Production and Optimization of L-Glutaminase from Endophytic Fungi

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By

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Dedicated to my
Late Mother

DECLARATION

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Sadaf Sajjad

Certificate

This thesis submitted by ***Sadaf Sajjad*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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List of Contents

Sr. #	Title	Page #
1.	List of Tables	ii
2.	List of Figures	iii
3.	List of Appendices	iv
4.	List of Abbreviations	v
5.	Acknowledgements	vi
6.	Abstract	vii
7.	Introduction	1
8.	Aims and Objectives	7
9.	Literature Review	8
10.	Material and Methods	26
11.	Results	33
12.	Discussion	47
13.	Conclusion	52
14.	Future Prospect	53
15.	References	54
16.	Appendices	66

List of Tables

No.	Title	Pg. No.
2.1	Therapeutic uses of some microbial enzyme	16
4.1	Qualitative test for L-glutaminase production by fungal isolates	37
4.2	Placket-Burman design matrix showing coding values for each factor and their corresponding response	40
4.3	ANOVA table [Partial sum of squares-Type III] of critical medium components identified from Plackett-Burman design for L-glutaminase production based on enzyme activity (IU)	44
4.4	LD^{50} of precipitated enzyme against brine shrimps	46

List of Figures

No.	Title	Pg. No.
1.1	Mechanism of action of L-glutaminase against tumor	5
1.2	Interconversion of L-glutamine and glutamate	5
1.3	Role of glutamine catabolism in overall metabolic process	6
2.1	Applications of therapeutic enzymes	16
4.1	Quantitative Estimation of Four strains NFW1, NFW6, NFW11, NFW16	35
4.2	Plate Morphology of *Epicoccum sp.* (NFW1)	36
4.3	Effect of Temperature on Production of L-glutaminase from *Epicoccum sp.* NFW1	36
4.4	Effect of pH on Production of L-glutaminase from *Epicoccum sp.* NFW1	37
4.5	Production of L-glutaminase on different days from *Epicocum sp.* NFW1	37
4.6	Effect of varying media composition on enzyme activity[IU]	41
4.7	Perturbation chart showing effects of most vital components on Enzyme Activity as function of deviation from reference point	41
4.8	Overall effect of each factor on response (enzyme activity IU)	42
4.9	Percentage contribution of each factor to response	42
4.11	Pareto chart showing t-value of effect by important factors, generated by Plackett-Burman design	43
4.12	Interactive effects of important factors on enzyme activity (IU)	45

List of Appendices

No.	Title	Pg. No.
Appendix 3	Quantitative Estimation of Four strains NFW1, NFW6, NFW11, NFW16	67
Appendix 4	Effect of Temperature on Production of L-glutaminase from *Epicoccum sp.* NFW1	67
Appendix 5	Effect of pH on Production of L-glutaminase from *Epicoccum sp.* NFW1	67
Appendix 6	Production of L-glutaminase on different days from *Epicocum sp.* NFW1	67
Appendix 7	Effect of varying media composition on enzyme activity[IU]	68
Appendix 8	Overall effect of each factor on response (enzyme activity IU)	68
Appendix 9	Percentage contribution of each factor to response	69

List of Abbreviations

ALL	Acute Lymphoblastic Leukemia
°C	Centigrade/Degree Celsius
Con.	Concentration
hrs	Hours
i.e	That is
IU	International unit
L	Liter
$lb/inch^2$	Pounds per square inch
M	Molar solution
min	Minute
mM	Milli molar
mCD	Modified czapek dox
OD	Optical density
PDA	Potato dextrose agar
rpm	Revolution per minute
Sp.	Species
U/mg	Units per milligram

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Abstract

L-Glutaminase can be effectively used for treating leukemia and other tumors. It is also used for the treatment of HIV. Besides its therapeutic purposes, it is well known for use in food and chemical industry as well. Present work mainly focuses on production and optimization of L-glutaminase from selected endophytic fungal strain. Six endophytic fungal strains were screened for L-glutaminase production using modified Czapek Dox's agar containing L-glutamine and phenol red. The plate assay was based on pink colour formation around fungal colony. Four strains found positive for L-glutaminase production were further screened by quantitative analysis and *Epicoccum sp.* NFW1 was found to exhibit the highest activity. This strain was further used to optimize fermentation parameters by shake flask fermentation, such as temperature, pH and incubation time. Results showed that 30°C temperature, pH 6 and 5 days of incubation were found to be optimum for production of L-glutaminase. Nutritional factors were optimized using statistical tool, Plackett-Burman design. Out of eleven nutritional factors nutritional factors potassium chloride, molasses, arginine and L-glutamine were found to be contributing in the increased production of L-glutaminase from *Epicoccum sp.* NFW1. Further under optimum conditions L-glutaminase production by epicoccum sp. NFW1 was carried out. Enzyme was purified by acetone precipitation and 56.24 units/mg of specific activity was recorded in the precipitate.

Introduction

Enzymes are the catalysts of biochemical reactions. They are biological macromolecules which catalyze all those interconvertible biochemical reactions which are necessary for life. Enzymes and their use have been known to mankind for a very long time. In ancient Egypt use of enzyme was in practice for preserving food and food related items. Cheese manufacturing which goes back to 400 BC has always involved enzymes (Neelam et al., 2013).

At present, enzyme industry is one of the rapidly growing industries. In 2010 global market for industrial enzymes was reported to be at 3.3 billion US dollars which is predicted to reach at 4 billion US dollars by 2015 (Neelam et al., 2013). There is a great demand of enzymes for use in food and beverage industry, textile and leather, cleaning products and pharmaceuticals.

All of the enzymes currently are either produced by microorganisms or by adding enzyme containing preparations such as papaya. Enzymes produced by microbes are preferred over other sources such as plants and animals due to several beneficial aspects. These include economic and efficient production, consistency and manipulation of process and strain improvement and optimization. In addition microbial enzymes are more stable than enzymes obtained from other sources. But only 2-3% of microbes have been explored as sources of enzymes. (Wiseman., 1978). These microbes include some species of fungi as well. More than 25% of all industrial enzymes are being produced by *Aspergillus*. Other important fungi include *Trichoderma, Penicillium, Rhizopus* and *Humicola*. These contribute to 20% of the industrial enzyme production. Another 14% of commercial enzymes come from representatives of 20 other genera each of which account for only one or two products (Lars and Hans., 2010).

Enzymes of fungal origin are particularly well suited for industrial application for several reasons. In order to utilize complex energy sources such as starch, pectin, lignin and waxes, they secrete extracellular enzymes. This secretion makes the isolation of enzymes relatively simpler on industrial scale. In addition, extracellular enzymes are naturally adapted to work under harsh conditions. Which makes them best candidate for industrial applications.

Endophytic fungi have emerged as valuable reservoir of bioactive metabolites and enzymes. Ever since the discovery of the world's first most expensive anticancer drug, Taxol, several other compounds have been reported from endophytic fungi from all over the world. A large number of compounds has been reported from endophytic fungi including several enzymes.

Enzymes reported from endophytic fungi include amylases, lipases, cellulases, laccases, glucosidases, pectinases, lignases and catalases (Demain, 1971, Akhter et al., 2011). Since the reports of endophytic fungi producing several enzymes have come up, researchers are pursuing the isolation of industrially important enzymes from endophytic fungi as well as optimization of production in order to get maximum yield. Different fermentation strategies are being tested and also efforts in terms of strain improvement are being made (Sabir et al., 2007).

Besides the use of enzymes in industry, enzymes are also equally important in therapeutics. Therapeutic enzymes are applied in a wide variety of specific fields such as oncolysis, thrombolysis or anticoagulants and as supplements for metabolic deficiencies. For example proteolytic enzymes are used as anti-inflammatory agent, ribonucleases as antiviral agents and streptokinases as anticoagulants. One important enzyme which has gained the attention of researchers is L-glutaminase.

L-Glutaminase is an enzyme that produces L-glutamate and ammonia by hydrolysis of amido group of L-glutamine (Fig.1.2) (Roberts., 1970). This enzyme besides deaminating L-glutamine also performs proteolysis by hydrolysis of the peptide bonds in the interior side of the proteins. L-Glutaminase is found in wide range of plants, animals and microorganisms that include bacteria, yeasts, moulds and fungi..

Physical properties of this enzyme vary greatly with respect to the microorganism from which it is isolated. The pH and temperature tolerance of glutaminase from various microorganisms differs greatly, while optimal activities of glutaminase have been reported to be at a broad range of (5-9). Glutaminase also showed a wide variation in terms of thermostability. Enzyme isolated from different microorganisms showed different behaviour towards temperature. Stability range extended from 37 to 70°C.

Sodium chloride was found to affect the activity of glutaminase from both fungi and bacteria of terrestrial origin. Glutaminase from *E.coli, P .fluorescence, Cryptococcus albidus* and *A.sojae* showed only 65, 75, 65 and 6% respectively of their original activity in presence of 18% NaCl (Yokotsuka., 1987). Similar results were obtained with glutaminase from *Candida utilis, Torulopsis candida* and *A.oryzae* (Nakadai et al., 1989; Yano *et al.,* 1988). Salt tolerant glutaminase have been observed in *Cryptococcus albidus* and *Bacillus subtilis* (Iwasa et al., 1987; Shimizu et al., *1991).*

L-glutaminase is the key enzyme present in mammalian cells which is involved in the breakdown of L-glutamine, which is the most abundant amino acid found in the body (Bergstrom J.et al, 1974). L-Glutamine is found in the mammalian blood at a concentration of 0.6-0.9 mmol/L, while in some tissues may be as high as 20 mmol/L. L-Glutamine has two nitrogen side chains one being an amino and the other an amide group, and for that reason, it is the most important nitrogen carrier which accounts for 30% to 35% of all amino acid nitrogen transported in the blood (Souba., 1987). Glutamine plays main role in cellular metabolism by delivering nitrogen which is essentially required for the biosynthesis of a variety of nitrogenous metabolic products (Fig.1.3). Tumor cells are foremost glutamine consumers, and they fight with the host cells for glutamine circulating in blood (Medina et al., 1992). Glutamine is the chief respiratory fuel and energy resource of the rapidly propagating tumor cells and that is why glutaminase has received much attention with respect to its therapeutic application for treatment of leukemia (Roberts et al., 1970).

L-Glutaminase, since its antitumor properties were reported, has attracted attention in this regard. Cancer is a global hazard that despite serious ongoing efforts is still growing and thus search for new and efficient drugs is persistently being pursued all over the world. Acute lymphocytic leukemia is a type of cancer for which an effective drug has been sought for many years. L-Glutaminase is being anticipated to include in the available remedies against leukemia. As leukemic cells are directly dependent upon the external supply of L-glutamine from human blood for their growth and survival. Hence, blood glutamine provides metabolic precursors for the synthesis of nucleotides and protein in tumor cells. As a result, L-glutaminase targets and specifically causes

catabolism of L-glutamine, which results in the death of L-glutamine dependent tumor cells as energy route for their propagation is blocked (Fig.1.1).

Another major application of L-glutaminase is in food industry. It enhances the flavor of fermented food by increasing its glutamic acid content (Yokotuska., 1997). Flavor enhancement comes from amino acids which are produced by the degradation of proteins brought about by enzymes. L-glutamic acid, a product of L-glutaminase reaction, is a well known flavor enhancing amino acid which contributes to the unique flavor of soy sauce (Ohshita et al., 2000).

Besides its applications as antitumor and flavor enhancing agent, L-glutaminase has applications in other areas as well. L-Glutaminase is being prospected as a therapeutic agent against HIV (Kumar and Chandrasekaran., 2003). It acts as antiretroviral agent by lowering the L-glutamine level in blood which results in reduced reverse transcriptase activity of human immune deficiency virus (HIV) (Sarada., 2013). Once nutritional requirements of viruses have been identified, this unique approach can be used against other viruses as well.

L-Glutaminase is also being used as biosensing agent for monitoring of glutamine levels in the cell cultures (Kashyap et al., 2002), also in the production of special chemicals like theanine by reaction of γ-glutamyl transferase.

Ability of the L-glutaminase to carry out degradation of L-glutamine provides new front in fight against leukemia and other fatal tumors. it can be a candidate for use as a replacement or in combination with L-asparaginase in the treatment of acute lymphocytic leukemia. Yet, the large scale application of glutaminase in cancer chemotherapy is still in experimental phase and not much information is available.

