SCREENING AND GROWTH KINETICS STUDIES OF MICROALGAE AS A NOVEL SOURCE FOR ANTITUMOUR ENZYMES PRODUCTION

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CERTIFICATE

This is to certify that project report entitled "**Screening and growth kinetics studies of microalgae as a novel source for Antitumour enzymes production**", submitted by Uttara Shankar in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma.

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SUMMARY

Microalgae as a source for antitumour enzymes have been positive and therefore can be used as a novel source for its production. L-asparaginase is an amidohydrolase enzyme which is used for acute lymphoblastic leukemia chemotherapy. In this experiment we have screened 3 microalgal isolates from different lakes of Himachal Pradesh, India. In this project, enzyme activity of both the enzymes L-Asparaginase and L-Glutaminase have been calculated using their substrates as an inducer for better enzyme production.Parallely, growth kinetics studies also have been carried out to check the growth of the microalgal species in modified medium and utilizing them as a nitrogen source for its growth. Growth parameters such as specific growth rate, doubling time have been calculated henceforth. We have found that *Chlorella speciesand Scendesmus quadricauda* can produce an inducible intracellular and extracellular -asparaginase in the presence of L-asparagine, and L-asparagine can induce more growth of this microalga.

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(Uttara Shankar) (Dr. Garlapati Vijay Kumar)
Date: Date:

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LIST OF ABBREVIATIONS

ALL	Acute Lymphoblastic Leukemia
BSA	Bovine Serum Albumin
DO	Dissolved oxygen
FC	Folin –Ciocalteau
FDA	Food and Drug administration
IB	Inclusion body
IU	International Unit
kDa	Kilodalton
L-ASNase	L-asparaginase
nm	nanometer
OD	Optical density
PEG	Polyethylene glycol
SmF	Submerged fermentation
SSF	Solid –state fermentation
TCA	Trichloroacetic acid

Chapter 1
INTRODUCTION

1.1 Enzymes

Microbial sources have been a major source for enzymes that are employed for the daily needs of humans. They are the biological catalysts that accelerate a chemical reaction without undergoing permanent changes in the structure. The history of modern enzyme technology era kick started in the 19th century when Louis Pasteur concluded while performing fermentation of sugar to alcohol by yeast that it was being catalyzed by a vital driving force called “ferments”, found within the yeast cells. The term *“enzyme”* was coined by a German physiologist Wiliam Kuhne in 1978, which comes from Greek word *ενζυμον* that means "in leaven", to describe this process. Currently a massive interest has been generated in the use of enzymes especially in food, pharmaceutical, environmental and detergent industries ranging from low purity to highly pure pharmaceutical grade enzymes.

1.2 Therapeutic enzymes

Therapeutic enzymes have been used since ages for treatment of many diseases safely such as cystic fibrosis, cancer, inflammation, gout; digestive disorders etc. either in combination with other existing therapies or as alone. They have been widely employed for “Enzyme therapy” and therefore have high specificity and are catalytic and convert multiple targeted molecules into desired products. This makes enzymes as highly potent and specific therapeutics for a plethora of disorders (Vellard et al., 2003). For example, a therapeutic enzyme was described as part of replacement therapies for genetic deficiencies in the 1960s (De Duve, 1966). There are many drawbacks as to why the use of enzymes as therapeutics is limited as they are very large to be evenly distributed within the body cells and another problem being the elicitation of immune response within the host cells after the injection of the foreign therapeutic protein (Ramandeep et al., 2012). This problem has been combated through polyethylene glycol (PEG) by attaching it covalently, by fishing out novel sources and through entrapment in synthetic microspheres, artificial liposomes, and red blood cell ghosts.

1.3 Sources of Therapeutic enzymes

Animal, plant, bacterial and fungal species are the main sources for production of therapeutic enzymes. Microbial enzymes as the major pool as they are cheaper and require constant composed raw materials but one disadvantage they pose is that they can be incompatible with the human body.

Phenolic compounds (from plants), endogenous enzyme inhibitors, proteases and other harmful materials are produced by plant and animal sources.

1.4 Production of Therapeutic enzymes

Different fermentation types ranging from submerged fermentation (SmF) to solid-state fermentation (SSF) has been reported for the production of therapeutic enzymes (Velasco et al., 2012). Many therapeutic enzymes mainly from microbial sources have been performed on large scale along with its downstream processing. Also these enzymes have been grown as heterologous proteins using recombinant DNA technology. Research has been carried primarily on microbes such as bacteria, fungus, and yeast and also on plants etc. Present investigation is focussed on the production of Antitumour enzymes from microalgal sources as recently a lot of interest is being shown on marine species (Anitha et al., 2012; Kim and Kim, 2012) especially microalgae because of the advantages that they show:

1) Very less research and therefore microalgae as source for therapeutic enzymes have been exploited very less. Only two have been reported so far for the production of Antitumour enzymes – Clamydomonas (Paul, 1982) and Chlorella vulgaris (Anitha et al., 2014).

2) They require simple and cheap media unlike plant or animal sources. The need for any complex carbon or nitrogen source is eliminated. They can also use sunlight as their energy source for their growth, the greenhouse gas carbon dioxide as their carbon source and in some cases atmospheric nitrogen as nitrogen source.

3) It is non-toxic and produces health benefits. Much purification is not required like in case of bacteria and fungi where inclusion body (IB formation) is a problem.

4) Its biomass can be used in other process, for example as a complete human and animal diet supplement (Kay and Barton, 1991; Spolaore et al., 2006), incorporation to cosmetics or use as a source of highly valuable molecules like polyunsaturated fatty acid oils, pigments as natural dyes (Spolaore et al., 2006; Shi et al., 1997) or even biodiesel can be produced from algae.

5) Allergy is a key problem when we talk about to bacterial enzymes as they produce toxic compounds such as endotoxins.

Table 1: Different sources, EC number and uses of therapeutic enzymes

Enzyme	EC number	Source	Use
L-Asparaginase	3.5.1.1	*Pseudomonas acidovorans, Acinetobactor sp*	Antitumour
L-Arginase	3.5.3.1	*Bacillus subtilis, E.coli*	Antitumour
L-Tyrosinase	1.14.18.1	*Streptomyces glausescens, Agaricus bisporus*	Antitumour
L-Glutaminase	3.5.1.2	*Beauveria bassiana, Vibrio costicola*	Antitumour
α-Glucosidase; ß-Glucosidase	3.2.1.20;3.2.1.21	*Aspergillus niger*	Antitumour
ß-Galactosidase	3.2.1.23	*E.coli*	Antitumour
Serratiopeptidase	3.4.24.40	*Serratia marcescens*	Anti–inflammatory
Superoxide dismutase	1.15.1.1	*Mycobacterium sp.,Nocardia sp*	Anti-oxidant, Anti–inflammatory
Collagenase	3.4.24.3	*Clostridium histolyticum*	Treatment of skin ulcers
Urokinase	3.4.21.73	Human urine	Blood clots, Anticoagulant
Uricase	1.7.3.3	*Candida utilis*	Gout
Streptokinase	3.4.22.10;3.4.24.29 b	beta haemolytic streptococci	Anticoagulant
Hyaluronidase	3.2.1.35	Bovine testicular tissue extracts	Heart attack
Lysozyme	3.2.1.17	Chicken egg white	Antibiotic

1.5 Cancer

Among the major human diseases, cancer is the most threatening lifestyle disease that is feared by major chunk of people. Cancer rates could increase by 50% to 15 million according to the World Cancer Report by 2020. The death rate is three times greater in developed countries than in developing countries (Kruisinger, 1969; Paymaster and Gangadharan, 1971). Almost 56 million deaths worldwide were caused by 12 percent of the malignant tumours in the year 2000. In a year, presently, 6.4 million deaths due to cancer have been estimated to occur globally and a little over 10 million new cases of cancer (Pisani et al., 1999; Parkin et al., 1999).

According to WHO report, 2003, 6.2 million died altogether out of which 5.3 million were men and 4.7 million were women who developed this malignant tumour. In India, the scenario is much different the incidence rate of cancer is about 70 per one lakh population as compared to 289 per one lakh population. Out of 5, 00,000 patients, 3, 00,000 lakh patients die every year because of cancer. The main reasons for the unabated growth of cancer are its late detection, origin and resistance development.

