SCREENING OF MICROALGAE FOR L-ARGINASE AND L-GLUTAMINASE ACTIVITIES

A Thesis Submitted in partial fulfillment of

BACHELORS IN TECHNOLOGY

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CERTIFICATE

This is to certify that **Ms. Harshita shukla (**Roll No: 121552) **and Ms. Priyanka kapil (**Roll No: 121567) final year students of Bachelor of Technology, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, HP-173234, have successfully completed the Project work entitled "Screening of Microalgae for L-Arginase and L-Glutaminase activities" under the supervision of "**Dr.Garlapati Vijay Kumar"** during their Bachelor's Curriculum. This work has not been reported earlier anywhere and can be approved for submission in partial fulfillment of the course work.

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ABSTRACT

The therapeutic enzyme L-Arginase and L-Glutaminase have received a greater attention as anti-tumor agents, for treatment of tumor cells particularly acute lymphoblastic leukemia. In this project undertaken, microalagal species namely Chlorella sp. and *Scendesmus dimorphus* were grown in BG-11 media were screened for L-Arginase and L-Glutaminase activities.

Keywords: Chlorella sp., *Scendesmus dimorphus*, L-Arginase , L-Glutaminase

CHAPTER 1

Introduction

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Catalysts are proteinaceous in nature. Every chemical is customized to do one exceptional undertaking. Like a key in a lock every chemical fits together with one particular substrate changing it in one appropriate way. The assembling or handling of catalysts for use as medications is a vital aspect of today's pharmaceutical industry. Endeavors to benefit from the upsides of compounds as medications are presently being made at for all intents and purposes each pharmaceutical exploration focus on the planet. Helpful catalysts have an expansive assortment of particular uses: as oncolytics, thrombolytics or anticoagulants and as substitutes for metabolic insufficiencies. Moreover there is a developing gathering of different proteins of assorted capacity. Proteolytic compounds have been broadly utilized as calming operators.

A noteworthy potential helpful utilization of chemicals is in the treatment of growth. L-asparaginase has turned out to be especially encouraging for the treatment of intense lymphocytic leukemia. Its activity relies on the way that tumor cells are inadequate in aspartate-alkali ligase movement, which confines their capacity to combine the regularly unimportant amino corrosive, L-asparagine. Along these lines, they are compelled to concentrate it from body liquids. The activity of Asparaginase does not influence the working of ordinary cells, which can blend enough for their own necessities, however decrease the free exogenous focus, thus impel a condition of deadly starvation in the vulnerable tumor cells. A 60% frequency of complete reduction has been accounted for in an investigation of very nearly 6,000 instances of intense lymphocytic leukemia.

This chemical is managed intravenously. Microbial chemicals are favored over plant or creature proteins because of their monetary generation, consistency, simplicity of procedure adjustment and improvement. They are moderately steadier than comparing proteins derived from plants or creatures. Further, they give more prominent differing qualities of reactant exercises. The dominant part of proteins at present utilized as a part of industry is of microbial starting point. Some reports are accessible on the generation of L-asparaginase from

microorganisms, parasites and creature sourcses. L-asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) is utilized as a part of the treatment of intense lymphoblastic leukemia and in numerous other clinical tests identifying with tumor treatment (D. zh. Wei and H. Liu, 1998). With the advancement of its new capacities, an awesome interest for L-asparaginase is normal in coming years. Along these lines, it is alluring to hunt down new bacterial disengages creating L-asparaginase with novel properties from whatever number distinctive sources as could be allowed.

The present study portrays seclusion and screening of microorganisms from soil for L-asparaginase action, and for the determination a high potential strain, Characterization and distinguishing proof of the potential strain by blend approach, Optimization of the procedure parameters by traditional and measurable methodology for expansion of L-asparaginase generation by the potential strain, Bench scale creation and unstructured active demonstrating of L-asparaginase generation by the potential strain, Purification of L-asparaginase from generation medium, Characterization and assessment of anticancer movement of the decontaminated L-asparaginase.

CHAPTER 2

Review of Literature

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2.1 Enzymes

Life relies on upon a very much organized arrangement of synthetic responses. Large portions of these responses, be that as it may, continue too gradually all alone to manage life. Consequently nature has planned impetuses, which we now allude to as enzymes, to incredibly quicken the rates of these synthetic responses. The reactant force of chemicals encourages life forms in basically all life-shapes from infections to man. Numerous compounds hold their reactant potential after extraction from the living being, and it didn't take long for humanity to perceive and abuse the synergist force of chemical for business purposes. Actually, the most punctual known references to compounds are from antiquated writings managing the production of cheeses, breads, and mixed refreshments, and for the softening of meats. Today compounds keep on playing key parts in numerous nourishment and refreshment fabricating forms and are fixings in various customer items, for example, clothing cleansers (which break down protein based stains with the assistance of proteolytic chemicals). Enzymes are also of fundamental interest in the health sciences, since many disease processes can be linked to the aberrant activities of one or a few enzymes (Copeland, 2000).

2.2 L-Glutaminase

L-Glutaminase (EC.3.5.1.2) is an amidohydrolase which catalyzes the hydrolyticaldeamidation of L-glutamine bringing about the generation of L-glutamic corrosive and alkali. L-Glutaminases are universal in the natural world (Ohshima et al., 1976a; Iyer and Singhal, 2010) and life forms going from microorganisms to people have the compound. L-Glutaminase has a focal part in mammalian tissues (Errera and Greenstein, 1949). They are by and large sorted as the kidney sort and liver sort glutaminases and both sorts have been purged and portrayed (Svenneby et al., 1973; Curthoys et al., 1976; Heini et al., 1987). Enthusiasm on amidohydrolases began with

the disclosure of their antitumor properties (Broome, 1961; El-Asmar and Greenberg, 1996; Santana et al., 1968; Roberts et al., 1970) and from that point forward, a considerable measure of endeavors have gone in broad studies on microbial L-glutaminases with the goal of creating them as antitumor operators. A parallel enthusiasm on microbial L-glutaminases originated from its applications in nourishment of biotechnology, microbial L-glutaminases discovered more up to date applications in clinical investigation and even in production of metabolites. This prompted the broad studies on L-glutaminase notwithstanding the way that present day biotechnological strategies propose option and particular techniques for the treatment of tumors, where amidohydrolases used to be utilized.