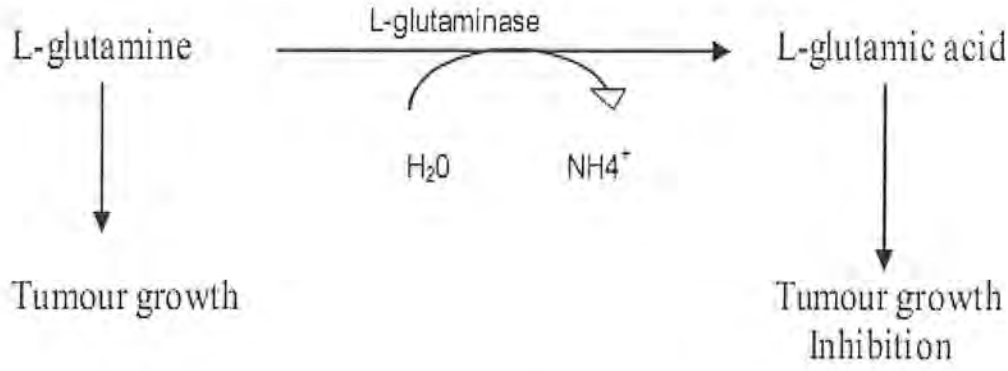

Figure 1.1: Mechanism of Action of L-glutaminase against tumor

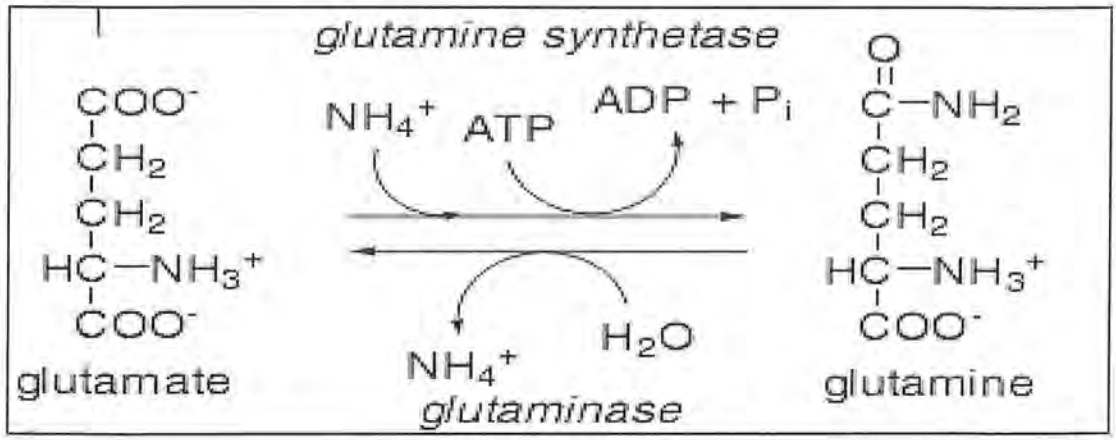

Figure 1.2: Interconversion of L-glutamine and glutamate

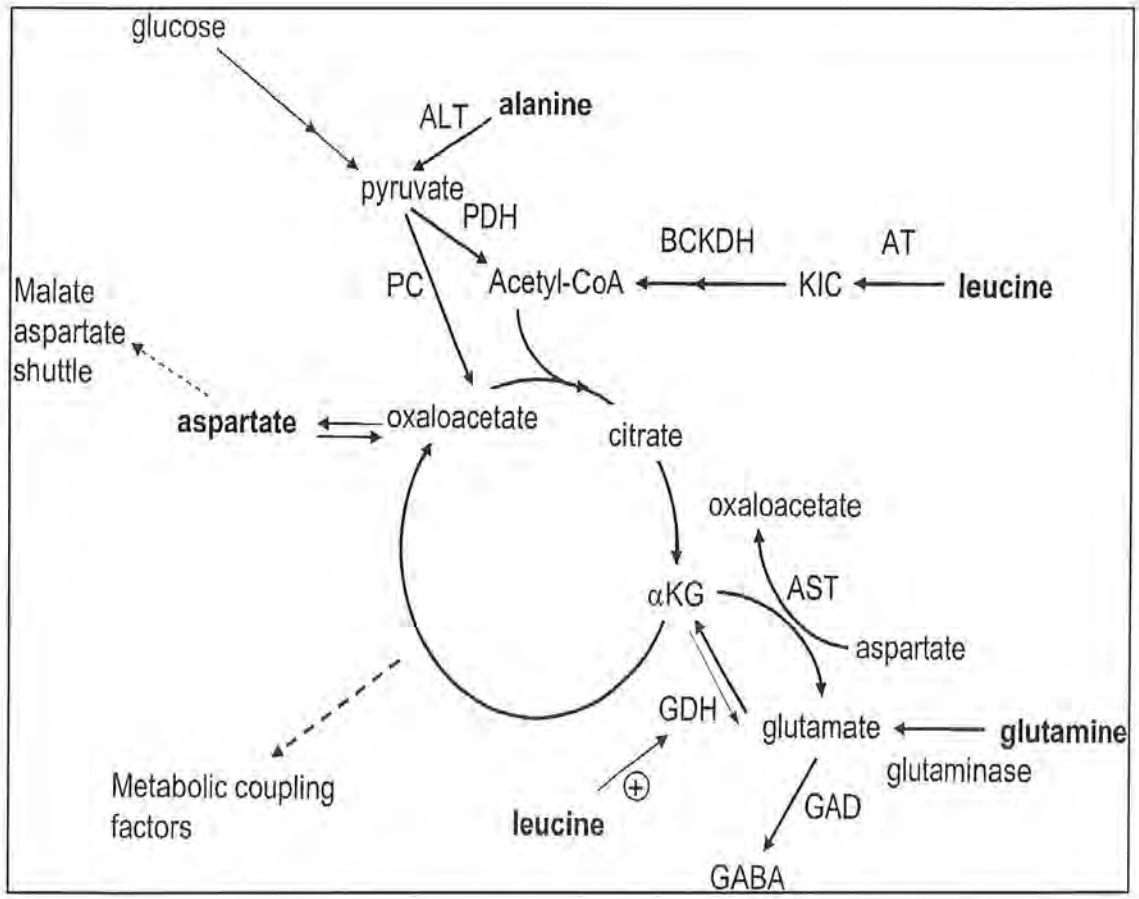

Figure 1.3: Role of Glutamine catabolism in overall metabolic process

AIMS AND OBJECTIVES

The main aim of the present research was production of L-Glutaminase from endophytic fungi. To fulfil this aim following objectives were put forward;

1. Screening of L-glutaminase producing endophytic fungal strains.
2. To study the ability of selected fungal strain *Epicoccum sp.* NFW1 to produce L-glutaminase in liquid medium.
3. To study the effect of different culture conditions i.e.; temperature, pH, incubation period on production of L-glutaminase from fungal strain.
4. To statistically optimize nutritional factors for the production of L-glutaminase from *Epicoccum sp.* NFW1 by Plackett-Burman design.
5. Production and precipitation of extracellular L-glutaminase under optimized conditions.
6. To test the precipitated enzyme for cytotoxicity.

Literature Review

Nature is an infinite spring of never ending chemicals and bioactive compounds. Chemistry of natural products has gone through robust and diverse growth making the efforts in drug designing much more promising. Natural products have largely contributed in pharmaceutical industry. Particularly areas of oncology and infectious diseases have sought much benefit as many new classes of drugs or templates have been discovered. In these areas, 60 to 70% of new drugs have been introduced which were obtained from natural sources. Natural products have continuously gained attention because of their salient features such as their structural diversity, larger chemical space and wider dispersion of properties than synthetic counterparts. Thus natural products obtained from plants and microorganisms (bacteria, yeast, and fungi) are an important reservoir which needs to be explored.

Endophytic Microorganisms as Potential Source of Novel Compounds

Endophytic fungi are microorganisms which live inside the tissues of plants and do not cause any negative effect on host (Joseph & Priya., 2011). They have been found in every plant species thus are ubiquitous in nature (Khan., 2007). Endophytic fungi have grabbed huge attention of researchers as single endophyte maybe able to produce many different types of metabolites. These bioactive metabolites can be used as templates for drug designing (Yu et al., 2010).

Bioactive products obtained from endophytic fungi are not only useful in drug discovery and pharmaceutics but also in many other industries. In order to get a hold on the biological aspects of endophytes researchers need to thoroughly study the ecological aspects related to endophytic fungi.

Host plant-endophyte relation

All features of the ecology and relation types of endophytes with their relevant hosts is a greatly under explored and stimulating field. Therefore, more background understanding about plants and their microbial biology would offer a better insight into leading the search for bioactive products. At present, no obvious facts about the nature of plant-endophyte relations are available.

Research shows that nature of plant–endophyte relations is determined by the methods of spreading, mode of infectivity, plant age, environmental conditions and overall genetic makeup (Saikkonen et al., 1998). Studies revealed that systemically transmitted endophytes, which grow inside seeds, are more expected to be symbiotic, non-systemically transmitted endophytes are liable to be more hostile to the host (Schardl et al., 1991; Saikkonen et al., 1998). The genetic and biochemical foundation responsible for the shift from symbiotic to parasitic lifestyle is distinguished by some discrepancy in exchange of nutrients that explains the reason for colonization of different hosts by divergent lifestyles.

Plenty of data shows that endophytic fungi play a vital role in host plant functioning. They obtain nourishment, shelter and growth chance from their respective hosts (Clay and Schardl., 2002; Thrower & Lewis., 1973). Fitness settlement presented by mutualistic fungi add to the ease of plant in adaptation to biotic and abiotic conditions by enhancing resistance to harsh nutrient and water conditions as well as tolerability to high temperature and mineral content (Redman et al., 2002; Arnold et al., 2003; Waller et al., 2005; Zhang et al., 2006). It is noteworthy in this regard that most of the natural products produced by endophytes possess antimicrobial activity, which also can be used to protect plants against phytopathogens. (Gunatilaka., 2006). A number of studies showed enhanced production of flavonoids and phenolic compounds, which possess antioxidant characteristics, by endophytes inhabited plants (Herrera-Carilloet al., 2009; Torres et al. 2009), this increase in antioxidant production was proposed assumed to be activated by endophytic production of some reactive oxygen species (ROS). ROS is assumed to be produced to tarnish and denature the membranes and cellular structures of host and in so doing they facilitate the leakage of nutrients from plant cells which then are taken up by fungal hyphae.

Endophytes can also play role in increasing the fitness of host and survival abilities by enhancing germination rate and growth facilities by developing metabolic pathways to produce different hormones including IAA, and cytokinins, or by enhancing the ability of absorption of nutrients of the host (Tan and Zou., 2001; Zhang et al., 2006). plants provide accommodation, protection from harsh conditions like drought, and spreading to the next generation of hosts (Saikkonen et al., 1998).

Endophyte Biodiversity

Evaluation of microbial assortment presents a great confront to present microbiology as a huge number of microbial species have already been explored and more of them are believed to exist. (Gamboa et al., 2001). As compared to all other organisms, microorganisms can be found in all habitats on earth (Gunatilaka., 2006). It has now become a well established fact that among 300,000 plant species, each specie host from one to many hundred endophytic strains (Strobel and Daisy., 2003; Huang et al., 2007a).

Generally the endophytic fungi are classified into two major groups, i.e. Clavicipitaceous (C-endophytes) endophytes which inhabit inside different populations of grasses and non-Clavicipitaceous (NC-endophytes) endophytes inhabit asymptomatically the internal tissues of higher plants like gymnosperms and angiosperms (Rodriguez et al., 2009).

Fungal endophytes are the most recurrent type of endophytes commonly isolated from medicinal plants. Dreyfuss and Chapela (1994) reported the probability of occurrence of almost 1 million species of mycoendophytes. Kharwar et al., (2008) reported 183 fungal endophytes which represented 13 fungal taxa. These were isolated from root, stem and leaf tissues of *C. roseus*. The leaf tissues showed more diversity of endophytes like, *Curvularia*, *Bipolaris*, *Aspergillus*, *Alternaria* and *Drechslera*. Kajula et al. (2010) studied the extracellular siderophore production as well as production of antioxidant and antibacterial compounds by endophytic fungi of *Pinus sylvestris* and *Rhododendron tomentosum*. The highest biological assortment in terrestrial ecosystems is in humid and moderate rainforests; amusingly though they also encompass the greatest number of fungal endophytes. These ecosystems encompass only 1.44% of the total land's surface, however they anchor more than 60% of the world's terrestrial biodiversity (Strobel and Daisy., 2003). A study conducted to evaluate diversity of endophytic fungi showed the trends of endophytic occurrence. 1,202 endophytic fungal strains were isolated from plants of different taxa. Sampling was carried out at six different locations, ranging from tropical forests of Panama to boreal forest of Canada. Endophytic variety decreased in a straight manner from the tropical range to boreal forests. Additionally, endophytic population from higher scope was distinguished by having decreased fungal diversity

containing fewer fungal species belonging to some classes of *Ascomycota*, while tropical endophyte collection was found to be subjugated by quite a few number of classes but relatively a large number of species of endophytes (Arnold and Lutzoni., 2007).

Basis for Plant choice and Isolation of Endophytes

It is important to be aware of the methods and basis that provides improved insight into isolation of endophytes which are prone to produce unique bioactive metabolites. Studies have helped to establish a criteria which enlists several helpful methodologies which suggest that plants which grow in the regions of immense biodiversities such as humid and moderate rain forests, special habitats such as declined environments or those environments which are covered by plants infected with pathogens can be endophyte repositories, supported by the fact that such plants gain survival benefits by accommodating endophytes (Strobel *et al.*, 2004; Yu *et al.*, 2010). In a similar manner, plants which humans have used for medicinal purposes or those which have background of an ancient habitat are more prone to harbor endophytes with bioactive natural compounds. (Strobel and Daisy, 2003; Yu *et al.*, 2010).

A comparison of different endophytic hosts suggests that medical plants, crops and plants in special environment have frequently been studied for endophytic screening (Yu *et al.*, 2010). Kumaresan and Suryanarayann (2001) studied Mangrove forest and were successful in the isolation of 12 endophytes with promising bioactive potential. Similarly, Tuntiwachwuttikul *et al.*, (2008) reported antimicrobial activity of an endophyte against phytopathogen *Colletotrichum musae* (You *et al.*, 2009).

Many suggestions have been presented regarding the mutualistic lifestyle of endophytic fungi, one suggestion is that endophytic fungi which grow within plant tissues and cause no harm to it may have been evolved from pathogenic fungi which later on switched to mutualistic mode of life. (Kogel *et al.*, 2006; Gao *et al.*, 2010). Once an equilibria is established between fungal and plant biochemical reactions, then a true state of mutualism is attained (Giménez *et al.*, 2007). Fungal endophytes provide benefits to plant by endorsing conditions for plant growth, improving stress resistance, providing pathogen protection, also repelling insects, diseases and grazing animals (Wilkinson *et al.*, 2000; Strobel, 2003; Malinowski *et al.*, 2004; Tanka *et al.*,

2005; Vega *et al.*, 2008; Joseph and Priya, 2011). These properties are regarded to the bioactive substances production by endophytes, which consequently is related to the independent evolution of mycoendophytes (Pimentel *et al.*, 2010). This also helps endophytes to adopt to the host plant in a better way (Joseph and Priya, 2011).

