Isolation, screening and characterization of asparaginase producing novel streptomyces strain from chilika lake and partial purification of asparaginase from potential strain

Dissertation submitted in partial fulfilment for the degree of

Master of Science in Biotechnology

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CERTIFICATE

This is to certify the dissertation entitled **"Isolation, Screening and Characterization of Asparaginase producing novel Streptomyces strain from Chilika lake and partial purification of Asparaginase from potential strain"**Submitted by ***Arpita Basak*** in partial fulfilment of the requirement for the degree of Master of Science in Biotechnology/Applied Microbiology, KIIT School of Biotechnology, Kalinga Institute of Industrial Technology(KIIT), Deemed to be University, Bhubaneswar-751024,Odisha bearing Roll No. ***1661010*** & Registration No. **16646151458** is a *bona fide* research work carried out by her under my guidance and supervision from **'01/01/2018'**to **'14.05.2018'**.

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Ref..... Date.....

CERTIFICATE

This is to certify that the dissertation entitled **"Isolation, Screening and Characterization of Asparaginase producing novel Streptomyces strain from Chilika lake and partial purification of Asparaginase from potential strain"** submitted by **'Arpita Basak, Roll No.1661010, Registration No.16646151458.'** to the School of Biotechnology, Kalinga Institute of Industrial Technology(KIIT), Deemed to be University, Bhubaneswar-751024,Odisha for the degree of Master of Science in Biotechnology is her original work, based on the results of the experiments and investigations carried out independently by her during the period from **01/01/2018** to **14/05/2018** of study under my guidance.

Further, it is also to certify that the above said work has not been previously submitted for the award of any degree, diploma, or fellowship in any Indian or foreign University.

Dipanwita Roy
(Supervisor signature)

Date: 15.05.2018

Place: Bhubaneswar

DECLARATION

I hereby declare that the dissertation entitled "***title of the work***" submitted by me, for the degree of Master of Science to KIIT University is a record of *bona fide* work carried by me under the supervision and guidance of **'Dr.Lopamudra Ray'**, *KIIT School Of Biotechnology, Bhubaneswar, Odisha,India*.

Date: 15.05.2018

Place: vBhubaneswar

Arpita Basak

'Your Name & Signature'

Abstract :

189 actinomycetes isolates obtained from chilika lake were screened for asparaginase activity. After secondary screening (Nesslerization experiment) four strains were selected for further studies. Out of them two strains HB6AG, HB3AG and MM1AG12 were studied for further characterization and shown to produce optimal amount of asparaginase(29,28,32micro gram/ml) respectively. HB3AG was further characterized by 16s rDNA sequencing as S*treptomyces cavourensis* NBRC13026. Asparaginase enzyme was then partially purified from HB6AG. The obtained enzyme showed optimum activoty at 1%(w/v) NaCl, pH7. Also the enzyme showed activity at 5%(w/v) NaCl pH 9 and different metal ions and inhibitors tested. This enzyme further can be characterized by kinetic studies. The obtained strains will be characterized by polyphasic taxonomy.

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GENERAL INTRODUCTION:

The lake Chilika is a natural brackish water lagoon of marine origin and a Ramsar site since 1981. It landscapes in Puri, Ganjam and Khurda districts of Odisha between latitudes $19^{O}28'$ to $19^{O}54'$ N and longitudes $85^{O}05'$ to $85^{O}38'$ E. The water spread area of the lagoon varies between 1165sq. km in monsoon to about 906 sq. km in premonsoon. The sea spread area of the lagoon varies between 1165sq. km in monsoon to about 906 sq. km in premonsoon. The surface temperature of Chilika lake varies from 17.5 to 32^{O}C. Salinity varies from traces to 36ppt. ph varies from 7.6 to 10. Chilika receives fresh water through Daya, Bhargavi, Nuna and Makra rivers, the distributaries of Mahanadi and many other small streams[5]. It is connected to the Bay of Bengal through a 25km long outer channel making it an a unique assembly of, brackish, marine and fresh water eco-system with estuarine characters and is a hotspot of biodiversity that shelters a number of rare species listed in the IUCN red list of threatened species.

Bacteria belonging to the genus streptomyces are well known as the largest antibiotic producing genus in the microbial world. Filamentous bacteria belonging to the genus *Streptomyces* are well-known as the largest antibiotic-producing genus in the microbial world discovered so far (Taddei et al., 2006; Jayapal et al., 2007). Most *Streptomyces* and other Actinomycetes produce a diverse array of anti-biotics including aminoglycosides, anthracyclins, glycol-peptides, β-lactams, macrolides, nucleosides, peptides,

Streptomyces is a genus of Gram-positive bacteria that grows in marine environments, with a filamentous form similar to fungi. The morphological differentiation of*Streptomyces* involves the development of a layer of hyphae that can differentiate into a chain of spores. This process is exclusive among Gram-positives, requiring a specialized and synchronised metabolism(de Lima Procópio, da Silva, Martins, de Azevedo, & de Araújo, 2012). The most attention-grabbing property of *Streptomyces* is the capability to produce bioactive secondary metabolites such as,

antitumoral, antivirals, antifungals, anti-hypertensivesand many enzymes such as asparaginase and tyrosinase those are well known ……..

The present study was conducted with the following objectives:

1. To isolate, identify and characterize novel micro-organisms from the estuarine Chilika lake using polyphasic approach for microbial systematics.
2. To purify and characterize asparaginase produced in a novel isolate *Streptomyces HB6AG strain* isolated from Chilika Lake.
3. Optimization of culture conditions for determining the application of novel *Streptomyces* isolate HB6AG with respect to asparaginase activity.

Chapter 2

2.1 INTRODUCTION AND REVIEW

2.1.1 CHILIKA LAKE

Estuarine habitats are among the most biodiverse and productive natural habitats in the world (Costanza *et al.,* 1993). Chilika Lake (19º 28' and 19 º 54' N and 85º 05' and 85º 38' E), a brackish water lagoon is situated on the east coast of India. It is one of the largest brackish water lakes in Asia with estuarine features. The lake is a designated first Indian wetland of international importance under the Ramsar convention, 1981 (http://www.ramsar.org). Extending from the southwest corner of districts of Puri and Khurdha to the bordering Ganjam district in the state of Odisha. The water-spread area of the lake fluctuates between 1165 and 906 sq. km during the monsoon and summer, respectively. The primary inflow is acquired by nearly 35 river streams, which outflow through the mouth at Satpada to merge into the Bay of Bengal. The lake is broadly divided into 4 ecological sectors based on salinity and depth: the south, north, central and outer channel. The lake is known for its rich biodiversity and is home to several rare, vulnerable and endangered species listed in the IUCN Red List of threatened animals inhabiting the lake area. This includes Irrawady dolphins, amphibians and reptiles. It is the largest wintering ground for seasonal migratory birds like migratory water-fowl on the Indian subcontinent. The highly productive lake supports fisheries and aquaculture for a sustainable livelihood. A large salinity gradient with alkaline pH across the lake also contributes to rich populations of macro and microalgae, marine seaweeds, marine sponges and sea grasses. This makes this habitat an excellent ground for isolation of micro-organims with a broad range of tolerance to saline (halophilic/halotolerant) and alkaline (alkalophilic/alkali-tolerant) environments.

Despite having tremendous biodiversity, the microbial diversity of Chilika Lake has been inadequately explored. Limited reports of phototrophic bacteria like *Shewanella chilikensis* (Sucharita *et al.,* 2009) and *Allochromatium phaeobacterim* (Srinivas *et al.,* 2009) have been isolated and taxonomically identified from this habitat. The habitat is presumed to be a repository of industrially important microorganisms with biotechnological potential. No reports of isolation of Streptomyces strains from the lake are known. Thus, Chilika Lake could be a favorable environment for bioprospecting of novel microbial isolates.

2.1.2 Bioprospecting of asparaginase producing streptomycetes strains from estuarine environments

Estuarine environments are transition zones between freshwater and marine or ocean environments. Due to marine tidal influences like waves, influx of saline water and river influences like freshwater input and sediment, estuarine environment is subjected to mix of nutrients from both freshwater and marine water. This results in high productivity in ecotone regions and is responsible for increased biodiversity. Estuarine lakes are often flooded with freshwater from rivers, which on draining into oceans, mixes with seawater resulting in water with salinity and pH midway, termed as brackish water. The salt content in brackish water varies between 0.5 to 30grams/Litre. Due to high biodiversity, brackish water estuarine environments have several species of vertebrates and invertebrates.

Estuarine habitats are good sources for bio-prospecting of micro-organisms with halophilic and alkaline microbes with industrial applications. Microbes which belong to genus *Streptomyces*, isolated from marine and estuarine habitat have been widely recognized as a potential source of antifungal, anti-tumour, anti-bacterial compounds (**Table 1**).

Sl no.	**Strain**	**Activity**	**References**
1	*Streptomyces thermophiles*	Antibacterial	Schone, 1951
2	*Streptomyces refuineus*	Antitumor & Antimicrobial	Hu *et al.,* 2007
3	*Streptomyces griseus*	Antimicrobial & Insecticidal activity	Nair et al., 1989
4	*Streptomyces werraerisi*	Antibacterial	Sanghvi*et al*., 2014
5	*Streptomyces lunaliharesii*	Antifungal	Gomes *et al., 2001*
6	*Streptomyces aureofaciens*	Antifungal	Darekn. M.A 1960
7	*Streptomyces thermoviolaceus*	Antifungal	Tsujibo*et al*., 2004
8	*Streptomyces halstedii*	Antifungal	Joo*et al*., 2005
9	*Streptomyces olivaceoviridis*	Antifungal	Romaguera*et al*., 1992
10	*Streptomyces venezuelae*	Antifungal	Mukhherjee.,*et al* (2004)

11	*Streptomyces coelicolor*	Antifungal	Saito., *et al* (1999)
12	*Streptomyces antibioticus*	Antifungal	Xu *et al*., 1986
13	*Streptomyces griseoruber*	Antifungal	Wang *et al*., 1984
14	*Streptomyces cinereoruber*	Antifungal	Okazaki *et al*., 1991

Table 1: Antagonisticc *Streptomyces* strains.

2.1.3 PHYLOGENY AND TAXONOMY

Phylogenetics is the study of the evolutionary relationships, development and diversification of a species or group of organisms, or of a particular feature of an organism. On a broader scale, phylogeny assists to unravel the interrelation of all living organisms in our immediate environment. In recent times, it has become a significant tool for understanding evolution and diversity of life, especially, for categorization of organisms into species, genera or families. Due to insufficient fossil records in the microbial world, evolutionary relationships of bacteria and the archaea are deduced from conserved gene sequences, which serve as 'evolutionary chronometer or molecular clocks' (Woese*et al*., 1990).

Taxonomy is a biological discipline dealing with classification of living organisms. In taxonomy, several phenotypic characteristics (i.e. morphological or physiological parameters) are measured and compared, which are used to group organisms into separate groups and hence into a classification system. Every taxonomic hierarchy for every organisms, begins with species as the smallest unit and domain as the highest taxonomic rank. The order is shown

Domain: Bacteria

Phylum: Actinobacteria

Class: Actinobacteria

Order: Actinomycetales

Family: *Streptomycetaceae*

Genus: *Streptomyces*

Species: *albus* (type species of genus*Streptomyces*)

2.1.4 Polyphasic Taxonomy

Classical methods of microbial taxonomy were based on phenotypic parameters like size, shape, texture, cell arrangement, motility etc. Later, physiological, metabolic, biochemical, antigenic, genetic (G+C content) and ecological parameters were also considered. Currently, molecular methods based on comparison between conserved gene sequences (ribosomal ribonucleic acid; rRNA) helps to establish evolutionary associations (Woese*et al*., 1990). The rRNA gene sequences function as an evolutionary chronometer due to its several advantageous features. rRNA gene sequences are found in all living organisms in different forms. The small subunit ribosome of 16S rRNA (~ 1500nt) is compared in prokaryotes and 18S rRNA in eukaryotes. The small subunit ribosome, is relatively large, functionally constant and contains several variable and conserved regions. Several databases (RDP: Ribosomal Database Project; Cole *et al*., 2008) exist where sequences of rRNA from different organisms are stored and used for phylogenetic analysis.

However, due to lack resolution of 16S rRNA gene sequences for precise description of micro-organisms at species level, in addition to genotypic parameters (DNA-DNA hybridization, phylogenetic trees), phenotypic variables like chemotaxonomic properties are also considered. In modern microbial taxonomy, this collective approach of using range of genotypic, phenotypic and chemotaxonomic methods for taxonomic identification of micro-organisms is known as “polyphasic approach” and is widely used for bacterial systematics. The term polyphasic taxonomy was coined for delineation of taxa (Colwell, 1970). The approach aimed at different kinds of data and information (phenotypic, genotypic and phylogenetic) on microorganisms and essentially indicates a consensus type of taxonomy (Vandamme*et al*., 1996).