The Greek physician Hippocrates (460-370 BC), the “Father of Medicine” was credited for origin of the word cancer. Non-ulcer forming and ulcer forming tumours were described by Hippocrates as *carcinos* and *carcinoma* respectively. *Oncos* word was used to describe tumours (Greek word for swelling) by Galen (130-200 AD), a Greek physician.

In the beginning of the 15^{th} century, scientists and surgeons developed greater understanding during the Renaissance period. Many surgeons such as John Hunter (1728-1793), Giovanni Morgagni laid the foundation for study of cancer. With the advent of modern microscope in 19^{th} century, scientific oncology took a major turn by study of the diseased tissues. The modern pathologic study of cancer was laid by Rudolf Virchow and linked pathology to illness.

Many cancer treatments have evolved through the years from the more invasive surgery, hormone therapy to radiotherapy, chemotherapy and recently immunotherapy. Cancer chemotherapy has primarily been directed at discovery of cytotoxic agents capable of inhibiting many aspects of mammalian cell division (Creasey, 1981). Therefore many naturally occurring compounds produced by microorganisms, plants and mammalian cells are being exploited for the development of human anticancer agents. Recent monographs and reviews by Glasby (1976); Aszalos and Berdy (1978); Cassady and Duros (1980); Dourans and Suffress (1981a) can be studied.

1.6 Antitumour enzymes

The present study aims at one of the approaches that certain tumours are auxotrophic for a particular amino acid and one of the methods to treat them is amino acid deprivation (Feun et al, 2008). L-asparaginase, L-Glutaminase are such enzymes that have been exploited as antitumour agents. They act on their respective substrates asparagine, glutamine, arginine and break them down which is essential for the cell survival and proliferation. Their mechanism of action is that normal cells can produce their amino acids therefore it becomes non-essential for them whereas cancer cells are auxotrophic for these as it is required for their survival and they need to acquire it from circulating sources. This difference is used as a key in cancer chemotherapy. By depriving the cells of the amino acid using their respective enzymes, it leads to tumour apoptosis.

Chapter 2

REVIEW OF LITERATURE

2.1 L-Asparginase

L-asparaginases **(E.C.3.5.1.1, L-ASNases**) are successfully used as anti-neoplastic agents in the chemotherapy of ALL (Ekert, 1982; Ali, 1989; Krasotkina et al., 2004). It catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia which is needed for cell survival and growth (Capizzi et al., 1971). L-asparagine is a non-essential amino acid which is synthesized in ordinary human cells by L-asparagine synthetase from aspartic acid. But neoplastic cells cannot synthesize L-asparagine due to the absence of this enzyme; hence, L-asparagine is an essential amino acid for these cells and should be obtained from circulating sources. Therefore, this difference is used as a key in ALL chemotherapy.

2.1.1 Distribution

L-ASNases are widely distributed and have been found in animal, plant, microbial, fungal and microalgal sources (Wriston and Yelli, 1973). Its presence was first reported in guinea pig serum (Clementi, 1922). A number of transplantable lymphomas in mice and rats were inhibited by guinea pig serum as found by Kidd Broome (1953). Deamidation of L-asparagine by extracts of *E. coli* was first reported in 1957(Cedar and Schwartz, 1968). Later, Mashburn and Wriston observed that L-asparaginase, purified from cell extract of *E. coli* has an antitumor activity similar to that of guinea pig serum (Tosa et al., 1971).

2.1.2 Assay methods

Numerous methods have been devised for the assay of L-ASNase activity. The standard unit is international unit (IU), micromoles of L-asparagine hydrolyzed per minute of the assay. One IU is the quantity of enzyme that catalyses the formation of 1 mole of NH_3 per min under the given conditions.

L-asparaginase activity can be measured by:

1) Disappearance of alternative substrate (Jackson and Handschumacher, 1970).

2) Direct nesslerization of ammonia (Wriston, 1970)

3) Determination of aspartic acid (Howard and Carpenter, 1972)

4) Determination of disappearance of asparagine (Howard and Carpenter, 1972)

Direct nesslerization is the most suitable method as it is highly sensitive and can detect enzyme concentrations as low as 0.1 IU/ml.

2.1.3 Biological effects of L-ASNase

2.1.3.1 Pharmacological aspects of L-ASNase

Mostly L-ASNase from *E.coli* have been studied for most of the L-ASNase sensitive neoplasms like that in Rat (Kidd, 1953), Mouse (Broome, 1961; Boyse et al., 1963; Mashburn and Wriston, 1964), Swiss albino mice (Ali et al., 1994) and in Man (Oettgen at al., 1967; Hill et al., 1967).

The activity can be detected in plasma when the enzyme is administered intravenously. Rapid absorption into blood is achieved through intra-peritoneal and intramuscular injections. No enzyme activity was observed when given orally. But it can cause anaphylactic reactions especially in case of *E.coli* (Elspar^{R}) as bacteria produce a number of endotoxins that could be harmful (1978). Thus enzyme preparation from *Erwinia chrysanthemi* (Erwinase^{R}) was approved by the FDA (2011) to combat with the side-effects produced by Elspar^{R}.

2.1.3.2 Km values

Two L-ASNase were isolated from E.coli K-12 designated as II and I (Schwartz et al., 1966). L-ASNase I doesn't inhibit tumour growth whereas L-ASNase located on the cell surface induced by anaerobosis is highly active against ALL tumours (Cedar and Schwartz, 1968). Km values are seen as responsible for the differences in their antitumour property. L-ASNase is saturated to 10^{-4} M L-asparagine whereas L-ASNase at this concentration has only like 5% of its maximum velocity. The clinically approved *Erwinia* L-ASNase has a Km value of 10^{-5} (Summers and Handschumacher, 1971). At physiological ph, L-ASNase has high affinity which is an absolute prerequisite for antitumour activity. Until and unless the concentration of L-asparagine does not drop till 10^{-5} or below, it is not severely rate limiting in protein synthesis (Sobin and Kidd, 1965; Broome, 1968).

2.1.4 Clinical aspects of L-ASNase

The first clinical use of L-ASNase was for the treatment for a human patient (Dowly et al., 1967). It has been successfully used as antineoplastic agent against ALL. Complete remission in ALL by Hill

et al, 1967 has made the use of L-ASNase widespread in the use of ALL. More than half of the patients with ALL achieved complete remissions when treated in the early stages of the disease. Three dogs who were suffering from lymphosarcoma at their advanced stage were treated with *E.coli* L-ASNase preparation. All three responded to therapy by marked regression in lymph nodes leading to drastic improvement in general condition without any evidence of toxicity (Old et al., 1967).

Brown et al (1986) showed the clinical pharmacology of polyethylene glycol (PEG) L-ASNase. In this experiment, PEG-L-ASNase was infused intravenously in 31 patients at doses ranging from 500-8000 units/m^2 out of which 27 were evaluated pharmacokinetically. A sudden disappearance of the enzyme levels was found in two patients which was preceded by anaphylactic reactions during subsequent treatment. And, a third patient developed severe bronchospasm 30mins after the first dose was given, but his enzyme levels were within the normal window.

Clarkson et al (2006) investigated the clinical results with E.coli L-Asparaginase in adults with leukemia, lymphoma and solid tumours.

2.1.5 Mode of action of L-ASNase

The mode of action of L-ASNase is interesting because most of the tumours are extra vascular and to exert the desired affect the enzyme has to penetrate from blood into the microenvironment of the tumour cells.

When the enzyme is administered into the vascular system it catalyses the hydrolysis of L-asparagine to aspartic acid and ammonia which prevents the supply of asparagine (Broome, 1968). The tumours which are dependent on the amino acid for their survival are hence starved leading to tumour apoptosis.

The enzyme is unique as chemotherapeutic agents in the sense as it uses the difference as a key in ALL chemotherapy; as its action is based on the fact that certain tumours are auxotrophic for specific amino acids.

The enzyme molecules pass into the intercellular spaces from fine capillaries and break down L-asparagine thereby preventing its free diffusion to the cancerous cells from the surrounding tissues (Putter, 1970). As a result, large amount of L-aspartic acid and ammonia are accumulated have been reported that have no such role in tumour regression (Hill et al., 1967; Broome, 1968; Miller et al., 1989).

