technique for the screening of significantly influencing medium components for the production of phytase (Singh and Satyanarayana, 2008), glucansucrase (Majumder and Goyal, 2008), alpha amylase (Gangadharan *et al.,* 2008), griseofulvin (Dasu and Panda, 2000; Dasu *et al.,* 2002a), lipase (Gaur *et al.,* 2008), pectin and pectate lyase (Gummadi and Kumar, 2006) etc., there is no information available for the screening of medium components for L-asparaginase production from *P. carotovorum* MTCC 1428 in submerged fermentation. However, the screening of significantly influencing medium components by PB experimental design for enhanced production of L-asparaginase from *P. aeruginosa* in solid state fermentation was reported (Abdel-Fattah and Olama, 2002). Recently, Sunitha *et al.,* (2010) has been successfully screened 67 nutrients for the production of L-asparaginase by PB design.

4.7. Optimization of the screened medium constituents for maximizing the L-asparaginase production

The experiments were performed according to the design matrix to optimize the levels of screened medium components (L-asparagine, glucose, KH_2PO_4 and $MgSO_4.7H_2O$) using central composite experimental circumscribed design. The design matrix and the corresponding results of observed and predicted responses (production of L-asparaginase) are shown in Table 4.8. The cell growth and L-asparaginase activity (U ml^{-1}) profiles inferred that the growth associated production of L-asparaginase (Table 4.8). The enzyme activity varied from 7.37 to 15.34 U ml^{-1}, where as the cell growth varied from 0.632 to 1.092 g l^{-1}. By applying the multiple regression analysis on the experimental data, the following nonlinear equation was found to explain the production of L-asparaginase from *P. carotovorum* MTCC 1428.

$$Y_{\text{specific activity}} = 25.909 - 0.969X_1 - 0.497X_2 - 1.321X_4 - 1.468X_6 - 0.771X_1^2 - 1.295X_2^2 - 0.701X_4^2 - 0.792X_6^2 + 0.471X_1X_2 + 0.433X_1X_4 + 0.079X_1X_6 - 1.119X_2X_4 + 0.058X_2X_6 - 0.353X_4X_6 \quad (4.3)$$

Where, X_1 = L-asparagine, X_2 = glucose, X_4 = KH_2PO_4 and X_6 = $MgSO_4.7H_2O$.

The results were analyzed using the analysis of variance (ANOVA) as appropriate to the experimental design used (Table 4.9). According to the ANOVA of the quadratic regression model, the model was highly significant, as is evident from the Fisher, *F*-test (mean square regression: mean square residual is 40.07) with a very low probability value ($P_{\text{model}} > F$ is 0.0001). This indicates that the combined effects of all the independent variables significantly contributed to maximize the production of L-asparaginase. The goodness of the model was checked by coefficient of determination, R^2, implies that the sample variation of 92.27% for

L-asparaginase production is attributed to the medium components and also only 7.73% of the total variation is not explained by the model.

Table 4.8. 2^4 full-factorial central composite design matrix in coded units and real values (parenthesis) with experimental and predicted values of L-asparaginase production from *P. carotovorum* MTCC 1428

Run Order	Coded levels				Specific Activity (U mg^{-1})		Enzyme activity (U ml^{-1})	Cell growth (g l^{-1})
	Glucose (X_1)	L-asparagine (X_2)	KH_2PO_4 (X_4)	$MgSO_4.7H_2O$ (X_6)	Observed[a]	Predicted		
1	-1(2.0)	-1(3.0)	-1(2.0)	-1(0.30)	26.98±0.08	25.99	14.71±0.34	0.993±0.04
2	-1(2.0)	1(7.0)	-1(2.0)	-1(0.30)	27.75±0.03	26.18	15.34±0.26	0.978±0.04
3	1(5.0)	-1(3.0)	-1(2.0)	-1(0.30)	23.03±0.35	22.09	12.74±0.12	0.937±0.02
4	1(5.0)	1(7.0)	-1(2.0)	-1(0.30)	24.06±0.11	24.16	13.29±0.23	0.962±0.01
5	-1(2.0)	-1(3.0)	1(5.0)	-1(0.30)	26.32±0.23	25.43	15.22±0.21	1.035±0.06
6	-1(2.0)	1(7.0)	1(5.0)	-1(0.30)	20.96±0.15	21.14	10.74±0.14	0.874±0.02
7	1(5.0)	-1(3.0)	1(5.0)	-1(0.30)	23.94±0.32	23.26	13.02±0.51	0.964±0.03
8	1(5.0)	1(7.0)	1(5.0)	-1(0.30)	21.25±0.26	20.85	11.41±0.46	0.889±0.03
9	-1(2.0)	-1(3.0)	-1(2.0)	1(0.80)	24.55±0.75	23.49	13.17±0.36	0.952±0.03
10	-1(2.0)	1(7.0)	-1(2.0)	1(0.80)	24.15±0.45	23.91	12.93±0.18	0.927±0.03
11	1(5.0)	-1(3.0)	-1(2.0)	1(0.80)	21.02±0.32	19.90	11.04±0.31	0.863±0.03
12	1(5.0)	1(7.0)	-1(2.0)	1(0.80)	22.77±0.32	22.20	12.36±0.21	0.955±0.08
13	-1(2.0)	-1(3.0)	1(5.0)	1(0.80)	22.54±0.41	21.51	12.15±0.04	0.927±0.01
14	-1(2.0)	1(7.0)	1(5.0)	1(0.80)	17.97±0.33	17.45	7.95±0.52	0.632±0.02
15	1(5.0)	-1(3.0)	1(5.0)	1(0.80)	19.54±0.28	19.66	8.53±0.37	0.779±0.03
16	1(5.0)	1(7.0)	1(5.0)	1(0.80)	17.43±0.33	17.48	7.37±0.27	0.687±0.01
17	0(3.5)	0(5.0)	0(3.5)	0(0.55)	26.18±0.33	25.74	14.21±0.42	1.092±0.01
18	0(3.5)	0(5.0)	0(3.5)	0(0.55)	26.13±0.40	25.74	14.35±0.19	0.997±0.02
19	0(3.5)	0(5.0)	0(3.5)	0(0.55)	25.71±0.11	25.74	13.98±0.13	1.056±0.04
20	0(3.5)	0(5.0)	0(3.5)	0(0.55)	26.23±0.18	25.74	14.27±0.37	1.079±0.05
21	0(3.5)	-2(1.0)	0(3.5)	0(0.55)	19.97±0.39	21.54	8.64±0.33	0.821±0.04
22	0(3.5)	2(9.0)	0(3.5)	0(0.55)	19.80±0.01	19.55	9.42±0.23	0.867±0.03
23	-2(0.5)	0(5.0)	0(3.5)	0(0.55)	23.25±0.47	24.58	12.68±0.18	0.981±0.02
24	2(6.5)	0(5.0)	0(3.5)	0(0.55)	20.72±0.13	20.71	10.55±0.40	0.925±0.01
25	0(3.5)	0(5.0)	-2(0.5)	0(0.55)	24.10±0.45	25.57	12.37±0.25	0.995±0.03
26	0(3.5)	0(5.0)	2(6.5)	0(0.55)	20.43±0.54	20.28	10.73±0.30	0.938±0.02
27	0(3.5)	0(5.0)	0(3.5)	-2(0.05)	24.62±0.18	25.49	13.84±0.28	1.047±0.03
28	0(3.5)	0(5.0)	0(3.5)	2(1.05)	19.18±0.34	19.62	8.32±0.24	0.837±0.04
29	0(3.5)	0(5.0)	0(3.5)	0(0.55)	25.99±0.39	25.74	14.56±038	1.058±0.05
30	0(3.5)	0(5.0)	0(3.5)	0(0.55)	25.27±0.39	25.74	14.29±0.25	0.989±0.04

[a]The observed values of L-asparaginase specific activity were the mean values of duplicates with standard deviation (Mean ± S.D.)

X_1, X_2, X_4 and X_6 are mentioned in g l^{-1}

Table 4.9. ANOVA of L-asparaginase production in the optimization study

Source	DF	SS	MS	F-value	Prob. (*P*)>F
Model	14	453.137	32.375	40.07	<0.0001
Residual (error)	16	37.971	0.808	-	-
Lack-of-Fit	10	33.127	3.313	25.30	<0.0001
Pure Error	6	4.844	0.131	-	-
Total	30	491.109			

R^2 = 92.27%; CV = 10.629 %; *R* = 0.8997; *DF* degrees of freedom; *SS* sum of squares; *MS* mean square

The Student *t* distribution and the corresponding *P* values, along with the parameter estimate are shown in Table 4.10. The *P* values of all linear and most of quadratic relationships between medium components and L-asparaginase production suggest that they are highly significant (*P* = 0.000). Also the effects of interactions of glucose with L-asparagine and KH_2PO_4, and L-asparagine with KH_2PO_4 were found to be highly significant on the enzyme production.

Table 4.10. Model coefficient estimated by multiple linear regressions

Model term	Parameter estimate	Standard error	t-value	*P*-value
Intercept	25.9090	0.2402	107.854	0.000
X_1	-0.9685	0.1297	-7.465	0.000
X_2	-0.4970	0.1297	-3.831	0.000
X_4	-1.3212	0.1297	-10.184	0.000
X_6	-1.4676	0.1297	-11.312	0.000
X_1^2	-0.7707	0.1189	-6.484	0.000
X_2^2	-1.2954	0.1189	-10.899	0.000
X_4^2	-0.7009	0.1189	-5.897	0.000
X_6^2	-0.7924	0.1189	-6.667	0.000
X_1X_2	0.4705	0.1589	2.961	0.005
X_1X_4	0.4331	0.1589	2.726	0.009
X_1X_6	0.0788	0.1589	0.496	0.622[a]
X_2X_4	-1.1189	0.1589	-7.042	0.000
X_2X_6	0.0579	0.1589	0.364	0.717[a]
X_4X_6	-0.3529	0.1589	-2.221	0.031

[a]Non-significant at P > 0.05

Three-dimensional response surface plots were constructed to predict the L-asparaginase production for different values of the tested variables and also to analyze the interaction between the variables. The response surface plots were constructed by plotting the response (L-asparaginase specific activity) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels as shown in Fig. 4.5a-f. As shown in the Fig. 4.5a-c, there was a steep increase in the production of L-asparaginase with increase in L-asparagine concentration and reached maximum at 5 - 6 g l^{-1} of L-asparagine. Similarly, glucose showed significant effect on the production of L-asparaginase and achieved maximum at 2 g l^{-1} (Fig. 4.5ade). The interaction of glucose with L-asparagine (P = 0.005) and KH_2PO_4 (P = 0.009) is very prominent as shown in Fig. 4.5a and d, respectively. Similar effect was observed for L-asparagine with KH_2PO_4 (P = 0.000) (Fig. 4.5b). However, the interaction of $MgSO_4$ $7H_2O$ with glucose (Fig. 4.5e, P = 0.622), L-asparagine (Fig. 4.5c), P = 0.717) and KH_2PO_4 (Fig. 4.5f), P = 0.031) is not prominent. As seen in Figs. 4.5cef, increase in $MgSO_4.7H_2O$ concentration has shown decrease in L-asparaginase production. The parity plot (Fig. 4.6) showed a satisfactory correlation between the experimental and predicted values (obtained from Eq. 4.3) of L-asparaginase production, wherein, the points cluster around the diagonal line which indicated the optimal fit of the model, since the deviation between the experimental and predicted values was minimum.

Maximization of the regression equation (Eq. 4.3) was carried out using an iterative technique to obtain optimum levels of concentrations of medium components. By substituting the corresponding coded concentration levels of the factors into the regression equation, the maximum predictable response for L-asparaginase production was calculated. The optimum levels of each variable were determined to be as follows: L-asparagine 5.202 g l^{-1}, glucose

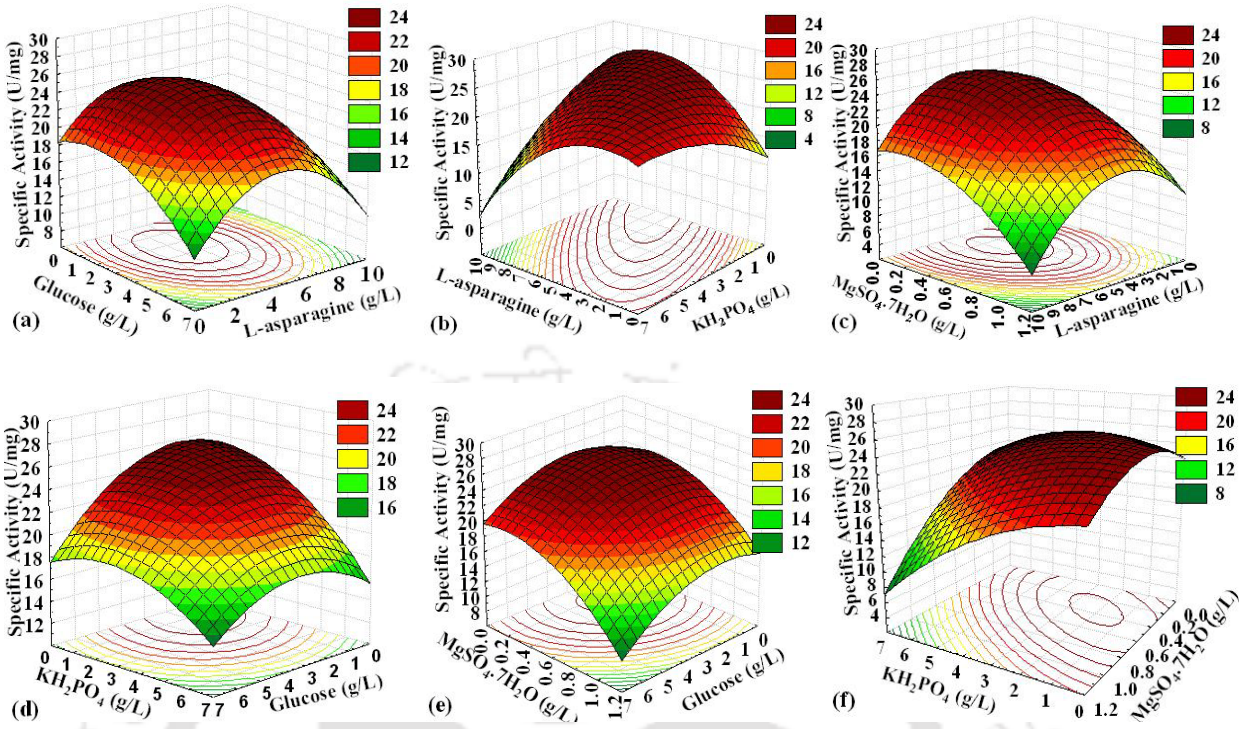


Fig. 4.5. Three-dimensional response surface plots for L-asparaginase production showing the interactive effects of (a) L-Asparagine and Glucose (b) L-Asparagine and KH_2PO_4 (c) L-Asparagine and $MgSO_4.7H_2O$ (d) Glucose and KH_2PO_4 (e) Glucose and $MgSO_4.7H_2O$ (f) KH_2PO_4 and $MgSO_4.7H_2O$

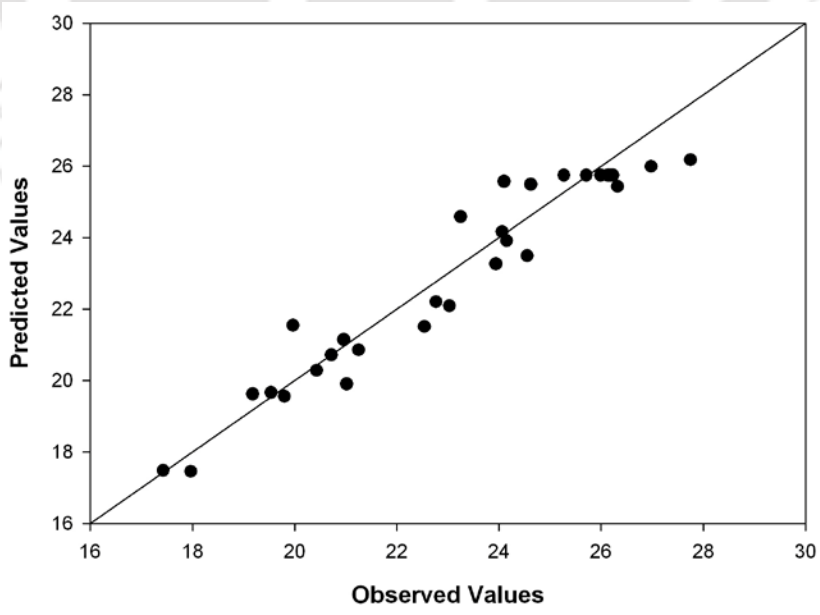


Fig. 4.6. Experimental *vs*. predicted values of L-asparaginase in the optimization study.

2.076 g l^{-1}, KH_2PO_4 1.773 g l^{-1} and $MgSO_4.7H_2O$ 0.373 g l^{-1}. The maximum production of glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428 in the optimized medium was found to be 27.88 U mg^{-1} of protein and it was in very good correlation with the predicted value. An overall 8.3 fold increase in L-asparaginase production was achieved using Plackett-Burman design followed by central composite experimental design technique.

Both Plackett–Burman and central composite designs have 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.,* 2002b), etc. To the best of our knowledge, we could not find any report in the literature on the screening and optimization of significantly influencing medium components for the production of glutaminase-free L-asparaginase form *P. carotovorum* MTCC 1428.

The range of specific activity of L-asparaginases from different microorganisms was reported in between 0.1 – 35.0 U mg^{-1} of protein (Raha *et al.,* 1990; Maladkar *et al.,* 1993; Manna *et al.,* 1995; Nawaz *et al.,* 1998; Abdel-Fattah and Olama 2002; El-Bessoumy *et al.,* 2004; Prakasham *et al.,* 2007). L-Asparaginases from *Erwinia carotovora, Erwinia crysanthemi, E. coli* and *S. cerevisiae* have been cloned and successfully expressed in bacterial and yeast expression systems (Khushoo *et al.,* 2004; Kotzia and Labrou 2005; Khushoo *et al.,* 2005; Maria *et al.,* 2006; Kotzia and Labrou 2007). However, the drug, obtained from *Escherichia 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. The specific

activity of recombinant L-asparaginases were varied from 7.7 to 95.7 U mg^{-1} of protein in crude extract (Khushoo *et al.*, 2004; Khushoo *et al.*, 2005; Kotzia and Labrou, 2007; Cappelletti *et al.*, 2008). The maximum production of glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428 was achieved, 27.88 U mg^{-1} of protein. This is very much comparable with the reported values. The time for L-asparaginase production was reported by various researchers varies from 36 h to 120 h (Raha *et al.*, 1990; Maladkar *et al.*, 1993; Manna *et al.*, 1995; Abdel-Fattah and Olama, 2002; El-Bessoumy *et al.*, 2004; Prakasham *et al.*, 2007). The productivity of L-asparaginase from *P. carotovorum* MTCC 1428 much higher due to short duration of fermentation time (12 h) than the reported values. The large quantities of the enzyme with the higher activity would obtain in a short period in the optimized medium. In addition, the enzyme from L-asparaginase from *P. carotovorum* MTCC 1428 also displays comparable catalytic activity, making it attractive for further screening as a potential anticancer agent.