2.1.5 Actinobacteria: genus *Streptomyces*

The family*Streptomycetaceae*, consists of three genera, *Streptomyces*, *Kitasatospora* and *Streptacidiphilus*. The genus *Streptomyces* (Waksman and Henrici, 1943), a type genus of the family *Streptomycetaceae*, is the largest of the phylum Actinobacteria (Kampfer, 2006; Lodders and Kampfer, 2007) and constitutes the most predominant component of micro-organisms found in soils, sediments and decaying vegetation invariably throughout the world (Labeda, 2012). They are aerobic, Gram positive,

high G+C content (69-78 %), produce extensively branched network of substrate and aerial mycelia and most species are sporulating. Almost more than 600 species of diverse genera have been described, many of which are officially published (Euzeby, 2012). *Streptomyces* have been extensively explored for multitude of secondary metabolites, bioactive compounds like antibiotics and industrially and commercially important enzymes (Anderson *et al.*, 1956; Watve, 2001; Chater*et al.*, 2010).

Although on the basis of 16S rRNA sequence, which is highly conservative and universally distributed, the genus *Streptomyces* forms a separate phylogenetic cluster, its resolution at species level is very low. Hence, establishing differences at species level when 16S rRNA sequence similarities are > 97 %, is challenging. Hence, distinction of novel species should is often done on basis of phenotypic features like colony morphology and colour on several defined media. In addition, chemotaxonomic markers maybe used, although, sometimes these tests may also provide conflicting results due to poor resolution at species level. Several recently developed techniques are valuable in classification of new affiliates to the taxon isolated from diverse geographical environments (Goodfellow, 2007; Kampfer and Labeda (2006); Rong and Huang, 2010, Labeda, 2012).

2.1.6.Taxonomy of genus *Streptomyces*

2.1.6.1 Phenotypic characterization

Members of genus *Streptomyces* can be phenotypically identified as Gram positive, filamentous bacteria with presence of well-developed vegetative hyphae or mycelia (0.5-2.0 µm diameter). They produce extensive branched network of substrate and aerial mycelia. The filamentous vegetative substrate mycelia assist in obtaining nutrients from dead organic matter. During growth, the aerial filaments differentiate into aerial mycelia, from which propagative spores develop at maturity. This gives the colony a powdery, granular or velvety appearance. The spores' surfaces may vary from smooth, rough, hairy, spiny, warty and rugose. The shape and arrangement of filaments and spores maybe rectiflexibiles, retinaculiaperti, spirales or verticilliati (Shirling and Gotlieb, 1968).

Previously, the description of streptomycete species mainly involved the phenotypic observations by comparisons between colour of aerial and substrate mycelia

(Krainsky, 1914; Waksman and Curtis 1916). Due to the sheer large number of described species in the genus *Streptomyces*, taxonomy has been a challenging task. These problems in taxonomy of *Streptomyces* were addressed by a series of co-operative studies among which the International Streptomyces Project (ISP) has been the most notable one. The project was planned and enacted by the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature and the Subcommittee of Actinomycetales of the Committee on Taxonomy of the American Society of Microbiology. The aim of the study was to provide consistent and reliable description of existing and genuine type strains of *Streptomyces* and *Streptoverticillium* species. The ISP study primarily involved description of type species based on identification and distinction of the strains using strictly standardized media (ISP media) and experimental conditions to determine morphology (spore chain, colony colour), pigmentation properties and carbon utilization profiles (Shirling and Gottlieb, 1966). The classification of genera *Streptomyces* and *Streptoverticillium* in the eighth edition of Bergey's Manual of Determinative Bacteriology was based on the results of the International Streptomyces Project (Pridham and Tresner, 1974).Further, more precise characterization of the species were supported by serological, physiological and biochemical methods (Goodfellow*et al.,* 1987; Ridell*et al*., 1986).

2.1.6.2 Numerical Taxonomy

The ISP method used extensively for the description of *Streptomyces* strains was largely based on morphology and pigmentation but attempts to compare and delineate Streptomyces strains were beyond its scope. ISP posed limitations for classification based on comparison of described species; hence, phylogenies could not be established. Application of numerical taxonomy was first proposed by Sneath, 1970 for reclassification of surplus of *Streptomyces* strains. The first comprehensive numerical taxonomic study was proposed by Williams *et al*.,1983 who assigned type strains of *Streptomyces* species to 23 major (6–71 strains), 20 minor (2–5 strains) and 25 single membered clusters. The minor and single membered clusters were considered to be species and the major clusters species-groups (Goodfellow*et al*., 1992). The major clusters defined by Williams *et al*., 1983 were recognized and compiled by Kampfer, 1991. The Bergey's Manual of Systematic Bacteriology, volume 4 (Williams *et al*., 1989) is a compilation of *Streptomyces* species based on

the numerical taxonomic study of Williams *et al*., 1983.

2.1.6.3 Molecular Taxonomy

With advent of computers and advancements in molecular biology, increased the amount of evolutionary information has become available for classification and systematics. In this light, nucleic acid sequence comparison of target genes, particularly the comparison of rRNA sequence, is useful for analyzing questions related to horizontal gene transfer of genes within the genus (Huddleston *et al*., 1997).

Due to the highly conserved nature of rRNA, 16S rRNA gene sequences (Woese, 1987) are used for observing comparison of sequence variations, useful for genus-specific or species-specific investigation within the genus *Streptomyces* (Stackebrandt*et al*., 1991). Several studies have revealed that phenetic classification better reflects the phylogenetic relationships between the examined strains (Gladek*et al.*, 1985; Witt and Stackebrandt, 1990). Currently, several databases like Ribosomal database project-II (Cole *et al*., 2008), GeneBank from NCBI (National Centre for Biotechnology Information) (Benson, 2011) and ez-taxon (Chun *et al.*, 2007) are available for sequence comparison.

Phylogenetic trees are constructed using Internet based online softwares like PHYLIP, MEGA 5 etc. These sequences are aligned manually against the corresponding sequences retrieved from the GeneBank using Clustal W program (Larkin *et al*., 2007). The aligned sequences are manually checked for gaps using online BioEdit Sequence Alignment Editor, and adjusted before construction of phylogenetic trees in tree making algorithms of neighbour joining method (NJ; Saitou &Nei, 1987); maximum likelihood (ML; Felsenstein, 1981) maximum parsimony method (MP; Fitch, 1971) and minimum evolution (ME; Rzhetsky and Nei, 1992) of MEGA software (version 5, Tamura, 2011). Evolutionary distance matrix are calculated using method of Kimura's two parameter model (Kimura, 1980). The topology of the phylogenetic tree is evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Determination of species identity within streptomycetes is done by DNA-DNA hybridization i.e. monitoring the reassociation of single-stranded DNAs from closely related organisms. DNA-DNA hybridization is a requisite for the description of a new species within a taxon when strains share more than 97 % 16S rRNA gene sequence

similarity (Stackebrandt& Goebel, 1994, Tindall*et al.*, 2010). This limit is however debatably discussed by Stackebrandt&Ebers, 2006.

The degree of relatedness is expressed as % homology and the genomic definition of species is considered to include strains with $\geq$ 70 % DNA–DNA relatedness and $\leq$ 5 ^{o}C difference in the melting temperature (ΔTm) between the homologous and heterologous hybrids formed using standard stepwise denaturation conditions (Wayne *et al*., 1987). DNA-DNA hybridization can also be used as a method for classification of streptomycetes at genus level.

Apart from this restriction fragment length polymorphism (RFLP) analysis and random amplified polymorphic DNA (RAPD) PCR (polymerase chain reaction) assays involving entire bacterial chromosomes can also be used for providing taxonomic information (Beyazova&Lechevalier, 1993; Williams *et al*., 1990).

Currently, new technologies such as automatic sequencers, pyrolysis mass spectroscopy and DGGE (denaturing gradient gel electrophoresis) have been successfully applied, resulting in an increase in taxonomic data and improvement of differential analysis (Anderson and Wellington, 2001).

AIMS AND OBJECTIVES

Hence the present study was conducted with the following objectives:

1. To isolate novel asparaginase producing Streptomyces strains using pure culture techniques..
2. To taxonomically identify and classify novel Streptomyces strains prodcucing asparaginase from the estuarine Chilika lake using polyphasic approach for microbial systematics

2.2 MATERIAL AND METHODS

2.2.1 SOURCE OF CHEMICALS, REAGENTS AND INSTRUMENTS

2.2.1.1 Media Chemicals, Cultures

All microbiological media, ISP media, stains, antibiotic discs, sugars and sugar discs used was purchased from Hi Media, Pvt. Ltd. Mumbai, India; Merck, Germany; SD Fine, India. Cultures of several type strain like *Escherichia.coli* MTCC 82, *Staphylococcus aureus* MTCC 96, *Salmonella typhi* MTCC 734, *Micrococcus luteus*, *Bacillus subtilis* used for antimicrobial activity were procured from, Microbial Type Culture Collection (MTCC, Chandigarh) and *Bacillus cereus* IP406 was procured from Pasteur Institute, France

2.2.1.2 Instruments

Research equipment used are listed in Table 1 of Appendix.

2.2.2 COLLECTION OF SAMPLES, ISOLATION, ENRICHMENT AND SCREENING OF ISOLATES

2.2.2.1 Sampling

Sediment were collected from the shore lines of 21 different sampling stations of Chilika lake. Sediment samples were collected as Streptomyces and Actinobacteria are genrally soil and sediment dwellers.(**Figure 1)**. Samples were collected aspetically in zip lock packets and stored at 4^{o}C until further analyzed. Temperature and pH of the water and soil samples were measured at the time of collection.

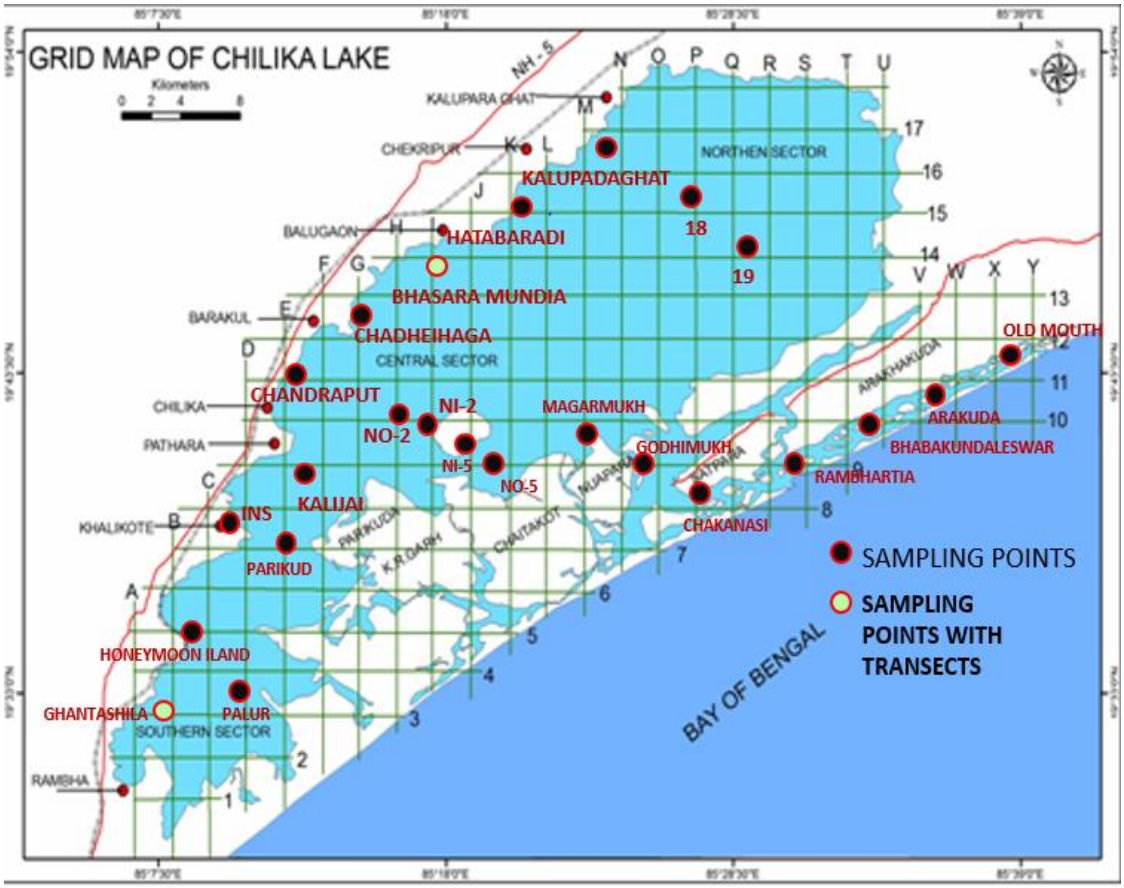


Figure 1: Map of Chilika lake showing sampling sites for isolation of *Streptomycete*s strains from sediment samples collected from sampling station Magarmukh from central zone of Chilika lake

2.2.2.2 Enrichment and isolation of Streptomyces

The enrichment was done on starch casein media (Table 4, Appendix I) broth supplemented with nystatin (stock: 50mg/L; working: 50ug/ml)) at 30^{o}C at 120 rpm. Five grams of sediment were added to 250 ml conical flasks containing 50 ml of sterile starch caesin medium. The flasks were incubated on a rotary shaker (120 rpm) at 30^{0}C for 7 days. 1ml of this culture was transferred to fresh broth and this was repeated 4 times for 1.5 months. Isolates were obtained by plating 100ul of serially diluted enrichment culture, after 7 days on starch casein agar medium supplemented with nystatin.