Voigt et al., 1970 have observed the cytomorphological effects in sensitive cells under light and electron microscope. The enzyme was found to exert changes in the histological structure of solid tumours as well as in single cells of leukemia after 48 hrs of the administration.

2.1.6 Physical and Chemical properties of L-ASNase

Size and shape of *E.coli* enzyme have been determined through physical studies. Crystallographic studies provide information on its tetrameric structure (Epp et al., 1971). Furthermore, the subunits are structurally similar.Swain et al., 1993 identified the crystal structure of both *E.coli* L-ASNase as well as *Erwinia chrysanthemi* L-ASNase. Both these enzymes are active in their homotetrameric form; each monomer containing 330 amino acids.

2.1.7 Microbial production of L-ASNase

L-ASNase can be produced by both submerged as well as solid state fermentation as discussed below.

2.1.7.1 Production of L-ASNase through submerged fermentation

Submerged fermentation is mostly preferred for bacterial species. Growth of fungi is not preferred in submerged fermentation (SmF) as their natural form of mycelia causes viscosity in the media in which they are growing, causing problems in mixing and aeration.

The following is a list of the workers who've reported L-ASNase production through SmF.

Heinemann and Howard, 1969 reported the production of L-ASNase by submerged growth of *Serratia marcescens* ATCC 60. Five strains of *S. marcescens* were screened in shake-flask studies and 48 hrs after inoculation were found to produce 0.8 to 3.7 IU/ml. The requirements for asparaginase production with *S. marcescens* ATCC 60, the highest producing strain, included the following: an incubation temperature of 26 degree Celsius, 4% autolyzed yeast extract medium (initial *p*H 5.0), and limited aeration to maintain a zero level of dissolved oxygen (DO) during the fermentation. Yields were not enhanced by addition of various carbohydrates to the fermentation medium. Highest enzyme yields were found when the *p*H of the fermentation cycle rose to approximately 8.5. Yields of asparaginase/ml (4 IU) of cell suspension have been obtained consistently in 40 to 42 hr from 10-liter volumes (500 ml/4-liter bottle) produced on a reciprocating shaker. Also yields of 3.1 IU/ml were obtained by scale-up to a 60-liter fermentor in 35 hr.

Maria et al., 2004 have investigated the filamentous fungi *Aspergillus tamarii* and *Aspergillus terreus* for L-ASNase production by SmF. Nitrogen source affect its growth rate.

Peterson and Ciegler, 1969 obtained maximum yields of 1,250 IU/g of L-ASNase from *Erwinia aroideae* NRRL B-138. They performed partial purification and concentration of the extracted L-asparaginase that yielded a preparation with an activity of 275 IU/ml.

Karamitros and Labrou, 2014 performed extracellular expression and purification of L-ASNase from *E.chrysanthemi* and *E.coli*. The strain E. coli Rosetta (DE3) exhibited the highest extracellular expression levels among all the strains tested and it was chosen for further optimization and the development of purification protocol.

Upadhyay et al., 2014 reported the refolding and purification of recombinant L-ASNase from inclusion bodies (IBs) of *E.coli* into active tetrameric protein. Protection of the existing native-like protein structure during solubilization of IB aggregates with 4M urea improved the propensity of monomer units to form oligomeric structure. Bioactive tetrameric form of L-ASNase was efficiently recovered by retaining the native-like structures through their mild solubilization technique.

2.1.7.2 Production of L-ASNase from Solid state fermentation

Solid state fermentation (SSF) is when a microorganism is allowed to grow on a solid matrix in absence or near absence of free water. Moisture level is optimised for such processes. SSF started initially as Koji fermentation and has gained importance in the recent years for the production of enzymes due to high yields.

Komathi et al., 2013 isolated, produced and partially purified L-ASNase from *Pseudomonas aeruginosa* by SSF. The isolated strain was subjected to SSF techniques along with Soya bean meal maker as substrate. The high amount of L- asparaginase production was observed in 30 mins incubation on optimal medium. Further, the maximum enzyme activity was recorded at 40ºC.

Abha Mishra, 2006 reported the production of L-ASNase from a new isolate of *Aspergillus niger* in SSF using agro wastes from three leguminous crops. *Glycine max* as a substrate is economically attractive as it is a cheap and readily available raw material in agriculture-based industries and therefore asparaginase production process should be developed on the basis of bran of *G.max*.

2.2 L-Glutaminase

L-Glutaminase (**L- Glutamine aminohyrolase EC 3.5.1.2**) that removes the amide group from L-Glutamine has a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes. The rates of enzymatic synthesis and degradation determine the intracellular levels of glutamine in microorganisms (Prusiner et al., 1976). Glutamine synthetase works antagonistically to the action of glutaminase (Meister et al., 1955).

L-glutaminase is also used in ALL chemotherapy with the fact that the tumours are dependent on glutamine for their survival. Ability of the enzyme to bring about deprivation of the amino acid glutamine poses it as a possible candidate for enzyme therapy which may soon replace or combined with L-ASNase in the treatment of ALL. However, the large scale application of glutaminase in cancer chemotherapy it's still under experimental conditions and not much information is available.

2.2.1 Distribution

Commercial production of L-glutaminase is mostly from mammalian tissue while it is widely distributed in animal tissues and microbes. The distribution of L-glutaminase in various microorganisms was studied extensively by Imada et al., 1973. 464 strains of bacteria, 1326 strains of yeast and 4185 fungal strains were tested for glutaminase activity.

2.2.2 Assay methods

It is the same as for L-ASNase. The standard unit is international unit (IU), micromoles of L-glutamine hydrolyzed per minute of the assay. One IU is the quantity of enzyme that catalyses the formation of 1 mole of NH_3 per min under the given conditions. The most preferred method being nesslerization of ammonia (Wriston, 1970).

2.2.3 Clinical aspects of L-glutaminase

Baskerville et al., 1980 found that the enzyme caused dysentery and diarrhoea that was fatal within 10 days when chemically modified microbial glutaminase was administered to mice, rabbits, marmosets, and rhesus monkeys at various doses. Hambleton et al., 1980 reported that treatment with chemically modified glutaminases was lethal to rabbits and rhesus monkeys and produced lesions in kidney; liver and intestine while treatment with unmodified glutaminase induced changes in rabbits but not in rhesus monkeys.

2.2.4 Physical and Chemical properties of L-glutaminase

X-ray diffraction was used to determine the structure of L-glutaminase. It is a dimeric protein that contains two chains containing 305 residues.

2.2.4.1 Molecular weight

P. aurantiaca was the source for higher molecular weight L-glutaminase (148 kDa) (Imada et al., 1973). *P. nitroreducens* is the source for the lowest molecular weight L-glutaminase (40 kDa) (Tachiki et al., 1996). Some of the bacterial L-glutaminases are reported as dimers and tetramers while most of them are monomers.

2.2.5 Microbial production of L-Glutaminase

Microbial L-glutaminases can be produced through both submerged and solid state fermentation as discussed below.

2.2.5.1 Production of L-glutaminase by SmF

Prasanth Kumar et al., 2009 performed the screening of extracellular L-glutaminase from marine bacterial strains isolated from sediment and water samples collected from different places along the beach of Bay of Bengal in Vishakhapatnam. LG24 among the thirty isolates showed the highest activity of 22.68 U/ml where in L-glutamine was supplemented as sole carbon and nitrogen source.

Padma Iyer et al., 2008 using statistical optimization through response surface methodology for the production of L-glutaminase from *Zygosaccharomyces rouxii*. The medium containing yeast extract; glutamine, and sodium chloride, that resulted to produce an enzyme activity of 149.98 U/l and 0.488 U/mg protein of specific activity.

Das Mousumi and Agsar Dayanand, 2013 reported the production of L-glutaminase from *Streptomyces enissocaesilis* DMQ-24. Starch and L-glutamine 1.0 % w/v were proven to be the best carbon and nitrogen sources for the enhanced production of L-glutaminase. Through SmF the maximum production of L-glutaminase (31.55 IU) was achieved.

Ahmed et al., 2015 optimized the synthesis of L-glutaminase by *Aspergillus oryzae* NRRL 32657 that proved to be the highest producer (24.19 U/ml). Effect of incubation period, initial pH level, sodium chloride concentrations, concentration of nitrogen and incubation temperature were tested on enzyme synthesis. The highest L-glutaminase synthesis (46.53 U/ml) was allowed to grow aerobically in a buffered medium containing 2% yeast extract, 2.5% lactose, 0.5% L-glutamine and sodium chloride.

