Bacillus subtilis as an expression host for the production of glutaminase free recombinant L-asparaginase II

A THESIS

Submitted by

SUSHMA CHITYALA
(126106027)

For the award of the degree

of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
GUWAHATI-781039, ASSAM, INDIA

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Dedicated to my Husband, Parents &

Grand Parents

INDIAN INSTITUTE OF TECHNOLOGY (IIT) GUWAHATI

Department of Biosciences and Bioengineering

Guwahati -781039

CERTIFICATE

It is certified that the work described in this thesis, entitled "***Bacillus subtilis* as an expression host for the production of glutaminase free recombinant L-asparaginase II**", done by **Sushma Chityala** for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in Department of Bioscience and Bioengineering, Indian Institute of Technology Guwahati, India.

The results embodied in this thesis have not been submitted in any other university or institute for the award of any degree.

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STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India, under the guidance of Prof. Venkata Dasu Veeranki.

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

November 2017
Guwahati

Sushma Chityala

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Date: **Sushma Chityala**

ABSTRACT

The bacterial L-asparaginase has been widely used as a therapeutic agent in the treatment of ALL (acute lymphoblastic leukemia). Moreover, it is used in food industry for the production of acrylamide free starchy/baked foods, L-asparagine biosensor for diagnosis of leukemia and as a model enzyme for the development of new drug delivery systems. The various side effects of L-asparaginases are mainly due to the presence of partial glutaminase activity. Hence, we made an attempt to produce glutaminase-free L-asparaginase II, which is highly desirable for its successful application.

The gene encoding glutaminase-free L-asparaginase II (*ans B*2) from *Pectobacterium carotovorum* MTCC 1428 was cloned into pHT43 vector and transformed in *Bacillus subtilis* WB800N. It was further optimized to maximize the expression levels of recombinant enzyme (rL-asp II). A three-fold higher enzyme production was observed with an efficient transformant as compared to native strain. Enzyme localization studies revealed that > 90 % of recombinant enzyme is secreted extracellularly. The expression of recombinant L-asparaginase II was confirmed by SDS-PAGE, IMAC (Immobilized metal ion affinity chromatography) purification followed by Western blotting. Process parameter optimization with OFAT (one factor at a time) revealed that an agitation (120 rpm), temperature (37 ºC), Isopropyl b-D-1-thiogalactopyranoside (IPTG) concentration (1 mM) and time of induction (at 0.8 OD600nm) plays a vital role in achieving a maximum of 55 IU/ml. Furthermore, consecutive induction by IPTG improved the enzyme production up to 105 IU/ml of protein.

The crucial components, which elevate the expression of rL-asp II from *Bacillus subtilis* WB800N were analysed. The Plackett-Burman tool identified sucrose, NH_4Cl, NaH_2PO_4 and $MgSO_4$ as the significant influencing factors ($p<0.05$). Further

investigations showed that artificial neural network genetic algorithm (ANN-GA) was more effective than central composite design (CCD) in optimizing the influencing factors. The maximum rL-asp II expression was found to be 389.56 IU/ml and 525.98 IU/ml using CCD (R^2=90.4%) and ANN-GA (R^2=96.2%), respectively. The validation experiments were carried out in a 3 L batch bioreactor where kinetic modelling of the obtained data was done.

The possible effect of growth inhibition caused due to initial sucrose concentration on *Bacillus subtilis* WB800N for the production of rL-asp II was evaluated. The experiments were carried out at different initial substrate concentrations of sucrose (2.5 - 180 g L^{-1}). The results obtained were used to develop and analyze the biokinetic growth models. Among different concentrations, 40 g L^{-1} of sucrose showed highest specific growth rate of 0.125 h^{-1}. Employing various 3 and 4 parametric Monod-variant models estimated that the 4- parameter growth model proposed by Luong was found to be best fit, to describe the dynamics for sucrose consumption and growth kinetics of *Bacillus subtilis* WB800N (R^2=0.979). The non-linear regression analysis was applied to estimate the model parameters as well as goodness of fit. The optimization of model parameters using parameter sensitivity analysis revealed that μ_{max}, K_I, and γ_S^* are the most sensitive parameters in modeling of growth inhibition kinetics of recombinant *Bacillus subtilis* WB800N.

The purified rL-asp II showed no glutaminase activity, which may reduce the possibility of side effects of the enzyme during the course of anti-cancer therapy. The rL-asp II from *B. subtilis* WB800N was purified using His-tag column. The yield of purified enzyme was found to be 42.91 IU indicating 86.9 % recovery with purification fold of 3.79. The molecular modelling of the L-asparaginase II was performed to analyze the residues involved in active site formation. The L-asparaginase II sequence was modelled

using the modeler 9.13 software. The structure-based sequence alignment of 1HG1.A, 2JK0.A pdb structures with modelled protein showed sequence similarity of 67.5 % and 67.3 % while the percentage of secondary structure similarity was observed to be 88 % and 87 %, respectively. The absence of GLU63 in rL-asp II was found to be the main reason behind the no glutaminase activity. It predicted that amino acids, GLY60, GLY119 and ALA252 in the active site are responsible for the glutaminase free L-asparaginase II activity.

The rL-asp II enzyme is very specific towards its natural substrate, L-asparagine. The activity of rL-asp II was activated by mono cations and various effectors including Na^+, K^+, L-histidine, L-cystine, 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 rL-asp II were found to be 0.65 mM, 4.018 IU μg^{-1} and 2.496×10^3 s^{-1}, respectively. Optimum pH of the purified enzyme for the hydrolysis of L-asparagine was in the range of 8.0 – 9.0, and its optimum temperature was found to be 40°C. The minimum deactivation rate constant (k_d) and maximum half life ($t_{1/2}$) were found to be 0.043 min^{-1} and 16.1 h, respectively at pH of 8.6 and 40ºC. Thermodynamic parameters (ΔG^*, ΔH^*, ΔS^*, and activation energy) were also evaluated for purified rL-asp II. The probable deactivation mechanism of rL-asp II was described in this study. The fluorescence spectral studies reveal that the enzyme retains its secondary structure at 40ºC. In addition, the effect of rL-asp II on acrylamide reduction was evaluated. It was observed that the acrylamide content of potato chips using mustard oil reduced up to 95 % by using 10 IU/ml of rL-asp II per gm of potato chips. *In vitro* anti-cancer activity assays using three different human cancer cell lines (HeLa, SAS and MCF 7) were done to evaluate the therapeutic potential of rL-asp II.

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Abbreviations

ALL	acute lymphoblast leukemia
AIC	Akaike's information criteria
ATTC	American Type Culture Collection
*ans B*2	L-asparaginase II gene from the *Pectobacterium carotovorum* MTCC 1428
ANOVA	analysis of variance
ANN-GA	Artificial neural network linked Genetic algorithm
aprE	Serine alkaline protease (subtilis E)
B. subtilis	*Bacillus subtilis*
bpr	Bacillopeptidase F
bsr	blasticidin
ble	bleomycin
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 (%)
E. coli	*Escherichia coli*
ErA	*Erwinia chrysanthemi*
epr	Minor alkaline extracellular serine protease
EDTA	Ethylene diamine tetra acetic acid
FMOC-Cl	9-fluorenylmethyl chloroformate chloride
GdnCl	guanidine hydrochloride
GRAS	Generally regarded as safe
HPLC	high performance liquid chromatography
HRP	HRP-labelled rabbit anti-mouse IgG H&L

Abbreviations

hyg	hygromycin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MS	mean square
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MSE	Mean square error
nprE	Extracellular neutral metalloprotease
neo	neomycin
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PB	Plackett-Burman
p-CMBA	*p*-chloromercuribenzoic acid
PCR	polymerase chain reaction
PEG	polyethylene
rDNA	Recombinant DNA technology
rL-asp II	Recombinant L-asparaginase II
RSM	response surface methodology
RPM	Rotations per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
sp.	species
SS	sum of square
SSE	sum of square of errors
SSD	sum of squares of the differences
SSF	solid state fermentation
TCA	tri-chloro acetic acid
TBST	Tris-Buffered Saline and Tween 20
TES	Trace element solution

Abbreviations

vpr	Minor extracellular serine protease

Notations

°C	degree centigrade
b	Variable of AIC analysis (number of parameter plus one)
C	integration constant
D	dilution factor
E	activation energy
g	gram
g	gravitational acceleration
h	hour
K	Webb constant (g L^{-1})
K_1	inhibition constant for sucrose (g l^{-1})
Ks	Half saturation constant for sucrose (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_s	Monod half saturation constant for sucrose (g l^{-1})
M	molar (mol l^{-1})
min	minute
ml min^{-1}	milliliter per minute
MW	molecular weight
M	molecular weight of enzyme (kDa)
n	constant in Loung model accounts the relationship between μ and sucrose
nm	nanometer
R^2	regression coefficient

Notations

s	second
S	Sucrose concentration (g l^{-1})
p	Number of experimental data points
P_{AIC}	Probability that model with given AIC_c value is correct
Prm	Number of parameters to be estimated in a model
Δ_{AICc}	Difference in AIC_c Value between two models
IU	International unit of enzyme activity
IU mg^{-1}	unit of enzyme activity per milligram
IU ml^{-1}	unit of enzyme activity per milliliter
v/v	volume/volume
Ve	elution volume
V_{max}	Maximal reaction velocity (IU 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})
Y	predicated response
$Y_{X/S}$	yield of product (rL-asp II) on sucrose (IU g^{-1} of sucrose)
qs/h^{-1}	specific sucrose uptake rate per hour

Greek letter

μ	specific growth rate (h^{-1})
μ_{Expt}	experimental specific growth rate (h^{-1})
µl	microlitre
γ_s	Initial sucrose concentration, g L^{-1}
γ_s^*	Critical sucrose concentration, g L^{-1}
μ_m	maximum specific growth rate (h^{-1})
µM	Micromolar (µmol l^{-1})
µmol	Micromoles
μ_{Pred}	theoretical specific growth rate (h^{-1})

Notations

α	growth-associated constant for L-asparaginase production (IU mg^{-1})
β	non growth-associated constant (IU mg^{-1} h^{-1})
β_i	Linear effect
β_{ii}	Square effect
β_{ij}	Interaction effect
β_o	Offset term

Chapter 1

Introduction

1.1. Background and motivation

Bacillus subtilis is a gram positive, nonpathogenic, endo and exotoxin free bacteria. The tag of GRAS (Generally regarded as safe) approved, made it an explorable platform for production of various heterologous proteins, vitamins, antibiotics, etc., [1], [2]. In recent days it got much attention as an attractive cell factory for the production of both intracellular and extracellular enzymes [3]. The second most important reason for wide use of *Bacillus* sp is, due to extracellular secretion of most of the recombinant enzymes into broth which reduces the cost of downstream processing as well as absence of codon bias [4]–[6]. *Bacillus subtilis* WB800N used in this study is an eight extracellular protease deficient strain (nprE aprE epr bpr mpr::ble nprB::bsr vpr wprA::hyg) [7] for the extracellular production of proteins [1], [4], [8], [9]. Improved methyl parathion hydrolase variants were constructed using *Bacillus subtilis* WB800 secretory expression system [10]. Thermo-stable β-1,3-1,4-glucanase from *Clostridium thermocellum* was successfully cloned and expressed in WB800 strain by Luo *et al.,* [11] . Nguyen *et al.,* [12] cloned and expressed nattokinase at high level (600 mg/L protein) into the culture medium under the control of acoA promoter which is very high compared to any extracellular-protease-deficient *B. subtilis* system.

L-asparaginase comes under the family of amidohydrolase that catalyzes the hydrolysis of L asparagine to aspartic acid and ammonia. The enzyme is widely used as anti-leukemic agent for treating cancer cells by making cells deprived of L-asparagine [13]. It has been used in food industries for the reduction of acrylamide in starchy products [14] and also studied in development of L asparagine biosensors for leukemia detection in blood samples [15]. Although L-asparaginase is produced by many bacteria, fungi and plants sources [16], only asparaginase produced by *E. coli* and *Erwinia chrysanthemi* are used in treatment of acute lymphoblastic leukemia (ALL) patients [17]. Circumstantially, the use of L-asparaginase from *E.Coli* and *ErA* caused some side effects like hypertriglyceridemia in acute

lymphoblastic leukemia patients with 11q23 abnormality, hepatotoxicity, impairments in blood coagulation, and neurotoxicity due to the presence of glutaminase activity that limits this enzyme usage [18], [19]. Hence, alternative sources of novel L-asparaginases without glutaminase activity were investigated [20], [21].

The aim of my project was to clone the L-asparaginase II gene (*ans B2*) of *Pectobacterium carotovorum* MTCC 1428 into *Bacillus subtilis* WB800N for the enhanced production of recombinant L-asparaginase II (here after referred to as rL-asp II) protein into broth medium which will reduce the cost of downstream processing compared to native strain. The bioprocess development of rL-asp II was studied from shake flask level to reactor level. Then the impact of produced rL-asp II on starchy food products as well as cytotoxicity effect on the cell lines were also carried out.

1.2. Objectives of the present study

- Cloning and Expression of L-asparaginase in *Bacillus subtilis*.
- Purification and characterization of recombinant L-asparaginase from *Bacillus subtilis* WB800N.
- Development of medium for enhanced expression of recombinant L-asparaginase.
- Production of recombinant L-asparaginase in a batch bioreactor.
- Evaluation of L-asparaginase role in reduction of acrylamide substrate in starchy material and cytotoxicity of L-asparaginase using cell lines.

1.3. Approach

B. subtilis WB800N and pHT43 vector were purchased from MoBiTec (Gottingen, Germany). *Bacillus subtilis* WB800N is an eight-protease-deficient strain (nprE aprE epr bpr mpr::ble nprB::bsr vpr wprA::hyg). Combined recombinant DNA technology and bioprocess development approaches were employed for the production of glutaminase free L-

asparaginase II enzyme from *Pectobacterium carotovorum* MTCC 1428, while choosing a desired feedstock for *Bacillus* based rL-asp II production (Fig 1.1).

Initially *ansB2* (asparaginase II gene) was cloned from the genome of *Pectobacterium carotovorum* MTCC 1428 into *E. coli* DH5α and subsequently into *Bacillus subtilis* WB800N using pHT43-*ans B2* plasmid. The process development commenced with preliminary expression of the strain under different physico-chemical parameters e.g. IPTG, temperature, agitation, biomass concentration at the time of induction and optical density to find out their respective values which support optimal growth with enhanced rL-asp II production. The initial characterization also includes a detailed characterization of the recombinant strain on wide range of carbon and nitrogen sources in order to screen the nutrient which could be growth supportive or rL-asp II induction or both, followed by optimization of these key nutrients via statistical optimization method (*viz*., CCD and ANN-GA). Further, a multi-nutrient mechanistic model was developed to predict growth kinetics of the organism under different nutritional conditions, which differ in terms of the type of nutrient limitation and their concentration. The model could predict growth kinetics and production kinetics both in nutrient sufficient and starvation condition.

Finally, the produced rL-asp II in batch reactor was purified using His-tag column and the purified enzyme was characterized for its stability studies. The purified rL-asp II was used to check the efficacy of the enzyme in inhibiting the formation of acrylamide and cancerous cell growth. In the broader context, current study was focused to develop a cost effective generation of minimal media for rL-asp II production by reducing secondary metabolite formation, enhanced extracellular production of rL-asp II using novel approaches.

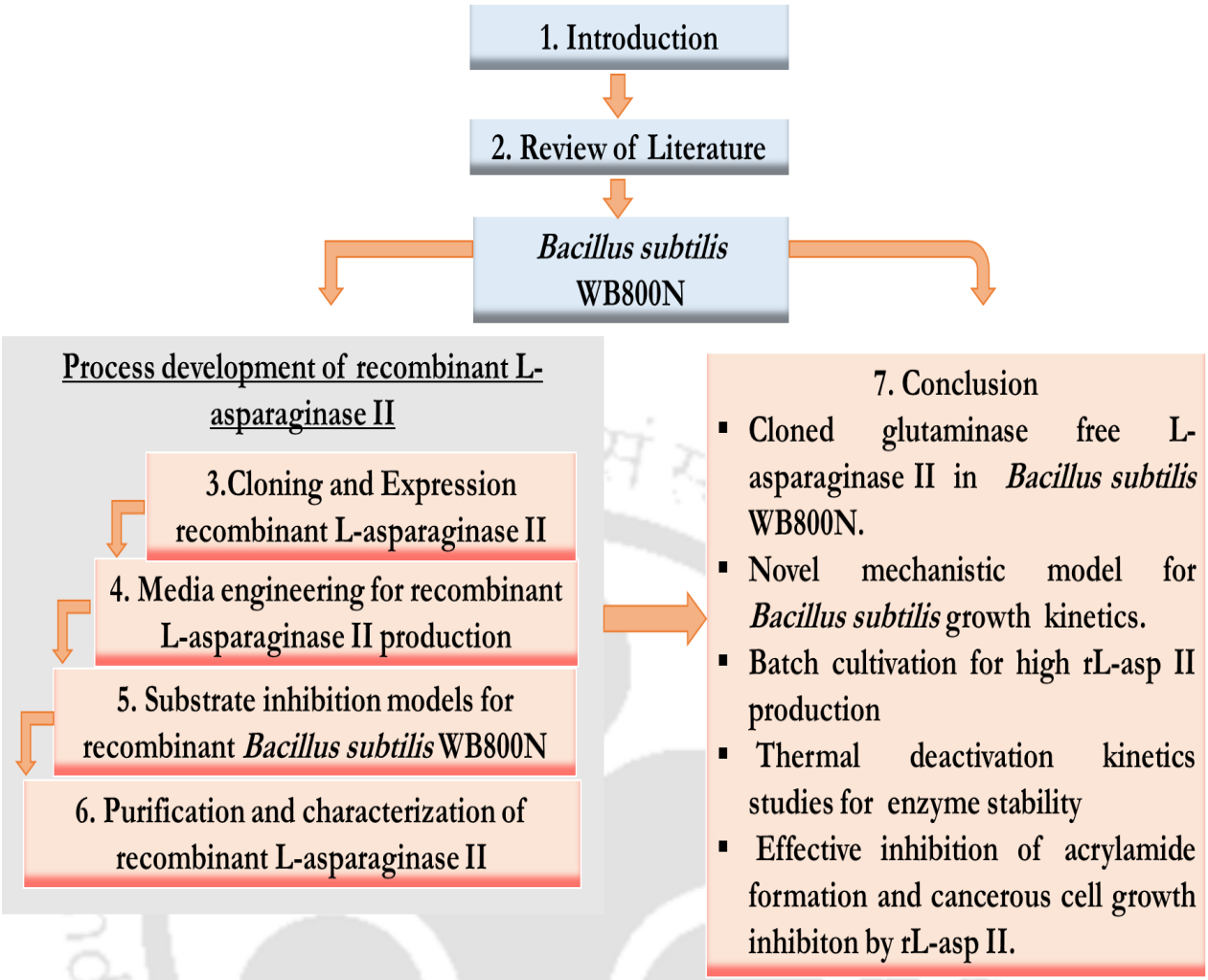

Fig. 1.1 Bioprocess development and statistical modeling approaches employed to achieve high rL-asp II production and to understand the effect of purified rL-asp II through characterization and application, from a novel strain *Bacillus subtilis* WB800N.

1.4. Organization of thesis

The thesis consists of seven chapters. Chapter 1 deals with general background, motivation, objectives of present study and approaches to resolve existing hurdles. Chapter 2 includes a detailed literature survey on *Bacillus subtilis* WB800N and L-asparaginase II, research advancements towards L-asparaginase II production and the existing bottlenecks associated with commercial *E.coli* and *Erwinia* asparaginases. Chapter 3 describes the cloning, expression and confirmation of novel glutaminase free L-asparaginase II of *Pectobacterium carotovorum* MTCC 1428 in *Bacillus subtilis* WB800N. Chapter 4 focuses

on media engineering for enhanced production of recombinant novel glutaminase free L-asparaginase II. Chapter 5 details on modelling and simulation of substrate inhibition models for recombinant L-asparaginase II producing *Bacillus subtilis* WB800N. Chapter 6 deals with purification, characterization and structural stability studies of recombinant L-asparaginase II from *Bacillus subtilis* WB800N. Chapter 7 summarizes key research highlights obtained from the present study with way out for further prospects.

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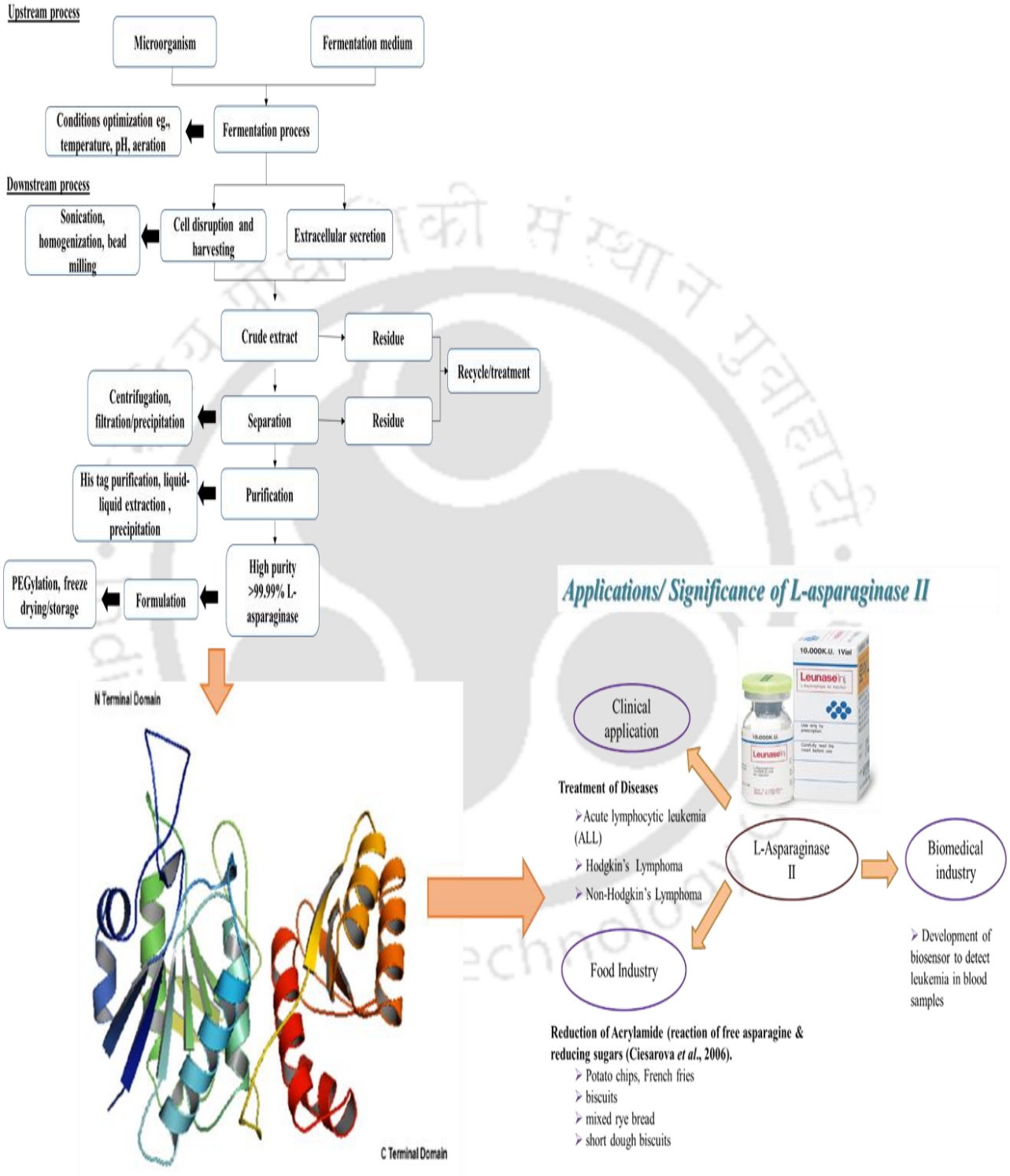
Chapter II

Review of Literature

Graphical abstract



2.1. L-asparaginase, its history and mechanism

L-asparaginase is an amidohydrolase (EC 3.5.1.1),which catalyzes the breakdown of the amino acid L-asparagine into aspartic acid and ammonia [1] (Fig. 2.1). It is widely distributed among living creatures, including animals, plants, and microorganisms except *Homo sapiens* [2]. The application of L-asparaginase came to lime-light, when Kidd., [3] discovered the anti-leukemic effect of guinea pig serum. Since then many researchers are trying to figure out the sources and side effects of the L-asparaginase produced from various sources. L-asparaginase is the first enzyme with antineoplastic properties that has been studied broadly by scientists and researchers. The enzyme was first observed by Lang and Uber [4]. The capabilities of enzyme was under way for more than half a century. It was Clementi [5] who revealed the presence of L-asparaginase in guinea pig serum. L-asparaginase that contains antitumor properties was later proved by Kidd [3]. Broome., [6] made a break through by demonstrating the substrate specificity of L-asparaginase. The development of L-asparaginase as an antileukemic agent is shown in Table 2.1 [7].

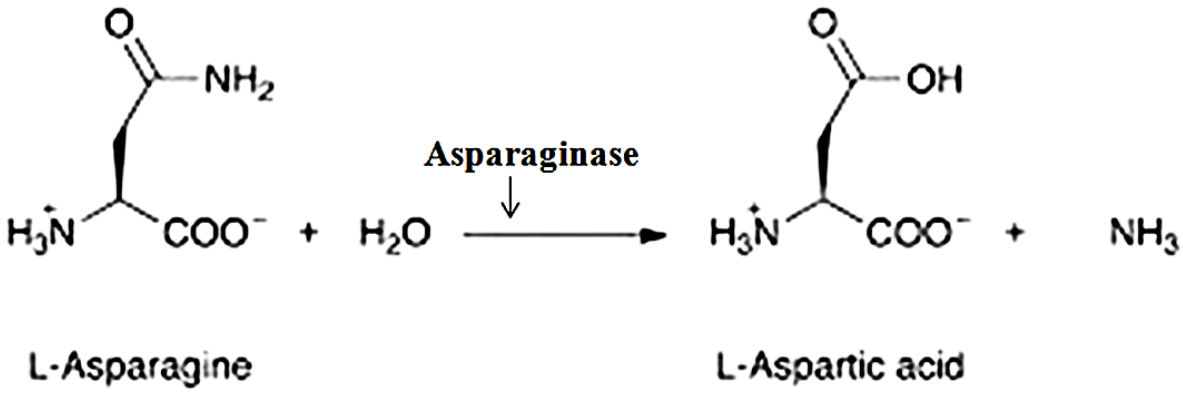


Fig 2.1: Mechanism of Biochemical reaction catalyzed by L-asparaginase II.

Cancer cells, mainly lymphatic cells, require high amount of asparagine (essential amino acid) for its growth. However, leukemic lymphoblast's and few other tumor cells do not have or possess low quantity of L-asparagine synthetase that is essential for L-asparagine syntheses. Thus, these malignant cells are dependent on asparagine from blood serum for their proliferation and survival [8] - [9]. L-asparaginase hydrolyzes asparagine from blood serum, thus starving the tumor cells to death by depriving of an essential factor for protein synthesis (p53-dependent apoptosis). However, healthy cells are not affected, because they are capable of producing asparagine using L-asparagine synthetase. Fig. 2.2 depicts the antineoplastic action of L-asparaginase.

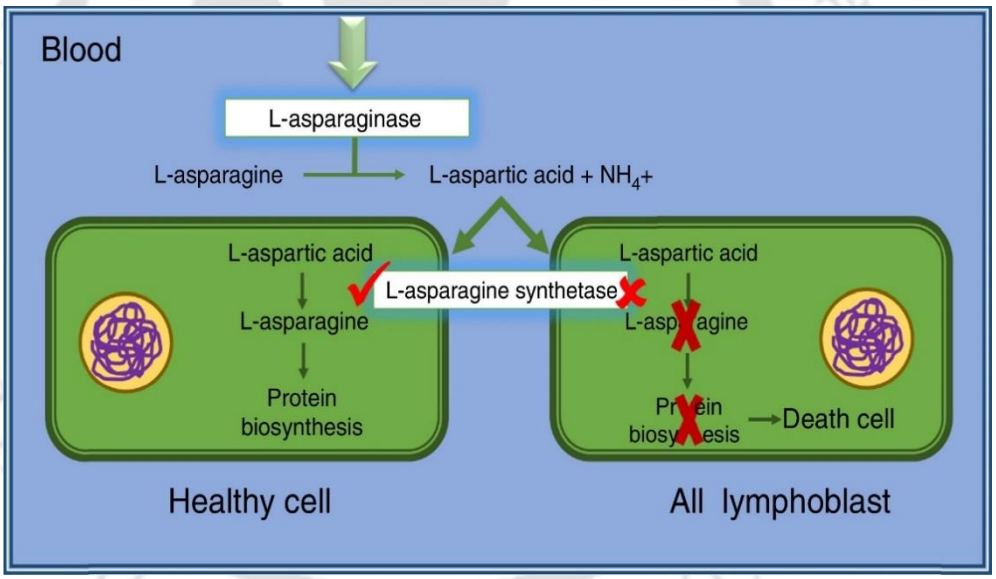


Fig. 2.2 the antineoplastic mechanism of L-asparaginase. Adapted from [7]

Table 2.1. Development of L-asparaginase (LA) [Adapted from [7], [10]]

Year	Advancement
1904	Lang observed first L-asparaginase primarily
1922	Clementi: revealed the presence of L-asparaginase in blood serum of guinea pig
1953	Kidd: discovery of antileukemic effect of guinea pig serum
1963	Broome: identification of L-asparaginase as antileukemic agent in guinea pig serum
1964–1967	Suppression of tumor cell growth by *Escherichia coli* derived L-asparaginase; isolation and purification of active *E. coli* isoform
1966	Dolowy: first clinical use of L-asparaginase
1968	Wade: isolation of L-asparaginase from *Erwinia carotovora* (chrysanthemi)
1978	Native *E. coli* asparaginase approved by FDA for use to treat ALL (Acute lymphoblastic leukemia)
1981	Kamisaki: initial development of pegylated *E. coli* derived asparaginase
1985	*Erwinia* asparaginase authorized in the UK for use to treat ALL
1993	Asselin: identification of distinct pharmacokinetic properties
1994	Pegylated *E. coli* asparaginase (Oncaspar) approved by FDA for use to treat ALL
2006	Pegylated *E. coli* asparaginase approved by FDA for first-line use to treat ALL
2008	Start of COG ALL07P2 and compassionate use EMTP trials to evaluate asparaginase *Erwinia chrysanthemi*
2011	Asparaginase *Erwinia chrysanthemi* approved by FDA for use in patients with hypersensitivity to *E. coli*-derived asparaginase

2.2. L-asparaginase structure

Many scientists tried to elucidate the structure of L-asparaginase at the molecular level. L-asparaginase usually exists as a tetramer but hexameric, dimeric, and monomeric forms are also found in different sources. Most bacterial L-asparaginases exhibit tertiary and quaternary structure [11]. Molecular structures of *E. coli* and *Erwinia* sp. are thoroughly investigated,

and their structural information is easily available [12] - [13]. Both *E. coli* and *Erwinia* sp. have similar three-dimensional structures [14]. *Erwinia carotovora* enzyme consists of two tetramers (ABCD and EFGH) made up of four identical monomers (A to H) each. Three hundred and twenty-seven amino acids assemble themselves in each monomer with 14 α-strands, eight β-helices [15], two domains, a big N-terminal domain and a small C-terminal domain [12]. The active site is positioned in between two adjacent monomers (A and C: B and D). The tetramer consists of four identical subunits. The whole molecule is considered as a dimer of dimers [16] - [17]. Every active site is shaped by the conveyance of amino acids in two adjacent monomers. Following amino acids constitute the active site: Thr15, Tyr29, Ser62, Glu63, Thr95, Asp 96, Ala120, and Lys168, while only one residue Ser254 is present in adjacent monomer.Thr15 and Thr95 are the residues responsible for the catalytic activity of the enzyme [11], [16], [18] - [23].

2.3. L-asparaginase side effects

L-asparaginase causes major side effects, in spite of its potential antileukemic activity. In some cases, the utilization of L-asparaginase by leukemic patients is found to cause lethality to normal cells. L-asparaginase may cause wide range of symptoms such as skin rashes, edema, hepatic dysfunction, fever, diabetes, leucopenia, pancreatitis, neurological seizures, and hemorrhage [24] - [25]. Moola reported that some hypersensitivity reactions, mild allergic reactions, and anaphylactic shock are also caused by the usage of asparaginase based drugs [26]. Adolescents appear to be at a higher risk of neurotoxicity caused by L-asparaginase, which results in depression, lethargy, fatigue, dizziness, and agitation [27]. The toxicity of L-asparaginases is believed to be due to its glutaminase activity [28].

2.4. Sources of L-asparaginase II

The L-asparaginase is present in wide varieties of organisms, including animals, plants, and microorganisms (bacteria, yeast, algae, fungi, and actinomycetes) except humans. Even though L-asparaginase exists in several animals and plants, owing to the difficulties in extraction procedures, researchers have explored other sources for enzyme production (bacteria, yeast, algae, fungi, and actinomycetes). Large-scale production of enzyme from microbes is much easier due to their facile production methods [29]. The different microbial sources of L-asparaginase is briefly outlined.

2.4.1. *Bacterial Sources*

L-asparaginase has been reported from both gram-positive and gram-negative bacterial population from the marine and terrestrial environment [30]. However, gram-positive bacteria have gained less consideration as compared to gram-negative [31]. Major species of both gram-negative and gram-positive classes studied intensively are shown in Table 2.2.

L-asparaginase produced by most of the gram-negative bacteria is classified into two types: type I and type II L-asparaginase. Type I L-asparaginase is expressed quantitatively and possesses enzymatic activity on both L-asparagine and L-glutamine amino acids, while type II L-asparaginase possesses high specific activity towards L-asparagine [17]. Type II L-asparaginase produced from *E. coli* (*Ec A II*) and *Erwinia chrysanthemi* (*Er A*) has been used as an commercial antitumor agent for the effective treatment of ALL for over 30 years [32]. L-asparaginase from *E. coli* and *E. carotovora* is currently in clinical use for the treatment of acute lymphoblastic leukemia [33].