2.2.1 Structure

The structure of Glutaminase has been resolved utilizing X-beam diffraction to a determination of up to 1.73 Å. There are 2 chains containing 305 deposits that make up the length of this dimeric protein. On every strand, 23% of the amino corrosive substance, or 71 buildups, are found in the 8 helices. Twenty-one percent, or 95 buildups, make up the 23 beta sheet strands [1]

2.2.2 Isoenzymes

People express 4 isoforms of glutaminase. GLS1 encodes 2 sorts of kidney-sort glutaminase with a high action and low Km. GLS2 encodes 2 types of liver-sort glutaminase with a low movement and allosteric regulation.

2.2.3 Related proteins

Glutaminases have a place with a bigger family that incorporates serine-subordinate beta-lactamases and penicillin-restricting proteins. Numerous microbes have two isozymes. This model depends on chose known glutaminases and their homologs inside prokaryotes, with the rejection of profoundly determined (long-branch) and compositionally fluctuated homologs, in order to accomplish preservationist assignments. A sharp drop in scores happens underneath 250, and shorts are set appropriately. The protein changes over glutamine to glutamate, with the arrival of smelling salts. Individuals have a tendency to be depicted as glutaminase A

(glsA), where B (glsB) is obscure and may not be homologous (as in Rhizobium etli; a few species have two isozymes that may both be assigned A (GlsA1 and GlsA2).

2.2.4 Occurrence and Distribution

L-glutaminase action is broadly appropriated in plants, creature tissues and in microorganisms including microscopic organisms, yeast and growths. Over a couple of decades, significant examination has been embraced with the microbial creation of extracellular L-glutaminase. The major favorable position of utilizing microorganisms for the creation of L-glutaminase is the sparing mass creation limit furthermore microorganisms are anything but difficult to control to acquire compounds of sought qualities. Among microorganisms, E.coliglutaminases has been contemplated in much detail. A portion of the case of bacterial strains delivering L-glutaminase incorporate E.coli, Pseudomonas species like P.aurantiaca, P.fluoroscens, P.aeroginosa, and P.aureofaciens; Acinetobacter sp, Bacillus sp, Erwiniacaratovora, Klebsiellaaerogens, Aerobacteraerogens and so forth. L-glutaminase from the individuals from Enterobacteriaceae family has been best portrayed among the bacterial genera. Among the contagious species, Aspergillus oryzae, Aspergillus sojae, Beauveriasp, Tilachlidium humicola, Trichoderma koningii, Verticillium and so forth., have been accounted for to create L-glutaminase. August 2013, Volume: I, Issue: VIII 2 Among yeast types of Hansenula, Rhodotorula, Candida scotii, and Crytococcusalbidus, Candida utilis, Torulopsis sp., Zygosaccharomycesrouxii and so forth have been accounted for to create the compound L-glutaminase.

2.2.5 Properties of L-Glutaminase

Properties of any protein decide its reasonableness and effectiveness for the application in bioprocesses. There have been wide varieties in the properties of glutaminases. Some of them are portrayed beneath:

pH:

The ideal exercises of L-glutaminase from Pseudomonas aeruginosa were at pH of 7.5-9.0 and 8.5 individually. L-glutaminase from Pseudomonas species was accounted for to be dynamic over an expansive scope of pH 5-9

with an ideal close pH 7.0. An intracellula L-glutaminase from Cryptococcus albidus favored an ideal pH of 5.5-8.5.

Temperature:

L-glutaminase had demonstrated a wide variety in its temperature strength. L-glutaminase from Pseudomonas species demonstrated greatest action at 37°C and was not steady at high temperature, where as the compound from Clostridium welchii held movement at 60°C. Glutaminase from Cryptococcus albidus held 77% of its movement at 70°C even after 30 minutes of brooding. Glutaminase I and II from Micrococcus luteus had temperature optima of 50°C and the nearness of sodium chloride (10%) expanded the thermo-strength. In any case, a significant number of the L-glutaminases have reported both an ideal and stable temperature of around 28-50°C. L-glutaminase likewise contrasted in their capacity towards L-glutamine furthermore has distinctive isoelectric focuses. Different substances and substantial metals repress the protein, L-glutaminase action.

2.2.6 Types of L-Glutaminase reactions

The group of amidohydrolase that catalyze the deamination of glutamine contains two classes. The top notch incorporates glutaminase, which is very particular for glutamine and catalyzes the hydrolysis of glutamine to glutamic corrosive. The menial contains the chemical that is less particular and catalyzes the hydrolysis of glutamine to glutamic corrosive and asparagine to aspartic corrosive with comparable effectiveness and wide substrate specificity. This is a class of amidohydrolase that has gotten significant consideration as some of them are being utilized as a part of the treatment of leukemia especially intense lymphocytic leukemia (ALL). The proportion of L-glutaminase to L-asparaginase action was roughly 1.5:1 on account of Pseudomonas boreopolis. Substrate comparability between L-glutaminase and L-asparaginase, has prompted the vague amino corrosive succession of L-glutaminase from those of L-asparaginase. The grouping homology between the two hydrolytic compounds, L-glutaminase and L-asparaginase is demonstrative of a typical system for deamination. Keeping in mind the end goal to separate the genome groupings between the above two compounds, exercises of the quality items must be measured.

Glutaminase has more business significance than L-asparginase. Microbial glutaminases hold a critical spot among industrially vital chemicals because of their demonstrated part as antileukaemic and as flavor-improving operators. Despite this, glutaminase or glutaminase–asparaginase chemicals have gotten sparse consideration from examiners when contrasted with other set up mechanical compounds or even their partner asparaginase. A real share of late research on glutaminase has concentrated on mammalian glutaminase, their organic chemistry, control and hereditary make-up, legitimately because of the part of glutaminase in August 2013, Volume: I, Issue: VIII 3 mammalian digestion system. Be that as it may, this situation is changing as various late studies are endeavoring being devoted to achieve an inside and out learning of administrative, basic and biochemical angles and in addition the quality articulation of glutaminases from different microbial sources. Still, there is a lot of space for exploration on glutaminases including the disconnection of salt and thermo-tolerant proteins, which would altogether improve their applications in the sustenance industry. Moreover, a point by point comprehension of the control of quality expression in view of atomic methodologies and different means would contribute gigantically towards creating fruitful methodologies for strain change which is an essential for any mechanically imperative catalyst.

2.2.7 Mechanism of action of L-Glutaminase

Not at all like typical cells, leukemic cells does not rely on upon L-glutamine synthetase, they straightforwardly rely on upon the exogenous supply of L-glutamine from the blood for their development and survival. Along these lines, blood L-glutamine serves as a metabolic forerunner for the nucleotide and protein blend of tumor cells .Consequently, L-glutaminase causes particular demise to Lglutamine subordinate tumor cells by hindering the vitality course for their multiplication.