2.2.5.2 Production of L-Glutaminase by SSF

Sabu et al., 2000 produced extracellular L-glutaminase from *Bauveria sp*. was isolated from marine sediment through SSF using polystyrene as an inert support. After 96 hrs incubation maximal enzyme production (49.89 U/ml) was observed in seawater based medium supplemented with L-glutamine as substrate.

Ashraf and Al- Shayd, 2009 conducted SSF for the production of L-glutaminase by *Trichoderma koningii* using different agro-industrial by-products such as wheat bran, soybean meal, rice hulls, groundnut residues, corn steep, lentil residues and cotton seed residues as solid substrates. For induction of L-glutaminase (121. U/mg) by *T. koningii,* wheat bran was the best substrate. Under SSF the productivity of L-glutaminase by *T. koningii* was increased by 2.2 fold in comparison to submerged culture.

Nathiya et al., 2011 optimised the production of L-glutaminase from *Aspergillus flavus* KUGF009 cultured on agro industrial residues. On the 5^{th} day of incubation high levels of glutaminase were produced at an optimum pH 4.0, temperature 30^{o}C and moisture content 50% by SSF.

2.3 Microalgae

In Germany during World War II, the large scale cultivation of microalgae and the potential use of its biomass were considered. At the Carnegie Institution of Washington, the scientists aimed at the use of *Chlorella* for large-scale production of food. In the course of time, techniques for the cultivation of algae on large scale were carried out along with its utilization in several developing countries (Venkataraman and Becker, 1985).

2.3.1 Therapeutic enzymes from Microalgae

J H Paul, 1982 purified L-ASNase from a marine *Clamydomonas* species, the first enzyme to be purified from a microlga. The purified enzyme possessed a Km value of $1.34x10^{-4}$ M.

Alireza Ebrahiminezhad et al., 2014 obtained a novel source for L-ASNase production –*Chlorella vulgaris*. In this experiment, 40 microalgal isolates from south of Iran were screened by examining their intra- as well as extracellular L-ASNase activity.

2.3.2 Growth kinetic studies of Microalgae

The following shows a list of the workers that have carried growth kinetics on microalgae which are as follows;

Yue Wang et al., 2014 characterized and studied the kinetics of bio-butanol production with *Clostridium acetobutylicum* using mixed sugar medium simulating microalgae-based carbohydrates. The hydrolyzed biomass of *Chlorella vulgaris* JSC-6 was used as the prime source for biobutanol production from *C. acetobutylicum* ATCC824. Using Michelis-menten and Monod –type models, the product formation and growth kinetics based on xylose, glucose and mixture of both the sugars were determined. The resulting maximum butanol production rate was 0.89-0.93 g/h/L which was similar in case of both glucose alone or when mixed sugars was used.

Guido et al., 2013 studied the effect of light, ph, intensity and temperature on triacylglycerols (TAG) accumulation induced by nitrogen starvation in *Scenedesmus obliquus* UTEX 93. To make the production of TAGs economical, effect of different parameters such as ph, temperature were studied under nitrogen deprived conditions. At the lowest intensity, pH 7 and 27.5 degree Celsius, the highest yield of fatty acids was achieved.

Shuo Yan et al., 2014 studied the modelling of microalgae growth in nitrogen –limited culture system for estimation of biomass productivity by comparing different coefficients and expressions in several models. And due to this the results of numerical modelling differ.

Worrarat et al., 2015 studied the kinetics of *Scenedesmus obliquus* microalgae growth utilizing carbon dioxide gas from biogas. The biogas was derived from an anaerobic digester utilizing animal wastes and also an anaerobic reactor utilizing wastewater, containing 60% CH_4 and 40% CO_2.The biomass productivity and the maximum specific growth rate for *S. obliquus* was 0.145 g/L/d and 0.56 d^{-1} respectively.

Rakesh Singh Gaur et al., 2014 carried out screening of the native predominant algal species of Himachal Pradesh, India for growth and lipid accumulation properties. The dominant species being *Chlorella sp.* which was followed by *Scenedesmus dimorphus* and *Scenedesmus quadricauda* in the sample collections. Urea can be used as an alternative nitrogen source for commercial scale – cultivation as the biomass productivity and biomass yield was greater in BG-11+0.1% urea as compared to BG-11 medium.

OBJECTIVES OF THE STUDY

The present study has been undertaken to utilize microalgae as novel source for production of the antitumour enzymes (L-asparginase, L-glutaminase) with the following objectives.

1. Cultivation, harvesting and extraction of microalgal species predominant in the state of Himachal Pradesh. The native species undertaken for the study are *Chlorella sp., Scenedesmus dimorphus* and *Scenedesmus quadricauda.*

2. Growth of the microalgal species in normal as well as modified medium (containing substrate). The modified medium along with acting as a nitrogen source for microalgal growth also induces the production of the enzyme.

3. Screening for enzymatic activity by performing assay.

4. Kinetic studies of the enzymes by comparison of growth parameters in normal and modified medium.

Chapter 3

MATERIALS AND METHODS

3.1 Materials and Chemicals

L-asparagine was obtained from LobaChemie (Mumbai) whereas L-glutamine was obtained from SRL (Mumbai). All the other chemicals used were analytical grade and obtained from Merck (Mumbai) and SRL (Mumbai). Nessler's reagent used for assay was procured from CDH (Central Drug House), New Delhi present already in the lab.

3.2 Equipments used

Autoclave

Weighing balance

Spectrophotometer;

Water bath

Centrifuge

Microscope

Magnetic stirrer

3.3 Media preparation

3.3.1 BG-11 medium (Control)

Stock Solution 1	**Amount (g/L)**
EDTA (disodium salt)	0.001
Ferric ammonium citrate	0.006
Citric acid	0.006
$CaCl_2.2H_2O$	0.036

Stock solution 2	**Amount (g/L)**
$MgSO_4.7H_2O$	0.075
Stock solution 3	**Amount (g/L)**
K_2HPO_4	0.04
Stock solution 4	**Amount (g/L)**
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$CuSO_4.5H_2O$	0.079
$ZnSO_4.7H_2O$	0.222
Na_2MoO_4	0.39
$Co\ (NO_3)_2$	0.494
$NaNO_3$	1.5
Na_2CO_3	0.02

Optimize pH 7.5 and autoclave.

3.3.2 Induced medium

To induce the production of the enzymes NaNO3 in the BG-11 media was replaced with their respective substrates- L-glutamine and L-asparagine (10g/L).

3.4 Preparation of reagents

3.4.1 Reagents for L-asparginase and L-glutaminase estimation

Nessler's reagent was used for the assay of both the enzymes.

3.4.1.1 Tris-HCl buffer

50mM Tris-HCl buffer was prepared in distilled water (pH 8.5).

3.4.1.2 L-asparagine and L-glutamine solutions

L-asparagine and L-glutamine solutions were prepared at a concentration of 10mM each in distilled water.

3.4.1.3 TCA solution

15% (w/v) TCA solution was prepared in distilled water.

3.4.2 Reagents for protein estimation by Lowry's method

FC (Folin-Ciocalteau) reagent was present in the lab.

3.4.2.1 Bovine Serum Albumin (BSA) solution

BSA solution was prepared at a concentration of 1mg/mL for the standard plot.

3.4.2.2 Preparation of Reagent C

Reagent A

Sodium carbonate 20 g/L

Sodium hydroxide 4 g/L

Reagent B-1

Copper sulfate 10 g/L

Reagent B-2

Potassium sodium tartarate 20 g/L

Reagent C

Reagent B-1 1ml

Reagent B-2 1ml

Reagent A 100ml

TO BE MIXED FRESH AT THE TIME OF ASSAY ONLY

3.5 Methodology

3.5.1 Isolation and maintenance of micro algae species

The algal samples were isolated from different ponds, streams and lakes from various geographical areas of Himachal Pradesh. Enrichment of 5-10 mL water samples was done with BG-11 medium at the time of sampling and they were also preserved by the addition of 10% formaldehyde. The culture flasks and plates were cultured in liquid, solidified and modified BG-11 medium and were incubated at 25+_2^{o}C, 3000 lux intensity with 16:8 hours light and dark phase (Rakesh Singh Gaur et al., 2009).