Endophytic Fungi as Source of Enzymes

Endophytic fungi have shown potential to produce several important enzymes. A single strain of endophytic fungi can produce more than one enzymes. These enzymes have diverse biotechnological applications such as applications in environment, industrial applications and biotransformation of several organic compounds which otherwise would have been difficult to transform and several medical applications (Pimentel et al., 2010). This potential has made them a potential candidate for use in industrial production of enzymes.

Enzymes

Enzymes are the large biomolecules that are required for numerous chemical interconversions that uphold life. They boost up the metabolic reactions in body by carrying out specific tasks. They are just like chemical catalysts which speed up chemical reactions, enzymes carry out biochemical reactions both inter and intracellularly. Thus generally are known as biocatalysts. Enzymes are highly specific in their reactions and they not only enhance the reaction rate but also the specificity of reactions. Almost every chemical reaction carried out in biological cells is carried out in the presence of enzymes; these reactions include from digestion of the food to the synthesis of DNA. Nearly every chemical reaction in a biological cell needs the presence of enzymes in order to take place at rates adequate for life. Enzymes are acknowledged to catalyze almost 4,000 biochemical reactions (Bairoch., 2000).

In view of the fact that enzymes are substrate specific and catalyze only a few selective reactions from amongst many available possibilities, thus the deposit of enzymes occurring in a biological cell establishes which metabolic pathway occurs in that particular cell. For example kreb's cycle, glycolysis and citric acid cycle denotes use of glucose as source of energy and thus enzymes produced indicate the presence of these pathways while in stress conditions GS/GOGAT pathway is adopted by

bacterial cells indicated by production of glutamine synthetase and glutamate synthase.

Use of enzymes has been known to mankind for very long time. History of their use is ancient but research and effort to characterize these molecules is not that old. Famous Italian catholic priest Lazzaro Spallanzani was first to mention the importance of this biomolecule in 1783 (Vallery et al., 2003). Gottlieb Sigismund Kirchhof investigated the procedure of conversion of starch into glucose in 1812. He also described the application of these biomolecules as catalyst (Asimov et al., 1982). First enzyme, diastase was discovered by Anselme payen in 1833 (Payen et al., 1955). It was not until 1926 when for the first time enzyme was purified, an accomplishment of James Sumner. Sumner in 1947 purified and crystallized the enzyme urease from the jack bean. Since then a rapid progress in developing new methods for identification, isolation and characterization of new and useful enzymes has been made. Enzyme industry has become one of the largest biomolecules industry which has its applications in nearly every field of life.

Enzymes show a wide range of applications in different industries whether it may be food, textile, medicine, dairy, or any other. In industry, enzymes are frequently used for process improvement, for instance to enable the utilization of new types of raw materials or for improving the physical properties of a material so that it can be more easily processed. They are the focal point of biotechnological processes.
The deliberate use of enzymes by man is central to the application of biotechnology, since enzymes are involved in all aspects of biochemical conversion from the simple enzyme or fermentation conversion to the complex techniques in genetic engineering.
In order to meet high demand of enzymes men had to concentrate on obtaining enzymes from sources other than plants and animals and microorganisms proved to be a window of opportunity.

Short fermentation time, inexpensive media, ease of developing simple screening procedures, fast growth of microbes, biochemical diversity, enzyme concentration may be increased by environmental and genetic manipulation, flexibility of choice of fermentation conditions, higher production rate, these qualities got microorganisms to replace other sources of enzymes.

Endophytic fungi have been reported to produce many useful enzymes. They produce these enzymes during the course of colonization in host or to defend against pathogens (Li et al., 2005). This ability of producing extracellular enzymes is being exploited to produce enzymes on industrial scale.

Fungal enzymes are more stable than enzymes obtained from plants and animals. They can tolerate harsh conditions such as high or low pH, temperature and salt concentration (Maria et al., 2005). Because of these reasons fungi are considered ideal for being used at industrial scale to produce enzymes. Furthermore other niches are being searched to isolate novel fungal strains that could produce enzymes with unique characteristics.

Amirita et al (2012) reported eight fungal strains that showed amylase activity. Amylases have applications in food, detergents, drinks, animal feed and baking. (Pandey et al., 2000; Fogarty et al., 1990). Two strains showed cellulase and two of them showed laccase activity. These strains belonged to *hyphomycetes, coelomycetes, xylariales*. Also lipase and protease activity of these strains was reported.

Gustavo et al., (2012) reported amylase and lipase production from five endophytic fungal strain isolated from Baru (*Dipteryx alata Vog.*). Urairuj et al (2003) reported production of peroxidase from endophytic *Xylariaceae* strain isolated from healthy tropical native plants of northern Thailand.

Besides the industrial and food biotechnological applications, enzyme have also found their applications in therapeutics (Fig.2.1). Enzyme therapy is found useful in the management of enzyme deficiency and was brought into light by Edward Howell in 1920s (Kaur et al., 2012). Therapeutic enzymes either digestive or metabolic, can be used medically alone or in combination with other treatments used against a range of diseases safely (Table.2.1). Digestive enzymes are those which are used to digest food and metabolic enzymes are indispensable for life. Digestive enzymes include lactase and lipase which can be used in supplementation therapies. (www.livestrong.com). Metabolic enzymes include Aglucerase, Imiglucerase, which are proposed to be useful against Gaucher's disease (Barton et al., 1991).

Two important features make use of enzymes as therapeutic agent suitable;

i) They show great affinity and specificity towards their target molecules.

ii) They catalyze and convert targeted biomolecules into the required products.

These are the features that make enzymes precise and powerful drugs against a wide range of diseases. (Vellard et al, 2003; Cooney et al, 1975; Margolis et al., 1972). Also combinatorial approach can be used in enzyme therapy.

In case of combination therapy, combination of two or more than two enzymes or combination of any other drug with enzymes can be used. This therapy is helpful in improving the effect of drugs. Many combinatorial drugs are currently in use. Trypsin is an example, available in combination with enzymes such as trypsin-chymotrypsin-diclofenac-paracetamol, trypsinchymotrypsin-aceclofenac and trypsin-chymotrypsin-aceclofenac-paracetamol. These combinations are in use for the treatment of pain, fever and soreness (prescriptiondrug-info).Aceclofenac-paracetamol-serratiopeptidase is another example of combination drug which is used for the treatment of pain and soreness coupled with the rheumatoid arthritis, osteoarthritis, tendinitis, ankylosing spondylitis and sprain (ww.healthkosh.com).This approach besides enhancing the efficacy of drugs also removes the toxicity and cell damage risks associated with some drugs.

Evolution of therapeutic enzymes has brought the thought to screen unique enzymes which can be used against one of the lethal diseases, cancer. Asparaginase is such an enzyme which has been used in treatment of leukemia for many years. But since the enzyme was being produced by bacteria it had toxic side effects. Recently another enzyme L-Glutaminase has gained parallel importance as potential antitumor agent (Roberts et al., 1970, 1979; Pal and Maity., 1992).

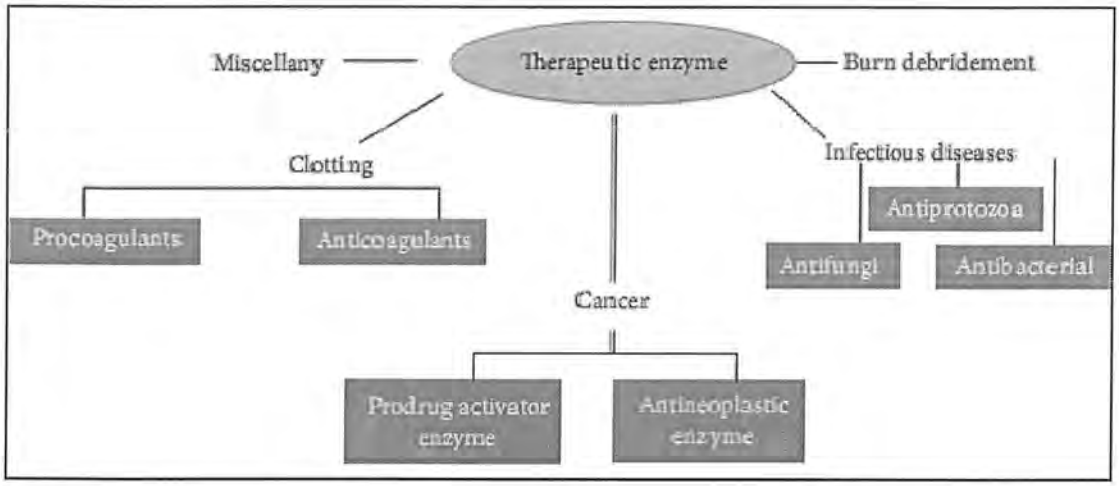

Figure 2.1:Applications of Therapeutic Enzymes

Table 2.1:Therapeutic uses of some microbial enzyme

Enzyme	Microbial source	Therapeutic Use
L-Asparaginase	Actinomycetes	Antitumor
L-Arginase	Bacillus subtilis	Antitumor
L-Tyrosinase	Agaricus bisporus	Antitumor
L-Glutaminase	Trichoderma koningii	Antitumor
α-Glucosidase	Aspergillus niger	Antitumor
Superoxide dismutase	Mycobacterium sp	Antioxidant, Anti-inflammatory
Uricase	Candida utilis	Gout
Lipase	Aspergillus oryzae	treatment of disorders of the pancreas
Laccase	Agaricus bisporus	Detoxifer
Rasburicase	Aspergillus favus	Hyperuricemia
Sacrosidase	(Saccharomyces cerevisiae	Congenital nutrient deficiency
Protease, Peptidase	Beauveria bassiana	Celiac disease
Acid protease	Aspergillis niger, oryzae	Stomach disorders
Maltase	Aspergillis oryzae	Pompe's disease
α-Amylase	Aspergillus sp	Easy digestion

L-Glutaminase

L-Glutaminase is an important enzyme which is gaining attention mainly as therapeutic agent against leukemia and HIV (Roberts and McGregor, 1991) and generally as flavor enhancer in food industry. This enzyme is identified as hydrolase and further characterized as amidohydrolase.

L-Glutaminase is abundantly found in nature. It is produced by bacteria, fungi and human cells as well. In mammals, the enzyme needs the presence of inorganic phosphate for its activity. This enzyme plays main role in a number of physiological processes, such as ammoniagenesis in kidney and the synthesis of glutamate in brain, which is a neurotransmitter (Curthoys and Watford., 1995). Glutaminase is an imperative supplier to the pools of Glutamate (Nicklas et al., 1987) and the main glutamine consuming enzyme of the brain (Kvamme., 1984).

Tumor cells are unable to synthesize glutamine so they have to rely on the exogenous supply of glutamine. This makes use of L-glutaminase an attractive strategy as L-glutaminase breaks down L-glutamine into glutamate and ammonia. Since the discovery of antitumor potential of L-glutaminase its production has been reported from many sources including bacteria and fungi. But use of bacterial enzymes has been reported to pose a toxicity risk so efforts are being made in exploring fungal sources. A search for potential strains that hyper produce this enzyme with novel properties under economically viable bioprocesses is pursued.

Imada et al (1972) reported production of L-glutaminase from several species of *penicillium* and *moniliaceae*. Dutt et al (2010) reported L-glutaminase from strain of *penicilium expansum*. Nathya et al (2011) reported production of L-glutaminase from *Aspergillus flavus*. Nathiya et al (2011) studied forty strains of endophytic fungi which could produce l-glutaminase. These strains belonged to different species of *Aspergillus, Trichoderma, Penicillium, Fusarium, Mucor* and *Basidiomycetes*. Nathiya et al (2012) reported cytotoxicity of L-glutaminase produced by *Aspergillus flavus* strain against MCF7 cancer cell line. *Zygosaccharomyces rouxii* was found to produce L-glutaminase (Iyer and Singhal., 2007). Production of this enzyme was reported from *Penicillium politans* NRC510 by Thanaa et al (2009). Sabu A (2000) reported production of L-glutaminase from marine fungi.

Production Strategies of enzymes from Microbes

Different techniques of fermentation have been employed for bulk production of therapeutic enzymes carried out on commercial scale, such as solid state fermentation, immobilization on inert solid support. These methods are preferred over liquid fermentation in huge bioreactors. The large-scale production of microbial enzymes with reference to various production techniques, and downstream dealing has been reported by many researchers (Sabu et al., 2005).

Commercially, L-Glutaminase has been produced by submerged fermentation technique, but in recent years, it is also being produced using solid state fermentation technique.

Solid state fermentation

This technique involves the production of enzymes on solid medium using natural (E.g. brans, husks, oil cakes etc) and inert solid materials (E.g. polystyrene beads).

Solid state fermentation offers several advantages over other traditional fermentation techniques; submerged fermentation etc. The major advantages include;

Higher yields, lower investment and persistent expenditure, lower waste water output, cheap energy requirements, absence of foaming, simple proceduring, high reproductivity, relatively simpler media of fermentation, lesser space requirement, absence of thorough control of fermentation parameters, economical to use even on smaller scales, easy organization of contamination, applicability in terms of using fermented solids directly, storage facility of dried fermented matter, lower cost of downstream handling . (Ramesh and Lonsane., 1990).

Problems which are generally confronted in solid state fermentation are;

Heat build-up, rapid spread of contamination, upscaling, growth evaluation and management of process parameters.

Submerged Fermentation

It is a type of fermentation where liquid medium is used for the production of enzymes and then product can be recovered from the medium.

In submerged fermentation (SmF) the cost of production and contamination problems are very low and it assists better process control. Disadvantages of submerged fermentation include;

Lesser productivity, problems in recovery, high expenses and energy requirements.

Optimization of production of Enzymes

Optimization of growth and production conditions is an important step in industrial production of compounds. Physical as well as nutritional parameters play a key role in the production of compounds from biological systems like microorganisms. Traditional optimization involves optimization of one parameter at a time. In the last few years use of statistical methods has emerged as promising technique.

