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L-glutaminase Producing Actinomycetes from Marine Sediments –Selective Isolation, Semi Quantitative Assay and Characterization of Potential Strain

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Abstract: Actinomycetes from terrestrial and marine ecosystems have long been recognized either as organism of academic curiosity and organism of antibiotic producers. L-glutaminase is an enzyme produced by various microorganisms which are currently used for the treatment of leukemia, as flavor enhancer and also as enzyme sensors. Even though L-glutaminase activity was reported in various microorganisms, L-glutaminase from actinomycetes in general and marine actinomycete in particular is very scanty. From the reviewed literature, information on L-glutaminase production from actinomycetes is very scanty. With this view, marine sediment sample was collected from mangrove rhizosphere of Parangipettai coastal area (Lat. 11°.29' N; Long. 79°.46'E). Totally 20 L-glutaminase producing actinomycete strains were isolated by using minimal glutamine agar medium supplemented with substrate 1% L-glutaminase, 0.012% phenol red and 3 % NaCl. All the 20 actinomycete strains were micromorphologically identified as *Streptomycetes*. L-glutaminase enzyme was produced from all the 20 actinomycete strains by submerged fermentation and tested by semi quantitative assay. Crude L-glutaminase produced from strain P2 produced 38 mm of pink color zone. L-glutaminase produced from strain P2 was purified further by precipitation and dialysis methods. In semi quantitative assay of dialysate showed 45 mm of zone of color change. Optimal condition for L-glutaminase production was determined at pH 7, temperature 30°C and salinity 3.5%. Based on the studied phenotypic characteristics the potential actinomycete strain was identified as *Streptomyces olivochromogenes* (P2). The selective isolation procedure and semi quantitative assay developed in this present study will be a suitable and best method for therapeutic enzymes in general and L-glutaminase in particular.

Key words: L-glutaminase, Marine actinomycetes, selective isolation, semiquantitative assay, *Streptomyces*, mangroves.

INTRODUCTION

In recent years, L-glutaminase has attracted much attention in both pharmaceutical and food industrial applications. In food industry, L-glutaminase is used as a flavor enhancer by increasing glutamic acid content in food through hydrolysis of L-glutamine to L-glutamic acid and ammonia. It also used in enzyme therapy for cancer especially for acute lymphocytic leukemia. Another important application of L-glutaminase is in biosensors for monitoring the glutamine levels in mammalian and hybridoma cells. In 1993, *Pseudomonas sp,* recombinant glutaminase was patented for its activity against cancer and HIV virus therapy (Sabu *et al.,* 2000). L-glutaminase synthesis was well reported from various terrestrial microorganisms such *as Escherichia coli, Pseudomonas species, Acinetobacter species, Bacillus species, Hansenula, Cryptococcus, Candida, Aspergillus oryzae* and *Beuveria bassiana* (Sabu, 2003). In addition to terrestrial sources, L-glutaminase activity was reported from few marine microorganisms such as *Pseudomonas fluorescens, Micrococcus luteus, Vibrio cholerae* and *Beuveria bassiana* (Chandrasekaran, 1997). Microbial sources like actinomycetes are well recognized to produce a variety of chemical structures, several which are most valuable pharmaceuticals,

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agrochemicals and industrial products like enzymes (Okami, 1986). Actinomycetes are aerobic gram positive filamentous bacteria with high G+C (Guanine + Cytosine) content which form asexual spores and which are widely distributed in both terrestrial and aquatic habitats including extreme habitats like deep sea, desert, mountain etc. Actinomycetes are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry and which are ecofriendly filamentous bacteria. The value of actinomycetes to society in terms of providing useful drugs especially antibiotics and anticancer agent and to the pharmaceutical industry for revenue generating discovery platform, is indisputable (Baltz, 2007). In recent literature revealed that atleast 4,607 patents have been issued on actinomycetes related product and processes (Berdy, 2005). Out of 22,500 total bioactive secondary metabolites 10,100 (45%) are reported to be produced from actinomycetes in which 7630 from *Streptomycetes* and 2470 from rare actinomycetes (Berdy, 2005). But, from the available literature, it is found that information on L-glutaminase production from actinomycetes is still in the stage of infancy (Sivakumar *et al.,* 2006). With this view, the present investigation designed for selective isolation of L-glutaminase producing actinomycetes from marine sediments.