3.5.2 Screening of micro algal isolates for L-asparaginase and L-glutaminase production

Screening for L-ASNase and L-glutaminase was done by measuring the rate of liberation of ammonia using L-asparagine and L-glutamine as substrate respectively (Imad et al., 1973). Assay is based on direct nesslerization of ammonia. Enzyme solution (30 µl) was added to Tris-HCl (pH 8.5, 50mM) to make up the volume till 1.5mL. 0.5mL L-asparagine solution (10mM, in 50mMTris–HCl, pH 8.5) was added to start the reaction and the reaction tubes were incubated for 20min in water bath at 37^{o}C. The reaction was terminated with addition of 0.5mL trichloroacetic acid 15 %(w/v) and the volume was made up to 4.5 mL with distilled water. Nessler's reagent (0.5mL, 45.5gHgI2 and 35.0gKI per litre distilled water containing 112g of KOH) was added and the tubes were incubated at room temperature for 15min. Using visible spectrophotometer, the absorbance was measured at 500nm. One International unit (IU) is the amount of enzyme which liberates 1µmol of ammonia per minute per ml [µ mole/ml/min].

3.5.3 Identification of uni-algal cultures

Serial dilution from 10^4 to 10^6 times of the algal culture was done and was plated on BG-11 medium with 1.5% agar. The isolated colonies were carefully picked that appeared after 2-3 weeks and were transferred to a glass slide containing a drop of BG-11 medium and observed under the microscope for morphological identification (Fig 1). Uni-algal cultures for all three microalgae *viz. Chlorella sp, Scenedesmus dimorphus* and *Scenedesmus quadricauda* were established (Rakesh Singh Gaur et al., 2014)

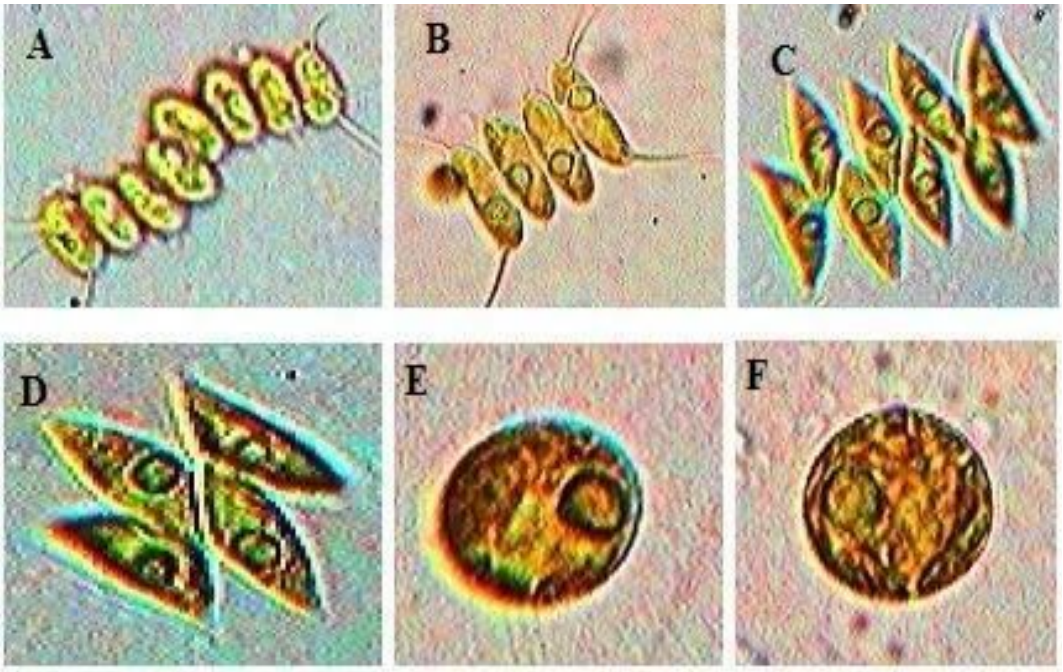


Fig 1 Cell morphology of micro algal species: A & B) *Scenedesmus quadricauda,* C & D) *Scenedesmus dimorphus,* E & F) *Chlorella sp*. (Rakesh Singh Gaur et al., 2014)

3.5.4 Ammonia Standard Curve

Ammonia standard curve was prepared by taking 1mM stock solution of $(NH)_2SO4$ in distilled water.

3.5.5 Standard Curve for Protein Determination

Protein estimation was carried out by Lowry's method using bovine serum albumin (BSA) as standard. The color change determines the concentration of protein in the sample solution which can be measured using colorimetric techniques. It employs the Biuret test followed by the oxidation of aromatic protein (tryptophan, phenylalanine, tyrosine) residues. It can measure protein concentrations of the range 0.01-1.0 mg/mL and is based on the reaction of FC (Folin –Ciocalteu) reagent with Cu^+ ions. The protein concentration is measured by taking absorbance at 750 nm which detects the reduced form of FC.

3.5.6 Fermentation for L-ASNase and L-glutaminase production

200 mL of BG-11 medium (control) and modified BG-11 medium (containing the respective substrates) were inoculated with 20 mL inoculum of each species and was kept at 18-25^{0}C and 3,000-4,000 lux light intensity with 16:8 (light: dark) phase with 3 times shaking each day.

3.5.7 Growth kinetics setup for microalgae

The same setup was put as for the enzyme production in normal as well as induced medium to check the growth response of microalgae to different substrates as nitrogen source. The readings were taken every 3 days i.e. day 0, 3, 6, 9… and so on followed by taking absorbance at 730 nm and cell counts using hemocytometer.

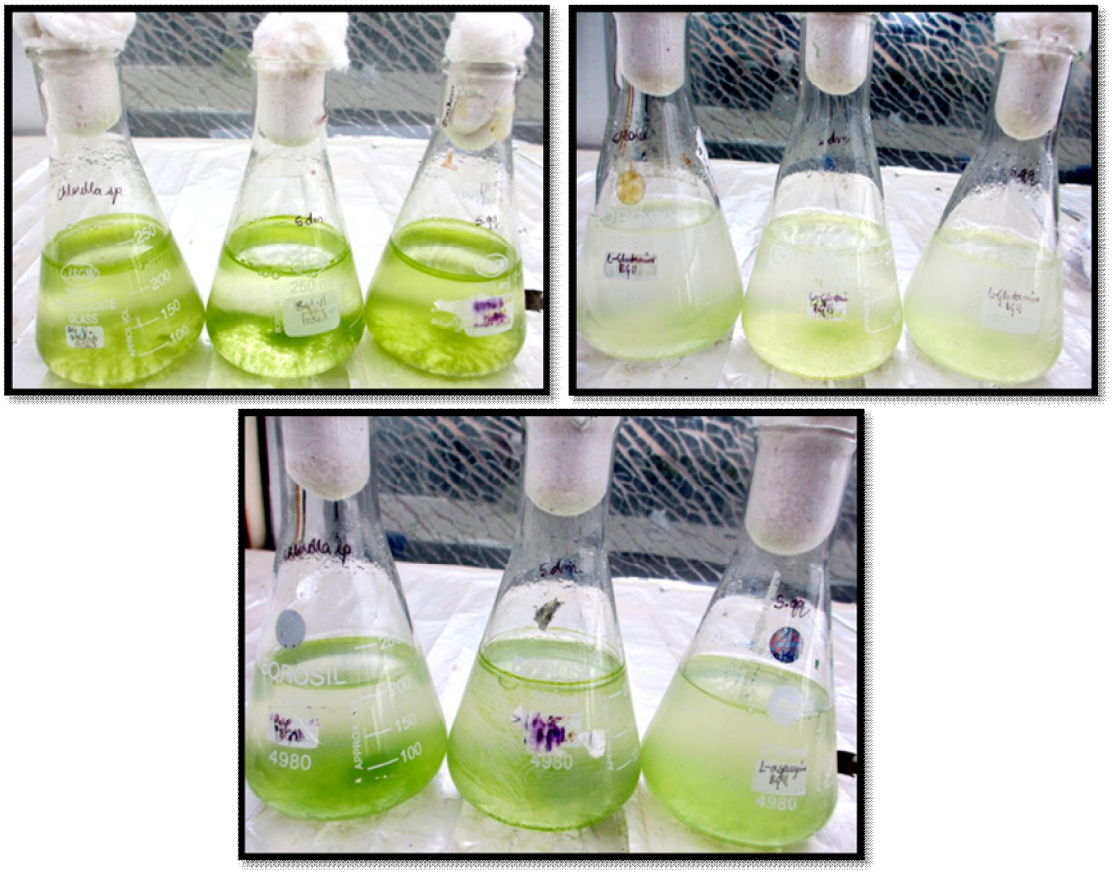


Fig 2 Growth kinetics setup for microalgae. Top left picture shows the growth in **control** media, top right corner picture shows the growth in **L-glutamine induced media** and the third picture shows the growth in **L-asparagine induced media**.