2.2.2.3 Primary Screening of isolates :

Primary screening by plate method :

Primary screening was done by adding the phenol red as an indicator dye in the media. The media asparagine glucose() agar was used. Twelve strains of previously isolated asparaginase positive Streptomyces sp. were spot inoculated in the asparaginase glucose agar plate and were incubated at 37°C for 7 days. Changing the colour of medium from yellow to pink indicates the production of asparaginase.

2.2.2.4 Secondary screening of isolates :

Secondary screening by checking the conc of ammonia released :

The cultures were grown in 20ml of starch caesin broth media at 37°C for a specific time. After the specific incubation time 2ml of culture supernatant was collected by centrifugation at 10,000 rpm for 5minutes. After centrifugation 0.5ml of supernatant,7ml of distilled water and 1ml of Nesslers reagent was taken and incubated at room temparature for 30minutes. A yellow colouration indicates the presence of ammonia. If the conc of ammonia is much, brown colour will formed. The yellow colour was read by using a UV-visible spectrometer at 480nm

Assay for asparaginase :

Asparaginase actiity was determined by the nesslerization according to the method that was described by Writson and Yellin, 1973. The reaction mixture contained 1.5ml of 0.04M asparagine prepared in 0.05M Tris-HCl buffer,pH 8.6 and an 0.5ml of an enzyme was taken to make up the total volume 2ml. The tubes were incubated at 37C for 30 minutes. The reaction was stopped by adding 0.5ml of 1.5m trichloroacetic acid(TCA). The blank was prepared wwithout adding the enzyme. The liberated ammonia was determined by adding 1ml of Nessler’s reagent into tubes which contained 0.5ml of supernatant and 7ml of distilled water and incubated at room temparature for 30 minutes. A yellow colouration indicates the presence of ammonia. If the conc of ammonia is much, brown colour will formed. The yellow colour was read by using a UV-visible spectrometer at 480nm. The amount of ammonia released was calculated by using standard curve. One unit (U) of asparaginase is defined as the amount of enzyme which catalyzed the formation of 1 micromole of ammonia from asparagine per minute at 37℃.

2.2.2.5 Screening of isolates by ARDRA (Amplified Ribosomal DNA Restriction Analysis):

Duplicate strains were excluded by performing ARDRA analysis of all the selected Streptomyces isolate after secondary screening. To perform ARDRA the genomic DNA of the strains were isolated by standard protocols , amplified and then it was digested using the restriction enzyme *KpnI* (Reference). The pattern obtained was then analysed to select the appropriate strain.

ARDRA:

16s rRNA PCR amplified products were further proceeded for restriction digestion. The reaction mixture was:

PCR amplified product	7µl
Kpn I	2µl
10X Buffer	3µl
Water	18 µl
Total	30 µl

Then samples were incubated at 37^{O}C for 5 hours for digestion. The reacttion was stopped by incubating at 70^{O}C for 5 minutes.. The samples were stored at 4^{O}C for further analysis. 10µl of samples were loaded to 1% agarose gel and banding patterns were observed in gel documentation system.

2.2.2.5.2 Genomic DNA isolation of Streptomyces: (By kit method)

Genomic DNA was isolated from strains by using HiPurA Streptomyces DNA purification kit (HiMedia, India). In brief, the cultures were inoculated in SCA media and incubated at 37^{O}C for 7 days. The cultures were then harvested by centrifuging at 6500 rpm for 15 minutes. The pellet were then resuspended in 300µl of lysis solution (AL). 20µl of RNase solution was added and incubated for 2 minutes at RT (25^{O}C). 20µl of proteinase K (stock-10mg/ml, working-20mg/ml) solution was added, mixed and the resuspended cell were transferred to Hibead tubes (DBCA05). Then it was left for 30 minutes of incubation at 55^{O}C. Hibead tubes were vertexed horizontally for 5-7 minutes and again incubated for 10 minutes at 95^{O}C followed by pulse vertexing once or twice. Tubes were centrifuged at 13000 rpm for 1 minute at RT and supernatant

were transferred to clean capped 2ml collection tubes. 200µl of lysis solution (C1), vertexed thoroughly for 15 seconds and incubated at 55^{O}C for 10 minutes. 200µl of ethanol (96-100%) was added to the lysates, mixed by pipetting/vertexing for 15 seconds. The lysates obtained were transferred into HiEluteMiniprep spin column (capped) and centrifuged at 10000rpm for 1 minute. Flow-through liquid were discarded and the spin column were placed in the same collection tubes. 500µl of prewash buffer solution (PWB) was added to the columns and centrifuged at 10000rpm for 1 minute. Again the flow-through liquid and same collection tubes with same columns. 500µl of diluted wash solution (WS) to the columns and centrifuged for 3 minutes at 13000-16000rpm to dry the column. Flow-through liquid were discarded and the same collection tubes were used. Columns were centrifuged for additional 1 minute at maximum speed if residual ethanol is seen. Flow-through was discarded and column were transferred into new capped collection tubes. 200µl of elution buffer (ET) was pippeted directly into columns without spilling to the sides and incubated for 1 minute. Then it was centrifuged at 10000 rpm for 1 minute to elute the DNA and the elutes were transferred to fresh capped 2ml collection tubes and stored at 2-8^{O}C.

2.2.2.5.3 Spectrophotometric quantitation of DNA

- DNA was diluted either in TE or Millipore water .
- OD_{260} was taken for the diluted sample against blank.
- DNA concentration was calculated as g/ml = OD_{260} X dilution factor X 50

2.2.2.5.4 PCR amplification of the 16S rDNA

PCR Conditions (for 34 cycles)

Initial denaturation	95^{0}C	5 min
Cyclic denaturation	94^{0}C	1 min
Cyclic Annealing	53^{0}C	30 sec
Cyclic Extension	72^{0}C	1 min
Final extension	72^{0}C	5 minutes
At hold	4^{0}C	infinity

Sequencing of 16S rDNA

Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with Forward and Reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Consensus sequence of **1501 bp** of *16S rRNA* was generated from forward and reverse sequence data using aligner software. The primer pairs used in the sequencing process were:

785F: GGA TTA GAT ACC CTG GTA

907R: CCG TCA ATT CCT TTR AGT TT

2.2.8.2 Sequence and Phylogenetic analysis

The identification of phylogenetic neighbors was initially conducted by subjecting the near complete 16S rRNA gene sequence (1501 bp) to NCBI BLAST (Altschul *et al.*, 1997) and mega BLAST (Zhang *et al.*, 2000) programs against the database of type strains with validly published prokaryotic names (Kim *et al.*, 2012). The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012).

To determine the phylogenetic relationships of HB6AG and its closest relatives, 16S rDNA sequence of the strains were aligned manually against the corresponding sequences retrieved from the GeneBank using Clustal W program (Larkin *et al*., 2007). The aligned sequences were manually checked for gaps using BioEdit Sequence Alignment Editor, version 7.1.3 (Hall, 1999) and adjusted before construction of phylogenetic trees in tree making algorithms of maximum likelihood (ML; Felsenstein, 1981) maximum parsimony method (MP; Fitch, 1971) and minimum evolution (ME; Rzhetsky and Nei, 1992) of MEGA software (version 5, Tamura, 2011). Evolutionary distance matrix was calculated using method of Kimura's two parameter model (Kimura, 1980) and tree was constructed using neighbour joining method (NJ; Saitou & Nei, 1987). The type strain *Actinomadura hibisca* JCM 9627^{T} was used as an outgroup. The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

2.2.4 MORPHOLOGICAL CHARACTERIZATION

2.2.4.1 Gram Staining

A loop full of culture was suspended in a drop of water on a clean glass slide. Then it was flooded with Crystal violet (2 min) and iodine for 2 min. The stain was then washed by using decolourizer (70% alcohol). The smear was then flooded with the counter stain saffranin for 30 sec and washed with distilled water. The smear was air dried and then observed under the phase contrast microscope (.Leica DM300, Leica microsystem, Germany).

2.2.4.3 Growth on ISP media (International Streptomyces Project; Shirling and Gotlieb, 1968)

Culture attributes of isolates, pigmentation and melanin production were recorded after 21 days incubation at 30^{o}C on several modified ISP [International Streptomyces Project; Shirling and Gottlieb, 1966] media, [Glucose yeast extract malt extract agar (ISP 2), Oatmeal agar (ISP 3), Inorganic salts starch agar (ISP 4), Glycerol aspargine agar (ISP 5), Peptone yeast Iron agar (ISP 6) and Tyrosine agar (ISP 7)]. The ISP media (Shirling and Gottlieb, 1966) were modified with seawater (50 % v/v) as basal medium instead of distilled water. Colour evaluations were determined by colour chips from ISCC-NBS Color Charts standards no. 2 2106 (Kelly, 1964),(table 2, appendix)

2.2.5 BIOCHEMICAL CHARACTERIZATION

2.2.5.1 Starch Hydrolysis (Amylase production)

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 1% soluble starch and incubated for 1 week at 30°C. Hydrolysis of starch was observed by flooding iodine solution (Apendix I, Table 1) and appearance of colourless zone around hydrolyzing colony on the plate.

2.2.5.2 Gelatin and Caesin Hydrolysis (Protease production)

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 1% casein and 1% gelatin, separetely and incubated for 1 week at 30°C. Hydrolysis of gelatin and caesin was observed by appearence of colourless zone after flooding the plates with acidic Mercuric chloride solution (Apendix I, Table 1)).

2.2.5.3 Lipids Hydrolysis (production of lipase)

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 1% tributyrin and 1% Tween 80, separetely and incubated for 1 week at 30°C. Hydrolysis of tributyrin and teen 80 was observed by appearence of precipitate zone around colony or culture on the plate.

2.2.5.4 Hydrolysis of Carboxy methyl cellulose (CMC) and Cellulose

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 1% CMC or cellulose, separetely and incubated for 1 week at 30°C. Degradation of CMC and cellulose were observed by appearence of colourless zone on flooding the plates with aqueous Congo red solution (Apendix I, Table 1).

2.2.5.5 Hydrolysis of Lecithin

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with egg yolk suspension (one egg yolk suspended in 0.9% saline), separetely and incubated for 1 week at 30°C. Appearance of opaque zone around colonies is indicative of lipolysis and a second colourless zone is indicative of proteolysis.

2.2.5.6 Hydrolysis of Chitin

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 1% colloidal chitin, separetely and incubated for 1 week at 30°C. Degradation of chitin were observed by appearence of colourless zone on the plate.

2.2.5.7 Pectin Hydrolysis

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 1% pectin and incubated for 1 week at 30°C. Plates were flooded with 1% hydroxyl trimethyl ammonium bromide (CTAB). A zone of precipitation of Ca pectate is indicative of pectin hydrolysis.

2.2.5.8 Catalase Test

Evaluation of catalase activity was determined by oxygen bubble formation on addition of 4 % H2O2 to well grown colonies on glass slide.

2.2.5.9 Citrate Test

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 1% sodium citrate and incubated for 1 week at 30°C. Colour change of medium from green to blue is indicative of citrate utilization.

2.2.5.10 Urease Test

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 1% urea and phenol red (few drops) as indicator and incubated for 1 week at 30°C. Change of colour of media to bright pink is indicative of positive urease test.

2.3.5.10 Oxidase Test

Grown culture were tested for oxidase activity by observing the change in colour (white to blue) of the oxidase disk (Hi Media, Pvt. Ltd. Mumbai, India).

2.2.5.11 Nitrate Reduction Test

The isolates were inoculated in seawater (50 % v/v) LB agar supplemented with 0.5% sodium nitrate with phenol red (few drops) as indicator and incubated for 1 week at 30°C. Appearance of pink colonies is indicative of nitrate reduction.