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

To verify the validity of the model, experiments were carried out at optimal levels of significantly influenced medium components and at middle level of other medium components. The observed specific activity of L-asparaginase was 27.88 U mg^{-1} of protein and this is very good agreement with the value predicted by the model, 27.62 U mg^{-1} of protein. The verification revealed a high degree of accuracy of the model of more than 98.6%, which is an evidence for the model predictions under the investigated conditions. The production of L-asparaginase was slightly higher (28.87 U mg^{-1}) in the fermenter than that in

the shake flasks at optimized conditions. The maximum production of L-asparaginase was achieved at 12 h in the fermenter as same the case in shake flask and resulted in an increase in the overall productivity from 1225.83 to 1282.50 U l^{-1} h^{-1}. A significant increase (21%) in biomass production (from 0.83 to 0.96 g l^{-1}) was also achieved in the fermenter.

4.9. Optimization of process parameters for maximization of L-asparaginase production

In order to optimize the physical process parameters (initial pH of the medium, temperature, rpm of the shaking incubator and inoculum size) for enhanced production of L-asparaginase, experiments were performed according to the design matrix using central composite experimental design (Box and Draper, 1987). The design matrix and the corresponding results of observed and predicted responses (production of L-asparaginase) are shown in Table 4.11. The cell growth and L-asparaginase activity (U ml^{-1}) data inferred that the growth associated production of L-asparaginase (Table 4.11). The enzyme activity varied from 2.37 to 17.45 U ml^{-1}, where as cell growth from 0.31 to 1.17 g l^{-1}. By applying the multiple regression analysis on the experimental data, the following nonlinear equation was found to explain the production of L-asparaginase from *P. carotovorum* MTCC 1428.

$$Y_{SA} = -541.370 + 80.820A + 15.326B + 0.606C + 16.258D - 5.870A^2 - 0.266B^2 - 0.002C^2 - 3.564D^2 + 0.095AD - 0.017AB + 0.078AC + 0.001BC - 0.087BD + 0.028CD \quad (4.4)$$

Where, Y_{SA} = response (enzyme production), A = initial pH of medium, B = temperature, C rpm of the shaking incubator and D = inoculum size

Table 4.11. 2^4 full-factorial CCD matrix in real values and coded units (in parenthesis) with experimental and predicted values of L-asparaginase production from *P. carotovorum* MTCC 1428

Run Order	Uncoded and Coded levels				Specific Activity (U mg^{-1})		Enzyme activity[a] (U ml^{-1})	Cell growth[a] (g l^{-1})
	A	*B*	*C*	*D*	Observed[a]	Predicted		
1	6.5(-1)	28(-1)	140(-1)	2(-1)	34.10 ± 0.25	33.62	17.45 ± 0.42	1.17 ± 0.02
2	7.5(+1)	28(-1)	140(-1)	2(-1)	33.85 ± 0.29	32.71	17.23 ± 0.37	1.12 ± 0.03
3	6.5(-1)	36(+1)	140(-1)	2(-1)	25.42 ± 0.42	24.39	14.17 ± 0.51	1.01 ± 0.06
4	7.5(+1)	36(+1)	140(-1)	2(-1)	24.35 ± 0.22	24.24	13.98 ± 0.27	0.96 ± 0.04
5	6.5(-1)	28(-1)	220(+1)	2(-1)	25.38 ± 0.18	25.92	14.18 ± 0.42	1.02 ± 0.01
6	7.5(+1)	28(-1)	220(+1)	2(-1)	24.36 ± 0.29	23.65	14.28 ± 0.25	0.97 ± 0.03
7	6.5(-1)	36(+1)	220(+1)	2(-1)	16.67 ± 0.12	17.24	9.40 ± 0.51	0.65 ± 0.05
8	7.5(+1)	36(+1)	220(+1)	2(-1)	15.42 ± 0.28	15.74	9.31 ± 0.19	0.62 ± 0.04
9	6.5(-1)	28(-1)	140(-1)	4(+1)	27.30 ± 0.22	27.30	15.38 ± 0.22	0.99 ± 0.07
10	7.5(+1)	28(-1)	140(-1)	4(+1)	28.55 ± 0.51	26.54	16.49 ± 0.59	1.05 ± 0.08
11	6.5(-1)	36(+1)	140(-1)	4(+1)	17.41 ± 0.16	16.68	10.60 ± 0.36	0.75 ± 0.05
12	7.5(+1)	36(+1)	140(-1)	4(+1)	16.91 ± 0.89	16.69	9.61 ± 0.53	0.69 ± 0.04
13	6.5(-1)	28(-1)	220(+1)	4(+1)	25.36 ± 0.30	24.03	14.75 ± 0.93	1.02 ± 0.01
14	7.5(+1)	28(-1)	220(+1)	4(+1)	20.57 ± 0.28	21.92	12.20 ± 0.26	0.86 ± 0.03
15	6.5(-1)	36(+1)	220(+1)	4(+1)	12.52 ± 0.43	13.97	7.02 ± 0.39	0.49 ± 0.04
16	7.5(+1)	36(+1)	220(+1)	4(+1)	13.58 ± 0.29	12.62	7.87 ± 0.88	0.53 ± 0.03
17	7.0(0)	32(0)	180(0)	3(0)	33.42 ± 0.42	34.60	17.38 ± 0.59	1.13 ± 0.02
18	7.0(0)	32(0)	180(0)	3(0)	33.40 ± 0.16	34.60	17.24 ± 0.73	1.08 ± 0.01
19	7.0(0)	32(0)	180(0)	3(0)	33.65 ± 0.48	34.60	17.33 ± 0.46	1.08 ± 0.03
20	7.0(0)	32(0)	180(0)	3(0)	33.51 ± 0.03	34.60	17.31 ± 0.70	1.09 ± 0.02
21	6.0(-2)	32(0)	180(0)	3(0)	26.63 ± 0.28	26.56	14.03 ± 0.63	0.98 ± 0.03
22	8.0(+2)	32(0)	180(0)	3(0)	23.13 ± 0.01	24.30	12.69 ± 0.26	0.97 ± 0.02
23	7.0(0)	24(-2)	180(0)	3(0)	22.19 ± 0.03	23.52	12.34 ± 0.14	0.95 ± 0.07
24	7.0(0)	40(+2)	180(0)	3(0)	5.21 ± 0.76	4.99	2.37 ± 0.59	0.31 ± 0.04
25	7.0(0)	32(0)	100(-2)	3(0)	22.97 ± 0.62	25.26	11.85 ± 0.41	0.96 ± 0.04
26	7.0(0)	32(0)	260(+2)	3(0)	14.67 ± 0.32	13.49	8.21 ± 0.57	0.54 ± 0.03
27	7.0(0)	32(0)	180(0)	1(-2)	21.31 ± 0.14	21.76	11.44 ± 0.30	0.86 ± 0.02
28	7.0(0)	32(0)	180(0)	5(+2)	11.67 ± 0.34	12.33	6.05 ± 0.38	0.43 ± 0.04
29	7.0(0)	32(0)	180(0)	3(0)	33.70 ± 0.28	31.30	17.32 ± 0.57	1.11 ± 0.03
30	7.0(0)	32(0)	180(0)	3(0)	33.35 ± 0.21	31.30	17.39 ± 0.48	1.07 ± 0.02

[a]The observed values of L-asparaginase specific activity, enzyme activity and cell growth were the mean values of duplicates with standard deviation (Mean ± S.D.).

According to ANOVA of the L-asparaginase production, the quadratic model was accurate, as is evident from the Fisher, *F*-test (mean square regression: mean square residual is 123.19) with a very low probability value ($P_{model} > F$ is 0.0001) (Table 4.12). This indicates that the combined effects of all the independent variables significantly contributed to maximize the production of L-asparaginase. The goodness of the model was checked by coefficient of determination, R^2, which implied that only 2.39% of the total variation is not explained by the model. The Student *t* distribution and the corresponding *P* values, along with the parameter estimate are shown in Table 4.13. The *P* values of all linear and quadratic relationships between medium components and L-asparaginase production suggest that they are highly significant (*P* = 0.000). Also the interaction effect between rpm and inoculums size was found to be highly significant on the enzyme production.

Table 4.12. Analysis of variance (ANOVA) for quadratic model

Source	DF	SS	MS	F-value	Prob. (*P*)>F
Block	1	145.55	145.55	71.83	0.000
Regression	14	3494.76	249.62	123.19	0.000
Linear	4	1728.00	91.98	45.39	0.000
Square	4	1718.04	429.51	211.96	0.000
Interaction	6	48.73	8.12	4.01	0.003
Residual Error	44	89.16	2.02		
Lack-of-Fit	10	80.61	8.06	32.04	0.000
Pure Error	34	8.55	0.25		
Total	59	3729.48			

R^2=97.61%; Adj R^2 = 96.79%; SS, sum of squares; DF, degrees of freedom; MS, mean square.

Table 4.13. Student *t* test of the model coefficients estimated by multiple linear regression

Model term	Parameter estimate	SE coefficient	Computed *t*-value	*P*-value
Intercept	-541.370	55.4847	-9.757	0.000
Block	1.652	0.1949	8.475	0.000
A	80.820	11.8166	6.840	0.000
B	15.326	1.2187	12.576	0.000
C	0.606	0.1120	5.407	0.000
D	16.258	4.3725	3.718	0.001
A^2	-5.870	0.7688	-7.636	0.000
B^2	-0.266	0.0120	-22.175	0.000
C^2	-0.002	0.0001	-15.518	0.000
D^2	-3.564	0.1922	-18.542	0.000
AD	0.095	0.1258	0.759	0.452
AB	-0.017	0.0126	-1.350	0.184
AC	0.078	0.5033	0.155	0.877
BC	0.001	0.0016	0.550	0.585
BD	-0.087	0.0629	-1.377	0.175
CD	0.028	0.0063	4.407	0.000

The three-dimensional response surface plots were constructed to predict the L-asparaginase production for different values of the tested variables and to analyze the interactions among the variables. The response surface plots were constructed by plotting the response (specific activity of L-asparaginase) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels as shown in Fig. 4.7a-f. As shown in the Fig. 4.7a, c and e, temperature have shown significant influence on the production of L-asparaginase and maximum level of L-asparaginase production was achieved at temperature range from 28 to 32 ºC. However, effect of initial pH of medium on variation was shown not much significant on the production of L-asparaginase with other variables and achieved maximum at 2-3 % (v/v) (Fig. 4.7a, b and d). The interaction of rpm and inoculums size (P = 0.000) is very prominent as shown in Fig. 4.7f. Similar effect was observed for temperature

with inoculums size (Fig. 4.7e). However, the interaction of temperature with rpm (Fig. 4.7c, P = 0.585) and initial pH (Fig. 4.7a, b and d, P>0.184) was not prominent.

The optimum levels of initial pH of the medium, temperature, rpm of the shaking incubator and inoculums size were determined by maximizing the regression equation (Eq. 4.4) and were found to be 6.90, 29.8ºC, 157 rpm and 2.61 (%, v/v), respectively. Fig. 4.8 showed a satisfactory correlation between the experimental and predicted values (obtained from Eq. 4.4) of L-asparaginase production, wherein, the points cluster around the diagonal line which indicated the optimal fit of the model, since the deviation between the experimental and predicted values was minimum.

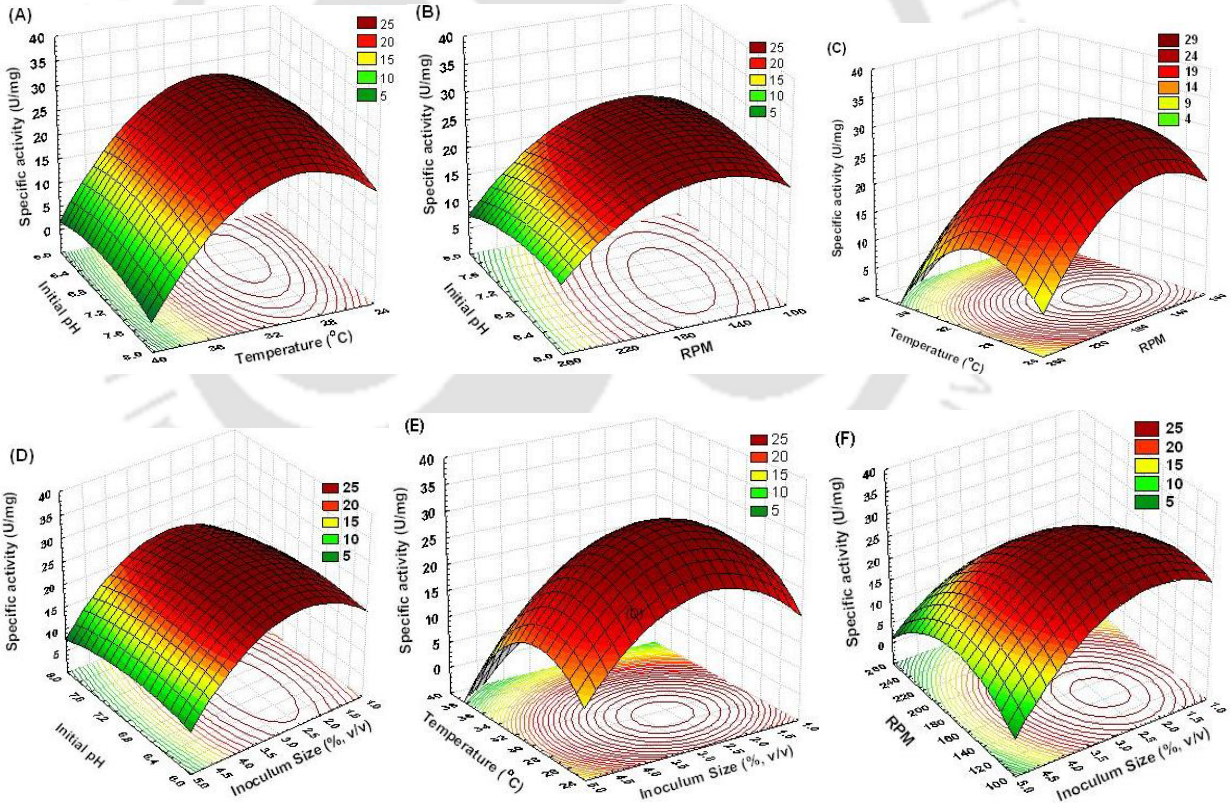


Fig 4.7. Three-dimensional response surface plot for L-asparaginase production, showing the interactive effects of (a) Initial pH and temperature (b) Initial pH and rpm (c) Temperature and rpm (d) Initial pH and inoculum size (e) Temperature and inoculum size (f) Rpm and inoculum size.

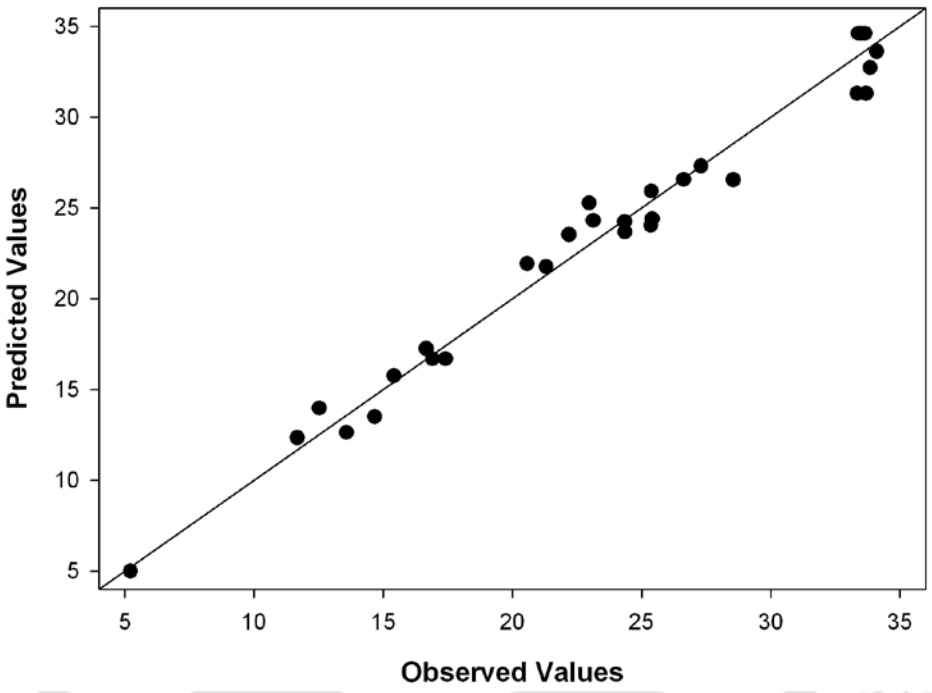


Fig. 4.8. Plot showing the distribution of experimental *vs*. predicted values of L-asparaginase

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) etc. To the best of our knowledge, we could not find any report in the literature on the optimization of significantly influencing physical parameters for the production of glutaminase-free L-asparaginase form *P. carotovorum* MTCC 1428 in submerged fermentation. However, a few reports are available for improved production of L-asparaginase using statistical methods. Abdel-Fattah and Olama, (2002) successfully used Plackett-Burman and Box-Behnken experimental designs for screening and optimization of process parameters to improve the production of L-asparaginase from *Pseudomonas aeruginosa* in solid state fermentation. Recently, 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.

4.10. Verification of model of optimized process parameters

Experiment was performed at these optimal levels of process physical parameters and production (specific activity) was found to be 35.24 U mg^{-1} of protein and it was in very good correlation with the predicted value (35.55 U mg^{-1} of protein). This is very much comparable with the reported values. The production of L-asparaginase under un-optimized level of physical parameters (initial pH 6.5, 30ºC, 180 rpm and inoculum size 2% (v/v)) and optimized medium was found to be 27.88 U mg^{-1} of protein (Kumar *et al.,* 2009). An enhancement of 26.39% was achieved in the production of L-asparaginase from *P. carotovorum* MTCC 1428 (Table 4.14). The verification revealed that a high degree of accuracy of the model of more than 99.0%, which is an evidence for the model prediction under the investigated conditions.

Table 4.14. Comparison of L-asparaginase production in the before and after optimization of chemical and physical process parameters.

Medium used	Shake flask/ fermenter	L-asparaginase production[a]		Enhanced Production (fold)	Productivity[a] (U l^{-1} h^{-1})
		Enzyme activity (U ml^{-1})	Specific activity (U mg^{-1})		
Un-optimized medium	Shake flask	0.76±0.01	3.35±0.32	--	63.33±0.85
RSM- chemically optimized	Shake flask	14.71±0.53	27.88±0.46	8.32	1225.83±44.23
RSM- chemically optimized	fermenter	15.39±0.33	28.87±0.37	8.62	1282.50±27.50
RSM- process optimized	Shake flask	17.81±0.67	35.24±0.71	10.52	1350.83±56.16
RSM- process optimized	fermenter	17.95±0.76	35.71±0.82	10.66	1404.16±63.35

[a]Mean values of duplicates with standard deviation (mean±SD)

The production of L-asparaginase was slightly higher (35.71 U mg^{-1} of protein) in the fermenter than in the shake flasks at optimal level of physical parameters. The increase in the overall productivity from 1350.83 to 1404.16 U l^{-1} h^{-1} was achieved (Table 4.14).