3.6 Enzyme production

After 15 days, the cells were harvested by centrifuging at 5,000xg at 4^0C for 10 mins. The supernatant thus obtained was used to assay extracellular L-ASNase and the pellets obtained were freeze-dried and 0.1 g of dry cell wt. was suspended in 80 µl of sonication buffer (10 mM and 50 mM tris, pH 7.5) in 1.5 mL eppendorf. Cells were disrupted using probe –sonicator on ice. The cell debris was centrifuged at 13,000xg at 4^0 C for 20 mins. The supernatant thus obtained was used for intracellular assay (Ebrahiminezhad et al, 2011).

3.7 Assay of enzymes

3.7.1 L-ASNase assay

L-ASNase assay estimates the liberation of ammonia generated by the action of L-ASNase on its substrate L-asparagine. A volume of 30µl of enzyme solution was added to Tris –HCl (pH 8.5, 50mM) to make up the volume till 1.5 mL. Addition of 0.5 mL of L-asparagine (10 mM) was done to start the reaction followed by incubation of tubes in 37°C water bath for 20 mins. Then the reaction was terminated by adding 0.5 mL trichloroacetic acid (TCA) 15% (w/v) and volume was attuned to 4.5 mL with distilled water. Nessler's reagent was added followed by 15 mins incubation at room temperature. The color development was measured using spectrophotometric method by taking absorbance at 500 nm. The assay was performed in duplicate with proper blank.

One enzyme unit (IU) was the amount of enzyme liberating 1µmol of ammonia per min under assay conditions.

$$\text{Enzyme activity (U/ml)} = \frac{\textit{Amount of NH3 liberated}}{\textit{Incubation time x mL of enzyme taken for test}}$$

3.7.2 L-Glutaminase assay

The assay procedure is the same as for L-ASNase and estimates the liberation of ammonia generated by the action of L-glutaminase on its substrate L-glutamine.

One enzyme unit (IU) was the amount of enzyme liberating 1µmol of ammonia per min under optimal conditions.

$$\text{Enzyme activity (U/ml)} = \frac{\textit{Amount of NH3 liberated}}{\textit{Incubation time x mL of enzyme taken for test}}$$

3.8 Estimation of protein

For estimation of protein content in the enzyme samples Lowry assay was done. The protein samples, blank, BSA standards were tested at 750 nm. Dilution of the protein sample sometimes was done for the resulting absorbance to fall within the linear range of the assay.

Chapter 4

RESULTS AND DISCUSSION

4.1 Screening of L-ASNase and L-Glutaminase producing isolates

The isolates were chosen according to their protein profiles with the species showing the maximum protein profile.

Isolate no.	**Place**	**Species**
12	Dehrian kuan	*Chlorella sp.*
15	Kapur Sagar	*Scenedesmus dimorphus*
19	Rewalsar lake	*Scenedesmus quadricauda*

All the three species showed L-ASNase production though only limited L-glutaminase production was observed.

4.2 Standard curve for Protein estimation

Protein standard curve was prepared by Lowry's method using BSA as standard.

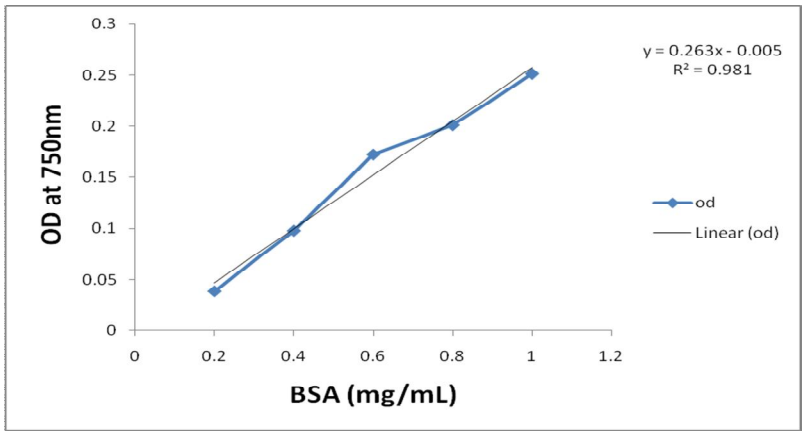


Fig 3 Standard protein curve

4.3 Standard curve for ammonia

In order to determine the L-ASNase and L-glutaminase activity, a standard curve was plotted for determining the concentration of ammonia using Nessler's reagent.

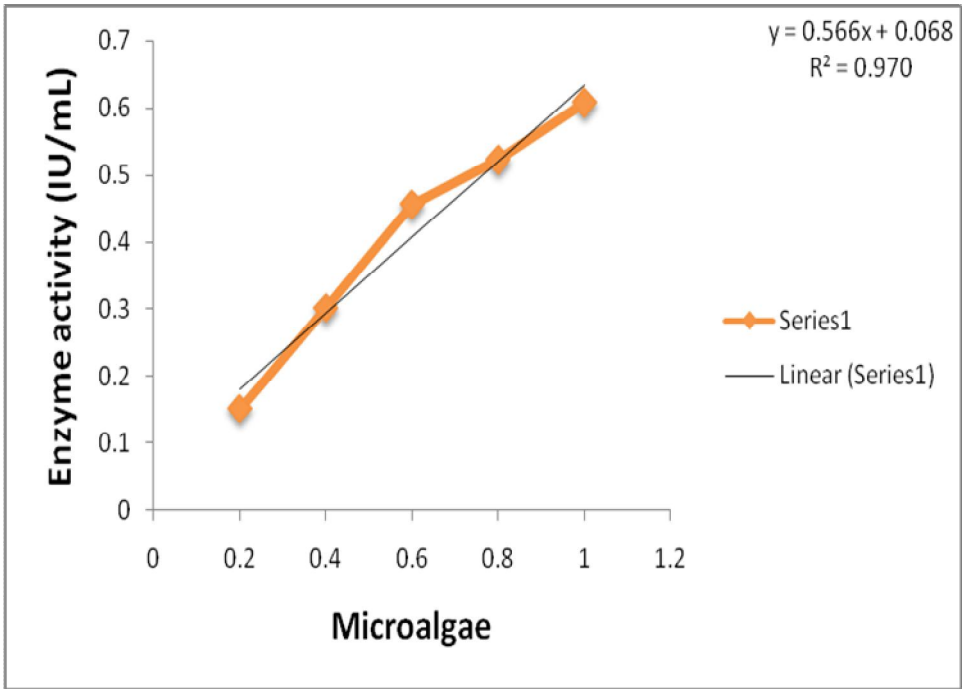
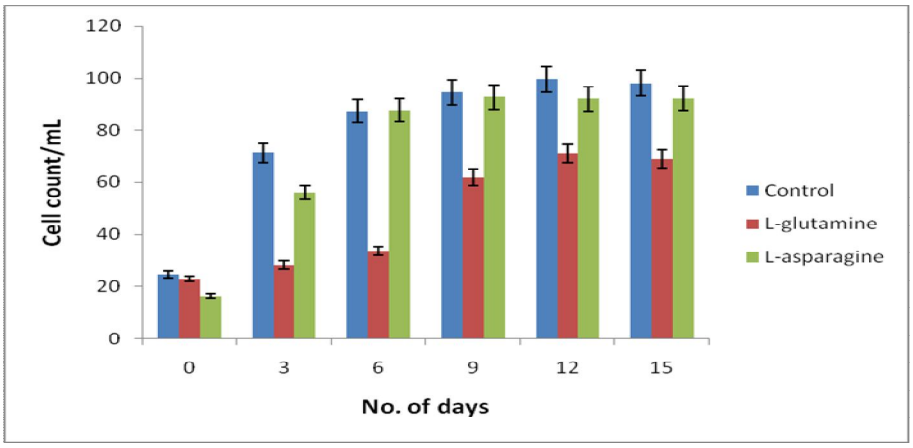