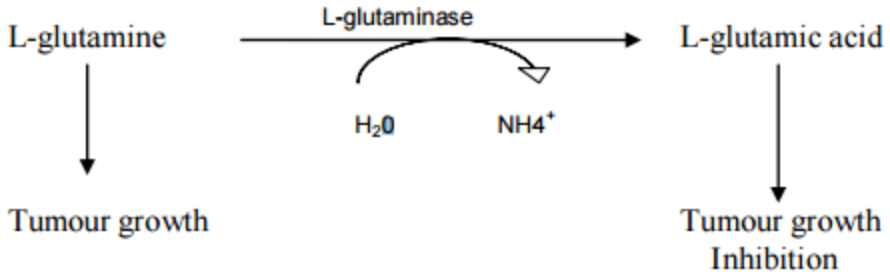

Fig.1. Mechanism of L-Glutaminase

2.2.8Applications of L-Glutaminase

L-Glutaminase in Acute Lymphocytic Leukemia (ALL)Under the brand name Elspar, E.coliasparaginase has been utilized for the treatment of hostile to lymphocytic leukemia for more than 20 a long time. A parallel enthusiasm for L-glutaminase has ascended from the shows that the catalyst, Lglutaminaseadditionally shows a hostile to leukemic movement. Not at all like ordinary cells, leukemic cells does not rely on upon L-glutamine synthetase, they specifically rely on upon the exogenous supply of L-glutamine from the blood for their development and survival. Accordingly, blood L-glutamine serves as a metabolic antecedent for the nucleotide and protein combination of tumor cells .Consequently, L-glutaminase causes specific passing to Glutamine subordinate tumor cells by obstructing the vitality course for their expansion. The component of L-glutaminase compound in intense lymphocytic leukemia.

2.2.8.1 L-Glutaminase in HIV therapy

L-glutaminase has been utilized as an effective hostile to retroviral operator in treating AIDS/HIV treatment as it brings down L-glutamine levels in both serum and tissues for delayed periods, accordingly bringing about significant diminishment in serum reverse transcriptase action (RTA) of the human immunodeficiency infection (HIV) with enhanced long haul survival advantages. This one of a kind methodology can be connected to other pathogenic infections which will rely on the distinguishing proof of dietary necessities for viral spread and the advancement of bioactive catalysts to drain these supplements in the region of tainted cells.

2.2.8.2 L-Glutaminase in food industries

L-glutaminase is for the most part viewed as a key chemical in improving the taste and fragrance of aged nourishments, for example, soy sauce, prominent in Asian nations. L-glutaminase upgrades the kind of aged nourishments by expanding their glutamic corrosive substance and in this manner are conferring a acceptable taste, stamping it as an essential sustenance compound amid any nourishment aging, for example, soy sauce aging. Under the low L-glutaminase action conditions, L-glutamine is artificially what's more, irreversibly changed over into pyroglutamic corrosive, which is a flavorless compound. Amino acids that are created by the enzymatic debasement of the proteins contained in the crude materials are surely understood as essential flavor segments of aged condiments.Lglutamic corrosive is one such flavor improving amino corrosive created by the hydrolytic activity of L-glutaminase on L-glutamine. Glutamic and aspartic acids are surely understood amino acids contributing not just fine taste, "umami" and sharp harsh taste additionally nutritious impacts to sustenance.

2.2.8.3 L-Glutaminase as a Bio-sensor

L-glutaminase compound has been utilized as a biosensor especially as a part of hybridoma and mammalian cell societies for observing the glutamine level without the need of independent estimation for glutamic corrosive.

2.2.9 Production of L-Glutaminase through SSF

Diverse techniques for aging innovation can be connected for the generation of Lglutaminase. Monetarily, L-glutaminase has been delivered by submerged aging procedure, however as of late; it is additionally being delivered under strong state aging strategy, utilizing regular (E.g. wheats, husks, oil cakes and so on) and dormant strong materials (E.g. polystyrene dabs).

Strong state maturation offers a few focal points over other customary maturations, for example, submerged maturation and so on. The real focal points incorporate higher item yields, lower capital and repeating consumption, lower waste water yield/less water need, diminished vitality necessity, nonattendance of froth

arrangement, straightforwardness, high reproducibility, less complex aging media, Lesser maturation space, nonattendance of thorough control of aging parameters, sparing to utilize even in littler scales, less demanding control of tainting, materialness of utilizing aged solids straightforwardly, capacity of dried matured matter, lower expense of downstream preparing.

Disadvantages

Issues regularly connected with strong state aging are warmth develop, bacterial pollution, scale-up, biomass development estimation and control of procedure parameters.

2.2.9.1 Effect of pH on L-Glutaminase production

The microbial L-glutaminase is created in the scope of pH from 6.0 to 9.0. Be that as it may, a larger part of the microbial species is known not the L-glutaminase in the unbiased pH or somewhat basic pH. Unbiased pH L-glutaminases are accounted for by Achromobacteraceae (Roberts et al., 1972), StenotrophomonasMaltophilia NYW-81 (Wakayama et al., 2005) and Zygosaccharomycesrouxii NRRL-Y 2547 (Iyer and Singhal, 2008) under submerged maturation conditions while Vibrio costicola (Prabhu and Chandrasekaran, 1997) and T. koningii (Sayed, 2009) under strong state maturation conditions. Creation of L-glutaminase in soluble base conditions (pH 9.0) in both submerged and strong state maturations is reported in Beauveria sp. (Keerthi et al., 1999; Sabu et al., 2000b). Notwithstanding, L-glutaminase creation at pH 6.0 is accounted for in Cryptococcus nodaensis (Sato et al., 1999) and pseudomonas sp. (Kumar and Chandrasekaran, 2003).

2.2.9.2 Effect of temperature on L-Glutaminase production

Microbial L-glutaminase creation is for the most part seen at gentle brooding temperature conditions running from 25 to 37 oC.Iyer and Singhal (2008; 2010) reported creation of L-glutaminase at 37 oC by Z. rouxii NRRL-Y 2547. In any case, reports are likewise accessible on creation of L-glutaminase at 35 ^{o}C by V. costicola (Prabhu and Chandrasekaran, 1997) and R. oligosporus (Han et al., 2003) understrong state

maturation. Powerful L-glutaminase generation at 25 oC is seen by Achromobacteraceae (Roberts et al., 1972), Providencia sp. (Iyer and Singhal, 2009) and *A. elegans* (Han et al., 2003).