4.11. Purification of L-asparaginase

In order to collect adequate amount of enzyme for the purification process, L-asparaginase production was carried out in a batch bioreactor and intracellular enzyme was isolated as described in the section 3.11.1. The crude intracellular protein in the Tris HCl buffer (50 mM, pH 8.6) was precipitated by the addition of ammonium sulfate (80% saturation). The precipitated enzyme was dissolved in the minimal volume of Tris HCl buffer (50 mM, pH 8.6) followed by dialysis. The results of purification procedure of L-asparaginase from *P. carotovorum* MTCC 1428 are summarized in Table 4.15. The purification of glutaminase-free L-asparaginase was involved ammonium sulphate precipitation followed by anion exchange chromatography using DEAE cellulose column (Fig. 4.9a). Final purification was achieved by gel filtration chromatography using Sephadex G-100 (Fig. 4.9b). The partially purified L-asparaginase was found to be stable at basic pH of above 8.0. Hence, we opted to use alkaline Tris HCl buffer system (50 mM, pH 8.6) in all the purification steps.

Table 4.15. Summary of steps employed and the results in purification of L-asparaginase from *P. carotovorum* MTCC 1428

Step	Collected volume (ml)	Total activity (IU)	Total Protein (mg)	Specific activity (IU mg^{-1})	Purification (fold)	Yield (%)
Crude extract	4080	62542	2233.64	28.02	--	100.00
$(NH_4)_2SO_4$ precipitation	408	43876	270.19	192.12	6.86	70.15
DEAE cellulose column	41	36260	24.21	1250.54	44.63	48.38

Sephadex G-100	4	26296	13.01	2020.91	72.12	42.05

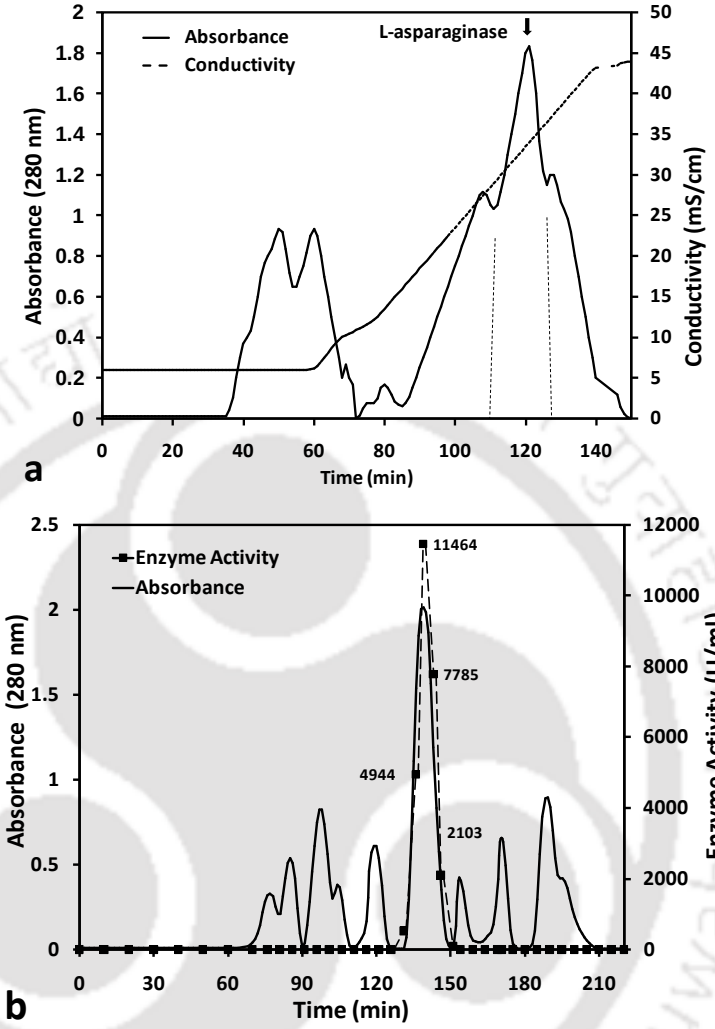


Fig. 4.9. Elution profile of ion exchange and gel exclusion chromatography employed in purification of L-asparaginase from *P. carotovorum* MTCC 1428 (a) Total protein and gradient used in DEAE cellulose column (b) Total protein and total activity from Sephadex G-100 column.

In most of the cases, ion exchange chromatography has been used for purification of L-asparaginase, since these enzymes have *pI* value of 8.7 (*Erwinia* species) or 5.0 (*E. coil*) (Muller and Boos, 1998). The retention of L-asparaginase on DEAE cellulose column is dependent on pH of the buffer used. The adsorbed L-asparaginase on DEAE cellulose column was eluted with gradient of 0-200 mM NaCl. Using Sephadex G-100 chromatography, glutaminase-free L-asparaginase was

purified to homogeneity with 42.02% yield, which is so far the first report on L-asparaginase purification from *P. carotovorum* MTCC 1428. The enzyme was purified approximately 72 fold with specific activity of 2020.91 U mg^{-1} and found to be homogeneous, as evident from SDS-PAGE. L-asparaginase from *Pseudomonas aeruginosa* 50071 has been purified in CM-Sephadex C-50 column up to 106-fold with 43% yield (El-Bessoumy *et al.,* 2004). L-asparaginase from *Streptomyces albidoflavus* has been purified 99.3-fold with 40% recovery in the final CM-Sephadex C-50 purification step (Narayana *et al.,* 2008).

4.12. Characterization of purified L-asparaginase

4.12.1. Effect of pH and temperature on activity and pH stability

Figure 4.10a shows that the purified L-asparaginase of *P. carotovorum* MTCC 1428 was active over a broad range of pH (7.5-9.0) with at an optimum pH of 8.5. Maximum L-asparaginase activity was obtained at pH 8.5 and 40 ºC, which decreased significantly (50%) when the pH was lowered to 6.5 (Fig. 4.10a 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 about 75% of the enzyme activity was retained at pH 7.4. The enzyme showed stability at alkaline pH range (pH 8.0–10.0) as it retained 90% of its original activity after incubation for 24 h (Fig. 4.10a). Majority of the L-asparaginases from *Erwinia* species showed alkaline pH optima (8.0-9.0) except L-asparaginase from *E. coli*, which exhibited acidic pH optimum of 5.0-6.0 (Muller and Boos, 1998). The purified enzyme exhibited maximum activity at a temperature of 40ºC. The activity decreased sharply above the optimum temperature range (35-45ºC) with almost 75% loss of its original activity at 60ºC (Fig. 4.10b). No significant enzyme activity was

lost when the purified enzyme was pre-incubated at 40ºC for 60 min, beyond this temperature the enzyme became increasingly unstable.

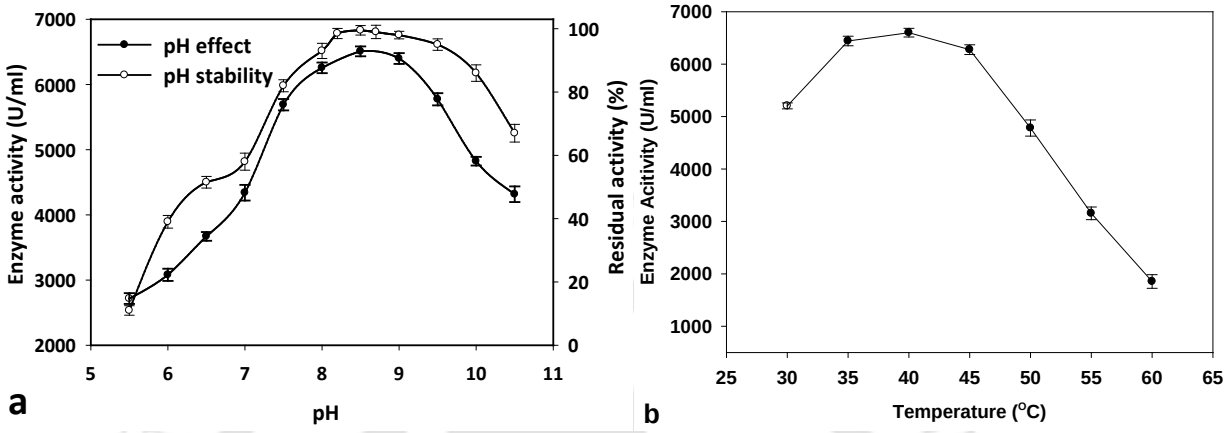


Fig. 4.10. Effect of physical conditions on L-asparaginase activity (a) pH of assay buffer and stability of purified L-asparaginase at different pH levels after incubation for 24 h at 4±1°C (b) Temperature of assay reaction.

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

The most important physical factors, which influence the enzymatic reaction rate, are pH and the temperature of incubation with the substrate. Each enzyme has a characteristic pH and temperature optima beyond which the reduction in activity is observed. The suitable temperature and pH for L-asparagine-L-asparaginase system was 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 L-asparaginase (keeping at constant temperature). The results clearly showed that the maximum activity was observed when the pH was varied from 8.0 to 9.0 (Fig. 4.10a). Similarly, experiments were performed to study the effect of temperature on the activity of L-asparaginase and found that the maximum activity was obtained when the temperature was varied between 35

to 45ºC (Fig. 4.10b). Hence, experiments were performed at various combinations of pH and temperature. The design matrix and the corresponding results of observed and predicted responses (L-asparaginase activity) are shown in the Table 4.16. The enzyme activity varies from 5237 to 6645 U ml^{-1}. By applying the multiple regression analysis on the experimental data, the following nonlinear equation was found to explain the dependence of L-asparaginase activity on pH and temperature of incubation.

$$Y_{\text{enzyme activity}} = -77954.2 + 15269.8A + 1008.9B - 927.7A^2 - 14.1B^2 + 11.7AB \quad (4.5)$$

Where, A = pH and B = temperature.

Table 4.16. Experimental design and results for the activity of L-asparaginase from *P. carotovorum* MTCC 1428 at various combinations of pH and temperature.

Run order	Uncoded and coded levels		Enzyme activity (U ml^{-1})	
	pH	Temperature (ºC)	Observed	Predicted
1	8.0(-1)	37(-1)	6244±14	6229
2	9.6(+1)	37(-1)	5759±20	5927
3	8.0(-1)	43(+1)	6069±20	6078
4	9.6(+1)	43(+1)	5669±25	5860
5	8.6(0)	40(0)	6598±09	6485
6	8.6(0)	40(0)	6604±07	6485
7	8.6(0)	40(0)	6607±15	6485
8	7.4(-2)	40(0)	5574±13	5621
9	9.8(+2)	40(0)	5237±11	5101
10	8.6(0)	34(-α)	6331±13	6298
11	8.6(0)	46(+α)	6137±14	6081
12	8.6(0)	40(0)	6645±14	6697
13	8.6(0)	40(0)	6635±21	6697

14	8.6(0)	40(0)	6634±21	6697

The results were analyzed using the ANOVA as appropriate to the experimental design used (Table 4.17). According to the ANOVA of the quadratic regression model, the model is highly significant, as is evident from the Fisher *F* test (mean square regression: mean square residual is 79.81) with a very low probability value ($P_{model} > F$ is 0.0001). This indicates that the combined effects of pH and temperature significantly contributed for maximizing the L-asparaginase activity. The goodness of the model was checked by coefficient of determination, R^2, which implies that the sample variation of 95.02% for the dependence of L-asparaginase activity is attributed to pH and temperature of incubation and also only 4.98 % of the total variation is not explained by the model.

The Student's *t* distribution and the corresponding *P* values, along with the parameter estimate are shown in Table 4.18. The *P* values of all linear and quadratic relationships between process parameters and L-asparaginase activity under assay conditions suggest that they are highly significant ($P<0.0004$). However, the interaction effect between pH and temperature is lower than linear effect ($P<0.0616$), but accounted appreciably. In addition, it was also found that the coefficient for pH is very much larger than coefficient for temperature. This suggests that any change in pH has large effect on enzyme activity of L-asparaginase than temperature in the range studied.

The 2D contour plot explains the behavior of the system and enzymatic activity over independent variables, pH and temperature are shown in Fig. 4.11. The enzyme activity is higher at a range of temperature (36-41ºC) and moderate pH (8.4-8.6). This equation was optimized and solved by MINITAB optimizer. The optimum levels of temperature and pH

were found to be 39.3ºC and 8.49, respectively. In order to verify the optimal conditions, the experiments were performed at central point and optimal levels of variables. These conditions have showed higher enzyme activity (6856 U ml^{-1}) value as compared to that used presently for the analysis of enzyme activity. After optimization, L-asparaginase activity was increased by 200 U ml^{-1} in the purified sample.

Table 4.17. Analysis of variance (ANOVA) for L-asparaginase activity: effect of pH and temperature

Source	DF	SeqSS	Adj SS	Adj MS	F	P
Regression	5	5492844	5492844	1098569	79.81	0.0001
Linear	2	476299	2657906	1328953	96.55	0.0001
Square	2	5012974	5012974	2506487	182.10	0.0004
Interaction	1	3570	3570	3570	0.26	0.0616
Residual Error	21	289046	289046	13764		
Lack-of-Fit	3	285089	285089	95030	432.35	0.0005
Pure Error	18	3956	3956	220		
Total	27	5799995				

R^2= 95.02%; R^2(adj) = 93.59%

Table 4.18 Model coefficient estimated by multiple linear regressions

Term	Coef	SE Coef	T	P
Constant	-77954.2	9610.25	-8.112	0.0001
pH	15269.8	1246.95	12.246	0.0001
Temperature	1008.9	252.38	3.998	0.0001
pH*pH	-927.7	48.77	-19.021	0.0004
Temperature*Temperature	-14.1	1.95	-7.228	0.0004

pH*Temperature	11.7	23.04	0.509	0.0616

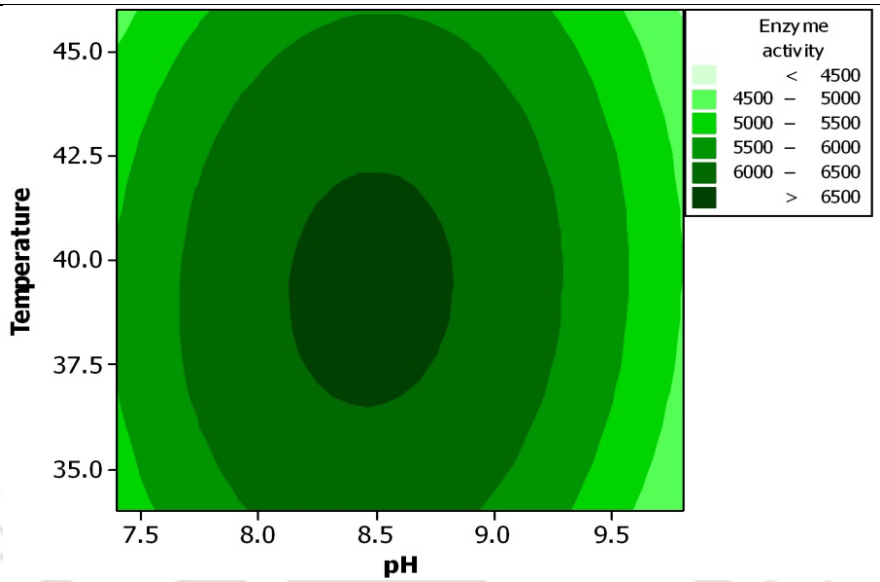


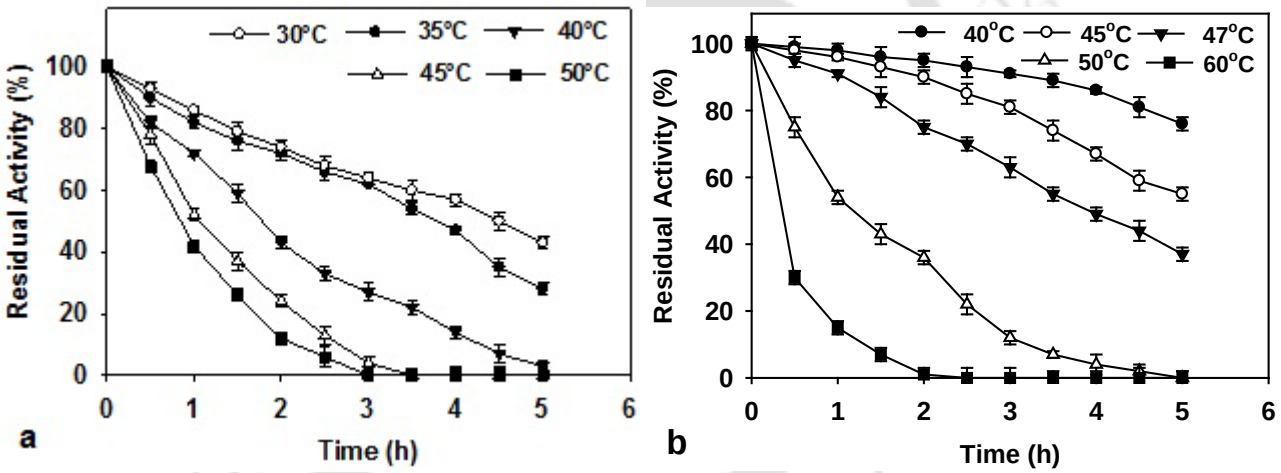
Fig. 4.11. 2D contour plot of L-asparaginase activity at different levels of pH and temperature of incubation.

4.12.3. Enzyme deactivation studies

In this study, L-asparaginase was deactivated under various combinations of pH and temperature as discussed in materials and methods section 3.12.11 and extent of deactivation is measured by deactivation rate. The deactivation rate is proportional to the active enzyme concentration, and k_d (deactivation rate constant) is the proportional constant. The deactivation process is modeled as first-order kinetics and the deactivation rate constant was evaluated. The effect of temperature on deactivation has been studied and the results are shown in Fig. 4.12a-c. The minimum value of k_d observed for L-asparaginase was 0.041 min^{-1} (Table 4.19). The combinations of pH and temperature at which the above mentioned minimum deactivation rate constant have been observed to be 8.6 and 40ºC, respectively. The deactivation process was found to be faster at pH 7.6 than at alkaline pH (8.6, 9.6) for

L-asparaginase. Naidu and Panda, (2003) observed that the similar effect of pH on deactivation rate constant reported in this study. This is possibly due to disulfide exchange,

which usually occurs at near neutral and alkaline conditions (Munch and Tritsch, 1990). 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 condition.



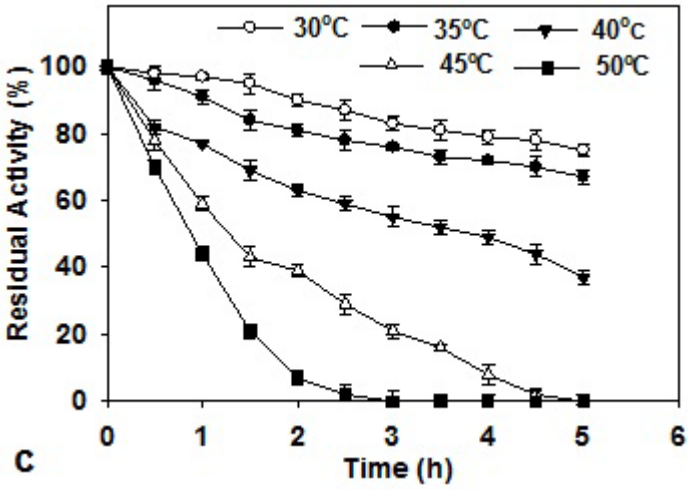


Fig. 4.12. Thermal stability of the purified L-asparaginase at (a) pH 7.6 (b) pH 8.6 and (c) pH 9.6.