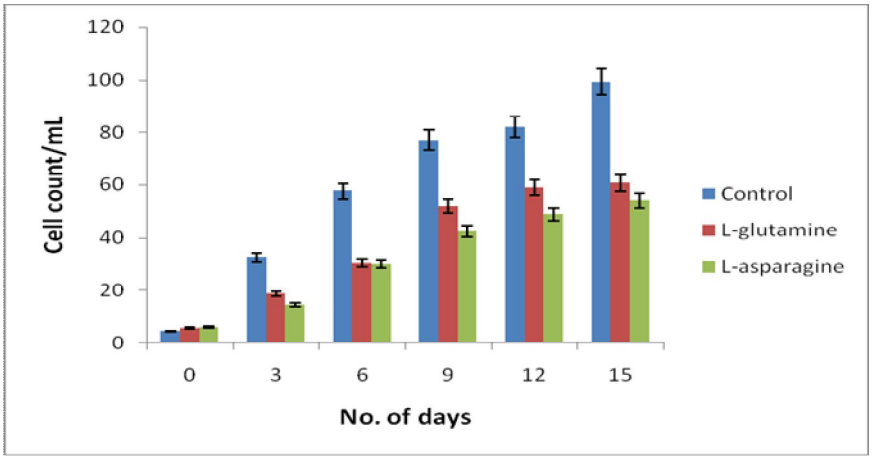
Fig 4 Standard curve of ammonia

4.4 Growth kinetics studies of Microalgal species in different media

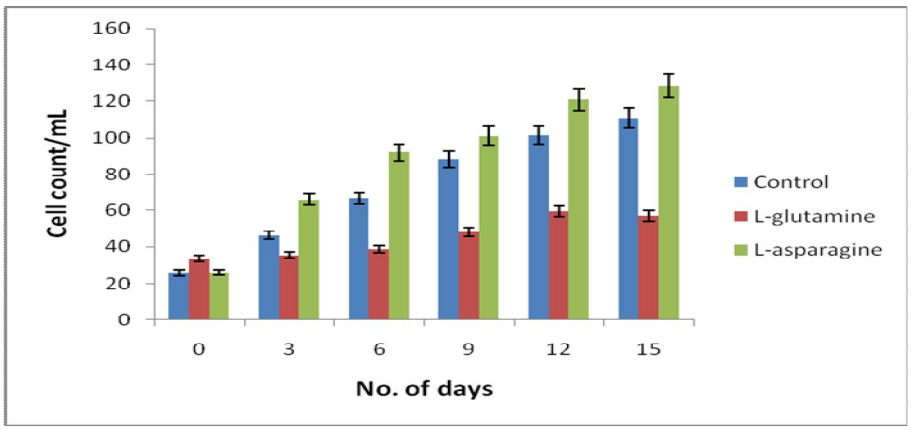
The following graphs show the growth curves for all the three species in different media measured as cell counts (10^4)/mL and also the growth curves as measured by optical density (OD) at 730 nm. *Chlorella sp.* the maximum growth in control media followed by media containing L-asparagine and least in L-glutamine contained medium (Fig. 5 (a)). *S. dimorphus* shows the maximum growth in control medium and comparatively lesser in L-glutamine and L-asparagine contained media (Fig. 5 (b)). *S. quadricauda* shows the maximum growth in L-asparagine containing media as N-source followed by control((Fig. 5 (c)).



(a)



(b)

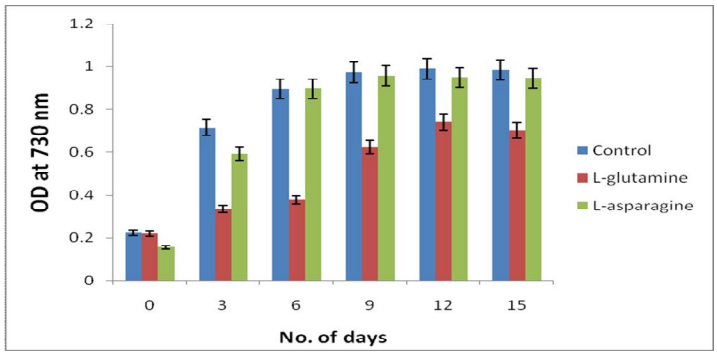


(c)

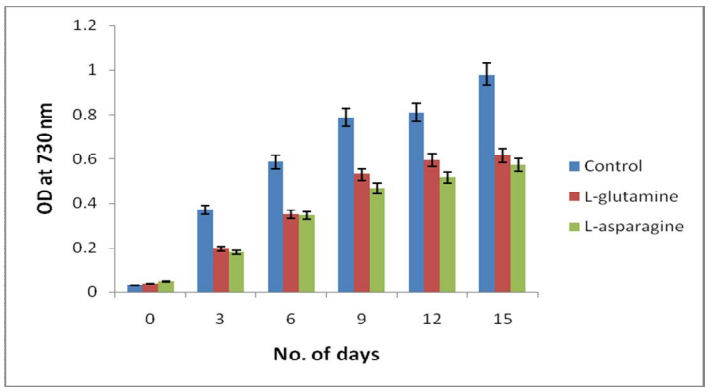
Fig 5 Growth response of (a) *Chlorella sp.(b) Scenedesmus dimorphus (c) Scenedesmus quadricauda* Growth response of *S. quadricauda,* measured as number of cells per mL of culture.

Calculation of growth kinetic parameters

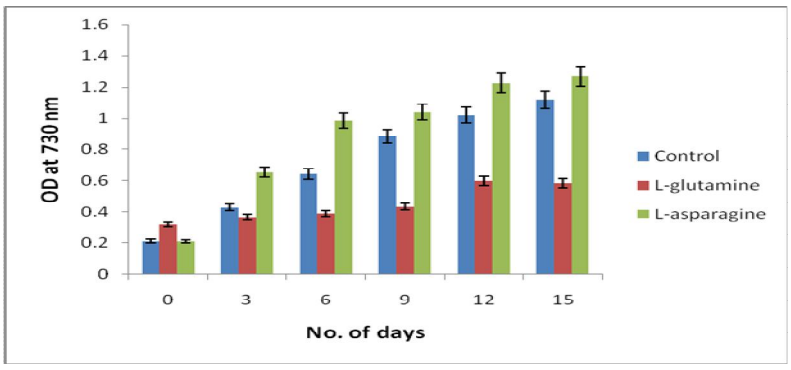
Following parameters were calculated from the observed data for the assessment of growth characteristics (Fig.6)



(a)



(b)



(c)

Fig.6 Growth response of (a) *Chlorella sp., (b) Scenedesmus dimorphus (c) Scenedesmus quadricauda* , measured as OD at 730 nm

- **Specific growth rate (μ)** was measured using the equation $\mu=\ln(N_t - N_0)/T_t - T_0$ where N_t is the number of cells at the end of log phase, N_0 is the number of cells at the start of log phase, T_t is the final day of log phase and T_0 is starting day of log phase.
- **No. of divisions per day (k)**= μ/0.6931
- **Doubling time (T_t)=** 0.6931/ μ

Table 2 Growth parameters of microalgal species in different media.

	Control			L-glutamine			L-asparagine		
	μ	k	T_t	μ	k	T_t	μ	k	T_t
Chlorella sp.	0.092	0.133	7.534	0.073	0.105	9.50	0.115	0.166	6.027
S. dimorphus	0.21	0.303	3.3	0.16	0.231	4.332	0.146	0.211	4.747
S.quadricauda	0.10	0.144	6.931	0.036	0.052	19.253	0.106	0.153	6.54

4.5 L-ASNase Production studies from control and induced medium

The graphs below depict the enzyme activity for L-ASNase in both control and induced medium (containing L-asparagine) both intra –and extracellular.