2.2.10 Production of L-Glutaminase through SmF

Microbial L-glutaminases were delivered in both submerged and strong state maturations. Table 2.2 and 2.3 give the different microorganisms what's more, their way of life conditions in both Smf and SSF were exhibited.

Roberts et al. (1972) disengaged the Achromobacteraceae from the dirt examples and watched that the chemical from this life form has L-glutaminase and L-asparaginase action in a proportion of 1.2:1. The most astounding yields of chemical are acquired at the point when cells are become vigorously in a basal engineered medium made out of L-glutamic corrosive, ammonium sulfate, follow minerals, and phosphate support. The creators watched that the temperature between 15 to 25 ^{o}C is positive to the life form development and protein creation. Extracellular L-glutaminase creating Beauveria sp. BTMF S10 was disconnected from marine residue (Keerthi

et al., 2006). The creators watched that this compound was inducible and development related. The most noteworthy yield (46.9 U ml^{-1}) is gotten in a medium supplemented with 1% yeast remove and sorbitol 9% sodium chloride what's more, 0.2% methionine at medium beginning pH 9.0 and 27 °C. L-Glutaminase creation by *Stenotrophomonas maltophilia* NYW-81 was upgraded by Wakayama et al, (2005). The most astounding yieldwas acquired at pH 7.0 and at 30 oC temperature. The creators watched that when glutamine is utilized a sole carbon and nitrogen source the creation is high. At the point when glucose is added to the medium it smothers the L-glutaminase generation in S. Maltophilia (Wakayama et al., 2005). Keerthi et al, (2006) watched that the L-glutaminase delivered from actinomycetes has a decent salt resilience. They detached the 20 strains from estuarine fish and watched that *Streptomyces rimosus* was indicated most noteworthy L-glutaminase movement. Ideal creation of Lglutaminase was seen at hatching temperature at 27 ^{o}C, pH 9.0 what's more, glucose and malt separate as carbon and nitrogen sources individually. In different examinations of Iyer and Singhal (2008; 2009) watched that the carbon and nitrogen hotspots for L-glutaminasecreation are differed with the living beings.

Supplementation of sucrose furthermore, yeast extracts as carbon and nitrogen source enhanced L-glutaminase creation from *Zygosaccharomyces rouxii*. While, higher L-glutaminase creation saw from Providencia sp. with supplementation of glucose and urea as carbon and nitrogen sources.

Renu et al. (1993; 1994) compared the L-glutaminase production in submerged fermentation with strong state maturation. The creators watched that strong state maturation was desirable over submerged aging for L-glutaminase creation as far as yield productivity, since 25 to 30 fold increment in chemical creation was gotten under strong state aging. For the generation of L-glutaminase in strong state maturation different agro mechanical materials were utilized as strong backing. Numerous creators reported that wheat grain was observed to be an ideal support for compound generation (Prabhu and Chandrasekaran, 1995; Kashayp et al., 2002; Sayad, 2009). Aside from the wheat grain, rice wheat, copra cake powder, ground nut cake powder and sesamum oil cake were utilized as strong substrates for compound creation (Prabhu furthermore, Chandrasekaran, 1995). However Polystyrene dots, impregnated with mineral salts and glutamine were utilized as strong substrate for glutaminase creation. Renu and Chandrasekaran (1992a; 1992b) watched that Pseudomonas fluorescens, Vibrio cholerae, and Vibrio costicola, from among the strains screened from marine situations of Cochin, delivered L-glutaminaseextracellularly in extensive sums. Process conditions for substantial scale generation of this catalyst were upgraded in strong state maturation. The creators concentrated on the effect of operational parameters on L-glutaminase creation by V. costicola, what's more, it was watched that most extreme catalyst creation was accomplished in a wheat grain medium containing particles 1.4 to 2.0 mm in size and under optimal conditions which were: a moisture content of 40 to 60 %, pH 6.0, incubation at 35°C, and with the addition of glutamine at 1.0 % (w w-1). Later Prabhu and Chandrasekaran (1995) observed that during solid state fermentation production of L-glutaminase by V. costicola also simultaneously produced alpha-amylases and cellulases. The authors inferred that wheat bran, which was used as solid substrate, may have been used as substrate and brought about the induction of synthesis of alpha-amylase and cellulase. L-Glutaminase was found to be induced by L-glutamine. The authors later observed that V. costicola could grow on an inert carrier such as Polystyrene beads, impregnated with mineral salts and glutamine and produce

enzyme under solid-state fermentation (Prabhu and Chandrasekaran, 1995). The ability to absorb onto polystyrene appears to be a basic property of marine bacteria. In their natural environment, many species of marine bacteria exist only under adsorbed conditions on detritus or solid substrates.

2.3 L-Arginase

Arginase (EC 3.5.3.1, arginine amidinase, canavanase, L-arginase, arginine transamidinase) is a manganese-containing protein. The response catalyzed by this compound is: arginine + H2O → ornithine + urea. It is the last compound of the urea cycle. It is omnipresent to all spaces of life.L-arginine (ARG) is a vital amino corrosive for both restorative and modern applications. For right around six decades, the examination has been continuing for its enhanced mechanical level generation utilizing distinctive microorganisms. While the underlying methodologies included arbitrary mutagenesis for expanded resistance to ARG and subsequently higher ARG titer, it is relentless and regularly prompts undesirable phenotypes, for example, hindered development. Disclosure of L-glutamate (GLU) overproducing strains and utilizing them as base strains for ARG generation prompted enhanced ARG creation titer. Proceeded with push to disclose sub-atomic instruments prompted the amassing of definite information on amino corrosive digestion system, which has added to better comprehension of ARG biosynthesis and its control. Additionally, frameworks metabolic designing now empowers researchers and architects to productively develop hereditarily characterized microorganisms for ARG overproduction in a more sane and framework wide way. In spite of such exertion, ARG biosynthesis is still not completely comprehended and a large number of the qualities in the pathway are mislabeled. Here, we audit the major metabolic pathways and its control required in ARG biosynthesis in various prokaryotes including late disclosures. Additionally, different procedures for metabolic building of microscopic organisms for the overproduction of ARG are portrayed. Moreover, metabolic building approaches for creating ARG subsidiaries, for example, L-ornithine (ORN), putrescine and cyanophycin are depicted. ORN is utilized as a part of restorative applications, while putrescine can be utilized as a bio-based forerunner for the

blend of nylon-4,6 and nylon-4,10. Cyanophycin is likewise a vital compound for the creation of polyaspartate, another imperative bio-based polymer.

