Studies on river water/sediment isolates of Actinomycetes as a source of novel secondary metabolites

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Introduction: Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites notably antibiotics, antitumor agents, immunosuppressive agents and enzymes. Because of the excellent track record of actinomycetes in this regard, a significant amount of effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds from terrestrial Actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased. Thus, it is crucial that new groups of actinomycetes from unexplored or underexploited habitats be pursued as sources of novel bioactive secondary metabolites.

Actinomycetes from freshwater habitats have been relatively neglected (Goodfellow and Haynes, 1984). There is also the crying need for new antibiotics and antifungal agents.

Freshwater habitats have been recently stressed as promising sources of bioactive metabolites (Cross, 1981). Terkina *et al*. (2002, 2006) reported that several lake Baikal actinomycetes inhibited the growth of several pathogens including antibiotic-resistant microbes. Elliah *et al*. (2002) observed that *Streptomyces strains* from Krishna river sediments in India had antibacterial and antifungal activities. Rifaat (2003) in his studies of actinomycetes from River Nile in Egypt, reported that several *Streptomyces strains* had significant antimycotic activity. New actinomycete strains or species have also been recovered from lakes and other aquatic environments. For example, Lango *et al*. (1999) identified *Streptomyces galbus* from sediment of La Caldera in Spain.

Actinomycetes are Gram-positive, free-living, saprophytic bacteria, widely distributed in soil, water, and colonizing plants. From the ~ 22,500 biologically active compounds that have obtained form microbes, 45% are produced by Actinomycetes. Genus *Streptomyces* is well known for producing a variety of bioactive secondary metabolites including antibiotics, immunomodulators, anticancer drugs & enzymes, antiviral drugs, antioxidants , herbicides , insecticides and many more
Actinomycetes were screened for production of antibiotic, anti-tumor enzymes.

Secondary metabolites from Actinomycetes:

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of an organism. Unlike primary

metabolites, absence of secondary metabolities does not result in immediate death, but it may result in long-term impairment of the organism. Antibiotics are one of the known major secondary metabolite by Actinomycetes.

Actinomycetes are known to be noteworthy as antibiotic producers, which make upto almost 75% of all known products; are without a doubt better equipped for their struggle for existence under the competitive complex conditions which exist among soil microflora. According to some estimates, the top 10 cm of global soil contains 1025-1026 actinomycetes, but only about 107 have been screened for antibiotic production in the past 50 years, leaving plenty of room for further screening. Among all actinomycetes; the *Streptomyces* are especially prolific produces a great number of antibiotics as both microbicidal and microbistatic agents; and other class of biologically active secondary metabolites (Kerry *et al* 2004).

Antibiotics of Actinomycetes comprise of a wide variety of chemical structures, including amino glycosides, anthracyclines, glycopeptides, β-lactams, nucleosides, peptides, polyenes, polyketides, actinomycins and tetracyclins (C. Manjula *et al* 2009). *Actinomycetes* cover around 80% of total antibiotic product, with other famillies trailing numerically. *Micromonospora*, which comes second to *Streptomyces.*, produces almost a tenth less number of antimicrobial agents than the latter.

Humans use secondary metabolites as medicines, flavorings, and recreational drugs. Actinomycetes are widely distributed in nature, and play an important role in the degradation of organic matter. They are also well known as a rich source of antibiotics and bioactive molecules, and are of considerable importance in pharmaceutical industries.

Recently, secondary metabolites obtained from some of the species of Actinomycetes have shown anti tumor activity against lots of human cancer cell lines (Carlos O. *et al* 2009) and also anti tubercle activity against *Mycobacterium tuberculosis* which causes tuberculosis, a life threatening disease (Radhakrishnan M. *et al* 2010).

Actinomycetes: Potent antibiotic producers

Antibiotics are low molecular-weight (non-protein) molecules, generally produced as secondary metabolites, mainly by microorganisms that live in the soil. An example of the variety of compounds synthesized by *Streptomyces* is the structural diversity of the antibiotics produced by *Streptomyces*, which can be derived from:

1. Aminoacids : α-lactamins (Ampicillin)
2. Polypeptidic (Pristinamycins)
3. Glucids: aminoglycosids (Streptomycin, Kanamycin)
4. Macrolides (Erythromycin, Spiramycin)
5. Naphtacen or cyclins:tetracyclins (Li *et al*, 2010 & Nedialkova *et al*, 2005)

Actinomycetes as the source of Anti-tumor enzymes

An enzyme that stimulates the degradation of a particular metabolite that cannot be synthesized by tumor cells, inhibits the synthesis of a metabolite needed by tumor cells, or inhibits tumor-specific DNA utilization, asparaginase. The examples of anti tumor enzymes are L-asparginase, pegglycated Arginine deaminase, (Lynn Feun *et al* 2006) L-lysine alpha oxidase, (Hitoshi Kusakabe *et al*, 1980) L-methionine gamma lyase (Daizou Kudou *et al*, 2007) etc.