2.2.5.12 Sugar Utilization Tests

The isolates were inoculated in basal salt medium (Apendix I, Table 3) supplemented with 1% sugar. Media without the respective sugars served as negative controls.The sugars tested include L-arabinose, D-mannose, D-cellobiose, D-fructose, D-glucose, D-galactose, raffinose, L-rhamnose, D-xylose, maltose, sucrose and inositol.

2.2.5.13 Antibiotic sensitivity assay

The isolates were cultured on seawater (50 % v/v) LB agar plates. Antibiotic discs of several antibiotics were placed on top of agar plates. The appearance of sensitivity zones were observed after 1 week incubation at 30^{o}CThe tested antibiotics are listedin Table 5, Appendix I).

2.2.6 PHYSIOLOGICAL CHARACTERIZATION

2.2.6.1 Determination of specific growth rate, doubling time and generation time for the selected isolates:

To determine the generation time and specific growth rate, preculture of isolates HB6AG was grown in Starch casein media. 1% (v/v) of precultures (containing approx.1.2-1.8 x10^{6} cells/ml) were inoculated to 50 ml of Starch casein broth. Aliquots of 1ml were taken at every 24h interval till 120h for determination of growth. The aliquots were centrifuged to obtain the supernatant. The supernatant was then evaluated for its antimicrobial property against *Micrococcus luteus* by well diffusion methods. on LB plate.. The viable cell count was determined on Starch casein agar plate. All the experiments were carried out in triplicates.

2.2.6.2 Growth on different NaCl conentration :

The isolate HB6AG was grown on different concentartion of NaCl (0.5%,1%,3%,5%) in solid media. Asparaginase glucose agar was prepared with different concentration of NaCl and the isolate were spot inoculated on the media fro checking the growth in time interval of 24 hour till 72hour.

2.2.6.3 Growth on different glucose concentration

The isolate HB6AG was grown on different concentartion of glucose (0.5%,1%,3%,5%) in solid media. Asparaginase glucose agar was prepared with different concentration of glucose and the isolate were spot inoculated on the media fro checking the growth in time interval of 24 hour till 72hour.

2.2.6.4 Growth on different pH

The isolate HB6AG was grown on different pH (5,7,9,11) in solid media. Asparaginase glucose agar was prepared with different pH and the isolate were spot inoculated on the media fro checking the growth in time interval of 24 hour till 72hour.

MOLECULAR TAXONOMICAL CHARACTERIZATION

2.2.8.1 16S rRNA Sequencing

2.2.8.1.1 Genomic DNA isolation of Streptomyces

16S RRNA sequencing was done by Xcelris Labs Ltd, India..

1. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with Forward and Reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

2. Consensus sequence of **1501 bp** of *16S rRNA* was generated from forward and reverse sequence data using aligner software.

2.3 RESULTS:

2.3.1 Enrichment and Isolation of Streptomyces strains :

A total of 189 strains were obtained by the enrichment of the sediment sample . The isolates were grown and maintained routinely on starch casein agar slants at 30^{o}C. 12 isolates were selected for primary screening on the basis of their pink colour formation on the asparaginase glucose agar medium and the secondary screening was carried out to select the potential strains.

Morphology of isolates selected for primary screening :

Morphology is an important characteristics of a taxa, though it is not adequate to differentiate between many genera. Aerial and substrate mycelia colour of 12 strains are shown in table

Sl no	Strain	Aerial mass colour	Substrate mycelia
1	MK11	Gy	Br
2	MK13	Gy	Br
3	MK4	Gy	Gy
4	MK20	W	Gy
5	HB2AG	W	Br
6	HB3AG	W	Br
7	HB6AG	W	W
8	MM1AG12	W	Br
9	BG2AG	Gy	Gy
10	BM4AG	W	Br
11	RB7AG	Gy	Gy
12	MK1	W	W

Table 2 : aerial and substrate mass colour of Streptomyces strains

Primary screening :

Strains	Pink colour formation
MK11	++
MK13	+
MK4	++
MK20	+
MK1	+
HB2AG	+
HB3AG	++
HB6AG	++
MM1AG12	++
BG2AG	+
BM4AG	++
RB7AG	+

Table 3 : Formation of pink colour on asparaginase glucose agar

(++) denoted as more pink colour (+)denoted as less pink colour

2.3.2 Secondary Screening

Secondary screening by checking the conc of ammonia released :

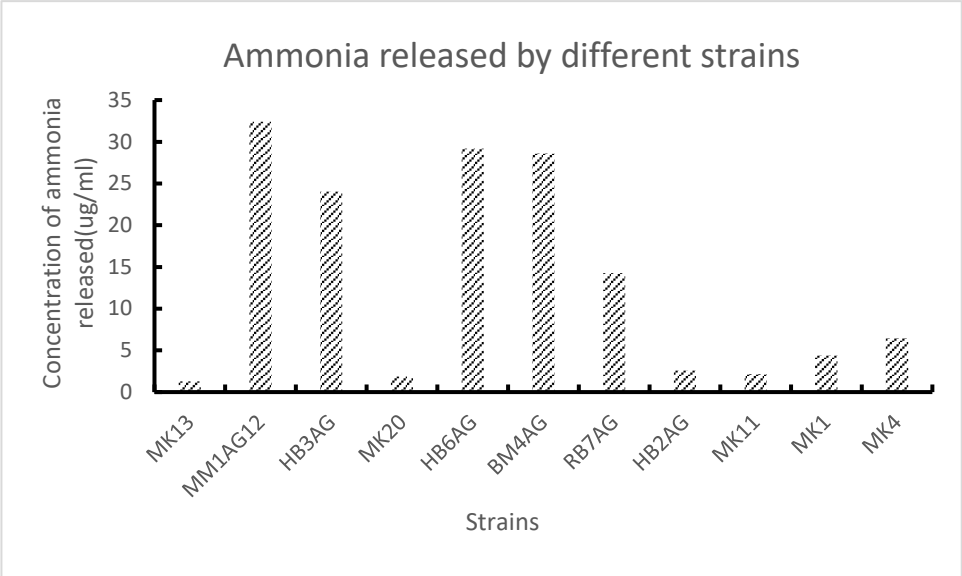


Fig 2 : Secondary screening - ammonia released by different streptomyces strains

2.3.3 Screening of isolates for ARDRA (Amplified Ribosomal DNA Restriction Analysis)

2.3.3.1 PCR amplification

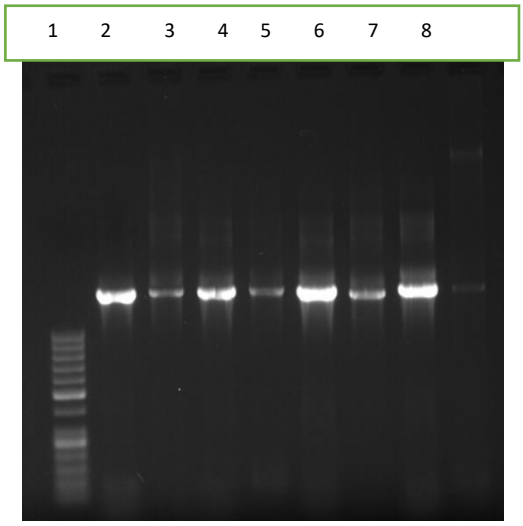


Figure 3 : Gel picture showing 16srRNA (1500bp) PCR amplified products of Streptomyces strains.

Lane 1- marker, lane 5- HB6AG, lane 7- MM1AG12, lane 8- BM4AG, lane 9- HB3AG.

2.3.3.2 ARDRA: Restricted fragments (ARDRA) of 16srRNA gene PCR amplified products of isolated strains.

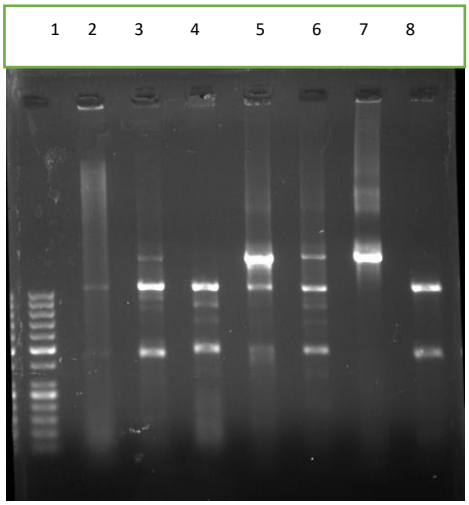


Figure 4: Gel picture showing restricted fragments (ARDRA) of 16srRNA gene PCR amplified products of isolated strains

Lane 1- marker, lane 2- PCR product lane 3- MM1AG12, lane 4- PCR product, lane 5- HB6AG.

2.3.3.3 Phylogenetic analysis:

Using a software PyElph 1.4 ARDRA gel pictures were proceeded for further phylogenetic study . Some strains are found to be so closely related that they might belong to a same species.

Phylogenetic tree

2.3.4 PHYSIOLOGICAL & BIOCHEMICAL CHARACTERIZATION:

The isolates HB6AG, MM1AG12 obtained were subjected to study physiological & biochemical characteristics (Table no. 5).

Gram staining :

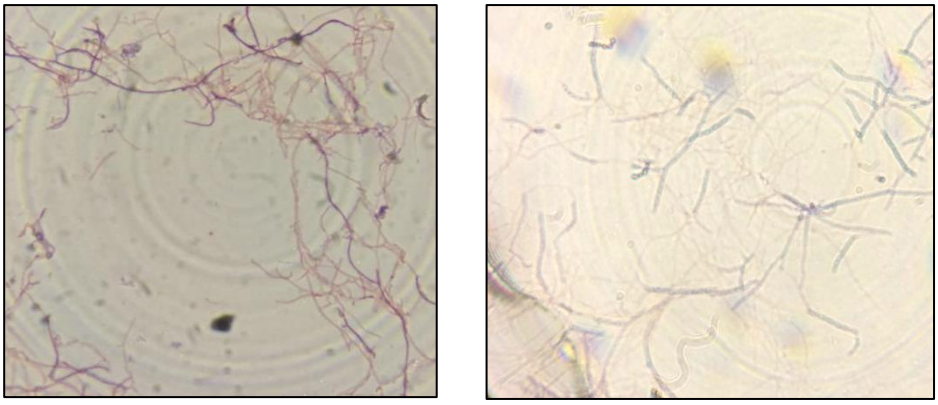


Fig 6 : 100x microscopic view of MM1AG12(A) and HB6AG(B) after gram staining

Colony morphology

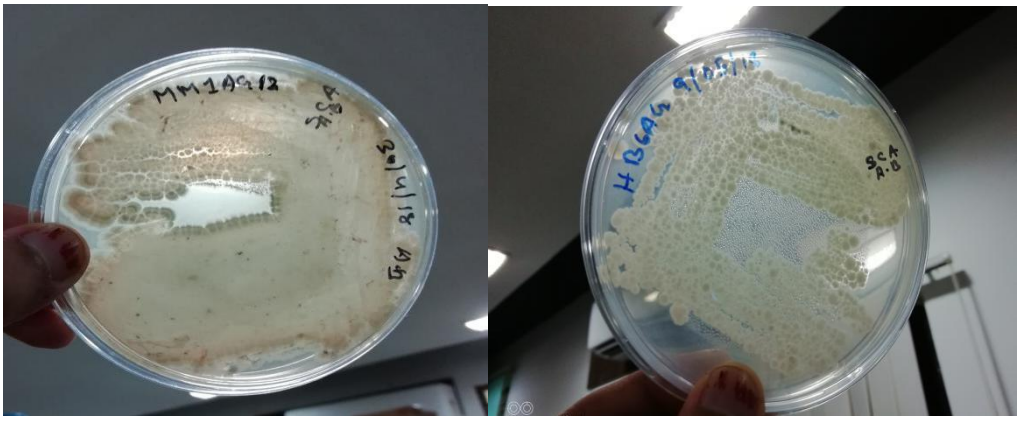


Fig 7 : Colony morphology of the isolate MM1AG12 & HB6AG on starch caesin plates.