Table 2.2 Bacterial sources of L-asparaginase

Gram-negative bacteria	References	Gram positive bacteria	References
Acinetobacter calcoaceticus	[34]	*Bacillus circulans*	[35]-[36]
Azotobacter agilis	[37]	*B. coagulans*	[38]
Brevibacillus brevis	[39]	*Bacillus sp.*	[40]
Citrobacter sp.	[41]	*B. mesentericus*	[42]
Escherichia coli	[43]	*B. polymyxa*	[44]
Enterobacter aerogenes	[45]	*B. subtilus*	[46]
E. cloacae	[47]	*B. licheniformis*	[48]-[49]
Erwinia aroideae	[50]-[51]	*Corynebacterium glutamicum*	[52]
E. cartovora	[53]	*Mycobacterium bovis*	[54]
E. chrysanthemi	[26]	*Staphylococcus* sp.	[55]
Helicobacter pylori	[56]	*S. aureus*	[57]
Klebsiella pneumoniae	[58]		
Pectobacterium carotovorum	[59]		
Pseudomonas sp.	[60]		
*P. fluorescens*AG	[61]		
P. geniculate	[37]		
P. ovalis	[62]		
P. stutzeri	[63]		
Pyrococcus horikoshii	[64]		
Serratia marcescens	[65]		
Thermus thermophiles	[66]		
T. aquaticus	[67]		
Citrobacter freundi	[68]		
Proteus vulgaris	[69]		
Zymomonas mobilis	[70]		
Vibrio succinogenes	[71]		

2.4.2. *Fungal Sources*

Fungi are another potential source of L-asparaginase along with bacteria. Several adverse side effects caused by bacterial asparaginase often restrain their application. This impediment requests a quest for new sources of L-asparaginase. Human beings are more closely related to fungi as compared to bacteria. Hence, the chances of immunological reaction against fungal L-asparaginase would be minimal [72]. Fungal L-asparaginase has acquired importance mainly based on the fact that it is produced extracellularly into the medium as it is easy to purify. Sources of L-asparaginase-producing fungi are enlisted in the Table 2.3.

Table 2.3 Fungal sources of L-asparaginase II

Fungi	References
Alternaria sp.	[73]
Aspergillus nidulans	[74]
A. niger	[75]
A. oryzae	[76]
A. tamarii	[77]
A. terreus	[78]
Cylidrocapron obtusisporum	[79]
Mucor sp.	[80]
Fusarium roseum	[81]

2.4.3. *Yeast Sources*

L-asparaginase with less toxic effects is reported from the yeast. Asparaginases reported from the yeast includes *Saccharomyces* sp., *Candida* sp., *Pichia* sp., *Rhodotorula*sp., *Hansenula* sp., and *Spobolomyces* sp., exhibits antitumor activity [82]. L-asparaginase producing yeasts are reported in Table 2.4.

Table 2.4 Yeast sources of L-asparaginase

Yeast	References
Candida utilis	[83]
C. bombicola	[84]
Pichia polymorpha	[85]
Rhodosporidium toruloides	[86]
Rhodotorula sp.	[84]
Saccharomyces cerevisiae	[87]

2.4.4. *Actinomycetes Sources*

Occurrence of L-asparaginase presence has also been reported in actinomycetes [88]. Actinomycetes are pervasive worldwide in soil, water, and nature, but only those found in living animals especially in fish are found to possess good enzymatic activity [89]. Moreover, actinomycetes are a better source of L-asparaginase when compared to bacteria and fungi [90]. L-asparaginase II producing actinomycetes are listed in Table 2.5.

Table 2.5. L-asparaginase II producing actinomycetes

Actinomycetes	References
Actinomyces sp.	[33]
Streptomyces albidoflavus	[91]
S. aurantiacus	[91]
S. collinus	[92]
S. griseus	[93]
S. gulbargensis	[94]
S. karnatakensis	[95]
S. tendae	[96]
S. venezuelae	[97]
Thermoactinomyces vulgaris	[98]
*Noccardia*sp.	[99]

2.4.5. *Algal Sources*

Reports are available on the production of L-asparaginase from algal sources such as a yellow green algae, *Vaucheria uncinata* [100] and *Chlamydomonas* sp. [101].

2.5. Available asparaginases

E. coli derived non-pegylated asparaginase preparations are the standard of enzyme in first-line therapy regimens of ALL in many countries. The non-pegylated 'native' *E. coli* product is marketed as Elspar[@] in the USA, Canada and also in some other non-European countries. In Europe, Paronal[@], Asparaginase Medac, and Kidrolase[@] are currently authorized, but differences exist based on the country. The selling and usage of Crasnitinis stopped. An Erwinase[@] derived from *Erwinia chrysanthemi* is authorized in the UK as a second-line of treatment, if the patients show hypersensitivity to either the pegylated or non pegylated *E. coli* produced enzyme products. It is also distributed in several other countries as well. A summary of different generic names of the drug available for clinical use is depicted in Table 2.6.

Table 2.6. Different generic names of L-asparaginase drug available in market (modified from [102].

Commercial product	Pharmaceutical company	Biological source	$t_{1/2}$
Asparaginase medac	Medac, Kyowa Hakko	*E. coli*	8-30 h
Crasnitin (no longer in use)	Bayer AGa	*E. coli*	
Ciderolase	Rhone-Poulenc Rorer	*E. coli*	
Elspar	MSD, Rhone-Poulenc Rorer	*E. coli*	
Erwinase	Speywood	*Erwinia chrysanthemi*	0.6 days; 4–22 h
Oncaspar	Enzon, Rhone-Poulenc Rorer, Medac	*E. coli*	5.8 days; 4.5 days in non-allergic patients with relapses; 2.3 days in allergic patients with relapses

2.6. Applications of L-asparaginase- II

2.6.1. *Anticancer drug*

L-asparaginase is widely used to treat ALL patients in combination with vincristine and a glucocorticoid (e.g., dexamethasone) [103]. Due to its antileukemic properties, it has been considered as a therapeutically important antitumor drug. It is used for the treatment of malignancies such as ALL (mainly in children), acute myelocytic leukemia, Hodgkin's disease, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lympho-sarcoma, reticulo-sarcoma, and melano-sarcoma in combination with other drugs [3], [6].

L-asparagine is an essential amino acid for tumor cell's growth and protein synthesis, while L-asparaginase has the ability to convert L-asparagine to aspartate. So, in the presence of L-asparaginase, tumor/malignant cells are deprived of an important growth factor which results in depletion of asparagine and ultimately leads to death of tumor cells [104]. L-asparaginase is widely reported in plants, animals and microorganisms, but only the asparaginase from *E. coli* and *E. chrysanthemi* has been approved to be used as a part of a multi-chemotherapy agent to treat ALL patients [10].

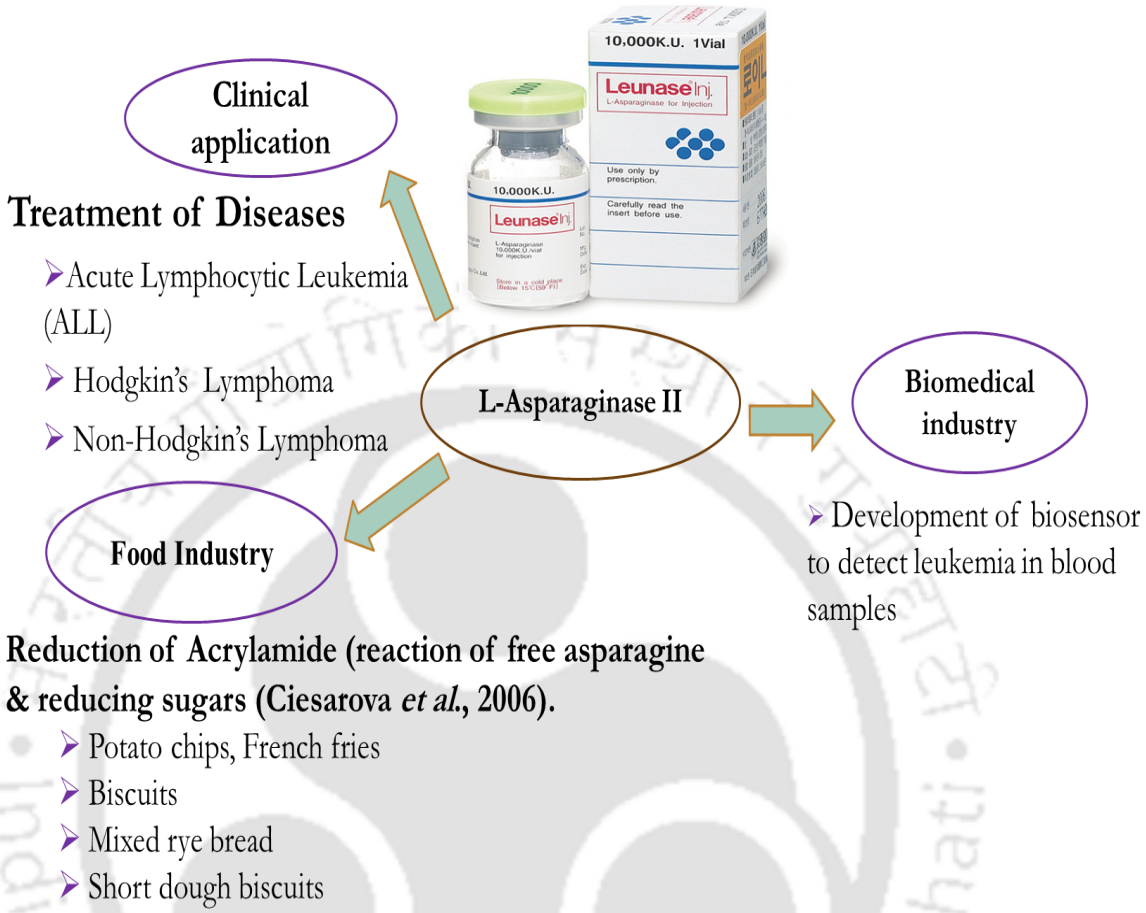


Fig. 2.3. Various applications of L-asparaginase II.

2.6.2. *Role of L-asparaginase in Biosensor*

Apart from the treatment of cancer, L-asparaginase is also used in the development of biosensors to analyze asparagine levels in leukemic samples and the food industry [10]. Several spectroscopic techniques such as XPS, XRD, TEM and SEM, were used for L-asparagine detection, but due to the high cost of the analysis and also tedious procedures make them less favorable [105]. Compared to the traditional methods, biosensor technology can be a reliable, cheap, and user-friendly. The mechanism of action of the biosensor is based on asparaginase activity, i.e., ammonium ions produced from the hydrolysis of asparagine which causes a change in pH that results in the change of absorption and color [106].

2.6.3. *Role of L-asparaginase in Amino Acid Metabolism*

L-asparaginase plays a vital role in the biosynthesis of an aspartate family of amino acids, namely threonine, lysine, and methionine. Besides Krebs cycle, aspartic acid which is a direct precursor of lysine, and threonine, is also formed by the action of L-asparaginase [107].

2.6.4. *Role of L-asparaginase in Food industry*

Furthermore, L-asparaginase is also widely used as a food processing aid. Recent developments in food industry demonstrated the presence of a colorless and odorless crystalline solid, *viz*., acrylamide (2-propenamide). It is, produced through Millard reaction when starchy foods are fried or baked at/above 120 °C [108]-[109]. Acrylamide is a neurotoxin and has been categorized as a carcinogenic to humans [110]. In the food industry, acrylamide is largely formed as a result of caramelization from heat-induced reactions [25] between the α-amino group of the free amino acid (i.e., asparagine) and carbonyl groups of reducing sugars such as glucose during baking and frying [111]. Due to its ability to convert L-asparagine to L-aspartate, L-asparaginase promises to be a possible way to reduce an ample number of precursors involved in Millard reaction by pre-treating the starchy foods (potato and bread dough), hence reducing the risk of acrylamide formation [112]. However, complete removal of acrylamide is not possible due to other independent asparagine formation [113]. Certain L-asparaginase from *Aspergillus oryzae* and *Aspergillus niger* are currently used in food industries [114].

2.7. Industrial production of l-asparaginase

The industrial production of L-asparaginase depends on factors pertaining to a process with economic viability and higher yield. These factors include type and concentration of carbon and nitrogen sources, fermentation time, pH, aeration, temperature and the microbial agents, which plays a major role in the process [7], [115]. As previously reported, several

microorganisms were able to produce L-asparaginase. However, L-asparaginase from *E. coli* and *E. chrysanthemi* are employed for industrial-scale production in pharmaceutical area, where as L-asparaginase from *Aspergillus oryzae* is used in food industry [114], [116]. Fig. 2.4 shows a schematic representation for L-asparaginase production in an industrial process. Different types of culture media have been explored for L-asparaginase production from different types of bacterial sources. However, carbon source and inductor (nitrogen source) are the most influencing components in the medium. For example, several studies have demonstrated the best inductor for reaching high yields to be L-asparagine [117]-[118], L-glutamine [118] and L-proline [77]-[78], [119]-[120]. The most common carbon source is glucose, in addition to alternative sources such as starch [121], sucrose [122] and maltose [117], [123].

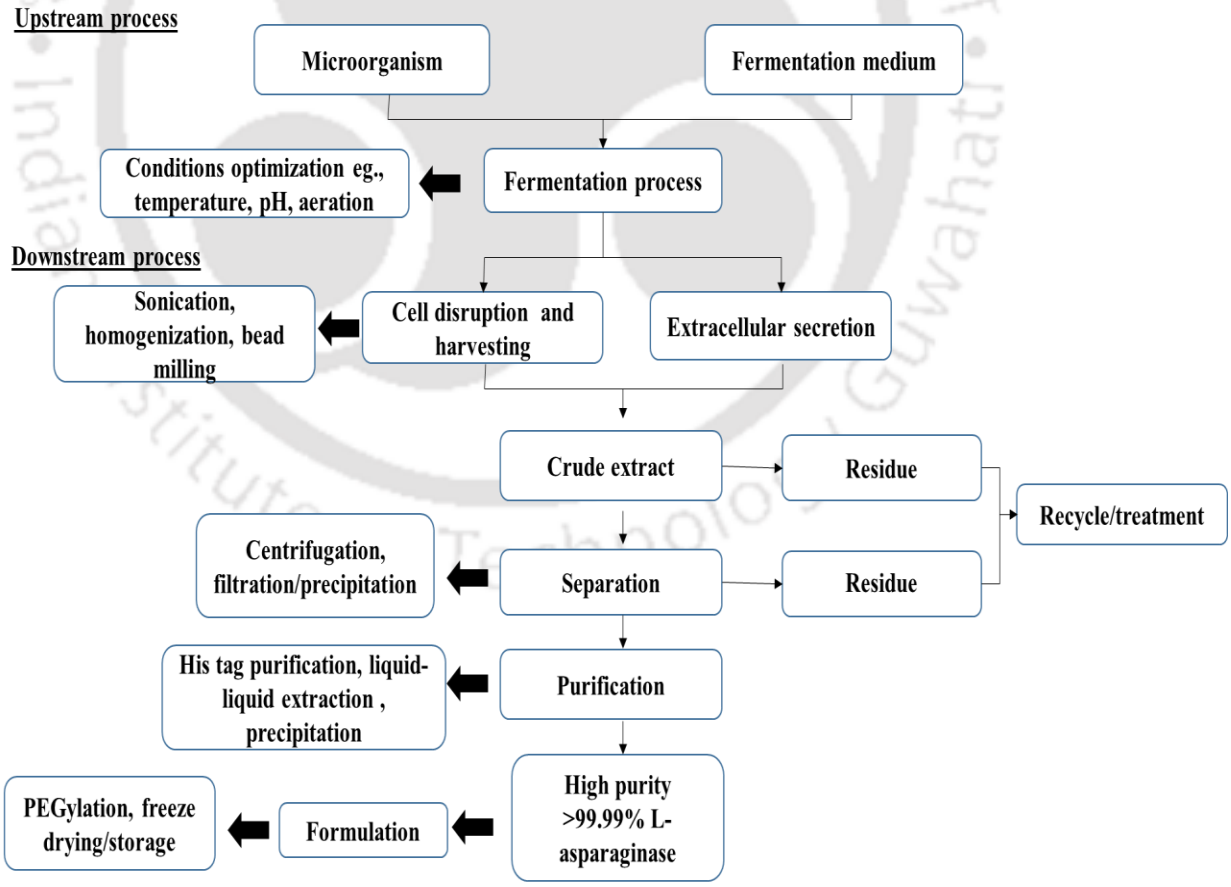


Fig. 2.4 Schematic representation for L-asparaginase production in an industrial process.

2.8. *Bacillus subtilis* as an expression host

Over the last few decades, numerous progress has been witnessed in the field of genetic engineering and cell engineering, where by various specific genes have been cloned and expressed in eukaryotic and prokaryotic cells to enhance heterologous protein production [124]-[125]. Recently, *Bacillus subtilis* expression system has been much focused as an attractive cell factory for the heterologous expression of proteins, vitamins and antibiotics [126]. *Bacillus subtilis* is a gram-positive, non-pathogenic endo and exotoxin free bacteria. It is GRAS (Generally regarded as safe) approved by FDA [127]. *Bacillus* system endowed with unique features such as no codon bias and has potential to secrete recombinant enzymes extracellularly [128]-[129]. Recently, a study was reported that *Bacillus subtilis* is used in fermentation of soybeans (Japanese traditional food Natto) [130]. Till date more than 60% of commercial proteins are produced by *Bacillus subtilis* [131]–[133].

Many products of *Bacillus* species have the status of generally recognized as safe. These unique characteristics and significant progress in genetic manipulation propels *Bacillus* species as a promising production platform for various metabolites [134]-[135]. Currently, the most commonly used bacterial host for industrial production of heterologous proteins is *Escherichia coli*. Since the *E. coli* can be grown easily in large fermentations vessels and is genetically amenable, it has the ability to produce large quantities of proteins. However, in *E. coli* the proteins produced usually accumulate within the cells as aggregates, resulting in the formation of inclusion bodies. To acquire the protein, the inclusion bodies need to be separated from the cell and subsequently the proteins need to be recovered from the inclusion bodies.

Moreover, the outer membrane of *E. coli*, and of gram-negative bacteria in general, contains lipopolysaccharide (LPS or endotoxin), which is highly pyrogenic and needs to be totally removed before using for clinical purposes. Although, most of the commercial proteins

produced by *B. subtilis* are secreted into the medium, there are also successful examples of cytoplasmic protein production in *Bacillus subtilis* [136]. Recently, *B. subtilis* was demonstrated to be an excellent model organism to apply systems biological analyses on metabolic response to various conditions [137]-[138]. Unraveling the metabolic flux regulation and control mechanisms in *B. subtilis* at the systems level further enhanced our understanding of this model gram-positive bacterium. Applying advanced knowledge of various regulatory networks will help to develop *B. subtilis* as a cell factory [139].

Bacillus subtilis has ability to reach high cell densities of 184 g dry cell weight l^{-1} [140], 100 g dcw l^{-1} [141]and 56 g dcw l^{-1} [142], respectively. It has ability to grow on wide varieties of carbon and nitrogen sources. In order to achieve high cell density and high level expression of heterologous protein, optimization of the medium components need to be performed [143]. Successful strategies for engineering *B. subtilis* to improve the protein production include knockout of extracellular and/or intracellular proteases [144]–[146], over-expression of chaperones and folding catalysts [147]-[148], overexpression of components such as secretion machinery, and/or modification of the cell wall micro-environment [149]-[150]. Besides engineering the host, protein can be modified to improve production and/or secretion.

2.9. Medium optimization and bioreactor studies

To increase production of the recombinant protein, we need to optimize the media components. Until now, there are very few reports available on the media optimization of recombinant *Bacillus subtilis* strains. Extracellular production of recombinant human bone morphogenetic protein-7 (rhBMP-7) was carried out through the fermentation of *Bacillus subtilis.* Three significant fermentation conditions and medium components were selected and optimized to enhance the rhBMP-7 production by using the response surface methodology (RSM). The optimum values of the three variables for the maximum

extracellular production of rhBMP-7 were found to be 2.93 g/l starch, 5.18 g/l lactose, and a fermentation time of 34.57 h [151]. Sucrose and ammonium chloride were optimized for the production of lipopeptide using *Bacillus subtilis* MO-01 [152]. The production of surfactin was considerably enhanced when xylose and arabinose were used over glucose as carbon source [153]. Westers *et al*., [144] had found that mineral super rich medium containing 1% xylose and 0.1% glucose enhanced the production of functional human interleukin-3 from *Bacillus subtilis* WB700. The supplementation of trace amounts of metal ions may act as folding catalysts for the secretion of certain proteins [127]. Hence, an optimized medium for the high level of recombinant protein production is very crucial. In order to optimize the media components various statistical approaches [154] were employed.

Optimization studies involving one factor at a time approach are not only tedious and time consuming, but also it overlooks the interaction effect among the variables and leads to the misinterpretation of the results. In contrast, statistical methodologies are preferred due to their advantages [154], such as reducing the number of experiments and interpretation of detailed information about possible interactions among the factors and thus reducing error in an economical manner [155]. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes [156]. RSM shows the effect of the independent variables alone and/or in combination of the processes. The most commonly used design in RSM is central composite design (CCD). The CCD is a full factorial design, composed of a cube part which allows determination of main and interaction effects and a star design (α) for quantifying main and quadratic effects [157].

Apart from statistical approaches such as RSM, other mathematical approaches such as Artificial Neural Network (ANN) coupled with Genetic Algorithm (GA) have also gained much of the research interest, because of their ability to optimize nonlinear modelling

problems with much more efficiency than previous methods [158], [159]. A neural network is an emerging non-linear computational modelling method used to solve engineering problems. Neural network is a collection of interconnecting computational elements which are simulated like neurons and they work well with large amounts of data and require no mechanistic description of the system [160]. The major advantage of ANN over the classical mathematical models is the fact that they allow the simultaneous identification of structure parameters, while they possess the ability to adapt by examples [161]. The concept of Genetic algorithms (GAs) is based on the evolutionary natural selection processes (Darwinism and Mendelism), where selection results in species that fit the best among the population. Genetic algorithm has proved to be extremely suitable for the optimization of highly non-linear problems with many variables and for identifying the global optimum of reported problems.

2.10. References

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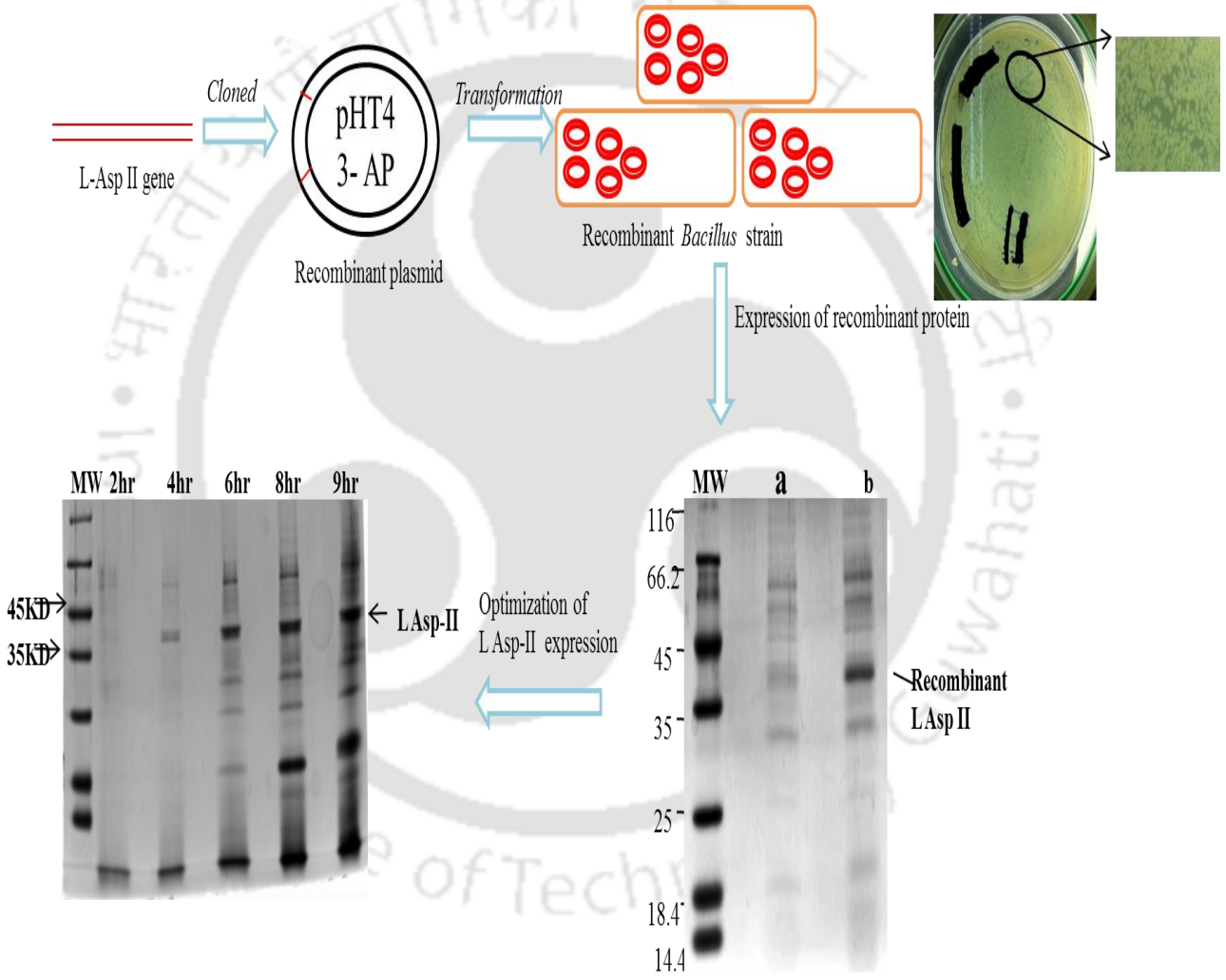
Chapter III

Cloning and Expression

Cloning and Expression of novel glutaminase free L-asparaginase II of *Pectobacterium carotovorum* MTCC 1428 in *Bacillus subtilis* WB800N

Graphical abstract:



3.1. Background and Motivation

L-asparaginase belongs to a family of amidohydrolases that catalyze L-asparagine to aspartic acid and ammonia. L-asparaginase II is widely used as an anti-leukemic agent for the treatment of cancer by making cells deprive of L-asparagine [1]. It also has the potential application in food industries for the reduction of acrylamide in starchy products [2]. L-asparaginase is also used for the development of L-asparagine biosensors in detection of leukemia in a blood samples [3]. Although L-asparaginases are produced by many bacteria, fungi and plants [4], only asparaginase produced by recombinant *E. coli* and *Erwinia chrysanthemi* were being used for the treatment of acute lymphoblastic leukemia (ALL) [5] because of their improved productivity. However, the L-asparaginase produced by *E. coli* and *Erwinia* contains glutaminase activity albeit at low level that limits this enzyme usage, as administration of this enzyme causes side effects like hyper-triglyceridemia in acute lymphoblastic leukemia patients with 11q23 abnormality, hepatotoxicity, neurotoxicity and impairments in blood coagulation [6]-[7]. Hence, alternative sources of novel L-asparaginases without glutaminase activity are being investigated [8]-[9]. *Bacillus subtilis* is gram-positive, non-pathogenic, endo- and exotoxins-free bacteria. *Bacillus subtilis* has GRAS (Generally regarded as safe) status [10]. In recent days, *B. subtilis* expression system is much focused as an attractive cell factory for the heterologous expression of proteins, vitamins and antibiotics [11]. In addition, this strain does not show codon bias and has the potential to secrete recombinant enzymes extracellularly that reduce the cost of downstream processing [12]-[13]. *Bacillus subtilis* WB800N is an eight-protease-deficient strain (nprE aprE epr bpr mpr::ble nprB::bsr vpr wprA::hyg) [14]. It is used as secretory expression system for the construction of improved methyl parathion hydrolase variants by Xie *et al.*, [11]. In another study, Luo *et al.*, [15] cloned thermostable b-1, 3-1, 4-glucanase from *Clostridium thermocellum* in *B. subtilis* WB800 strain and expressed successfully where the yield of the

enzyme was maximized up to 1.18 U/g cell mass. While, Nguyen *et al.*, [16] cloned and expressed nattokinase in *B. subtilis* WB800N under the control of acoA promoter and observed very high productivity (600 mg/L) compared to other extracellular-protease-deficient *B. subtilis* expression systems, such as WB700 and WB600.

In the chapter, we cloned, confirmed and optimized the expression of gene encoding glutaminase free L-asparaginase II of *Pectobacterium carotovorum* MTCC 1428 into *B. subtilis* WB800N. We also determined the enzyme localization and productivity improvement by modulation of inducer supplementation.

3.2. Materials and methods

3.2.1. Chemicals reagents, plasmids and strains

B. subtilis WB800N and pHT43 vector were purchased from MoBiTec (Gottingen, Germany). Primers were obtained from Eurofins (Bangalore). DNA polymerase, oligonucleotides, restriction enzymes and DNA ligase were purchased from New England Biolabs (England). Plasmid isolation and gel elution kits were from Roche (UK). All other chemicals and reagents used were of the highest purity obtained from Hi-Media (India). The amount of DNA used in the experiments was quantified using a Nano-Drop spectrophotometer (ND-1000; USA). The final concentration (100 µg/ml) of antibiotics used for the culturing of strain includes chloramphenicol and neomycin (Sigma-Aldrich). Chemi-luminescent peroxidase substrate-3 was purchased from Sigma-Aldrich.

3.2.2. Cloning of L-asparaginase II

The forward primer (5'-GAA GGATCC GAT GAC GCC AAA CCT GGC GTC ACT ATC-3') with a *BamHI* restriction site and reverse primer (5'-GTA GAC GTC TTA **ATG GTG GTG GTG ATG ATG** *CTTGTCGTCGTCGTC* CTGCTCGAAATAGGTACGGAT TTT-3') with an *AatII* restriction site (underlined letters shows the restriction sites, bold letters indicate 6X-His residues sequence and italic letters indicates enterokinase sequence)

were used in this study. The primers were designed according to the L-asparaginase II gene (*ansB2*) of *Pectobacterium carotovorum* MTCC 1428 (Accession Number: JN638885) available at National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The L-asparaginase II signal peptide was predicted using Signalp 4.1 and the primers were designed excluding signal peptide. The optimized PCR conditions for amplification of the gene were denaturation at 94 ºC for 70 s followed by 30 cycles of 50 s at 94 ºC, 50 s at 70.2 ºC and 1.40 min at 72 ºC followed by a final extension time of 72 ºC for 5 min carried after the 30 cycles. The amplified L-asparaginase II and plasmid pHT43 was digested with *BamHI* and *AatII* then ligated using T4 DNA ligase. The ligated pHT43-*ans B2* (L-asparaginase II cloned) transformed into *E. coli* DH5α and propagated in the medium supplemented with 50 µg/mL ampicillin. The ligation of pHT43-*ans B2* plasmid was confirmed by restriction digestion. The gene sequence of the L-asparaginase II insert was analyzed using EMBOSS Needle program for pairwise alignment of the sequence.

3.2.3. Development of recombinant strain and expression studies

3.2.3.1. Competent cells preparation

B. subtilis WB800N cells were streaked onto neomycin antibiotic agar plate from glycerol stock and the plate was incubated overnight at 37 ºC. According to the modified protocol of Vojcic *et al.*, [17], starting culture was prepared by inoculating single colony into LB media and grown overnight at 37 ºC. Competent cells were prepared by inoculating overnight seed culture into fresh SM1 media (yeast extract 1 g/L, casamino-acids 0.2 g/L, glucose 4 g/L, ammonium sulphate 2 g/L, dipotassium hydrogen phosphate 13.97 g/L, dihydrogen potassium phosphate 6 g/L, tri-sodium citrate 1 g/L, magnesium sulphate hepta-hydrate 0.2 g/L, L-tryptophan 1.1 g/L), with initial OD_{600nm} of ~ 0.1. Growth profile of cells was monitored for every 25 min. When the OD_{600nm} reached 1.9 in SM1 medium, 50 µl of

cells from SM1 medium was transferred to pre-warmed 500 µl of fresh SM2 medium (SM1 medium including calcium chloride 0.05 mM/ml and magnesium chloride 0.25 nM/ml). The cells were incubated for one and half hour and competency of the cells was checked under the microscope. When the cells were highly motile, 1 µg of pHT43-*ans B2* plasmid was added to the SM2 culture medium and incubated for 40 min. Fresh sterile LB media (500 µl) were added to the cells and incubated for another 45 min. Later, cells were centrifuged at 2500 × g for 5 min and the cell pellet was re-suspended in the remaining 100 µl medium and spread over LB agar plate containing chloramphenicol. The plate was incubated at 37 ºC overnight. The transformed colonies were further selected using double antibiotic plate (neomycin and chloramphenicol).

3.2.3.2. Expression of rL-asp II

The pHT43-*ans B2* plasmid transformed into *B. subtilis* WB800N cells were further analyzed for the expression of recombinant protein. Single colony was inoculated in 5 ml Luria–Bertani medium (LB) containing chloramphenicol (100 µg/mL) and incubated at 37 ºC, with 180 rpm overnight. Fresh LB media (100 ml) was inoculated with overnight culture, adjusting the initial OD_{600nm} to 0.1. The cells were grown till they reached 0.7–0.8 OD_{600nm} and were induced with 0.5 mM IPTG. After 8 h of induction, samples were collected and centrifuged at 12,000 × g for 10 min at 4 ± 1 ºC. Supernatant was stored at 4 ºC for analysis of enzymatic assay. The pHT43 vector without L-asparaginase II gene transformed into *B. subtilis* WB800N cells was taken as control.

3.2.3.3. Effect of process parameters on the expression of rL-asp II

Optimization of process parameters was performed using one factor at a time technique to maximize the rL-asp II production. Various parameters at different levels such as temperature (18, 25, 37 and 45 ºC), agitation (100, 120, 140, 160, 180, 200 and 240 RPM), IPTG concentration (0.1, 0.25, 0.5, 1 and 2 mM) and biomass concentration at the time of

induction (IPTG addition), optical density (OD) @ 600nm, OD_{600nm} (0.2, 0.4, 0.6, 0.8 and 1.0 OD_{600nm}) were studied. Fresh sterile LB media (25 ml) were inoculated with overnight grown pre-culture in order to set initial OD_{600nm} of ~ 0.1. The culture was induced with IPTG when it reached an OD_{600nm} of 0.8. The samples were collected up to 8 h of post-induction, centrifuged and stored at 4 ºC for analysis of the recombinant enzyme. Effect of consecutive induction by IPTG experiments were carried out to check the effect of intermittent addition of IPTG on enhancing the expression level of rL-asp II. IPTG induction was performed by adding the IPTG at different growth periods (0, 2 and 4 h). The samples were collected every two hours and the L-asparaginase II expression was analyzed.

3.2.3.4. Effect of consecutive induction by IPTG

Experiments were carried out to check the effect of intermittent addition of IPTG on enhancing the expression level of rL-asp II. IPTG induction was performed by adding the IPTG at different growth periods (0, 2 and 4 h). The samples were collected every two hours and the L-asparaginase II expression was analyzed.