L-Asparginase:

L-asparaginase (L-Aspargine amido hydrolase E.C.3.5.1.1) enzyme which converts L-asparagine to L-aspartic acid and ammonia has been used as a chemotherapeutic agent (Fisher and Wray, 2002). Several terrestrial *Streptomyces* like *S. karnatakensis*, *S. venezualae*, *S. longsporusflavus* and *S. albidoflavus* are capable of producing detectable amount of L-asparaginase (Narayana *et al*., 2007). Extra-cellular asparaginases are more advantageous than intracellular since they could be produced abundantly in the culture broth under normal conditions and could be purified economically. L-Asparginase production using microbial system has attracted considerable attention owing to cost effective and eco-friendly nature. Wide range of organisms such as filamentous fungi, yeast and bacteria has proved to be a beneficial source of this enzyme (Andrulis I L *et al* 1989)

L-Asparginase: Anti-tumor agent

L-asparaginase is an anti-Neoplastic agent used in the lymphoblastic leukemia chemotherapy (Selvakumar Dharmaraj *et al* 1977). Neoplastic cells cannot synthesize L-Aspargine because they lack the enzyme L-Aspargine synthetase (Ali S S *et al* 1994) which is essential for L-Aspargine synthesis. Use of enzyme like L-Asparginase in this case will result in cleavage of Aspargine making it unavailable for growth of the tumor cells. On the other hand normal cells are capable of synthesizing L-Aspargine because of the presence of the enzyme L-Aspargine synthetase (Savitri *et al* 2003).

Amino acid-degrading enzymes are known to inhibit the growth of tumor cells in culture by depleting amino acids in the medium. Therefore this enzyme can be used as a potential Anti-tumor agent. It has received increased attention in recent years for its anti-carcinogenic potential (Manna *et al* 1995). The clinical action of this enzyme is attributed to the reduction of L-asparagine, since tumor cells unable to synthesize this amino acid are selectively killed by L-asparagine deprivation**.**

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Limitations of L-Asparginase as an Anti-tumor agent

The native enzyme has a high immunogenicity and very short half life which creates obstacle in its use as an effective Anti-tumor agent. Thus these limitations needs to be overcome by use of certain modifications which will result in decreasing its immunogenicity and increasing its half life making the use of this enzyme as a safe anti-tumor agent (Savitri *et al* 2003).

Actinomycetes as the novel source of L- Asparginase

L-Asparginase is broadly distributed among animals, plants and microorganisms. Obtaining L-Asparginase from microorganisms like bacteria, yeasts, fungi and Actinomycetes is convenient owing to easy culturing condition along with easy purification and extraction of the enzyme.

Aims and Objectives:

1. Isolation of Actinomycetes from River water/sediment

2a). Screening of Actinomycetes for production of secondary metabolites like showing antibacterial activity against Gram positive & Gram negative test organisms:
e.g.: *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli.*

2a) Screening for ability to produce anti-tumor enzymes like L-Asparginase

3a). Selection of isolates showing antimicrobial activity against test organisms,

3b). Selection of isolate showing L-Asparginase production

4. Studies on Morphological and Biochemical characterization of selected isolates

And Identification based on 16 s r-DNA sequencing

5a). Laboratory scale fermentation for production of antimicrobial agent by selected strain, and
5b) Production of L Asparginase as an antitumor enzyme.

6. Optimizing the conditions for maximum production of antibiotic and L-Asparaginase.

Materials and Methods:

Sample collection, Isolation of Actinomycetes:

Sediments from various rivers like Krishna, Koyana, Mula, Mutha, Pavana from Maharashtra, Zelum from Kashmir and Mandavi and Zuari rivers from Goa were obtained. The samples were used for isolation of Actinomycetes on Bennett's agar. The plates were incubated for 4-5 days at 30 °c and colonies of Actinomycetes were picked up and re-streaked on Bennett's agar for isolation.