MATERIALS AND METHODS

Sample Collection and Pretreatment:

Marine sediment sample was collected from rhizosphere region of the mangrove *Rhizophora apiculata* of Parangipettai coastal area (Lat. 11.29' N; Long. 79.46'E), Tamilnadu. The surface layer of the sediment was removed and central portions of sediment, approximately 0.5 kg was collected and transferred in to sterile plastic bag. The collected sediment sample was dried at room temperature for a week. 10 gm of sediment sample was transferred to sterile petriplate and kept at 55°C for 10 minutes. The pretreated sample was used for the isolation of actinomycetes (Pisano *et al.,* 1986).

Selective Isolation of L-glutaminase Producing Actinomycetes:

About 5 gm of sample was taken and suspended in 95 ml of sterile distilled water in a 250 ml conical flask and kept in a rotary shaker with 120 rpm for 30 minutes for the thorough mixing of the sediment sample. About 1 ml of mixed sediment suspension from conical flask was transferred in to 9 ml of sterile distilled water. The sample was serially diluted up to 10^{-5} dilutions. Minimal glutamine agar (MGA) medium was prepared and used for the isolation of L-glutaminase producing marine actinomycetes. Components of MGA (gram/litre) include 0.5 KCl; 0.5 MgSo4; 1.0 KH2Po4; 0.1 FeSo4; 0.1 ZnSo4; 25 NaCl; 10 L-glutamine; 0. 012 phenol red in which L-glutamine act as carbon and nitrogen source and phenol red act as pH indicator.

After sterilization, the minimal glutamine agar medium was supplemented with two filter sterilized antibiotics *viz*., cycloheximide (20 µg/ml) and nalidixic acid (100 µg/ml), in order to retard the growth of fungi and bacterial populations (Balagurunathan & Subramanian, 1993). 0.1 ml of aliquot from 10^{-3} to 10^{-5} dilutions were taken and spreaded on minimal glutamine agar medium by using sterile L-rod. Plating was done in triplicates. All the plates were incubated at 28°C for 1 month. One uninoculated plate was kept as control. All the plates were observed from second day of incubation.

Selection of L-glutaminase Producing Actinomycetes:

During incubation, morphologically different actinomycete colonies which showed powdery or leathery consistency were selected. To obtain pure culture, the selected colonies were streaked on minimal glutamine agar medium by phase streaking and incubated at 28ºC for 7 days. After incubation, morphologically different 20 actinomycete colonies were selected and sub cultured on yeast extract malt extract medium (ISP medium 2) slants. In addition, spores of all the 20 strains were inoculated on sterile 20 % glycerol broth. Both the slant culture and glycerol stock cultures were stored at 4ºC until further study.

L-glutaminase Production from Actinomycetes by Submerged Fermentation:

For the preparation of actinomycete spores, all the actinomycete strains were inoculated on sporulation agar medium and incubated for 7 days at 28ºC. After incubation actinomycete spores were scrapped and inoculated in to 50 ml of mineral salt glutamine (MSG) medium (pH 7) in 250 ml conical flask. Components of MSG medium include (grams/litre) 1.0 KH2Po4; 0.5 MgSo4; 0.1 CaCl2; 0.1 NaNo3; 0.1 tri sodium citrate; 25 NaCl; 10 glucose. All the flasks were incubated at 28ºC for 72 hours in a rotary shaker at 120 rpm.

Each 100 ml of MSG medium with phenol red (0.012 %) at pH 7 was prepared in 500 ml Erlen Meyer flask and used for the production of L- glutaminase enzyme. After sterilization by autoclaving, 5% of actinomycete inoculum was transferred in to MSG production medium and incubated at 28ºC in rotary shaker

for 120 hours. The above procedure was applied for all the actinomycete isolates. After incubation, fermentation medium was removed from shaker and centrifuged by using cooling centrifuge at 10,000 rpm for 30 minutes at 4ºC. The clear supernatant was collected in screw cap tube and stored at 4ºC to use as a crude enzyme.