3.2.4. Enzyme and protein analysis

3.2.4.1. L-asparaginase activity and L-glutaminase activity

The enzymatic assay was performed according to Kumar *et al.*, [9], by nesslerization of one of the reaction products (ammonia). L-Glutaminase activity was determined using glutamine as substrate as per the modified method of Mashburn *et al.*, [18]. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of ammonia per minute at 37 ºC. Specific activity was expressed as units per milligram of protein.

3.2.4.2. Protein estimation

The total protein of the samples was analysed according to the method described by Bradford [19] using bovine serum albumin (Sigma) as standard.

3.2.4.3. Western blot analysis

Total of 5 ml crude broth sample containing rL-asp II was precipitated with TCA (Trichloroacetic acid) then electro-blotted onto nitrocellulose membrane using Bio-Rad apparatus at 15 V for 30 min. The membrane was blocked and incubated with mouse anti-his IgG antibodies in a ratio of 1:1500. The membrane was washed thrice with TBST (Tris-Buffered Saline and Tween 20) and incubated with 1: 5000 diluted HRP-labelled rabbit anti-mouse IgG H&L (HRP) at room temperature for 1 h. The membrane was then washed with TBST and blotting was performed using chemi-luminescent peroxidase substrate-3 for the immunological detection of recombinant protein [20].

3.3. Results and discussions

3.3.1. Cloning of rL-asp II

The pHT43 vector used in this study consists of *Bacillus* signal peptide (amy Q) fused to Shine-Dal-garno sequence, hence L-asparaginase II gene was amplified without native signal peptide. The amplified L-asparaginase II (1011 bp) and pHT43 (8057 bp) were double digested, gel eluted (Fig. 3.1A) and ligated. The recombinant plasmid isolated from *E. coli* DH5α cells was confirmed by PCR using gene specific primers and restriction digestion with *BamHI* and *AatII* enzymes (Fig. 3.1B). Concurrently, the sequenced L-asparaginase II was aligned with *Pectobacterium carotovorum* MTCC 1428 L-asparaginase II using EMBOSS Needle program (http://www.ebi.ac.uk/Tools), where the gene sequence showed 100 % identity to *Pectobacterium carotovorum* MTCC 1428 L-asparaginase II gene without signal peptide. The first 24 amino acids (72 bp) of L-asparaginase II from *Pectobacterium carotovorum* MTCC 1428 were predicted as signal peptide and confirmed using SignalP server (http://www.cbs.dtu.dk/services/SignalP/). The size of the amplified gene was observed to be 1011 bp (including C-terminal 6x-His tag and enterokinase designed in the reverse primer). *Bacillus subtilis* (native strain) contains *ans-B2* gene encoding for L-

asparaginase [21]. In order to measure the rL-asp II activity in *B. subtilis* WB800N pHT43-*ans B2*, a control experiment with empty vector (PHT43) transformed in *B. subtilis* WB800N was carried out in all cases. As the native strain has one copy of L-asparaginase, L-asparaginase activity of native strain is subtracted from L-asparaginase activity of recombinant strain.

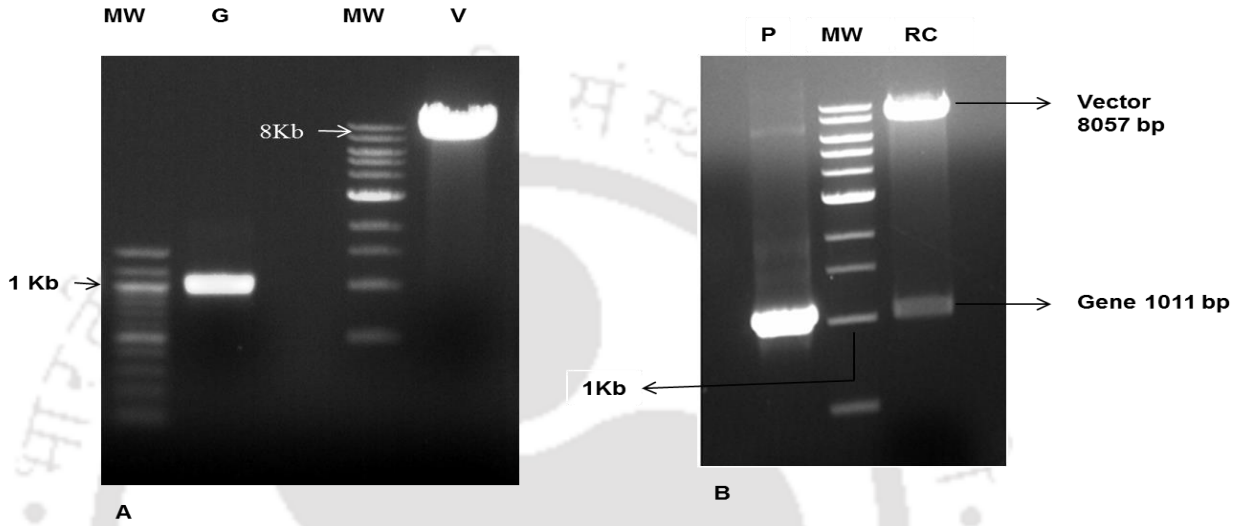


Fig 3.1:- A: First lane (MW) 100 bp ladder, second lane (G) double digested gene, third lane (MW) 1 Kb ladder, fourth lane (V) double digested vector.

B: First lane (P) PCR confirmation of transformed plasmid, second lane (MW) 1 Kb ladder third lane (RC) release check of transformed plasmid.

3.3.2. Optimization of parameters for generation of active competent cells

To understand the formation of competent cells, the growth profile of *B. subtilis* WB800N cells was monitored for every 25 min in SM1 medium. Competency of the cells was determined by microscopic observation of cell motility. To optimize the transformation efficiency, cells of different growth phases from late logarithmic phase to early stationary phase (OD $_{600nm}$ 0.9, 1.5, 1.7, 1.9, 2.1, 2.3) were collected to prepare competent cells. It was noticed that cells started showing competency when the culture OD_{600nm} reached to 1.4 and continued until it reached 2.3 OD_{600nm} (fig 3.2A). This was further confirmed by the plasmid uptake (Fig. 3.2B). These data further confirmed that the competency of cells varied with

growth pattern and maximum competency was observed when the cells reached an $OD_{600\ nm}$ of 1.9 (Fig. 3.2A). Comparative evaluation of competent cell formation and growth curve of the cells suggested that competency developed at the end of the exponential phase or starting of stationary phase was similar to that noticed by Hamoen *et al.*, [22] and Dubnau [23]. A maximum of $4.59x10^6$ transformants (per µg of DNA) were noticed at the end of the exponential phase (Fig. 3.2B).

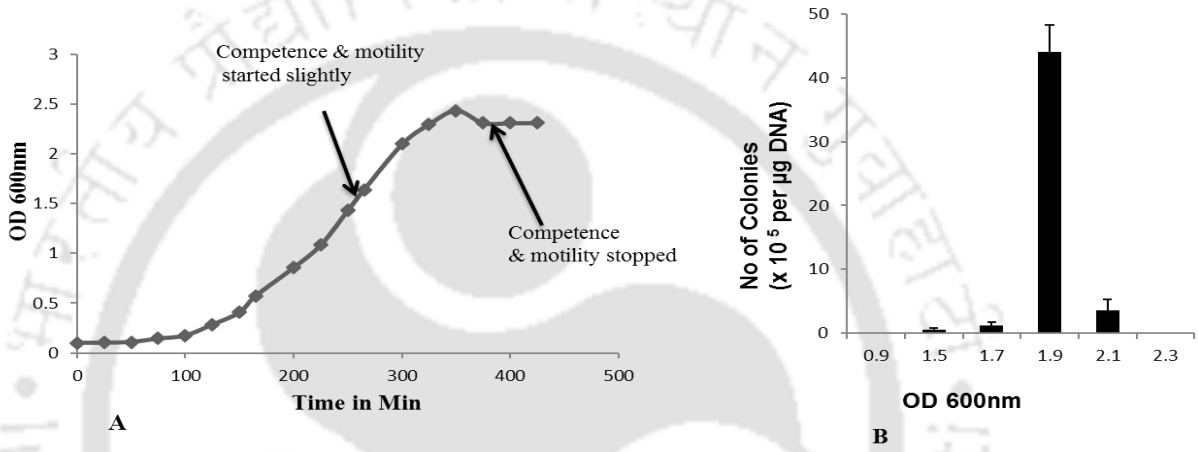


Fig 3.2:- A: Growth profile of *Bacillus subtilis* WB800N in SM1medium (i.e. development of competency). **B:** plasmid uptake profile at different OD_{600nm}.

3.3.3. Expression of rL-asp II

The rL-asp II expression in transformed *B. subtilis* WB800N with pHT43-*ans B2* was investigated against pHT43 vector as control. Induction of the rL-asp II expression was evaluated with the supplementation of 0.5 mM IPTG in transformed *B. subtilis* WB800N. The IPTG was added to the fermentation broth when the culture OD600nm reached 0.7 – 0.8, keeping the literature reports in view, where most of the recombinant proteins were induced with addition of IPTG at log phase [24]. The extracellular protein expression was observed after 4 h of IPTG post-induction and continued to express the same till 12 h in pHT43-*ans B2* only. However, the pHT43 samples without L-asparaginase gene did not show any expression of L-asparaginase II suggesting that transformation is successful and this transformant can be

used for production of rL-asp II extracellularly. Further L-asparaginase activity in the cell free extracts (collected after 8 h of IPTG post induction) of pHT43-*ans B2* was observed to be 33.26 IU/ml while the native strain of L-asparaginase II, *Pectobacterium carotovorum*, produced only 14 IU/ml [25]. These data further denote that the transformants have approximately 2.2-fold higher L-asparaginase II production. The expression of L-asparaginase II was further confirmed in crude broth samples of pHT43 and pHT43-*ans B2* by SDS-PAGE analysis and observed a band having molecular weight of ~ 36.5 KDa (Fig. 3.3), which was similar to that observed with L-asparaginase II produced by *Pectobacterium carotovorum* MTCC 1428.

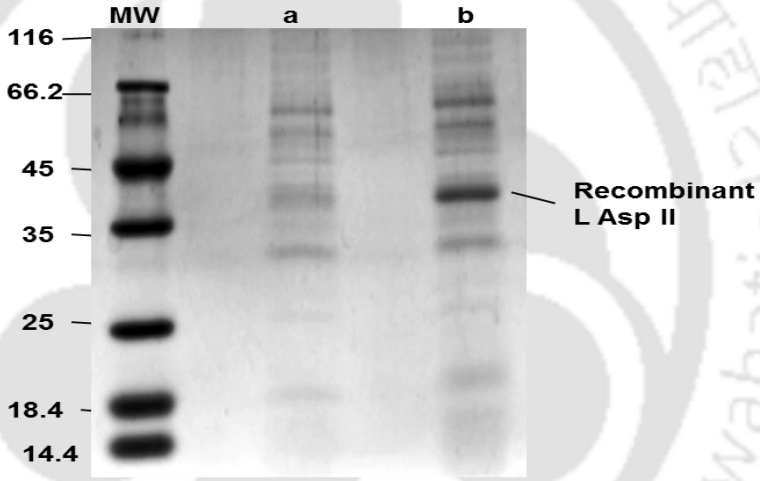


Fig 3.3: Molecular weight analysis of rL-asp II protein. Lane (1) Molecular marker, Lane (a) crude broth sample of pHT43 sample, Lane (b) crude broth sample of pHT43-*ans B2* showing rL-asp II protein.

3.3.4. Effect of process parameters on the expression of rL-asp II

3.3.4.1. Effect of temperature

The production of the recombinant enzyme from the host organism was investigated at different incubation temperatures ranging from 18 to 45 ºC by incubating the cultures at 180 rpm. The rL-asp II production in this temperature range was observed to be in the range of 1.95 – 31.26 IU/ml. The enzyme production data revealed that enzyme yield increased with

increase in incubation temperature, and maximum enzyme production (31.264 IU/ml) was noticed at 37 ºC. Further increase of temperature up to 45 ºC decreased the enzyme production (23.9 IU/ml) (Fig. 3.4A). These data are in accordance with Durban *et al.,* [26] who achieved high-level of recombinant phospholipase C in *B. subtilis* WB800 at 37 ºC. Mahajan *et al.*, [27] and Prakasham *et al.,* [28] also reported similar results showing that 37 ºC as an optimum temperature for the production of L-asparaginase from *Bacillus licheniformis* (32.26 IU/ml) and *Bacillus circulans* (85 IU/ml), respectively.

3.3.4.2. Effect of agitation

Singh *et al.*, [29] demonstrated that agitation speed helps in maintaining homogenous mixing of the culture for proper uptake of substrate and also mass balance of dissolved oxygen. However, each microbial strain has agitation optima to produce metabolic product/enzyme. The production of acetoin by *B. subtilis* 168Δupp was found to be optimum at 220 rpm [30] while in case of L-asparaginase production by *Escherichia coli* (ATCC 11303), it was noticed to be at 150 rpm [31]. In order to determine optimum agitation speed, the protein expression by transformant was evaluated at different agitation conditions (100–240 rpm) by supplementing 0.5 mM IPTG when culture OD_{600nm} reached 0.8. Highest expression of protein (38 IU/ml) was noticed when the culture incubated at 120 rpm, while any change in this resulted in reduced enzyme expression (Fig. 3.4B). RPM variation data revealed that agitation of culture is one of the key influential parameters in L-asparaginase II expression in this transformant. Similar experimental findings were reported for the proper growth of the *Bacillus* sp. and subsequent enzyme production [32], [33]. Further increase in rpm rate beyond 160 rpm has decreased the recombinant enzyme production and it drastically dropped at 200 (17.35 IU/ml) and 240 (14.12 IU/ml) RPM which may be attributed to the increased shear stress on cells with increased agitation. Similar trend was observed for optimum protein expression in *Bacillus subtilis* [34] and in *Bacillus amyloliquefaciens* [35].

3.3.4.3. Effect of cell density

To improve the expression of rL-asp II, IPTG induction strategy was adopted at different cell densities as the cloned gene was under the control of lac operon, which is induced by IPTG. Initially, 0.1 – 0.2 OD_{600nm} culture was inoculated into fresh medium and IPTG (1.0 mM) was added at selected cell densities (0.2 – 1.0 OD_{600nm}) individually (separate flasks) followed by measurement of enzyme expression after 8 h of IPTG addition. Figure 3.4C shows the effect of cell density with IPTG induction. The recombinant protein expression was found to be enhanced as cell density of the culture increased from 0.1 to 0.8 OD_{600nm} and then decreased at 1.0 OD_{600nm}. The expression of the recombinant protein was found to be maximum (54.88 IU/ml) at 0.8 OD_{600nm}. These data are in accordance with the reported recombinant enzyme production of antimicrobial peptide cathelicidin- BF, where the authors used the expression system of *B. subtilis* WB800N [36].

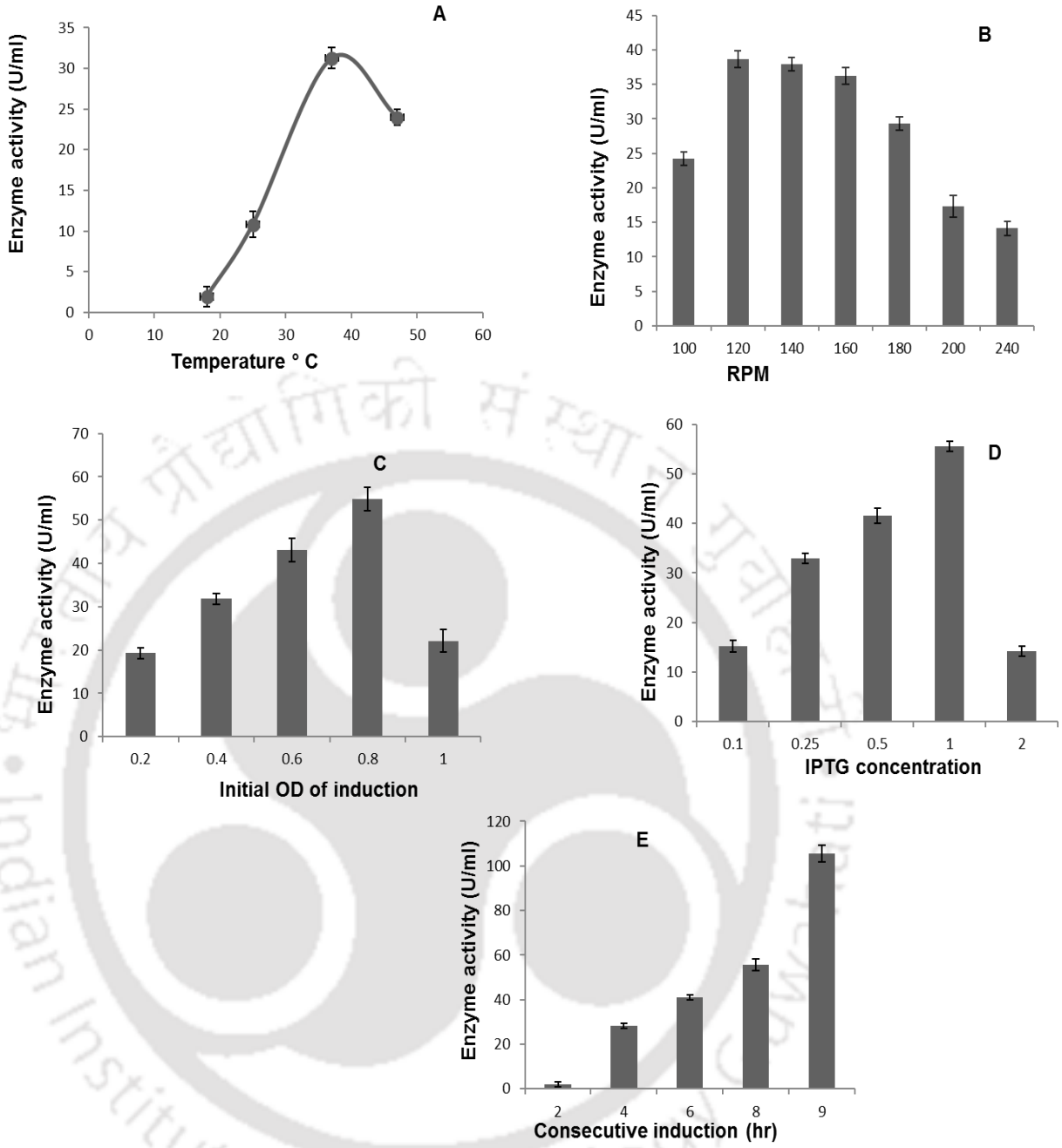


Fig. 3.4 Effect of process parameters on the expression of rL-asp II. **A**) Effect of different temperatures. **B**) Effect of different RPM. **C**) Effect of initial OD_{600nm} of induction. **D)** Effect of different IPTG concentrations. **E)** Effect of consecutive intermittent IPTG addition [all experiments were performed in triplicates and standard deviation is significant ($p< 0.5$)]

3.3.4.4. Effect of IPTG concentration

In the subsequent step, experiment was performed to evaluate the impact of IPTG concentrations (0.1 – 2 mM) for optimal expression of rL-asp II. The IPTG was added to the fermentation flask when the cell density reached to 0.8 OD_{600nm}. Evaluation of enzyme after 8 h of IPTG post-induction revealed that increasing of the IPTG concentration resulted in increase of rL-asp II production until IPTG concentration of 1.0 mM (55 IU/ml). These data are similar to the reports where 1 mM of IPTG was found to be optimum for the expression of recombinant enzymes, such as antimicrobial peptide cathelicidin-BF and procerain B [36] - [37]. Further increase of IPTG to 2.0 mM resulted in decrease of expression (14.25 IU/ml) (Fig. 3.4D) indicating a four-fold decrease in enzyme expression in this transformant, *B. subtilis* WB800N.

3.3.4.5. Effect of consecutive induction by IPTG

The reduced enzyme production at higher concentrations of IPTG (2.0 mM) may attribute to IPTG-induced metabolic burden as suggested by Glick [38]. In order to reduce the metabolic burden on transformant metabolism and faster uptake of IPTG for enhanced expression of rL-as II, lower concentrations of the IPTG was added consecutively to the cell culture thrice and the enzyme production pattern by the transformant was studied. Consecutive intermittent addition of IPTG (i.e. 0 h with 0.25 mM and 2 h, 4 h with 0.5 mM IPTG) enhanced the recombinant protein expression more than 1.9-fold i.e. 105 IU/ml with a specific activity of 101 IU/mg of protein (Fig. 3.4E) compared to single induction of IPTG at OD_{600nm} ~0.8 with 1.0 mM (55 IU/ml with specific activity of 52 U/mg) (Fig. 3.4D). These results are in harmony with Norsyahida *et al.*, [39] where authors reported increased recombinant BmR1 antigen production by addition of IPTG twice to the culture. After optimizing all the parameters, the production of the rL-asp II by *B. subtilis* WB800N containing pHT43-*ans B2* plasmid, was found to be 105 IU/ml in shake flask, which is 5.6-

fold higher than L-asparaginase produced from *Nocardiopsis alba* (18.47 IU/ml) [40] and 1.2-fold higher than L-asparaginase produced by recombinant *B. subtilis* B11–06 (89.48 IU/ml in a 5 L fermenter) [41].

The observed decrease (55 – 14 IU/ml) of rL-asp II enzyme with the increase in IPTG from 1.0 to 2.0 mM (Fig. 3.4C), further confirms that supplementation of 2.0 mM IPTG at one time may be resulted in alteration of physiology of the recombinant strain similar to that observed by Bentley *et al.*, [42]. However, the same group also reported that higher concentration of IPTG causes the metabolic burden leading to the alteration of the expression of recombinant proteins [42]. In another study, Lecina et al. [43] had reported that the addition of limited amount of IPTG avoids metabolic burden on *E. coli* cells thus increases the recombinant protein production. While Fernández-Castané *et al.*, [44] reported that higher biomass concentration leads to faster uptake of IPTG into the cell. This enhanced rL-asp II production was further confirmed by SDS analysis where addition of a small amount of IPTG at short intervals of time has found to enhance the protein expression (Fig. 3.5).

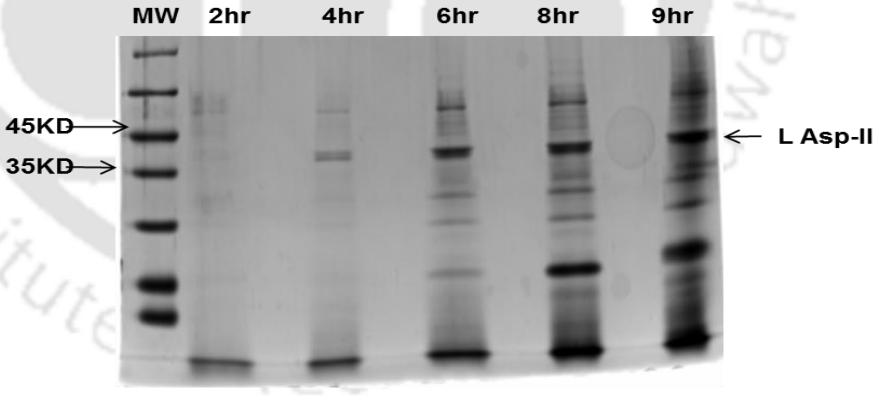


Fig. 3.5 Crude sample analysis of consecutively induced broth samples at different time intervals.

3.3.5. Localization of rL-asp II

rL-asp II expression at optimized fermentation conditions was used to evaluate the localization studies (extracellular, intracellular, membrane bound recombinant protein and inclusion bodies). The culture samples were collected at ninth hour after consecutive induction and analyzed according to the Smith *et al.*, [45] for enzyme localization. It was observed that 91.1 ± 2.2 % of the recombinant enzyme was secreted into broth; 2.6 ± 1 % was accumulated intracellularly and 6.29 ± 2.92 % was attached to the membrane as membrane bound (Fig. 3.6). It was observed that there was no formation of inclusion bodies. The observed maximum (>90 %) enzyme secretion into fermentation broth may be attributed to the presence of signal peptide (amy Q gene) fused to Shine-Dal-garno sequence in the pHT43 vector. Similar expression of foreign proteins extracellularly was observed with the vector pHT43 [46].

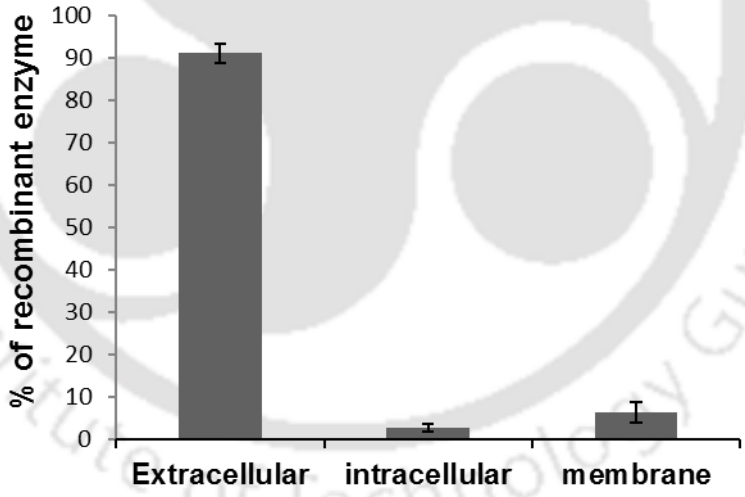


Fig. 3.6 Localization studies of rL-asp II expression

3.3.6. Western blot analysis.

To further confirm the rL-asp II expression in the broth samples, western blot was performed. Since, the rL-asp II contains 6x-His tag at C-terminal, 5 ml of crude broth sample was precipitated with TCA and used for rL-asp II confirmation. Electro-blotted recombinant protein onto nitrocellulose membrane was incubated with mouse anti-His IgG antibodies, which in turn was incubated with rabbit anti-mouse IgG H&L antibodies. Chemi-luminescent peroxidase substrate-3 was used for the recombinant protein detection. The observed band on the nitrocellulose membrane confirmed the presence of rL-asp II protein (Fig. 3.7).

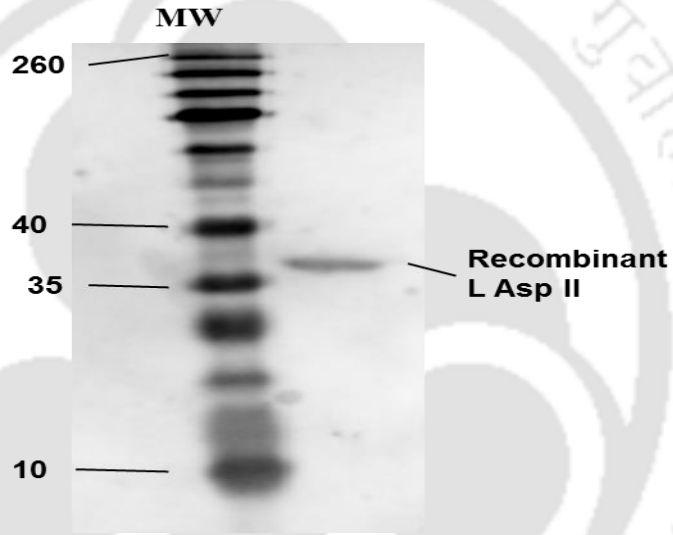


Fig 3.7: Western blot analysis of 5 ml crude broth sample.

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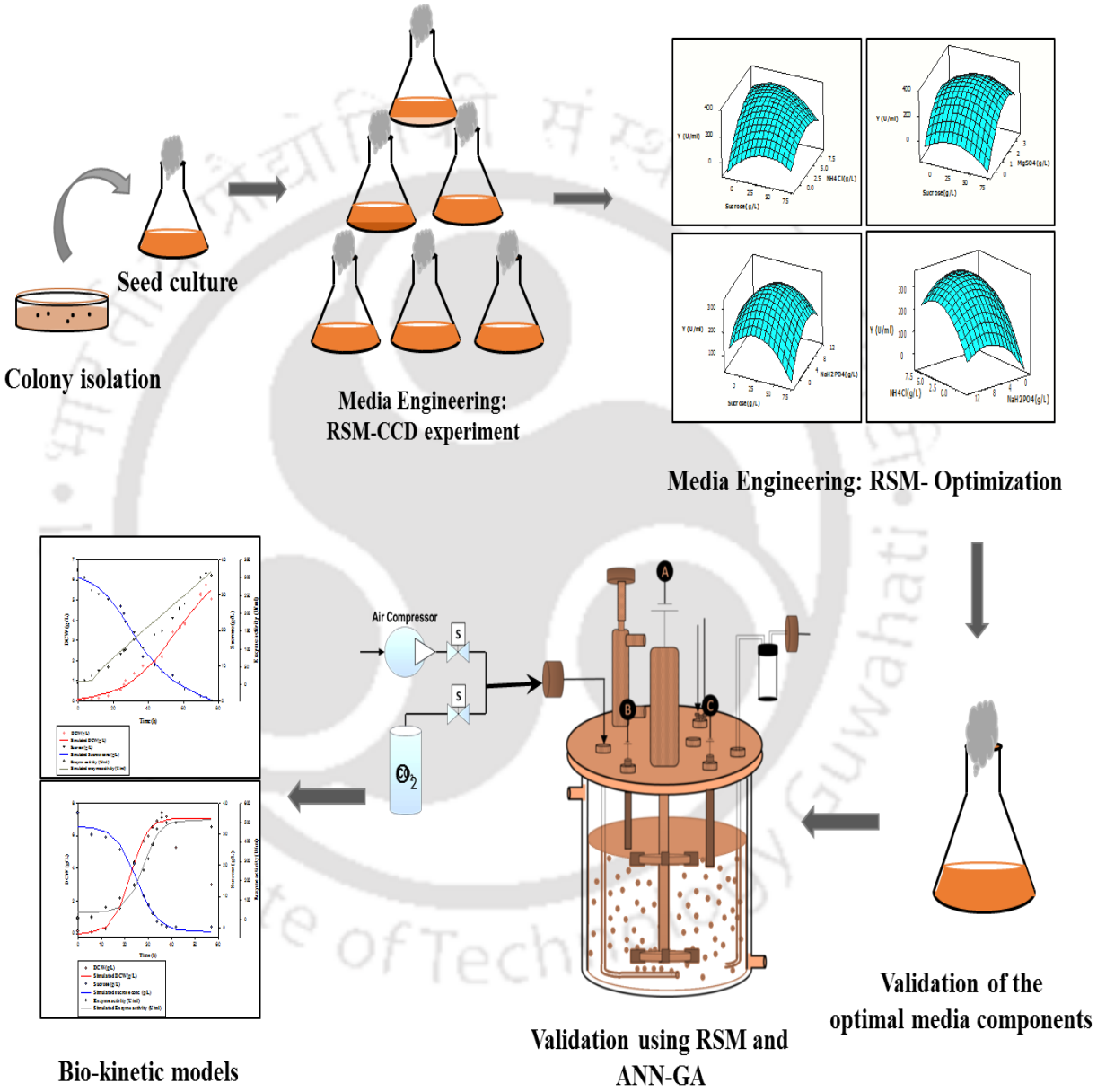
Chapter IV

Media Engineering and Optimization

Media engineering for enhanced production of rL-asp II

Graphical abstract:



4.1. Background and Motivation

Over the last few decades, numerous progress has been witnessed in the field of genetic engineering and cell engineering, where various genes have been cloned and expressed in eukaryotic and prokaryotic cells to enhance heterologous proteins production [1]–[3]. Recently, a study reported that *Bacillus subtilis* is used in the fermentation of soybeans (Japanese traditional food Natto) [4], which shows that this strain is more eco-friendly and can be used for the production of various heterologous proteins.

Till date, more than 60% of commercial proteins are being reported to be produced by *Bacillus* strains [5]–[8]. It can grow on wide variety of carbon, nitrogen sources and has the ability to reach high cell densities of 100 g dcw $l^{-}1$, 184 g dcw l^{-1} and 56 g dcw l^{-1} respectively [9]–[11]. Hence, it is considered as the best strain for the production of glutaminase free L-asparaginase II. Therefore, optimization of media components for the enhanced production of proteins is a valuable addition to the current research of heterologous protein production [12]–[15]. Hence, a medium that supplies defined quantity of nutrients to the developed strain (*Bacillus subtilis* WB800N pHT43-ans B2) for the production of recombinant novel glutaminase L-asparaginase II is very crucial. In order to improve supply of nutrients, the media components were modified from Wenzel et al., [16], through various statistical approaches [17].

Optimization studies involving one factor at a time approach are not only tedious but also time consuming and hence overlooks the interaction effect between the variables. In contrast, statistical methodologies are preferred due to their advantages such as reducing the number of experiments and interpretation of possible interactions among the factors and thus reducing error in an economical manner [29] - [30]. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving

and optimizing processes [19]. The most commonly used design in RSM is central composite design (CCD).

Apart from statistical approaches such as RSM, other mathematical approaches like Artificial Neural Network (ANN) coupled with Genetic Algorithm (GA) have also gained much of the research interest, because of their ability to optimize nonlinear modelling problems with much more efficiency than previous methods [20]–[22]. The major advantage of ANN over the classical mathematical models is the fact that they allow the simultaneous identification of structure parameters, while they possess the ability to adapt by examples [23]. The concept of Genetic algorithms (GAs) is based on the evolutionary natural selection processes (Darwinism and Mendelism), where selection results in species that fit the best among the population [22].

In this chapter, we achieved high level of rL-asp II expression by optimizing the media components effecting the production with respect to our previous chapter, where we cloned the L- asparaginase II gene in *B. subtilis* WB800N [24]. In order to further improve the rL-asp II expression, we applied consecutive induction strategy at the batch reactor level, which showed significant improvement. Furthermore, this strategy also minimized the production of overflow metabolites such as acetic acid and acetoin, which are major bottlenecks in the production of recombinant proteins.

4.2. Materials and Methods

4.2.1. Chemicals and reagents

All the chemicals purchased were of high-grade quality from Himedia. The antibiotics used for the culturing of strain includes chloramphenicol and neomycin (Sigma-Aldrich).