Confirmation of Actinomycetes:

The primary confirmation of their presence was done by observing the colony morphology and smell of geosmin. Further confirmation was done by performing monochrome staining of suspected colonies and was observed under 45X and 100X

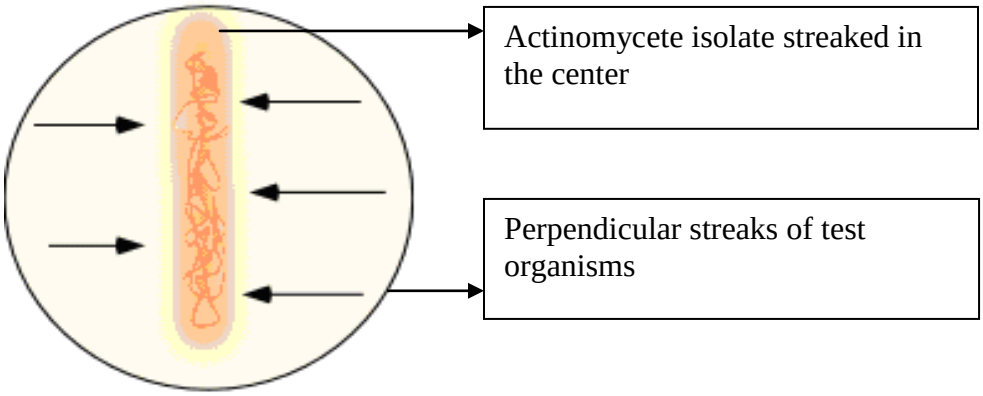
Antibiotic production testing by Perpendicular streak method:

The cultures which were confirmed as actinomycetes were then checked for their antibacterial activity against Gram Positive and Gram Negative bacterial pathogens.

The test cultures included:

1. Gram Positive bacteria : *Staphylococcus aureus, Bacillus subtilis*
2. Gram Negative bacteria : *Escherichia coli, Pseudomonas aeruginosa*

All these four bacterial cultures were the laboratory isolates.
Isolates of actinomycetes were streaked on Bennett's Agar plates at the corner of the plate in the form of lines. The plates were incubated at 37^0 C for 5 days. On the 4^{th} day of incubation, all the four test organisms were streaked on the different Nutrient agar plates to get 24 hour old culture of test organisms. On the 5^{th} day, a circular cellophane paper was put on the layer of agar. Onto that paper a coat of Nutrient Agar was layered and all test organisms were streaked. Then, these plates containing growth of actinomycete isolates and 24 hours old test organisms were incubated at 37^0 C for 24 hours.



Screening for L-Asparginase production:

Isolates obtained were screened for the presence of enzyme L-Asparginase along with previous isolates. The medium used for screening was Kuster's medium. The principle on which this screening was based is as follows- the medium contained phenol red indicator which turns pink in color under alkaline conditions, the enzyme L-Asparginase will act on the substrate L-Aspargine leading to production of ammonia which shifts the pH towards alkaline therefore the medium around the colony turns pink this indicates that the organism is able to produce L-Asparginase. Thus this is the screening test for L-Asparginase production.

Enzyme assay:

L-Asparginase activity was measured by following the method of (Imada *et al* 1973*)*.
The cultures in broth were centrifuged at 8000rpm for 30 mins and the resultant supernatant was then considered as the crude extract and the activity was then determined. Reaction was started by adding 0.5ml of crude extract into 0.5ml of 0.04M L-Aspargine solution and 0.5ml of 0.05M Tris HCL buffer pH 8, incubated at 37°C for

30 mins. The reaction was stopped by addition of 0.5ml of 1.5M TCA (tri chloro acetic acid). After centrifugation at 8000rpm for 15 mins, 0.1ml of supernatant was added to 3.75ml of distilled water, followed by addition of 0.2ml of Nessler's reagent and was incubated at room temperature for 10 mins after which the absorbance was taken at 425nm.

Identification of selected isolates based on cultural, Biochemical and 16 S r -DNA sequencing:

Slide culture technique was used to observe morphological and cultural characters of the isolates.

Biochemical tests were performed as per Bergey's Manual of Determinative Bacteriology (8^{th} Ed) and following tests were performed.