Table 4.19. Effect of temperature on deactivation constant (k_d) and half life time ($t_{1/2}$) of the purified enzyme

pH	Temp (°C)	K_d (h^{-1})	$t_{1/2}$ (h)
7.6	30	0.20	3.33
	35	0.15	4.50
	40	0.54	1.27
	45	0.87	0.79
	50	1.04	0.66
8.6	40	0.04	16.9
	45	0.09	7.07
	47	0.17	3.96
	50	0.75	0.91
	60	2.09	0.33
9.6	30	0.08	8.15
	35	0.05	12.37
	40	0.19	3.57
	45	0.64	1.08
	50	1.34	0.51

R^2 of plot of $\ln(E_d/E)$ verus t is 0.979.

4.12.4. Estimation of thermodynamic parameters

The change in enthalpy and entropy was calculated by transition state theory (Eq. 3.8). The results are shown in Table 4.20 for purified L-asparaginase from *P. carotovorum* MTCC 1428. The entropy value was found to be negative for L-asparaginase at pH 7.6. The negative values of entropy of deactivation of L-asparaginase suggested that the denaturation may be due to the compactation of the reacting enzyme molecule and the ordering of solvent molecules (Foster, 1980). 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 incubated at higher levels of pH. The probable reason is that the enzyme gets unfolded during deactivation and the negative sign is due to the formation of charged particles around the enzyme molecule with increase in pH or it may be due to the ordering of solvent molecules. The increase in ΔS^* indicates

an increase in the number of protein molecules in a transition activated state, which in turn, gives lower values of ΔG^*. The values of ΔG^* (calculated from Eq. (3.9)) are given in Table 4.20 for the enzyme. The decrease in entropy and enthalpy values was observed with increase in pH. Probably, at higher pH, the stable three dimensional structure of enzyme gets compressed, resulted in decrease in residual activity. To gain a deeper insight into the mechanism and specificity of L-asparaginase, the temperature-dependence of the catalytic activity was investigated. The temperature dependency of first-order deactivation rate constant was studied by Arrhenius equation (Eq. 3.11). The activation energy E and frequency factor k_0 were estimated from equation (Eq. 3.11) and they are shown in Table 4.20. It was found that the maximum deactivation energy at optimum pH and starts decreasing at higher range of pH for this enzyme.

Table 4.20. Estimated thermodynamic parameters during the thermal deactivation of the purified L-asparaginase from *P. carotovorum* MTCC 1428[a]

pH	ΔH (KJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	E (KJ mol^{-1})	k_0 (hr^{-1})	ΔG (KJ mol^{-1})
7.6	78.14	− 70.66	80.75	1.0×10^{6}	99.56-100.97[b]
8.6	175.66	221.57	178.67	2.2×10^{21}	106.27-101.84[c]
9.6	126.35	78.41	128.87	6.1×10^{13}	102.58-101.01[d]

[a]R^2 of plot of $\ln(k_d/T)$ verus 1/T is 0.965; R^2 of plot of $\ln(k_d)$ versus 1/T is 0.952; [b]The temperature range is 30-50°C; [c]The temperature range is 40-50°C; [d]The temperature range is 30-50°C.

Kapat and Panda, (1997) and Naidu and Panda, (2003) were also reported the similar observation on temperature dependency of deactivation rate constant for thermal deactivation of chitinase from *Trichoderma harzianum* and pectolytic enzymes from *Aspergillus niger*, respectively. For L-asparaginase, the deactivation energy increased at higher temperature suggesting that L-asparaginase require more amount of energy to deactivate. This is in agreement with the result that the L-asparaginase is more stable at pH 8.6 with lower temperature rather than pH 9.6 and 7.6 at higher temperature.

4.12.5. Effect of various effectors and specificity

The enzyme activity was determined in the presence of different modifiers. The results are presented in Table 4.21a. Different metal ions, *viz.*, Hg^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, and Zn^{2+} were observed to be very detrimental for enzymatic activity, whereas Na^{+} and K^{+} acted as a moderate enhancer. Among the amino acids tested, only L-cysteine and L-histidine proved to be stimulators of the enzyme activity, while others had no effect. Inhibition of enzyme activity with different metal ions and stimulation with the metal chelators, *viz.*, EDTA and cysteine, inferred that the enzyme is not a metalloprotein. Moreover, inhibition of enzyme activity in the presence of Hg^{2+}, Cd^{2+}, Zn^{2+} etc. might be indicative of essential vicinal sulfhydryl group(s) of the enzyme for productive catalysis. Furthermore, stimulation of the enzyme activity with the reducing agents, 2-mercaptoethanol and gltutathione, and inhibition in the presence of thiol group blocking reagents such as p-chloromercuribenzoic acid and iodoactamide provided additional proof for the role of sulfhydryl group(s) in the catalytic activity of the enzyme. Similar type of variation in the activity of purified L-asparaginase from *Erwinia carotovora* was observed in the presence of thiol protecting and thiol blocking reagents (Warangkar and Khobragade, 2010). The enzyme completely lost its activity at 2.0 M urea, and only 21% activity was retained at 2.0 mM of SDS.

The substrate specificity of the purified enzyme is presented in Table 4.21b. No positive hydrolysis was observed when 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, N- α-acetyl-L-asparagine 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 and no glutaminase activity was observed with L-glutamine as substrate (Table 4.21b). Therefore, the novel purified glutaminase-free L-asparaginase reported in this study will be an advantageous and value-added product.

Table 4.21. (a) Influence of different effectors on L-asparaginase activity[a] (b) Substrate specificities of purified L-asparaginase from *P. carotovorum* MTCC 1428

a

Addition	Conc. (mM)	Relative activity (%)[a]
No Addition	--	100
Na^+ (NaCl)	50	113
K^+ (KCl)	150	129
Mg^{2+} ($MgCl_2$)	40	74
Ca^{2+} ($CaCl_2$)	150	28
Mn^{2+} ($MnCl_2$)	100	21
Zn^{2+} (ZnCl)	100	11
Fe^{3+} ($FeCl_3$)	100	52
Ni^{2+} ($NiCl_2$)	10	57
Cu^{2+} ($CuCl_2$)	10	0
Cd^{2+}($CdCl_2$)	10	0
Hg^{2+}($HgCl_2$)	10	0
EDTA	5	101
Iodoacetamide	5	77
L-Cystine	25	127
L-Histidine	25	112
Gltutathione	0.5	105
2-Marceptoethanol	0.5	117
*p*CMBA	0.5	0
SDS	2.0	21
Urea	2.0 (M)	0

[a] 100% of activity correspondent to 0.5 U of enzyme;
*p*CMBA = *p*-Chloromercuribenzoic acid

b

Substrate added	Conc. (mM)	Relative activity (%)[a]
L-asparagine	10	100
D-asparagine	10	3
DL-asparagine	10	2
L-glutamine	10	N.D.
D-glutamine	10	1
D-aspartic acid	10	N.D.
DL-aspartic acid	10	1
L-glutamic acid	10	N.D.
Succinamic Acid	10	1
L-aspartic acid amide	10	N.D.
L-asparagine-t-butyl ester HCl	10	1
BOC-L-asparagine	10	N.D.

[a] 100% of activity correspondent to 0.5 U of enzyme

N.D.- Not detected

4.12.6. Kinetics of purified enzyme

The kinetic study of the purified L-asparaginase from *P. carotovorum* MTCC 1428 was performed as described in section 3.12.5. The values of K_m and V_{max} of L-asparagine were determined by steady state kinetic analysis (Fig. 4.13). Plots of the reaction velocities versus substrate concentration (0-2.0 mM) displayed typical hyperbolic saturation curves, which were fitted to the Michaelis-Menten equation (R^2=0.98), yielding the kinetic constants. The K_m and V_{max} values of purified L-asparaginase from *P. carotovorum* MTCC 1428 were 0.657 mM and 4.45 U μg^{-1}, respectively. This indicates that the high affinity of the enzyme towards the substrate, L-asparagine. The substrate affinity in terms of K_m is very low (0.657 mM), which is 5–9 times lower than the reported cytosolic L-asparaginase (Kumar *et al.,* 2010).

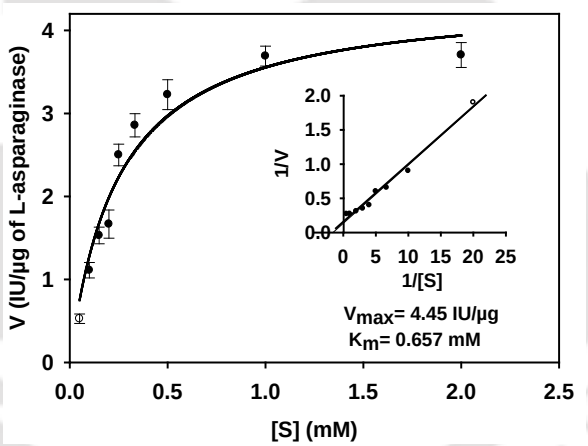


Fig. 4.13. Plot of the reaction velocities (*V*) *vs.* substrate concentration (S: 0.05-2.0 mM) fitted to the Michaelis–Menten equation (R^2=0.98) and determination of K_m and V_{max} of purified L-asparaginase for L-asparagine by non-linear regression analysis of experimental steady-state data. (*Inset*) The corresponding Lineweaver-Burk plot for L-asparaginase catalyzed reaction

L-Asparaginase of different microorganisms has different substrate affinities and probably plays different physiological roles in the enzyme activity. Higher K_m values (2.5 mM and 3.5 mM) for L-asparaginase from *C. glueamicum* and *E. coli*, respectively, have been reported (Willis and Woolfolk, 1974). On the other hand, a lower K_m value (0.074 mM) was obtained for

L-asparaginase from *Vibrio succinogenes* (Willis and Woolfolk, 1974). Turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) of purified L-asparaginase from *P. carotovorum* MTCC 1428 for L-asparagine were found to be 2.751×10^3 s^{-1} and 4.187×10^6 $M^{-1}s^{-1}$, respectively. The kinetic parameters determined in this study were comparable with those reported for many bacterial L-asparaginases (Kotzia and Labrou, 2005; Kotzia and Labrou, 2007).

4.12.7. Molecular mass determination

The molecular weight of subunit of L-asparaginase was found to be approximately 36.5 kDa by SDS-PAGE analysis. The purified L-asparaginase had an approximate molecular weight of 146±2.4 kDa as assessed by Native PAGE (Fig. 4.14ab). The isoelectric focusing showed that the *pI* of purified L-asparaginase from *P. carotovorum* MTCC 1428 is nearly 8.4 (Fig. 4.14c). The enzymes from *Erwinia* species and *E. coil* have *pI* value of 8.7 and 5.0, respectively (Muller and Boos, 1998). Recently, Abakumova *et al.,* (2009) reported that the *pI* of L-asparaginase isolated from *Yersinia pseudotuberculosis* was 5.4. 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 dimers. Despite this fact, the active enzyme is always a tetramer (Khushoo *et al.,* 2004; Swain *et al.,* 1993). Native molecular weight of L-asparaginase was determined by gel filtration on Sephdex G-100 column and was found to be 146 ± 2 kDa for purified L-asparaginase (Fig. 4.15a). The intact L-asparaginase was found to be homotetramer of size 144.42 kDa and its subunit size of 36.10 kDa from MALDI-TOF mass spectrum analysis (Fig. 4.15b). The data obtained from MALDI-TOF MS and gel exclusion chromatography were supported by the SDS and Native PAGE analysis.

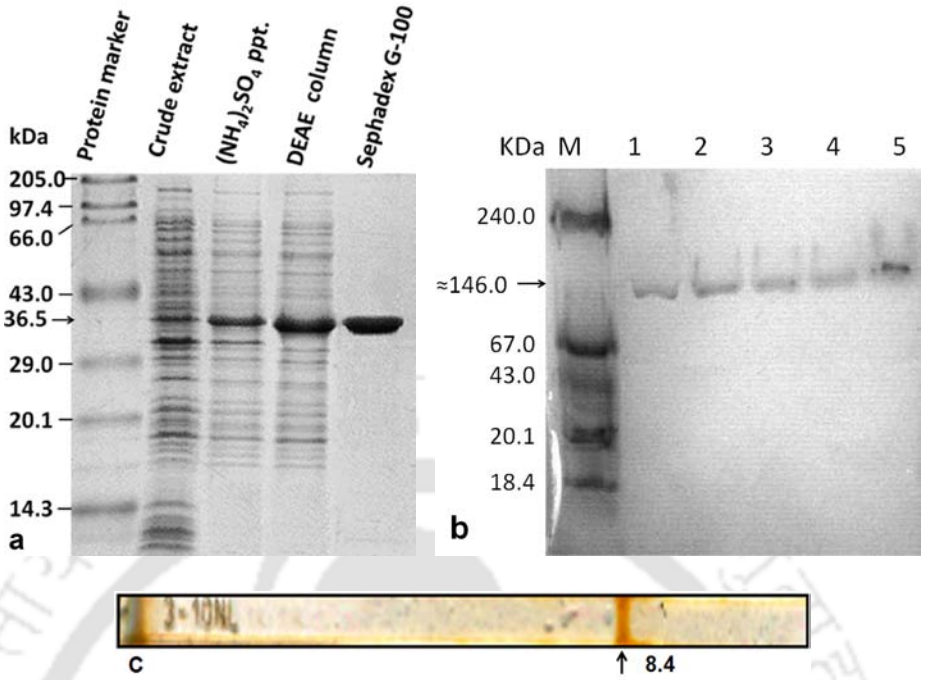


Fig. 4.14. Molecular weight and *pI* analysis of purified L-asparaginase from *P. carotovorum* MTCC 1428 (a) SDS PAGE on 12.5% gel of stained with coomassie brilliant blue (CBB) R-250 (b) Native PAGE on 7.5% gel of stained with CBB R-250: M, Native PAGE marker; 1 and 2, 15 µg purified L-asparaginase; 3 and 4, 10 µg purified L-asparaginase; 5, 20 µg purified L-asparaginase (c) Silver stained IEF strip containing purified L-asparaginase for determination of *pI*.

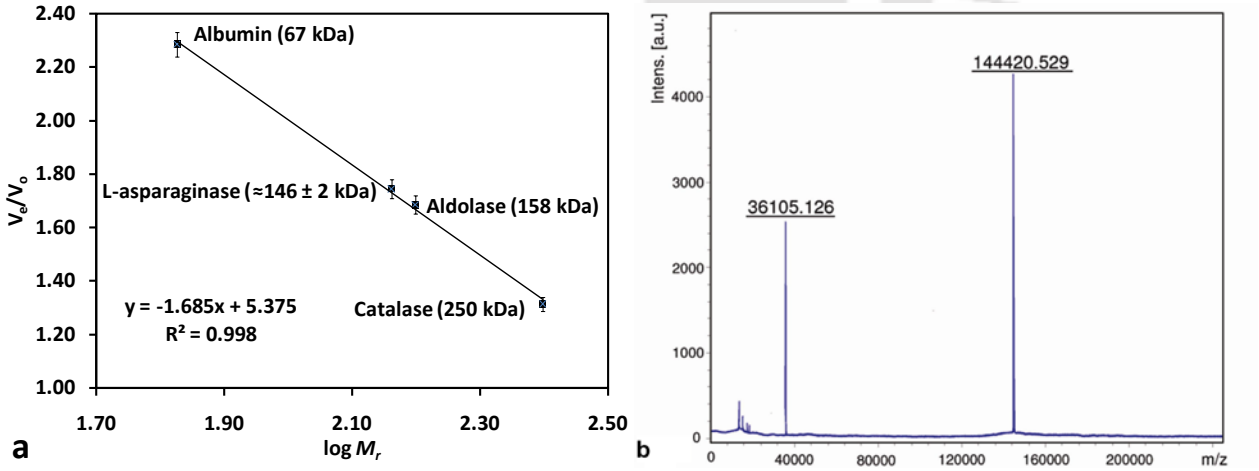


Fig. 4.15. Molecular weight estimation of purified L-asparaginase from *P. carotovorum* MTCC 1428 (a) Sephadex G-100 gel exclusion chromatography (b) MALDI-TOF mass spectrum of purified L-asparaginase from *P. carotovorum* MTCC 1428. First peak due to single subunit size of L-asparaginase (36105.126 Da) and second peak homotetramer size of L-asparaginase (144420.529 Da)

4.12.8. N-terminal sequence

The purity of the final L-asparaginase preparation was evaluated by SDS-PAGE, which showed the presence of a single polypeptide chain. Furthermore, the purity of the enzyme preparation was assessed by N-terminal amino acid sequence analysis. N-Terminal sequencing of L-asparaginase showed that 10 amino acid residues in the order of NLPXIVIXAT (X, are the unidentified residues at positions 4 and 8 from the N-terminal). N-terminal amino acid sequence of cytosolic L-asparaginase from *P. carotovorum* MTCC 1428 almost matched by protein database blast with L-asparaginases of *Erwinia* species (Table 4.22). Figure 4.16 shows the amino acid sequence alignments resulting from the BLAST search of L-asparaginases by ClustalW. L-Asparaginase from *P. carotovorum* MTCC 1428 showed a maximum of 80% N-terminal sequence identity with the L-asparaginase I of *E. carotovora* enzyme, whereas significantly lower identity was observed with L-asparaginase II of *E. carotovora* and L-asparaginases from other *Erwinia* species. On the contrary, no match was found with L-asparaginase I of *E. carotovora*. On analysis of the ClustalW result, the predicted amino acid at 4^{th} and 8^{th} place from N-terminal would be N and L, and corresponding gene sequence would be AAC and CTG.

Table 4.22. N-terminal sequence of purified cytosolic L-asparaginase blast matched of the various L-asparaginases

Accession	Protein names	organism	Gene
Q6D4Q0	L-asparaginase I	*E. carotovora* subsp. *atroseptica*	ansA (ECA2340) (1017 bp)
B2VKW1	L-asparaginase 1	*E. tasmaniensis* (DSM 17950)	ansA (ETA15640) (1011bp)
Q6Q4F4	L-asparaginase	*E. carotovora* subsp. *atroseptica*	ansB1(ECA1102) (1041 bp)
Q7WWK9	L-asparaginase	*E. carotovora* subsp. *atroseptica*	lanS (1050 bp)
Q6Q4F3	L-asparaginase	*Erwinia chrysanthemi*	(1038bp)
P06608	L-asparaginase	*Erwinia chrysanthemi*	ansB (asn) (1042 bp)
Q6CZM5	L-asparaginase II	*E carotovora* subsp. *atroseptica*	ansB2 (ECA4126) (1050 bp)
Q6DAX0	L-asparaginase	*E carotovora* subsp. *atroseptica*	ECA0132 (948bp)

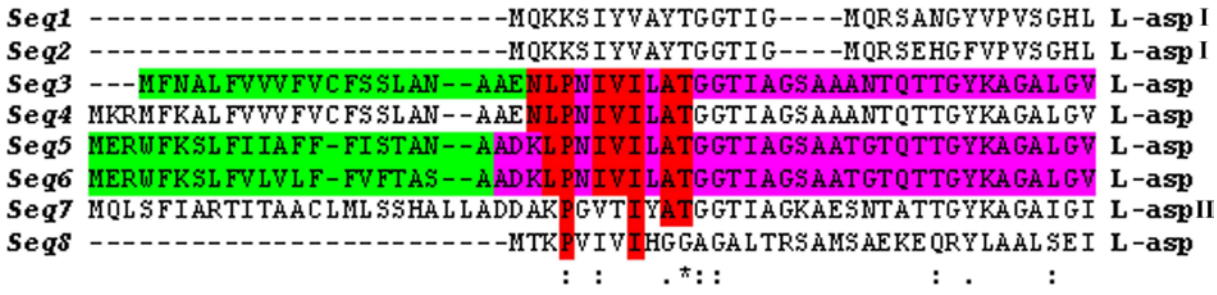


Fig. 4.16. N-Terminal Sequence alignments of L-asparaginases from *Erwinia* species. The alignments were produced using ClustalW. NCBI accession number for L-asparaginases are in brackets: Seq1 – *E. carotovora* subsp. *atroseptica* (Q6D4Q0); Seq2 – *E. tasmaniensis* (B2VKW1); Seq3 – *E carotovora* subsp. *atroseptica* (Q6Q4F4); Seq4 – *E. carotovora* subsp. *atroseptica* (Q7WWK9); Seq5 – *Erwinia chrysanthemi* (Q6Q4F3); Seq6– *Erwinia chrysanthemi* (P06608); Seq7 – *E. carotovora* subsp. *atroseptica* (Q6CZM5); Seq8 – *E carotovora* subsp. *atroseptica* (Q6DAX0). L-asp I : L-asparaginase I (cytoplasmic); L-asp : L-asparaginase (location not known); L-asp II : L-asparaginase II (periplasmic).