Morphological and biochemical characterization of HB6AG and MM1AG

Aerial spore mass colour Spore surface Ornamentation		W Smooth	Grey Smooth
Yeast extract malt extract agar (ISP 2)	A	G++	G++
	S	W++	B++
Oat meal agar (ISP 3)	A	-	W++
	S	BR+	G++
Inorganic Salt Starch agar (ISP 4)	A	G++	G+
	S	B++	B++
Glycerol asparagine agar (ISP 5)	A	W+	G+
	S	W++	B+++
Peptone yeast extract iron agar (ISP 6)	A	-	-
	S	-	W+
Tyrosine agar (ISP 7)	A	G++	W+
	S	B++	B++
Tryptone -Yeast extract (ISP 1)	A	W+	G+
	S	BR+++	B+++
Degradation of [#]			
Starch		+	-
Caesin		-	+

Tributyrin	-	+
Gelatin	+	+
Chitin	+	+
Pectin	+	+
Carboxymethyl cellulose	+	-
Tween80	+	+
Utilization of [♯]		
Citrate	+	+
Growth on sole carbon source (1% w/v)[∞ ♯]		
Sucrose	+	+
Glucose	+	+
Mannose	+	+
Xylose	+	+
Fructose	+	+
Fermentation/utilization of sugars[∞]		
Arabinose	+	-
Cellobiose	+	-
Mellibiose	+	-
Mannitol	+	-
Rhamnose	+	-
Salicin	-	-
Trehalose	+	-
Tolerance to antibiotics		
Amikacin (Ak)	+	-
Amoxycilin (Am)	-	-
Bacitracin(B)	+	NA
Carbenicilin(Cb)	+	-
Co-trimazole(Co)	-	+
Cephalexin(Cp)	-	-
Cephadroxil(Cq)	-	-
Erythromycin€	+	+
Furazalidone(Fr)	-	+
Nalidixic acid(Na)	-	-
Nitrofuratoin(Nf)	+	+
Norfloxacin(Nx)	-	-
Oxytetracyclin(O)	+	NA
Growth in Presence of NaCl (%, w/v)[§]		
1,2	+	+
Growth in pH[♯]		
5, 7, 9, 11, 13	+	-

Table 4: comparative study of physiological &biochemical characterization of selected Streptomyces strainHB6AG

Strain	Starch	Gelatin	Caesin	TBT	Tween-80	Pectin	CCA	Catalase	Citrate	Urease
MM1AG12	-	+	-	-	+	-	+	-	+	+
HB3AG	-	+	-	-	-	+	+	-	-	+

Table 6 : Biochemical characterization of selected strain MM1AG12 and HB3AG

2.3.5 Growth curve & generation time:

The organisms were grown in 250 ml of conical flasks with 50 ml of medium in it. Well grown pre culture at 120 rpm and 30° C was used for growth kinetic studies. The experiments were carried out in triplicates. 1% w/v of the inoculum was used to inoculate the flasks. Viable cell count of the inoculum was carried out by drop method. At the time of inoculation sample was taken out at 0h for viable cell count and dry weight /wet weight wet estimation. The flasks were then incubated at 120 rpm and 30° C. Time course sampling was carried out at 24h interval till 120h.

Growth curve was constructed and generation time was calculated by counting the viable cells grown on the plates.

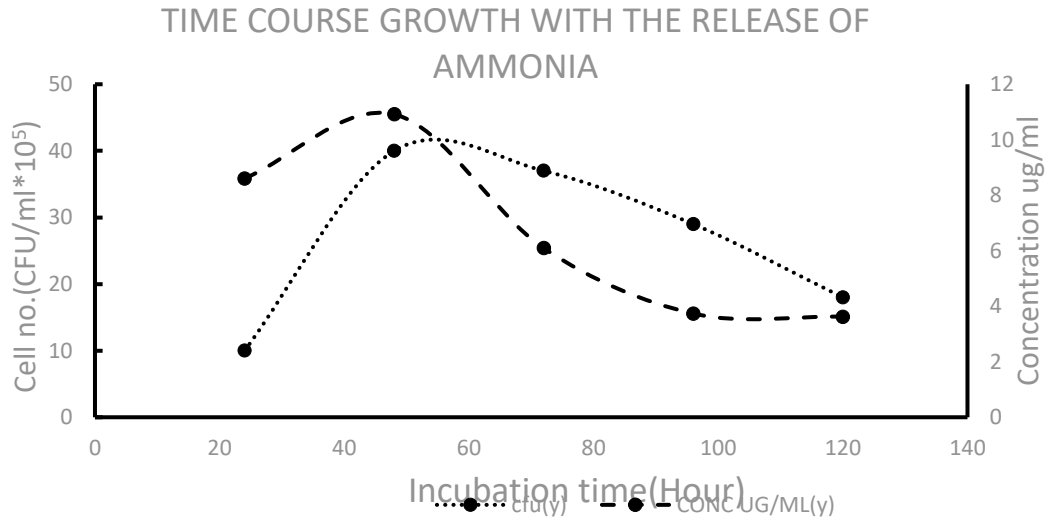


Figure 8: Time course growth & activity of HB6AG

N = generation time (n) = (log 10 Nt – log10 N0)/log2

n = 8.671513

No = population size at certain time of exponential phase

Nt = Population size at the subsequent time

T = time lapse between Nt and No

Mean growth rate constant k= n/t

K = 8.671513/24 =0.361313 generation /h

Mean generation time g= 1/k

g=1/0.361313 generation/ h

g= 2.76768h/ generatio

2.3.6 16s sequencing :

CONSENSUS SEQUENCE (1501 bp) Of HB3AG :

TGATCAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAACCCGGGG
AAACCCGGGCTAATACCGGATACGACCCTCCAGGGCATCTTGGGGGGTGGAAAGCTCCGGCGGTG
CAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCCACCAAGGCGACGACGGGTA
GCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGGCCCAGACTCCTACGGGAGGC
AGTAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGG
CCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCA
CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGG
GCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTCGTGAAAGCCCGGGGCTTAACCCCGGGTCT

GCGGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAAT
GCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGA
GCGAAACGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTA
GGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGT
ACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGAGGCGGAGCATGTGGCT
TAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACTGGCAGAGATGTC
AGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGG
ACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCC
TTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGA
GCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAG
TCTCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTGGTACACACCGCCC
GTCACGTCACGAAAGTCGGTACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGTCGTCG
A

Table: 6 showing percentage of sequence similarity with *Streptomyces* strains

Rank	Name	Accession	Pairwise Similarity(%)
1	Streptomyces thermolineatus strain 9-4	KJ571064.1	99.
2	Streptomyces sp. OAct 90	JX047047.1	99.
3	Streptomyces thermolineatus strain NBRC 14750	NR_112442.1	99.
4	Streptomyces sp. strain JJ19	KX352767.1	99.
5	Streptomyces sp. CPE510	JN969037.1	
6	Streptomyces sp. CPE184	JN968995.1	99.
7	Streptomyces thermolineatus strain A1484	NR_026529.1	99.
8	Streptomyces sp. strain JJ16	KX352765.1	98.
9	Actinobacterium ZXY005	JN049454.	98.
10	Streptomyces sp. strain JJ124	KX352810.1	98.

Phylogenetic tree :

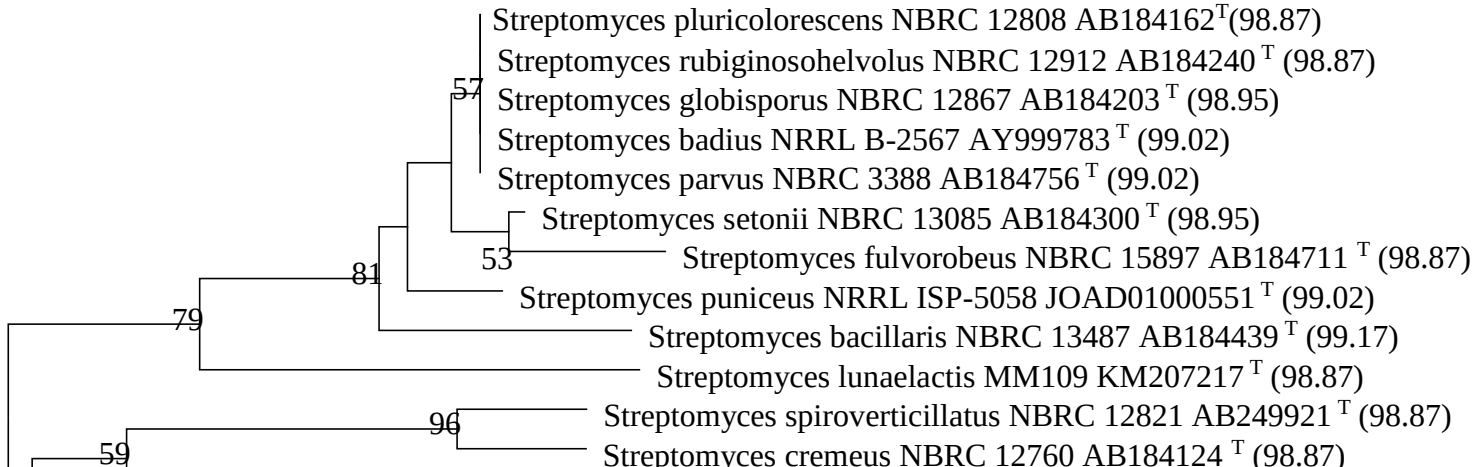


Figure : Evolutionary relationships of taxa

2.4 DISCUSSION:

189 Actinomycetes strains were isolated from the collected samples.. Out of these 12 strains were selected from the primary screening. These strains were then screened by secondary screening and for further characterization. 4 strains were selected for further evaluation..

The result revealed that two strains HB6AG and MM1AG12 were further characterized. These strains showd positive result for cellulose, amylase, protease, gelatinase and caesinase activity. This indicated that the actinomycetes are potential source of several hydrolysing enzymes, which may be the result from natural selection of microorganism in order to survive in a completing environment. However The strain HB6AG showed distinct zone of pink colour against different pH, NaCl and glucose concentration in time interval . Thus Streptomyces strain HB6AG was selected for further analyses. Evidently, based upon a range of phenotypic, and genotypic characterization, the strain was designated as a member of the genus Streptmyces.

2.5 CONCLUSION:

1. HB6AG is a novel Streptomyces strain
2. It possess distinct antimicrobial activity against *M.luteus*

   HB6AG was chosen for further studies on purification and characterization of the enzyme activity.

CHAPTER-3

Purification and Characterization of asparaginase produced by HB6AG

3.1 INTRODUCTION AND REVIEW:

3.1.1 Asparaginase producing Streptomyces

Asparaginase(L-asparagine aminohydrolase, E.C 3.5.1.1), belongs to an amidase group that hydrolyses the amide bond in L-asparagine to L-aspertic acid and ammonia (kumar and verma, 2012). It is the first enzyme that has anti-leukemic activity as well as it is an antineoplastic agent(Savitri and Azmi, 2003). It is used for treatment of acute lymphoblastic leukemia(ALL). This enzymes are naturally occuring enzymes that expressed and produced by animal tissues, bacteria, plants, and in the serum of certain rodents but not in mankind. Large number of microorganisms like ***Erwinia carotovora, Pseudomonas aerugenosa, and E.coli*** are included that has been knowing to produce L-asparaginase. Different types of asparaginase is used for different industrial and pharmaceutical approaches.

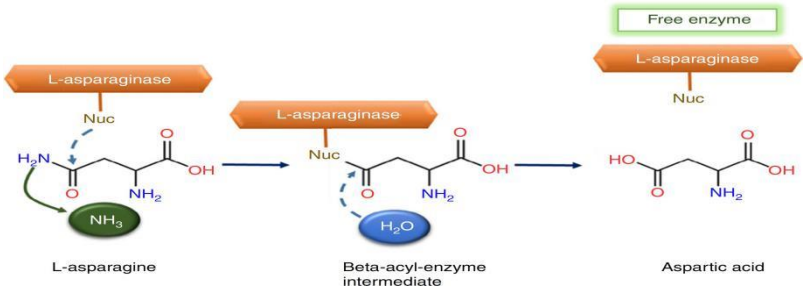


Fig 12: General mechanism of asparaginase reaction catalyzed. Dashed arrow nucleophilic attack.

Source : Based on Hill (1967)[31] cited by El-Bessoumy et al.(2004)[32], and Shrivastava et al.(2016)[33]

3.1.2 History of asparaginase :

Asparaginase is the therapeutic enzyme that was first discovered and it has antineoplastic properties. Lang first discovered this enzyme in 1904[34]. After so many years of the first discovery, it was again leap forward in 1922 by Clementi when he found the presence of asparaginase in guinea pig serum. In 1953, Kidd was experimented to prove the ability of guinea pig serum as tumor inhibitor[35]. He carried out different exmeriments where one part of animals were given guinea pig serum and other part of animals were left untreated. As a result the mice which were subcutaneously treated with serum failed to proliferate lymphosarcoma but the untreated mice were died because of carcinoma. In the second experiment the mice with different types of lymphomas that are mammary carcinoma and fibrosarcoma were treated with the guinea pig serum. As a result the mice were failed to proliferate the carcinoma. In 1961, another discovery was revealed by Broome when he stated that asparaginase as an antitumor agent with substrate specificity of guinea pig serum[36]. Other substrate specific asparaginses were found to inhibit tumours on later(37-39). Centrality of amino acid for asparaginase was proved by McCoy for Walker carcinoma256[40]. The presence of antitumor activity of asparaginase in bacteria and yeast was found by Altenbern in 1954 and Broome in 1965, respectively[41,42]. though asparaginase is present in mice and rodents but absence in humans[43].