Statistical methods offer efficient way of optimizing different components at the same time and also provide an insight into the interactive effects of all the parameters. One such design is Plackett-Burman design which provides statistical assistance to optimize the process of biological compounds.

Levin et al (2004) reported the optimization of parameters for production of ligninolytic enzyme using Plackett-Burman design. Palvannan and Sathishkumar (2010) reported optimization of laccase production from *Pleurotus florida* based on Plackett-Burman design. Evaluation of nutritional components was done by Plackett-Burman design for lipase production from *Penicillium citrinum*, reported by Salihu et al (2011). Evaluation of medium components using Plackett-Burman design for Lipase Production by *Candida rugosa* was reported by Arvindan et al., (2008).

Other Important Compounds from Endophytic Fungi

Antimicrobial

Metabolites that are active against microorganisms at low concentration are low molecular weight organic substances and are termed as antimicrobial metabolites (Guo et al, 2008). Endophytic fungi that have potential to produce secondary metabolites are candidates for use as antimicrobial agents. The discovery of new antimicrobial metabolites from endophytes is a significant substitute to overcome the problem of increasing drug resistance by plant and human pathogens. The bioactive metabolites can be used not only as pharmaceutical agents by humans but these also have ability to be used as food preservatives to control food spoilage and food-borne diseases, a serious concern in the world food chain (Liu et al., 2008). A large number of antimicrobial compounds obtained from endophytes belonging to different structural groups like alkaloids, peptides, steroids, terpenoids, phenols, quinines, and flavonoids are reported in many studies.

Three compounds, melleolides K, L and M. from *Armillaria mellea* which showed antimicrobial activity against grampositive bacteria, yeast and fungi were reported (Li et al., 2010; Momose et al., 2000). Donnelly et al., 1985) isolated two new sesquiterpene aryl esters, 4-O-methylmelleolide and judeol, both of strong antibacterial activity against gram positive bacteria. Bioactive compound 7-amino-4-methylcoumarin isolated from the culture extracts of the endophytic fungus *Xylaria sp.* YX-28 isolated from *Ginkgo biloba* L. by (Liu, Dong et al., 2008) having an activity against several food-borne and food spoilage microorganisms and was suggested to be used as natural preservative in food. Three steroids namely,ergosta-5,7,22-trienol, 5α,8α-epidioxyergosta-6,22-dien-3β-ol, ergosta-7,22-dien-3β,5α,6β-triol and one nordammarane triterpenoid helvolic acid were isolated for the first time from the endophytic fungus *Pichia guilliermondii* Ppf9 from the medicinal plant *Paris polyphylla* var. *yunnanensis* showing the strongest antibacterial activity against all test bacteria (Zhao et al., 2010). Two compounds pestalachlorides A and B, from the endophytic fungus *Pestalotiopsis adusta*, displayed a significant antifungal activity against three phytopathogenic fungi, *Fusarium culmorum,Gibberella zeae*, and *Verticillium alboatrum* (Li et al., 2008). Chaetomugilin A and D which showed antifungal activities, were isolated

from an endophytic fungus *Chaetomium globosum* isolated from *Ginkgo biloba* (Qin et al., 2009). Cytosporone B and C were isolated from a mangrove endophytic fungus, *Phomopsis sp.* which inhibits the activity of two fungi *C. albicans* and *F. oxysporum*. (Silva et al 2005).

Pestalotiopsis theae an endophytic fungus isolated from branches of a tree in China, yielded four new metabolites named pestalotheols A–D. (Li et al.2008). Enfumafungin which is a hemiacetal triterpene glycoside, was isolated from cultures of a *Hormonema sp.* Endophytic fungi inhabiting inside leaves of *J. communis* . The compound showed inhibitory effect to the incorporation of [14 C]-glucose into the cell wall extract which was alkaline insoluble in whole cells of both *Candida* and *Saccharomyces* without showing any effect on mannan or chitin synthesis, thus indicating that enfumafungin could be a specific inhibitor of glucan synthesis in fungal cell wall. (Garcia-Effron et al., 2009; Onishi et al., 2000; Schwartz et al., 2000; Peláez et al., 2000).

Many bioactive compounds have been isolated from endophytic fungi belonging to genus *Xylaria*. These compounds include "sordaricin" which showed antifungal activity against *Candida albicans* (Phongpaichit et al.2006). Also multiplolides which showed activity against *Candida albicans* (Boonphong et al., 2001). Another strain F0010 of the specie belonging to same genus was reported. It was isolated from *Abies holophylla* and was recognized as a producer of "griseofulvin" which is a spirobenzofuran, characterized as antifungal antibiotic agentcommonly used for the treatment of human and veterinary animals mycotic diseases (Park et al., 2005). Cytosporone B and C were isolated from a mangrove endophytic fungus, *Phomopsis sp.* They inhibited two fungi *C. albicans* and *F. oxysporum* (Huang et al., 2008). an endophytic fungus *Phomopsis cassia* was isolated from *Cassia spectabilis* and was reported to produce two antifungal compounds; ethyl 2,4-dihydroxy-5,6-dimethylbenzoate and other one was phomopsilactone. Both of these compounds exhibited strong activity against plant pathogenic fungi (Silva et al., 2005). An endophytic fungi *Muscodor albus* which belongs to non spore forming xylaria genera, was reported to show both inhibitory and detrimental effect against fungi and bacteria. This endophyte was isolated from *Cinnamomum zeylanicum* and inhibits microorganisms by producing certain volatile compounds (Strobel., 2006).

Until now, several compounds bearing antimicrobial activity have been reported to be produced by endophytes belonging to diverse genera of endophytes. Conversely, the endophytes which have been explored and studied for their antimicrobial potential encompass a very smaller proportion of endophytic population. This implies that a large opportunity is waiting ahead in the form of unexplored endophytic species which are a potential treasure chest of novel and efficient antimicrobial compounds. (Yu *et al.*, 2010).

Antioxidant

Molecules known as reactive oxygen species (ROS) are produced in the course of overall metabolism. These reactive oxygen species are indispensable for several processes of body such as cell signaling, programmed cell death, gene expression and transportation of biomolecules. ROSs play a vital role in deteriorating conditions such as cancer, neurodegenerative diseases, and diseases related to the bones and inflammations (Aruoma, 1991). These set of chemicals can become a cause of oxidation stress if mount up in the body in surplus amount. Consequently, certain structures of body and biomolecules which necessarily support vital functions of life can e damaged. These prone structures and molecules involve proteins and lipids along with DNA and RNA. The irregularity in the performance of these structures thus caused can result in the elevated risk of some life threatening diseases such as, cancer, cardiovascular problems and autism. Thus, the nutritional management to maintain the supply of antioxidants is essential in order to lessen the harmful effects caused by free radicals.

A compound's antioxidant activity is the efficient contra effect against the damage caused by ROSs to the cells (Huang et al., 2007). Antioxidants are thought to be the potent way of treatment in preventing and treating the disorders linked to ROSs (Valko et al., 2007).in nature antioxidants are frequently found in many vegetables, fruits and also a load of medicinal plants. Nevertheless, endophytic fungi have been reported to be a prospective source of antioxidants with novel structures and enhanced effectiveness.

Compounds have been isolated from endophytic fungi belonging to *Xylaria sp.*, which show antioxidant activity. Endophytic fungi which is reported was isolated from *Ginkgo biloba*, a plant of medicinal importance (Liu et al., 2007). Antioxidant

aptitude of endophytic fungi isolated from Chinese medicinal plant were studied by Huang et al (2007). They proposed that the total phenolic content was the main antioxidant bearing component of endophytes. "Pestacin" (C15H14O4) and "isopestacin", are two compounds which were acquired from endophytic fungus isolated from a plant *Terminalia morobensis* (Harper et al. 2003; strobe et al., 2002). These compounds mainly isopestacin which has similar structure to that of the structure of flavonoids exhibits antioxidant activity. It scavenges both of the superoxide and hydroxy free radicals in solution (Strobel et al., 2002).

A phenolic compound was isolated from *Cephalosporium sp* of endophytic fungi. This compound known as Graphislactone A exhibited antioxidant activity in vitro. Activity exhibited by this compound was stronger as compared to that of the standards tested in the experiment (Song et al., 2005).

Anticancer

Cancer is a described as a set of diseases illustrated by unrestrained propagation of cells which eventually results in the death of organism (Pimentel *et al.*, 2010). There has been a A incessant increase in the scale as well as death rate caused by cancer since last two decades. This points toward the incompetence of already present treatment resources. Also proposes the call for discovering fresh and potent anticancer drugs. Substantial data is there about production of bioactive compounds from endophytic fungi which could prove to be substitute for discovery of novel drugs.

Taxol is a novel diterpenoid originally obtained from the stem bark of yew tree (*Taxus brevi folia Nutt*. The supply of taxol from the bark is limited (0.01–0.05 %) because the plant is not abundantly found in nature (Cragg et al., 1993), and it also grows slowly. Since the production of Taxol from endophyte *Taxomyces andreanae* has been reported, an immense amount of work has been put forward in attempt to obtain this compound from endophytes (Stierle etal., 1993). various endophytic fungi that belong to different genera such as *Bartalinia robillardoides, Pestalotiopsis microspora, Pithomyces sp., Alternaria alternata, Monochaetia sp. Periconia sp., Taxomyces andreanae, Chaetomella raphigera, Seimatoantlerium Nepalense,* and *Botryodiplodia theobromae,* have been reported to produce Taxol. Another endophytic fungi *Pestalotiopsis terminaliae* which was isolated from the plant

Terminalia arjuna, was reported to produce the highest quantity of Taxol (Gangadevi & Muthumary., 2009).

CPT, which is an alkaloid, is a potent antitumor agent. It was first isolated by Wall and coworkers in 1966 from the wood part of a native Chinese plant named *C. acuminate*. This unique alkaloid has been reported to be produced in highest amounts from *Nothapodytes nimmoniana* and in substantial amounts from numerous other plants such as *Merrilliodendron megacarpum* and *Ervatamia heyneana*. (Govindachari and Viswanathan 1972). CPT is known to restrain the reproduction of HIV in vitro. It has also reported to show effectivity in the absolute reduction of several types of cancers including cervical, lung, uterine and breast cancer (Kusari et al., 2009b). *Entrophospora infrequens*, an endophyte isolated from *Nothapodytes foetida*, was able to produce camptothecin (Puri et al., 2005), chemotherapeutic agent proficient against ovarian, lung and uterine cancer, it was isolated for the first time from *Camptotheca acumina* (Amna et al., 2006). The anticancer properties of the microbial products CPT and two other analogues of it; 9-methoxycamptothecin and 10-hydroxycamptothecin, were reported to be produced from the endophytic fungi *Fusarium solani* which was isolated from plant *Camptotheca acuminata* (Kusari et al., 2009)

Podophyllotoxin, which belongs to aryltetralin lignans, naturally are produced by *Podophyllum sp*. Research showed that *Trametes hirsute*, a novel endophytic fungi showed ability to produce podophyllotoxin and several other associated aryltetralin lignans (Puri et al., 2006). Other novel endophytic sources of Podophyllotoxin include *Aspergillus fumigatus* which was isolated from the tree *Juniperus communis* (Kusari et al., 2009).

Ergoflavin" (C30H26O14), belongs to the class of compounds known as ergochromes. It is a dimeric xanthene which is linked in position 2, and has been depicted as a novel anti tumor agent. This compound was isolated from an endophytic fungus that grows on the leaves of a medicinal plant of Indian origin, *Mimusops elengi* (Deshmukh et al.2009). Another endophytic fungi *Penicillium brasilianum*, inhabiting in roots of *Melia azedarach*, was found to promote the formation of phenylpropanoid amides which have attracted attention for their anticancer potential (Fill et al., 2010). Finally, some other compounds that exhibit anticancer features

isolated from endophytic microbes have been reported such as cytoskyrins (Brady et al., 2000) phomoxanthones A and B reported by Isaka and coworkers (2001), photinides A-F reported by Ding et al (2009) and finally rubrofusarin B reported by Song et al. (2004).

Materials and Methods

MATERIALS & METHODS

Microorganisms

Endophytic Fungal strains (NFW1, NFW6, NFW7, NFW8, NFW11, NFW16) were obtained from microbiology research lab, previously isolated by Dr. Nighat Fatima (2013). Strains were inoculated on PDA slants and incubated for 7 days.

Materials

L-Glutamine (Sigma-Aldrich, Germany), modified Czapek Dox's medium (mCD) (Oxoid, England).

Methods

Initial Screening for L-Glutaminase Production

To identify the fungal strains able to produce L-glutaminase, strains were inoculated on medium (Oxoid., England) agar plates and incubated at 25°C for 96 h. The mCD medium contained (g/L of distilled water): Sucrose; 30.0, K_2SO_4; 0.35, KCl; 0.5, $MgSO_4.7H_2O$; 0.5, $FeSO_4$; 0.01, $NaNO_3$; 2.0, agar; 15. The pH of the medium was adjusted to 6.2 by using 1N HCl and autoclaved at 121°C, 15 lb/inch^2 for 20 min. Following autoclavation, L-gplutamine (10 g/L) was added separately through syringe filters. This medium was then supplemented with phenol red (0.3 ml of 2.5% w/v dye at a final concentration of 0.009% w/v). Fungal strains that imparted pink color to the media were selected for further studies.

Preparation of Phenol red stock solution (2.5%, w/v)

2.5% stock of dye was prepared in ethanol and pH was adjusted to 7 by using 1 mol/L NaOH.

Quantitative estimation of L-Glutaminase Activity

Selected fungal strains were inoculated on modified Czapek Dox's agar. From these petri plates agar plugs were inoculated in 250 ml Erlenmeyer's flasks containing 100 ml mCD

broth in sterile conditions. Flasks were incubated at 30°C on shaker for 144 hrs at 150 rpm. The enzyme activity was determined by nesselerization.

