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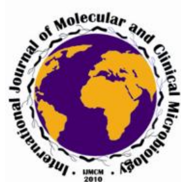

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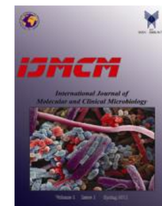

Isolation of L-Glutaminase Producing Marine Actinomycetes

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ABSTRACT

L Glutaminase, a therapeutic enzyme obtained from marine Actinomycetes gains importance because of their adaptability to varied environmental factors. An experimental study was carried out to isolate the L glutaminase producing actinomycetes from the marine sediment of Thoothukudi. Marine sediment samples were collected from six different locations of the Thoothukudi coastal ecosystem, enriched with glutamine broth to enhance the population of Actinomycetes. After enrichment, selective media were used for isolating the Actinomycetes. Ninety four isolate was obtained and all the isolates were screened for L Glutaminase activity in a rapid plate assay method. Three isolates showed strong activity with enzyme production to the level of 9.42 IU to 16.94 IU /ml. The isolate DSG 18 had produced 16.94± 0.62 IU and it could be used a good source of glutaminase.

1. Introduction

Marine microorganisms holds a diverse range of enzyme activities which has the capacity of catalyzing various biochemical reactions and also a source of vast array of biologically active compounds (Debashish *et al*., 2005; Samuel *et al.,* 2011). Many scientific workers demonstrated the marine organisms as potential sources of pharmaceutical compounds (Blunt *et al.,* 2004; Zhang *et al.,* 2005; Laatsch, 2006 and Mayer *et al.,* 2011). It is widely believed that enzymes and other compounds obtained from extreme environments will be stable and a good source of industrial applications (Ravikumar *et al.,* 2010). The search for novel therapeutic agent with antileukemic activity received much attention, especially from marine organisms. Mangroves of marine ecosystem are considered as a treasure of microbes with novel properties (Bungi *et al.,* 2004; Konig *et al*., 2006).

As the availability of anticancerous drugs used for the treatment of cancer is often unsatisfactory to normal cells, there is an increasing need for search of new anticancerous compounds. Modern biotechnological techniques were used to develop alternative and accurate method of cancer treatment, where amidohydrolases also employed. In recent years clinical research focused on enzyme therapy, especially on the utilization of glutaminase, asparaginase and other amino acid degrading enzymes as anticancerous agents (Nathiya *et al.*, 2012; Lekshmi *et al.,* 2014). Nowadays, Glutaminase paid more attention due to its antileukemic activity. It catalyses the deamidation of L-glutamine to L-glutamic acid and ammonia and it has received significant attention as a therapeutic agent against cancer and HIV (Tapiero *et al.,* 2002; Rajeev Kumar and Chandrasekaran, 2003).

L Glutamine is used as sole nitrogen donor for the biosynthesis of purine and pyrimidine nucleotides in a living cell. Glutaminase catalyzes the conversion of glutamine in the presence of water to glutamate and ammonia.

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Tumor cells depends completely on exogenous sources of L Glutamine, since these cells have no mechanism the same. L Glutaminase causes selective death of tumor cells by depleting the glutamine and hence it is used as an effective agent in the treatment of acute lymphocytic leukemia and HIV (Singh and Banik, 2013).

Marine Actinomycetes *S. olivochromogenes* P-2 strains with maximum activity of L-glutaminase was reported by Balagurunathan *et al*., 2010 and various culture conditions of *Streptomyces* sp strain SBU1 for l-glutaminase production was suggested by Krishnakumar *et al*., 2011 and Teja *et al.,* 2014. Jey Prakash *et al.,* 2009 reported the increased production of glutaminase by marine bacteria than the terrestrial sources and they also reported that glutaminase production was growth independent. Chidambara Rajan *et al., (*2015) isolated marine Streptomyces produces L Glutaminase from West Coat, Kerala. Even though Glutaminases are ubiquitous in nature, it is very difficult to obtain sufficient quantity of glutaminases, so not much work has been carried out (Iyer and Singhal, 2010). Hence an experimental study was carried out to isolate, L Gluataminase producing Actinomycetes from marine sediments.

2. Materials and Methods

2.1. Collection of marine sediments

Marine sediment samples were collected from six different places from the Thoothukudi coastal ecosystem of Tamil Nadu (Table 1) during the month of January 2013. The sediment samples were collected by using Core sampling (Dhevendaran and Annie, 1999) and Peterson Grab Sampling (RISC, 1999) methods. Grab sampler was used for collecting samples from DS1 and DS2 and Core sampler was used in case of TS and MK. The samples RP and HC were collected from the rhizosphere zone of mangrove plants (Gunter *et al.,* 2009). The depth of sample collection varied from 1 to 20 feet.