1. Indole production test
2. Urease
3. Catalase test
4. Gelatinase
5. Cellulase
6. Oxidase test
7. Citrate utilization test
8. Starch hydrolysis

16 S r -DNA sequencing of selected isolates was performed using ABI sequencer at NCCS, Pune

Cultivation of selected isolates by shake flask culture:

Shake Flask culture method was used for production of the antimicrobial agent. Selected cultures were inoculated in Bennett's broth (200 ml) in Erlenmeyer flask (500ml). These flasks were kept on rotating shaker at 120 rpm for 8 days (at 30° C).

Bioassay of cell free supernatant by Agar Well Method:

Cell free supernatant of selected isolates was subjected to Agar Well Assay. The innoculum density for 24 hours old laboratory isolate of *S. aureus ATCC 29737* was decided by adjusting the absorbance of the saline suspension of *S. aureus* to 0.6 at 545 nm .The plates were incubated for 48-96 hrs and the effectiveness of the samples was checked by measuring their respective zones of inhibition in mm. The zones were measured inclusive of the well.

Solvent Extraction:

The isolates in their aqueous phase after fermentation were centrifuged and the cell free extract was subjected to solvent extraction. Extraction was performed using

solvents ranging from lower polarity to higher polarity. The extracts in organic phase which might contain the active antibacterial compound were checked by Agar Well Assay.

Thin Layer Chromatography:

The samples which were subjected to solvent extraction were taken and the thin layer chromatography was performed. Here the solvent system was decided on the basis of the assay results of the solvent extracts. G4 showed a single spot in chloroform, while G1 showed single spots in hexane.

Optimization of conditions for maximum production of L- asparginase:

Following parameters were standardized, Initial pH amd temperature of incubation, aeration, time of incubation. Medium optimization included carbon and nitrogen source.

Results and Discussion:

24 isolates were obtained from the collected river sediment samples. These isolates were as follows:

Mandvi river, Goa: G1, G2, G3, G4

Zelum river Kashmir: K1

Krishna river, Maharashtra: Kr1

Koyna river, Karad , Maharashtra: Ko1, Ko 2

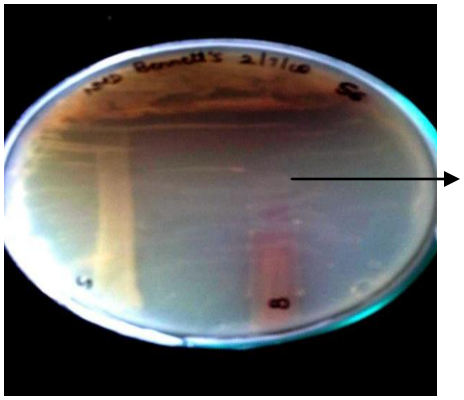
Lake from Gulmerg, Kashmir: g1, g2, g3, g4

Madhya Pradesh NT, NT1, NT2, NT3, NT4, NT5, NT6, NT7

Mula, Mutha River : M1,M2

Pavana river : Pa1, Pa2

Out of these 16 isolates, 2 isolates showed significant activity against the test organism, *Staphylococcus aureus* in Perpendicular streak Method

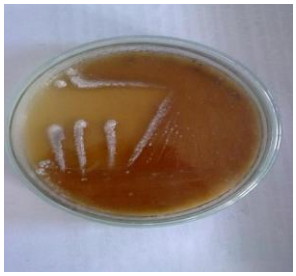


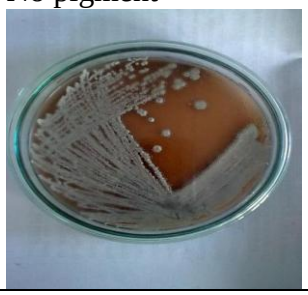
Inhibition of *S. aureus ATCC*29737

G1 and G4 were selected Identification of G1 and G4

Colony Characters:

Isolates	G4	G1
Size	>2mm	>2mm
Shape	Circular	Circular
color	White	White
margin	Entire	Entire
elevation	Low convex	Low convex
opacity	Opaque	Opaque
Pigment	Wine red	No pigment





Biochemical test results:

Sr. No.	**Test**	**G1**	**G4**
1	Cellulase production	- ve	- ve
2	Urease production	- ve	- ve
3.	Citrate utilization	+ ve	+ ve
4	Cyanide utilization	- ve	- ve