4.12.9. In vitro cytotoxicity of L-asparaginase

The therapeutic potential of purified L-asparaginase from *P. carotovorum* MTCC 1428 was evaluated by *in vitro* cell culture experiments using three different human caner cell lines (HepG2, MCF-7 and HeLa). The qualitative determination of anti-cancer activity was assessed by inverted light microscopic observations, where it was seen that the cells treated with L-asparaginase have under gone drastic morphological changes (Fig. 4.17). While the morphology of the untreated cells remained unaffected. The morphological features of the treated cells suggested that the cells were prone to apoptosis. The quantitative estimation of L-asparaginase activity against the selected cell lines was done by a simple, rapid and efficient spectrophotometry based MTT assay (Mosmann, 1983). The water soluble yellow coloured MTT was reduced to a water insoluble purple coloured formazan complex by mitochondrial reductases that were active only in living cells. The complex was subsequently dissolved in DMSO and the absorbance of the coloured solution was read in a spectrophotometer at 570

nm. The intensity of purple colour complex can be directly correlated to the number of viable cells. The viability (%) was estimated using Eq. (3.12) and a dose response curve was plotted (Fig 4.17). From the trend line the IC_{50} values were calculated, which were found to be 103.65, 88.81 and 82.21 U ml^{-1} against HepG2, MCF-7 and HeLa, respectively. It was seen that the purified L-asparaginase was active against all the three cancer cell line tested.

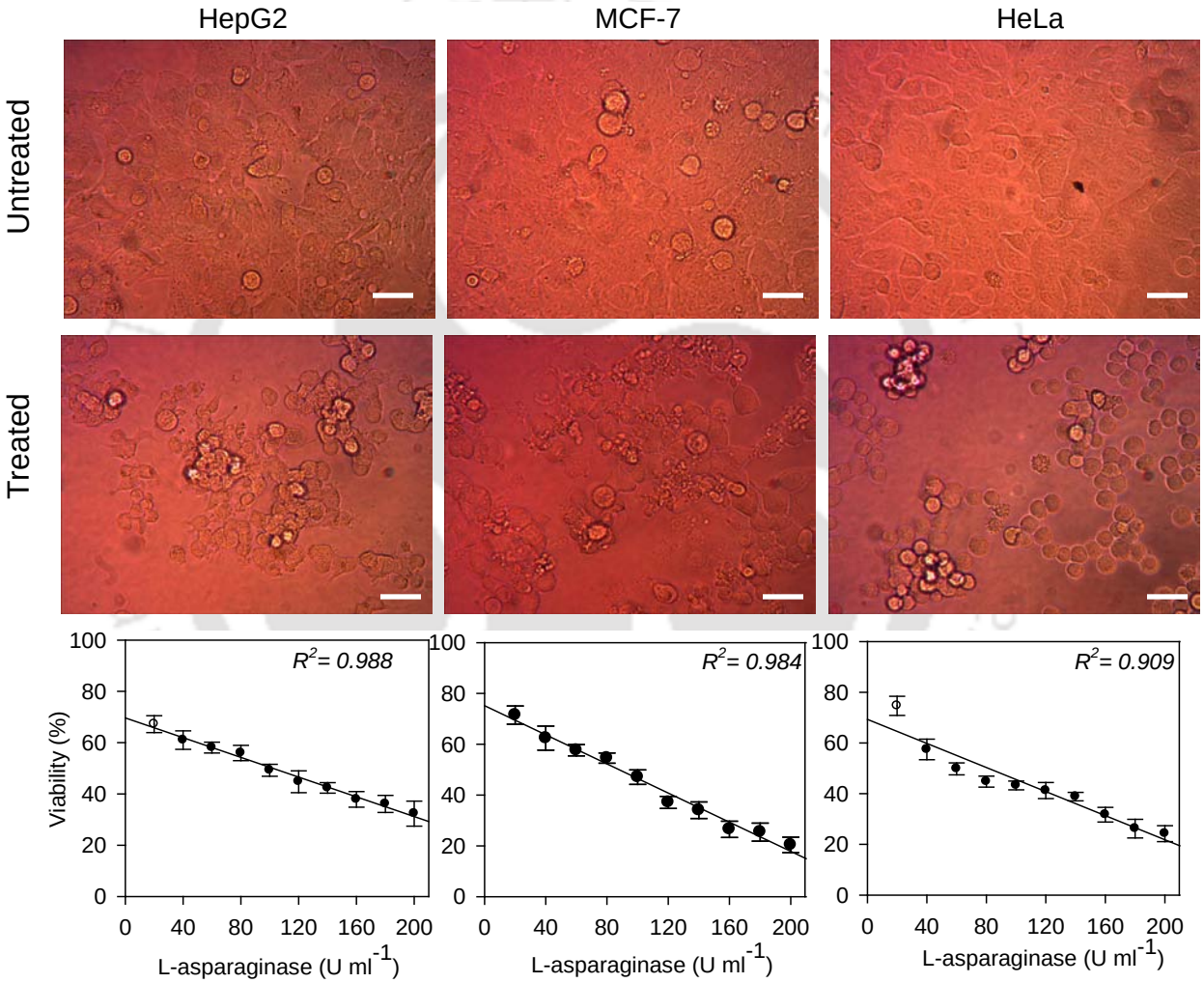


Fig. 4.17. Qualitative and quantitative determination of anti-cancer activity of cytosolic purified L-asparaginase from *P. carotovorum* MTCC 1428 on three different cancer cell lines. As observed under inverted light microscope, in comparison to untreated cells, the L-asparaginase treated cells have undergone drastic morphological changes which resembled apoptotic mode of cell death. The viability (%) was estimated by MTT assay and a dose response curve was drawn. Bar represents 25 µm size.

Such data are in accordance with the results obtained by Asselin *et al.*, (1989) who quantified cell killing both *in vitro* and *in vivo* in patients with ALL undergoing treatment with L-asparaginase as a single agent. L-asparaginase can be effective in chemotherapy when the IC_{50} value less than 15000 U ml^{-1} (Cappelletti *et al.,* 2008). Cytotoxicity results of purified L-asparaginase from *P. carotovorum* MTCC 1428 against these three cell lines comparable with the recently reported IC_{50} values of L-asparaginase from *E. coli*, *Erwinia carotovora*, *Bacillus circulans* and *Helicobacter pylori* (Cappelletti *et al.,* 2008; Abakumova *et al.,* 2009; Prakasham *et al.,* 2010). Pritsa *et al.,* (2001) was tested antiproliferative activity of the purified L-asparaginase from *T. thermiphilus* against the various type of human cell lines and found most effective against HeLa (cervical cancer) and SK-N-MC (primitive neuroectodermal tumor) cell lines.

4.13. Production of L-asparaginase by *P. carotovorum* MTCC 1428 in batch bioreactor

To study the effect of pH controlled/uncontrolled and DO level on the production of L-asparaginase by *P. carotovorum* in bioreactor, experiments were carried out at different DO levels (uncontrolled, 10, 20 and 30%) and pH (controlled and uncontrolled). All these experiments were performed either at an agitation and aeration rate of 160 rpm and 1.5 vvm, respectively or cascading mode (agitation 160-600 rpm, aeration rate 1.5 vvm). The activity of L-asparaginase, cell growth, DO, and glucose and L-asparagine consumption profile by *P. carotovorum* were monitored for each experiment (Fig. 4.18a-f). Highest activity of L-asparaginase was found to be 17.97 U ml^{-1} at 12 h of fermentation at 20% DO level (Fig. 4.18a, Table 4.23).

Table 4.23. Strategies used to evaluate the effect of controlled pH[a] (8.5) after 12 h and DO level on L-asparaginase production[b] from *P. carotovorum* MTCC 1428 in batch bioreactor.

	DO level (%)	pH	Agitation (rpm)	Maximum L-asparaginase activity (U ml^{-1})
Batch 1	Uncontrolled	Uncontrolled	160	16.24
Batch 2	Uncontrolled	Controlled[a]	160	16.44
Batch 3	10	Controlled[a]	160-600 (Cascading mode)	12.43
Batch 4	20	Controlled[a]	160-600 (Cascading mode)	17.97
Batch 5	30	Controlled[a]	160-600 (Cascading mode)	13.73

[b]Production conditions: Initial pH 7.0; aeration -1.5 vvm; inoculum size – 2.61% (v/v); volume of broth 1.5 L; optimized medium under shake flask conditions

At uncontrolled pH (Batch 1), the enzyme activity was decreased with decrease in pH of the medium after 12 h of fermentation (Fig. 4.18b). Therefore, the subsequent experiments were carried out with controlled pH (8.5) after 12 h to verify the effect DO level on the production of L-asparaginase. Under controlled pH (Batch 2), sharp decline in enzyme activity was discontinued after 12 h of fermentation. The cells grew to a maximum range of 1-1.35 g l^{-1} (Fig. 4.18c). The level of glucose and L-asparagine reduced to 0.2-0.6 and 1.0-2.0 g l^{-1}, respectively at 12 h of fermentation in all conditions (Fig. 4.18de). In the Initial 4-5 h of fermentation, L-asparagine was consumed very slowly and after that it was also assimilated simultaneously along with glucose, and more than 90% of L-asparagine was exponentially consumed with in 16 h of fermentation. Maximum production (enzyme activity), substrate utilization and cell growth were observed at 20% of DO level in the medium. An increase in DO level (30%) of fermentation shows a decrease in production of L-asparaginase. DO level was also decreased rapidly to

minimum in 4 h of fermentation in all conditions and increased again ~22% in uncontrolled DO experiments (Batch 1 and 2) at nearly 12 h of fermentation (Fig. 4.18f). Specific growth rate was calculated at different experimental conditions and found to be maximum at 20% of DO level (~0.3 h^{-1}), which is clearly elucidate from growth profile (Fig. 4.18c). At optimal DO (20%), the productivity was increased to 1497.50 U l^{-1} h^{-1} of L-asparaginase, which corresponded to enzyme activity of 17.97 U ml^{-1} (36.28 U mg^{-1} of protein) of L-asparaginase. The production of L-asparaginase was increased by 10.8% when compared with the production under optimized process parameters (16.21 U ml^{-1}) in shake flask. The best combination of DO level and pH were found to be 20% and 8.5 (controlled after 12 h of fermentation), respectively for enhanced production of L-asparaginase from *P. carotovorum* MTCC 1428.

The effect of various fermentation variables on the production of L-asparaginase in shake flasks have been reported in the literature (Heinemann *et al.,* 1970; Khan *et al.,* 1970; Barnes *et al.,* 1977; Raha *et al.,* 1990; Maladkar *et al.,* 1993; Manna *et al.,* 1995; Nawaz *et al.,* 1998; Abdel-Fattah and Olama, 2002; El-Bessoumy *et al.,* 2004; Neto *et al.,* 2006; Prakasham *et al.,* 2007; Kumar *et al.,* 2009). However, reports on the production of L-asparaginase in batch bioreactors are very limited. It is very difficult to carry out experiment in shake flasks under controlled pH and DO level. But experiments can be performed under controlled parameters *viz.*, pH and DO level in the bioreactors to enhance the production and productivity. Hence, in this study, the effect of DO level and controlled pH (8.5) of the medium after 12 h on the production of L-asparaginase by *P. carotovorum* was studied in a batch bioreactor. The enzyme production was increased with increasing the pH of medium until 10 h of fermentation. As the system seems to be growth associated, the cell growth is also plays a vital role for the production of L-asparaginase (Fig. 4.18a).

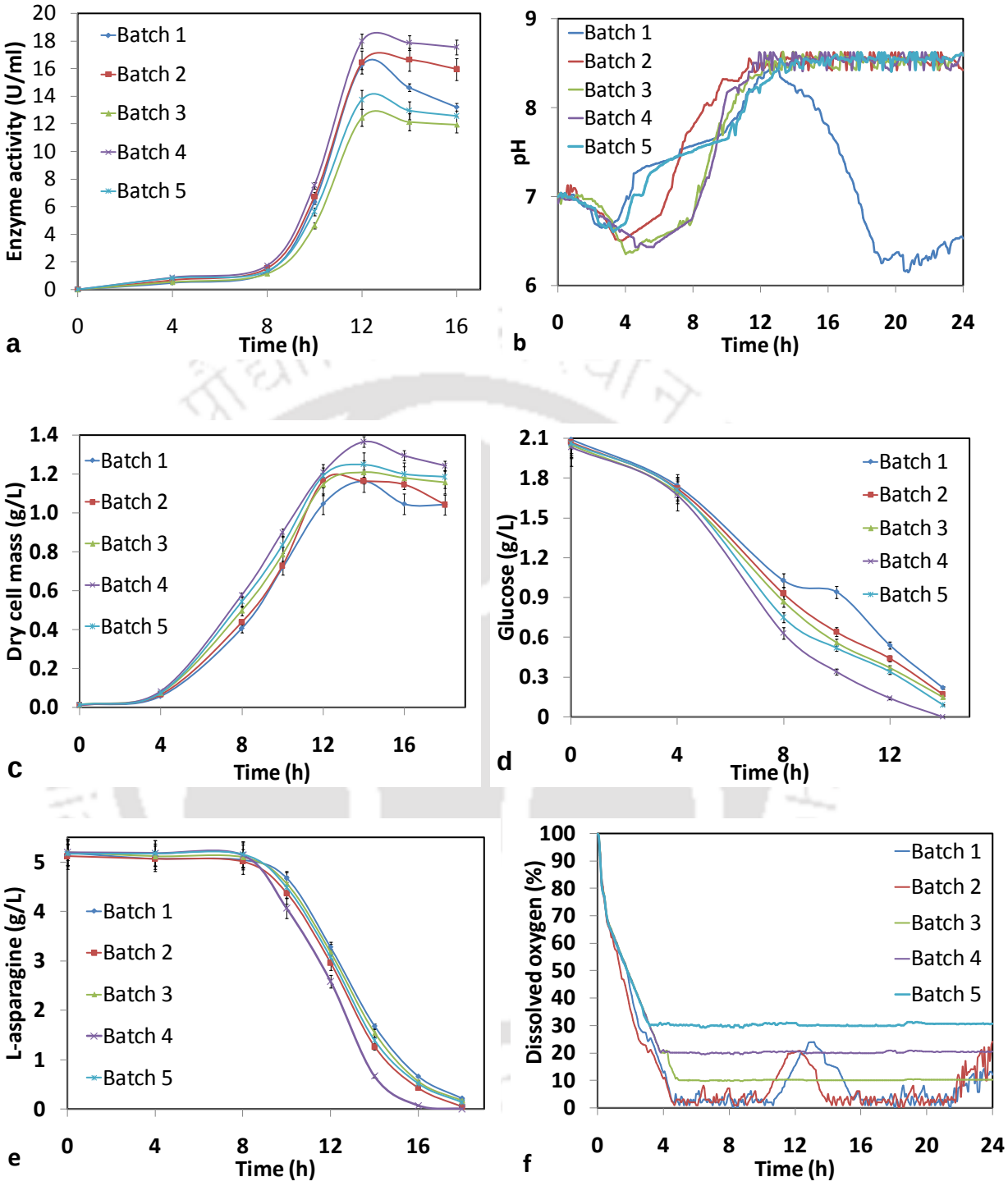


Fig. 4.18. Effect of DO level and controlled pH (8.5) after 12 h on L-asparaginase production by *P. carotovorum* MTCC 1428 batch in bioreactor: (a) Enzyme activity profile, (b) pH profiles, (c) DCW, (d) L-Asparagine utilization, (e) Glucose utilization and (f) DO profile.

The growth of the culture was retarded after 10 h of fermentation due to low level of DO and substrates (L-asparagine and glucose) in the medium. In this study, initial pH for optimum growth was observed to be 7.0 and maximum production was observed when pH of the medium raised to 8.5±0.2. The decrease in pH of the culture broth was observed during the initial time of fermentation due to the preferred utilization of glucose in place of nitrogenous substrates, thus contributing to acid production. After 10 h of fermentation, the increase in pH of the medium was observed, due to release of NH_3 by degradation of substrate containing amino groups (such as amino acids) (Geckil and Gencer, 2004). In general, L-asparaginase production was higher at late exponential phase (24-48 h) and enzyme activity was also a function of cell age (Mukherjee *et al.*, 2000). In our study, the maximum activity was observed at ~12 h in a batch bioreactor (Fig. 4.18a). It was found that controlling DO by cascade mode showed more enzyme production than the uncontrolled DO level. Few reports are available where it was seen that the L-asparaginase production was enhanced when the initial pH of medium was maintained in the range of 5.0–7.5 (Heinemann and Howard, 1969; Barnes *et al.*, 1977; Prakasham *et al.*, 2007). Geckil and Gencer, (2004) showed that the cultures grown under low aeration and low agitation had an about 2-fold higher L-asparaginase activity than cultures grown under high aeration/high agitation and no aeration/low agitation conditions. Various workers have also reported the similar observations for the maximum production of L-asparaginase from *Erwinia aroideae* (Liu and Zajic, 1973ab), *Serratia marcescens* (Heinemann *et al*, 1970) and *Citrobacter spp.* (Bascomb *et al.*, 1975) when the DO level dropped to zero during the process. In contrast, Mukharjee *et al.*, (2000) reported that the maximum production of L-asparaginase from *Enterobacter aerogenes* was achieved in the presence of sufficient level (~60%) of oxygen in the broth. It was observed that the glucose medium supported cell growth rather than enzyme production in the shake flask experiments.

4.14. Production of L-asparaginase by *P. carotovorum* MTCC 1428 in bioreactor operated under fed-batch mode

The objective of fed-batch studies is to enhance the production of L-asparaginase from *P. carotovorum* MTCC 1428 and to develop economically feasible bioprocess by feeding L-asparagine (essential substrate for L-asparaginase production) and/or glucose (carbon source for growth). Initially cultures were grown in the optimized medium containing L-asparagine and/or glucose in a batch mode and feeding of substrate(s) were started at 12 h of fermentation. The four different fed-batch strategies were applied to enhance the L-asparaginase production in fed-batch bioreactor as shown in the Table 4.24. The feeding of substrate(s) was regulated by keeping the concentration of L-asparagine and glucose in range of ~0.3-2.0 and ~0.5-4.0 g l^{-1}, respectively in the culture broth. This is the first investigation on the production of L-asparaginase by *P. carotovorum* MTCC 1428 in bioreactor under fed-batch mode.