L-Glutaminase Assay

L-Glutaminase assay was performed according to the method described by Imada et al (1973);

Reagents

- 0.05 M Tris HCl Buffer (pH 8.6)
- 0.04 M L-Glutaminase
- 1.5 M Trichloroacetic acid
- Nesseler's reagent
- Enzyme extract
- Distilled water

Procedure

A reaction mixture containing 0.5 ml of enzyme extract, 0.5 ml of L-Glutaminase, 0.5 ml of Tris HCl buffer and 0.5 ml of distilled water was incubated at 37^0C for 30 minutes. Reaction was stopped by adding 0.5 ml of Trichloroacetic acid. Then 0.1 ml of above mixture was added to 3.7 ml of distilled water along with 0.2 ml of nesseler's reagent. Colored reaction was allowed to develop and absorbance was measured at 450nm with a spectrophotometer. Liberated ammonia (µmol/ml) quantitatively determined by using ammonium sulphate standard curve.

International Unit

1 international unit of L-Glutaminase (IU) is defined as the amount of enzyme which liberates 1 µmol of ammonia per minute at 37^0C (µmol/ml/min).

Protein Estimation

Protein estimation was carried out by using the method of Lowry et al. (1951) and BSA (Bovine Serum Albumin) was taken as standard.

Four solutions were prepared:

Solution A

Ingredients	g/100 ml
Na_2CO_3	2.0
NaOH	0.4
NaK Tartarate	0.1

Na_2CO_3 was dissolved in 100 ml distilled water then NaK tartarate and finally NaOH was added.

Solution B

$CuSO_4.5H_2O$	0.5g
Distilled water	100 ml

Solution C

- Sol C was freshly prepared by mixing 25 ml sol A and 0.5 ml sol B.

Solution D

- Folin phenol in ratio of 1:1 with distilled water.

Procedure

1 ml of solution C was mixed with 1 ml of sample, shaken and kept for 10 minutes. Folin phenol in ratio of 1:1 with distilled water was added, 0.1 ml in each tube. O.D was taken at 650nm using spectrophotometer. Standard curve of BSA was prepared and amount of protein was determined using this curve.

Optimization of Operational Parameters for Production of L-Glutaminase

Factors influencing the production of L-Glutaminase like temperature, pH and incubation period were optimized.

Effect of Temperature on Production of L-Glutaminase:

The effect of temperature on maximum enzyme production of L-glutaminase was studied by taking 250ml Erlenmeyer flasks containing 100 ml of MCD broth, inoculated under sterile conditions, then incubated at different temperatures (15, 25, 30, 37°C) for 144 hrs at 150 rpm. Enzyme assay was performed at different time intervals.

Effect of pH on Production of L-Glutaminase:

Effect of pH was optimized by shake flask fermentation using mCD broth at different pH (5.5, 6, 6.5, 7, 7.5). Flasks were incubated at 30^0C for 144 hrs. at 150 rpm. Samples were drawn at different time intervals for enzyme assay.

Effect of Incubation Time on Production of L-Glutaminase:

Incubation time was optimized for maximum enzyme production. 100 ml mCD broth was inoculated under sterile conditions with selected strain, incubated at 30°C for 144 hrs. at 150 rpm. Samples were drawn at 72, 96, 120 and 144 hrs and enzyme assay was performed.

Optimization of Nutritional Factors for Production of L-Glutaminase by Plackett–Burman Design:

Plackett–Burman (PB) design was used for screening of important medium components which could enhance the production of L-glutaminase. 100 ml mCD broth pH 6 was prepared in 250 ml Erlenmeyer flasks which was used as basal medium. Eleven medium components used for screening were dissolved in different concentrations designed by Design Expert 9 (Stat-Ease Inc.) Eleven medium components included: KNO_3, NH_4NO_3, NH_4Cl and urea as nitrogen sources, wheat bran, molasses, glucose and maltose as carbon sources and three amino acids L-glutamine, arginine and glutamic acid. The components of the production medium were tested at two levels, high (+1) and low (−1).

Design Expert 9 (Stat-Ease Inc.) generated a set of 15 experimental runs based on Plackett-Burmann design. The submerged batch fermentation was conducted in shake flasks (in duplicate) at 30°C and the response was measured in terms of enzyme activity (IU).

Growth for Production of L-Glutaminase under Optimized Conditions

Optimum operational and nutritional parameters were determined later from Plackett-Burman design, under these optimum conditions endophytic fungal strain was grown to yield maximum activity of L-glutaminase in crude form. This crude enzyme was used for further experiments.

Protein Precipitation

Enzyme extract was placed in acetone compatible tube and pre cooled acetone (-20°C) was added in a volume four times to that of enzyme extract. Tube was vortexed and incubated at -20°C for 60 minutes. Then the whole liquid was microcentrifuged at 14,000x g for 10 minutes. Supernatant was discarded and pellet was collected, suspended in 0.01 M phosphate buffer pH 8 and stored at -20°C for further studies.

Brine Shrimp Cytotoxicity Assay:

Cytotoxicity of the precipitated enzyme was checked by Brine Shrimp Assay (Maridass., 2008)

a. Sample preparation

Sample was prepared by dissolving 10 mg of precipitated enzyme in 1 ml of phosphate buffer. The concentration of 10,000µg/ml was taken as a stock. Further dilutions were made from the stock solution.

b. Preparation of artificial seawater

By dissolving 34 g commercial sea salt in 1 liter distilled water artificial seawater was prepared (Harvest Co. H. K.). It was aerated by vigorous shaking on magnetic stirrer.

c. Shrimps Hatching

Brine shrimp (*Artemia salina*) eggs (Sera, Heidelberg, Germany) were hatched in a rectangular dish (22 x 32 cm) that was filled with artificial seawater. An artificial divider of 2 mm containing a number of pores was used to divide the dish in unequal compartments.

The eggs (20 mg approximately) were scattered in the larger compartment and was covered with aluminum foil to make it dark, while the smaller compartment was enlightened. Hatching began within 24 hrs after which, phototropic nauplii (brine shrimp larvae) were collected by Pasteur pipette from the lightened compartment.

d. Assay procedure

Two-dram vials were used for this bioassay. The final volume of reaction mixture was made upto 5 ml and assay was run in triplicate. About 0.5 ml of sample was taken from stock to make concentration 1000µg/ml, 50µl for 100µg/ml and 10µl for 10µg/ml. The nauplii were counted in the stem of pasture pipette against a light set. Ten shrimps were

added to each vial. After adding shrimps, volume in the vials was raised up to 5 ml. The final concentration of each vial became 100, 10 and 1 ppm, respectively.

The vials were kept under light lamp at room temperature 25- 28°c. Survivors were counted with the aid of 3x magnifying glass after 24 hours and 48 hours. Then LD_{50} was calculated by Finney probit software.

Results

Initial Screening for L-Glutaminase Production

Initially six strains (NFW1, NFW6, NFW7, NFW8, NFW11, NFW16) were tested for the production of L-glutaminase by agar plate method. Four strains imparted pink colour to the medium indicating the pH change due to the production of L-glutaminase. Endophytic fungal strains NFW7 and NFW8 were negative for L-glutaminase activity in quantitative test (Table 4.1).

Quantitative Estimation of L-Glutaminase Activity

Four strains selected by initial screening were further screened through quantitative method to identify the highest L-glutminase yielding strain. NFW1 was identified as the highest enzyme yielding strain which produced 21 IU of L-glutaminase on 5^{th} day of incubation. After 3 days NFW11 showed highest activity which reduced after 4 days and further decline was observed at 6^{th} day.

In case of NFW1 initially at 3^{rd} day activity was recorded which elevated at 4^{th} day and reached to its maximum on 5^{th} day. A sudden decline in activity was observed in 6^{th} day (Fig. 4.1).Based on that the strain NFW1 was selected for further studies, which has previously been identified as *Epicoccum sp.* based on 18s rRNA sequence data.

Optimization of Operational Parameters for Production of L-Glutaminase

Effect of Temperature on the Production of L-Glutaminase:

Effect of temperature on production of L-glutaminase from *Epicoccum sp.* was studied on 15, 25, 30, 37°C. Results showed the highest enzyme activity (IU) and specific activity (units/mg) at 30°C. Activity decreased by both lowering and increasing the temperature (Fig.4.3).

Effect of pH on Production of L-Glutaminase

Effect of various pH levels on the production of L-glutaminase from *Epicoccum sp.* was studied. Results showed that highest enzyme activity and specific activity was supported by pH 6. Activity rapidly decreased on both sides of optimum pH level (Fig. 4.4).

Effect of Incubation Time on Production of L-Glutaminase :

Effect of incubation period on the production of L-glutaminase from *Epicoccum sp.* was studied on 3rd, 4th, 5th and 6th day of incubation. Results indicated highest enzyme and specific activity on 5th day of incubation. Minimum activity was seen on 3rd and 6th day of incubation (Fig. 4.5).

Table 4.1:Strains showing colour change on agar medium

Strains	Colour change
NFW1	+ive
NFW6	+ive
NFW7	-ive
NFW8	-ive
NFW11	+ive
NFW16	+ive

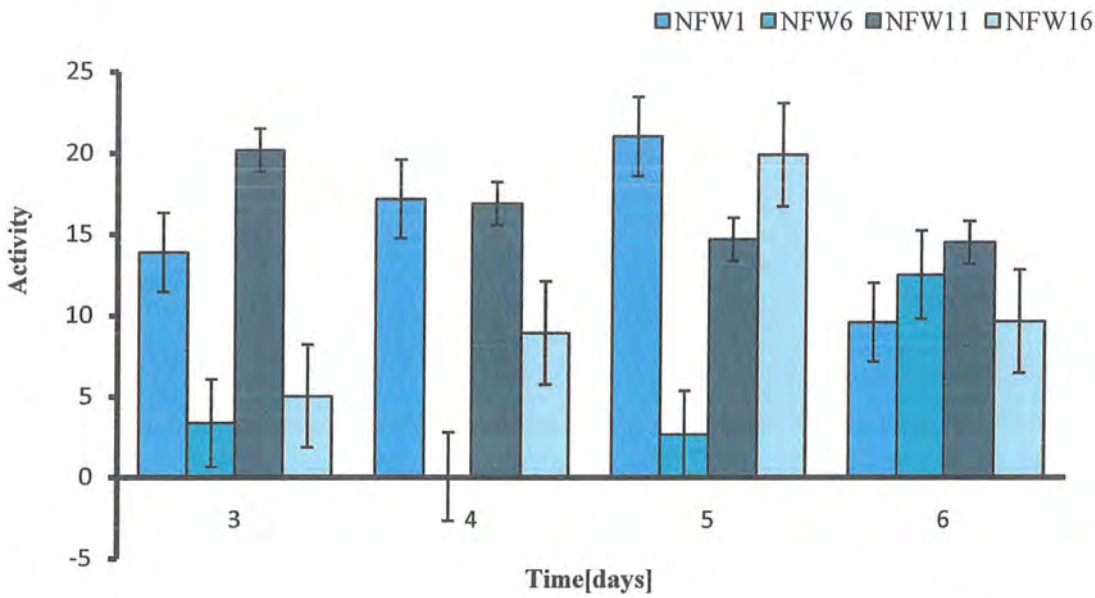

Figure 4.1: Quantitative Estimation of Four strains NFW1, NFW6, NFW11, NFW16

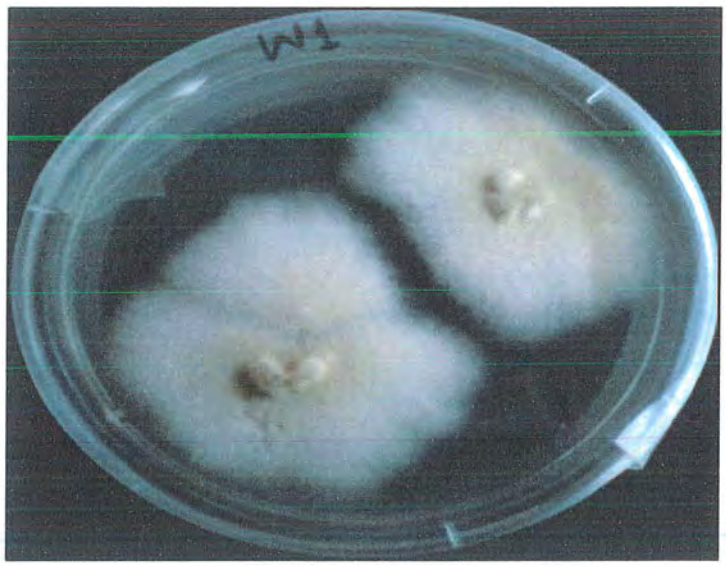

Figure 4.2: Plate Morphology of *Epicoccum sp.* (NFW1)

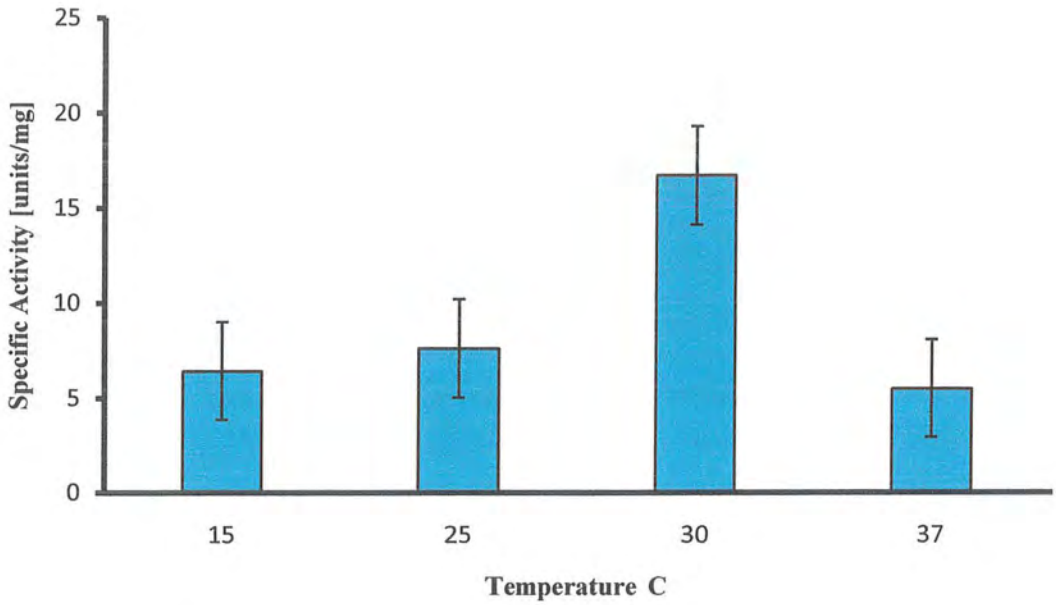

Figure 4.3: Effect of Temperature on Production of L-Glutaminase from *Epicoccum sp.*