2.3.1 Structure and function

Arginases have a place with the ureohydrolase group of proteins. Arginase catalyzes the fifth and last stride in the urea cycle, a progression of biochemical responses in warm blooded animals amid which the body discards destructive smelling salts. In particular, arginase changes over L-arginine into L-ornithine and urea. Mammalian arginase is dynamic as a trimer, however some bacterial arginases are hexameric. The compound requires a two-particle metal group of manganese keeping in mind the end goal to keep up appropriate capacity. These Mn^{2+} particles coordinate with water, orientating and balancing out the atom and permitting water to go about as a nucleophile and assault L-arginine, hydrolyzing it into ornithine and urea.

In many vertebrates, two isozymes of this compound exist; the to start with, Arginase I, capacities in the urea cycle, and is found essentially in the cytoplasm of the liver. The second isozyme, Arginase II, has been ensnared in the direction of the arginine/ornithine fixations in the cell. It is situated in mitochondria of a few tissues in the body, with most wealth in the kidney and prostate. It might be found at lower levels in macrophages, lactating mammary organs, and brain. The second isozyme might be found without other urea cycle enzymes.

2.3.2 Mechanism of action

The dynamic site holds L-arginine set up through hydrogen holding between the guanidiniumbunch with Glu227. This holding situates L-arginine for nucleophilic assault by the metal-related hydroxide particle at the guanidinium bunch. This outcome in a tetrahedral transitional. The manganese particles act to balance out both the hydroxyl bunch in the tetrahedral transitional and the creating sp3 solitary electron pair on the NH2 bunch as the tetrahedral middle is shaped.

Arginase's dynamic site is remarkably particular. Changing the substrate structure and/or stereochemistry seriously brings down the active action of the catalyst. This specificity happens because of the high number of

hydrogen securities amongst substrate and compound; direct or water-encouraged hydrogen securities exist, soaking both the four acceptor positions on the alpha carboxylate gathering and every one of the three positions on the alpha amino gathering. N-hydroxy-L-arginine (NOHA), a middle of NO biosynthesis, is a moderate inhibitor of arginase. Precious stone structure of its complex with the protein uncovers that it dislodges the metal-crossing over hydroxide particle and extensions the binuclear manganese bunch.

Moreover, 2(S)- amino-6-boronohexonic corrosive (ABH) is a L-arginine simple that likewise makes a tetrahedral middle of the road like that framed in the catalysis of the common substrate, and is a powerful inhibitor of human arginase I.

2.3.3 Therapeutic uses of L-arginase

- **STROKE**

  Intravenous L-arginine dosed somewhere around 50 and 250 mg/kg enhanced the survival rate amid heatstroke (54 to 245 minutes). In particular, L-arginine lessened intracranial hypertension and expanded the levels of nitric oxide metabolite in the hypothalamus

- **DIABETES**

  L-arginine organization empowers insulin emission and improves insulin-intervened glucose transfer.

- **GYNECOLOGY**

  L-arginine might be advantageous in keeping the decrease in muscle power taking after menopause. Intrauterine development limitation treatment with L-arginine 3 g/day for 20 days showed a change in the heaviness of babies contrasted and no mediation.

- **OPTHALMIC**

. It prompts lessening in mean blood vessel weight and an expansion in retinal and choroidal blood stream proposing a part for arginine in visual illnesses connected with endothelial brokenness, for example, in diabetes or glaucoma

2.4 Therapeutic use of enzymes

Enzymes	Therapeutic Use	Basis	Additional Information
Prolactazyme	Lactose Intolerance	Prolactazyme is a proenzmye that produces lactase in stomach.	About 75% of the world's population is intolerant to lactose in adulthood. It occurs due to lack of lactase in digestive system.
Beta-Lactamase	Penicillin Allergy	Penicillin is converted to penicillioate	Learn more about penicillin allergy
Aglucerase	Gaucher's Disease type I	Enzyme replacement therapy	This disease is characterized by the lack of enzyme glucocerebrocidase.
Streptokinase	Heart Attacks (Myocardial Infarction)	Used as "clot blusters" to dissolve clots in the arteries of heart wall. Plasminogen is converted to plasmin which is fibrinolytic.	Administered i.v. to patients as soon as possible after the onset of a heart attack
Asparaginase	Acute Childhood Leukemia	Decreased level of serum asparagine and inhibition of aspargine dependent multiplication of tumor cells.	Tumor cells cannot synthesize L-asparagine due to lack of aspartate-ammonia ligase.
Collagenase	Skin ulcers	Causes collagen hydrolysis	Break up and remove dead skin and tissue
DNAse	Cystic Fibrosis (CF)	DNAse hydrolyses extracellular DNA responsible for Cystic Fibrosis.	DNA present in the mucous, which arises from dead WBCs and bacterial cells, serves to cross link the mucous, changing it from a fluid

			gel to a semi-solid.
Lysozyme	Antibiotic Therapy	Causes Bacterial cell wall hydrolysis	
Ribonuclease	Antiviral Therapy	Causes RNA hydrolysis	
Trypsin	Inflammation	Causes Protein hydrolysis	
Uricase	Gout	Converts Urate to allantoin	
Enzyme inhibitors	To increase the efficacy of drugs	Against resistant bacterisa	Example: Beta lactamase inhibitor like clavulanic acid along with amoxicillin.

2.5 Importance of therapeutic enzymes in cancer treatment

2.5.1 Mechanism of action

The particular initiation of prodrug(s) in tumor tissues by exogenous enzyme(s) for growth treatment can be proficient by a few ways, including quality coordinated protein prodrug treatment (GDEPT), infection coordinated compound prodrug treatment (VDEPT), and counter acting agent coordinated catalyst prodrug treatment (ADEPT). The focal piece of compound/prodrug growth treatment is to convey drug-initiating catalyst quality or utilitarian protein to tumor tissues, trailed by systemic organization of a prodrug. Albeit every methodology (GDEPT, VDEPT, and ADEPT) has been tried in clinical trials, there are some potential issues utilizing the present conveyance frameworks. In this article, hindrances and points of interest connected with every methodology (GDEPT, VDEPT, and ADEPT).