Table 4.24. Feeding strategies used to enhance L-asparaginase production[a] by *P. carotovorum* MTCC 1428 in bioreactor using fed-batch mode of operation.

	Batch (12 hr)		Feeding in Fed-Batch		Maximum DCW (g)	Volume of the culture at 24 h (l)	Maximum L-asparaginase activity (U ml^{-1})
	Initial glucose	Initial L-asparagine	glucose	L-asparagine			
Fed-batch 1	N	Y	N	Y	4.35	2.36	21.25
Fed-batch 2	Y	N	Y	Y	5.50	2.41	27.64
Fed-batch 3	Y	Y	Y	Y	6.53	2.47	33.52
Fed-batch 4	Y	Y	N	Y	6.97	2.43	38.78

[a]Production conditions: Initial pH - 7.0; DO level - 20%; aeration - 1.5-2.0 vvm; inoculum size - 2.61% (v/v); agitation - cascading mode 160-600 rpm; broth volume - 1.5 L; feeding substrate(s) concentrations – 30 g l^{-1} each; pH maintained 8.5±0.1 after 12 h. Y - present; N – absent.

In the first experiment, medium containing L-asparagine in the growth phase (batch) and feeding of L-asparagine started from 12 h of fermentation. The maximum L-asparaginase production was observed to be 21.25 U ml^{-1} (27.94 U mg^{-1} of protein) at 24 h of fermentation (Fig. 4.19). The productivity of L-asparaginase was obtained to be 885.4 U l^{-1} h^{-1}. The time profiles of cell growth and L-asparagine utilization profiles upon feeding of L-asparagine in fed-batch 1 are shown in Fig. 4.20a. In the second experiment, medium containing glucose was used in the growth phase (batch fermentation) and feeding of combination of L-asparagine and glucose was performed simultaneously, after 12 h of batch fermentation. The enzyme activity of L-asparaginase was improved to 27.64 U ml^{-1} in 24 h of fermentation (Fig. 4.19) and an enhancement of 53.8% L-asparaginase activity was observed in comparison to batch fermentation. The productivity of L-asparaginase was 1151.6 U l^{-1} h^{-1}, which corresponded to specific activity to 31.65 U mg^{-1} of protein. The time profiles of cell growth and both substrates utilization profiles upon feeding of L-asparagine and glucose in second experiment are presented in Fig. 4.20b. In the experiment 3, feeding stream containing both substrates, enhanced the L-asparaginase activity to 33.52 U ml^{-1} (35.87 U mg^{-1} of protein) of L-asparaginase in 24 h of fermentation (Fig. 4.19). The productivity of L-asparaginase in the experiment was found to be 1396.6 U l^{-1} h^{-1} and it was not improved as compared to the previous batch studies. The time profiles of cell growth and substrates (L-asparagine and glucose) utilization profiles are shown in Fig. 4.20c.

Previous fed-batches strategies did not shown much influence on L-asparaginase productivity. Hence, the experiment was performed in the medium containing both substrates (L-asparagine and glucose) in the growth phase (batch fermentation) and L-asparagine in the feeding stream (Fed-batch 4). The production of L-asparaginase was increased significantly to 38.78 U ml^{-1}

(37.56 U $mg^{-1}$of protein) in 24 h of fermentation (Fig. 4.19). An enhancement of 115.8% of enzyme activity was achieved in comparison to production in batch bioreactor. The productivity of L-asparaginase increased to 1615.83 U l^{-1} h^{-1} and an improvement of 7.9% was achieved when compared to batch culture (Table 4.25). The time profiles of cell growth and substrates (L-asparagine and glucose) utilization profiles upon feeding of L-asparagine are presented in Fig. 4.20d.

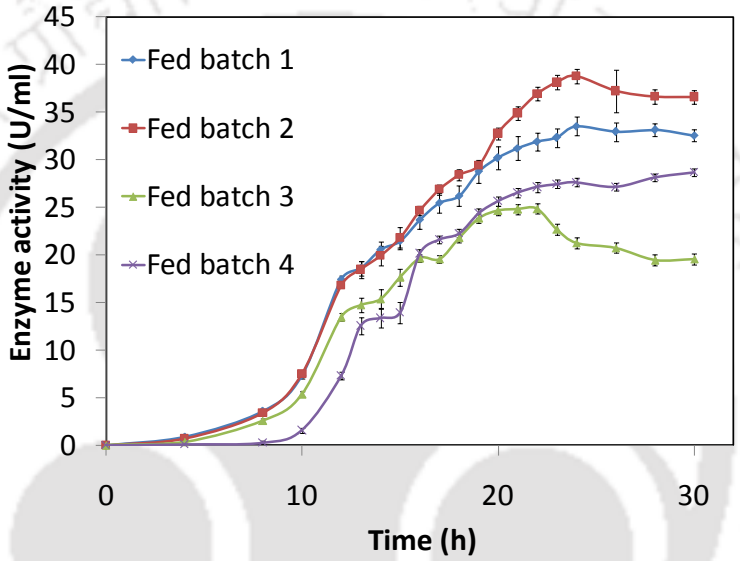


Fig. 4.19. Effect of fed-batch strategies on production of L-asparaginase by *P. carotovorum* MTCC 1428 in bioreactor using fed-batch mode of operation.

Batch operations suffer from low cell concentration and product recovery because of limited initial carbon source concentration. Fed-batch cultures have been successfully used to increase the yields of pectinases from *Debaryomyces nepalensis* (Gummadi and Kumar, 2008), PHB from *Methylobacterium* sp. ZP24 (Nath *et al.*, 2008) and the production of ergosterol by *Saccharomyces cerevisiae* (Shang *et al.*, 2006). Fed-batch strategy was investigated in the present study by feeding L-asparagine (essential substrate for L-asparaginase production) and/or glucose (carbon source for growth) from 12 h of batch fermentation. The productivity and production was increased by 7.9% and 115.8%,

respectively as compared to batch fermentation. Although considerable cell mass and volumetric activity was achieved in Fed-batch 2 and 3, but feeding stream containing both substrates of these fed-batches did not have much influence on the productivity of L-asparaginase and specific activity. This clearly suggests that the presence of glucose in the growth phase (batch mode) and feeding stream containing L-asparagine enhanced the production and productivity of L-asparaginase from *P. carotovorum* MTCC 1428.

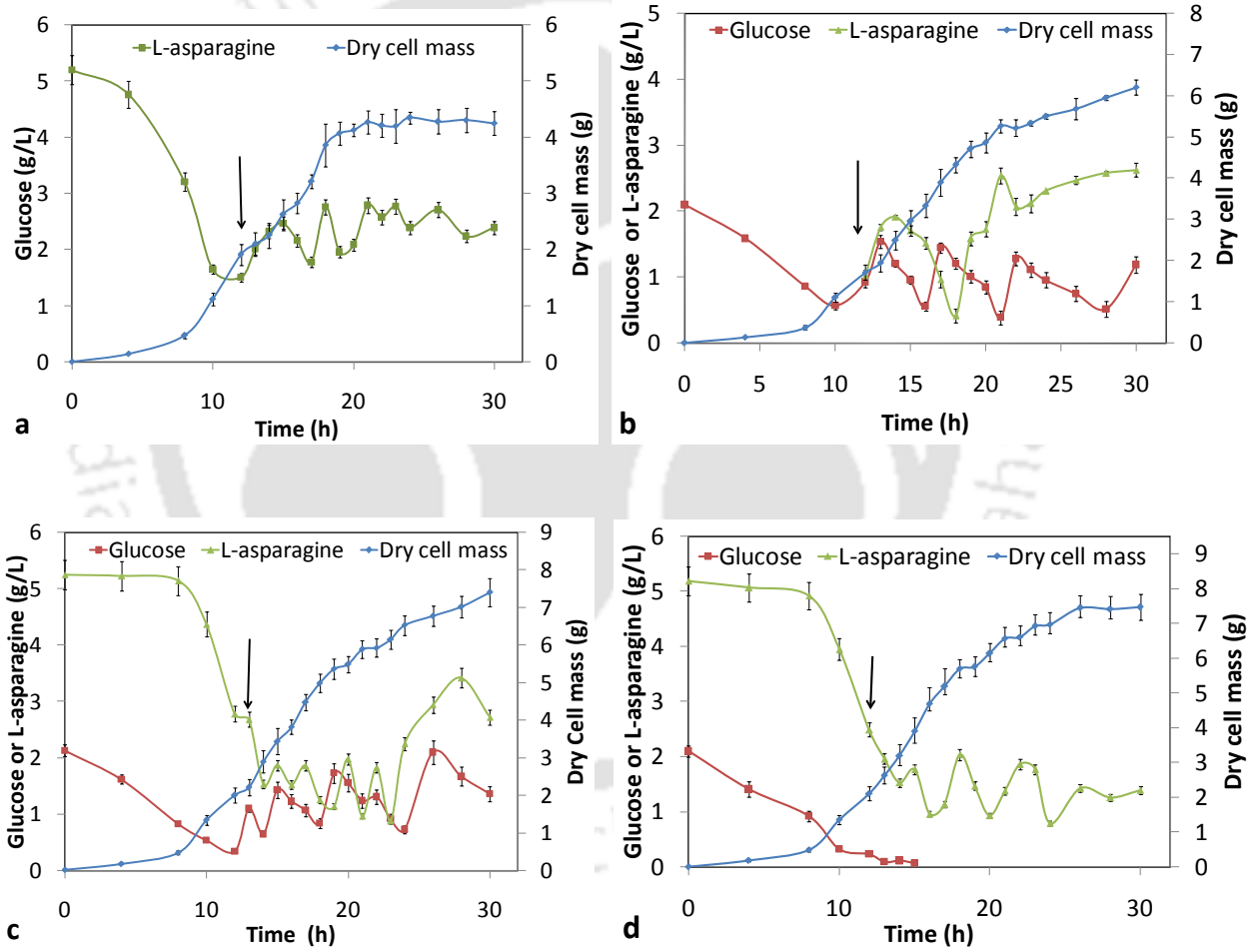


Fig. 4.20. Cell growth and substrates (L-asparagine and glucose) utilization profiles of fed-batch strategies used to L-asparaginase production by *P. carotovorum* MTCC 1428 in bioreactor. (a) Fed-batch 1, (b) Fed-batch 2, (c) Fed-batch 3, (d) Fed-batch 4. Arrow indicates the point of addition of L-asparagine and/or glucose to the production medium after 12 h of fermentation.

Table 4.25. Comparison of L-asparaginase production and productivity in different level and process conditions.

Process conditions	Shake flask/ Bioreactor	L-asparaginase production		Productivity ($U\ l^{-1}\ h^{-1}$)	Enhanced Productivity (fold)	Enhanced L-asparaginase production[a] (fold)
		Enzyme activity ($U\ ml^{-1}$)	Specific activity ($U\ mg^{-1}$)			
Before optimization	Shake flask	0.8±0.01	3.4±0.3	63.3±0.9	--	--
Optimized medium	Shake flask	14.7±0.5	27.9±0.5	1225.8±44.2	19.4	19.4
Optimized process parameters	Shake flask	16.2±0.7	35.2±0.7	1350.8±56.2	21.3	21.3
Optimized DO and pH	Batch	18.0±0.6	36.3±0.6	1497.5±49.7	23.6	23.6
Optimized feeding strategies for dual substrates	Fed-batch	38.8±0.6	37.6±0.8	1615.8±51.2	25.5	51.0

[a]Enhanced L-asparaginase production calculated from L-asparaginase activity

4.15. Effect of initial substrates concentration on L-asparaginase production and specific growth rate in batch bioreactor

In order to evaluate the effects of initial concentration of substrates on the production and specific growth, experiments were conducted in a batch bioreactor under optimal level of medium components and physical parameters (DO level and pH controlled after 12 h) at various concentrations of glucose and/or L-asparagine (Table 4.26).

Table 4.26. Production of L-asparaginase and specific growth rate from *P. carotovorum* MTCC 1428 at different concentration of glucose and L-asparagine in batch bioreactor.

Batch bioreactor run	Initial substrate concentration		Maximum L-asparaginase activity		Specific growth rate (μ) (h^{-1})
	Glucose (S_g)(g L^{-1})	L-asparagine (S_a) (g L^{-1})	Enzyme activity (U ml^{-1}) (h)	Specific activity (U mg^{-1})	
1	0	7	12.73 ± 0.37 (14)	26.67 ± 0.64	0.184 ± 0.05
2	7	0	1.95 ± 0.73 (12)	3.35 ± 0.39	0.169 ± 0.02
3	2	5	16.92 ± 0.54(12)	36.87 ± 0.83	0.344 ± 0.07
4	1	2.5	9.89 ± 0.49(12)	22.17 ± 0.43	0.292 ± 0.08
5	4	7.5	13.83 ± 0.32(18)	29.07 ± 0.49	0.321 ± 0.06
6	5	10	10.32 ± 0.65 (22)	21.73 ± 0.61	0.279 ± 0.04
7	2	0	1.23 ± 0.19 (12)	2.73 ± 0.58	0.175 ± 0.07
8	0	5	13.21 ± 0.39 (12)	25.43 ± 0.25	0.199 ± 0.08

Process condition: T_m, 30ºC; pH controlled when reaches < 8.5; aeration, 1.5 vvm; agitation, 160 – 600 rpm (cascading mode); DO level, 20%.

The cells grew to a maximum concentration range of 0.56-1.36 g l^{-1} at different concentration of glucose and/or L-asparagine in batch bioreactor (Fig. 4.21a). The maximum DCW was observed at 2 g l^{-1} and 5 g l^{-1} of glucose and L-asparagine, respectively in batch 3. Furthermore, *P. carotovorum* MTCC 1428 was exhibited shorter lag periods at lower concentration of substrates and longer lag at higher concentrations of substrates (Fig. 4.21a).

This might be due to presence of high concentration of substrates in medium (run 5 and 6) (Fig. 4.21a). More than 90% of glucose was exponentially assimilated in 14 h of fermentation by *P. carotovorum* MTCC 1428 in all conditions (Fig. 4.21b). L-asparagine was consumed very slowly until 4-8 h and after that assimilated simultaneously along with glucose in 16 h of fermentation (except run 5 and 6) (Fig. 4.21c). The maximum L-asparaginase production (16.92 U ml^{-1}) was observed in 12 h of fermentation at 2 g l^{-1} and 5 g l^{-1} of glucose and L-asparagine, respectively (Table 4.26). The maximum L-asparaginase production was observed at 12 h in all batch conditions except batch 5 and 6 (Fig. 4.21d).

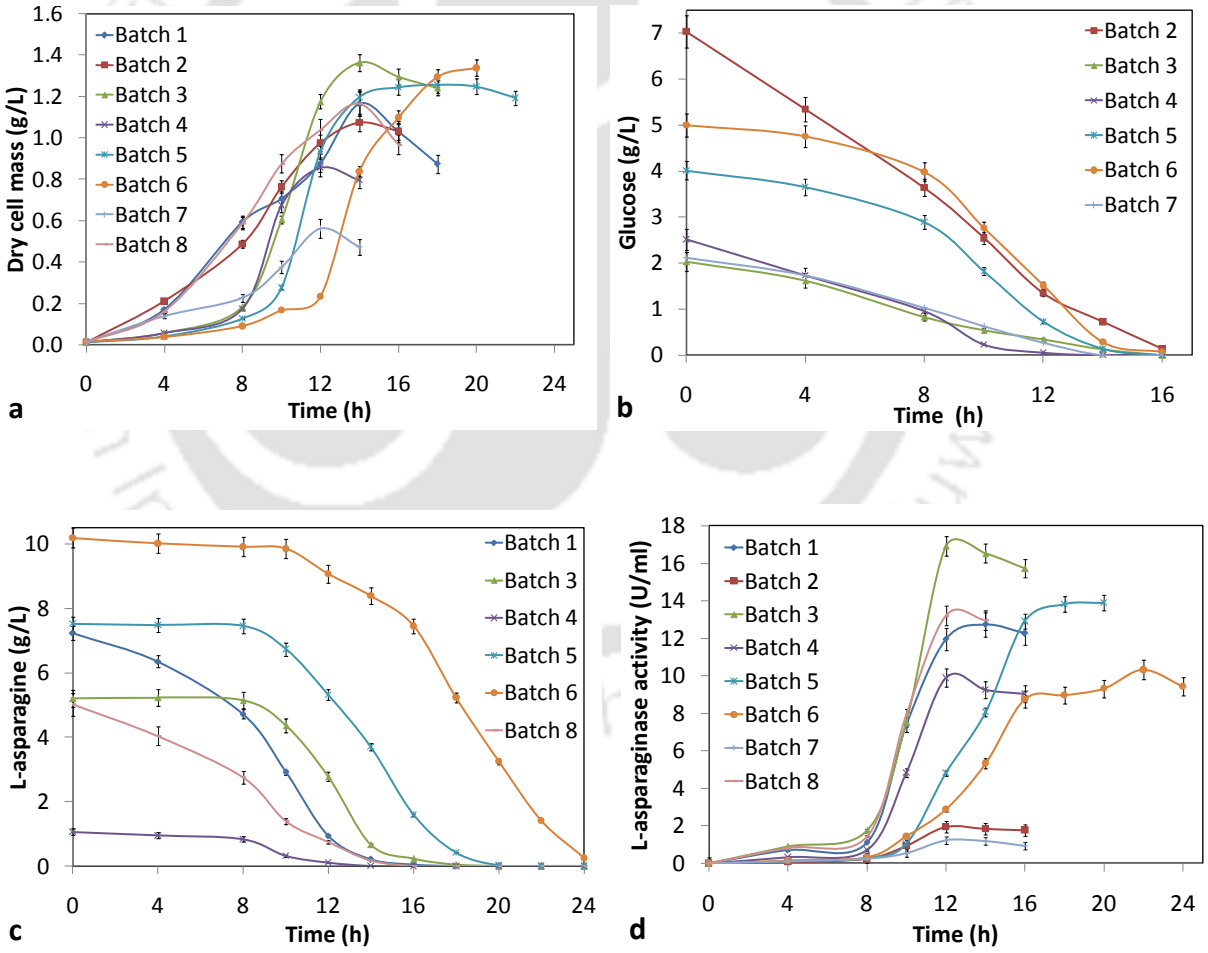


Fig. 4.21. Profiles of batch bioreactors runs. (a) Biomass production, (b) Glucose utilization. (c) L-Asparagine utilization, (d) L-Asparaginase enzyme production.

Specific growth rate (μ) for different combinations of initial concentration of glucose and/or L-asparagine (S_g and S_a) were calculated from the plot of ln(X) *vs*. time in logarithmic phase as discussed in section 3.15.1. The slope of the line during the exponential phase gives the specific growth rate and varies in each run (Fig.4.21a). Specific growth rate of *P. carotovorum* MTCC 1428 in the medium containing glucose was found to be 0.175 h^{-1} at 2 g l^{-1}, whereas decrease in specific growth rate was observed at higher initial concentration (7 g l^{-1}) of glucose (Table 4.26). Similarly, growth rate was found to be 0.199 h^{-1} at 5 g l^{-1} of L-asparagine in the medium. However, slightly decrease in the specific growth rate was observed at 7 g l^{-1} of initial L-asparagine concentration. Specific growth rate was found to be ~1.5-2.0 times higher when both substrates (L-asparagine and glucose) present in the medium. Specific growth rate was increased with increase in initial concentrations of glucose and L-asparagine until 2 g l^{-1} and 5 g l^{-1}, respectively and then decreased with increase in substrates concentrations suggesting that the inhibition of both substrates simultaneously (Table 4.26).