5	Gelatinase production	- ve	+ve
6	Amylase production	+ ve	+ ve
7	Catalase production	- ve	- ve
8	Indol production	- ve	- ve

16 S r-RNA sequencing results:

By Blast analysis G4 was identified as *Streptomyces purpeofuscus* OR Kitasatospora *azatica* (99% similarity)

By Blast analysis *G1 was identified as Streptomyces ginsengisoli* (99% similarity)

Agar Well Method:

G1 and G4 samples were subjected to Agar Well Assay. The innoculum density for 24 hours old laboratory isolate of *S. aureus* was decided by adjusting the absorbance of the saline suspension of *S. aureus* to 0.6 at 545 nm .The plates were incubated for 48-96 hrs and the effectiveness of the samples was checked by measuring their respective zones of inhibition in mm. The zones were measured inclusive of the well.4 isolates showed significant inhibitory zones. Here the test organism used was laboratory isolate of *S. aureu*s.

Zone of Inhibition obtained against S. *aureus ATCC* 29737

Isolate	G4	G1
Zone of inhibition (mm)	40	20

Agar well method of G4

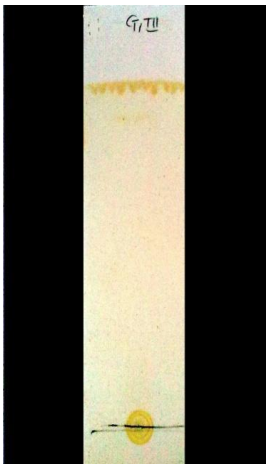
Result of solvent extracts:

Sample	Solvents in which sample extracted
G1	Ethyl acetate
G 4	Chloroform

Thin Layer Chromatography:
The samples which were subjected to solvent extraction were taken and the thin layer chromatography was performed. Here the solvent system was decided on the basis of the assay results of the solvent extracts. G4 showed a single spot in chloroform, while G1 showed single spots in hexane

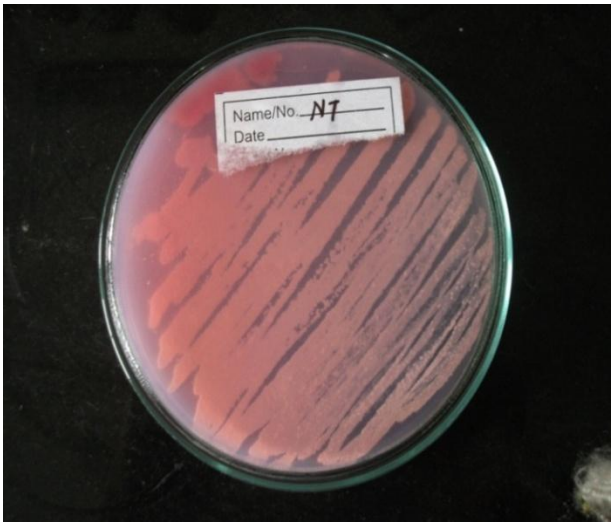
G4

G1



Samples	G4	G1
Rf Value	0.88	0.85

Screening for L-Asparginase production (Plate assay)



Out of the six cultures that showed change in color for the plate assay, cultures namely NT, NT-3 and G1 showed high intensity pink color.

Enzyme activity for isolated cultures:

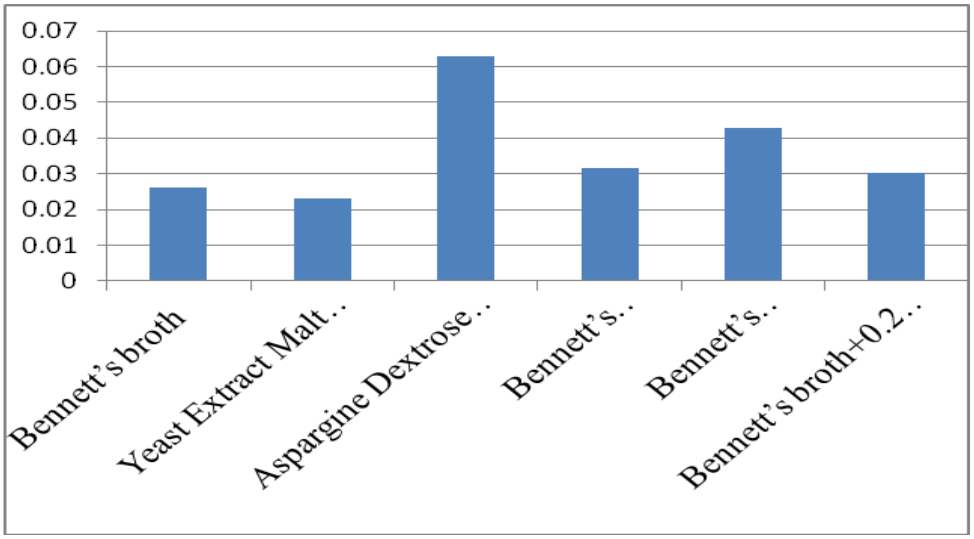
Name of the culture	Enzyme activity(µmol/ml/min)
NT	1.523
NT-3	1.396
G1	2.664