Enzyme activating prodrug treatment is a two-stage approach. In the initial step, a medication enacting protein is focused on and communicated in tumors. In the second step, a nontoxic prodrug, a substrate of the exogenous catalyst that is presently communicated in tumors, is regulated systemically. The net addition is that a systemically regulated prodrug can be changed over to high nearby convergence of a dynamic anticancer medication in tumors. To be clinically effective, both proteins and prodrugs ought to meet certain prerequisites for this technique. The compounds ought to be both of nonhuman birthplace or human protein that is missing or communicated just at low fixations in ordinary tissues. The protein must accomplish adequate expression in the tumors and have high reactant action. The prodrug ought to be a decent substrate for the communicated compound in tumors however not be actuated by endogenous protein in nontumor tissues. It must have the capacity to cross the tumor cell film for intracellular enactment, and the cytotoxicity differential between the prodrug and its relating dynamic medication ought to be as high as could be allowed. It is favored that the actuated medication be exceptionally diffusible or be effectively taken up by contiguous nonexpressing malignancy cells for an "onlooker" slaughtering impact, the capacity to execute any neighboring nonexpressing cells . Moreover, the half-existence of dynamic medication ought to be sufficiently long to incite an observer impact yet sufficiently short to stay away from the medication spilling out into the systemic dissemination

2.6 Advantages of Microalgae over other microbes

2.6.1 Algae Grow Fast

Green growth can twofold their numbers at regular intervals, can be reaped every day, and can possibly create a volume of biomass and biofuel commonly more noteworthy than that of our most gainful products.

2.6.2 Algae Can Have High Biofuel Yields

Green growth store vitality as oils and starches, which, consolidated with their high efficiency, implies they can create from 2,000 to upwards of 5,000 gallons of biofuels per section of land every year.

2.6.3 Algae Consume CO_2

Like some other plant, green growth, when developed utilizing daylight, expend (or assimilate) carbon dioxide (CO_2) as they develop, discharging oxygen (O_2) for whatever is left of us to relax. For high profitability, green growth requires more CO_2, which can be supplied by emanations sources, for example, power plants, ethanol offices, and different sources.

2.6.4 Algae Do Not Compete With Agriculture

Green growth development utilizes area that as a part of numerous cases is unacceptable for conventional horticulture, and water sources that are not useable for different harvests, for example, ocean, bitter and wastewater. Thusly, green growth based fills supplement biofuels produced using conventional rural procedures.

2.6.5 Microalgal Biomass Can Be Used for Fuel, Feed and Food

Microalgae can be developed to have a high protein and oil content, for instance, which can be utilized to create either biofuels or creature nourishes, or both. What's more, microalgal biomass, which is rich in micronutrients, is now utilized for dietary supplements to propel human wellbeing.

2.6.6 Algae Can Be Used to Produce Many Useful Products

Green growth can be developed to create an assortment of items for extensive to little markets: plastics, substance feedstocks, greases, composts, and even beauty care products. See different items green growth is utilized for here.

2.6.7 Algae can develop on non arable area

The greater parts of them don’t require crisp water, and their healthful worth is high. Broad R&D in progress on green growth as crude material around the world, particularly in North America and Europe with a high number of new businesses creating diverse choices.

2.6.8 Non sustenance crop

Non-rivalry with customary sustenance crops for fuel generation, giving a long haul manageable fuel source not got from nourishment source vegetable oils.

2.7 Previous works done on microalgae based therapeutic enzyme production from microalgae

- Actinomycetes were segregated from marine dregs of Cape Comorin coast (Lat.8° 21' N and Long 77° 30'E), India for extracellular L-glutaminase creation. Out of 25 separates screened, the strain Streptomyces sp.- SBU1 indicated promising L-glutaminase action. Maximal L-glutaminase movement (18.0 U/ml) was seen in a medium supplemented with 2% NaCl (w/v) and 1% malt remove (w/v) as nitrogen source, 1% glucose (w/v) as carbon source, the underlying pH of 9.0 at 30°C after 96 h of hatching was observed to be ideal society condition by the strain Streptomyces sp.- SBU1. So for just couple of marine actinomycetes have been investigated for their L-glutaminase generation. Consequently the present study was started to advance the media organization for L-glutaminase generation. (S.Krishnakumar,R.Alexis Rajan and S.Ravi Kumar)
- Glutaminase chemical generation via kelp endophytic growths. Absolutely 50 contagious endophytes confined from kelp tests, just 10 indicated glutaminase generation as clear by change of the shade of the medium from yellow to pink because of progress in the pH of the medium. The strain 7 of Penicillium sp., indicated greatest protein generation (31.62 U/ml) and strain S1 the base chemical creation (10.24 U/ml). (N. Sajitha, S. Vasuki, M. Suja, G. Kokilam and M. Gopinath).
- Expression and aggregation of monomeric and dimeric immunotoxin proteins in algal chloroplasts. These combination proteins contain an immunizer area focusing on CD22, a B-cell surface epitope, and the enzymatic space of exotoxin A from Pseudomonas aeruginosa. They exhibited that algal-delivered immunotoxins collect as solvent and enzymatically dynamic proteins that predicament target B cells and proficiently murder them in vitro. They likewise demonstrated that treatment with either the mono-or dimeric immunotoxins altogether draws out the survival of mice with embedded human B-cell tumors. (Miller Tran,Christina Van,Daniel J. Barrera,Jack Bui,Par L. Petterson and Stephen P. Mayfield)..

Objectives:

- Collection of Microalgae
- Reviving of the microalgal cultures
- Screening of Microalgae for L- Glutaminase production
- Screening of Microalgae for L-Arginase production

CHAPTER 3

Materials and methods

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3.1 Equipments used

The following equipments were used for the growth of micro algal cultures and for making of the standard curve for ammonia.

- Autoclave
- Weighing balance
- Spectrophotometer
- Microscope

3.2 Media preparation

Following are the components of the stock solutions that were used in media preparation.

- **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 Induced medium

To induce the production of the enzymes $NaNO_3$ in the BG-11 media was replaced with their substrate- L-asparagine (10g/L).

3.4 Methodology

3.4.1 Screening and isolation of Microalgal species

The algal examples were bound from different lakes, streams and lakes from various land domains of Himachal Pradesh. Change of 5-10 mL water tests was done with BG-11 medium at the period of assessing and they were moreover spared by the development of 10% formaldehyde. The lifestyle containers and plates were refined in liquid, set and changed BG-11 medium and were agonized at 25+_2oC, 3000 lux power with 16:8 hours light and dull stage (Rakesh Singh Gauret al., 2009).