4.16. Modeling of microbial growth kinetics

Although the production of L-asparaginase has been studied by several authors, the development of kinetic models for growth and L-asparaginase production has not been considered previously. We could not able to find any report related to kinetic models describing growth, substrate utilization and production of L-asparaginase. Mathematical modeling is necessary to predict the results of industrial fermentations and to choose optimized conditions. Additionally, it helps in understanding the complex mechanisms of growth and product formation of this microorganism (Zinn *et al.*, 2004). Tested growth-limiting substrates (glucose and/or L-asparagine) influence the growth kinetics of *P.*

carotovorum MTCC 1428 was demonstrated and given these observations in Table 4.26. The growth rate of *P. carotovorum* MTCC 1428 in the absence of L-asparagine and glucose was very less (< 0.075 h^{-1}). The sharply decrease in growth of microorganism, substrate utilization rate and L-asparaginase production was observed when the initial concentration of glucose and L-asparagine in the medium exceeds to 15 g l^{-1} and 10 g l^{-1}, respectively. Hence, the growth of *P. carotovorum* MTCC 1428 have represented by a kinetic expression by considering the dual-substrate limitations (glucose and L-asparagine).

Table 4.27 shows possible growth models using combinations of Eqs. (3.17) - (3.19), calculated kinetic parameters of various growth models with SSD and regression coefficients (R^2). The minimum SSD were found for model number 11, with R^2 0.971. Model 11 additive combined form of Luong and Luong model kinetics. The best growth model selected the additive form of double Luong model:

$$\text{Double Luong model: } \mu = \mu_m\left[\frac{S_g}{K_{Sg}+S_g}\left(1-\frac{S_g}{S_{mg}}\right)^{ng}+\frac{S_a}{K_{Sa}+S_a}\left(1-\frac{S_a}{S_{ma}}\right)^{na}\right] \qquad (4.6)$$

Where, subscript *g* and *a* represents glucose and L-asparagine, respectively. For the selected model, the kinetic parameters are μ_m = 0.355 h^{-1}, K_{sg} = 1.668 mg l^{-1}, K_{sa} = 1.464 mg l^{-1}, S_{mg} = 16.190 g l^{-1}, n_g = 0.841, S_{ma} = 11.28 g l^{-1}, n_a = 0.476. Fig. 4.22 shows that the experimental specific growth rate from batch experiments fitted over simulated surface using Origin® Pro 8.0 from additive form of double Luong model for specific growth rate (Eq. 4.6). The high correlation coefficient (R^2 = 0.971) demonstrates that the growth model accurately represents growth of *P. carotovorum* MTCC 1428. Luong model has been used in various studies to explain growth rate model using multiple substartes (Luong *et al.*, 1986).

Table 4.27. Estimated kinetic parameters of various growth models with SSD and regression coefficients (R^2).

Model	Equation number for Glucose	Equation number for L-asparagine	μ_m	K_{sg}	K_{sa}	K_{1g}	K_{1a}	$K_{2g}{}^2$	$K_{2a}{}^2$	S_{mg}	n_g	S_{ma}	n_a	SSD×10^{-2}	R^2
1	1	--	0.361	0.348	--	18.380	--	--	--	--	--	--	--	2.348	0.149
2	2	--	0.369	0.234	--	--	--	--	--	7.103	0.144	--	--	1.808	0.959
3	3	--	0.346	0.273	--	--	--	78.24	--	--	--	--	--	2.163	0.221
4	--	1	0.796	--	1.068	--	5.327	--	--	--	--	--	--	0.479	0.795
5	--	2	0.540	--	0.627	--	--	--	--	--	--	11.146	1.048	0.593	0.959
6	--	3	0.581	--	0.960	--	--	--	22.535	--	--	--	--	0.486	0.801
7	1	1	0.491	2.433	2.049	7.094	6.823	--	--	--	--	--	--	0.421	0.955
8	1	2	0.363	1.958	1.391	9.937	--	--	--	--	--	11.957	0.625	0.125	0.963
9	1	3	0.352	0.019	6.142	5.191	--	--	457.914	--	--	--	--	1.864	0.492
10	2	1	0.367	2.436	1.599		8.552			8.328	0.309			0.141	0.936
11	2	2	0.355	1.668	1.464	--	--	--	--	16.190	0.841	11.280	0.476	0.121	0.971
12	2	3	0.371	2.181	2.145	--		--	49.144	13.869	0.843	--	--	0.149	0.959
13	3	1	0.391	2.870	1.699	--	8.076	42.515	--	--	--	--	--	0.191	0.946
14	3	2	0.347	2.060	1.289	--	--	67.213	--	--	--	11.424	0.541	0.139	0.963
15	3	3	0.736	9.180	7.619	--	--	18.749	14.498	--	--	--	--	0.764	0.802

Dual substrate kinetics model 7-15 are additive form of column second and third respectively.

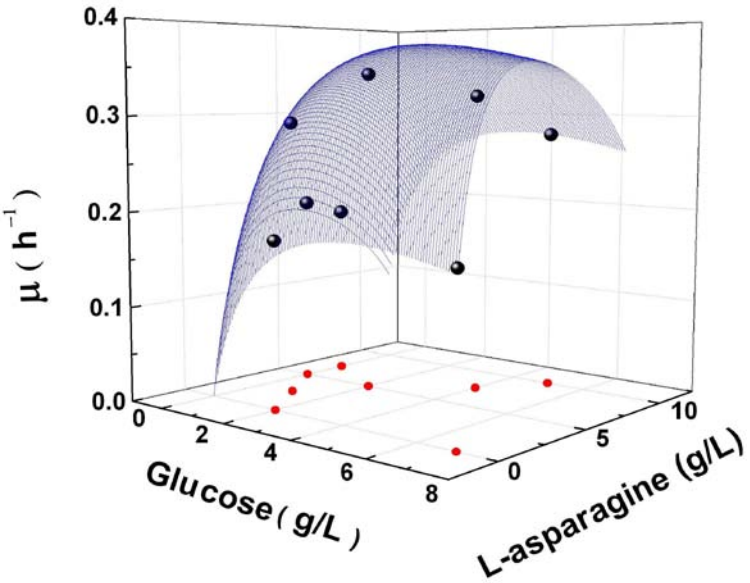


Fig. 4.22. The specific growth rate from batch bioreactors experiments fitted over simulated surface from dual substrate growth kinetic model (Eq. 4.6) (R^2=0.971). Projections of specific growth rates in XY plane (red points).

To have more insights to understand the growth kinetics, selected empirical models in additive form describing the substrate inhibition kinetics were employed to fit the kinetic data (Table 4.27). Initially growth data of *P. carotovorum* MTCC 1428 was tested for dual substrates kinetics with various single substrate models (Moser, Tessier, Monod, Contois, Yano and Kago 1 and 2, Andrew, Haldane, Double exponential, and Luong model) in non-interactive, multiple and additive form of these models and only additive form of Double exponential, Luong and Yano and Kago 2 model is found to be fitted very well with the experimental data. However, the remaining models did not fit well in any combination of forms (non-interactive, multiple and additive) and showed very low regression coefficient (R^2<0.80). This may be due to the fact that the other than selected models and its different combinations are not able to predict decrease in specific growth rate at high concentration of substrates. Therefore, Luong, Double exponential and Yano and Kago 2 model was used in the present study. Luong model was used extensively to explain the inhibition growth kinetics

of various microorganisms for various carbohydrates and nitrogenous sources (Luong *et al.*, 1986; Gokulakrishnan *et al.*, 2006). The values of S_{mg} and S_{ma} predicted by double Luong model (16.19 and 11.28 g l^{-1}) was close to the experimental observed values (15.0 and 10 g l^{-1}).

4.17. Modeling of L-asparaginase production using the modified form of Luedeking and Piret model

Previous studies in batch culture showed that the glucose is not sufficient to maximize the production of L-asparaginase from *P. carotovorum* MTCC 1428, and L-asparagine was found to be essential for higher production of L-asparaginase. When pH of the medium reached to above 8.5, the maximum production of L-asparaginase was observed. In the current study, the maximum production was observed at 12-14 h in batch bioreactor (Fig. 4.21d) except at high substrate concentration (run 5 and 6).

L-asparaginase production kinetics in batch bioreactor from *P. carotovorum* MTCC 1428 showed that the L-asparaginase production was clearly mixed growth-associated ($\alpha \neq 0$, $\beta \neq 0$). In addition, the values obtained for pH_{op} oscillated between 8.5 and 8.7 ($P < 0.05$) for maximum production of L-asparaginase. The kinetic parameters from the modified form of Luedeking and Piret model for L -asparaginase production are α = 3.27 U mg^{-1}, β = 6.421 U mg^{-1} h^{-1} and κ = 6.681. Fig. 4.23 shows that the correlation between production rate observed from experiments and predicted from Eq. 3.20. The correlation between model Eq. 3.20 and experimental values obtained from L-asparaginase production was very satisfactory (R^2>0.96). Hence, the production was classified as a pH dependent secondary metabolite.

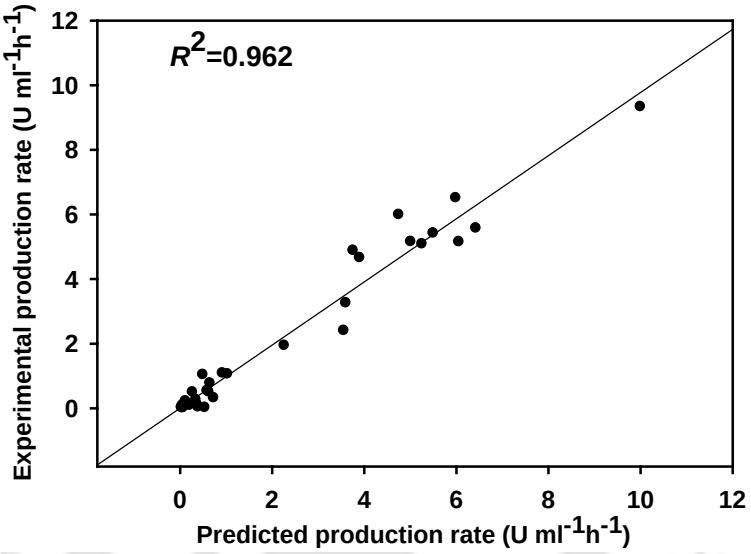


Fig. 4.23. The experimental batch bioreactor L-asparaginase production rate and predicted from production kinetics model.

In general, L-asparaginase production was higher at late exponential phase (24-48 h) and enzyme activity was also a function of cell age (Mukherjee *et al.*, 2000). Growth of L-asparaginase producing microorganism is often inhibited not only by nutrient limitation, but also by substrate inhibition and the production of ammonia which have toxic and antimicrobial effects, even for the producer cells. In such cases, the classic Monod model must be modified by including terms for accounting the inhibitory effects of substrate inhibition and end product formation (Callewaert and De Vuyst, 2000).

Although L-asparaginase production has been reported by various researchers to be growth-associated (Albanese and Kafkewitz, 1978; Khamna *et al.*, 2009; Shah *et al.*, 2010), in some cases, the lack of proportionality between L-asparaginase production rates and bacterial growth rate has been observed (Liu and Zajic, 1972ab; Wei and Liu, 1998; Geckil and Gencer, 2004; Mukherjee *et al.*, 2000). This was observed due to the specific influence of several key variables of the culture (like pH or some nutrient limitation) on L-asparaginase

synthesis (Heinemann and Howard, 1969; Abdel-Fattah *et al.*, 2002; Prakasham *et al.*, 2007) and bacterial growth rate makes difficult to fit the experimental L-asparaginase data using classical Luedeking and Piret model. Similar type of adaptation also seen for the bacteriocin production from *Lactococcus lactis* subsp. *Lactis* by Guerra and Pastrana, (2002) and Nelson *et al.*, (2007).

The alkalinization of the medium at low glucose concentration and complete utilization of this carbohydrate during the initial incubation periods, followed by the release of nitrogen as NH_3 through the degradation of substrates containing amino groups (such as amino acids) at further incubation, as often observed during the fermentation processes (Geckil and Gencer, 2004). It is known that accumulation of ammonia in the ionized form (NH_4^+), which further contributes to the acidity of the medium. These results are in good accordance with others showing that ions have an inhibitory effect on L-asparaginase production (Tosa *et al.*, 1971, Sun and Setlow, 1991).

4.18. Modeling of L-asparagine and glucose utilization

The considered models for both substrates (L-asparagine and glucose) were solved using non-linear regression analysis by Microsoft Excel 2007 Solver®. The predicted evolutions of the glucose and L-asparagine utilization rate by Eqs. 3.21 and 3.22 during the fermentation process together with the experimental data shows the fitting of results was satisfactory (correlation coefficient, $R^2 > 0.93$). The values of parameters of substrates uptake model were calculated as follows for glucose $Y_{X/Sg}$,$Y_{P/Sg}$, m_g are 0.624 g g^{-1}, 0.808 U g^{-1}, 0.011 g g^{-1} h^{-1} and for L-asparagine $Y_{X/Sa}$, $Y_{P/Sa}$, m_a are 0.286 g g^{-1}, 10.910 U g^{-1}, 0.029 g g^{-1} h^{-1}, respectively.

4.19. Effect of initial glucose and/or L-asparagine concentration on substrate(s) uptake rate

The variation in specific substrate uptake rate ($Y_{S/X}$) was observed at various concentration(s) of L-asparagine and/or glucose in batch bioreactor. This deviation of substrate uptake rates from *P. carotovorum* MTCC 1428 at different concentration(s) of glucose and/or L-asparagine in batch bioreactor are presented in Fig. 4.24. The specific substrate uptake rate ($Y_{Sg/X}$) for glucose was varied in the range of 0.271-0.551 g of glucose g^{-1} of cells h^{-1}. However, specific substrate uptake rate ($Y_{Sa/X}$) for L-asparagine is very high, closely 2 fold in all combinations. The constant n_i estimated by Luong model for both substrates are suggesting that the non linear relationship between μ and S exists during inhibition (Fig. 4.24) (Luong *et al.*, 1986).

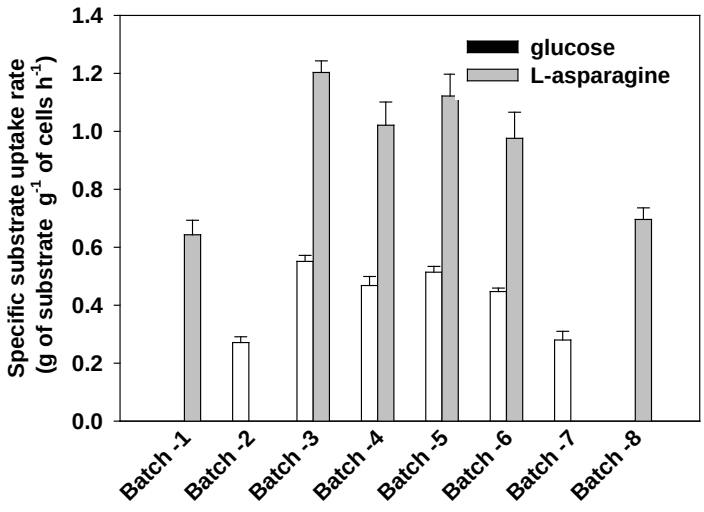


Fig. 4.24. Experimental specific substrate uptake rate from *P. carotovorum* MTCC 1428 at different concentration of glucose and L-asparagine in batch bioreactor.

So far, no reports are available on the kinetics of dual substrate growth, substrates utilization and production of L-asparaginase from *P. carotovorum* MTCC 1428 in batch bioreactor. It has been shown that the strain used in this study followed the mixed growth associate product

formation kinetics. There are three different equations derived to describe the detailed kinetic behavior of dual substrate growth, substrate(s) utilization and L-asparaginase production from *P. carotovorum* MTCC 1428. The kinetic parameters were estimated in this study and correlation coefficient are presented in Table 4.28.

Table 4.28. Estimated kinetic parameters obtained in the study from dual-substrate growth, L-asparaginase production and substrate utilization models.

	Kinetic parameters							R^2
Dual substrate growth model	μ_m	K_{sg}	K_{sa}	S_{mg}	n_g	S_{ma}	n_a	0.971
	0.355	1.668	1.464	16.190	0.841	11.280	0.476	
L-asparaginase production model	α	β	κ					0.962
	3.27	6.421	6.681					
Substrate utilization model	$Y_{X/Sg}$	$Y_{P/Sg}$	m_g	$Y_{X/Sa}$	$Y_{P/Sa}$	m_a		0.935
	0.624	0.808	0.011	0.286	10.910	0.029		

CHAPTER 5
SUMMARY AND CONCLUSIONS

- A novel glutaminase free L-asparaginase producing strain, *Pectobacterium carotovorum* MTCC 1428 was screened. Localization of L-asparaginase was studied in various microorganisms for the development of bioprocess.
- Medium was successfully developed for L-asparaginase production form *P. carotovorum* MTCC 1428. Statistical experimental design including PB design and central composite design (CCD) were employed for optimization of chemical and physical parameters to maximize the production of L-asparaginase fro*m P. carotovorum* MTCC 1428. The production of L-asparaginase (enzyme activity) was enhanced by 21.33 fold under optimal level of chemical and physical parameters.
- A three step purification process was developed and achieved a native L-asparaginase from *P. carotovorum* MTCC 1428. The molecular mass (144.42 kDa) and homotetramer structure of L-asparaginase was revealed by SDS-PAGE, Native PAGE, gel exclusion chromatography and MALDI TOF MS analysis.
- Studies on physical conditions, which influence the performance of purified L-asparaginase revealed that the purified L-asparaginase was active over a broad range of pH (7.5-9.0) and temperature (35-45ºC). The enzyme showed stability at alkaline range of pH (pH 8.0–10.0) and it retained 90% of its original activity when incubated at 4±1ºC for 24 h. Maximum L-asparaginase activity was obtained at pH of 8.5 and 40ºC.

- Studies on effect of different metal ions and reagents on the performance of purified L-asparaginase 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 L-asparaginase with various structures analogues of L-asparagine suggested that the enzyme was very specific for its natural substrate, L-asparagine.
- The optimal level of pH and temperature to maximize the rate of enzymatic reaction under assay condition using purified L-asparaginase were found to be 8.49 and 39.3°C, respectively.
- Thermodynamic parameters (K_d, $t_{1/2}$, ΔH, ΔS, ΔG and E_a) were determined to evaluate the probable mechanism of deactivation of this enzyme.
- The substrate affinity of the purified L-asparaginase in terms of K_m was found to be very low (0.657 mM), which is 5–9 times lower than the reported cytosolic L-asparaginase. Maximal velocity (V_{max}), Turnover number (K_{cat}) and specificity constant (K_{cat}/K_m) of purified L-asparaginase for L-asparagine were found to be 4.45 IU μg^{-1}, 2.751×10^3 s^{-1} and 4.187×10^6 $M^{-1}s^{-1}$, respectively.
- The *pI* of the purified L-asparaginase was determined by Iso electric focusing (IEF) and it was found to be 8.4.
- N-terminal sequence of this purified L-asparaginase found to be a novel enzyme. L-Asparaginase from *P. carotovorum* MTCC 1428 showed 80% sequence similarity with *E. chrysanthemi* enzyme.