G1 showed highest activity and therefore selected for optimization studies.

Optimization for maximization of enzyme production:

Media shortlisted for growth and production of enzyme from G1

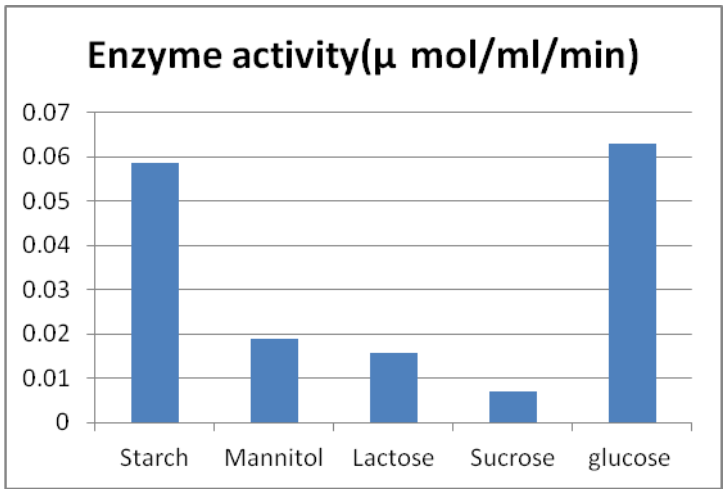
1	Bennett's broth	1.04
2	Yeast Extract Malt Extract	0.92
3	Aspargine Dextrose Salt broth	2.53
4	Bennett's broth+0.05L-aspargine	1.268
5	Bennett's broth+0.05L-aspargine(casein hyrolysate replaced)	1.712
6	Bennett's broth+0.2 L-aspargine(casein hyrolysate replaced)	1.206



Different media were tried for getting more growth and enzyme production, out of these media, ADS showed more enzyme activity compared to all other.

Carbon source optimization

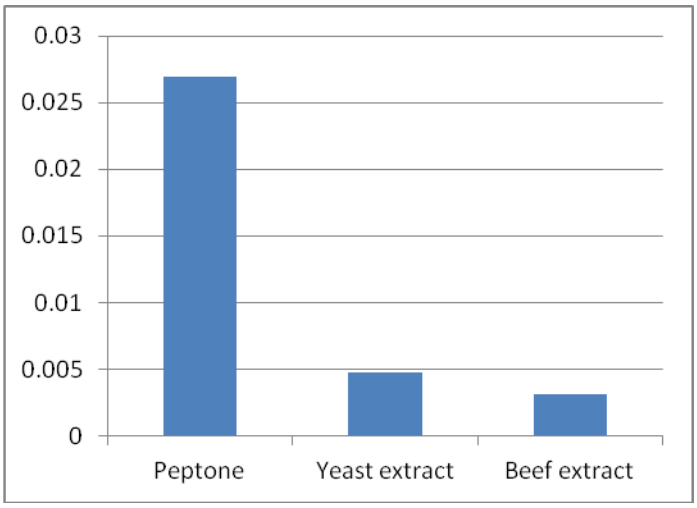
Carbon source	Enzyme activity(µmol/ml/min)
Starch	2.348
Mannitol	0.76
Lactose	0.632
Sucrose	0.28
Glucose	2.52



Starch showed enzyme activity that was found to be comparable to glucose which is a normal constituent of ADS.