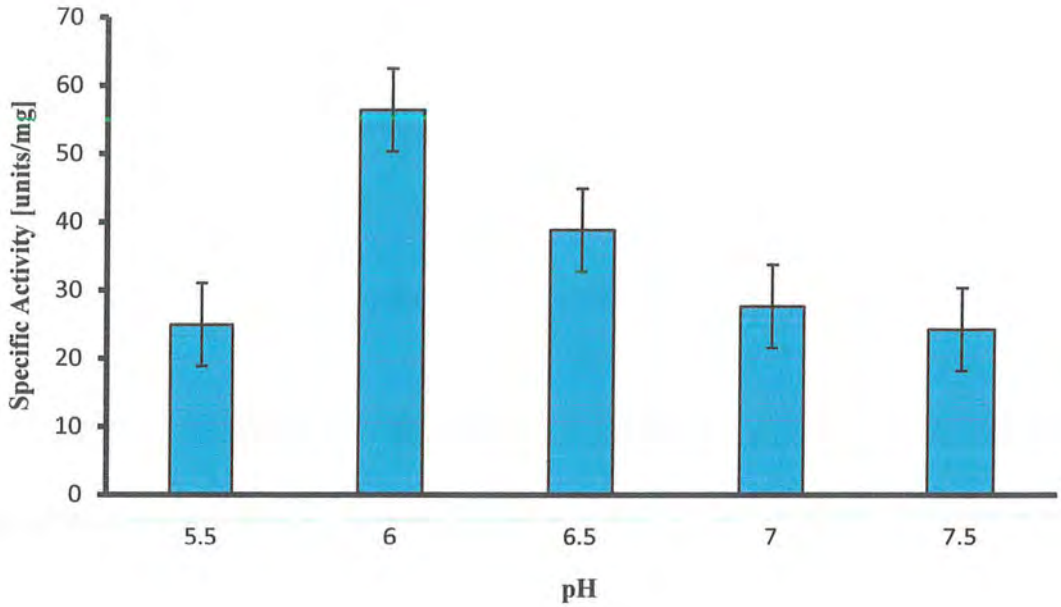

Figure 4.4: Effect of pH on the production of L-Glutaminase from *Epicoccum sp.*

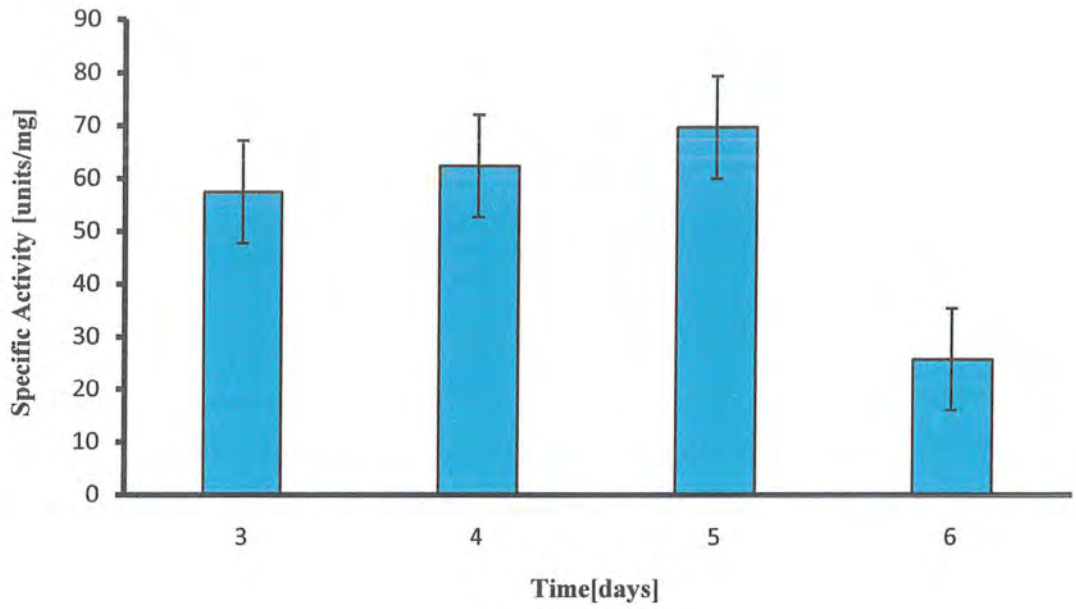

Figure 4.5: Production of L-Glutaminase on different days from *Epicoccum sp.*

Optimization of Nutritional Factors for Production of L-Glutaminase by Plackett–Burman Design:

The effect of eleven medium components on the fermentation for L-glutaminase production by NFW1 was evaluated using Plackett-Burman design. Experiment was designed using eleven selected medium components; potassium nitrate, ammonium nitrate, ammonium chloride, urea, wheat bran, molasses, glucose, maltose, L-glutamine, arginine and L-glutamic acid. Table 4.2 shows design matrix generated by Placket-Burman design. For each experimental run experiment value of enzyme activity is shown in the table as well. PBD1, PBD6, PBD9, PBD12, PBD14 and PBD15 showed maximum enzyme activity. The highest enzyme activity was observed 25.85 IU for experimental run 12 and lowest enzyme activity 8.72 IU for experimental run 7 (Fig. 4.6).

Effect of selected components on L-glutaminase production was evaluated on next step. Perturbation chart shows the positive and negative effects of different components on the enzyme activity (Fig. 4.7).

Figure 4.8 shows that out of four nitrogen sources only potassium nitrate had negative effect on the production of L-glutaminase. Three out of four carbon sources had negative effect on enzyme activity with only maltose as positively effecting carbon source. None of the selected amino acids caused negative effect on enzyme production.

Figure 4.9 shows the percentage contribution of each factor either positive or negative, to the enzyme activity. Major contributing factors were found to be Arginine which contributes to 42% of the positive effect while L-glutamine contributes to the 23%. Molasses had 9% effect on the enzyme production. Figure 4.10 shows the comparison of experimental values of enzymes activity with the values predicted by Placket-Burman design.

The Pareto chart shown in Fig. 4.11 offers a convenient view of the results obtained by Plackett-Burman design. The Pareto chart illustrates the order of significance of the factors affecting the production of L-glutaminase. The order of significance as indicated by Pareto chart is; Arginine, L-glutamine, Molasses, Maltose, Ammonium chloride, Wheat bran, Ammonium nitrate, Potassium nitrate.

The first order model equation developed by PB design in terms of coded factors for enzyme activity is:

$$Y = 17.26073 + (-0.89925A) + 1.005583B + 1.498083C + (-1.35108E)$$

$$+ 1.752417F\ (-1.53608H) + 2.70525J + 3.671417K$$

Table 4.3 shows ANOVA for linear regression model. The model F-value 25.10421 shows that model was significant also the p-value 0.00126 confirmed the significance of model. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case six factors; Ammonium chloride, Wheat bran, Molasses, Maltose, L-Glutamine and Arginine were significant terms with respect to $p < 0.05$ while Ammonium nitrate was found to be at margin as its p-value was at 0.053528. High p-value and low F-value makes lack of fit non significant.

The R^2 value for the response indicated that ~89% of the sample variation attributed towards the factors for enzyme activity and only ~11% of the variation could not be elucidated by the model.

Figure 4.12 illustrates the 3D surface plots of interactive effects of some critical factors evaluated by Plackett-Burman design. These plots show the trend of enzyme activity at different concentrations of factors with respect to each other. Ammonium nitrate: ammonium chloride and ammonium chloride: molasses, these two combinations enhance enzyme activity when they are at their centre points. While comparing arginine and L-glutamine with ammonium chloride increasing the concentration of amino acids supported enzyme activity where ammonium chloride concentration should be decreased. Interactive effect of molasses and wheat bran depicts that by increasing molasses concentration enzyme activity can reach to optimum level while wheat bran concentration had opposite effect. Other three combinations molasses:arginine, molasses:glutamine and arginine:glutamine depict enhanced enzyme activity at centre points.

Table 4.2: Plackett-Burmann Design Matrix showing coded values for each factor and their corresponding response

Run	Factors											Response
	A	B	C	D	E	F	G	H	J	K	L	Enzyme Activity (IU)
1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	19.832
2	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	11.05
3	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	11.067
4	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	14.46
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	9.74
6	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	24.74
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	8.72
8	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	14.76
9	0	0	0	0	0	0	0	0	0	0	0	21.96
10	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	15.968
11	0	0	0	0	0	0	0	0	0	0	0	18.65
12	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	25.85
13	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	18.74
14	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	23.22
15	0	0	0	0	0	0	0	0	0	0	0	20.154

[1]

[1] A;Potassium nitrate, B;Ammonium nitrate, C;Ammonium chloride, D;Urea, E;Wheat bran, F;Molasses, G;Glucose, H;Maltose, J;L-glutamine, K;Arginine, L;Glutamic acid.

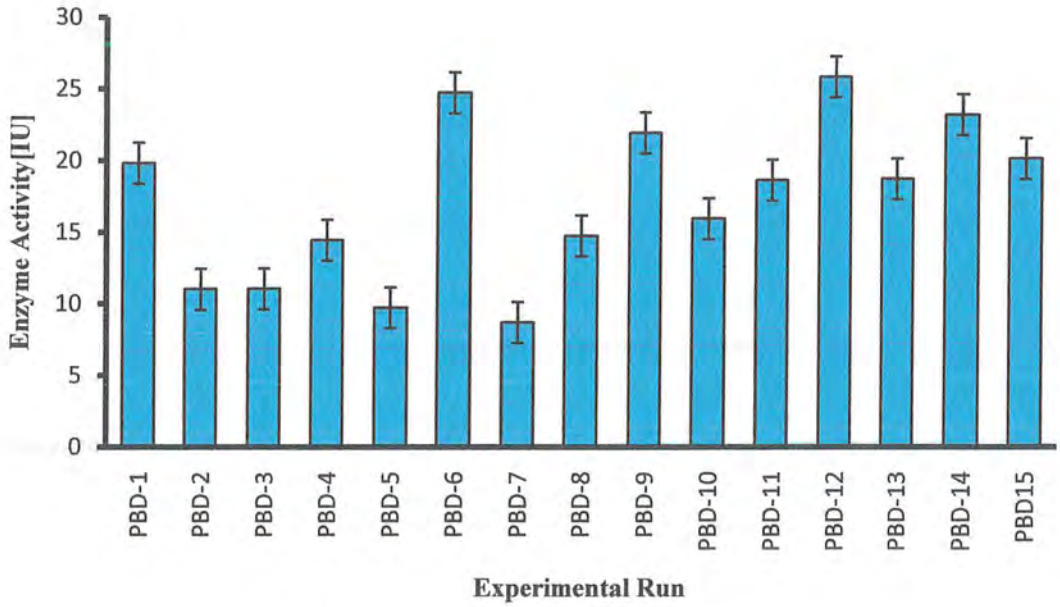

Figure 4.6: Effect of varying media composition on Enzyme Activity[IU]

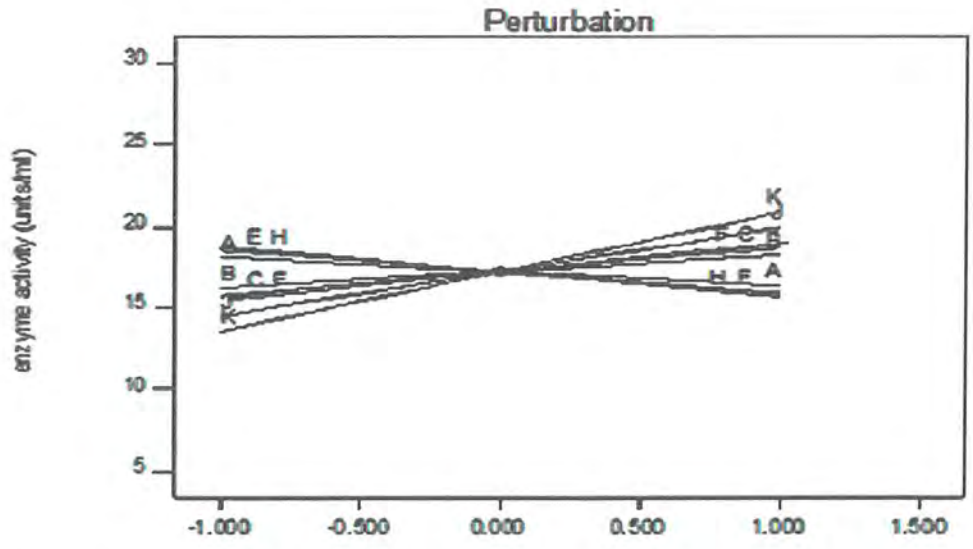

Figure 4.7: Perturbation chart showing effects of most vital components on Enzyme Activity as function of deviation from reference point