4.2.2. Media development for the optimized expression of rL-asp II

4.2.2.1. Strain and Media formulation

Bacillus subtilis WB800N harboring *ans B2* in pHT43 (pHT43- *ans B2)* vector was used for the optimization of recombinant L-asparaginase II (rL-asp II) expression [24]. The maintenance and sub-culturing of recombinant strain was performed on LB media (yeast extract 5 g/L, tryptone 10 g/L and NaCl 5 g/L) with 100 µg/ml chloramphenicol for plasmid selection (pHT43- *ans B2)* and 100 µg/ml neomycin for strain selection (WB800N). The production of rL-asp II has been studied in mineral salt media modified from that of Wenzel et al., [16], which consists of Na_2SO_4 (2.0 g L^{-1}), $(NH_4)_2SO_4$ (2.68 g L^{-1}), NH_4Cl (0.5 g L^{-1}), K_2HPO_4 (14.6 g L^{-1}), NaH_2PO_4 . H_2O (4.0 g L^{-1}), $MgSO_4$. $7H_2O$ (1.0 g L^{-1}), tryptophan (1 g L^{-1}) (as the strain is auxotroph for tryptophan), trace element solution (TES) 3 ml L^{-1} with carbon source 5 g L^{-1} for pre-culture and 25 g L^{-1} carbon source for the fermentation. TES contains $CaCl_2$ (0.5 g L^{-1}), $ZnSO_4$. $7H_2O$ (0.18 g L^{-1}), $MnSO_4$. H_2O (0.1 g L^{-1}), Na_2-EDTA (10.05 g L^{-1}), $FeCl_3$ (8.35 g L^{-1}), $CuSO_4$. $5H_2O$ (0.16 g L^{-1}), and $CoCl_2$. $6H_2O$ (0.18 g L^{-1}). All the media components were autoclaved separately and reconstituted aseptically prior to inoculation in order to avoid precipitation of media components.

4.2.2.2. Fermentation conditions

The inoculum was prepared by inoculating a single colony of WB800N/pHT43-*ans B2* into 10 ml of pre-culture (aforementioned media composition) followed by incubation at 37 °C, 120 rpm for about 12 h. The overnight pre-culture was inoculated aseptically into 150 ml shake flasks with a production medium of 25 ml. The culture sample was induced by adding the IPTG consecutively and intermittently at different growth periods [24].The samples were then collected at regular intervals of 12 h and measured for rL-asp II expression and dry cell weight (DCW). All experiments were performed in triplicates.

4.2.3. Initial screening of carbon, nitrogen sources and their effect on rL-asp II expression

Eleven carbon sources (Glucose, glycerol, sucrose, sorbitol, mannitol, galactose, maltose, lactose, gluconate, pyruvate, ethanol) and eight nitrogen sources (NH_4SO_4+NH_4Cl, NH_4SO_4, NH_4Cl, glycine, L-asparagine, peptone, casaminoacids, yeast extract) were screened based on the expression of rL-asp II in the above-mentioned media.

4.2.4. Screening of crucial medium components by Plackett–Burman design

Plackett–Burman design was employed to screen the significant media components effecting the rL-asp II expression [25]. A total of eight parameters, *viz.*, Sucrose, NH_4Cl, Na_2SO_4, K_2HPO_4, $NaH_2PO_4.H_2O$, $MgSO_4$ $7H_2O$, Tryptophan and TES (trace element solution) have been considered for the screening variables based on the previous reports [26]. The data range was varied based on the original medium components and represented at two levels i.e., high and low which are denoted by (+1) and (−1) respectively as shown in Table 4.1. Twelve experiments were performed in triplicates and the average of results were taken as the response. The observed and predicted values are given in Table 4.3. The significance of each variable was determined by student's t-test. Plackett-Burman experimental design is based on the first order polynomial model, which is given as:

$$\boldsymbol{Y_{rL-asp\ II} = \beta_0 + \sum \beta_i X_i} \quad \text{----------} \quad (\mathbf{1})$$

Where, $Y_{rL\text{-}aspII}$ is the response (rL-asp II activity), β_o is the model intercept, β_i is the linear coefficient and X_i is the level of the independent variable. The significance of each variable was determined *via* student's t-test.

Table 4.1: Experimental variables at different levels used for the expression of rL-aspII by *Bacillus subtilis* WB800N/pHT43-ans *B2* using Plackett–Burman design

Variables	Symbol code	Experimental values (g/L)	
		Lower (-1)	Higher (+1)
Sucrose	X1	5	50
NH_4Cl	X2	1	6
Na_2SO_4	X3	1	5
K_2HPO_4	X4	5	25
NaH_2PO_4	X5	1	8
$MgSO_4$	X6	0.5	2.5
Tryptophan	X7	0.5	2.5
TES	X8	1	5

4.2.5. Optimization of crucial medium components using different statistical designs

4.2.5.1. Response Surface Methodology (RSM)

Central composite design (CCD) was adapted with the view of further optimizing the screened variables and to explain the combined effect of the variables *viz.*, sucrose, NH_4Cl, NaH_2PO_4 and $MgSO_4$ on rL-Asp II production [27]. The screened significant variables and their ranges are given in the Table 4.2, where each of the variables were assessed with five coded levels (-2, -1, 0, +1, +2). According to CCD, 30 ($=2^k + 2k + 6$) experimental combinations were performed, where k is the number of independent variables [28], [29]. Twenty-four experiments with six replications at the center points were augmented to evaluate the pure error. All experiments were performed in triplicates and the average values with standard errors are reported in Table 4.5.

Table 4.2: Experimental codes, ranges and levels of the independent variables for RSM experiment

Variables	Symbol coded	Range and levels				
		-2(-α)	-1	0	+1	+2(+α)
Sucrose(g/L)	*X1*	17.5	5	27.5	50	72.5
NH_4Cl(g/L)	*X2*	1.5	1	3.5	6	8.5
NaH_2PO_4(g/L)	*X5*	2.5	1	4.5	8	11.5
$MgSO_4$(g/L)	*X6*	0.5	0.5	1.5	2.5	3.5

The data obtained from CCD on rL-asp II expression was subjected to analysis of variance (ANOVA). The second order polynomial for predicting the optimal levels was expressed according to the equation (2).

$$\boldsymbol{Y_{rL-aspII} = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j} \quad (\mathbf{2})$$

Where $Y_{rL\text{-}asp\ II}$ is the predicted rL-asp II expression, k is the number of factor variables. *Xi and Xj* are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the i^{th} quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient. The statistical significance of the model equation and the model terms were evaluated via the Fisher's test. The model efficiency was expressed via the coefficient of determination (R^2). Regression analysis and response surface graphs was plotted with the aid of statistical software package MINITAB® Release 16.1.1, PA, USA.

4.2.5.2. Artificial neural network linked genetic algorithm (ANN-GA)

In this protocol, we employed feed forward back propagation method to train the network. The input and output of CCD was used as input and output neurons. The network architecture consists of four input layers *viz*., sucrose, NH_4Cl, NaH_2PO_4 and $MgSO_4$, three hidden layers and one output layer representing rL-asp II expression (Fig 4.5A). In feed forward system, the data flows from input layer to output layer *via.*, the hidden layer. The

input, hidden and output layers are connected with weights (w) (real number quantity associated with the connection between two neurons) and biases (b) that are considered parameters of the neural network (NN) [42] - [43]. The system performs its work by summing up all weighted inputs including biases and transfers it to the first non-linear transfer function (tansig) which is situated between the input and hidden layer. The output produced by the hidden layer will then be transferred to the linear transfer function namely purelin, from where the output will be transferred to the output layer.

$$\boldsymbol{Purelin = sum} \quad (3)$$

$$\boldsymbol{tansig = \frac{1+\exp(-sum)}{1-\exp(-sum)}} \quad (4)$$

The total experimental data was divided in to three different sets: 20, 5 and 5 of data sets were used as training, validation and testing, respectively. The error function is calculated based on the difference between actual output and predicted output. ANN is an iterative method, which is pre-specified to minimize error function and adjust weight appropriately. The commonly used error functions i.e., mean squared error (MSE) was used in the present study and is given by the equation (5):

$$\boldsymbol{MSE = \frac{1}{N}\sum_{i=1}^{N}(Y_a - Y_p)^2} \quad (5)$$

Where Y_a is the actual output, Y_p is the predicted output and N is the number of data points. The MSE is minimized by adjusting the weights and biases appropriately. During training step, the weight and biases iterated by Levenberg–Marquardt algorithm, until the convergence to the certain value is achieved. In this work, a Neural Network Toolbox of MATLAB (2010a) mathematical software was used to predict the rL-asp II expression [32].

4.2.5.3. Genetic algorithm (GA)

The genetic algorithm (GA) is a global optimization procedure, where the convergence is independent of the initial value. Once the ANN is developed, the input space is further optimized using GA. GA follows four steps to find global solution. In the first step, initialization of the solution for the population will take place followed by fitness computation, which in turn is dependent on an objective function and in the next step the better individual will be selected. The selected individual will then undergo crossing over and mutation, which leads to the creation of new sets of individuals with better performance. This process will be repeated until a maximum output result was achieved [30].

4.2.6. Unstructured bio-kinetic models for Batch fermentation studies

Inoculum for the bioreactor was prepared using the previously mentioned modified mineral salt medium. The seed culture was prepared by inoculating sterile medium (100 mL) with loop of recombinant *Bacillus subtilis* WB800N/pHT43-*ans B2* in a 250-mL Erlenmeyer flask as explained above. Later the flask was incubated at 37 ^{0}C, 120 rpm for 16 hrs. The entire contents of the flask with an average $O.D_{600nm}$ (optical density) of 5-6 were used for inoculating the bioreactor. Batch fermentations were performed using a 3 L ez-control (Applikon, Netherlands) stirred tank bioreactor. Initially, the bioreactor contained 1 L of the liquid medium. The incubation temperature and the agitation speed were maintained at 37 ^{0}C and 300 rpm, respectively. The aeration rate was 1.5 L min^{-1}. The dissolved oxygen level was maintained at 30 % of the air saturation value.

Kinetic modelling is regarded as an indispensable step while developing an industrial fermentation process, since it helps to determine the optimal operation conditions for the production of the target metabolite. The growth analysis was done using a logistic equation, whereas, the Luedeking–Piret equation and the Modified Luedeking-Piret equation were used for analysis of product formation and substrate utilization [33] .

Logistic Equation

The exponential growth phase can be characterized by the following first order equation which states that the rate of the increase of cell biomass is proportional to the quantity of viable cell biomass at any instant [34], [35].

$$\frac{dX}{dt} = \mu X \quad ---------- (6)$$

The growth of cell is governed by hyperbolic relationship and there is a limit to the maximum attainable cell biomass concentration. Such growth kinetics is described by logistic equation

$$\frac{dX}{dt} = \mu_{max} X\left(1 - \frac{X}{X_{max}}\right) ---------- (7)$$

Where, X is the biomass concentration (g L^{-1}), X_{max} is the maximum biomass concentration predicted by model (g L^{-1}), dX/dT is the rate of biomass production (g L^{-1} h^{-1}), μ is the specific growth rate and μmax is the maximum specific growth rate (h^{-1})

The integrated form of equation (7) is

$$X = \frac{X_o \exp(\mu_{max} t)}{1 - \left(\frac{X_o}{X_{max}}\right)(1 - \exp(\mu_{max} t))} ---------- (8)$$

The kinetic parameter μ_{max} in this equation is determined by rearranging the above equation and plotting $\ln\left(\frac{X'}{1-X'}\right)$ **v/s t** should give a straight line of slope μ_o and intercept - $\ln\left(\frac{X_{max}}{X_o} - 1\right)$.

Where X_0 is the initial biomass concentration (g L^{-1}), t is time (h), μ_0 is specific initial specific growth rate, X_0 is the biomass concentration when t = 0 and $X' = \frac{X}{X_{max}}$.

$$\mu_o t = \ln\left(\frac{X_{max}}{X_o} - 1\right) + \ln\left(\frac{X'}{1 - X'}\right) ---------- (9)$$

Luedeking–Piret equation

The kinetics of rL-asp II production was described by Luedeking-Piret equation which states that the product formation rate depends upon both the instantaneous biomass concentration (X) and growth rate (dX/dt) in a linear fashion [36]–[38].

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \quad ----------(10)$$

Where, α is a growth associated constant (mg g^{-1}) and β is a non-growth associated constant (mg $g^{-1}h^{-1}$). The values of α and βdepends mainly on batch fermentation conditions.

Integrating equation (10)

$$P_t = P_0 + \alpha A(t) + \beta B(t) \quad ----------(11)$$

Where, P_0 and P_t are the product concentrations at initial time and at any time (at time t) respectively

$$A(t) = X_o\left[\frac{e^{\mu_o t}}{1 - \frac{X_o}{X_{max}}(1 - e^{\mu_o t})} - 1\right] \quad ----------(12)$$

$$B(t) = \frac{X_{max}}{\mu_0} ln\left[1 - \frac{X_o}{X_{max}}(1 - e^{\mu_o t})\right] \quad ----------(13)$$

The parameters α and β in equation (11) are determined by plotting **$(P_t - P_0)$ / B(t)** vs **A(t) /B(t)** which is a straight line with slope 'α' and intercept 'β'

Modified Luedeking–Piret equation

The substrate utilization kinetics is given by the following equation, which considers substrate conversion to cell mass, product and maintenance energy [35], [39].

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} - \frac{1}{Y_{P/S}}\frac{dP}{dt} - k_e X \quad ----------(14)$$

Where $Y_{X/S}$ and $Y_{P/S}$ are yields of cell mass and product with respect to substrate and K_e is the maintenance coefficient for cells. Rearranging the substrate material balance equation

$$\frac{dS}{dt} = -\gamma\frac{dX}{dt} - \eta X \quad ----------(15)$$

Where, $\gamma = \frac{1}{Y'_{X/S}} + \frac{\alpha}{Y'_{P/S}}$ and $\eta = \frac{\beta}{Y'_{P/S}} + k_e$ ---------- (16)

Equation 15 is the modified Luedeking- Piret equation for substrate utilization kinetics.

Substituting for μ from equation (7) and integrating gives

$$S_t = S_0 - \gamma m(t) - \eta n(t) \quad \text{----------} (17)$$

Where, S_0 and S_t are the substrate concentrations at initial time and at any time 't' respectively,

$$m(t) = X_o \left[\frac{e^{\mu_o t}}{1 - \frac{X_o}{X_{max}}(1 - e^{\mu_o t})} - 1 \right] \quad \text{----------} (18)$$

$$n(t) = \frac{X_{max}}{\mu_0} ln \left[1 - \frac{X_o}{X_{max}}(1 - e^{\mu_o t}) \right] \quad \text{----------} (19)$$

Kinetic parameters (γ, η) in equation 17 is determined by plotting $\frac{S_o - S_t}{n(t)}$ vs $\frac{m(t)}{n(t)}$ which is a straight line with slope γ and intercept η.

4.2.7. Analytical Methods

The growth profile of *Bacillus subtilis* WB800N/pHT43-*ans B2* was monitored by measuring the absorbance of cells at 600 nm (OD_{600nm}) with a UV–visible spectrophotometer (Cary 50, Varian, Australia) and were expressed in terms of dry cell weight (DCW) using the correlation equation DCW = 0.45 × OD_{600nm}. The sucrose was estimated using high-pressure liquid chromatography, (HPLC) (Agilent 1220 Infiniti HPLC, USA) equipped with SUPELCOGEL Ca, 300 mm × 7.8 mm I.D., 9 μm particle. The acetate and acetoin were estimated using Rezex ROA-organic acid H+ (8%) column (300 mm × 7.8 mm, Phenomenex, USA) linked to a guard column (50 mm × 7.8 mm, Phenomenex, USA) using 0.005 N H_2SO_4 as mobile phase at a flow rate of 0.5 ml min^{-1}.

4.3. Results and Discussion

L-asparaginase is gaining importance due to its industrial, clinical, economic and immunological suitability. From the industrial perspective, high level of production and process economics are of major concerns. Many studies have shown that the microbial growth, metabolism and productivity is influenced by nutritional and physiological properties [41]–[46]. With respect to biotechnological perspective, optimization of such parameters is of central importance since a small improvement in the production titre will be crucial for a commercial success. Considering the above aspects, we have focused on optimization of nutritional parameters to enhance the expression of rL-asp II.

4.3.1. Media engineering for the optimized expression of rL-asp II

4.3.1.1. Effect of carbon source

As carbon source has major role on productivity by influencing the carbon flux, till date many investigations have been carried out for selecting the best carbon sources which maximizes the L-asparaginase II production [41], [59] - [60]. In the present study, we made an effort to screen the best carbon sources, which influence the rL-asp II expression in *Bacillus subtilis* WB800N. Based on the literature survey, we have selected eleven different carbon sources effecting the rL-asp II production as mentioned earlier within the range of 25 g L^{-1}. It was interesting to know that sucrose tends to show higher specific growth rate of 0.043 h^{-1} and 57.9 IU/ml of rL-asp II production using *Bacillus subtilis* WB800N/ pHT43-*ans B2* expression system. This findings are comparable with Jia et al., [49], where they found maximum L-asparaginase production from *Bacillus subtilis* B11–06 using sucrose as the major source of carbon. Similar results were reported by Gu et al., and Jacques et al., [13], [50], who reported a substantial effect on lipopeptide production from *Bacillus subtilis* MO-01 and *Bacillus subtilis* S499 respectively using sucrose. The expression profile of different carbon sources effecting specific growth rate along with the production of over-flow

metabolite's such as acetate and acetoin has been recorded in Fig 4.1. It was observed that after sucrose, glucose tends to show high specific growth rate with high rL-asp II production followed by mannitol, pyruvate and sorbitol, with less acetoin and acetate production vice versa respectively.

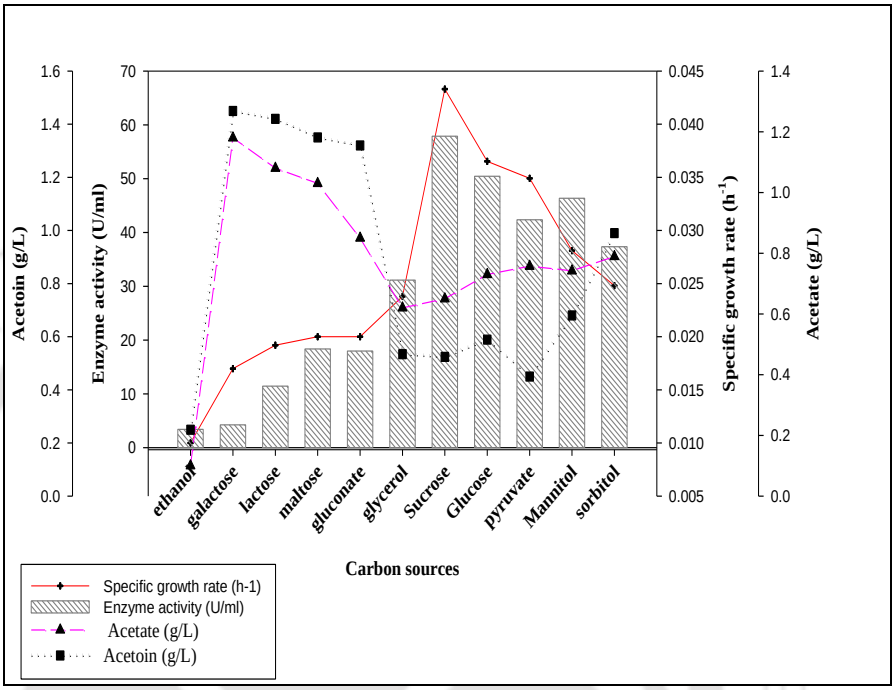


Fig 4.1: Effect of carbon source on the expression of rL-asp II.

4.3.1.2. Effect of nitrogen source

In microorganisms, nitrogen source plays a crucial role in protein expression during the transcription of carbon metabolizing gene which depends on the source of nitrogen [51]. To understand the effect of nitrogen, we studied various organic (yeast extract, casamino acids and peptone), inorganic (NH_4SO_4+NH_4Cl, NH_4SO_4 and NH_4Cl) and amino acids (glycine and L-asparagine) as a nitrogen sources on the expression of rL-asp II. Among the tested sources, ammonium chloride showed maximum rL-aspII expression (74.1 IU/ml), with a specific growth rate of 0.0445 h^{-1}. These findings are comparable with previous report of Hymavathi et al., [52], who found maximum L-asparaginase production using ammonium chloride as a sole source of nitrogen with *Bacillus circulans* (MTCC8574) as an expression

host. In another report, maximum amount of bio-surfactants production was reported using ammonium chloride as nitrogen source with the *Bacillus subtilis* strains [53]. When organic nitrogen sources such as yeast extract was added, the specific growth rate was very high but the expression of rL-asp II was very low. This may be due to high production of acetate and acetoin where the metabolic flux might be diverted towards the less efficient pathways [54], which is depicted in Fig 4.2. These results are in accordance to the previous reported findings where, when the yeast extract is used as nitrogen source high acetoin was produced but using ammonium chloride less acetoin was produced by *B. subtilis* SF4-3 [55]. This is the first study to show expression of novel glutaminase free L-asp II using sucrose and NH_4Cl as a carbon and nitrogen sources in *Bacillus subtilis* WB800N respectively with less acetoin and acetate.

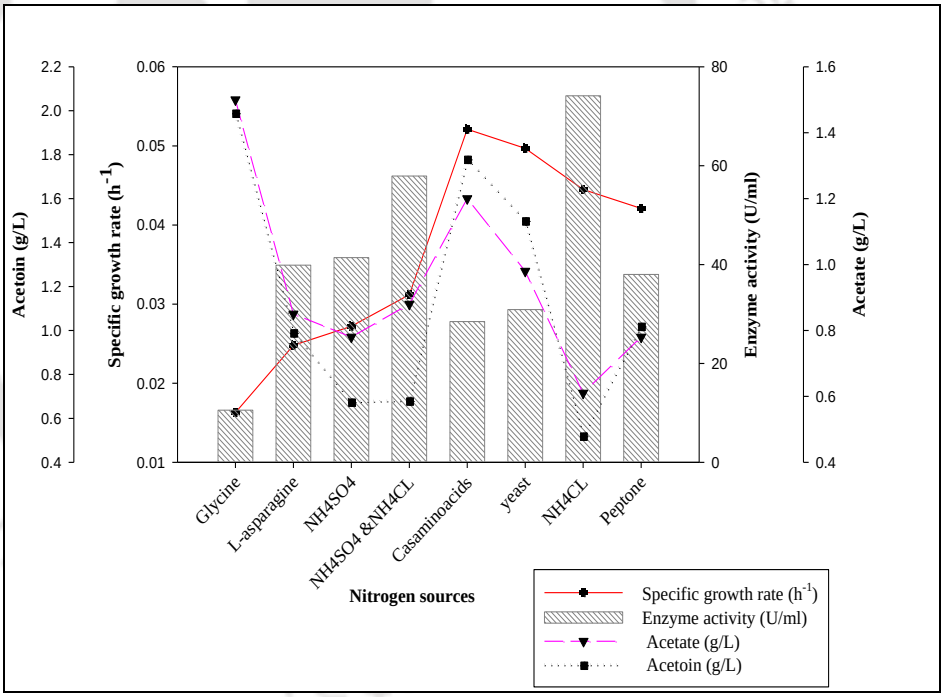


Fig 4.2: Effect of nitrogen source on the expression of rL-aspII.

4.3.2. Evaluation of significant medium components by the Plackett–Burman experimental design

Plackett-Burman design is a very first basic and useful statistical tool for screening of significant media components [25]. In the current investigation, with the aim of maximizing the expression of rL-asp II, eight variables were selected and their effect were studied using predetermined matrix using MINI Tab@ 16 statistical software. The design matrix selected for the screening of significant variables for rL-asp II expression and the corresponding responses are represented in Table 4.3. The experiments based on Plackett-Burman design showed a wide variation from 2.15 to 33.019 IU/ml of enzyme activity. This variation reflects the significance of medium components optimization to achieve higher productivity. The adequacy of the model was calculated and the variables with statistical significance were screened via student's t-test (Table 4.4).

Table 4.3: Plackett–Burman design matrix for eight variables with coded values along with the observed and predicted rL-asp II expression

Run order	Experimental values								Enzyme activity (IU/ml)	
	X1	X2	X3	X4	X5	X6	X7	X8	[a] observed values	Predicted values
1	1	-1	1	-1	-1	-1	1	1	28.72 ± 0.99	30.7221
2	1	1	-1	1	-1	-1	-1	1	24.928 ± 0.56	24.144
3	-1	1	1	-1	1	-1	-1	-1	3.982 ± 0.265	4.864
4	1	-1	1	1	-1	1	-1	-1	29.876 ± 0.125	29.53
5	1	1	-1	1	1	-1	1	-1	15.192 ± 0.85	15.97
6	1	1	1	-1	1	1	-1	1	2.152 ± 0.872	1.27
7	-1	1	1	1	-1	1	1	-1	11.892 ± 0.452	12.22
8	-1	-1	1	1	1	-1	1	1	20.072 ± 0.452	18.07
9	-1	-1	-1	1	1	1	-1	1	13.891 ± 0.24	15.89
10	1	-1	-1	-1	1	1	1	-1	21.161 ± 0.124	20.377

11	-1	1	-1	-1	-1	1	1	1	11.572 ± .356	11.23
12	-1	-1	-1	-1	-1	-1	-1	-1	33.0194 ± 0.124	32.138

[a] The observed values of rL-asp II activity, were the mean values of duplicates with standard deviation (mean±SD)

Generally, factor with higher t value and lesser p value are considered as significant model term. Factors evidencing P-values of less than 0.05 were considered to have significant effects on the response, and were therefore selected for further optimization studies. In the present study, sucrose, NH_4Cl, NaH_2PO_4 and $MgSO_4.7H_2O$ emerged as the significant variables affecting the production of rL-asp II. Among these variables NH_4Cl, NaH_2PO_4, $MgSO_4.7H_2O$ showed negative effect, while sucrose was found to show positive influence. A positive symbol signifies that higher level of the variable have higher response, whereas the negative sign indicates that lower level of variable is responsible for higher response [56], while rest of the parameters showed no significant influence. Hence, were maintained at their middle level (centre point) as these variables are also crucial for growth of the organism. Thus, neglecting the terms that were insignificant, the model equation for rL-asp II enzyme activity can be written as:

$$\boldsymbol{Y_{rL-asp\ II} = 18.038 - 6.418X_2 - 5.296X_5 - 2.948X_6 + 2.3X_1 ----(20)}$$

Where X_1, X_2, X_5 *and* X_6 are sucrose, NH_4Cl, NaH_2PO_4 and $MgSO_4$ respectively.

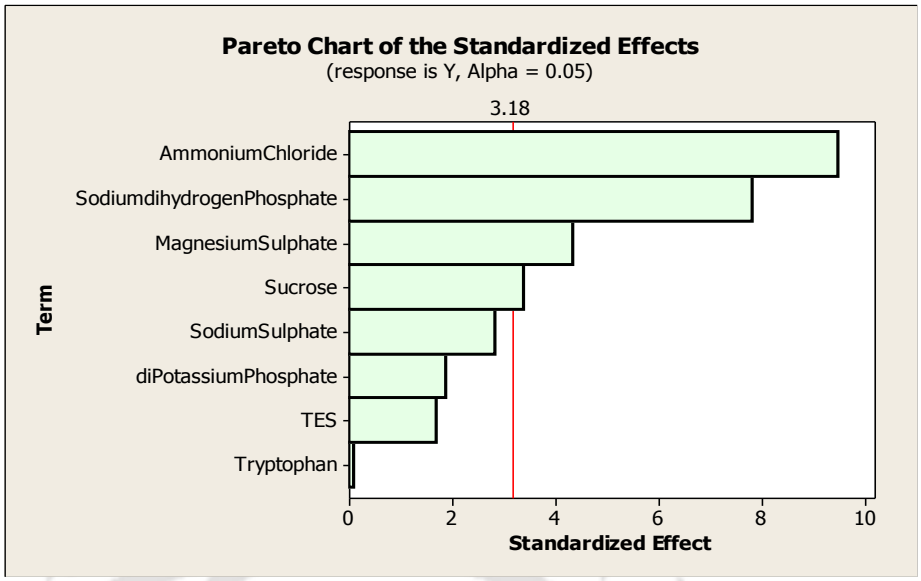


Fig 4.3: Pareto chart of standardized effects of the factors on rL-asp II expression. Media components denoted in g/L.

The Pareto chart (Fig 4.3) is one of the convenient method of representing the effect of variables on responses in Plackett- Burman design. It indicates the ranking of the variables based on the absolute values of standardized effect. The reference line (3.18 in Fig 4.3) indicates that effects were significant with α value of 0.05. The standardized effects were the t statistics shown in Fig 4.3. It is quite evident from the figure that variables such as sucrose, NH_4Cl, NaH_2PO_4 and $MgSO_4$ were influencing the production of rL-asp II significantly. The Plackett-Burman design allowed us to define a new medium composition for the production of rL-asp II [50]. Similar results were observed for the production of novel lipopeptide by *Bacillus subtilis* MO-01 using sucrose and ammonium chloride along with zinc sulphate [13]. Kim et al., [57] also observed that screening of media components using combined statistical designs has enhanced the production of extracellular proteolytic enzyme by *Bacillus subtilis* FBL-1.

Table 4.4: Statistical analysis of Plackett–Burman design showing effect, coefficient values, t and P-value for each variable

Term	Symbol code	Effect	Coef	T	P
Constant			18.038	26.61	0.000 [a]
Sucrose(g/L)	X_1	4.6	2.3	3.39	0.043[a]
NH_4Cl(g/L)	X_2	-12.837	-6.418	-9.47	0.002[a]
Na_2SO_4(g/L)	X_3	-3.845	-1.922	-2.84	0.066[b]
K_2HPO_4(g/L)	X_4	2.541	1.27	1.87	0.158[b]
NaH_2PO_4(g/L)	X_5	-10.592	-5.296	-7.81	0.004[a]
$MgSO_4$(g/L)	X_6	-5.895	-2.948	-4.35	0.022[a]
Tryptophan(g/L)	X_7	0.127	0.063	0.09	0.931[b]
TES(ml/L)	X_8	-2.298	-1.149	-1.7	0.189[b]

[a]Significant
[b]Nonsignificant at P>0.05
R-Sq = 98.49% R-Sq(pred) = 75.83% R-Sq(adj) = 94.46%

4.3.3. RSM optimization for rL-asp II expression

The CCD is a full factorial design, composed of a cube part which allows determination of main and interaction effects and a star design(α) for quantifying main and quadratic effects [58]. The experiments were performed as mentioned in the materials and methods section. The design matrix and the corresponding responses of CCD experiments are shown in Table 4.5, along with the mean predicted values. The results were analysed using ANOVA (Table 4.6). According to ANOVA, the quadratic model proved to be highly significant with the Fisher F test (mean square regression: mean square residual is 53.51) with a very low probability value ($P_{model} > 0.05$). The residuals were plotted against the predicted values of $Y_{rLasp\ II\ activity}$ in Fig 4.4E.

Table 4.5: A 24 full-factorial central composite design matrix of four variables in coded units with experimental and predicted values of rL-asp II expression.

Run Order	Coded levels				Enzyme Activity (IU/ml)	
	X1	*X2*	*X5*	*X6*	Observed Values [a]	Predicted Values
1	-1	-1	-1	-1	105.8797 ±1.79	108.508
2	1	-1	-1	-1	99.3891 ±1.68	101.627
3	-1	1	-1	-1	196.782 ±2.58	187.532
4	1	1	-1	-1	108.7012 ±3.87	112.451
5	-1	-1	1	-1	111.6224 ±1.99	127.259
6	1	-1	1	-1	138.2836 ±1.79	154.431
7	-1	1	1	-1	182.7376 ±3.56	174.352
8	1	1	1	-1	146.9928 ±0.61	133.324
9	-1	-1	-1	1	115.6162 ±1.77	123.771
10	1	-1	-1	1	141.51 ±1.96	149.362
11	-1	1	-1	1	381.425 ±3.64	364.745
12	1	1	-1	1	343.286 ±4.00	322.136
13	-1	-1	1	1	153.8898 ±1.46	149.608
14	1	-1	1	1	205.5154 ±1.92	209.252
15	-1	1	1	1	366.4016 ±3.50	358.65
16	1	1	1	1	353.2558 ±3.58	350.095
17	0	0	0	0	320.3629 ±3.55	323.025
18	0	0	0	0	318.574 ±3.00	323.025
19	0	0	0	0	320.3731 ±4.17	323.025
20	0	0	0	0	308.6056 ±4.00	323.025
21	-2	0	0	0	205.15 ±2.55	212.092
22	2	0	0	0	197.5507 ±2.72	196.655
23	0	-2	0	0	128.45 ±0.64	99.371
24	0	2	0	0	284.1124 ±3.55	319.238
25	0	0	-2	0	195.21 ±2.92	203.416
26	0	0	2	0	252.286 ±3.12	250.126
27	0	0	0	-2	29.896 ±2.84	22.325

28	0	0	0	2	240.7421 ±3.11	254.359
29	0	0	0	0	320.3731 ±4.11	322.279
30	0	0	0	0	348.372 ±1.28	322.279

The "horizontal band" indicates no unusual behavior or abnormality [13], [59], which explains the adequacy of the model. The goodness of fit for the model was determined through co-efficient of determination value and it was evident that the model is highly significant with R^2 of 0.9817. This shows that the model is capable of explaining 98.17 % of the variation in response. Also, the lack of fit of the model has shown an insignificant value ($p>0.05$) with F value of 3.87. This represents the lack of fit which measures the failure of the model to represent data in the experimental domain at points which are not included in the model [60]. By applying the multiple regression analysis on the experimental data, the following second order polynomial was given by:

$$Y_{rL-asp\ II\ activity} = 322.652 - 3.859\,X_1 + 54.967X_2 + 11.678X_5 + 58.008X_6 - 29.476X_1^2 - 28.244\,X_2^2 - 23.877\,X_5^2 - 45.984\,X_6^2 - 17.05X_1X_2 + 8.513\,X_1X_5 + 8.118X_1X_6 - 7.983X_2X_5 + 40.487X_2X_6 + 1.771X_5X_6 \quad (21)$$

Table 4.6: Analysis of variance (ANOVA) for quadratic model

Source	DF	SS	MS	F-value	P-value
Model	14	278218	19872.7	53.51	<0.000
Residual (error)	14	5199	371.4	-	-
Lack-of-Fit	10	4712	471.2	3.87	0.102
Pure Error	4	488	121.9	-	-
Total	29	283421			

R^2 = 98.17%; Adj R^2 = 88.39 %; pred R^2 = 96.2 %
SS, sum of squares; DF, degrees of freedom; MS, mean square.

The student's t distribution and the corresponding P values, along with the parameter estimate are shown in Table 4.7. It was evident from the Table 4.7, that all linear and square

terms except sucrose, have shown a significant effect on rL-asp II expression, whereas the interaction term between sucrose and NH_4Cl, NH_4Cl and $MgSO_4$ were highly significant on the production. Similar observations were reported by Liu et al., [61], where including the interaction effects of parameters in the model showed significant improvement in the expression of HAS/lL 1Ra in *Pichia pastoris*. In order to understand the effect of variables and interaction among them on rL-asp II expression better, three-dimensional response surface plots were constructed by MINITAB® Release 16.1.1, PA, USA. In Fig. 4.4A, it was observed a steep increase in rL-asp II expression with increase in ammonium chloride and sucrose concentration but the rL-asp II expression decreased at higher concentration of sucrose which may be due to the substrate inhibition [62].