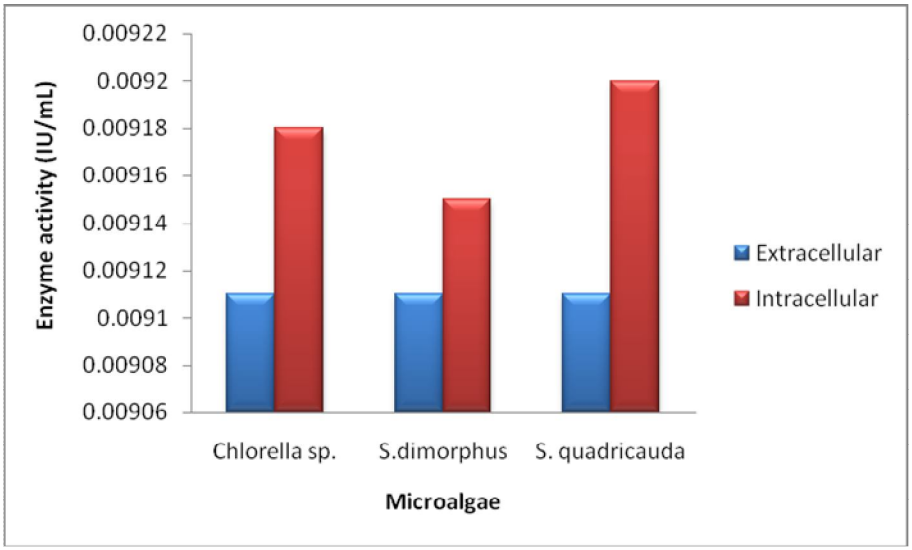


Fig 7 Enzyme activity of L-ASNase in normal BG-11 (control)

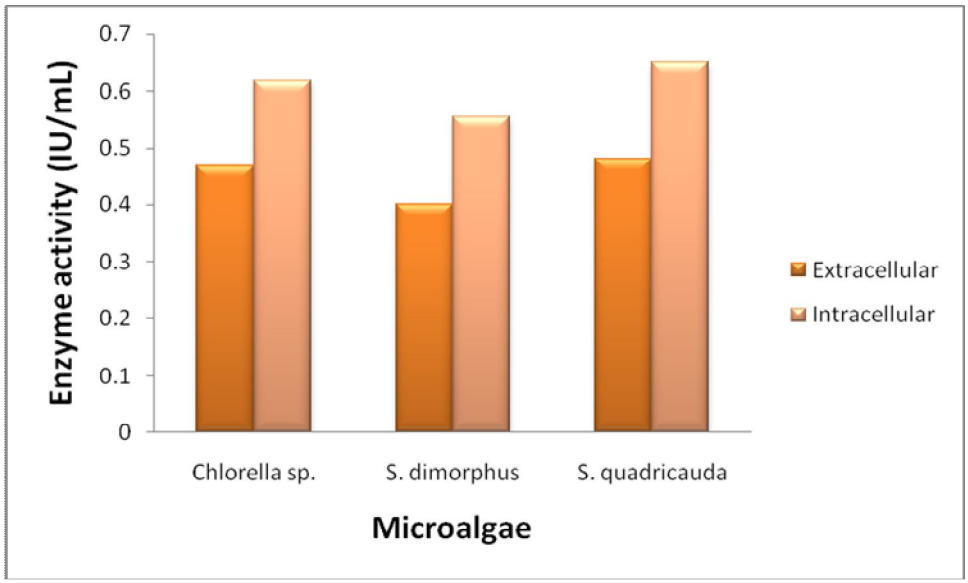


Fig 8 Enzyme activity of L-ASNase in induced BG-11 (containing L-asparagine)

Enzyme activity of **L-ASNase** was **highest** for *Scenedesmus quadricauda* in induced medium.

4.6 L-Glutaminase Production Studies from Control and Induced medium

The graphs below depict the enzyme activity for L-Glutaminase in both control and induced medium (containing L-glutamine) both intra –and extracellular.

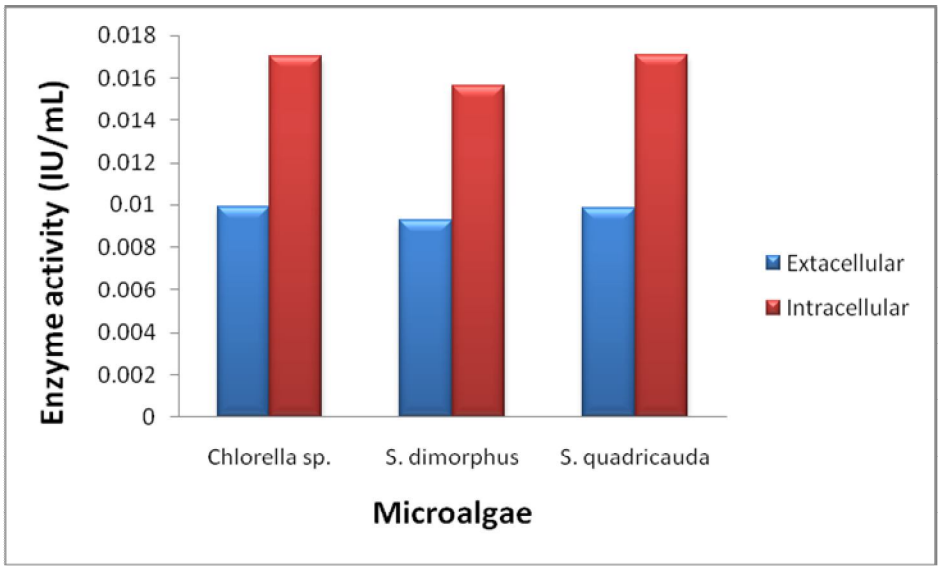


Fig 9 Enzyme activity of L-glutaminase in normal BG-11 (control)

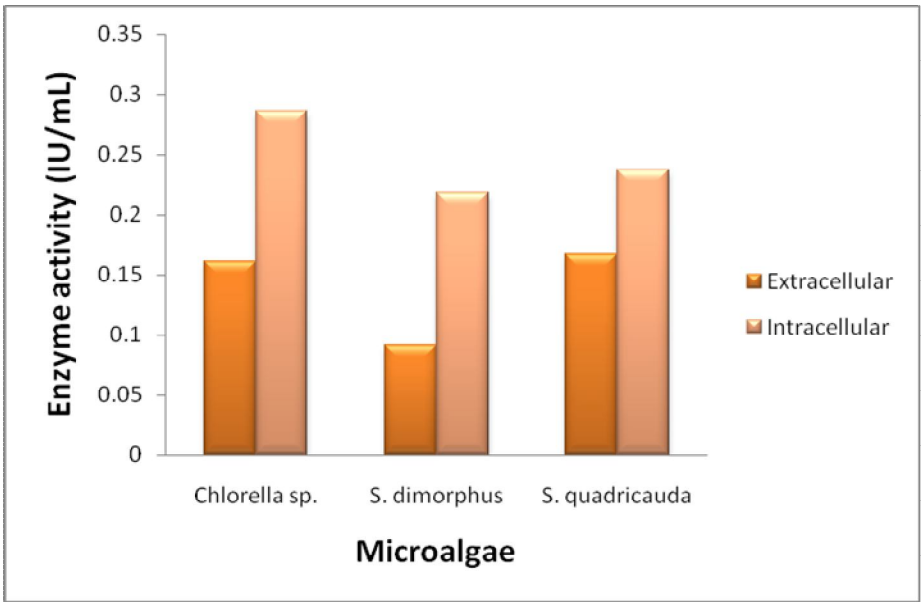


Fig 10. Enzyme activity of L-glutaminase in induced medium (containing L-glutamine)

Enzyme activity observed for L-glutaminase was very low and was highest for *Chlorella sp*. produced intracellularly.

Conclusion and future prospects

L-ASNase and L-Glutaminase are important anti-cancer enzymes and continued research is being carried out on them. Present study showed its production through microalgae –a novel source for both of them which offers many advantages. Maximum L-ASNase activity was shown by *Scenedesmus quadricauda* in induced medium (containing L-asparagine) while maximum L-glutaminase activity was shown by *Chlorella sp.* in induced medium, both of them intracellularly. Also, growth kinetics study revealed that both the substrates can act as N-source for the growth of microalgae, L-asparagine being a better one and also thereby inducing better production of enzyme.

Further studies such as purification and optimization of parameters can be carried out on the crude extract of the enzyme obtained. Research on L-asparaginase from microalgae can be exploited therefore as a novel source

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