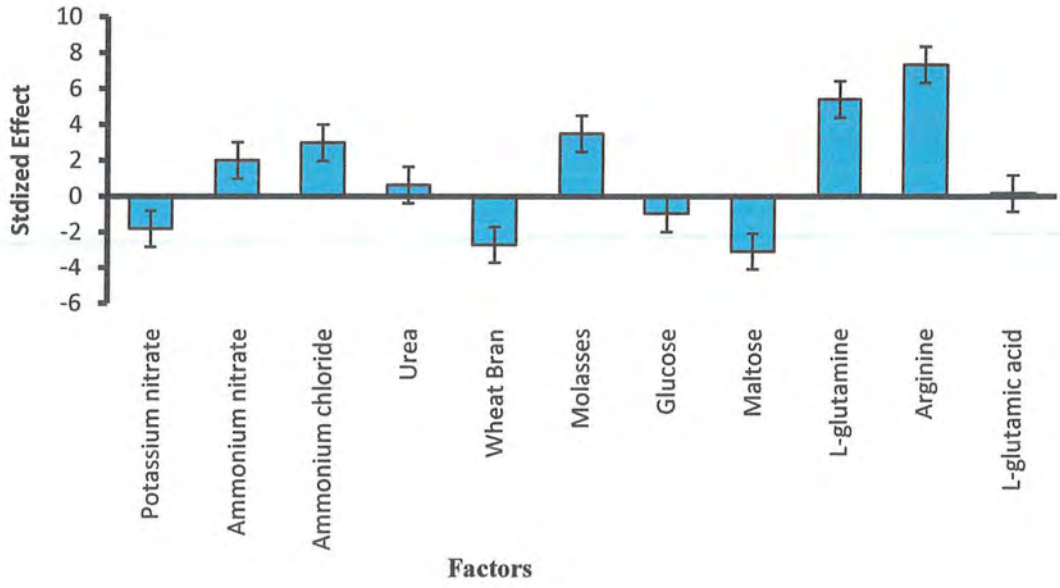

Figure 4.8: Overall effect of each factor on Response (Enzyme Activity IU)

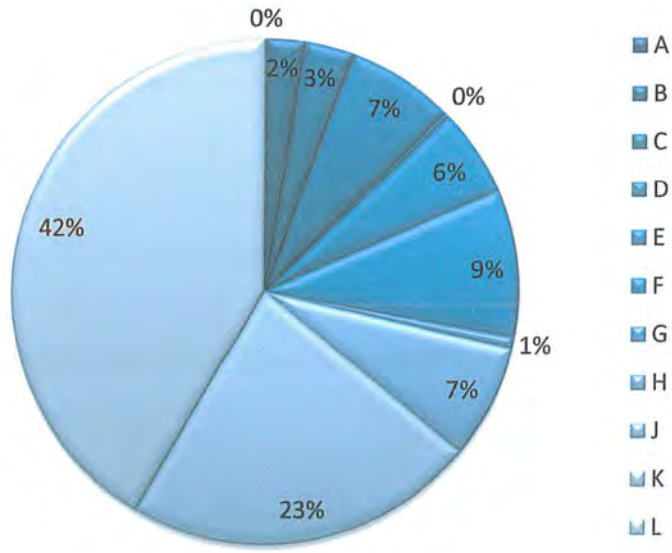

Figure 4.9: Percentage contribution of each factor to response

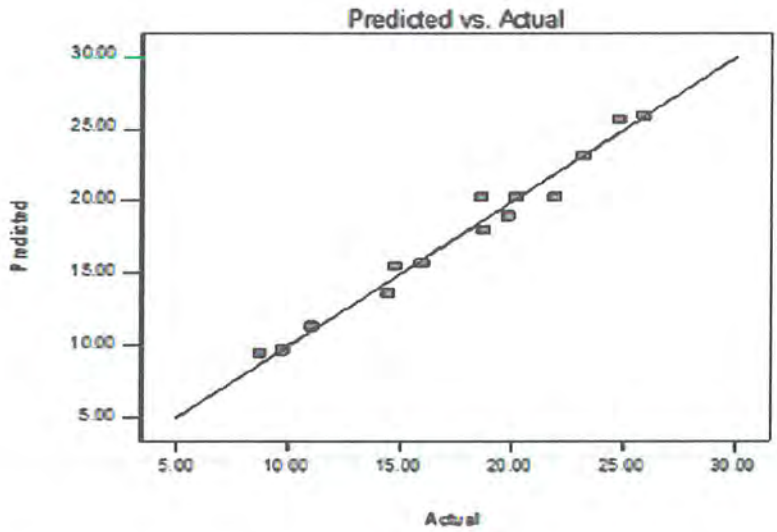

Figure 4.10: Comparison of Predicted vs. Actual values of Enzyme Activity (IU)

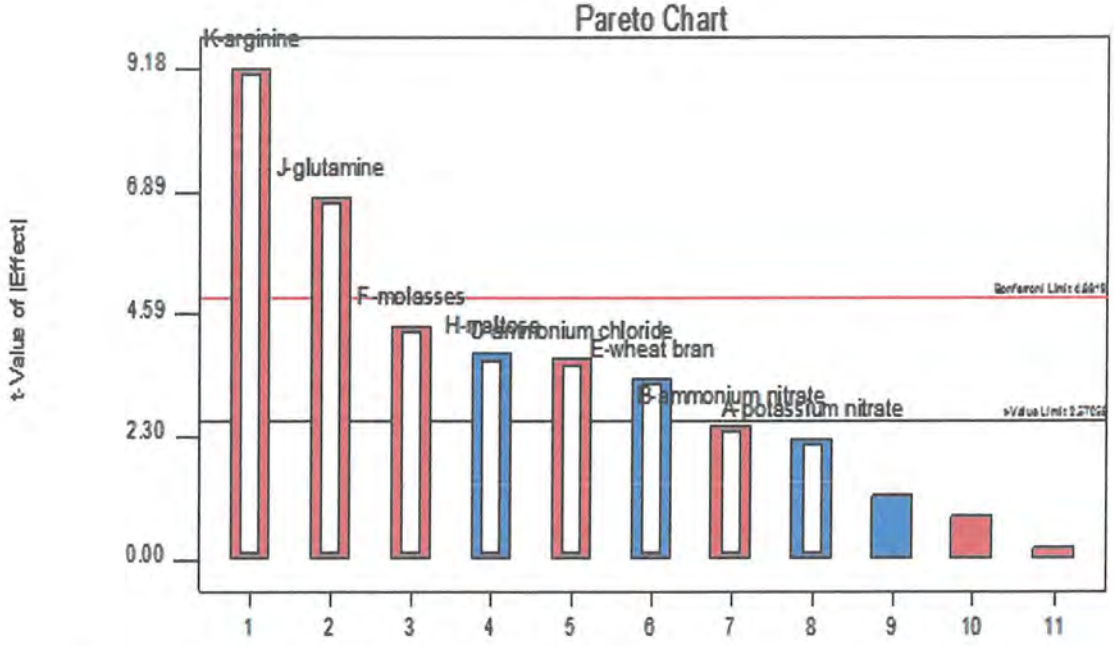

Figure 4.11: Pareto chart showing t-value of effect by important factors, generated by Plackett-Burmann design.

Table 4.3: ANOVA table [Partial sum of squares-Type III] of critical medium components identified from Plackett-Burman design for L-glutaminase production based on enzyme activity (IU)

Source	Sum of squares	df	Mean Square	F Value	p-Value Prob > F	
Model	385.4127	8	48.17658	25.10421	0.00126	**significant**
A-Potassium nitrate	9.703807	1	9.703807	5.056532	0.074401	
B-Ammonium nitrate	12.13437	1	12.13437	6.32307	0.053528	
C-Ammonium chloride	26.93104	1	26.93104	14.03343	0.013347	
E-Wheat bran	21.90511	1	21.90511	11.41448	0.019706	
F-Molasses	36.85157	1	36.85157	19.20289	0.00714	
H-Maltose	28.31462	1	28.31462	14.75439	0.01211	
J-Glutamine	87.82053	1	87.82053	45.76217	0.001072	
K-Arginine	161.7516	1	161.7516	84.28673	0.000257	
Curvature	33.61364	1	33.61364	17.51564	0.008612	
Residual	9.595319	5	1.919064			
Lack of Fit	4.102068	3	1.367356	0.497831	0.720479	**Not significant**
Pure Error	5.493251	2	2.746625			
Cor Total	428.6216	14				

[2]

[2] Std. Dev; 2.68356, Mean; 17.26073, C.V. %;15.54719, PRESS; 189.3994, R-Sqd; 0.899191, Adj R-Sqd; 0.764779, Pred R-Sqd; 0.55812, Adeq Precision; 7.932051.

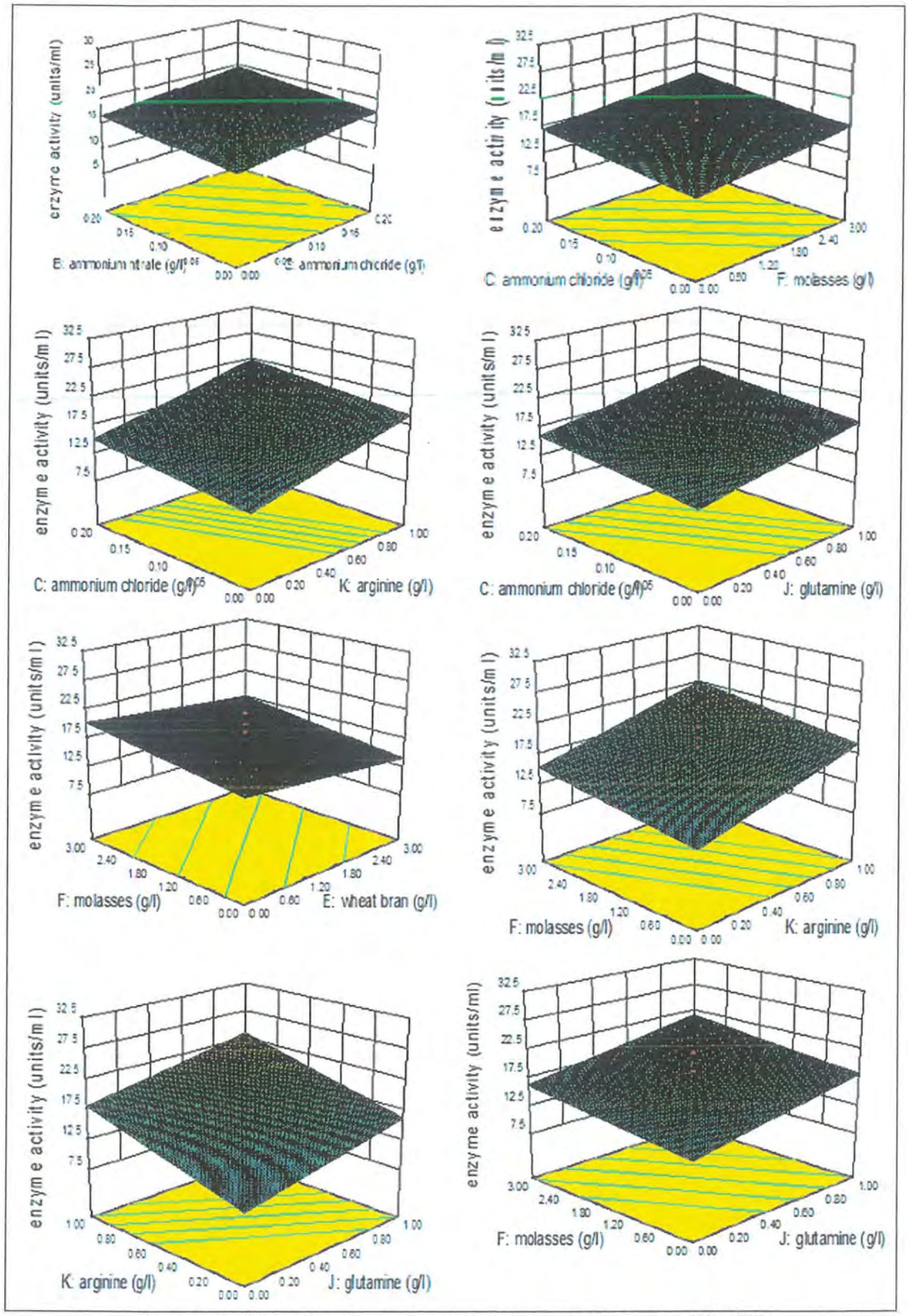

Figure 4.12: Interactive effects of important factors on enzyme activity (IU)

Production of L-Glutaminase from *Epicoccum sp.* NFW1 under Optimized Conditions

L-Glutaminase production under optimized conditions was carried out and enzyme was precipitated. Precipitated enzyme showed enzyme activity of 13 IU and specific activity 56.24 units/mg.

Brine shrimp cytotoxicity assay:

Precipitated enzyme was tested for cytotoxicity against brine shrimps. Enzyme was found to have cytotoxicity thus LD50 was calculated which is given in table 4.4;

Table 4.4: LD_{50} of precipitated enzyme against brine shrimps

Time Period	LD50 µg/ml
24 hrs	620
48 hrs	100

Discussion

Endophytic fungi are the organisms residing within the intracellular matrix of almost every plant species found on earth without causing harm to host. This group of microorganisms is considered untouched due to their wide range of habitats. Endophytic fungi especially those from medicinal plants are excellent source of bioactive metabolites and extracellular enzymes. After the discovery of taxol production from endophytic fungi several studies regarding the production of bioactive metabolites have been conducted with promising results. The present study aimed at the production and optimization of commercially important enzyme L-glutaminase.

L-Glutaminase plays a vital role in the cellular nitrogen metabolism of prokaryotes and eukaryotes. L-Glutaminase has gained attention as a therapeutic against cancer and HIV (Roberts et al., 1970), as a biosensing agent in monitoring glutamine levels (Zhao et al., 2004), for the production of important chemicals like theanine by reaction of γ-glutamyl transfer and as a flavor enhancer in food industry.