Table 4.7: Model coefficient estimated by multiple linear regressions

Model term	Coef	SE Coef	Computed t-value	*P*-value
Constant	322.652	7.965	40.507	0.000
X_1	-3.859	3.934	-0.981	0.343
X_2	54.967	3.934	13.973	0.000
X_5	11.678	3.934	2.969	0.010
X_6	58.008	3.934	14.746	0.000
X_1^2	-29.476	3.680	-8.011	0.000
X_2^2	-28.244	3.680	-7.676	0.000
X_5^2	-23.877	3.680	-6.489	0.000
X_6^2	-45.984	3.680	-12.497	0.000
X_1X_2	-17.05	4.818	-3.539	0.003
X_1X_5	8.513	4.818	1.767	0.099
X_1X_6	8.118	4.818	1.685	0.114
X_2X_5	-7.983	4.818	-1.657	0.120
X_2X_6	40.487	4.818	8.404	0.000
X_5X_6	1.771	4.818	0.368	0.719

A similar profile was observed in Fig 4.4B, Fig 4.4C and Fig 4.4D, with sucrose and $MgSO_4$ (p value 0.114), sucrose and NaH_2PO_4 (p value 0.099) and NaH_2PO_4 and NH_4Cl (p

value 0.120) respectively. The experimental data were fitted into the aforementioned equation (21), and the optimum levels of each variable were determined to be as follows: sucrose 17.0455 (g/L), NH_4Cl 8.5 (g/L), NaH_2PO_4 4.146 (g/L), $MgSO_4$ 2.974 (g/L) with an over-all yield of 389.56 U/ml of rL-asp II production. An overall yield of 5.25-fold increase in rL-asp II production was achieved using the Plackett–Burman design followed by the central composite experimental design technique compared to un-optimized medium. Similar reports of enhanced production of L-asparaginase II enzyme were noted while optimizing the culture conditions with different strains [43], [63]–[68]. However, very few studies deal with statistical optimization of the process for recombinant L-asp II expression in *Bacillus subtilis* [49]. To our best knowledge, there are no reports available on optimization of media for the production of rL-asp II production using sucrose and ammonium chloride as carbon and nitrogen sources from *Bacillus subtilis* WB800N.

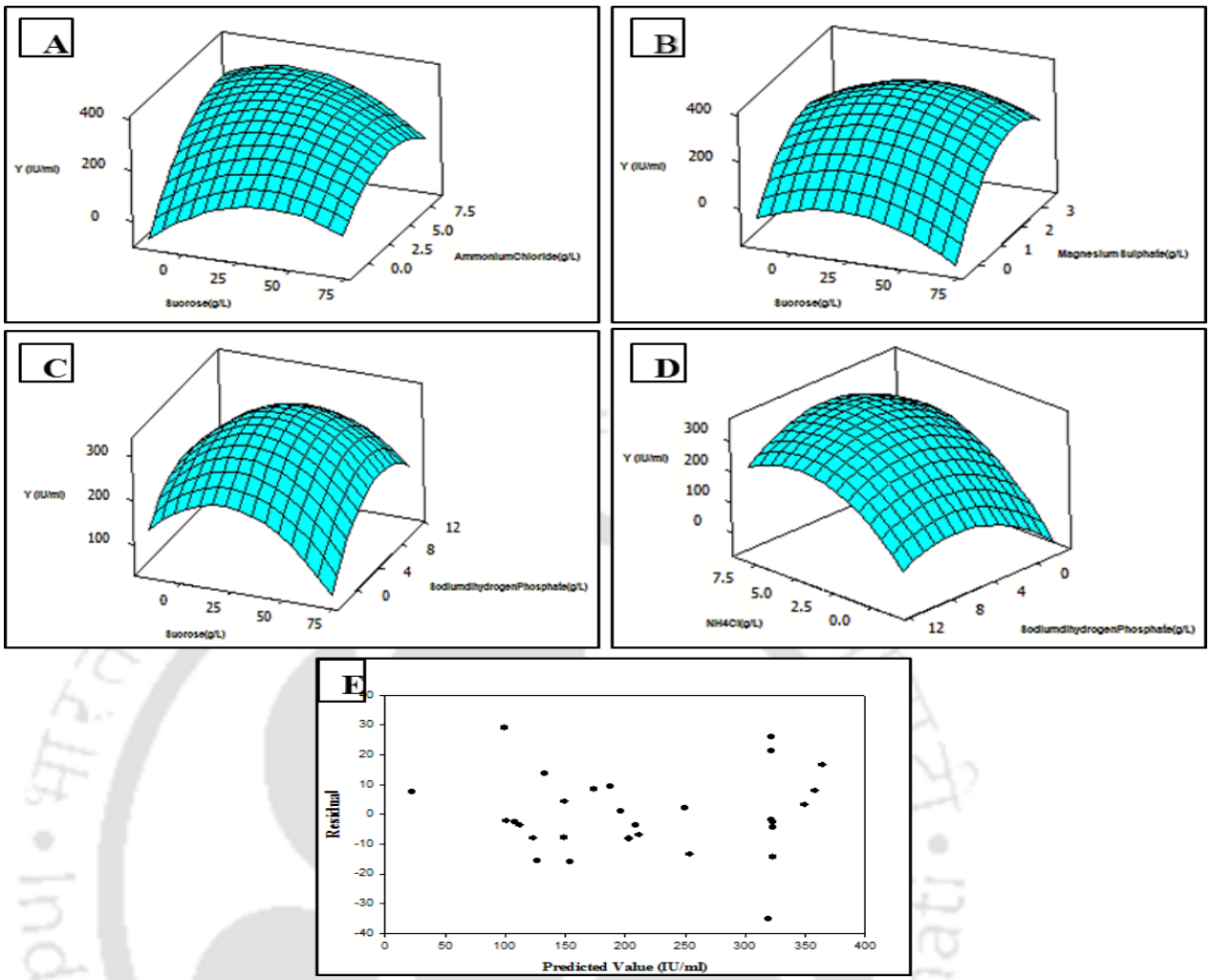


Fig 4.4: Three-dimensional response surface plot for rL-asp II expression showing the interactive effects of **(A)** Sucrose and NH_4Cl **(B)** Sucrose and $MgSO_4$ **(C)** Sucrose and NaH_2PO_4 **(D)** NaH_2PO_4 and NH_4Cl **(E)** Residuals plotted against predicted values of $Y_{rLasp\ II\ activity}$ (IU/ml) from RSM.

4.3.4. Hybrid model of statistical analysis using Artificial neural network linked genetic algorithm (ANN-GA)

Artificial neural network

In this study, we used feed forward back propagation algorithm. The input neuron represents sucrose, NH_4Cl, $MgSO_4$ and NaH_2PO_4, while the output layer represents rL-asp II expression. The training was done for 1000 epoch. The optimal results were found at 9 epochs with 4 inputs, 3 hidden layers and 1 output layer (Fig 4.5A). The MSE and determination

coefficient (R^2) for training, validation and test are shown in Table 4.8. The parity plot of experimental output versus prediction output is shown in Fig 4.5B. The model R^2 and adjusted R^2 was found to be 0.971 and 0.989 respectively.

Table 4.8: Artificial Neural Network Architecture MSE and R^2 Prediction of rL-asp II expression.

	Samples	MSE	R^2
Training	20	0.64	0.99
Validation	5	0.81	0.89
Testing	5	0.58	0.97

Genetic algorithm based optimization

Once the Neural network was trained, GA was used to further optimize the input spaces for maximizing rLAsp II expression. The values of GA specific parameters used in the optimization technique were as follows: population size = 20, cross over probability = 0.8, mutation probability = 0.01, no. of generations = 100. In order to achieve optimum global solution, GA was repeated several times with different initial values. The maximum predicted value of 546.46 IU/ml rL-asp II was achieved by maintaining the parameters at *viz*., sucrose 36.12 (g/L), NH_4Cl 5.99 (g/L), NaH_2PO_4 1.19 (g/L), $MgSO_4$ 1.58 (g/L), after 100 iterations and the probability of optimal variable solution was found (Fig 4.5C).

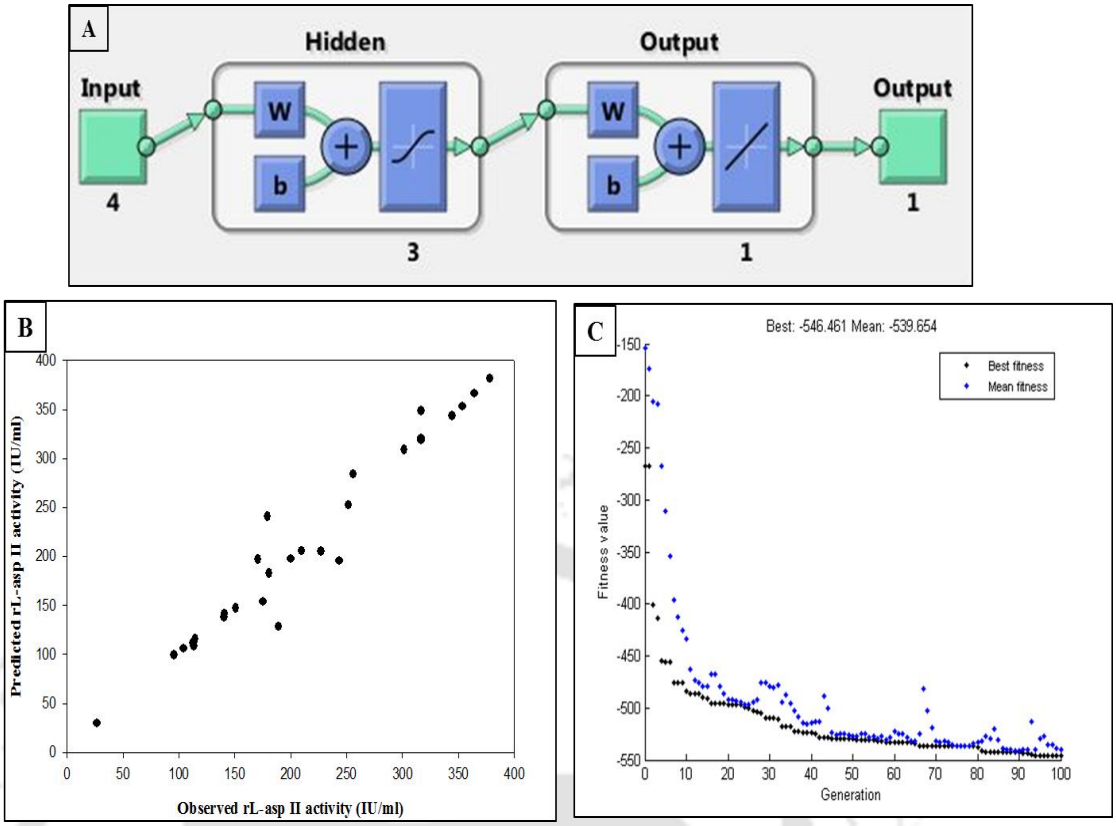


Fig 4.5: A: Artificial Neural Network architecture showing the input, hidden and output layer. **B:** Parity plot showing the goodness-of-fit for the ANN model (* R^2 = 97.1 % and $Radj^2$ = 98.9 %), **C:** Representative plots generated from the optimization by GA using MATLAB (2011 b) Best and average fitness values with successive generations showed gradual convergence to the optimum value for rL-asp II expression.

4.3.5. Verification of model from RSM and ANN linked GA

In order to validate the above proposed experimental models, the validation experiments were performed for RSM and ANN linked GA. The experiments were carried out at optimum conditions predicted by RSM, then the experimental and the predicted output data from the model were evaluated. It was found that the experimental output data (rL-asp II activity) was 389.56 ± 1.89 IU/ml, whereas the predicted value from the polynomial model was 430.602 IU/ml. The verification of the model revealed a high degree of accuracy, more than 90.46 %, which is an evidence for the good model validation under the investigated

conditions. The validation experiment for ANN linked GA showed that 525.98 ± 2.04 IU/ml which is in close agreement with the hybrid ANN-GA output data of 546.46 IU/ml. The accuracy of the model was found to be 96.25 %. The production of rL-asp II from WB800N is higher than the L-asparaginase produced from *B. subtilis* WB600 through a combined strategy during fed batch conditions (407.6 IU/mL) [69]. It was observed that ANN-GA was more efficient than that of CCD with an $R^2_{ANN\text{-}GA}$ 96.26 % > R^2_{CCD} 90.46 % (Table 4.9).

Table 4.9. RSM and ANN linked GA for modelling and optimization of rL-asp II expression

Variable	**Optimum Concentration, (g/L)**				**rL-asp II activity (IU/ml)**		**R^2 Value**
	X1	X2	X5	X6	Predicted	Observed	
RSM model	17.0455	8.5	4.146	2.974	430.602	389.56	0.9046
ANN - GA	36.12	5.99	1.19	1.58	546.46	525.98	0.9626

The results are in accordance with the recent research articles where many scientists reported that ANN-GA was found to be more precise than RSM model [68], [70]–[74]. The maximum biomass was found to be 7.52 DCW/L with production of rL-asp II 525.98 IU/ml during batch bioreactor. An overall increase of 7.098-fold in rL-asp II expression was observed compared to un-optimized with ANN-GA. In this study, the rL-asp II expression was observed to be higher than the reported values, from different strains such as, *Bacillus subtilis* WB600 407.6 U/ml [69], *Aspergillus terreus* MTCC1782 36.97 IU/ml [75], *Pectobacterium carotovorum* MTCC1428 14.53 IU/ml [76], *Pichia pastoris* (PichiaPink) 2.5 IU/ml [77], *Bacillus aryabhattai* ITBHU02 6.35 IU/mg [68] and *Streptomyces ginsengisoli* 3.23 µ mol/mL/min [78]. This shows the efficacy of the WB800N strain.

4.3.6. Unstructured Bio-kinetic Models for Prediction of rL-aspII Fermentation

The experimental and predicted profiles for the production of rL-asp II in batch bioreactor under un-controlled and controlled conditions are shown in Fig 4.6A and 4.6B

respectively. It was observed that under uncontrolled conditions the rL-asp II expression in batch fermentation increased up to 73 h (310.20 IU/ml), after which it declined. While under controlled conditions the rL-asp II expression enhanced up to 36 h (525.98 IU/ml) and then declined gradually. The kinetic parameters involved in the process were estimated using different models mentioned in equations (8), (11) and (17). The fitting of experimental data with the bio-kinetic models, nonlinear regression using the least-square method was employed to predict the model simulated data using Microsoft Excel Solver 2003.

Table 4.10: Parameters estimated by logistic and Leudeking-Piret model equation.

Model	Bioreactor	Parameters	R^2
Logistic model	Un-optimized Bioreactor	X_0 (g/L) = 0.033 , X_{max} (g/L) = 5.79, μ (h^{-1}) = 0.0802	0.983
LP model		α (IU/ml) = 0.0916, β (IU/ml.h) = 0.0161	0.941
Logistic model	optimized Bioreactor	X_0 (g/L) = 0.046 , X_{max} (g/L) = 7.52, μ (h^{-1}) = 0.2190	0.99
LP model		α (IU/ml) = 52.511, β (IU/ml.h) = 0.86	0.945

The estimated kinetic parameters from these models were mentioned in Table 4.10. The coefficients of determination (R^2) values obtained by fitting various models to experimental data were found to be highly significant. The Leudeking- Piret model for rL-asp II expression suggested that the rL-asp II expression is mixed growth associated in uncontrolled bioreactor, while under controlled conditions it showed growth associated production (Table 4.10) [56]. The modified Leudeking- Piret model for substrate consumption profile of model simulated and predicted are shown in Fig 4.6A and 4.6B. The maximum biomass, rL-asp II expression, specific growth rate, total protein expression acetate and acetoin was found to be 7.52 g/L, 525.98 U/ml, 0.21 h^{-1}, 8.83 g/L, 0.79 g/L and 0.645 g/L respectively. The acetate and acetoin has been maintained at very low levels in ANN

optimized experiment. The total protein production was also enhanced when the parameters were optimized. This clearly shows that optimization of physical and media components for high yield expression of rL-asp II is very essential in the biotechnological prospective.

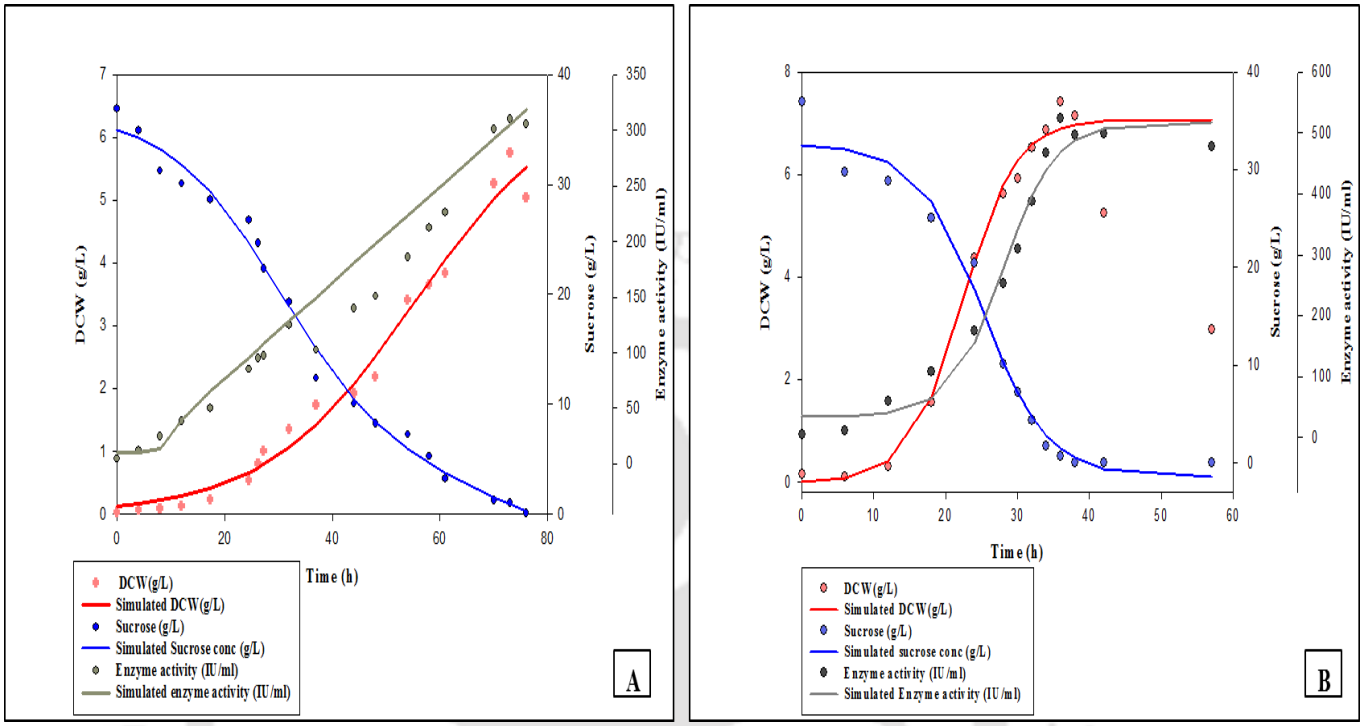


Fig 4.6: Experimental and model simulated growth kinetics, production kinetics and substrate consumption kinetics. (**A):** Un-optimized bioreactor during rL-asp II production. (**B):** Optimized bioreactor rL-asp II production.

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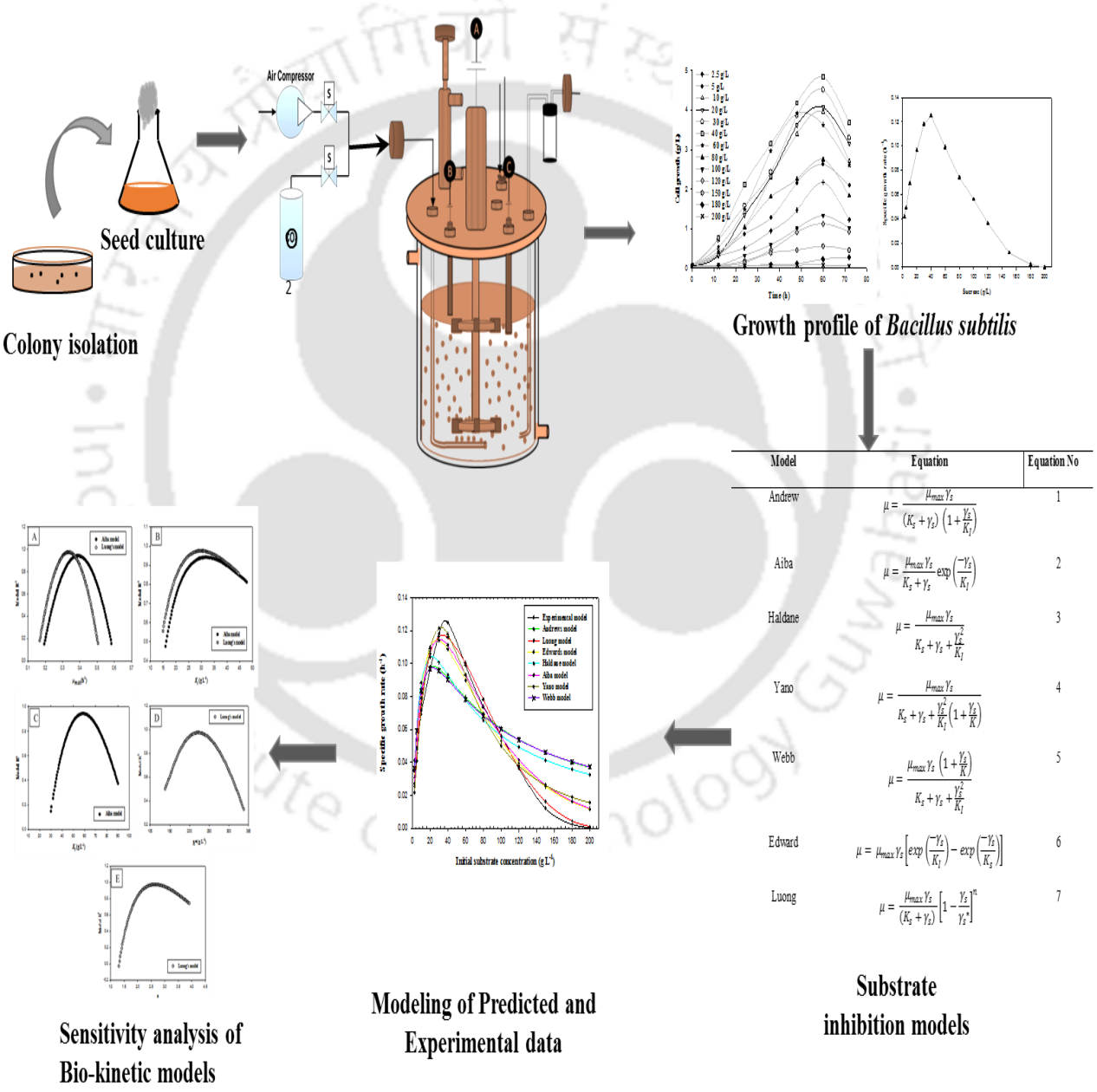
Chapter V

Mathematical modeling and Simulation

Mathematical modelling of Bio-kinetic growth models for rL-asp II producing *Bacillus subtilis* WB800N

Graphical abstract:



Model	Equation	Equation No
Andrew	$\mu = \frac{\mu_{max} Y_S}{(K_S + Y_S)\left(1 + \frac{Y_S}{K_I}\right)}$	1
Aiba	$\mu = \frac{\mu_{max} Y_S}{K_S + Y_S} \exp\left(\frac{-Y_S}{K_I}\right)$	2
Haldane	$\mu = \frac{\mu_{max} Y_S}{K_S + Y_S + \frac{Y_S^2}{K_I}}$	3
Yano	$\mu = \frac{\mu_{max} Y_S}{K_S + Y_S + \frac{Y_S^2}{K_I}\left(1 + \frac{Y_S}{K}\right)}$	4
Webb	$\mu = \frac{\mu_{max} Y_S \left(1 + \frac{Y_S}{K}\right)}{K_S + Y_S + \frac{Y_S^2}{K_I}}$	5
Edward	$\mu = \mu_{max} Y_S \left[\exp\left(\frac{-Y_S}{K_I}\right) - \exp\left(\frac{-Y_S}{K_S}\right)\right]$	6
Luong	$\mu = \frac{\mu_{max} Y_S}{(K_S + Y_S)}\left[1 - \frac{Y_S}{Y_S^*}\right]^n$	7

5.1. Background and Motivation

L-asparaginase II is gaining more importance than chemotherapy for treating cancer patients and reducing acrylamide formation in starchy/baked food products [1], [2]. Extensive studies have been carried out, on production, purification and characterization of L-asparaginase II from different plants, fungi and bacterial sources [3], [4]. On the other side, even though few studies were available on the production of L-asparaginase II [5], [6], studies on substrate inhibition were not available on *Bacillus subtilis* producing recombinant glutaminase free L-asparaginase II. Unlike *E.coli* and *Erwinia chrysanthemi* L-asparaginases, which contains slight glutaminase activity causing side effects in ALL (Acute lymphoblastic Leukemia) patients [7], [8], rL-asp II (recombinant L-asparaginase II) from *Bacillus subtilis* WB800N/pHT43-*ans B2* does not show any glutaminase activity [9]. In continuation with our previous studies, we are trying to estimate the parameters of substrate inhibition profile which will be further used in future fed batch studies.

The *Bacillus* expression system was much focused than *E.coli* for heterologous production of various proteins, antibiotics and vitamins [10], [11]. *Bacillus subtilis* is considered as an efficient host strain for synthetic [12] and systems biological analyses on gene regulation due to early sequencing of its whole genome and genome wide gene function analysis [13], [14]. During industrial microbial synthesis of products under fed-batch operations, substrate inhibition and catabolite repression occurs, due to the change of feed flow rate that controls the substrate concentrations [15]. The success of fed-batch mainly depends on feeding strategy and also on medium composition for the synthesis of desired product, thus reducing the byproducts formation [14], [16]. Hence, various researchers tried to understand the substrate inhibition effects on products formation [17], [18]. Very few studies available on substrate inhibition effects for the L-asparaginase II production by *Pectobacterium carotovorum* [19].

The heterologous protein production depends on various process conditions which are in turn influenced by environmental factors [20]. In order to grasp and minimize the influence of environmental factors and optimize the process parameters, mathematical modeling plays a crucial role [21]. Till date, there are no reports available on mathematical modeling of rL-asp II production by *Bacillus subtilis* WB800N. Hence, in this chapter we attempted to study the growth models of sucrose consumption kinetics of rL-asp II production from *B. subtilis* WB800N.

5.2. Materials and Methods

5.2.1. Chemicals and reagents

All the chemicals purchased were of high-grade quality from Himedia. The antibiotics used for the culturing of strain includes chloramphenicol and neomycin (Sigma-Aldrich).

5.2.2. Strain maintenance

Recombinant *Bacillus subtilis* WB800N/pHT43-*ans B2*, expressing rL-asp II under the control of *lac* promoter was used for kinetic studies [9]. Stock culture was maintained on LB media (yeast extract 5 g/L, tryptone 10 g/L and NaCl 5 g/L) with 100 µg/ml chloramphenicol.

5.2.3. Media and seed preparation

The seed culture was prepared by inoculating a single colony of *Bacillus subtilis* WB800N/pHT43- *ans B2* in 25mL chemically defined media modified from that of Wenzel *et al.,* [22]. The medium used for seed culture consists of (g L^{-1}): Na_2SO_4 2.0, $(NH_4)_2SO_4$ 2.68, NH_4Cl 0.5, K_2HPO_4 14.6, $NaH_2PO_4.H_2O$ 4.0, MgSO4.$7H_2O$ 1.0, tryptophan 1, sucrose 5, trace element solution (TES) 3 ml L^{-1}. TES contains $CaCl_2$ 0.5, $ZnSO_4$. $7H_2O$ 0.18, $MnSO_4$. H_2O 0.1, Na_2-EDTA 10.05, $FeCl_3$ 8.35, $CuSO_4$. $5H_2O$ 0.16, and $CoCl_2$. 6H2O 0.18. The culture was then incubated at 37^0C, 120 rpm for 10-12 h.

5.2.4. Bio-kinetic experiments

The production medium for rL-asp II expression contained (g L^{-1}): Na_2SO_4 2.0, $(NH_4)_2SO_4$ 2.68, NH_4Cl 0.5, K_2HPO_4 14.6, $NaH_2PO_4.H_2O$ 4.0, MgSO4.7H_2O 1.0, tryptophan 1, trace element solution (TES) 3 ml L^{-1} and different concentration of sucrose (2.5 - 180 g L^{-1}), and the initial pH was adjusted to 7.0. About 1 % of the inoculum from the above said seed culture was added to 50 mL of the fermentation medium in 250 mL shake flasks. The flasks were then incubated in an incubator at 37°C and 120 rpm. The samples were collected at regular intervals of time and for each initial concentration of substrate, specific growth rate (μ) was calculated in exponential phase. The specific growth rate (μ) in the exponential phase was calculated as the slope of plot drawn between ln (γ_x) vs t [23]. The γ_x is the dry cell mass obtained at a particular time. All the experiments were performed in triplicates for each sample and average value was noted.

5.2.5. Substrate inhibition growth models for recombinant *B. subtilis* WB800N/pHT43-*ans B2*

The correlation between initial substrate concentration and specific growth rate (μ) is the basic parameter for the formation of bio-kinetic models [24]. Any fermentation process is operated under certain substrate limiting conditions. Generally, higher substrate concentration causes the substrate inhibition. In order to deduce different substrate inhibitions models mathematically and understand the inhibitory mechanism of the substrate, various extension models of Monod's kinetics have evolved [25]. The *Andrew* model is capable of explaining inhibitory effect of substrate at higher concentrations; however, when the inhibition constant is beyond certain range the model will behave like Monod's equation [26]. The *Aiba* model clearly shows a decrease in specific growth rate with an increase in product concentration. This model is also known as growth inhibition model. The exponential term, which depicts the product inhibition, could be replaced with substrate concentration.

However, this model fails to give the critical value of inhibitory substrate/product concentration [27].

The *Haldane* model is the most widely used which consists of both the substrate affinity constant and the substrate-inhibition constant. This model explains growth kinetics under inhibitory substrate concentration when the inhibition constant is boundlessly large [28]. The *Yano* model was initially used for estimating kinetics of product expression at high sugar concentration [29]. The *Webb* model is the modified form of Yano model, where (1+ γ_s/K) term is present in numerator rather than denominator [30]. The *Edward* model explains the protective diffusional-limitation at high and inhibitory substrate concentrations [31]. The *Luong* model is a form of Monod's model that includes an additional term, which accounts for complete growth inhibition above certain critical substrate concentration. The relation between the specific growth rate and the initial substrate concentration could either be concavity downward ($n < 1$), linear ($n = 1$) or a concavity upward ($n > 1$) depending on the value of the constant parameter (n) [32]. The equations of different substrate inhibitory models are depicted in Table 5.1.

Table 5.1: Different substrate inhibition models used in this study

Model	Equation	Equation No
Andrew	$\mu = \frac{\mu_{max}\gamma_s}{(K_s + \gamma_s)\left(1 + \frac{\gamma_s}{K_I}\right)}$	1
Aiba	$\mu = \frac{\mu_{max}\gamma_s}{K_s + \gamma_s}\exp\left(\frac{-\gamma_s}{K_I}\right)$	2
Haldane	$\mu = \frac{\mu_{max}\gamma_s}{K_s + \gamma_s + \frac{\gamma_s^2}{K_I}}$	3
Yano	$\mu = \frac{\mu_{max}\gamma_s}{K_s + \gamma_s + \frac{\gamma_s^2}{K_I}\left(1 + \frac{\gamma_s}{K}\right)}$	4
Webb	$\mu = \frac{\mu_{max}\gamma_s\left(1 + \frac{\gamma_s}{K}\right)}{K_s + \gamma_s + \frac{\gamma_s^2}{K_I}}$	5
Edward	$\mu = \mu_{max}\gamma_s\left[exp\left(\frac{-\gamma_s}{K_I}\right) - exp\left(\frac{-\gamma_s}{K_s}\right)\right]$	6
Luong	$\mu = \frac{\mu_{max}\gamma_s}{(K_s + \gamma_s)}\left[1 - \frac{\gamma_s}{\gamma_s{}^*}\right]^n$	7

5.2.6. Statistical validation of model for acceptability

Different substrate inhibition models were fitted and analysed based on the complexity of the model (degree of freedom). Therefore, it is necessary to mathematically infer the data consistency by comparing one model to the others. As the fitting of kinetic models were improved, the complexity of the models increases (lower model sum of square or highest regression coefficient). Thus, the fitted models must be compared with *F* test [33] for nested models or Akaike information criterion (AIC) for both nested and non-nested pairs [23]. The Akaike information criterion (AIC) is defined by the following equation:

$$\mathbf{AIC} = \boldsymbol{m}\ln\left(\frac{SS}{m}\right) + 2\boldsymbol{b} \qquad \textbf{(8)}$$

Where, "*m*" is the number of data points, "*b*" is the number of parameters "*prm*" to be fitted by the regression plus one (*b* = *prm* + 1).When, there are few data points, the corrected AIC (AIC*c*) will be used, which is given by following equation:

$$AIC_c = AIC + \left(\frac{2b(b+1)}{m-b-1}\right) \quad (9)$$

The model with lower AIC*c* value is more likely to be correct and the probability (*pAIC*) that the more complex model is correct, is given by

$$P_{AIC} = \left(\frac{e^{-0.5\Delta AIC_c}}{1+e^{-0.5\Delta AIC_c}}\right) \quad (10)$$

Where, Δ_{AIC_c} is the difference between AIC_c values of complex and simple model.

The *F* test is an alternate for ANOVA analysis. It assumes that the simpler model to be correct (null hypothesis), if the relative increase in the degree of freedom and in the sum of squares of the model are approximately equal. Otherwise, the complex model (alternate hypothesis) is justified, if the relative increase in the sum of squares of the model largely exceeds the relative increase in the degree of freedom. The *F* ratio is given by following equation:

$$F = \frac{\frac{(SS_{simple} - SS_{alternative})}{SS_{alternative}}}{\frac{(df_{simple} - df_{alternative})}{df_{alternative}}} \quad (11)$$

Non-linear regression analysis

The parameters of different models were estimated from experimental results, using MATLAB© v.7.13.0.564. Levenberg-Marquardt nonlinear least squares algorithms was used to estimate the parameters iteratively since the models had non-linear coefficients [34]. The equations is as follows:

$$S = \sum_{i=1}^{n} (f_{exp} - f_{pre})^2 \quad (12)$$

5.2.7. Analytical Methods

The growth profile of *Bacillus subtilis* WB800N/pHT43-*ans B2* was monitored by measuring the absorbance of cells at 600 nm (OD_{600nm}) with a UV–visible spectrophotometer (Cary 50, Varian, Australia) and were expressed in terms of dry cell weight (DCW) using the correlation equation $DCW = 0.45 \times OD_{600nm}$. The sucrose was estimated using high-pressure liquid chromatography, (HPLC) (Agilent 1220 Infiniti HPLC, USA) equipped with SUPELCOGEL Ca, 300 mm × 7.8 mm I.D., 9 µm particle.