Nitrogen source optimization (ADS+0.1g% nitrogen source)

Nitrogen source	Enzyme activity(µmol/ml/min)
Peptone	1.76
Beef extract	0.126
Yeast extract	0.1904



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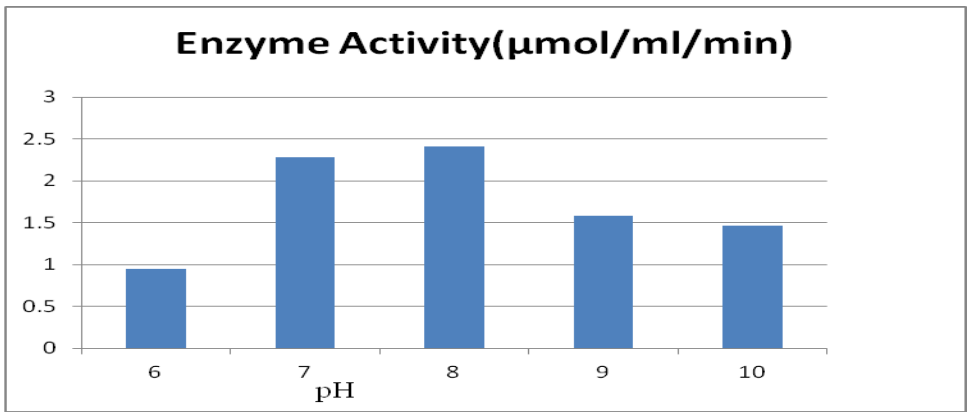
Out of the three nitrogen source that were considered, peptone showed higher enzyme activity compared to yeast extract and beef extract.

pH optimization

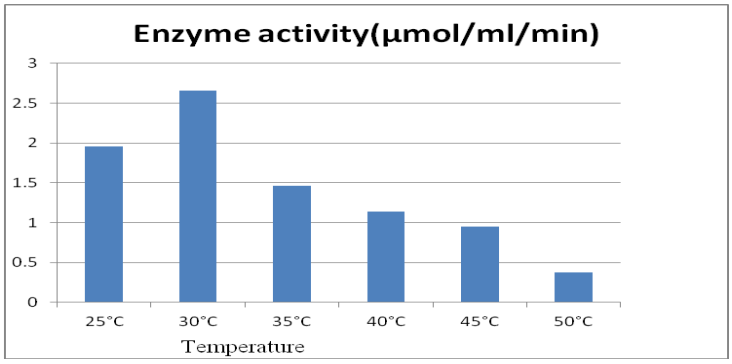
pH	Enzyme activity(µmol/ml/min)
6	0.95
7	2.28
8	2.412
9	1.584
10	1.46



Different pH was used out of which at pH 8 Maximum activity was seen.

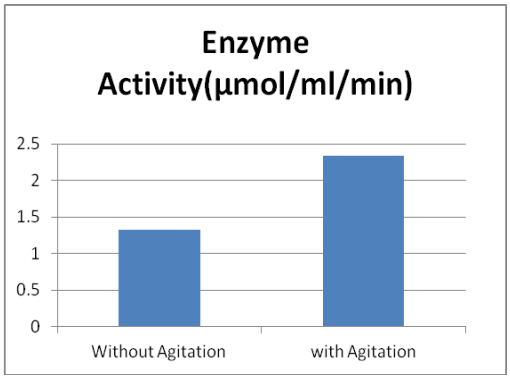
Temperature:

Temperature	Enzyme activity(µmol/ml/min)
25°C	1.96
30°C	2.66
35°C	1.46
40°C	1.14
45°C	0.95
50°C	0.38



Activity was found to be greater at 30°C, after which increase in temperature resulted in lowering the enzyme activity.

Effect of Agitation



Comparison between after and before optimization

	Before Optimization	After Optimization
enzyme activity (μ mol/ml/min)	2.52	3.23

Optimization has increased enzyme activity by 28.17%

Discussion: Among the 24 isolates two isolates namely G4 and G1 were selected for further studies on antibiotic production and L-Asparginase production respectively.
G4 which is identified as *Streptomyces purpeofuscus* showed 40 mm zone of inhibition for *Staphylococcus aureus* ATCC 29737 as test organism.
G1 which was identified as *Streptomyces ginsengisoli* showed very good L-Asparginase activity (2.52 μ mol/ml/min) among the reported *Streptomyces sp.* The optimization studies increased the yield og enzyme production by 28.17%. Further studies on enzyme purification and antitumor testing are to be done.

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