3.1.3 Type :

There are two types of asparaginase has been discovered in *E.coli*- type 1 and type 2 asparaginase. Type 1 asparaginase is a low afinity enzyme found in cytoplasm but type 2 is a high affinity enzyme that found in periplasm in *E.coli*[44]. Most of the microbial asparagine enzyme is intracellular in nature but there are some extracellular enzyme that are secreted outside the cell. Extracellular enzyme is advantageous because of its higher accumulation in culture broth, easy extraction and purification process[44].

3.1.4 Classification :

Asparaginase sequences can be divide into three groups based on their sequence homology analysis (Borek and Jaskólski, 2001) as well as biochemical(Campbell et al., 1968; holmium et al., 1970; Dunlop et al., 1980) and crystallographic

data(Aghaiypour et al., 2001; Jaskólski et al., 2001; Kolyani et al., 2001; Lubkowsky et al., 2003; Sanches et al., 2003; Yao et al., 2005). The bacterial type is the first family, second family is plant-type and third is the enzymes that are similar to Rhizobium etli asparaginase(Bonthron and Jaskólski, 1997).

Bacterial type asparaginase are divided into two subtypes that are type I and type II. Type I has lower affinity than type II for substrate taht is asparagine. Plant type asparaginases are different from bacterial type by its evolutionary origin(Michalska et al., 2006).

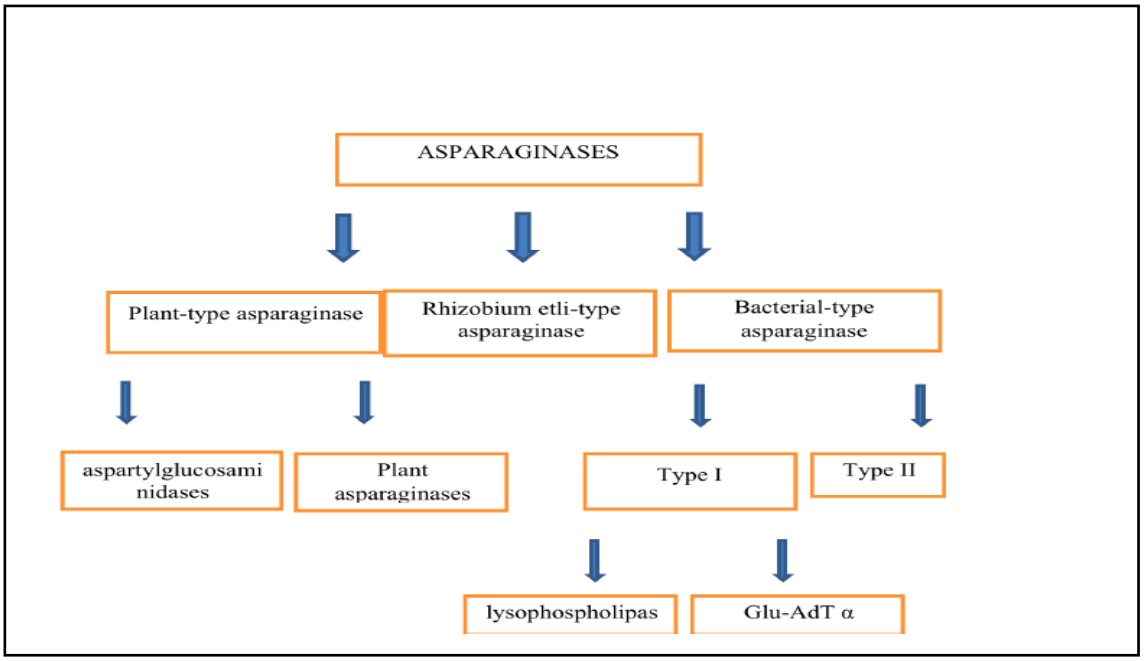


Fig 13 : Classification of asparaginase enzyme

3.1.5 Mechanism of action :

As asparaginase has hydrolytic property it is the keystone for treatment of ALL. Normal and leukemic cells need this amino acid for their metabolic requirements. In normal cells asparagine can be synthesized by using transaminase enzyme which converts oxaloacetate into an intermediate aspartate but later aspertate transfers its an amino group from glutamate to oxaloacetate producing α-ketoglutarate and aspertate[45]. Aspertate is converted to asparagine by asparagine synthatase. But the cancer cell lack the enzyme asparagine synthatase, so it has not the ability to synthesize the amino acid asparagine[45]. So cancer cells are dependent on the exogenous supply of asparagine. Lacking of asparaginase to cancer cells leads to

removal of all circulating asparagine. So cancer cells lacks asparagine and they die. Asparaginase has also the potential role in the food industry as food processor[45].

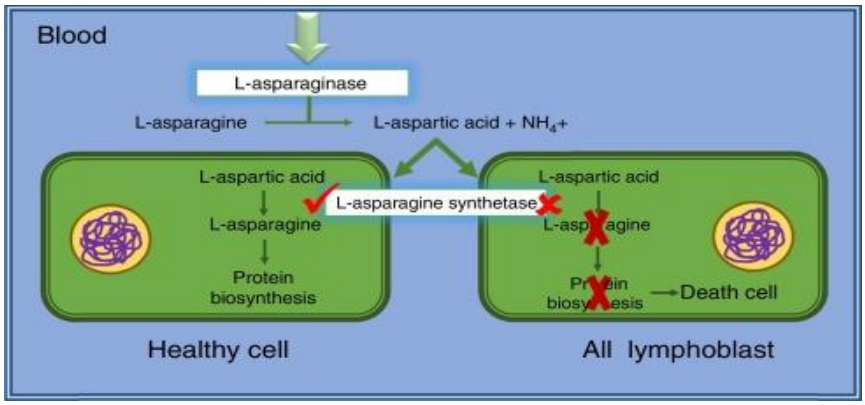


Fig 14 : antineoplastic action of asparaginase

Source : Based on Van den Berg(2011) [46]

3.1.6 Structure of asparaginase :

Many research has been sugested that asparaginase from different microbial source has different structure. Generally it is tetramer but it can be hexameric, monomeric, dimeric[47]. The molecular structure of E.coli and Erwinia sp. are already discovered[48,49]. The two species have the three dimensional structure. In both species the enzyme made up of two tetramers that is made up of four identical monomer. It has 14 α- strands, eight β- helics[50], a big N-terminal domain and a small C-terminal domain along with three hundred twenty seven amino acids[49]. The active site of the enzyme is present between the two adjacent monomers. Four identical subunits are present in the tetramer.

3.1.7 Enzyme kinetic properties of asparaginase :

L-asparaginase production and purification were studied to minimize the impurities that is responsible for allergic reactions[51,52]. With microbial source the biochemical and enzymatic kinetic properties of asparaginase varied. Physiochemical

and biochemical properties like optimum pH, temparature, substrate specificity, inhibition pattern etc is well documented. For example, asparaginase from Erwinia is considered as less toxic than E.coli. But the half life of Erwinia asparaginase is less than the asparaginase from E.coli[53].

3.1.8 Stability of Asparaginase :

As the anti-tumour activity of asparaginase has the function of half life in blood[54] so in recent there have been taken several attempts to increase its half life. In recent to increase the half life of asparaginase there are several methods like entarpment of the enzyme in liposome[55] or microcapsule[56] and in macromolecules by covalent coupling[57] are proposed. But as the approaches are fail to eliminate the disadvantages of asparaginase enzyme it is need to identify and characterize the new enzyme with better properties.

3.1.9 Comaparative studies of asparaginase of E.coli and Erwinia :

Different microbial source reaveled that the potential activity of asparaginase differ with the source of strain. As eg. Asparaginase from E.coli and Erwinia chrysanthemi are used as effective drugs in the treatment of ALL. They are also used in the treatment of other diseases like Hodgkin's disease, acute myelotic leukemia, chronic lympholytic leukemia, reticulosarcoma etc [58]. If a patient becomes hypersensitive to any one of the enzymes of E.coli and Erwinia, they can be used as alternative therapy as it both has different immunological specificities. Comparative studies reaveled that E.coli asparaginase can be used as the first line of treatment and Erwinia asparaginase is used if a patient is allergic to E.coli asparaginase. The toxicity is partially attributable to the glutaminase activity of these enzymes[59].

3.1.10 Sources of microbial asparaginase :

Bacterial sources of asparaginase :

Asparaginase has been reported to be pesent in both gram positive and gram negative bacteria. This species are being found in terrestrial and marine enviroment. The bacterial sources are listed below :

Table 7 : Bacterial sources

Gram-negative bacteria	References	Gram-positive bacteria	References
Acinetobacter calcoaceticus	[60]	*Bacillus circulans*	[86,87]
Azotobacter agilis	[61]	*B. coagulans*	[88]
Brevibacillus brevis	[62]	*Bacillus* sp.	[89]
Citrobacter sp.	[63]	*B. mesentericus*	[90]
Escherichia coli	[64]	*B. polymyxa*	[91]
Enterobacter aerogenes	[65]	*B. subtilus*	[92]
E. cloacae	[66]	*B. licheniformis*	[93]
Erwinia aroideae	[67]	*B. circulans MTCC 8574*	[86]
E. cartovora	[68]	*Corynebacterium glutamicum*	[94]
E. chrysanthemi	[69]	*Mycobacterium bovis*	[61]
Helicobacter pylori	[70]	*M. phlei*	[95]
Klebsiella pneumoniae	[71]	*Staphylococcus* sp.	[96]
Pectobacterium carotovorum	[72]	*S. aureus*	[97]
Pseudomonas sp.	[73]	*Streptococcus albus*	[98]
P. fluorescens AG	[74]		
P. geniculate	[75]		

P. ovalis	[76]
P. Stutzeri	[77]
Pyrococcus horikoshii	[78]
Serratia marcescens	[79]
Thermus thermophiles	[80]
T. aquaticus	[81]
Vibrio succinogenes	[82]
Citrobacter freundi	[83]
Proteus vulgaris	[84]
Zymomonas mobilis	[85]

Fungal sources of asparaginase :

Another potential source of asparaginase is fungi. Bacterial sources have some adverse side effects. Fungi are closely related to human beings as compare to bacteria(batool). The chances of immunological reaction from fungi is lesser(batool). Fungal asparaginase also has gained importance because of their production of asparaginase extracellularly and the purification process is also easy. The fungal sources of asparaginase are listed below :

Table 8 : Fungal sources

Fungi	References
Alternaria sp.	[99]
Aspergillus nidulans	[100]
A. niger	[101]
A. oryzae	[102]
A. tamarii	[103]
A. terreus	[104]
Cylidrocapron obtusisporum	[105]
Mucor sp.	[106]

Fusarium roseum [107]

Actinomycete source :

Actinomycetes are easily available in soil, water. Actinomycetes are better source of asparaginase. From the actinomycetes group the species of streptomyces are the best source for the production of streptomyces. Streptomyce sis essily available species. It can easily found in the soil, marine water, soil sediments etc. Actinomycetes are the better source for asparaginase production than bacteria, fungi, yeast etc.

Table 9 : Actinomycetes source

Actinomycetes	References
Actinomyces sp.	[108]
Streptomyces albidoflavus	[109]
S. aurantiacus	[109]
S. collinus	[110]
S. griseus	[111]
S. gulbargensis	[112]
S. karnatakensis	[113]
S. longsporusflavus	[114]
S. plicatus	[115]
S. tendae	[116]
S. venezuelae	[117]
Thermoactinomyces vulgaris	[118]
Noccardia sp.	[119]

3.1.11 Current status of asparaginase research in worldwide and India :

In recent years asparaginase has gained importance for its clinical value. In world wide and in India the researchers working on asparaginase. Recent studies showed that many researchers working on the isolation of asparaginase from different marine environments and study the morphology of asparaginase. Also they are working on the characterization and optimization of the asparaginase. Prodction of asparaginase from a potential strains and their purification also reported. They also checked the cytotoxicity of the asparaginase to the cancer cell to check its working potential.

In India research on asparaginase also reported. From different coastal area of India researchers isolate the strains which produce potential asparaginase. They working on the strain to get a good quantity and quality of asparaginase. They also working on the isolation identification strategy and the screening strategy of the strains which produce the potential asparaginase. They are also working on the large scale production and purification of asparaginase to apply it indutrially. Also checked for the toxicity of this enzyme on the cancer cell line.