5.3. Results and discussion

While developing any bioprocess model, the fermentation biologist should consider all the operational parameter's influencing the productivity such as medium composition, pH, temperature, aeration, agitation, induction and feeding strategy [35]. *Bacillus subtilis* has the capacity of using wide variety of carbon sources for fermentation [22], [36]. Hence, it is very crucial to understand the critical sucrose concentration for maximum growth rate and rL-asp II production.

5.3.1. Performance of *B. subtilis* WB800N, effect of various sucrose concentrations and rL-asp II production

The growth profile of recombinant *Bacillus subtilis* WB800N/pHT43-*ans B2* on different initial sucrose concentration is depicted in Fig. 5.1. It was observed that, there was a gradual increase in the growth of *B. subtilis* from 2.5 – 40 g L^{-1} followed by gradual decrease in growth was noted from 60 – 150 g L^{-1} of substrate. While the growth was completely ceased or very less in case of 180 g L^{-1} of sucrose. The inhibition of biomass at high substrate is may be due to osmotic shock at higher substrate concentration. Irrespective of initial sucrose concentration in different sets, the consistency of 12 h lag period was observed before the cells entered in log phase. As the *Bacillus* is a spore-producing organism, a well demarcated stationary phase was not observed, as it enters into spore producing phase [37].

The plot of cell growth rate against initial sucrose concentration revealed that the maximum DCW of 4.84 g L^{-1} was achieved at 40 g L^{-1} of sucrose (Fig. 5.1).

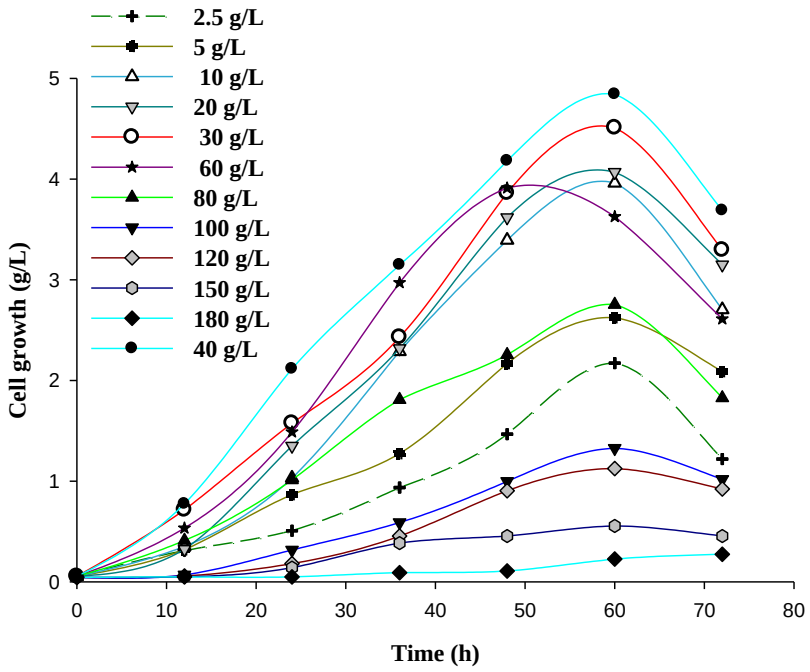


Fig 5.1: Growth profile of *Bacillus subtilis* WB800N/pHT43-*ans* B2 at different initial concentrations of sucrose.

Complete utilization of sucrose was observed within 60 h for initial sucrose concentration of 2.5 – 10 g L^{-1}, while it took 72 h for 20 – 60 g L^{-1} and with an exception for 80 - 120 g L^{-1} of sucrose concentration, where it took more than 72 h for complete utilization of substrate. When the initial sucrose concentration was 150 to 180 g L-1, led to lower consumption of sucrose and the rL-asp II production was very low, this may be attributed to substrate inhibition at higher concentrations. The yield and specific sucrose uptake rate was calculated at different initial substrate concentration and the results were recorded in Table 5.2. The highest yield of $Y_{X/S}$ = 0.4231 was obtained at initial substrate concentration of 40 g L^{-1} comparable with results of Heryani *et al.,* who observed highest yield of bio surfactant at 50 g L^{-1} of glucose [38].

Table 5.2: Yield and specific sucrose uptake rate at different initial substrate concentrations.

Y_{s0}/g L^{-1}	2.5	5	10	20	30	40	60	80	100	120	150
$Y_{X/S}$	0.137	0.164	0.182	0.241	0.265	0.423	0.164	0.123	0.073	0.053	0.071
qs/h^{-1}	0.240	0.184	0.132	0.358	0.487	0.912	0.615	0.599	0.758	0.674	0.166

The extracellular L-asparaginase activity was measured at different time points with respect to various initial substrate concentrations (Fig. 5.2). The rL-asp II production continues to increase until 60 h, where the highest activity was noted (116.64 U/ml) with initial sucrose concentration of 40 g L^{-1}. This could suggest the possible substrate inhibition when sucrose concentration increased above 40 g L^{-1}. The profile of specific growth rate shows that increase in sucrose concentration up to certain level, increases specific growth rate but further increase in sucrose concentration lead to decline in specific growth rate (Fig. 5.3). The results are comparable with Singh and Srivastava, who reported maximum specific growth rate at 15 g L^{-1} of glucose but maximum substrate consumption and best specific productivity were noted at 5.0 g L^{-1} of glucose [39]. This could also suggest possible substrate inhibition leading to decrease in growth beyond 40 g L^{-1} of sucrose.

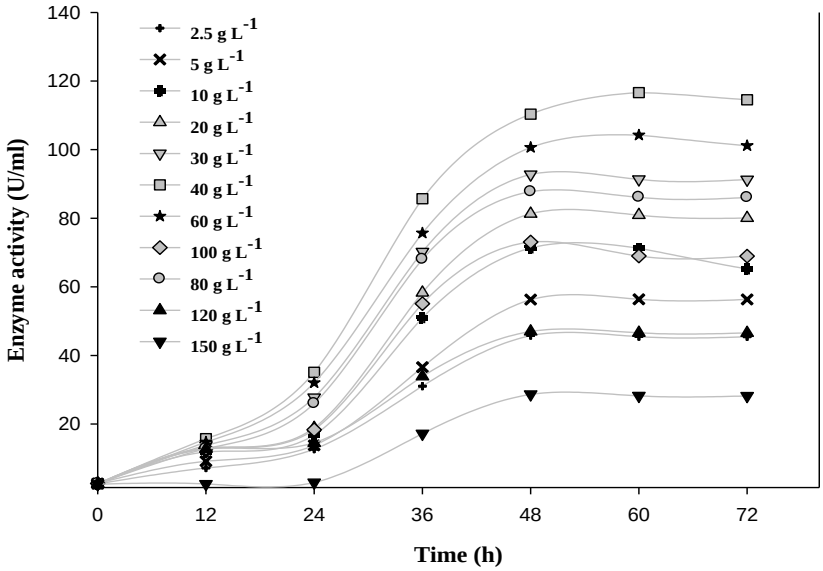


Fig 5.2: L-asparaginase II activity of *Bacillus subtilis* WB800N/pHT43-*ans* B2 at different initial concentrations of sucrose.

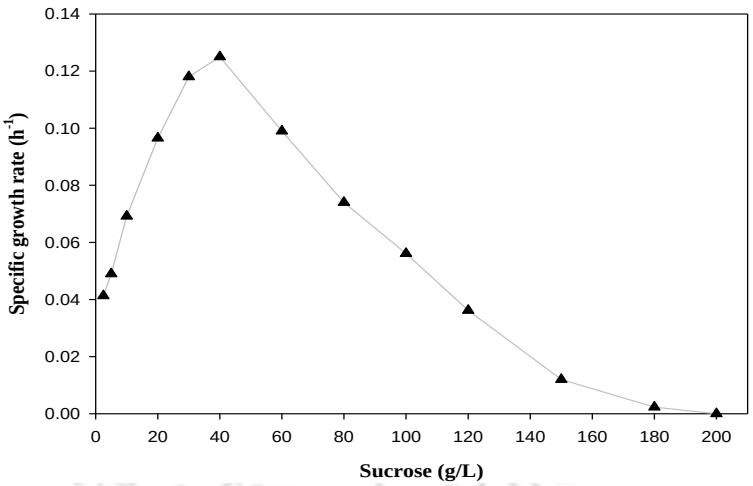


Fig 5.3: Specific growth rate as a function of initial sucrose concentration

5.3.2. Mathematical modeling of growth kinetics of substrate on *B. subtilis* WB800N

The cell growth kinetics of *B. subtilis* WB800N/pHT43-*ans B* was modeled using substrate inhibition models. Even though the growth profiles of WB800N/pHT43-*ans B* at lag phase were similar with respect to different initial concentration, the experimental specific growth rate profile was different. It was observed that specific growth rate was dependent on

initial sucrose concentration (Fig 5.3). It was noted that with increasing the initial sucrose concentration, the specific growth rate decreased, which shows possible substrate inhibition (Fig 5.3). Similar results were reported by Agarwal *et al.*, and Dutta *et al.*, [23], [40].

In this study, various inhibition models were chosen to understand the behavior of growth kinetics of recombinant *B. subtilis* WB800N/pHT43-*ans B2* under different concentrations of initial substrate. The regression analysis was employed on the experimental specific growth rate and model predicted specific growth rate using MATLAB v.7.13.0.564. The profile of experimental *vs* predicted specific growth rate, depicted in Fig 5.4. The biokinetic models used in this study showed a precise fit between experimental and modeled specific growth rate profiles with respect to different substrate concentration. The biokinetic parameters estimated using the non-liner regression analysis of various models for sucrose are recorded in Table 5.3.

Table 5.3: Estimated bio-kinetic parameters from different models

Model fit	**parameter**	**dF**	**Estimated parameters**							
			μ_{max}	K_I	K_s	n	γ_s*	K	R^2	RMSE
Aiba	3	6	0.389	59.9	31.798	-	-	-	0.978	0.0096
Andrews	3	6	0.268	30	10	-	-	-	0.6842	0.0224
Edward	3	6	0.326	62.01	22.94	-	-	-	0.941	0.0105
Haldane	4	5	0.256	29.46	15.62	-	-	-	0.733	0.0207
Luong	4	5	0.336	-	29.773	2.6	225	-	0.979	0.0063
Webb	4	5	0.184	41	10.082	-	-	10000	0.676	0.0228
Yano	4	5	0.336	245	36.28	-	-	7.89	0.928	0.0351

dF- degree of freedom

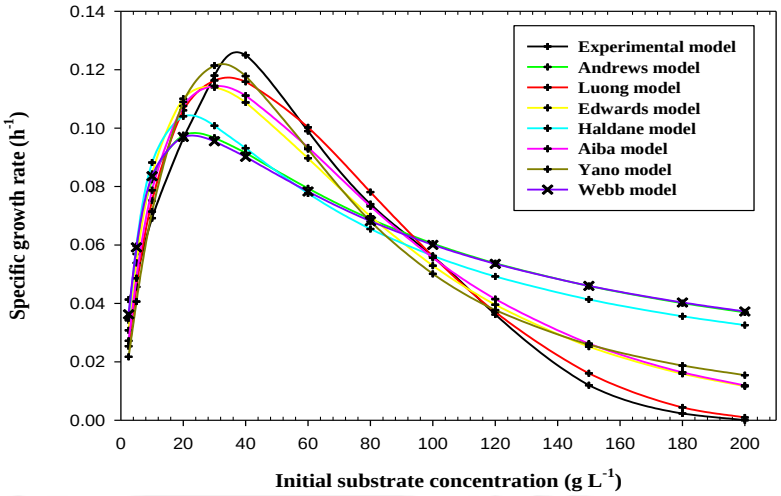


Fig 5.4: Predicted and Experimental data fitted in various substrate inhibition models predicted as a function of initial sucrose concentrations

Among the various models fitted, it was observed that except Webb, Andrew and Haldane models, most of the models had very close resemblance of experimental and predicted specific growth rate profiles in sucrose concentration regime. The Parity plot showing predicted specific growth rate by different models that fit to the entire data *vs* experimental specific growth rate is shown in Fig 5.5. Among the several models used, it was noted that Luong had the highest R^2 value of 0.979, followed by Aiba model (R^2 = 0.9785), Edward model (R^2 = 0.941), Yano model (R^2 = 0.928) were showing good fit between predicted and experimental values. The root mean square errors (RMSE) between experimental and model predicted values were 0.0063, 0.0096, 0.0105 and 0.035 respectively, for four different models. The highest μ_{max} was predicted by Aiba (0.389), followed by Luong (0.336), Yano (0.336) and Edward (0.326). According to the Luong model, the critical sucrose concentration at which the cell growth completely ceased was 225 g L^{-1}.

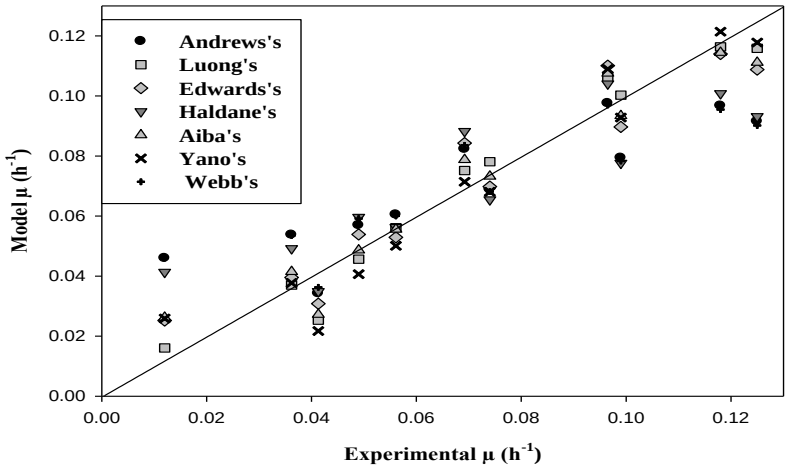


Fig 5.5: Parity plot for the predictions of specific growth rate by various substrate inhibition models

The Edward and Aiba model showed inhibition constant (K_I) of 62.01 and 59.9 respectively. The other models were not considered, as the K_I was insensible. From the results, it could be inferred that Luong model best fits to explain the substrate inhibitions kinetics of *B. subtilis* WB800N. While Dutta *et al.,* observed that Andrews and Webb models fits best to the experimental values for cutinase production from *P. cepacia* [23], Whereas, Agarwal *et al.,* found that Han Levenspiel model shows proper fit to the experimental data for producing cellulose by *C. cellulans* [40]. Vital Jacome *et al.,* studied the degradation of 4-chlorophenol by aerobic granules and observed that the experimental data was a good fit to the Haldane model [41]. It is very important to model the growth kinetics, as different model/models will fit for different strains with different carbon sources.

The *Bacillus subtilis* WB800N/pHT43-*ans B2* showed growth associated protein production during the same fermentation phase. The feeding of sucrose is necessary to enhance the biomass production along with simultaneous intermittent induction with IPTG, which enhances the rL-asp II production [13]. The addition of IPTG only once at particular period of optical density has showed less protein production compared to consecutive intermittent IPTG addition [9]. In few studies it was observed that adding IPTG at higher

concentration causes metabolic burden [42] and thus lead to inclusion body formation [43] or may lead to spore formation [37] . Hence, we induced the culture with IPTG in small quantities consecutively, which enhanced the rL-asp II production drastically. During fed batch fermentation, design of medium components along with the feeding strategy developed directs the flux towards the intracellular reaction network, which helps in production of desired products [14]. The success of fed batch operation mainly depends on yielding high productivity, preventing substrate inhibition. Thus, in this study we tried to understand the substrate inhibition mechanism of sucrose on WB800N/pHT43-*ans B2.*

5.3.3. Model acceptability and Sensitivity analysis of estimated kinetic parameters

The sensitivity analysis and mode acceptability analysis was carried out according to Sobie to assess the changes in parameters which would affect the model R^2 (regression co-efficient) [44]. In this current study, we chose the biokinetic parameters of Aiba (3-parameter) and Luong (4-parameter) for sensitivite analysis. The input parameters of both the models were varied ±50 % of their estimates and keeping all others values constant. The results of sensitivity analysis for μ_{max}, K_S and K_I values are shown in Fig. 5.6a, 5.6b and 5.6c respectively. It was apparent that among all other parameters, maximum specific growth rate (μ max) appeared as the most sensitive parameter for both Aiba and Luong models. Any changes of μ max (± 50%) caused a severe variation in R^2 for both Aiba and Luong models. The very high sensitivity of maximum specific growth rate suggests requirement of its precise measurement/estimate for further predictability of the model. It was evident from the figure that K_S for both Aiba and Luong models showed a high sensitivity in upper variations. The K_I also showed high sensitivity in Aiba model, where as 'n' constant parameter in Luong's model showed more sensitivity in downside variations from their standard estimate. The (γ_s^*) critical sucrose concentration also showed quite high sensitivity. Thus pointing towards the necessity of finding the sensitivity analysis of parameters in bio-kinetic models of the bio-

systems [45]. Aiba (3-parameter) and Luong (4-parameter) model showed a regression coefficient of (0.978 and 0.979). In general, if the model is more complex then it shows better fit. Theodore and Panda reported the similar findings, where they used the Luong model to explain the growth of *Trichoderma harzianum* during production of *β*-1,3glucanase [46].

The model acceptability analysis of two models was performed using Akaike's information content criteria or extra sum of square *F* test. The probability value of extra sum-square *F* test was >0.05. At 95% confidence, the complex model (alternate hypothesis) is justified, as the relative increase in the sum of squares of the model exceeds the relative increase in the degree of freedom. Hence, the complex model was accepted. Using Akaike's information criteria (*AIC*) the 3 and 4 parameters model were compared. The result of AIC and *F* test are recorded in Table 4. It was observed that SSE values were not as large as expected from change in number of parameters. Hence, the Δ_{AICc} showed a positive value. The model with lower AIC*c* value is more likely to be correct. Thus, the probability of choosing complex model (Luong model) was >99%, with an overwhelming evidence ratio of 82.251 was noted. Similar results were observed by Zhang *et al.*, who studied the influences of temperature and light, on cyanobacterial growth and bio-hydrogen production simultaneously [47].

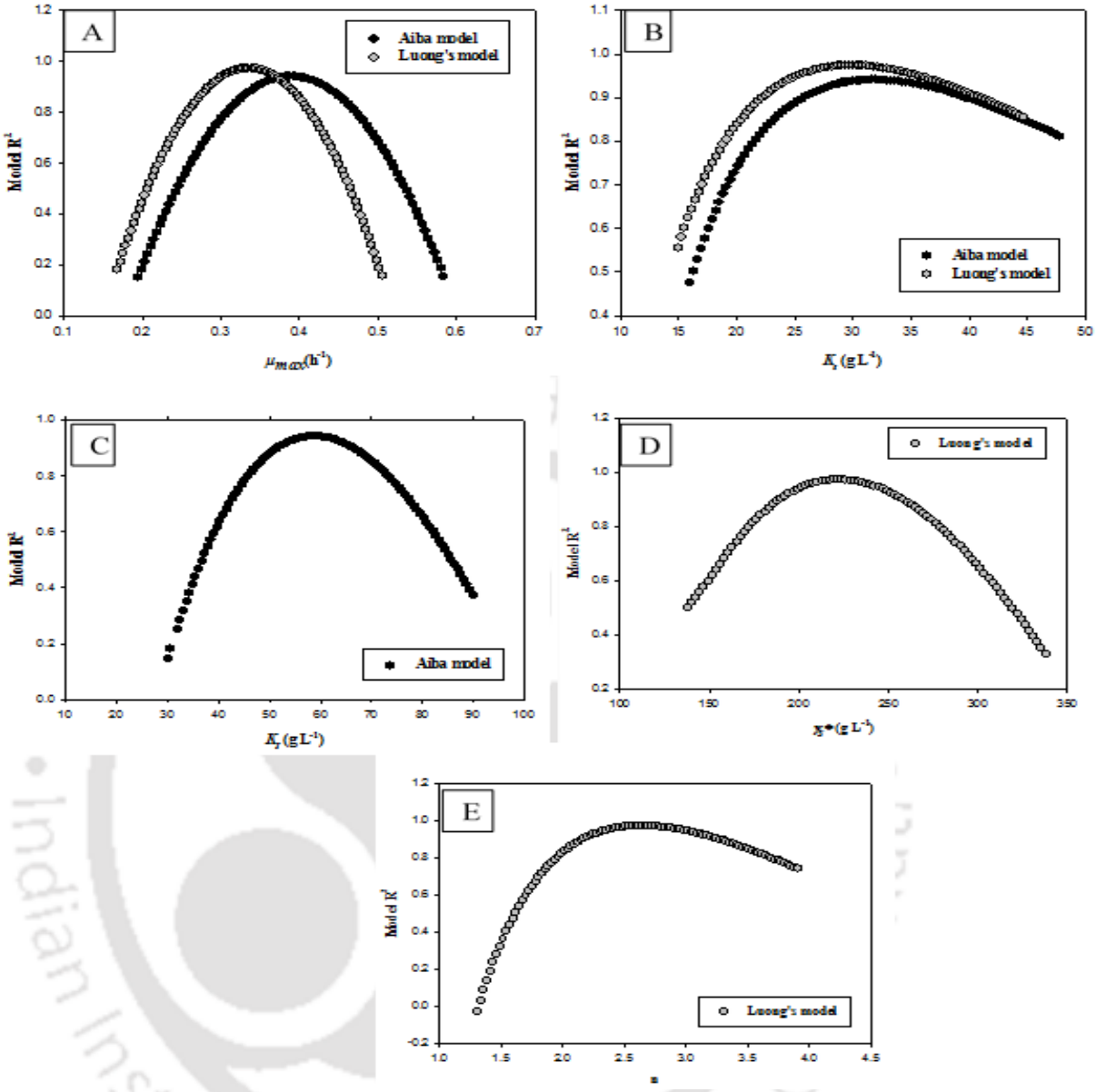


Fig 5.6: Sensitivity analysis of A) maximum specific growth rate, B) Monod half saturation constant, C) substrate inhibition constant (Aiba model only) and D) Critical substrate concentration (only for Luong's model) E) 'n' constant parameter (only for Luong's model) as estimated from the Aiba and Luong models toward model regression coefficients.

Table 5.4: Summary of model discrimination using Akaike's information criterion and extra sum-of-square F-test

Models	model specific information				Akaike's information criteria			Extra sum of square F test				
	prm	*p*	SS (10^{-3})	df	AICc	Δ_{AICc}	P_{AICc}	evidence ratio	X	▼	F ratio	p value
Aiba	3	13	16.35	6	-73.816	8.819	0.987	82.251	0.221	0.24	1.105	0.466
Luong	4	13	20.99	5	-64.997		0.012					

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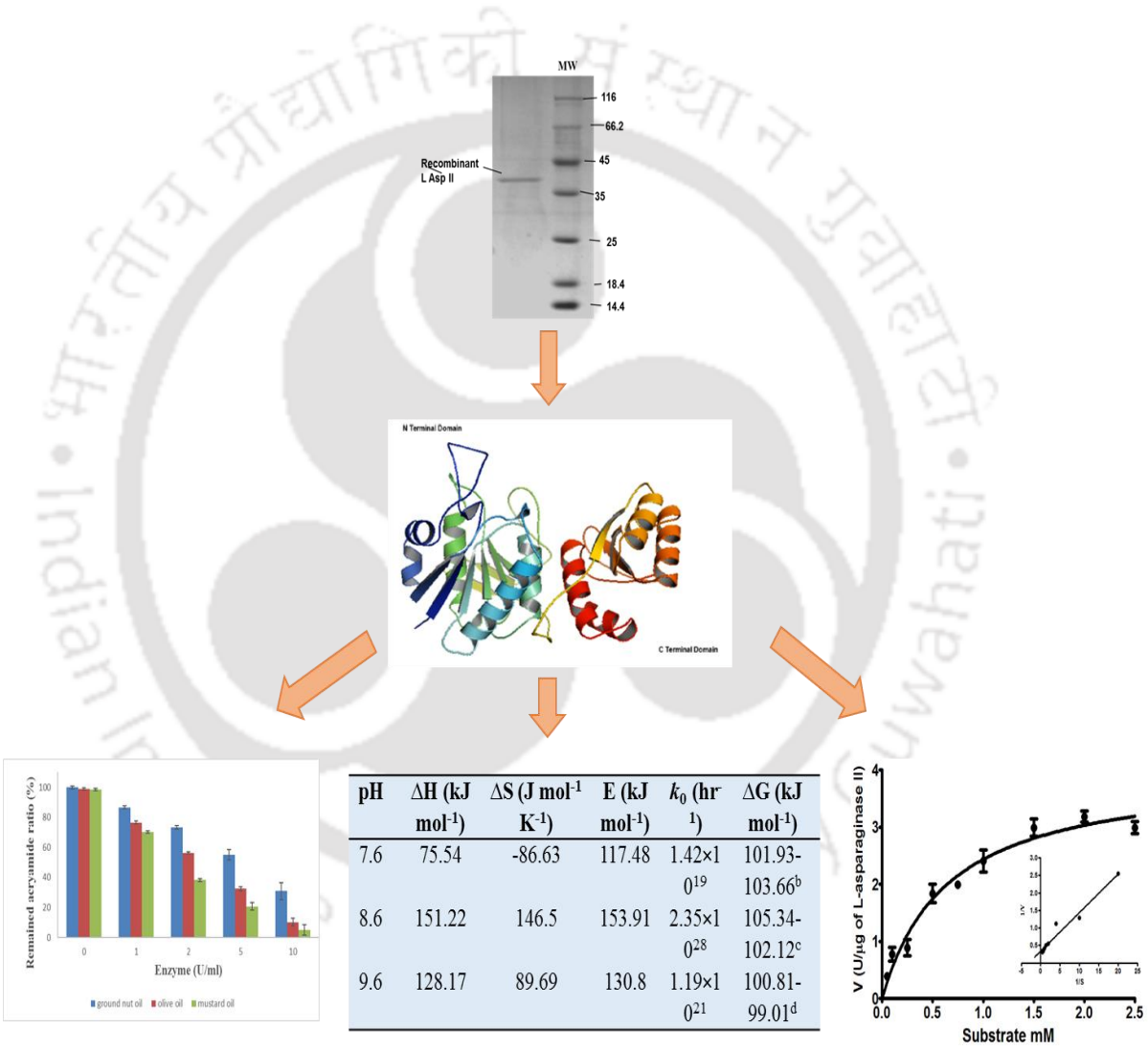
Chapter VI

Purification and Characterization

Purification and characterization of rL-asp II from *Bacillus subtilis* WB800N

Graphical abstract:



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	75.54	-86.63	117.48	1.42×10^{19}	101.93-103.66[b]
8.6	151.22	146.5	153.91	2.35×10^{28}	105.34-102.12[c]
9.6	128.17	89.69	130.8	1.19×10^{21}	100.81-99.01[d]

6.1. Background and Motivation

A protein's function depends on its ability to acquire a native structure. Measurement of protein stability and how a protein folds into a well-ordered native structure are essential for elucidating protein function *in vivo*, engineering the improved stability of bio catalytic enzymes, and formulating proteins for therapeutic delivery [1]. Studies on the molecular structure, catalysis, clinical aspects, genetic determinants involved in regulation and stabilization to enhance biological half-life of L-asparaginase have been reported [2]. However, very few studies have been reported on thermodynamic aspects of this enzyme [3], [4]. Thermal stability studies would help to understand the relation between structure and function of a particular enzyme [5], [6]. L-asparaginase deactivation plays a vital role in cancer therapy. 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.

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 [7]. Both of these properties are modified to a large extent 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 [8]. It is well known that the activity and stability of an enzyme is strongly influenced by pH and temperature [9].

In this, current study we purified and characterized the rL-asp II from *Bacillus subtilis* WB800N. It was observed that the purified rL-asp II was effective in inhibiting the acrylamide formation.

6.2. Materials and Methods

6.2.1. Purification of L-asparaginase II

All the purification steps were carried out at 4 ºC. The cell-free supernatant stored at 4 ºC was used for the purification of 6x- His-tagged extracellular rL-asp II by Ni–NTA affinity chromatography. A column packed with 5 ml of 50 % Ni–NTA resin (Sigma) was equilibrated with equilibration buffer (20 mM potassium phosphate, pH 8.0, 50 mM NaCl and 20 mM imidazole). The final concentration of the supernatant was maintained as 50 mM NaCl and 20 mM imidazole. Treated culture supernatant (100 ml) from the process above was concentrated using protein concentrators to 10 ml and loaded on to the Ni–NTA column. The column was washed with 5 column volumes of equilibration buffer (20 mM potassium phosphate, pH 8.0, 50 mM NaCl and 20 mM imidazole) and the protein was eluted with elution buffer (20 mM phosphate buffer, pH 8.0, containing 250 mM imidazole and 50 mM NaCl) [10]. Fractions of 1 ml were collected and analysed by SDS–PAGE and the eluted protein concentration was measured using Bradford assay. Fractions containing rL-asp II protein were pooled and concentrated using a protein concentrator (centrifugal concentrator) MWCO 10 KDa (Sartorius, Germany). The crude and purified rL-asp II samples, assayed by SDS-PAGE (12 % gels) under reducing conditions were stained with Coomassie Brilliant Blue R250 by the Laemmli method.

6.2.2. Modelling of the protein

The three-dimensional structure of L-asparaginase II monomer was modelled using the x-ray crystal structures of L-asparaginases from *Erwinia chrysanthemi* and *Erwinia carotovora* (PDB ID: 1HFJ, 1O7J, 2JK0, 1ZCF) as a template using Modeller 9.13. The initial models generated were further, prioritized based on MOLPDF, discrete optimized protein energy (DOPE) and GA341 score. The final model (MOLPDF and DOPE) was validated in SAVES server (http://nihserver.mbi.ucla.edu/SAVES/) and analysed using PROCHECK and

ERRAT plot. The modelled protein structure was superimposed onto a crystal structure of 1HG1 to determine the active site residues involved in ligand binding using PyMOL (http://pymol.org/). Protein and ligand interactions of the superimposed 3D structure were analysed with the help of PyMOL viewer.

6.2.3. Effect of pH on activity and stability of recombinant enzyme

The influence of pH on the activity and stability of the rL-asp II was determined under the standard assay conditions over a pH range of 5 – 10.5. For the determination of stability and residual activity of the purified rL-asp II, enzyme preparations were incubated at different pH for 24 h at 4˚C ± 1˚C in the absence of substrate.

6.2.4. Effect of temperature on recombinant enzyme activity.

To check the effect of incubation temperature on the activity of rL-asp II, the enzyme assay was done at different temperatures (25, 30, 37, 42, 47, 52, 57, 62, 67 ˚C). To check the effect of temperature on the stability, the enzyme was pre-incubated for 30 min at the temperatures above mentioned and residual activity was estimated by standard protocol given before. At each temperature, a control assay was performed without adding enzyme and considered as blank.

6.2.5. Effect of incubation time on activity of purified enzyme

In order to study the effect of incubation time on the enzyme activity, recombinant enzyme was incubated separately with the substrate at different time intervals (15, 25, 30, 40, 50, 60, 70, 80, 90 min) and enzyme assay was performed under the standard conditions.

6.2.6. Effect of ionic strength of the buffer

To check the effect of ionic strength of the buffer (Tris-HCL) on the activity of the enzyme, enzyme assay was performed at different concentrations (5, 10, 15, 30, 40, 50, 60, 70, 80, 90 and 100 mM) of the buffer at standard conditions.

6.2.7. Substrate specificity of rL-asp II

The activity of the rL-asp II was determined using different structure analogs of L-asparagine viz., DL-asparagine, D-asparagine, L-glutamine, D-aspartic acid, DL-aspartic acid, succinamic acid, L-glutamic acid, L-asparagine-t-butylester HCl, N-a-acetyl-L-asparagine and BOC-L-asparagine as substrate at a final concentration of 10 mM [11]. The relative activity was expressed as the percentage ratio of the enzyme activity determined against different substrates to that of enzyme activity with L-asparagine.

6.2.8. Influence of various modulators on rL-asp II activity

The influence of various modulators on rL-asp II enzyme activity was analysed according to Kumar et al., (2011). The enzyme was exposed to each effector (Table 6.3) for about 30 min and then the residual enzyme was calculated as a percentage ratio of the enzyme activity incubated with effectors to that of untreated samples using standard protocol.

6.2.9. Determination of kinetic parameters of enzyme.

The parameters like K_m, V_{max}, K_{cat} and (K_{cat}/K_m) of the purified rL-asp II from *Bacillus subtilis* WB800N was determined using L-asparagine as a substrate (0.05 to 2.5 mM) with 1 mg/ml concentration of enzyme. The kinetic parameters were calculated by nonlinear regression analysis using computer program Graph Pad PRISM 5 Software. Turnover numbers were calculated on the basis of one active site per 37.5 KDa subunit by SDS-PAGE.

6.2.10. Deactivation studies

Experiments at different combinations of pH and temperature were conducted to study the thermostability of purified rL-asp II. The various range of temperature and pH selected to study the deactivation kinetics of rL-asp II were 7.6, 8.6, 9.6 and 32-52°C, 40-62°C, 32-52°C, respectively. The enzyme samples were deactivated at different combinations of pH and temperature (table 6.5.) and the aliquots of deactivated samples were collected at different

intervals of time. All experiments were performed in triplicates and average of the results were mentioned.