3.4.2 Identification of the algal culture

Serial weakening from of the algal society was done and was placed on BG-11 medium with 1.5% agar. The separated states were intentionally lifted that appeared taking after 2-3 weeks and were traded to a glass slide containing a drop of BG-11 medium and saw under the amplifying lens for morphological conspicuous verification .Uni-algal social orders for each one of the three microalgae viz. Chlorella sp, *Scenedesmus dimorphus* were set up.

3.4.3 Procedure for making Standard curve

1. Label five test tubes according to the concentrations of the standard solutions (S1 to S5). Pipette 2.0 ml of the substrate solution to each tube. Incubate in the water bath for 10 minutes. To each tube, add 100 µl of the appropriate standard solution and mix. Incubate the tubes in the water bath exactly for 30 min. Add 0.4 ml of the TCA stop solution to stop the reaction. Add 2.5 ml of water and mix. This is the reaction mixture.

2. Prepare five test tubes (labeled S1 to S5). Add to each tube 800 µl of water and 20 µl of the appropriate reaction mixture. To develop colour, add 170 µl of the phenol nitroprusside solution, mix and add 170 µl of the alkaline sodium hypochlorite solution. Mix and incubate in the water bath for 10 min. Transfer the content of each tube to the spectrophotometer cuvette and measure the absorbance at 600 nm after zeroing the instrument against air.

3. Use linear regression to prepare the standard curve. Plot the absorbance against the concentration of ammonium sulfate in the standard solutions (mg/ml). Use the slope of the standard curve (ml/mg) to calculate the activity of the control and test samples.

3.5 L- Glutaminase assay

3.5.1 Materials required

Algal extract,centrifuge,L-glutamine,Distilledwater,Phosphatebuffer,waterbath,Trichloroaceticacid,Nesslers reagent,spectrophotometer,BG-11 media,testtubes, test tube stand,pipette

3.5.2 Reagents preparation

3.5.2.1 Phosphate buffer preparation

- Solution A-0.2 M solution of $Na_2HPO_4.7H_2O$
- Solution B- 0.2 M solution of $NaH2PO_4.2H_2O$
- pH 8.0- 94.4 mL of solution A+5.3 mL of solution B+100 mL of distilled water

3.5.3 Assay method

- Algal extract was taken and centrifuged at 10,000rpm for 20 minutes at 4 degree Celsius.
- Pellet was discarded and pellet was used for the assay
- 0.5 ml of algal supernatant was taken
- It was added to 0.5 ml of 0.4M L-Glutamine and 0.5ml distilled water
- It was mixed with 0.5 ml of 0.1M phosphate buffer with pH 8.0
- This mixture was kept on water bath for 15 minutes at 37 degree Celsius
- Further 0.5ml of trichloroacetic acid was mixed
- 0.1 ml of this mixture was added in 3.57 ml of distilled water and 0.2 ml nesslers reagent
- OD was taken at 450 nm

3.6 L-Arginase assay

3.6.1 Materials required

Glycine, Sodium hydroxide,Distilledwater,Arginine, Magnesium chloride, 10% per chloric acid, Algalsupernatant, waterbath,spectrophotor, tet tubes, test tube stand, pipette.

3.6.2 Chemicals needed

- 100 mL of 0.1 M glycine /NaLOH (pH 9.5)

  (a) 0.2M solution of glycine =0.75g in 50mL of water

  (b) 0.2M NaOH

  25mL of (a)+8.49mL of(b)

- 50mL of 0.25M arginine (pH 9.5)= 2.18 gm in 50mL distilled water
- 50mL of 10mM $MnCl_2$= 0.629gm in 50mL distilled water
- 10%perchloric acid=1mL of perchloric acid+ 9mL distilled water

3.6.3 Methodology

3.6.3.1 Sodium carbonate buffer preparation

- Solution A- 0.2M anhydrous sodium carbonate
- Solution B- 0.2M sodium bicarbonate
- pH 9.5-13mL of solution A+37 ml of solution B +150mL distilled water

3.6.4 Assay methodology

- O.2mL of glycine buffer was taken in a test tube

- 0.5 mL of algal supernatant was added to it
- 0.1 mL of magnesium chloride was added in the above mix
- It was incubated for 10 minutes at 37 degree Celsius
- After 10 minutes 0.1 mL arginine was added and incubated for 30 minutes at 37 degrees Celsius
- After 30 minutes 1 mL of 10%perchloric acid was added to the above mixture
- Absorbance was taken at 520 nm

CHAPTER 4

Results and Discussion

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4.1 Microscopic examination of algal cultures

4.1.1 *Scendesmus dimorphus*

Scenedesmus is a genus of green algae, specifically of the chlorophyceae. They are colonial and non-motile.

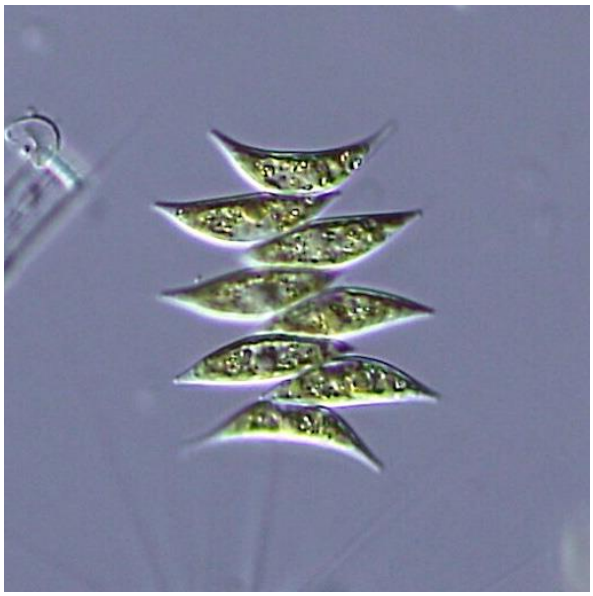

Fig 2. Microscopic examination of *Scendesmus dimorphus*

4.1.2 Chlorella

Chlorella is a genus of single-cell green algae belonging to the phylum chlorophyta. It is spherical in shape, about 2 to 10μm in diameter, and is without flagellla.

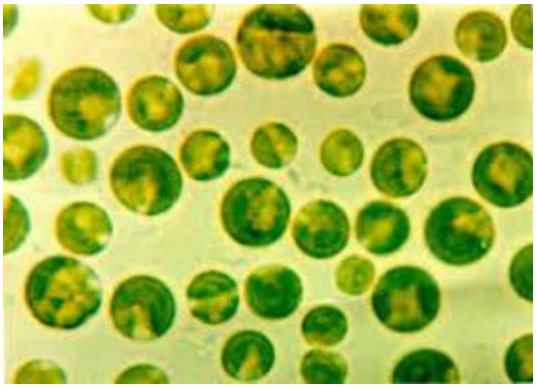

Fig 3. Microscopic examination of Chlorella sp.