The vast experience mounted up in the course of the use of microorganisms for the production on industrial scale of biologically active compounds has been put to use to a substantial degree for the microbial synthesis of enzymes. Collectively with this, the biosynthesis of several enzymes and the techniques of recovering and purifying these enzymes has a number of important aspects which can be beneficially considered while carrying out the production of one of these enzymes for medical purposes, the antitumor enzyme L-glutaminase (Roberts et al., 1970).

Screening of endophytic fungi for the production of L-glutaminase is based on the semi-qualitative method described by (Gulati et al., 1997). The plate assay performed was advantageous and time efficient as well, as it can be visualized directly from the plates without performing any laborious assay. The given endophytic fungi could grow on mCD agar with phenol red, a dye indicator that changes its color from yellow (acidic condition) to pink (alkaline condition). The pink zone around fungal colony indicates the pH alteration which results from ammonia accumulation in the medium. The plate assay was rapid as well as reliable. Those strains which produced pink zone were further selected for quantitative studies.

After initial screening, the endophytic fungal strains were cultured in mCD broth. Enzyme activity was determined using nesselerization method described by Imada et al. (1971). *Epicoccum sp.* NFW1 was found to be the most efficient producer of L-glutaminase. Nathya et al., (2011) reported production of L-glutaminase from *Aspergillus flavus*. Once strain with highest yield was identified, optimization of operational parameters was next step.

Optimization is important in large scale production of any compound. By optimizing the conditions and substrate we try to achieve almost optimum yield of product. In this case initially operational parameters were optimized. Optimum temperature, pH and incubation period were identified, one parameter used to optimize the other one by one.

As large number of microorganisms including bacteria, yeast and fungi are able to produce L-glutaminase, the optimum temperature varies widely for its production. In present study the production of L-glutaminase was checked at different temperatures and was observed that *Epicoccum sp. Epicoccum sp.* NFW1 was able to produce maximum enzyme 15.216 IU at 30°C (Fig 4.3). Maximum specific activity 16.72 u/mg was also observed at the same temperature. Upon increasing and decreasing the temperature yield of enzyme decreased. This finding is approximately in range of the results reported by Chanakya et al (2010). They observed that yield of enzyme increased significantly with increase in temperature from 25-33°C, but at temperatures higher than this range enzyme activity decreased significantly. El-Sayed (2009) also reported the optimum temperature for the production of L-glutaminase from *Trichoderma koningii* at 30° C. He found that production of L-glutaminase decreased gradually above this temperature and a decline in enzyme activity of 77% with reference to that of optimum temperature was seen. Nathya et al (2011) also reported similar results. They found production of enzyme to be at maximum level within the range of 30-40°C by *Aspergillus flavus*.

Medium pH is an important factor contributing to the growth of microorganisms and production of biological compounds. Fungal strains are known for their best performance in the range of 3.5-7.0 (Pandey et al., 2001). Effect of pH was tested on different pH levels: 5.5, 6, 6.5, 7, 7.5. Maximum L-glutminase was observed to be produced at pH 6 (Fig 4.4).

At pH 6 enzyme activity of 13.322 IU and specific activity of approximately 57 u/mg was observed. Substantial amount of enzyme was obtained at other pH levels as well. This result is in agreement with the report of Sivakumar et al (2005) who reported two pH optima, at 6 and 9. El sayed (2009) reported maximum enzyme activity at pH 7 and drastic change of enzyme yield below and above this level. Sabu et al. (2000) reported maximum L-glutaminase productivity by *Beauveria sp.* under SSF at two pH optima, one at pH 6.0 and other at pH 9.

Enzyme production shows direct relation with growth. This might be explained as the growth of microorganism with increasing time causes an imbalance in nutrients and other physicochemical conditions of the medium which effects the production of enzyme. In the present study production of L-glutaminase with reference to the enzyme was studied. Enzyme activity was tested at 3^{rd}, 4^{th}, 5^{th} and 6^{th} day of incubation. Maximum enzyme activity was observed at 4^{th} and 5^{th} day, 17 and 21 IU respectively (Fig. 4.5). Specific activity was also at maximum on these two days, 62 and 69 u/mg. This result is in agreement with Chanakya et al (2010). They reported the production of L-glutaminase to be maximum at 5^{th} day of incubation of *T.koningii*. Also Sivakumar et al (2005) reported maximum L-glutaminase activity at 4^{th} day of incubation. Nathya et al (2011) also reported maximum L-glutaminase yield from *Aspergillus flavus* on 5^{th} day of incubation. They observed a rapid decrease in enzyme activity at 6^{th} and 7^{th} day of incubation which supports the findings of present study. This indicates that as the growth increases production of enzymes decreases may be because of the production of secondary metabolites starts.

Interactions among medium components and cellular metabolism with reference to the production of bioactive compounds and enzymes are significant. Processes used to optimize the conditions ruling these interactions can be designed by use of standard experiment design tool. Over past few years, media optimization for industrial purposes has been evolved through statistical means. Traditionally media components are optimized one by one and physical parameters are optimized one leading to another. This approach does not illicit the total effect caused by interactions of all components included in media (Rathi et al., 2001). Optimization using statistical tools allows quick screening that

covers a large area of experimental domain. It also depicts the effect of each component separately on production of desired components.

In the present study Plackett-Burman design was used to evaluate important carbon and nitrogen sources as well as amino acids for enhancing the production of L-glutaminase. Overall effect of each experimental run, each component and percentage contribution of these components was illustrated by design. Results showed that four out of eleven components (potassium nitrate, wheat bran, glucose and maltose) had negative effect on L-glutaminase production (Fig. 4.8). In previous studies (Chanakya et al., 2010) both glucose and maltose were reported to enhance the production of L-glutaminase by *T.koningii.* Also Sivakumar et al (2005) reported positive effect of glucose on the production of L-glutaminase. This might be explained by the fact that these reports are based on the study of individual effect while present study involves the interactive effects of the components.

In the next step of statistical evaluation order of importance of critical components was obtained in the form of pareto chart. This chart illustrated the order as follow; arginine, L-glutamine, molasses, maltose, ammonium chloride, wheat bran, ammonium nitrate, potassium nitrate (Fig. 4.11). Padma and Rekha (2007) also found L-glutamine as important factor in production of L-glutaminase. They optimized the production of L-glutaminase using response surface methodology.

ANOVA chart showed significance of model and components on the basis of F-value and *p* value. Model was significant and so were the most of the components, thus overall experiment showed significant results. 3D surface plots of critical important factors illustrated the trend of enzyme activity with respect to the increasing or decreasing concentration of interacting factors. Thus overall important factors; molasses, arginine, L-glutamine and ammonium chloride which showed major contribution, were used for optimized production of L-glutaminase for further studies.

To produce enzyme in a concentrated form, acetone precipitation was used. This method is efficient and provides less laborious way to precipitate enzyme. Precipitated enzyme's activity was found to be 13 IU. Cytotoxicity assay was performed using this precipitated

enzyme. For testing cytotoxicity, brine shrimps (Artemia) were used. Concentrations of 1000, 100 and 10 µg/ml of precipitate were tested against brine shrimps and LD_{50} was calculated. LD_{50} of precipitate calculated was 620 and 100 µg/ml at 24 and 48 hrs respectively. High LD_{50} shows that enzyme is less cytotoxic and thus can be used in food and drugs within approved ranges.

As for the wide range of applications of L-glutaminase, it is necessary to explore more diverse and less harmful sources of this enzyme. Bacterial glutaminases prove to be toxic and allergic when used in food industry. Also in medicines they pose a toxicity risk to humans. This calls for a need to explore eukaryotic sources of L-glutaminase. Endophytic fungi prove to be promising source of these enzymes because of their ubiquitous nature and diversity of metabolism.

FUTURE PROSPECTS

- The screened fungal strain can be used to check the production of other valuable compounds and enzymes.
- The fungal strain producing L-glutaminase can be used in industry as well as in medical applications.
- The L-Glutaminase from strain of *Epicoccum sp.* can be studied in detail so that it might be used for the treatment of acute lymphoblastic leukemia.

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Appendix 1:

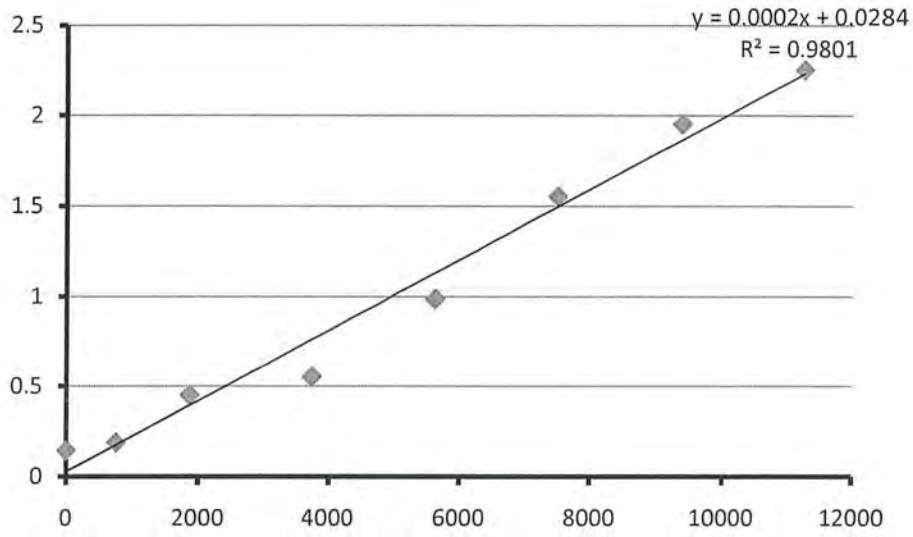

Figure 1: Ammonium sulfate standard curve

Appendix 2:

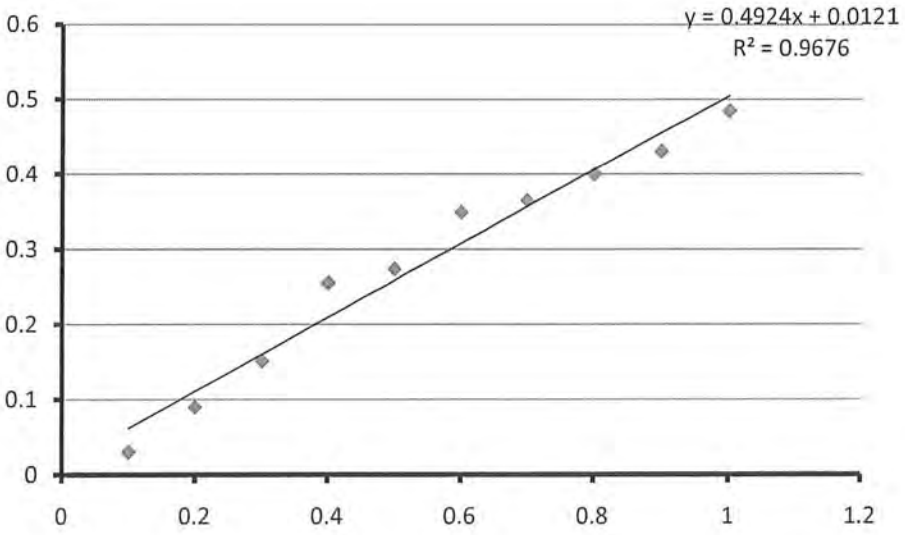

Figure 2: BSA standard curve

Appendix 3:

Figure 3: Quantitative Estimation of Four strains NFW1, NFW6, NFW11, NFW16

Time of Incubation (Days)	Enzyme Activity (IU) NFW1	NFW6	NFW11	NFW16
3rd	13.894	3.364	20.194	5.033
4th	17.188	0.057	16.91	8.943
5th	21.052	2.658	14.71	19.92
6th	9.603	12.52	14.52	9.67

Appendix 4:

Figure 4: Effect of Temperature on Production of L-glutaminase from *Epicoccum sp.* NFW1

Temperature (°C)	Enzyme Activity (IU)	Specific Activity (units/mg)
15	4.5	6.43
25	5.2244	7.62
30	15.216	16.72
37	3.56	5.5

Appendix 5:

Figure 5: Effect of pH on Production of L-glutaminase from *Epicoccum sp.* NFW1

pH	Enzyme Activity (IU)	Specific Activity (units/mg)
5.5	11.2016	25
6	13.3221	56.45
6.5	12.01325	38.88
7	11.16465	27.7
7.5	9.6576	24.33

Appendix 6:

Figure 6: Production of L-glutaminase on different days from *Epicocum sp.* NFW1

Incubation Time(Days)	Enzyme Activity (IU)	Specific Activity (units/mg)
3rd	13.894	57.5
4th	17.188	62.432
5th	21.052	69.77
6th	9.603	25.76

Appendix 7:

Figure 7: Effect of varying media composition on enzyme activity[IU]

Experimental Run	Enzyme Activity (IU)
PBD-1	19.832
PBD-2	11.05
PBD-3	11.067
PBD-4	14.46
PBD-5	9.74
PBD-6	24.74
PBD-7	8.72
PBD-8	14.76
PBD-9	21.96
PBD-10	15.968
PBD-11	18.65
PBD-12	25.85
PBD-13	18.74
PBD-14	23.22
PBD-15	20.154

Appendix 8:

Figure 8: Overall effect of each factor on response (enzyme activity IU)

Factors	Stdized effect
Potassium nitrate	-1.7985
Ammonium nitrate	2.0111667
Ammonium chloride	2.9961667
Urea	0.6418333
Wheat Bran	-2.702167
Molasses	3.5048333
Glucose	-0.962167
Maltose	-3.072167
L-glutamine	5.4105
Arginine	7.3428333
L-glutamic acid	0.1721667

Appendix 9:

Figure 9: Percentage contribution of each factor to response

Factors	% contribution
A	2.263956
B	2.831022
C	6.283174
D	0.288331
E	5.110595
F	8.597693
G	0.647959
H	6.605972
J	20.48906
K	37.73762
L	0.020747