6.2.11. Thermodynamic studies

A thermodynamic study was conducted to assess the spontaneity or non-spontaneity, heat of reaction, and irreversibility and reversibility of reaction process [6]. The deactivation of L-asparaginase II is a major constraint in the efficiency of chemotherapy. Hence, a better understanding of deactivation mechanism is mandatory. The deactivation of rL-asp II is assumed to follow first-order kinetics, which is known as single step two-stage theory [11], [12]. The mechanism of two-state enzyme reaction is as follows:

$$E \rightarrow E_d \text{ ---------- (6.1)}$$

The assumption is that, the active enzyme state E directly converts to E_d (inactive enzyme) without forming any significant amount of intermediates. The first-order deactivation kinetics is represented as:

$$\frac{dE}{dt} = -k_d[E] \text{ ---------- (6.2)}$$

Integrating the equation (2) leads to:

$$\alpha = exp(-k_d\, t) \text{ ---------- (6.3)}$$

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

The value of deactivation rate constant k_d can be calculated from the plot of ln(α) *vs* t. The half-life of an enzyme is considered as the time required for the enzyme to loose half of its initial activity, which is as follows:

$$t_{\frac{1}{2}} = \frac{\ln(2)}{k_d} \text{ ---------- (6.4)}$$

Calculation of thermodynamic parameters

The enthalpies (ΔH^*) and entropies (ΔS^*) of deactivated rL-asp II, can be estimated using absolute reaction rates [6], [13]. The deactivation constant is expressed in terms temperature dependency as follows:

$$k_d = \frac{kT}{h} exp\left(\frac{\Delta S^*}{R}\right) exp\left(-\frac{\Delta H^*}{RT}\right) \text{ ---------- (6.5)}$$

or,

$$ln\left(\frac{k_d}{T}\right) = ln\left(\frac{k}{h}\right) + \frac{\Delta S^*}{R} - \left(\frac{\Delta H^*}{R}\right)\frac{1}{T} \text{ ---------- (6.6)}$$

The plot of ln(k_d/T) versus 1/T gives the values of ΔH^* and ΔS^*, which are calculated from slope and intercept respectively. Whereas, ΔG^* was estimated by the following equation:

$$\Delta G^* = \Delta H^* - T\Delta S^* \text{ ---------- (6.7)}$$

The activation energy is calculated from the Arrhenius equation as:

$$k_d = k_0 exp\left(-\frac{E}{RT}\right) \text{ ---------- (6.8)}$$

or,

$$ln(k_d) = ln(k_0) - \left(\frac{E}{R}\right)\frac{1}{T} \text{ ---------- (6.9)}$$

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

6.2.12. Fluorescence spectroscopy

Fluorescence intensity was measured at 25ºC with a Fluoromax-Spex (Jobin et Yvon, France). The samples were excited at 275 nm with excitation and emission slit width 5nm. While emission spectra were recorded from 290 to 340 nm. Baseline corrections were performed with appropriate buffer without protein in all the cases. All the experiments were performed in triplicates and each spectrum was the average of three data sets. One milliliter

of 1 µg/ml of rL-asp II was poured in the cuvette containing phosphate buffer for analysis unless otherwise mentioned [14], [15].

6.2.13. Effect of acrylamide inhibition in potato chips using rL-asp II

Acrylamide was analyzed by high performance liquid chromatography (HPLC) with ultraviolet detection at 200 nm, as described previously Barber et al., and Paleologos and Kontominas [16], [17]. Briefly, 1 g of a sliced potato sample was crushed by a lab mixer homogenizer and was homogenized with 1 mL of distilled water. Then, 2 mL of distilled water was added, and the homogenates were incubated in a water bath at 70 °C with agitation for 30 min. The homogenates were then centrifuged at 10000g for 30 min and were filtered through a 0.45 µm microporous membrane. The filtrates were transferred to a 10.0 mL volumetric flask and diluted till mark with distilled water. Hexane (3 mL) was added to 1 mL of the sample solution to extract long-chain fatty acids. The mixture was shaken thoroughly, and the upper layer was removed with a pipette. The lower aqueous layer was transferred into glass vials for autosampler analysis. Sulfuric acid solution (0.01 M) was used as mobile phase. Samples were then run on an Aminex HPX-87H (300 mm × 7.8 mm) column at a temperature of 30 °C, flow rate of 0.5 mL/min, and with a sample volume of 20 µL, while ultraviolet detection was maintained at 200 nm. All the experiments were carried out in triplicates.

6.2.14. Cytotoxicity assay

The human breast adenocarcinoma cancer cell line (MCF 7), Human tongue squamous cell carcinoma (SAS) and Human cervical adenocarcinoma cell line (HeLa) were obtained from National Centre for Cell Science (Pune, India). The cell lines 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 – antimitotic solution (1,000 IU ml^{-1} penicillin G, 10 mg ml^{-1} streptomycin sulfate, 5 mg ml^{-1} gentamycin, and 25 µg ml^{-1} amphotericin B).

The cell lines at 1 × 10^5 cells per well were treated with different concentrations of rL-asp II from *Bacillus subtilis* WB800N and commercial L-asparaginase and then incubated for 24 h in humidified CO_2 incubator (Healforce) with 5% CO_2 at 37°C. 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 5 to 50 IU ml^{-1} in serum-free DMEM) of commercially available *E.coli* L-asparaginase (Elspar®) and purified L-asparaginase to study the dose dependent response and to calculate the IC_{50} values.

After 24 h of treatment, 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 recorded at 570 nm in a multi-mode microplate reader (infinite m200, Tecan) and the cell viability (%) was calculated by the Equation 6.1 [18] and plot of inhibitory rate was plotted.

$$Viability\ rate\ (\%) = \left[\frac{A}{B}x100\right] \quad \text{---------- (6.10)}$$

Where, A is mean absorbance at 570 nm of test samples and B is mean absorbance of control cells at the same wavelength.

6.3. Results and discussion

6.3.1. IMAC purification of rL-asp II

Table 6.1 shows the purification profile of the rL-asp II from transformed *B. subtilis* WB800N while Fig. 6.1 depicts the SDS-PAGE analysis of 5 µg of purified protein using His-tag column. The yield of purified enzyme was found to be 42.91 IU indicating 86.9 % recovery with purification fold of 3.79. This purified enzyme denoted specific activity of 153.25 IU/mg.

Table 6.1. Purification profile of the rL-asp II from *Bacillus subtilis* WB800N

Step	Enzyme activity (IU/ml)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Yield %
Crude enzyme	49.33	1.22	40.43	1	100
His-tag column	42.91	0.28	153.25	3.79	86.9

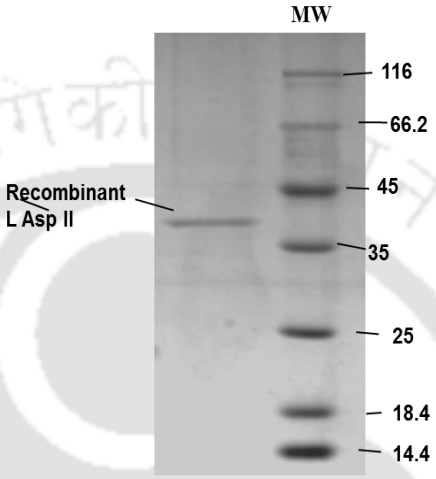


Fig 6.1. SDS-PAGE analysis of 5 µg of purified protein.

6.3.2. Molecular modelling of the L-asparaginase II

The molecular modelling of the L-asparaginase II was performed to analyze the residues involved in active site formation. The L-asparaginase II sequence was modelled using the modeler 9.13 software by taking L-asparaginase crystal structures from *Erwinia* species as template sequence. The PDB ID structures 1HFJ, 1O7 J, 2JK0, 1ZCF were taken as templates. The first two structures (1HFJ and 1O7J) have sequence similarity of 53 % with coverage of 92 % while the latter two structures (2JK0 and 1ZCF) revealed similarity of 52 % with 91 % sequence coverage. Since, the L-asparaginase II protein of *Pectobacterium carotovorum* MTCC 1428 is a homotetramer, the modelling was performed using monomer of L-asparaginase II and generated using modeler 9.13 software. Figure 4.2 depicts the modelled L-asparaginase II monomer that is generated for *Pectobacterium*. The generated model was energy minimized and validated by Ramachandran plot using PROCHECK and

ERRAT plot. The overall quality factor of ERRAT plot was found to be 77.71 indicating the model is acceptable. The Ramachandran plot showed two residues in generously allowed regions and there were no residues of amino acids in disallowed regions. The generated model was submitted to PMDB database (https: // bioinformatics .cineca.it/PMDB/) with an accession number: PM0079916.

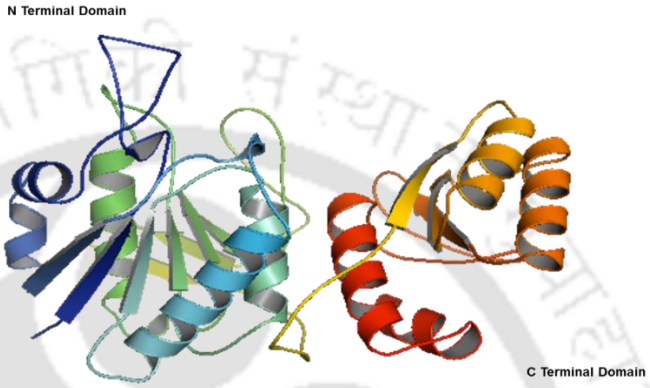


Fig 6.2. Modeled rL-asp II monomer showing N terminal and C terminal domains

The generated model of the rL-asp II in the present study was superimposed onto 1HG1 structures of *Erwinia chrysanthemi* using the PyMOL (Fig. 6.3) to understand the variation of residues at active site. The rmsd value calculated using multiprot was found to be lower than 0.43A° [19] indicating the generated model is acceptable. The structure-based sequence alignment of 1HG1.A, 2JK0.A pdb structures with modelled protein showed sequence similarity of 67.5 and 67.3 % while the percentage of secondary structure similarity was observed to be 88 and 87 %, respectively (Fig. 6.4). Thus, denoting that the active site involved in ligand binding was similar except that the GLU63 ERA was replaced by GLY60, ALA116 ERA was replaced by GLY119 and SER254 ERA was replaced by ALA252 (Fig. 6.5, 6.6) in L-asparaginase II of present study. The absence of glutaminase activity in the rL-asp II may attribute to observed difference in active site amino acid sequence of present study (GLY60) to that of ERA L-asparaginase (GLU63). This is because, the presence of glutamine in the active site that forms the framework for substrate binding introduces additional negative

charge leading to increased interaction of positively charged aamino group of the substrate [20] resulting in more affinity towards glutamine. This was further confirmed by Aghaiypour et al. [8] where the authors showed that GLU63 ERA and SER254 ERA play a significant role in L-glutaminase activity and their replacement by Gln and Asn, respectively, leads to low glutaminase activity.

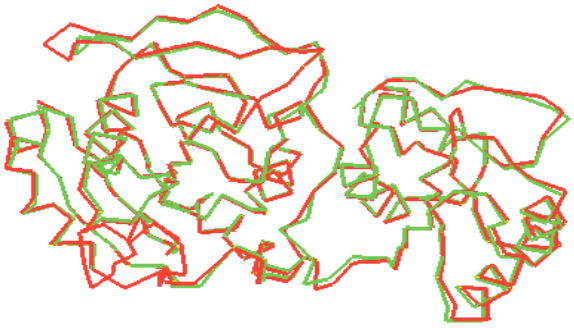


Fig 6.3. Superimpose of 1HG1.A structure on to modelled rL-asp II using pymol. (red color template: modelled rL-asp and green color template: 1HG1.A)

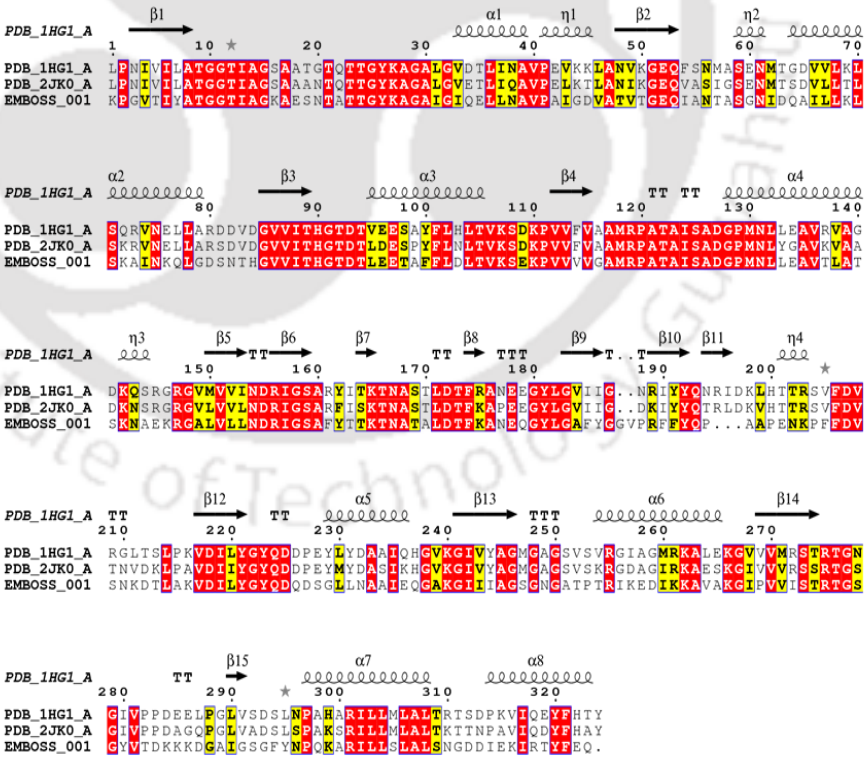


Fig 6.4. Structure-based sequence alignment of structures 1HG1.A, 2JK0.A and modelled L-asp II of Pectobacterium. Red colored regions shows the conserved regions.

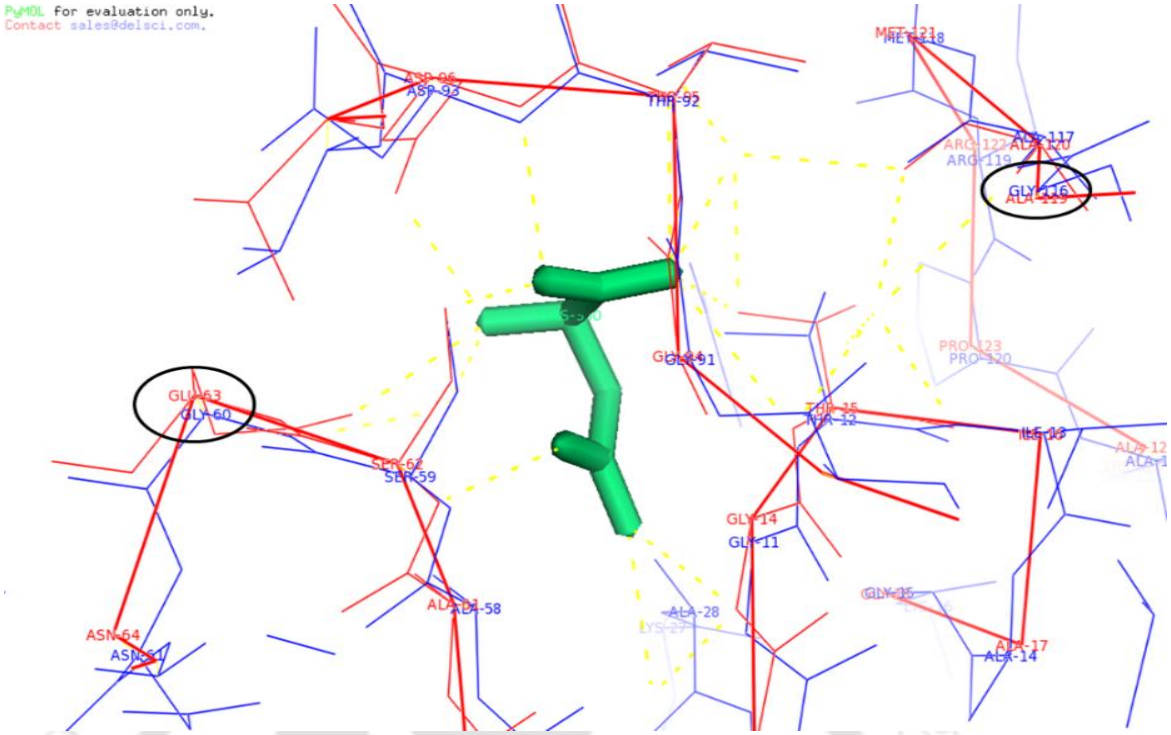


Fig 6.5. Superimposed strucutre of modelled rL-asp II onto 1HG1 pdb structure containing ligand DAsp using pymol

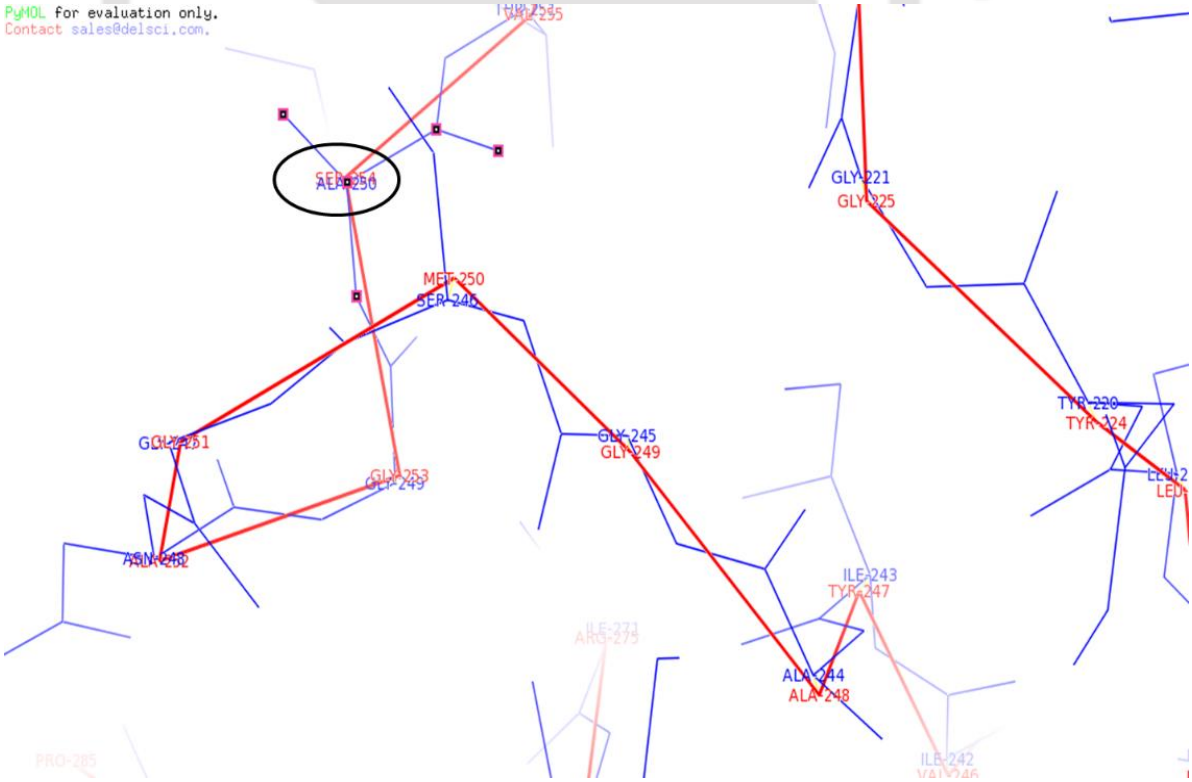


Fig 6.6. Showing superimposed structure of modelled rL-asp II onto 1HG1 pdb structure in which SER 254 was replaced by ALA 252.

6.3.3. Effect of pH on activity and stability of the recombinant enzyme.

The stability of the enzyme at harsh environmental conditions is a significant factor for its ultimate application. The rL-asp II retained more than 60 % activity over the pH 7.5 – 10. The maximum enzyme activity was obtained at pH 8.5 and temperature 40 °C whereas activity decreased more than 50 % when the pH was decreased to 6.5 (Fig 6.7). A total of, 79 % of enzyme activity was retained at pH 7.4, which is the pH of blood in human body. The enzyme is more stable at alkaline pH (8 -10.0) as it retained 90 % of its original activity when incubated up to 24 h (Fig 6.7). Most of the L-asparaginases from *Erwinia sp*. exhibited alkaline pH optima (8.0–9.0) whereas from *E. coli* showed an acidic pH optimum of 5.0–6.0 [21].

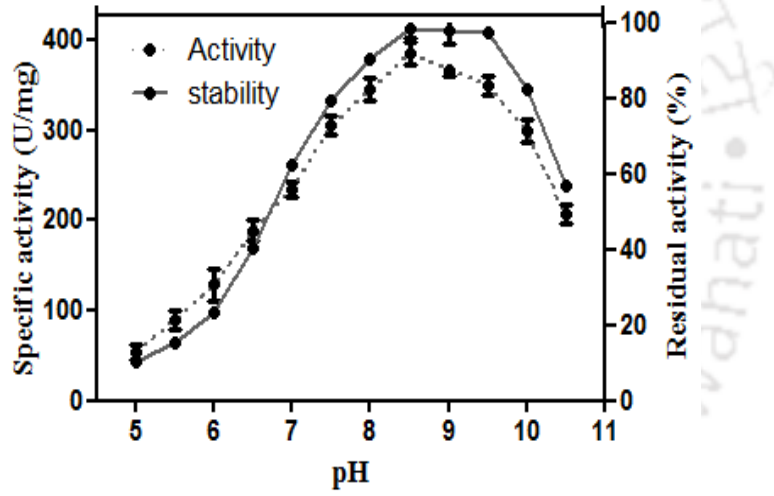


Fig 6.7. Influence of pH on the activity and stability of purified rL-asp II at different pH after incubation for 24 h at 4˚C ± 1˚C

6.3.4. Effect of temperature on recombinant enzyme activity.

The purified rL-asp II showed maximum enzyme activity in the range of 25 to 42 ˚C and its residual activity reduced drastically at 67 ˚C, retaining only 10.48 % of the initial residual activity (Fig 6.8). The results are similar to the native L-asparaginase II purified from *Pectobacterium carotovorum* MTCC 1428 which showed maximum activity at 40 ˚C [11].

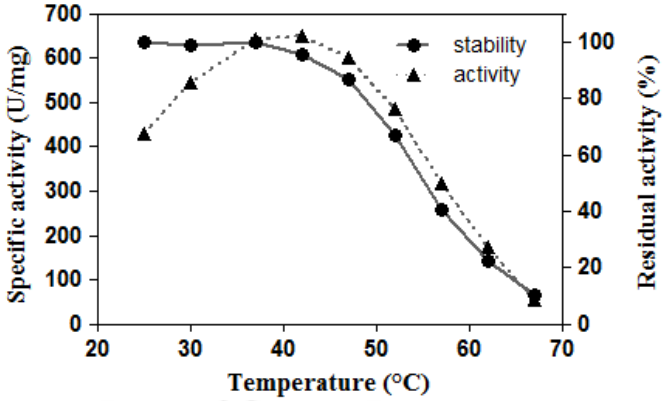


Fig 6.8. Influence of different temperature on activity and stability of enzyme.

6.3.5. Effect of incubation time on activity of purified enzyme

The studies on incubation time showed that the highest enzyme activity was observed at 30 min time interval. Fig 6.9 clearly depicts that the enzyme activity was reduced as the time of incubation is increased from 30 min to 90 min. The results are similar to [22] and [23], who reported that the prolonged incubation of enzyme with substrate lead to decrease in enzyme activity. The decrease in the activity of the enzyme was may be due to the inhibition of the substrate by product formed i.e., product inhibition [24].

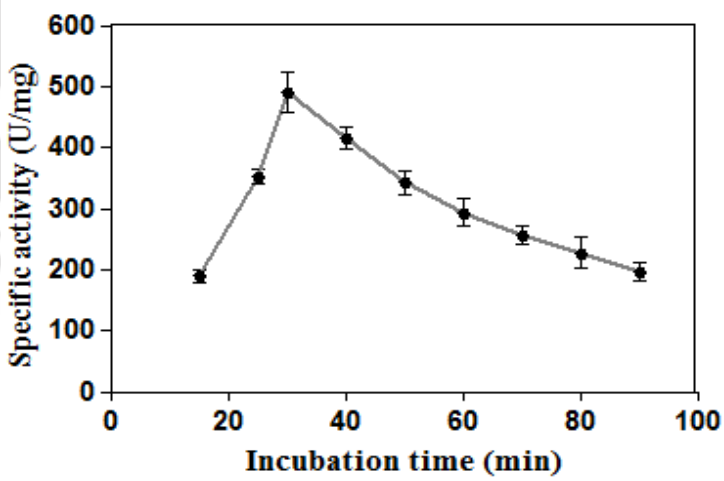


Fig 6.9. Influence of incubation time on enzyme activity

6.3.6. Effect of ionic strength of the buffer

Ionic strength of the buffer possibly alters the enzyme activity by electrostatic interactions of the substrate and enzyme but does not change the three dimensional structure of the enzyme [25]. Keeping in view this effect we studied the effect of different concentration

of Tris-HCL buffer on enzyme activity. The enzyme activity gradually increased from 5 mM and reached maximum at 50 mM concentration of buffer and gradually reduced from 60 mM to 100 mM (Fig 6.10). Minimum and maximum enzyme activity was observed at 5 mM and 50 mM respectively which are similar to the reported values of [22]. Reduction in the activity of the enzyme at lower or higher ionic strength of the buffer may be due to the inability of the enzyme to form non-covalent interaction with the substrate [25].

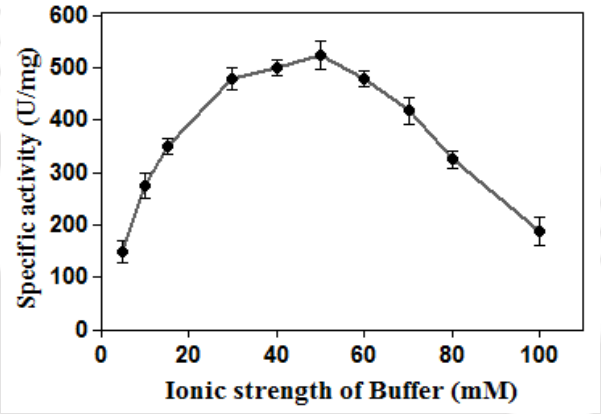


Fig 6.10. Influence of ionic strength of buffer on enzyme activity.

6.3.7. Substrate specificity of rL-asp II

The rL-asp II expressed in *Bacillus subtilis* WB800N has showed more specificity towards L-asparagine but not with glutamine, which is similar to the native L-asparaginase II produced by *Pectobacterium carotovorum* MTCC 1428 [11]. The specificity of substrates towards the rL-asp II were shown in Table 6.2.

Table 6.2. Substrate specificity of rL-asp II from *B. subtilis* WB800N.

Substrate	Conc (mM)	Relative activity(%) [a]
L-asparagine	10	100
DL-asparagine	10	3.2±0.5
D-asparagine	10	2.8±0.3
L-glutamine	10	N.D
L-aspartic acid	10	N.D
D-aspartic acid	10	N.D
DL-aspartic acid	10	N.D
Succinmaic acid	10	1±0.2
L-glutamic acid	10	N.D
L-asparagine-*t*-butyl ester HCl	10	2±0.8
N-α-acetyl-L-asparagine	10	1.6±0.4
BOC-L-asparagine	10	N.D

N.D. Not detected

[a]Residual activity shown in table as mean ± SD (n=3)

6.3.8. Influence of various modulators on rL-asp II activity

The influence of various modulators on the enzyme activity was determined (Table 6.3). Among the various ions tested, Na^{+} and K^{+} was found to enhance the enzyme activity but significant loss of activity was observed with other ions, viz., Ca^{+2}, Hg^{+2}, Mg^{+2}, Cu^{+2}, Fe^{+3}, Ni^{+2}, Co^{+2}, Zn^{+2}, Mn^{+2}. The addition of EDTA as a metal ion chelating agent improved the stability of recombinant enzyme which, explains that the rL-asp II was not a metaloenzyme. The enzyme activity was enhanced by 2-mercaptoethanol and glutathione, and activity was inhibited by thiol group blocking agents viz., p-CMBA and iodoacetamide indicates it contains sulfhydrl group(s) [26]. The enzyme activity was reduced significantly when SDS (22.5 ± 0.18 %) was used, these results are similar to the reports of Huang et al., [27]. The amino acids like L-cystenine and L-histidine did not show any effect on enzyme activity [28]. The addition of urea, lead to the complete loss of enzyme activity. Over all the

results of the rL-asp II are comparable with that of the native L-asparaginase II enzyme from *P. carotovorum* MTCC 1428 [11].

Table 6.3. Influence of different modulators on enzyme activity.

Addition	Conc. (mM)	Relative activity (%)[a]
NaCl	50	116 ± 1.5
KCL	150	127.5 ± 2.1
$MgCl_2$	40	72 ± 2.5
$CaCl_2$	150	30.5 ± 1.25
$MnCl_2$	100	17.5 ± 3.5
$ZnCl_2$	100	15 ± 4.15
$FeCl_3$	100	50.5 ± 2.48
$NiCl_2$	10	59.5 ± 1.8
$CoCl_2$	10	31 ± 0.99
$CuCl_2$	10	13 ± 0.356
$HgCl_2$	10	22 ±0.53
2-mercaptoethanol	0.5	119.5 ±0.25
SDS	2.5	22.5 ± 0.18
Urea	2.5	N.D
EDTA	5	104.5 ±1.25
Iodoacetamide	5	76.5 ± 1.96
L-Cystenine	25	125.5 ± 2.56
L-Histidine	25	112.5 ± 0.26
p-CMBA	0.5	N.D
Glutathione reduced	0.5	100.5 ± 4.56

p-CMBA = *p*-Chloromercuribenzoic acid.

N.D not detected

[a] Residual activity shown in table as mean ±SD (n=3)

6.3.9. Determination of kinetic parameters

The kinetic parameters of the L-asparaginase II was calculated by Lineweaver Burk plot in Fig 6. The K_m and V_{max} of purified L-asparaginase II from *Bacillus subtilis* WB800N using L-asparagine as a substrate was found to be 0.65 mM and 4.018 U μg^{-1} respectively.

The comparative analysis of K_m values of different L-asparaginase enzymes isolated from different sources is given in Table 6.4. The Turnover number (K_{cat}) and specificity constant (K_{cat}/K_m) were calculated to be 2.496 x 10^3 S^{-1} and 3.84x 10^6 M^{-1} S^{-1} respectively.

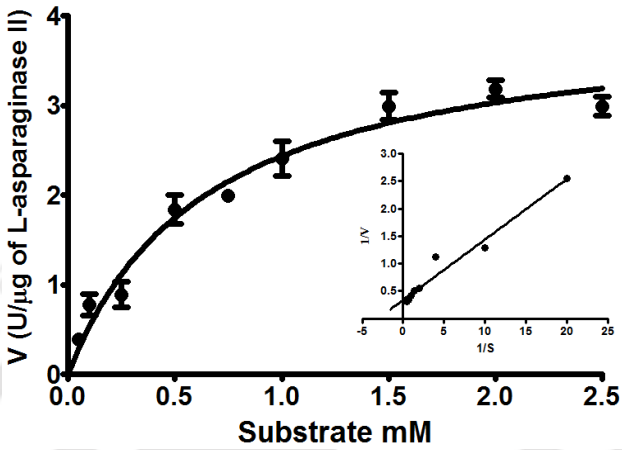


Fig 6.11. Plot showing kinetic parameters of purified L-asparaginase II fitted to Michaelis-Menten equation (R^2= 0.97) and determination of Km and V_{max} by non-linear regression analysis. Reaction velocities (V) *vs.* substrate concentration (S: 0.05-2.5 mM). (Inset) The corresponding Lineweaver-Burk plot for the catalyzed reaction. (V_{max}= 4.018 U µg^{-1} and K_m= 0.65 mM).

Table 6.4. Comparative analysis of Kinetic parameters of L-asparaginases from different sources.

Organisms	K_m	K_{cat}	K_m/K_{cat}	References
Recombinant L-asp II	0.65 mM	2.496 x 10^3 S^{-1}	3.84x 10^6 M^{-1} S^{-1}	This study
Pectobacterium carotovorum MTCC 1428	0.657 mM	2.751 x 10^3 s^{-1}	4.187 x10^6 M^{-1} S^{-1}	[11]
Escherichia coli	3.5 mM	N.R.	N.R.	[29]
Bacillus Licheniforms	0.014 mM	2.68 x10^3 s^{-1}	1.503 x10^6 M^{-1} s^{-1}	[30]
Enterobacteriaceae	0.89 mM	N.R.	N.R.	[31]
Thermococcus kodakarensis KOD1	2.6 mM	694 s^{-1}	N.R.	[32]
Rhizomucor miehei	0.0253±0.0024 mg/ml	676± 26.7 s^{-1}	N.R.	[27]
Erwinia carotovora	0.085 ± 0.02 mM	31.4 ± 1.44 ×10^3 s^{-1}	36.9 ×10^7 M^{-1} s^{-1}	[3]
Yersinia pseudotuberculosis	17 µM,	0.22 C^{-1}	N.R.	[33]
Penicillium brevicompactum NRC 829	1.05 mM	N.R.	N.R.	[34]

N.R. Not Reported.

6.3.10. Thermodynamic analysis

The deactivation rate of purified rL-asp II enzyme is proportional to the rL-asp II enzyme concentration. The deactivation process of rL-asp II was modeled as first-order reaction kinetics and k_d (deactivation rate constant) was calculated. The effect of different temperatures were studied and the results are depicted in fig 6.12a - 6.12c . The minimum k_d value of 0.043 min^{-1} was observed for rL-asp II (Table 6.5). The conditions of pH and temperature at which, minimum k_d was found to be 8.6 and 40^{o}C respectively. These results

were similar to the native L-asparaginase II enzyme purified from *Pectobacterium carotovorum* [7]. The deactivation rate was observed to be faster at pH 7.6 than pH 8.6 or 9.6 for rL-asp II enzyme. Similar results of deactivation rate constant was reported by [12], who observed that this might be due to disulfide bond exchange which occurs near alkaline and neutral conditions. Hegde et al., [35] observed that maximum stability was achieved close to the pI value of enzyme, thus leading to lower thermo-stability below and above the pI value respectively. It is also observed that in naturally occurring enzymes, the conformational stability and enzyme activity cannot be stable at temperature far more than organism's growth conditions [36], [37]. The results observed in this study also explains that the optimum temperature and pH are near to the growth conditions of organism.