4.2 L-Arginase assay

4.2.1 Standard curve of urea

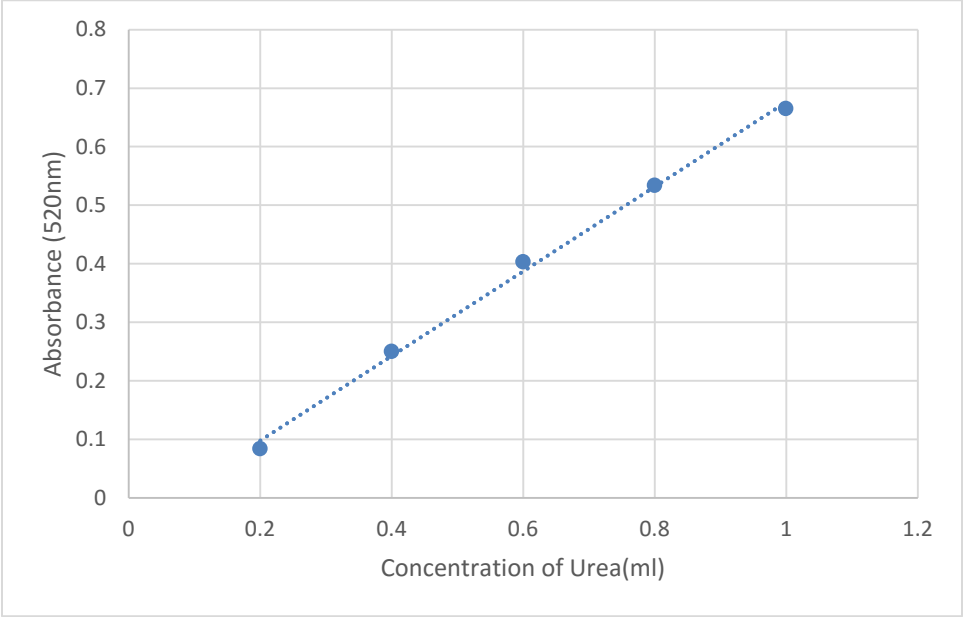

Fig 4. Standard curve of Urea

4.2.2 Calculation of L-arginase activity

Arginase (micromole/ml)= absorbance at 520nm/0.5*0.2634*10 = 1.1009 micromole per ml.

4.3 L-Glutaminase assay

4.3.1 Standard curve of ammonia

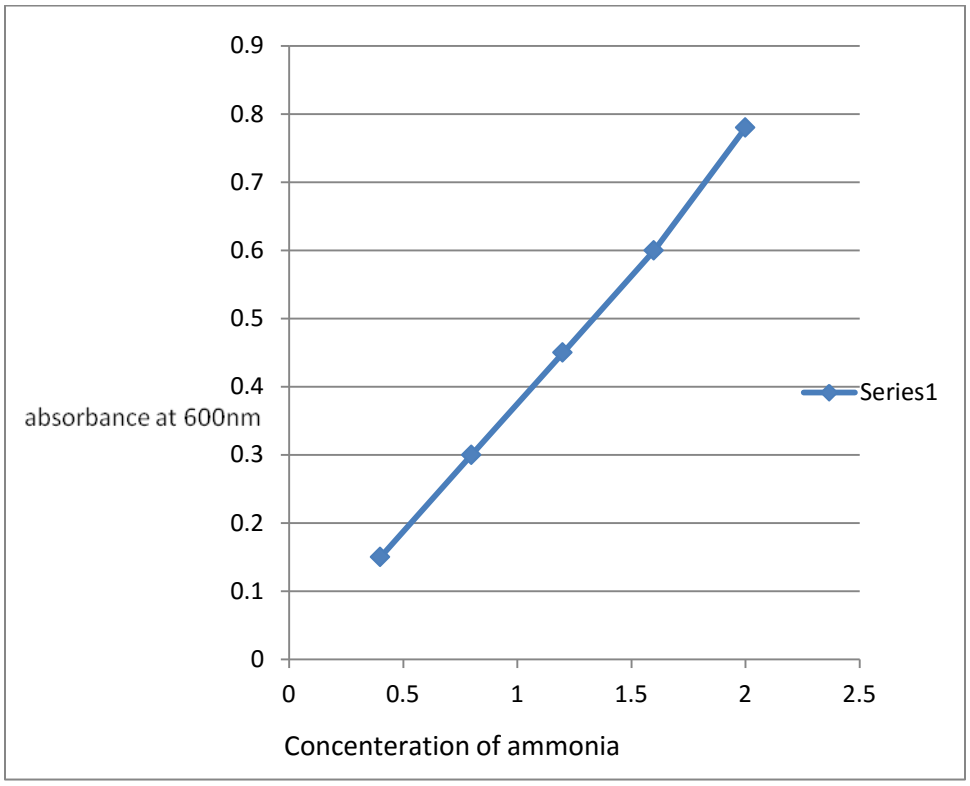

Fig 5. Standard curve of Ammonia

X-axis-concenterationof ammonia

Y-axis-absorbance at 600nm

The absorbance came out to be 1.388

4.3.2 Calculation of L-glutaminase activity

L-glutaminase activity (units/ml/min)= micromoles of ammonia liberated*2.50/0.1*30*0.5 units/ml/min

Where, 2.50 = volume of step 1; = volume of step 1 used in step 2

30 = time of assay in minutes,

0.5 = volume of enzyme used (1 mole of ammonium sulphate corresponds to 2 moles of ammonia.)

Activity of L-glutaminase=152.8 u/ml/min

CHAPTER 5

Conclusions & Future Prospects

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Conclusions:

- Two species of microalgae have been successfully grown by overcoming the contamination problems
- Screening of microalgal cultures revealed that these microalgae can able to produce L-Arginase enzyme
- The species also found to be exhibited L-Glutaminase activity
- Based on these prelinary studies these microalgae are the potential candidates for L-Arginase and L-Glutaminase production.

Future Prospects:

- Have to select the appropriate growth conditions for L-Arginase and L-Glutaminase production.
- Have to test the microalgae under stress conditions for L-Arginase and L-Glutaminase production

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