Semi Quantitative Assay of L- Glutaminase:

For this study, minimal glutamine agar medium was prepared and a well with 5 mm diameter was made at the centre of the agar medium by using well puncher. About 50 µl of crude enzyme was added in to the well and plates were incubated at 37°C for 24 hours. One uninoculated plate was kept as control (Gulati *et al.,* 1997). Actinomycete strain with its crude L-glutaminase enzyme showed maximum zone of color change (from yellow to pink) on minimal glutamine agar medium was selected as potential strain for further studies. Crude L-glutaminase enzyme was produced in large quantities from potential actinomycete strain by submerged fermentation method described earlier.

Separation, Dialysis and Activity of L-glutaminase from Potential Strain:

Finely powdered ammonium sulphate (Himedia) was slowly added in to crude enzyme preparation so as to reach 40% saturation. The whole content was stirred at 4°C on a magnetic stirrer. The precipitated protein was removed by centrifugation at 10,000 rpm at 4°C for 20 minutes. Fresh ammonium sulphate was added to the supernatant to increase the concentration to 50%. The obtained precipitate was resuspended in a minimal volume of 0.01M phosphate buffer (pH 8). Precipitated protein was removed by centrifugation as described earlier. Once again the fresh ammonium sulphate was added to the supernatant to increase the concentration to 80%. The obtained enzyme precipitate was resuspended in a minimal volume of 0.01M phosphate buffer (pH 8) and precipitated protein was recovered by centrifugation (Sabu *et al.,* 2005).

Dialysis:

The enzyme precipitate obtained after ammonium sulphate precipitation was dialyzed against 0.01 M phosphate buffer (pH 8) for 24 hours at 4°C with stirring and the buffer was changed occasionally. Finally, L-glutaminase activity of dialysate was semi quantitatively assayed by adopting the method described earlier.

Effect of pH, Temperature and Salinity on the Production of L-glutaminase:

Actinomycete inoculum was prepared by inoculating spores of potential actinomycete strain on ISP2 broth and kept in shaker for 48 hours with 120 rpm. Each 5 ml of ISP2 broth culture was used as inoculum. 5 ml of potential actinomycete strain was inoculated in to series of flasks containing 100 ml of MSG medium with different pH (6, 6.5, 7, 7.5 and 8). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours. To study the effect of temperature, 5 ml of inoculum was inoculated in to 100 ml of MSG medium and incubated at different temperature (25°C, 30, 35, 40 and 45°C) for 120 hours in rotary shaker with 120 rpm. To study the effect of salinity on glutaminase production, MSG medium was prepared with different concentration of sodium chloride (0, 1, 2, 2.5, 3 and 3.5 %). 5 ml of inoculum was added in to each flasks and incubated at 28°C in rotary shaker with 120 rpm for 120 hours. All the extracts obtained from above parameters are studied for its L-glutaminase activity.

Phenotypic Characterization of Potential Actinomycete Strain:

Microscopic, cultural, carbon utilization and physiological characterization of potential actinomycete strain was studied by method described by Shirling and Gottileb (1966).

Micromorphology:

Spore morphological characteristics of potential actinomycete strain was studied by suspending a loopful of a week old culture in the semi solid agar medium and one or two drops of medium were aseptically pipetted on to a sterile glass slide. The agar drop was spread well on the slide and allowed to solidify in to a thin film so as to facilitate direct observation under light microscope. The culture was incubated at 28ºC and examined periodically for the formation of aerial mycelium, spore structure and spore morphology.

Cultural Characteristics:

Cultural characteristics of potential actinomycetes was studied by inoculating in to ISP1, ISP2, ISP3, ISP4, ISP5, ISP6 ISP 7 media and incubated at 28ºC for 7-14 days. The color of the mature sporulating aerial mycelium was recorded in a simple way (white, grey, red, green, blue and violet). When the aerial mass color

fell between two colors series, both the color was recorded. The strains were divided in to two groups by their ability to produce soluble pigments other than melanin. The color was recorded (red, orange, green, yellow, blue and violet).