3.1.12 Isolation strategies of asparaginase producing microbes :

In recent years the screening technology of enzyme has undergone massive development. Within the 1990s, the high-throughput screening of enzyme activities were perceived as a crucial bottleneck. Today, a giant repertoires of economical screening method are on the market that permit testing of virtually any reaction with high-throughput. Several investigators have evolved in screening of asparaginase producing microbes using different methods. Novel microorganism isolation procedures and screening of their natural product and bioactive compounds and therapeutic enzymes in medicines are necessary during this direction. Among the bacterium, streptomyces gain a special importance as a result of belong to the group of actinomycetes that are known to provide a wide variety of enzymes and metabolites(Annie et. al.,1997; Balagurunathan,2004).

3.1.13 Screening of asparaginase producing Streptomyces by rapid plate assay :

Microbial strains that producing aspraginase were known by forming pink coloured colony on changed M9 agar medium with phenol red as indicator dye. As asparaginase cleaved the asparagine in aspartic acid and ammonia, an alkalyne by

product increase the pH of the medium, so it change from yellow to pink. The intensity of the colour is directly proportional to the amount of asparaginase produced. Gulati et al., 1997 developed a rapid plate technique in which modified Czapek Dox's medium is used along with phenol red 0.009 per cent(Sabu et al.,2003)

3.1.14 Isolation and identification of asparaginase producing Streptomyces :

Actinomycetes were isolated by soil dilution plate technique on starch caesin agar medium, starch nitrate agar medium, glycerol glycine agar medium, and chitin agar medium(Haefner et al., 2003).

The morphological identification methods were done by the macroscopic and microscopic characterization. Macroscopic identification were done by differentiating their colony characters e.g size, shape, colour etc. Microscopically, the isolates were fully grown by glass culture technique(Kawato and Sinobu,1959). they were then determined for their mycelial structure and conidiospore and anthrospore arrangement on the mycelia under the microscope(1000X).

Biochemical tests were done for identifying the isolates(Howell and Pine, 1956 and Howell et al., 1959). Different biochemical test like catalase test, starch hydrolysis, nitrate reduction, litmus milk reacton, H_2S production and acid from carbohydrates(dextrose, xylose, mannitol, raffinose and mannose) were performed. In the fermentation tests, the carbohydrate were added to the basal medium with a concentration of 0.5 per cent. Starch hydrolysis test were dtermined by flooding the gram iodine solution to the growing culture plate. Nitrate reduction were tested by adding KNO_3 to the medium. Catalase tests were done according to the standard protocol.

The actinomycetes posses many important antibiotics and other therapreutic compounds. They play a major role in the cycling of organic matter in soil(Lacey,1978; Williams, 1978). The isolation of actinomycetes from mixed microflora is complicated because of their slow growth tham other soil bacteria. This has resulted within the development of selective isolation procedures primarily based totally on one or each of the subsequent approaches: (i) nutritionary choice within which media are developed with nutrients which are preferentially utilised by

actinomycetes (El-Nakeeb, 1963; Kuster, 1964; Tan 2006), (ii) selective inhibition, during which compounds like antibiotics area unit incorporated into media to by selection inhibit non-actinomycete bacterium (Ara et al., 2007; Huang et al., 2008). the key difficulties with every of those approaches area unit that neither is strictly selective for actinomycetes which every have the inherent potential to truly inhibit the expansion of some actinomycetes.

3.1.15 Optimization of culture conditions for L-asparaginase production :

Asparaginase formation has been shown to possess a firm link to active cell growth. Savitri and Azmi, (2003) have found most L-asparaginase activity of actinomycete plicatus at pH scale 7.0, whereas Narayana et al., (2007) has reportable most L-asparaginase production of S. albidoflavus at pH scale 7.5. Gulati et al. (1967) have reportable that 6.2 was the optimum pH scale for L-asparaginase producing Aspergillus nidulans strain.

Sarquis et al.,(2004)reportable that 30°C is that the appropriate temperature for L-asparaginase production through submerged fermentation by employing A.terreus and A.tamarii. S.albidoflavous created high amount of asparaginase at 28°-30°C.

The best pH scale determined for L-asparaginase activity of Streptomyces gulbargensis was 9.0. Dhevagi and Poorani,(2006) reportable the utmost L-asparaginase activity of Streptomyces sp. PDK7 between pH scale 8.0 and 8.5. concerning the pH scale stability. Moreover the pH stability range of more than 80 per cent activity of asparaginase lies between 7.0-10.0. The temaparature 40°C at which the enzyme shows its maximum activity.

Production of L-asparaginase began when 24hrs of cultivation and reached to most levels when 72hrs of incubation. Most biomass production obtained with 72hrs recent culture of Streptomyces albidoflavus might even be correlate with high levels of L-asparaginase production. L-asparaginase formation has been shown to possess a firm link to the active cell growth (Savitri et al., 2003).

The acidic nature of fermentation medium of Streptomyces albidoflavus might inhibit L-asparaginase synthesis. Glucose is reportable to be a represser of asparaginase synthesis owing to the acid production (Geckil et al., 2006). Production levels of

L-asparaginase by Streptomyces griseus ATCC 10137 was 0.36 IU per mg of cell dry weight as reportable by Jong (1971).

Mostafa and Salma (1979) reportable that the six day recent culture of Streptomyces collinus created high quantity of L-asparaginase once fully grown at pH scale 8.5 and temperature 28°-30°C. However Narayana et al. (2008) study reportable that 3-day recent culture of S. albidoflavus was found to provide most quantity of enzyme at pH scale 7.5 and temperature 35°C.

3.1.16 Asparaginase purification and properties :

Asparaginase from marine Streptomyces sp. PDK2 has been sublimate 85-fold with a pair of.18 per cent recovery within the final Sephadex G - two hundred purification step (Dhevagi and Poorani, 2006).

The foremost active fractions for catalyst activity with specific activity 132.4 IU/ mg, and purification fold of concerning 35 and 63 per cent yield were pooled along, dialyzed against 0.01 M Tris - HCl buffer (pH 8.0), and focused by lyophilisation (-50°C). The extraction profile of the foremost active fractions collected from Sephadex G - 100 and loaded on Sephadex G - 200 column. A pointy distinctive peak of L-asparaginase activity, which inserts with just one macromolecule peak, was noticed . the foremost active fractions (F7 - F9) with specific activity 574.24 IU/ mg and concerning 151 - fold purification and 40 per cent catalyst recovery were pooled along, focused with lyophilizer and keep at -20°C (Elshafei et al., 2012).

Dhevagi and Poorani (2006) UN agency reportable the largest L-asparaginase activity of actinomycete sp. PDK7 that was between pH scale eight.0 and 8.5, and therefore the best L-asparaginase activity extracted from actinomycete gulbargensis was nine.0 (Amena et al., 2010). Asparaginase which belongs to amidase group, are generally active and it is stable at neutral and alkaline pH that is pH 5.0 to 9.0 which were reported earlier to be optimum amidase activity (Ohshima et al., 1976). L-asparaginase, sublimate from marine eubacterium, exhibited most activity between pH scale 7.0 and 8.0 (Basha et al., 2009). Maximum activity of purified Streptomyces gulbargensis showed at 40°C.

AIMS & OBJECTIVES:

1. Qualitative determination of HB6AG
2. Partial purification of the extracellular asparaginase enezyme

MATERIAL AND METHODS

SOURCE OF CHEMICALS, REAGENTS AND INSTRUMENTS

Media, Chemicals, Reagents

All microbiological media, chemicals for buffers and components of Ion exchane chromatography like column [aniline diphenyl amine] used were purchased from Hi Media, Pvt. Ltd. Mumbai, India; Merck, Germany; SD Fine, India. All the chromatography resins were purchased from Sigma-Aldrich, USA. All the solvents were purchased from Merck.

Compositions of all media used in the current study are listed in Table 3 of Appendix I.

Instruments (table 1, Appendix)

Optimization of culture conditions for asparaginase production by the isolate HB6AG:

For large scale production of antimicrobial compound by MM1AG7, the experiments were conducted to determine the optimum culture condition for the same including the effect of incubation period, temperature, pH, inhibitor, metals, NaCl concentration. The enzyme activity was observed.

Profile of the enzyme in different NaCl

The isolate was exposed to the different NaCl concentration that is 0.5%,1%,3%,5% and 7%. and incubated for 30 mins. The specific activity of every conccentration was calculated.

Profile of the enzyme in different temperatures

The isolates was expose to different temparature at 20°C, 30°C, 40°C, 50°C. and 60°C and incubated for 30 minutes. The specific activity of different temparature was calculated.

Profile of the enzyme in different pH

The isolates was expose to different pH buffer that is citrate buffer for pH 5, Potassium Phosphate buffer for pH 7 tris-HCl for pH 8 and Glycine-NaOH for pH 9 and incubated for 30 minutes. The specific activity of different pH was calculated.

Profile of the enzyme in different metals

The isolate was expose to different metals like Hg,Mn,Cu,Fe,Zn. Incubated for 30 mins and the The specific activity of every conccentration was calculated.

Profile of the enzyme in different inhibitor

The isolate was exposed to different inhibitor like SDS, β-marcaptoethanol and Triton-X and incubated for 30 minutes. The specific activity of different inhibitor was calculated.

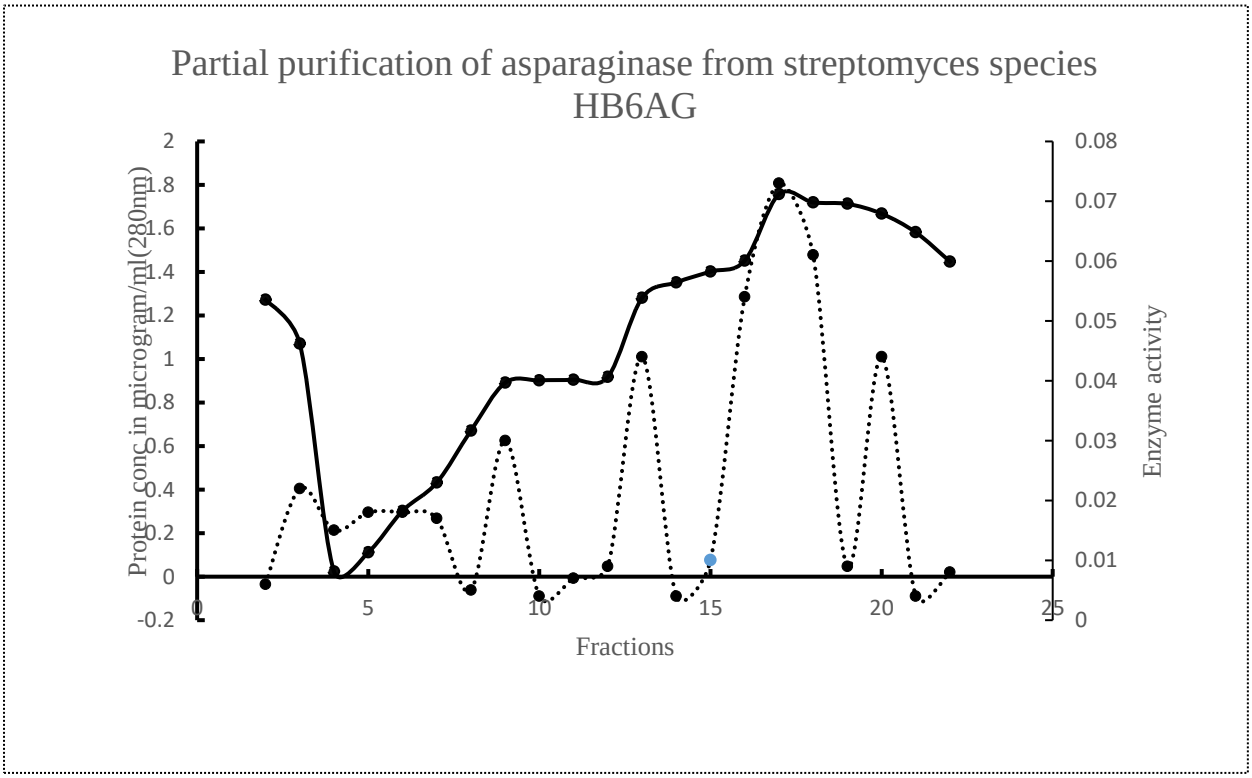
Purification of the asparaginase by column chromatography:

HB6AG strain was inoculated in 500ml SCA media and incubated at 37^{O}C for 2 days. Fully grown culture was centrifuged at 10,000rpm for 5 minutes. The culture supernatant obtained was precipitated with 45% of ammonium sulphate at 4℃ overnight. The pellet was obatained by centrifugation at 10,000rpm for 15 minutes. The pellet was dissolved in minimul amount of 50mM Tris-HCl buffer pH 8.6 and was dialyzed against the same buffer for 12hours. After dialysis the samples were used for prtein estimation by the Lowry et al method and Nesslerization method, respectively.