2.2. Enrichment and isolation of actinomycetes

The collected sediment samples were analyzed for its physico - chemical properties as per the standard procedure (APHA, 1989). Bacteria, fungi and actinomycetes population were enumerated to assess the total microbial load (Cappuccino and Sherman, 2002).

Pre treatment of sediment was done to improve the population of Actinomycetes (Ellaiah and Reddy, 1987). The sediment samples were enriched in Glutamine broth for 14 days and after enrichment, the cultures were isolated using different media. Several different combinations of media have been suggested for the isolation of actinomycetes (Waksman, 1961). Loopful of inocula from the pre-enriched broth was streaked on different media. A total of eight different media was selected to determine the isolation, growth and activity of actinomycetes from the marine sediment samples. Kenknight agar, Nutrient Agar, Sea water complex agar, Sea water Glutamine Agar (Parsons *et al*., 1984), Actinomycetes isolation agar, Starch casein agar, Modified M_9 media (Dharmaraj, 2011) and Modified Nutrient Agar were used for the isolation. All the media were added with cycloheximide 50 µg/mL and nalidixic acid 35 µg/mL in order to selectively isolate the Actinmycetes. The plates were incubated at 37°C for 7 days and after incubation pure cultures were obtained. A total of 94 individual colonies with different macroscopic characteristics, colony size and texture were selected and all these isolates were tested for the presence of glutaminase activity.

2.3. Screening of therapeutic enzymes

The screening for glutaminase enzyme activity was done with sea water glutamine agar media spiked with phenol red. In the sea water glutamine medium L glutamine acts as the sole carbon and nitrogen source and phenol red as a pH indicator. The concentration of 0.018% of dye was added to the glutamine medium, plates were prepared, with a control (without dye and without glutamine). The plates were inoculated with 72 hr old cultures for rapid screening of glutaminase. The production of extra cellular L glutaminase by the isolate was inferred from the change of colour from yellow to pink. All the isolates were streaked on glutamine agar medium. After 96 hours of incubation at 32°C, the change of medium colour around the colony as pink indicates the positive reaction.

Secondary screening of the positive isolate was done by the same method but in liquid medium and the colour as well as pH changes

were measured. Loopful of isolates showing glutminase positive reaction in seawater glutamine broth was streaked and the plates were incubated at 37°C for 7 days. From the inoculum prepared, 5ml was transferred aseptically to 45ml of the Sea water glutamine medium. The flasks were kept on an orbital shaking incubator at 35°C at 120rpm for120h. The samples withdrawn were centrifuged at1500rpm for 30min and the clear supernatant was used for enzyme assay.

The assay mixture containing 0.25 ml of crude enzyme extracts was added with 1.25 ml of 0.2 M borate buffer (pH 8). Then 0.5 ml of 0.04 M, L-glutamine in borate buffer was added and the mixture was incubated at 35°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 15 % TCA and the assay mixture was subjected to centrifugation at 4,000 rpm for 20 min. After centrifugation, one ml of the supernatant was mixed with 4 ml of sterilized distilled water free from ammonia. To this 0.5 ml Nessler's reagent was added and the colour intensity read in a spectrophotometer at 450 nm. Ammonia content was estimated using standard ammonium chloride solution and is expressed as International units (IU) per mg of protein (Benny and Ayyakkannu, 1992). One IU is the amount of enzyme that liberates one micromole of ammonia in one min under optimum condition and expressed as IU/ml.

2.4. Identification of Actinomycetes

Pure colonies were subjected to morphological and biochemical characterization to identify the organisms (Bergey, 2000) and in accordance with the guidelines established by the International *Streptomyces* Project (Shirling and Gottlieb,1969). The utilization of different carbon and nitrogen sources were also analyzed (Pridham and Gottlieb, 1948).

3. Results

3.1. Characterization of marine sediments

The sediment samples collected from Thoothukudi were analyzed for its physico - chemical properties. All the samples had an alkaline pH ranging from 8.41 to 8.52. The electrical conductivity of the samples varied drastically (1.96 to 9.36 dSm^{-1}). The Organic carbon content of the sediments ranged between 1.5 to 13.3 % (Table 2).

The collected sample had very low bacterial (30 to 170 x 10^2 g-1 sediment) and fungal population (20 to 110 x 10^1 g-1 sediment). The entire sample recorded very low actinomycetes population (6 to 32 x 10^1 g-1 sediment).