Utilization of Carbon Source

The ability of actinomycete strain in utilization of various carbon compounds as source of energy was studied following the method described by international Streptomyces Project (Shirling & Gottileb, 1966). Carbon sources used for this test were arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose. These carbon sources were sterilized by ether sterilization without heating. The media and plates were prepared and inoculated according to the convention of ISP (Shirling & Gottileb, 1966).

Physiological Characteristics:

Growth of potential actinomycete on different pH (5, 7, 9 and 11), temperature (20, 30, 37 and 45°C) and salinity (0, .5, 1, 2 and 3 % NaCl) was studied by using ISP 2 medium. All the plates were incubated at 28° C for 7-14 days.

Identification of potential actinomycete strain

Based on the phenotypic characteristics the potential actinomycete strain was identified with the help of Nonomura's keys (1974) and ACTINOBASE database (Ugawa *et al.,* 1989).

RESULTS AND DISCUSSION

Selective Isolation of L-glutaminase Producing Marine Actinomycetes:

On isolation agar plates, colonies with powdery consistency which appeared in different colors were observed. Pink color zone was observed around the colonies and such colonies were further purified by streaking on ISP2 medium. From this, 20 morphologically different actinomycete colonies were selected. Based on the cultural and microscopic appearance all strains were suspected as streptomycetes. Microscopic and cultural characteristics of all the 20 actinomycete strains were given in table 1. In most of the microbial screening programmes for enzyme production, the microorganisms are first isolated from certain environments by routine isolation procedures and then screened for enzymatic activity by methods like zone of color change or zone of clearance on agar medium supplemented with suitable substrate (Ranjekar & Sridhar, 2002). Rajeswari & Shome (2001) reported microbial asparaginase from mangrove sediments of Andaman Islands. In that study, they screened 200 bacterial isolates by plate method by using asparaginase as substrate and found that 108 (54%) were asparaginase producers. But in the present study isolation and screening is a single step process which reduced the cost and time needed for primary screening.

The isolation medium used in this study was selective for the isolation of L-glutaminase producers since the absence of carbon and nitrogen source other than L-glutamine in the isolation medium. The presence of pH indicator in this media showed the L-glutaminase production by change in the color from yellow to pink. Finally the presence of 2.5 % NaCl in the isolation media, make it as a native environment for the growth of marine isolates rather than terrestrial strains. The method described in this study is speedy, consistent and reproducible method when compared to the previously described method which is resource wasting and time consuming. Thus the use of selective media and the presence of antibiotics, NaCl and pH indicator makes the media suitable for direct and selective isolation of L-glutaminase producing marine actinomycetes.

Production of L-glutaminase by Submerged Fermentation:

After 96 hours of fermentation, most of the strains changed the color of the medium from yellow to pink which shows that the extracellular L-glutaminase production by the actinomycete strains. The intensity of pink color was increased up to 120 hours of incubation. Submerged fermentation is the routinely used method for L-glutaminase production from various microorganisms (Dura *et al.,* 2002 and Sabu, 2003). But there is no encouraging report on L-glutaminase production from actinomycetes in general and marine actinomycetes in particular by adopting submerged and solid state fermentation. For this reason, in this present study actinomycete strain was inoculated in two flasks of mineral salt glutamine media in which one is added with phenol red as a pH indicator. During incubation, color of the phenol red supplemented media was changed from yellow to pink after 96 hours and increased up to 120 hours but not latter. Based on this observation, it is easy to conclude the color change was due to the production of L-glutaminase which liberated ammonia from L-glutamine. Thus the color change from yellow to pink indicated the production and increase of L-glutaminase level respectively.

Semi Quantitative Assay of L-glutaminase and Selection of Potential Strain:

In semi quantitative assay, pink color zone with varying size was observed around the well on minimal agar medium (table 1). Three strains *viz.* P2, LG1 and LG2 produced maximum of 38 mm, 31 mm and 30 mm diameter zone of color change, respectively. Based on the results of semi quantitative assay, actinomycete strain P2 was selected as potential strain for further studies. In general enzymes are measured by estimation of enzyme activity by qualitative estimation (Gulati *et al.*, 1997) and also by using UV spectrophotometer (Sabu *et al.,* 2000). There is also one more semi quantitative method recommended by Gulati *et al.,* (1997) for asparaginase activity in which the enzyme producing organism was spotted on Czepek's Dox medium and M-9 medium supplemented with L-asparagine and phenol red as pH indicator. Based on the changes in the size of zone of color change around the spot, the enzymatic activity was assayed semi quantitatively. But this is not accurate since the variations in the number of cells when placing the inoculum. So, to overcome this problem, in this present study the same principle was adopted for glutaminase activity with slight modifications. Instead of using Czepek's Dox medium and M-9 medium, minimal salt glutamine agar medium supplemented with phenol red was used. Instead of inoculating the test organisms, known quantity of crude enzyme (50 µl) on well (5 millimeter in diameter) made on minimal glutamine agar medium supplemented with phenol red. The zone size of color change around the well is directly proportional to the amount of active enzyme present in the crude enzyme preparation. Actinomycete strains screened by this method showed varying size of zone of color change on this media especially strain P2 produced maximum of 38 mm of pink color zone around the well. The present study reported a rapid and reliable semi quantitative assay method for L-glutaminase. This method is recommended for semi quantitative assay of L-glutaminase from any microorganisms.

***Separation, Dialysis and Semi Quantitative Assay of L- Glutaminase*:**

After precipitation and dialysis, the dialysate was tested for L-glutaminase assay by semi quantitative assay. The dialysate produced 45 mm diameter of pink color zone around the well. L-glutaminase produced from P2 strain was purified by ammonium sulphate precipitation & dialysed and tested for L-glutaminase activity by semi quantitative assay. The dialysate produced 45 mm of pink color zone around the well. The zone size produced by dialysate was 7 mm higher than the pink color zone produced by crude L-glutaminase produced from actinomycete strain P2. Further chromatographic purification was needed to study its full activity and also for its chemical characterization and structure elucidation.

Optimized Effect of pH, Temperature and Salinity on L-glutaminase Production and its Activity by Semi Quantitative Assay:

In semi quantitative assay, crude L-glutaminase produced at pH 7, temperature 30ºC and salinity of 3.5 % showed optimal enzymatic activity (table 2). Three physicochemical parameters were (pH temperature and salinity) studied for optimal L-glutaminase production from P2 strain. The maximal L-glutaminase production was at pH 7, temperature 30°C and 3.5 % NaCl concentration. These results are same as glutaminase from *Lactobacillus rhamnosus* reported by Alexandra *et al.,* (2003). The maximal activity of L-glutaminase at 3.5 % NaCl also confirms the marine nature of the actinomycete strain P2.

Characterization and Identification of Actinomycete Strain:

Under microscopic observation, actinomycete strain P2 showed both aerial and substrate mycelium. The spore chain arranged in spirals with smooth surfaces (figure 1 and 2). Nearly 30-50 spores are present in spore chain. In cultural characterization good growth was observed on ISP1, ISP3 and ISP6 medium. In carbon utilization test, growth of potential actinomycete strain was observed on medium supplemented with most of the sugars tested except raffinose and cellulose. Good growth was observed at pH 7 and 9, temperature 28ºC and 37ºC and salinity 2, 3 and 5 % NaCl. Moderate growth was observed under anaerobic conditions (table 3). The potential L-glutaminase producing marine actinomycete strain was tentatively identified as *Strepotomyces olivochromogenes* (P2) based on their phenotypic characteristics and with the help of key characteristics proposed by Nonomura (1974) and Actinobase database developed by Ugawa *et al.,* (1989). Molecular characterization was needed for the strain level identification of *Streptomyces olivochromogenes* (P2). This may be the first report on L-glutaminase producing *S. olivochromogenes* in general and marine strains in particular.

The present study concludes that, selective isolation procedure and semi quantitative assay developed in this present study will be a reproducible method for selective isolation of marine actinomycetes from any coastal ecosystems and for semi quantitative assay of L-glutaminase from any microorganisms. Further investigation on the *S. olivochromogenes* (P2) was needed to prove its industrial significance.