- The optimal level of DO was found to be 20% at pH of 8.5 after 12 h of fermentation in batch bioreactor. Under these conditions, *P. carotovorum* MTCC 1428 produced 17.97 U ml^{-1} corresponding to a productivity of 1497.50 of U L^{-1} h^{-1} of L-asparaginase.
- The feeding stream containing L-asparagine was found to be the best strategy for enhanced production of L-asparaginase in fed-batch studies. Under these conditions, production of L-asparaginase increased to 38.78 U ml^{-1} corresponded to a productivity of 1615.83 U L^{-1} h^{-1} of L-asparaginase. Enhancement in the enzyme activity and productivity from batch to fed-batch were observed to be 115.8 % and 7.9 %, respectively.
- Kinetic models were developed for dual substrate growth, substrates utilization and L-asparaginase production by *P. carotovorum* MTCC 1428 in batch bioreactor. The proposed models fits adequately the experimental results and able to simulate the growth, substrate(s) consumption, and L-asparaginase production of *P. carotovorum* MTCC 1428 accurately.

Future scope of work

1. *In vivo* studies of purified L-asparaginase from *P. carotovorum* MTCC 1428 to established its full potential as a chemotherapeutic agent.
2. Genetic improvement through cloning and expression encoding genes of L-asparaginase from *P. carotovorum* MTCC 1428. Development of high level expression system for the production of recombinant L-asparaginase.
3. Studies on biophysical properties of this novel glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428.

4. Immobilization studies of purified L-asparaginase to increase stability by physical entrapment and conjugation with low immunogenicity, antigenicity and biodegradable bioconjugates.

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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 50mM Tris buffer of pH 8.6. $(NH_4)_2SO_4$ 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.*, 0.2 to 2.0 of interval 0.2 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 section 3.6.1. The points were fitted with a linear regression model with the help of Microsoft Excel® software (Fig. A.1). Experiments for L-asparaginase activity in test samples were performed by modified Nessler`s method as described in section 3.6.1. Then amount of ammonia in the test sample was calculated with the help of OD 425 nm data and the slope of calibration 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 = 3.891 µmol of ammonia).

Calculation for L-asparaginase activity

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

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

Where, A_c = amount of ammonia released during reaction in µmol (test sample absorbance at 425 nm (Abs_{425}) × 3.391 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 putting the all values in Eq. A.1, it simplify into Eq. A.2

L-asparaginase activity (U ml^{-1}) = Abs_{425} × 14.039 (A.2)

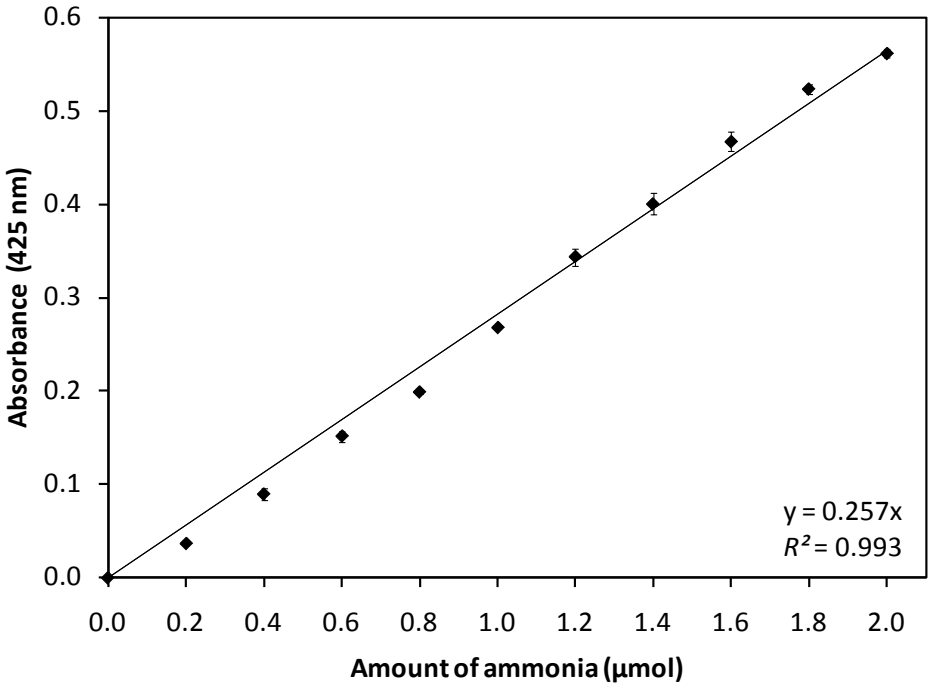

Fig A.1. Standard curve drawn between known amount of ammonia and the corresponding OD measured at 425 nm.

A.2. Sample calculation for the estimation of glucose

Preparation of standard plot for glucose

Stock solution of 1 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 of interval 0.1 as shown in X-axis of Fig A.2. Experiments

were performed for standard curve in triplicates and absorbance of the standard samples was measured at 540 nm against the appropriate blank as described for test samples in section 3.6.4. The points were fitted with a linear regression model with the help of Microsoft Excel® software (Fig. A.2). Glucose concentration in test sample was measured by DNS method as described in section 3.6.4. Then concentration of glucose (mg ml^{-1}) in the test sample was calculated with the help of OD 540 nm data and the slope of standard curve (1 unit OD at 540 nm = 0.636 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_{540} × 0.636 (A.3)

Where, Abs_{540} = test sample absorbance at 540 nm against appropriate blank.

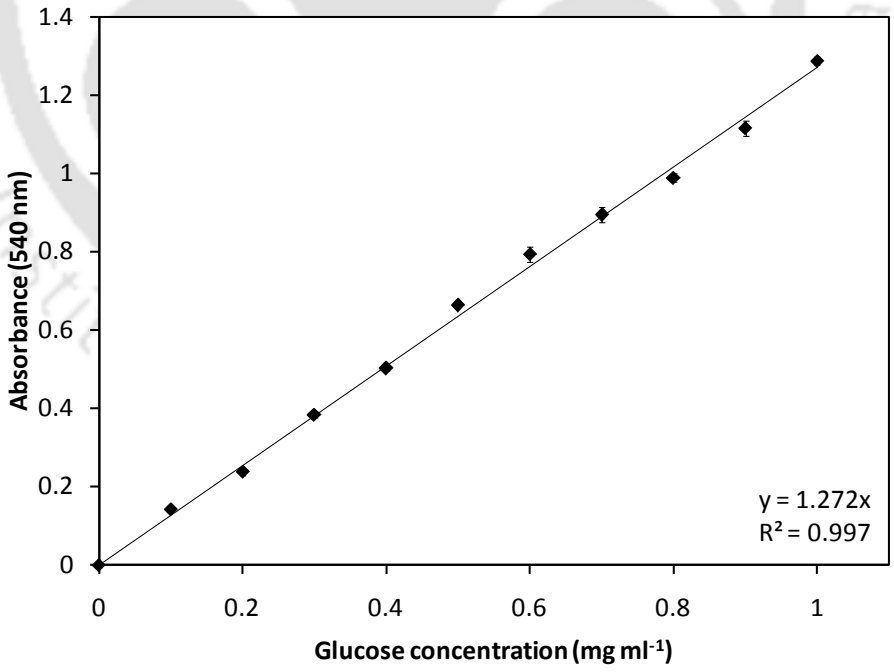

Fig A.2. Standard curve drawn between known glucose concentration and the optical density measured at 540 nm.

A.3. Sample calculation for the estimation of protein

Preparation of standard plot for protein

Stock solution of 0.1 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*., 0.01 to 0.1 of interval 0.01 as shown in X-axis of Fig A.3. Experiments were performed for standard curve in triplicates and absorbance of the standard samples was measured at 660 nm against the appropriate blank as described for test samples of protein in section 3.6.2. The points were fitted with a linear regression model with the help of Microsoft Excel® software (Fig. A.3). 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 with the help of data at OD 660 nm and the slope of standard curve (1 unit OD at 660 nm = 0.370 mg ml^{-1} of protein).

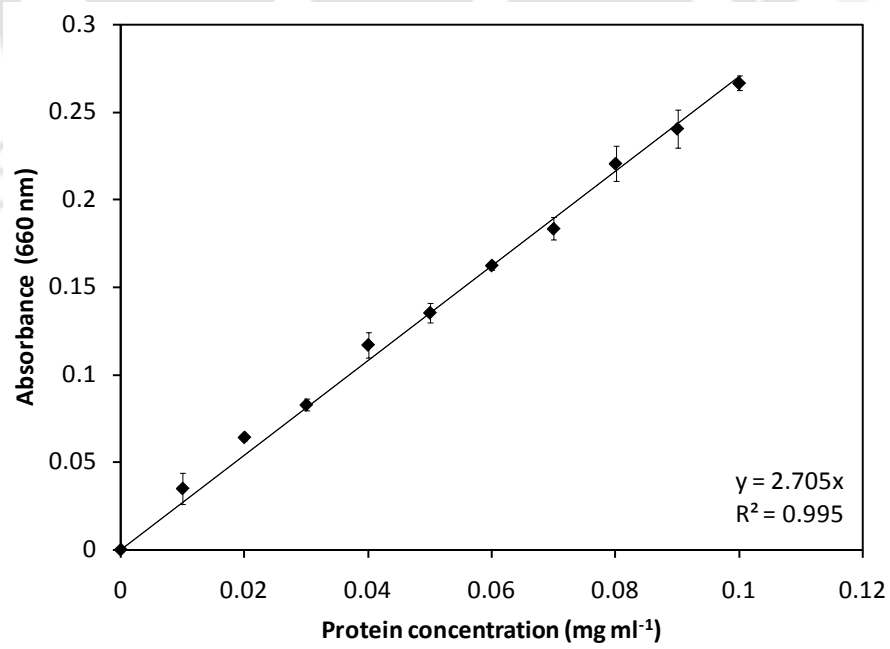

Fig A.3. Standard curve drawn between known protein concentration and the optical density measured at 660 nm.

Calculation for protein concentration

Protein concentration in test sample was calculated by the following Eq. A.4.

Glucose concentration (mg ml^{-1}) = Abs_{660} × 0.370 (A.4)

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

A.4. Sample calculation for the estimation of 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 section 3.6.3. Different dilution of cell samples were used for measuring cell OD (~0.1-1.0) at 600 nm and corresponding DCW (g l^{-1}) determined at 105°C for 24 h (Fig. A.4). DCW of the unknown sample was determined by measuring the OD of the culture broth at 600 nm using UV-visible spectrophotometer and compared with standard curve between OD at 600 nm vs. DCW (1 unit OD at 600 = 0.272 g l^{-1} DCW).

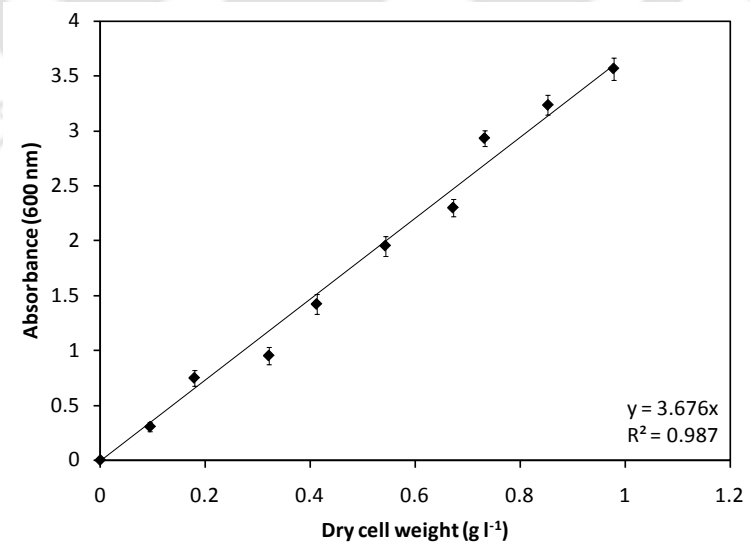

Fig A.4. Standard curve drawn between cell dry weight of *P. carotovorum* MTCC 1428 and the optical density measured at 600 nm.

Calculation for DCW

Protein concentration in test sample was calculated by the following Eq. A.5.

Dry cell weight concentration ($g\ l^{-1}$) = $Abs_{600} \times 0.272$ (A.5)

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

A.5. Sample calculation for the estimation of L-asparagine

Preparation of standard plot for L-asparagine

Experiments were performed for standard curve in duplicates and injected the known L-asparagine concentration sample into the HPLC column and L-asparagine peak was detected using UV (263 nm) detection system as described in section 3.6.5. The L-asparagine concentration in the sample was determined based on a standard curve obtained with L-asparagine concentration (10-500 µM) *vs*. peak area as standard (Fig. A.5). L-asparagine sample was diluted in range of 10-500 µM with boric acid buffer (pH 8.5, 200 mM). The points were fitted with a linear regression model using Microsoft Excel® software. (Peak area 1 (mAU.min) at 263 nm = 0.707 µM of L-asparagine)

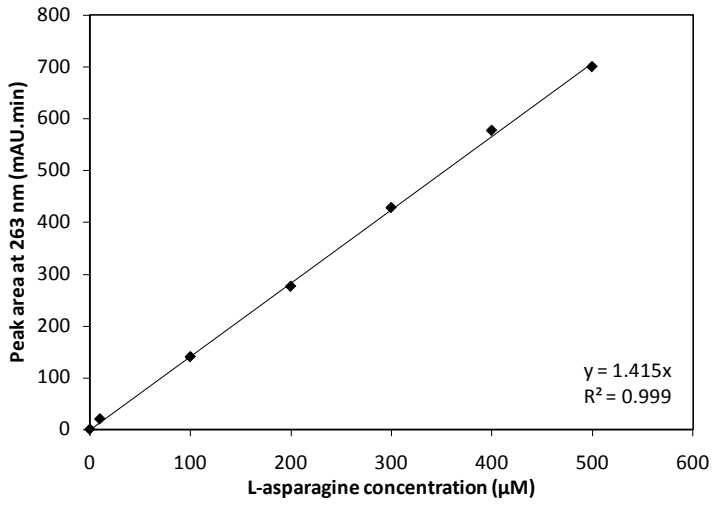

Fig A.5. Standard curve drawn between known L-asparagine concentration (µM) and peak area.

Calculation for L-asparagine concentration

L-asparagine concentration ($C_{L\text{-}asp}$) in test sample was calculated by the following Eq. A.5.

$C_{L\text{-}asp}$ (µM) = Peak area at 263 nm×0.707×dilution factor (D)

(A.5)

$$\text{L-asparagine concentration (g l}^{-1}) = \frac{C_{L-asp} \times 10^{-6}}{MW_{L-asp}} \quad \text{(A.6)}$$

Where, $MW_{L\text{-}asp}$ = L-asparagine molecular weight (132.11)

A.6. 16S rRNA sequence of *Serratia marcescens* SK 07

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   1 AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC TTAACACATG CAAGTCGAGC GGTAGCACAG GGGAGCTTGC TCCCCGGGTG ACGAGCGGCG
 101 GACGGGTGAG TAATGTCTGG GAAACTGCCT GATGGAGGGG GATAACTACT GGAAACGGTA GCTAATACCG CATAACGTCG CAAGACCAAA GAGGGGGACC
 201 TTCGGGCCTC TTGCCATCAG ATGTGCCCAG ATGGGATTAG CTAGTAGGTG GGGTAATGGC TCACCTAGGC GACGATCCCT AGCTGGTCTG AGAGGATGAC
 301 CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT
 401 GTGAAGAAGG CCTTCGGGTT GTAGAGCACT TTCAGCGAGG AGGAAGGTGG TGAGCTTAAT ACGCTCATCA ATTGACGTTA CTCGCAGAAG AAGCACCGGC
 501 TAACTCCGTG CCAGCAGCCG CGGTAATACG GAGGGTGCAA GCGTTAATCG GAATTACTGG GCGTTAAGCG CACGCAGGCG GTTTGTTAAG TCAGATGTGA
 601 AATCCCCGGG CTCAACCTGG GAACTGCATT TGAAACTGGC AAGCTAGAGT CTCGTAGAGG GGGGTAGAAT TCCAGGTGTA GCGGTGAAAT GCGTAGAGAT
 701 CTGGAGGAAT ACCGGTGGCG AAGGCGGCCC CCTGGACGAA GACTGACGCT CAGGTGCGAA AGCGTGGGGA GCAAACAGGA TTAGATACCC TGGTAGTCCA
 801 CGCTGTAAAC GATGTCGATT TGGAGGTTGT GCCCTTGAGG CGTGGCTTCC GGAGCTAACG CGTTAAATCG ACCGCCTGGG GAGTACGGCC GCAAGGTTAA
 901 AACTCAAATG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGATGC AACGCGAAGA ACCTTACCTA CTCTTGACAT CCAGAGAACT
1001 TTCCAGAGAT GGATTGGTGC CTTCTGGAAC TCTGAGACAG GTGCTGCATG GCTGTCGTCA GCTCGTGTTG TGAAATGTTG GGTTAAGTCC CGCAAGGAGC
1101 GCAACCCTTA TTCTTTGTTG CCAGCGGTTC GGCCGGGAAC TCACAGGAGA CTGCCAGTGA TAAACTGGAA GAACGTGGGG ATGACGTCAA GTCATCAAGG
1201 CCCTTACGAG TAGGGCTACA CACGTGCTAC AATGGCATAT ACAAAGAGAA GCGACCTCGC GAGAGCAAGC GGACCTCATA AAGTATGTCG TAGTCCGGAT
1301 TGGAGTCTGC ATCTCGACTC CATGAAGTCG GAATCGCTAG TAATCGTAGA TCAGAATGCC ACGGTGAATA GGTTCCCGGG CCTTGTACAC ACCGCCCGTC
1401 ACACCATGGG AGTGGGTTGC AAAAGAAGTA GGTAGCTTAA CCTTCGGGAG GGCGCTTACC ACTTTGTGAT TCATGACTGG GGTGAAGTCG TAACAAGGTA
```

Fig. A.6. 16S rRNA sequence of the isolated bacterial strain *Serratia marcescens* SK 07 (GenBank accession no. FJ612597).

LIST OF PUBLICATIONS

Published in Referred International Journals

Kumar S, Venkata Dasu V, Pakshirajan K. 2010. Localization and production of novel L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. *Process Biochemistry* **45**: 223-229.

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Agarwal[#] A, **Kumar[#] S,** Venkata Dasu, V. 2010. Optimization of process conditions to maximize the production of L-asparaginase from a newly isolated *Serratia marcescens* SK-07. *Letters in Applied Microbiology* DOI: 10.1111/j.1472-765X.2011.03006.x. ([#]Equal contribution)

Kumar S, Venkata Dasu V, Pakshirajan K. 2010. Studies on pH and thermal stability of purified novel L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. *Microbiology* (Accepted).

Submitted in Referred International Journals

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Kumar S, Venkata Dasu V, Pakshirajan K. 2007. Studies on localization and production of bacterial L-asparaginase. *48th Annual conference of AMI*, Dec 18-21, IIT Madras, Chennai, India. IN15, p 22.