Glutamine broth was used for enriching the sediment samples for 14 days and after enrichment, the cultures were isolated with different selective media. Among the media used for screening of actinomycetes, sea water glutamine media, actinomycetes isolation agar showed more number of colonies as compared to other media. Least population was observed in Nutrient agar and Kenknight agar. Among the different media used, large sized, white colony growth was observed in sea water glutamine media.

The number of isolates obtained from various locations are presented in table 3. Isolates obtained from the selective media were purified and maintained. A Total of 94 individual colonies with different growth morphology were subjected to L glutaminase activity.

3.2. Screening for Glutaminase activity

The isolates were subjected to a semiquantitative plate assay method by measuring the diameter of the colonies and the clear halo zones. The glutaminase positive isolates showed pink coloration due to increase in pH of the filtrates. So whenever pH increases, the media turns to pink which in turn indicates the glutaminase production. The different isolates exhibited pink halo zone around them. Nearly 41 isolates had enzyme activity, but many islaoates did not produced glutaminase at a significant level (Fig. 1). Only three isolates showed strong glutaminase activity which was visualized by a change in color of the whole medium in the plates.

The isolates TSG4, HCSG9, DSG18 showed strong glutaminase activity and used for crude enzyme extraction (Table 4). Simultaneously pH of the crude enzyme extract was also measured and it varied from 8.03 to 8.97.

3.2. Identification of Actinomycetes

The characteristics of the three isolates are given in table 5. The colonies are white, dull white and gray colored growth without any motility. Isolate TSG4 produced white colored colonies HCSG 4 produced dull white colored colonies without pigmentation and DSG8 produced gray colonies.

All the isolates are gram positive and acid fast negative. Temperature required for growth ranged from 37 to 40^{0}C and grew well at 5 % NaCl without any pigment production. The isolates prefer to grow at neutral pH, reduced nitrate and showed an MR positive reaction. All the isolates showed gelatinase, amylase activity and utilized tyrosine, not able to utilize cellulose. All the isolates preferred glucose as carbon source, but also grow in mannitol containing media. None of the isolates utilised Arabinose as carbon source. Differences were observed between the isolates in case of nitrogen utilization. L asparagine was utilized by all the isolates and L Phenylalanine was not utilized. DSG18 strongly utilizes L glutamine.

4. Discussion

Mangrove ecosystem is largely unexplored region for Actinomycetes with the potential to produce biologically active metabolites. It is a well known potent area for distribution of microbes (Gupta *et al.,* 2007). The sediment analyses indicated the presence of very low Actinomycetes population, which necessitated to carry out enrichment before enumerating the actinomycetes population. Hence the marine sediment samples were enriched by method standardized for coastal ecosystem to improve the population for screening purposes. Many studies had indicated that enrichment of samples in selective media led to the isolation of new actinomycetes strains from marine sediment samples. Mixture of antibiotics (Porterj *et al.,* 1960), addition of Nalidixic acid (Takizawa *et al.,*1993) in an isolating medium was experimented for selective development of actinomycetes counts of marine sediments. In the present study also incubating the sample for 14 days at 37°C was found to be good for isolating Actinomycetes from the marine sediments.

Twenty one types of media were suggested for the isolation of actinomycetes from soil (Waksman, 1961). Rathna kala and Chandrika (1993) observed glucose asparagine agar, Grein and Meyer's agar and Kusters Agar were found to be the most suitable for isolation of actinomycetes from air dried sediment samples. The present study confirms that among the eight media used for isolation, Sea water glutamine agar and Actinomycetes isolation agar supported a good number of Actinomycetes than the other media. Savitha *et al.*, 2016 purified L-Glutaminase enzyme from Streptomyces species from soil with glucose as carbon source and Lglutamine as nitrogen source. In the present study also, Sea Water Glutamine agar was found to be highly suitable for isolating glutamine positive marine Actinomycetes.

Abdallah *et al.,* 2013 reported that the optimum pH was 7.0 to 8.0 and temperature was 30°C. They reported 4 % salinity requirement, whereas Teja *et al*, 2014 reported 3.5 % requirement. In the present study, the isolates showed good growth at slightly higher temperature ranged from 37 to 40^{0}C and grew well at 5 % NaCl and neutral pH. Not much variation was observed in the various carbon sources, but differences were observed in the nitrogen utilization. It was identified by morphological, biochemical, carbon utilization and nitrogen utilization pattern as *Streptomyces* sp. but needs molecular confirmation.