Table 1: L – glutaminase producing actinomycetes from Parangipettai mangrove sediments

Strain No	Aerial mycelium	Substrate mycelium	Mycelial color	Reverse side pigment	Soluble pigment	L-glutaminase activity (zone size in millimeter)
P1	+	+	Gray	-	-	10
P2	+	+	Gray	+	+	38
P3	+	+	Gray	+	-	15
P4	+	+	White	-	-	7
P5	+	+	Gray	-	-	8
P6	+	+	Gray	+	+	12
P7	+	+	Black	+	+	7
P8	+	+	Dirty white	+	+	20
P9	+	+	Dirty white	+	+	9
P10	+	+	Blue	-	-	15
P11	+	+	Gray	+	+	19
P12	+	+	White	-	-	14
P13	+	+	White	+	-	7
LG1	+	+	Dirty white	+	-	30
LG2	+	+	Black	+	+	31
LG3	+	+	Pink white	+	+	8
LG8	+	+	White	-	-	11
LG9	+	+	Black	+	-	26
LG10	+	+	White	+	-	12
LG11	+	+	White	+	-	17

+ - Present; - - Absent

Table 2: Effect of pH, temperature and salinity on L-glutaminase production by actinomycetes

Parameters	Activity (zone size in millimeter)
pH	
6	29
6.5	35
7	39
7.5	40
8	36
Temperature (ºC)	
20	28
30	40
35	34
40	32
45	28
Salinity (NaCl %)	
1	20
2	24
2.5	32
3	35
3.5	38

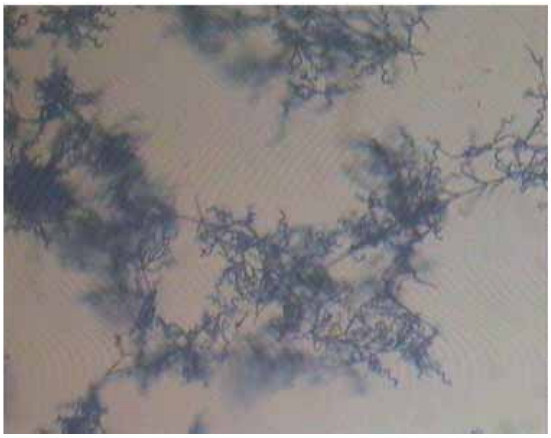

Fig. 1: Micro morphology of actinomycete strain P2 under light microscope (400 magnifications)

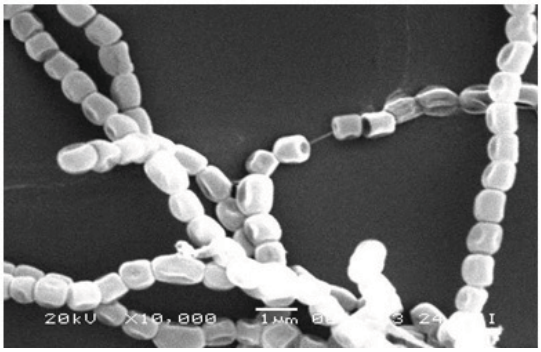

Fig. 2: Micro morphology of actinomycete strain P2 under Scanning Electron Microscope

Table 3: Cultural characteristics of potential actinomycete strain P2

Characteristics	Result	Characteristics	Result
Cultural characteristics		Enzymatic activity	
ISP 1	Good	Lipase	-
ISP2	Moderate	Amylase	+
ISP3	Good	Protease	+
ISP 4	Poor	L- asparaginase	+
ISP 5	Poor	pH tolerance	
ISP6	Good	5	Poor
ISP7	Good	7	Good
Micromorphology		9	Good
Aerial mycelium	+	11	Poor
Substrate mycelium	+	Temperature tolerance (ºC)	
Spore chain	Spiral	15	No growth
Spore surface	Smooth	28	Good
Number of spores	30 – 50	37	Good
Carbon utilization		45	Moderate
Sucrose	+	Salinity (%)	
Raffinose	-	0	No growth
Fructose	+	0.5	No growth
Arabinose	+	1	Moderate
Xylose	+	1.5	Moderate
Glucose	+	2	Good
Inositol	+	3	Good
Cellulose	-	5	Good
Rhamnose	+	Growth at anaerobic conditions	Moderate
Mannitol	+		

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