The dialyzed enzyme solution was loaded into a diethylaminoethyl(DEAE) Sepharose column which was pre-equilibrated with 50mM Tris-HCl buffer. The column was washed with the same buffer and the eluted protein was gradiently eluted using 100-700mM of NaCl in 50mM Tris-HCl. Fractions were collected at a flow rate of 4.28ml/min(each fraction conatain 4ml). All fractions were used for enzyme activity and protein estimation by procedures described earlier. Fractions showing high activity were collected for further use.

RESULTS :

Partial purification of asparaginase enzyme from HB6AG



Characterization of partially purified asparaginase enzyme from HB6AG

Profile of the enzyme in different NaCl concentration(%,wt/vol) :

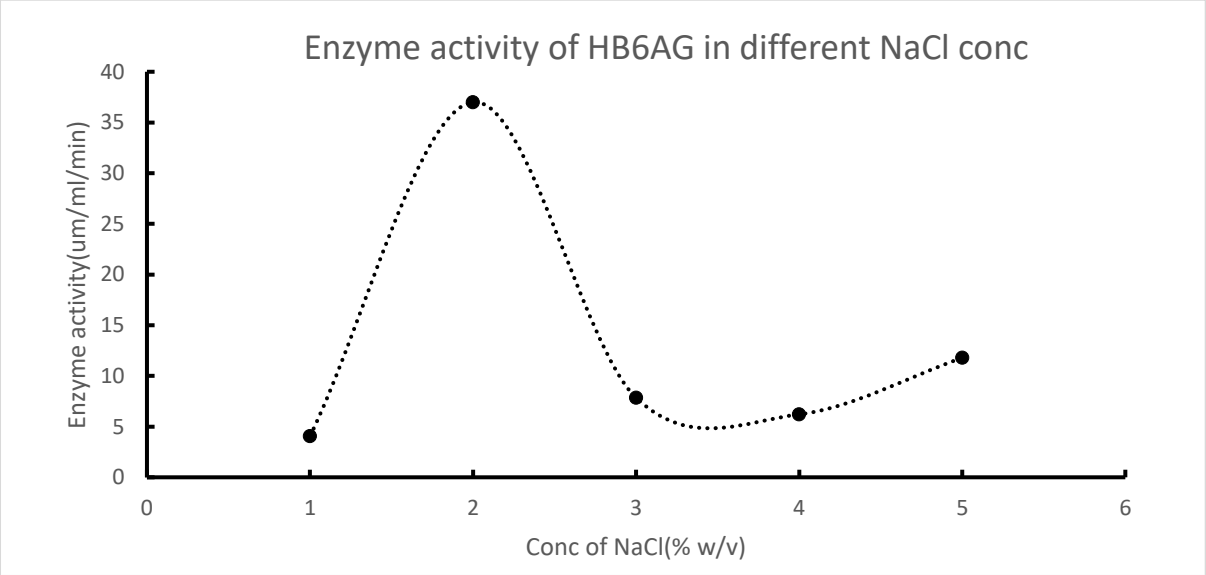


Fig 15 : Enzyme activity in different NaCl concentration

Profile of the enzyme in different temp :

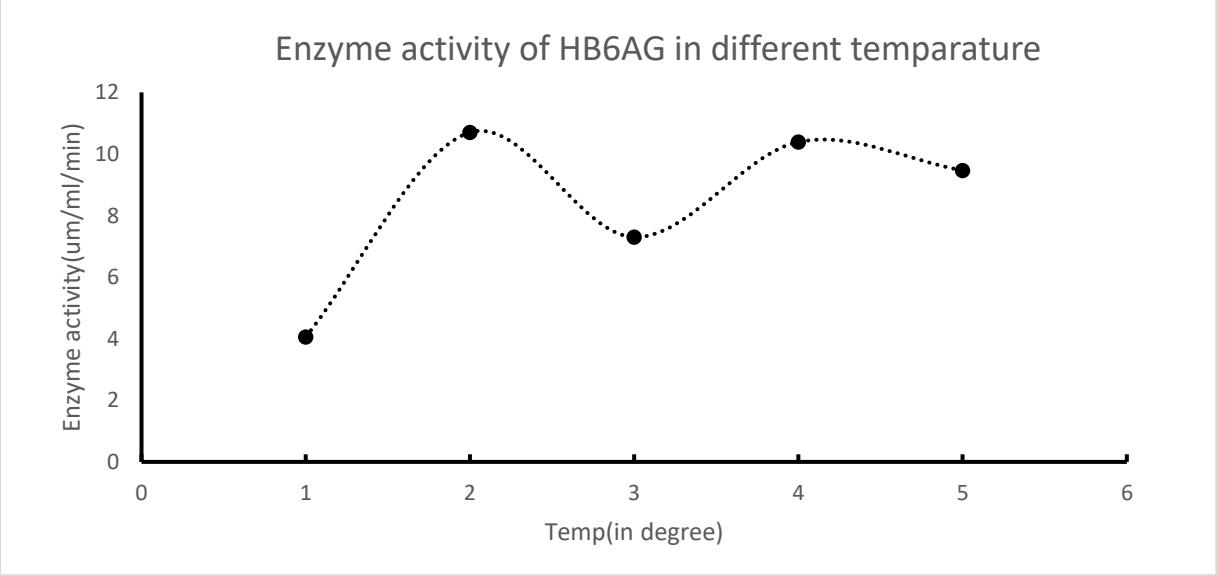


Fig 16 : Enzyme activity in different temparature

Profile of the enzyme in different pH :

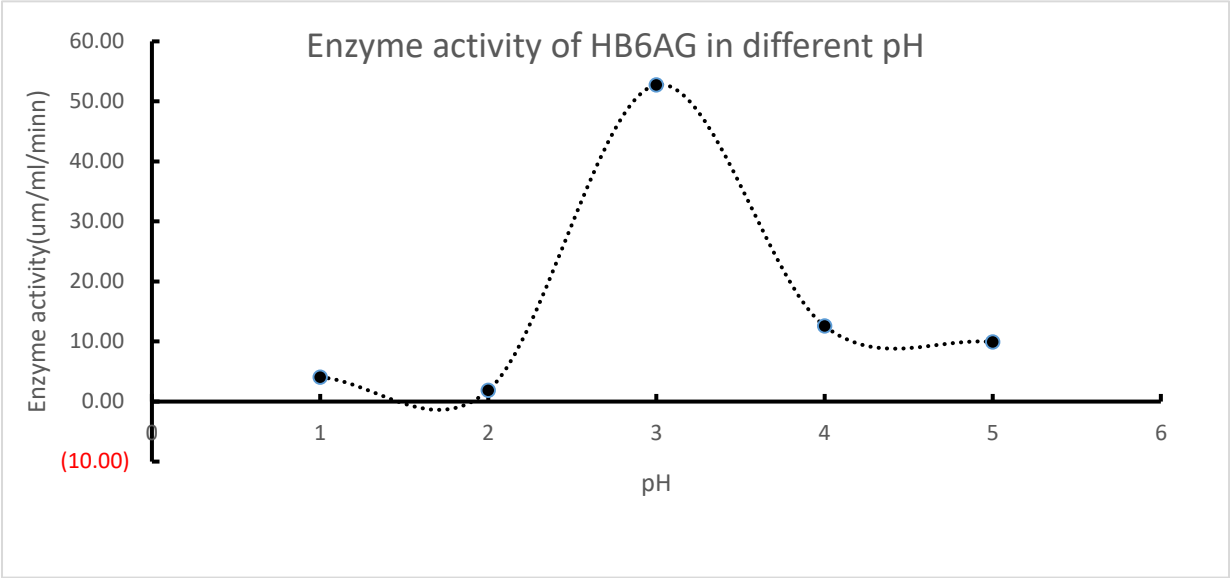


Fig 17 : Enzyme activity in different pH

Profile of the enzyme in different Metals :

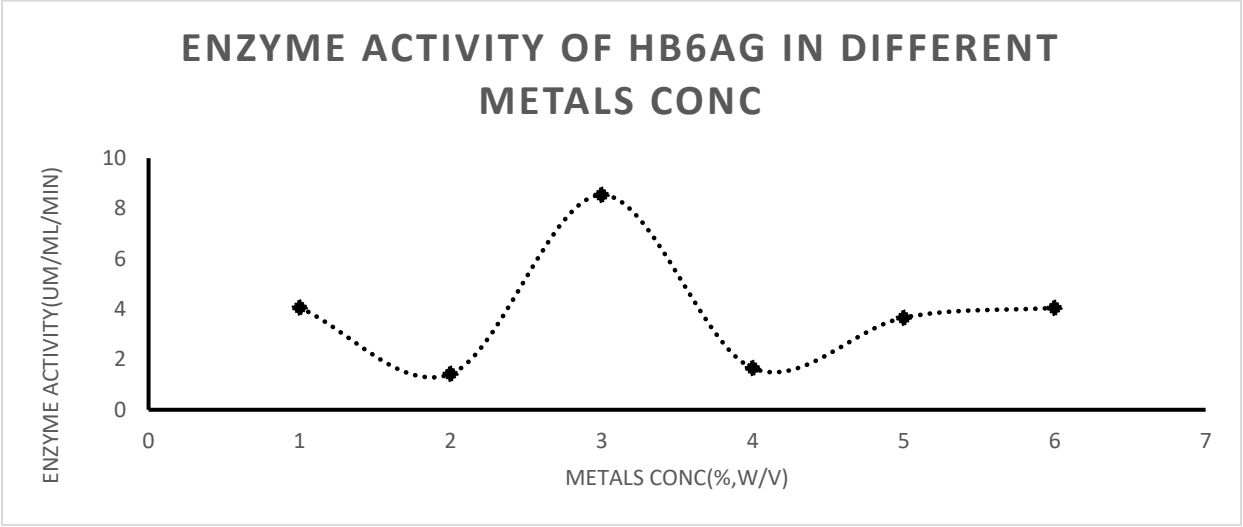


Fig 18 : Enzyme activity in different metals

Profile of the enzyme in different Inhibitor :

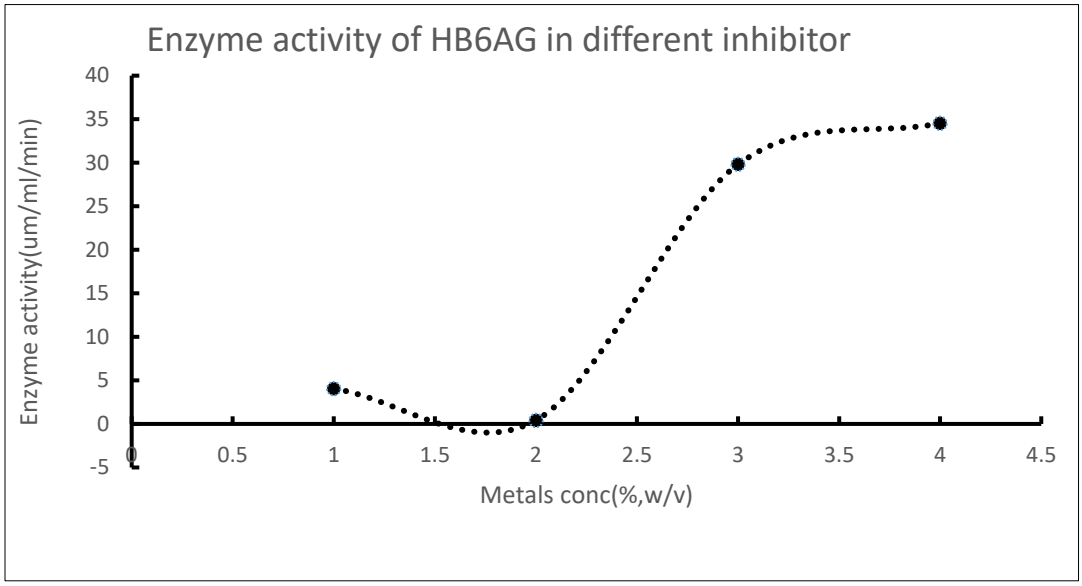


Fig 19 : Enzymme activity in inhibitor

Discussion :

Asparaginase enzyme was then partially purified from HB6AG. The obtained enzyme showed optimum activoty at 1%(w/v) NaCl, pH7. Also the enzyme showed activity at 5%(w/v) NaCl pH 9 and different metal ions and inhibitors tested. This enzyme further can be characterized by kinetic studies.

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