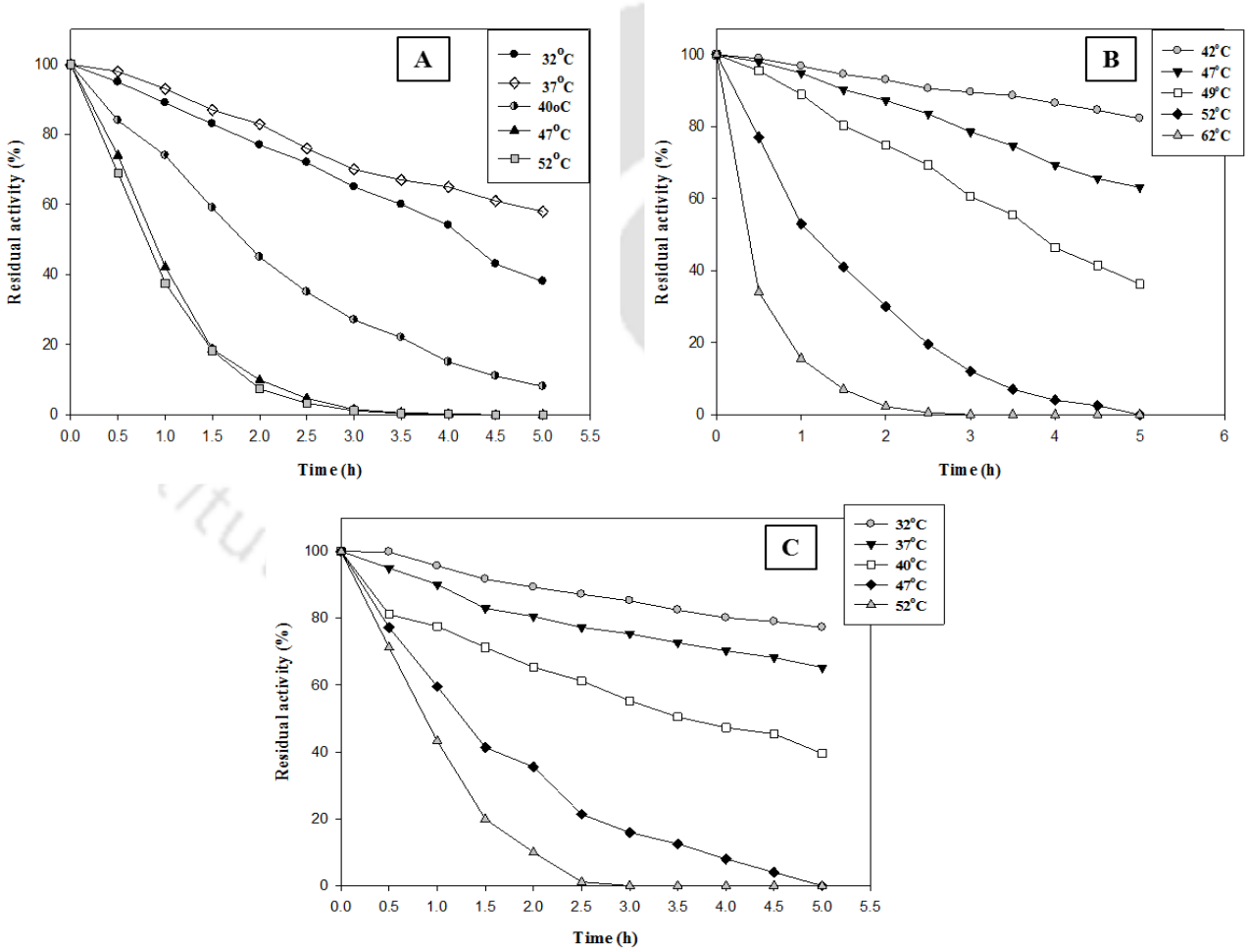


Fig 6.12. Thermal stability of rL-asp II at (a) pH 7.6 (b) pH 8.6 and (c) pH 9.6.

Table 6.5. Effect of temperature at different pH on deactivation constant (k_d) and half life time ($t_{1/2}$) of the purified rL-asp II enzyme

pH	Temp (°C)	K_d (h^{-1})	$t_{1/2}$ (h)
7.6	32	0.1433	4.836008374
	37	0.109	6.357798165
	40	0.52	1.332692308
	47	1.4046	0.493378898
	52	1.5075	0.459701493
8.6	40	0.043	16.11627907
	47	0.21868	3.169014085
	49	0.1832	3.782751092
	52	0.7542	0.918854415
	62	1.9661	0.352474442
9.6	32	0.0534	12.97752809
	37	0.0906	7.649006623
	40	0.1903	3.641618497
	47	0.6357	1.090136857
	52	1.071	0.647058824

R^2 of plot of $\ln(E_d/E)$ verus t is 0.986.

6.3.11. Calculation of thermodynamic parameters

The change in entropy and enthalpy was calculated according to Eqs. (6) and (7) by transition state theory. The estimated thermodynamic parameters of purified rL-asp II from *Bacillus subtilis* WB800N was shown in Table 6.6. The entropy value (ΔS^*) was found to be negative for rL-asp II at 7.6 pH. The negative value of ΔS^* suggests that denaturation may be due to reduced porosity of the reacting enzyme molecule and the arrangement of molecules in solvent [38], [39]. The values of ΔH^* and ΔS^* were majorly influenced by solvent and structural effects. At higher pH, increased values of enthalpy and entropy was observed. This may be attributed to the unfolding of the enzyme during deactivation process and negative

sign is due to formation of charged particles around rL-asp II enzyme. At higher pH values, the increase in ΔS^* implies an increase in number of molecules entering in transition state, which leads to the lower ΔG^* values. The table 6.6. represents the ΔG^* values calculated from Eq. (7).

Table 6.6. Calculated thermodynamic parameters during the thermal deactivation of the purified rL-asp II from *Bacillus subtilis* WB800N[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	75.54	-86.63	117.48	1.42×10^{19}	101.93-103.66[b]
8.6	151.22	146.5	153.91	2.35×10^{28}	105.34-102.12[c]
9.6	128.17	89.69	130.8	1.19×10^{21}	100.81-99.01[d]

[a]R^2 of plot of ln(k_d/T) verus 1/T is 0.976; R^2 of plot of ln(k_d) versus 1/T is 0.982; [b]The temperature range is 32-52°C; [c]The temperature range is 40-62°C; [d]The temperature range is 32-52°C.

When the pH increases, the entropy and enthalpy values tends to decrease, this may be due to the compression of stable three dimensional structure, which results in reduction of residual activity. In order to understand specificity and mechanism of rL-asp II, the temperature dependency of catalytic activity was investigated. The temperature dependency deactivation rate constant was a first -order reaction, calculated from Arrhenius Eq. (Eq. (8)). The E (activation energy) and k_0 (frequency factor) were calculated from Eq. (8) and estimated parameters are shown in Table 6.6. It was observed that deactivation energy was maximum at optimum pH for rL-asp II and tends to decrease as the pH increases. Kumar et al., [7] and Naidu and Panda [12] also observed similar results for thermal deactivation of L-asparaginase from *Pectobacterium carotovorum* and pectolytic enzymes from *Aspergillus niger*. For rL-asp II, at higher temperature the deactivation energy increases implying that rL-asp II requires more energy to deactivate itself. This is in accordance with the results, where

rL-asp II is more stable at 8.6 pH with lower temperature rather than 7.6 and 9.6 pH at higher temperature.

6.3.12. Conformational change of rL-asp II during thermal deactivation

Tyrosine proved to be an important intrinsic fluorescent probe (amino acid), which is used to estimate the nature of the microenvironment of the residue. From the fluorescence spectra (Fig. 6.13) a red shift of $\lambda_{max} \approx 10$ nm, and decrease in intensity was observed with an increase in temperature for all combinations of pH levels. At room temperature and 40ºC there was no change in spectra, which suggests that the protein retains its native form at these temperatures, whereas further increase in temperature results in a gradual decrease in intensity and red shift in wavelength. This observation suggests that the enzyme retains its secondary structure at 40ºC but loses the same when incubated at 47ºC and above. This trend is similar for all four pH levels. Previously, Upadhyay et al., [2] had also observed the loss of helix structure upon unfolding of asparaginase enzyme at higher temperatures and in the presence of guanidine hydrochloride.

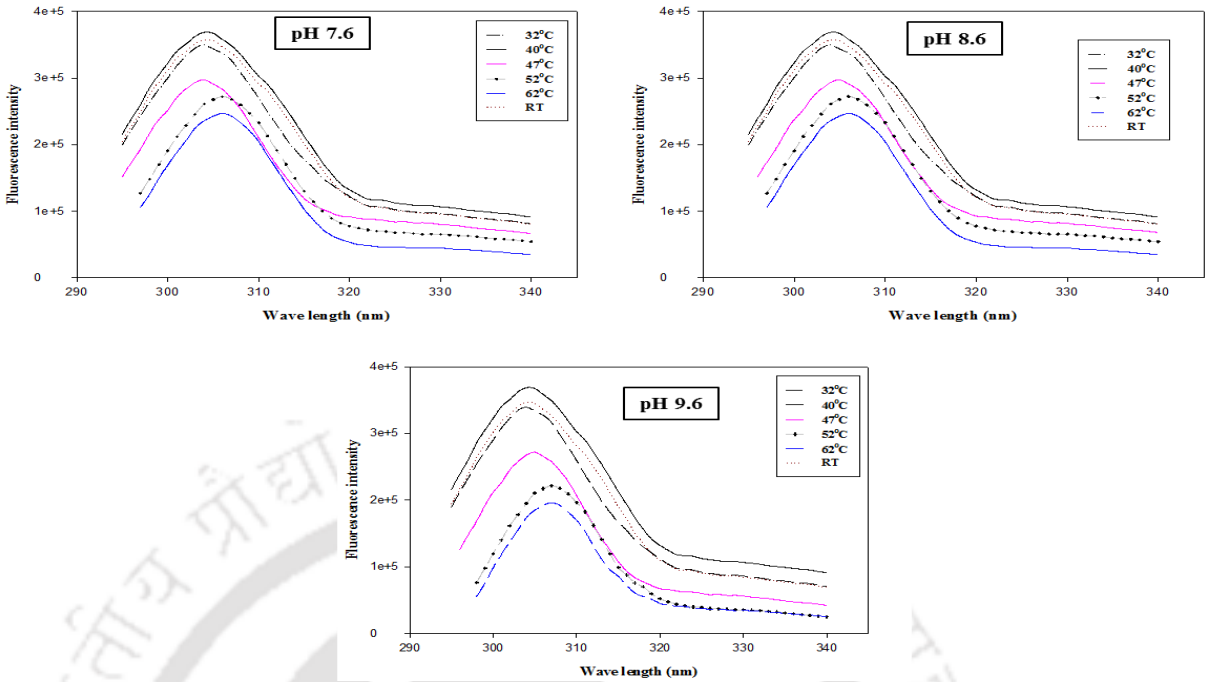


Fig 6.13. The fluorescence spectra of the rL-asp II enzyme at different pH and temperature.

6.3.13. Application of rL-asp II in potato samples

Pretreatment of potato chips using rL-asp II resulted in significant acrylamide decrease. The acrylamide content of raw potato chips bought from local market was estimated using different types of oils and different concentration of enzyme units. It was observed that the acrylamide content of potato chips using mustard oil reduced up to 95 % by using 10 IU/ml of rL-asp II per gm of potato chips. The effect of rL-asp II on acrylamide reduction is depicted in Fig. 6.14.

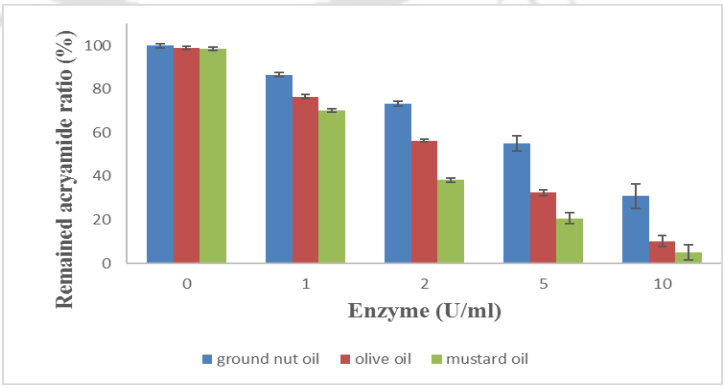


Fig 6.14. Acrylamide levels in potato chips as a function of L-asparaginase (rL-asp II) from *Bacillus subtilis* WB800N. For estimation, potatoes were sliced and incubated at

37ºC for 20 min in solutions with different amount of rL-asp II (0–10 IU/mL). After that, the raw or pre-treated potato chips were deep-fried at 170ºC for 5 min.

6.3.14. *In vitro* cytotoxicity of rL-asp II

The effect of rL-asparaginase II from *Bacillus subtilis* WB800N was tested against three different cell lines. The rL-asp II and commercial L-asparaginase significantly inhibited the proliferation of MCF 7, SAS and HeLa cells in a dose dependent manner (Fig 6.15). It was observed that rL-asp II from WB800N and commercial L-asparaginase effectively inhibited the cell growth. The quantitative estimation of L-asparaginase activity against the selected cell lines was done by a simple, rapid and efficient spectrophotometry based MTT assay [40]. The water soluble yellow colored MTT was reduced to a water insoluble purple colored formazan complex by mitochondrial reductases that were active only in living cells. The complex was subsequently dissolved in DMSO and the absorbance of the colored solution was read in a spectrophotometer at 570 nm. The intensity of purple color complex can be directly correlated to the number of viable cells. The viability (%) was estimated using Eq. 6.1 and a dose dependent response curve was plotted using GraphPad Prism 5.04. The IC_{50} values were calculated, for rL-asp II and commercial L-asparaginase were found to be 71.90 and 58.78C for HeLa cell lines, 68.69, 61.86 for SAS cell lines and 57.335, 45.13 for MCF 7 cell lines respectively. It was observed that the purified rL-asp II was active against all the three cancer cell lines tested. The data obtained were in accordance with Asselin et al., [41] who studied cell death both *in vitro* and *in vivo* in ALL patients undergoing treatment with L-asparaginase as a single agent. The L-asparaginase is considered effective in chemotherapy when the IC_{50} value was less than 15000 IU ml^{-1} [42]. The *in vitro* cytotoxicity results of purified rL-asp II from *Bacillus subtilis* WB800N against these three cell lines were comparable with the recently reported IC_{50} values of L-asparaginase from *E. coli*, *Erwinia carotovora*, *Bacillus circulans* and *Helicobacter pylori* [42]–[44]. Pritsa and Kyriakidis [45]

studied the anti-proliferative 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 neuro-ectodermal tumor) cell lines.

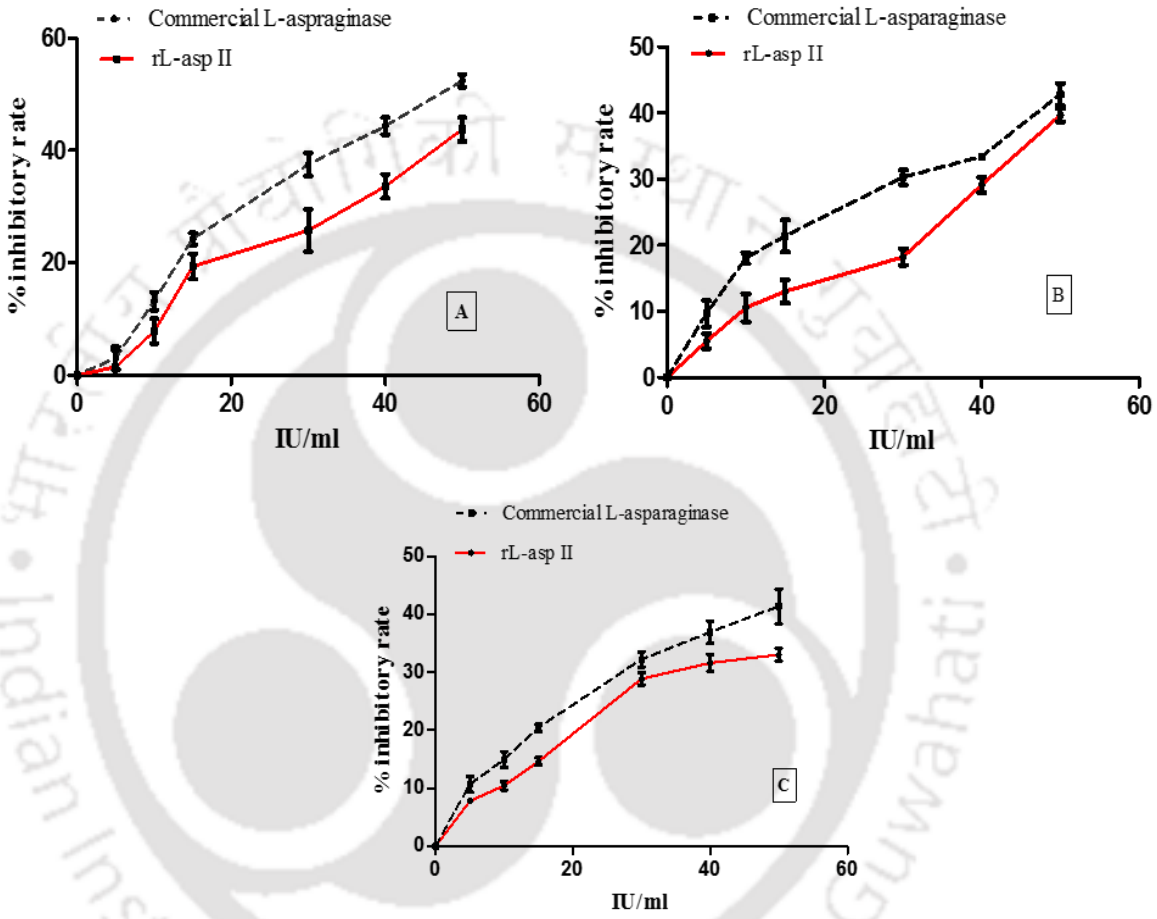


Fig 6.15. Reduced proliferation of **A:** MCF 7 **B:** SAS **C:** HeLa cell lines induced by purified rL-asp II and commercial L-asparaginase. The cell lines were treated with purified rL-asp II and commercial L-asparaginase (0- 50 IU/ml) and evaluated using MTT assay. The data represents the L-asparaginase induced decrease in the percentage of viable cells compared to the respective valvues observed in controls. The results were mean of the data, with error bars representing the standard deviation of triplicates ($P<0.05$).

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Chapter VII

Conclusion

Conclusion

- A 1011 bp *ans* B2 gene encoding rL-asp II is cloned in *Bacillus subtilis* WB800N under the control of *groE* promoter with lac operator in pHT43 vector.
- The process parameters such as temperature, agitation, cell density, IPTG concentration, consecutive induction of IPTG were optimized and it resulted in 105 IU/ml of rL-asp II.
- The localization studies revealed that > 90% rL-asp II is secreted extracellularly.
- Sucrose and NH4Cl were found to be potent carbon and nitrogen sources for the expression of rL-asp II. Plackett-Burman screening revealed that components such as sucrose, NH_4Cl, NaH_2PO_4 and $MgSO_4.7H_2O$ played a significant role on rL-asp II production.
- Further, RSM and ANN-GA resulted in a maximum production of 310.20 IU/ml of rL-asp II and in batch reactor studies 525.98 IU/ml of rL-asp II was produced.
- In substrate inhibition studies maximum growth rate was observed with 40 g/L sucrose. The Aiba model was found to be the best fit among the various models tested.
- The rL-asp II expressed was purified using Ni–NTA affinity chromatography resulted in 86.9% recovery of protein and the expression was confirmed using western blotting technique.
- The molecular modelling of rL-asp II was analyzed using modeler software and the active sites involved in the protein were estimated.
- The rL-asp II stability studies were performed using different pH, incubation time, ionic strength of the buffer, substrate specificity of the enzyme and effect of modulators.

- The kinetic parameters of rL-asp II was calculated. The K_m and V_{max} of purified rL-asp II using L-asparagine as a substrate was found to be 0.65 mM and 4.018 U µg-1 respectively.
- The thermodynamic paramters of rL-asp II were analysed and (ΔS^*) and ΔG^* were calculated.
- The structural stability studies with fluorescence spectroscopy revealed that the rL-asp II was stable at 40 °C and pH 8.6.
- The acryalamide inhibition using rL-asp II in potato chips revealed that using mustard oil the formation of acrylamide was reduced up to 95 % by using 10 IU/ml of rL-asp II per gm of potato chips.
- The cytotoxicity analysis on different cell lines revealed that around 50 – 70 IU of rL-asp II is required to inhibit the cell growth more that 50 %.

Future scope of work

- Fed batch studies for enhanced production of rL-asp II from *Bacillus subtilis* WB800N.
- Purification of rL-asp II from crude broth using novel downstream processess such as., Reverse micellar and Aqueos two phase seperation.
- *In vivo* studies of purified recombinant L-asparaginase II from *Bacillus subtilis* WB800N to established its full potential as a chemotherapeutic agent.

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 milliQ 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 in triplicates and absorbance of the standard samples was measured at 425 nm against the appropriate blank. 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 3.2.4.1. 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 (IU) is defined as the amount of enzyme that liberates 1 µmole of ammonia per min at 37°C (1 unit OD at 425 nm = 4.382 µmol of ammonia).

Calculation for L-asparaginase activity

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

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

Where, A_c = amount of ammonia released during reaction in µmol (test sample absorbance at 425 nm (Abs_{425}) × 4.382 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 simplifies into Eq. A.2

L-asparaginase activity (IU ml^{-1}) = Abs_{425} × 16.067 (A.2)

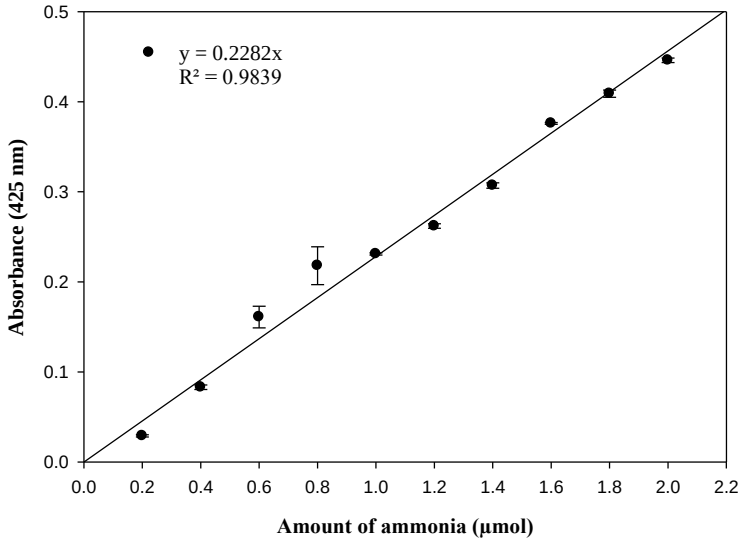

Fig A.1. Standard curve drawn between known amount of ammonia and the corresponding OD measured at 425 nm.

A.2. Sample calculation for the estimation of Sucrose

Preparation of standard plot for Sucrose

Stock solution of 1 mg ml^{-1} sucrose was prepared in milliQ water. The stock solution was appropriately diluted with same water to get standard solutions of various concentrations of sucrose (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 sucrose was estimated using high-pressure liquid chromatography, (HPLC) (Agilent 1220 Infiniti HPLC, USA) equipped with SUPELCOGEL Ca, 300 mm × 7.8 mm I.D., 9 µm particle as described for test samples in section 4.2.8. The points were fitted with a linear regression model using Microsoft Excel® software. (Peak area 1 (mAU.min) at R_t 11.9 = 4.4451× E^{-06} mg ml^{-1} of sucrose)

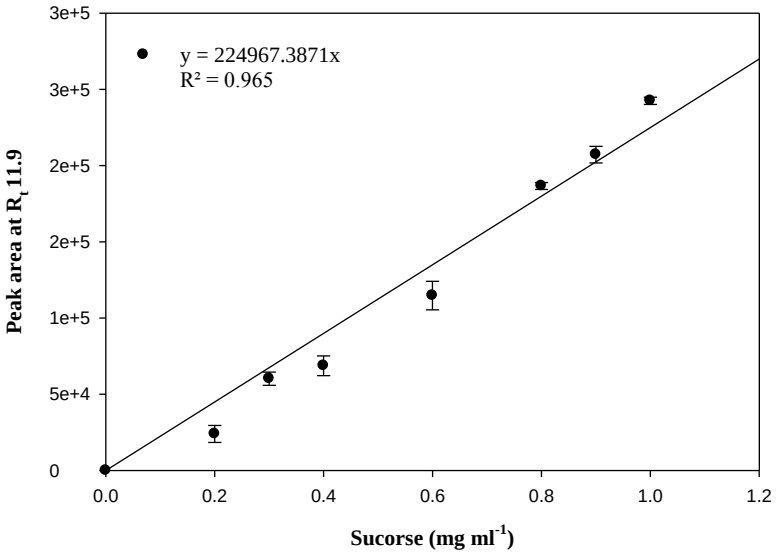

Fig A.2. Standard curve drawn between known sucrose concentration and the HPLC measured peak area

Calculation for sucrose concentration

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

Sucrose concentration (mg ml^{-1}) = Peak area at R_t 11.9 × 4.4451× E^{-06} ×dilution factor (A.3)

Where, Peak area at R_t 11.9 = test sample peak area at retention time (R_t) 11.9 min.

A.3. Sample calculation for the estimation of protein

Stock solution of 0.1 mg ml^{-1} protein (BSA) was prepared in milliQ 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 595 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 Braford method as described in section 3.2.4.2. Then concentration of protein (mg ml^{-1}) in the test sample was calculated with the help of data at OD 595 nm and the slope of standard curve (1 unit OD at 595 nm = 0.305 mg ml^{-1} of protein).

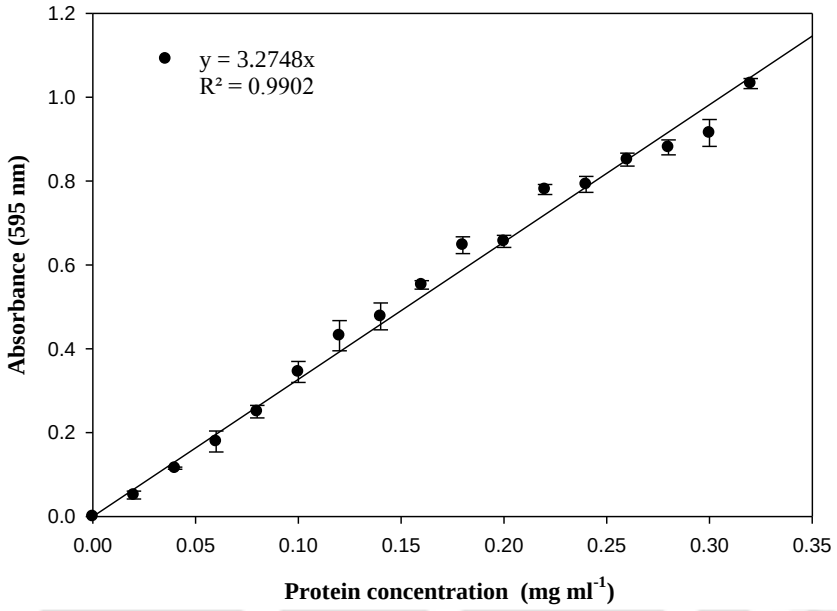

Fig A.3. Standard curve drawn between known protein concentration and the optical density measured at 595 nm.

Calculation for protein concentration

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

Protein concentration (mg ml^{-1}) = $Abs_{595nm} \times 0.305$ (A.4)

Where, Abs_{595nm} = test sample absorbance at 595 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 4.2.8. Different dilution of cell samples were used for measuring cell OD (~0.1-2.0) 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 = 0.4501 g l^{-1} DCW).

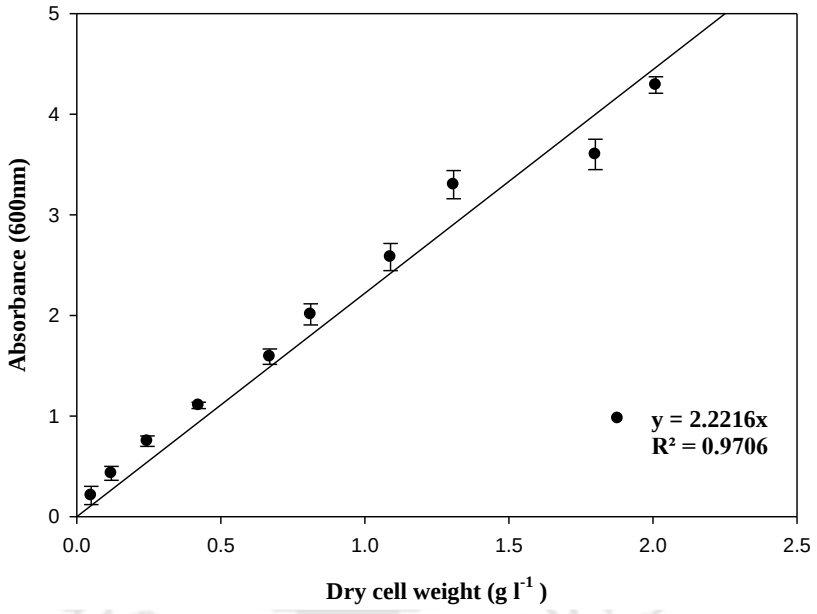

Fig A.4. Standard curve drawn between cell dry weight of *Bacillus subtilis* WB800N and the optical density measured at 600 nm.

Calculation for DCW

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

Dry cell weight concentration (g l^{-1}) = Abs_{600} × 0.4501 (A.5)

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

A.5. L-asparaginase II gene sequence from *Pectobacterium carotovorum* cloned in *Bacillus subtilis* WB800N [GenBank accession no: JN638885]

>JN638885.1 Pectobacterium carotovorum culture-collection MTCC:1428 L-asparaginase II gene, complete cds

ATGCAACTCTCATTTATCGCCCGCACCATCACCGCCGCTTGCCTGATGCTGTCGTCTCATGCGCTGCTAG
CCGATGACGCCAAACCTGGCGTCACTATCTACGCTACCGGCGGCACCATTGCTGGAAAGGCAGAATCCAA
TACGGCCACGACAGGTTATAAGGCGGGCGCTATCGGCATCCAGGAACTGTTGAACGCCGTTCCGGCTATT
GGCGATGTCGCTACGGTGACAGGCGAGCAAATCGCCAATACCGCCAGCGGAAATATCGATCAAGCGATTC
TGTTAAAGCTATCCAAAGCGATTAACAAACAGCTAGGCGATTCAAATACGCACGGCGTCGTCATTACTCA
CGGCACCGATACGTTGGAAGAGACCGCGTTCTTTTTGGATCTGACGGTAAAAAGCGAGAAACCAGTGGTT
GTCGTCGGCGCAATGCGTCCCGCCACCGCCATCAGCGCGGATGGTCCTATGAACCTGCTGGAAGCCGTCA
CGCTGGCAACCAGTAAGAACGCGGAAAAACGCGGCGCGCTGGTGTTGCTGAACGATCGCATCGGCTCCGC
ATTCTACACCACCAAAACCAATGCCACGGCGCTGGACACGTTCAAAGCCAATGAACAAGGCTATCTGGGC
GCATTCTACGGCGGCGTTCCGCGCTTCTTTTATCAGCCTGCTGCCCCCGAAAACAAACCGTTCTTTGACG
TAAGCAATAAGGACACGCTGGCGAAGGTCGACATTCTCTACGGCTATCAGGATCAAGACAGTGGTCTGCT
GAATGCCGCCATTGAACAGGGCGCAAAAGGCATCATTATCGCGGGTAGCGGTAACGGTGCGACGCCCACG
CGTATCAAAGAAGACATCAAGAAAGCGGTAGCCAAAGGCATTCCGGTGGTGATCAGTACGCGCACCGGCA
GCGGCTATGTTACCGATAAGAAGAAAGACGGTGCGATCGGCAGCGGATTCTACAACCCACAAAAAGCACG
CATTCTGCTTTCGCTAGCGCTGTCTAACGGCGACGACATCGAGAAAATCCGTACCTATTTCGAGCAGTAA

Fig. A.5. L-asparaginase II sequence of the isolated bacterial strain *Pectobacterium carotovorum* (GenBank accession no. JN638885).

LIST OF PUBLICATIONS

Published in Referred International Journals

Sushma Chityala, Ashish A Prabhu and Veeranki Venkata Dasu (2017) Media engineering and Kinetic modelling of *Bacillus subtilis* WB800N for rL-asp II production and it's in vitro effect on acrylamide formation. *Korean journal of chemical engineering*. 1-15. Doi:10.1007/s11814-017-0211-1.

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Publications from outside PhD work

Ashish A Prabhu, **Sushma Chityala**, Yachna Garg, V Venkata Dasu **(2016).** Reverse micellar extraction of papain with cationic detergent based system: An optimization approach. *Preparative Biochemistry and Biotechnology*. DOI:10.1080/10826068.2016.1201685.

Ashish A Prabhu, Yachna Garg, **Sushma Chityala**, V. V. Dasu **(2016).** Improvement of phytonutrients and antioxidant properties of wheat bran by Yeast fermentation. *Current Nutrition Food science*. Vol: 12, issue: 4,pg 249-255.

Book chapter

Sushma Chityala, Vidyadhar Nandana, Dharanidaran Jayachandran, Ashish Prabhu, Venkata Dasu Veeranki, **(2017).** "Biotechnology of commercial microbial products: A focus on plant secondary metabolites" Springer publications. Doi: 10.1007/978-981-10-6593-4_21

Ashish Prabhu, **Sushma Chityala**, Dharanidaran Jayachandran, Venkata Dasu Veeranki (2017). "Rhizoremediation: A potent bioremediation strategy" Springer publications. Doi: 10.1007/978-981-10-6593-4_17

Communicated/Under Review in Referred International Journals

Sushma Chityala and Venkata Dasu V (2017). Mathematical modeling of Bio-kinetic growth models for rL-asp II producing *Bacillus subtilis* WB800N *3 Biotech.* (Submitted)

Sushma Chityala, Venkata Dasu V (2017). Purification and characterization of novel glutaminase free recombinant L-asparaginase II from *Bacillus subtilis* WB800N. (Under preparation).

Published in National/International Conference Proceedings

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Sushma Chityala, Ashish A Prabhu, Yachna Garg, Veeranki Venkata Dasu, (2015). Screening of carbon, nitrogen sources and media components for the production of recombinant glutaminase free L-asparaginase II from eight protease free *Bacillus subtilis* WB800N. CHEMCON 2015, 27-30 Dec.

Sushma Chityala, Dharanidaran Jayachandran and Venkata Dasu Veeranki, (2016). "Purification of recombinant Novel Glutaminase free L-Asparaginase II Using Reverse Micellar Extraction for Usage in Food Processing Industries", "International Conference on

New Approaches in Agriculture, Food and Environmental Sciences", Dec, Andhra Pradesh, India

Dharanidaran Jayachandran, **Sushma Chityala**, Ashish Prabhu, Venkata Dasu Veeranki, (2017) "Modelling and Kinetics of Reverse Micellar Extraction for L-Asparaginase system", "Reflux", Mar. 2017, IIT Guwahati, India.

Vitae

The author was born on August 29th 1988 in Warangal, Telangana, India. She passed the secondary school examination conducted by Andhra Pradesh secondary Education Examination Board, Warangal, in 2003. She qualified the higher secondary school Examination conducted by Andhra Pradesh Board of Examination, Vijayawada, in 2005. She completed her Bachelor of Engineering in Biotechnology from Vinayaka Missions Kirupanada Variyar Engineering College, Salem, Tamilnadu, in 2010. She did her Master of Technology in Biotechnology from National Institute of Technology Durgapur, West Bengal, in 2012.

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