L glutamine are used as substrate for determining the activity of L Glutaminase and the end product ammonia released by Berthelot reaction was measured. It was determined by estimating the ammonia liberated from glutamine. Glutaminase production was visualized by an increase in pH of the culture filtrates which in turn changes the color of the broth. This colour change is due to changes in pH of the medium, as L-glutaminase breaks the amide bond of glutamine and releases ammonia. The different isolates exhibited pink halo zone around them. The diameter of the zone visually indicted the LGutaminase production by different isolates. The results were those in agreement with earlier workers like Gulati *et al*., (1997) and Sunil Dutt *et al*., (2010) who reported that isolates exhibited higher zone of diameter was considered as a potential strain of L-glutaminase producer. Katikala *et al*., 2009 used glutamine as therapeutic agents in the treatment of cancer and HIV as an analytical agent in the determination of glutamine.

Glutaminase has gained wide attention nowadays owing to its potential applications in medicine as an anticancer agent and in the food industries. In the present study the isolate DSG 18 showed maximum Glutaminase activity, which could be utilized commercially after further substantial research to determine the therapeutic potential with cytotoxic studies.

Acknowledgements

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Table 1. Details of sediments and sampling locations

Sample Number	Locations	Depth and method	Sediment type
TS	Thoothukudi – Threspuram	25 cm core sampling	Clay with fine organic material
MK	Thoothukudi - Meenavar Kuppam	6 feet with Peterson sampler	Muddy sand
RP	Thoothukudi - Beach road near Roach park	Rhizosphere Zone	Sandy loam soil
HC	Thoothukudi - Harbor camp	Rhizosphere Zone	Sandy loam soil
DS1	Deep sea sediment Thoothukudi	20', Grab sampling	Muddy sand
DS2	Deep sea sediment Thoothukudi	20', Grab sampling	Muddy sand

Table 2. Physico – Chemical analysis of the sediment samples

Sample No	Name of the place	pH	EC (dSm-1)	OC (%)	N %	P %	K %
TS	Thoothukudi - Threspuram	8.48	9.36	7.7	0.78	0.90	0.003
MK	Thoothukudi - Meenavar Kuppam	8.47	8.63	14.3	0.61	0.11	0.004
RP	Thoothukudi - Beach road near Roach park	8.46	7.51	1.5	0.72	0.28	0.005
HC	Thoothukudi - Harbor camp	8.41	1.96	12.3	1.29	0.03	0.09
DS1	Deep sea sediment Thoothukudi	8.52	2.86	6.1	0.56	0.13	0.007
DS2	Deep sea sediment Thoothukudi	8.43	4.20	6.7	0.28	0.14	0.006

Table 3. Isolates Showing L Glutaminase activity

Sample No	Total no of isolates obtained	Total no of positive isolates obtained	Glutaminase positive isolates
TS	12	4	TSG4
MK	13	13	-
RP	28	8	-
HC	10	2	HCSG9
DS1	15	5	-
DS2	16	9	DSG18

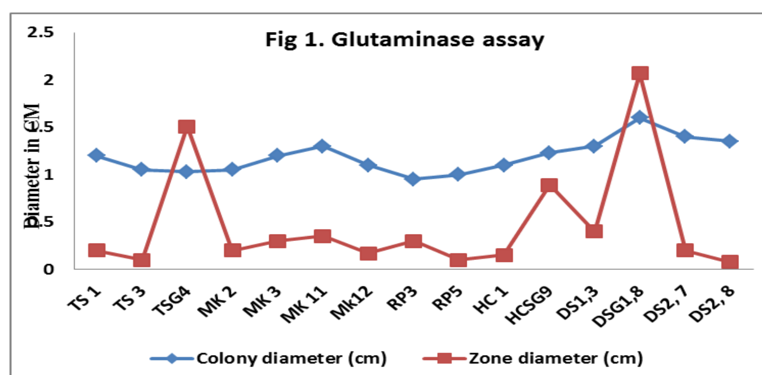
Table 4. Colony diameter and zone of clearance of the isolates

Isolate	Colony diameter (cm)	Zone diameter (cm)	Enzyme in IU/ml	pH (Culture Filtrates)
TSG4	1.03	1.51	12.70 ± 0.48	8.54
HCSG9	1.23	0.89	9.40 ± 0.94	8.97
DSG18	1.60	2.07	16.94 ± 0.62	8.03

Table 5. Morphological, Cultural and Biochemical Characteristics of the Promising isolates

Characteristics	Isolates		
	TSG4	HCSG9	DSG18
Morphology			
Colony color	White	Dull white	Gray
Growth	+	++	++
Diffusable Pigment	-	-	-
Gram staining	+	+	+
Acid fast	-	-	-
Physiological Properties			
NaCl requirement 5% (w/v)	+	+	+
Optimum temperature	37- 40°C	37- 40°C	37- 40 °C
Optimum pH range	7-8	7-8	7-8
Catalase activity	-	-	-
Oxidase	+	+	-
Nitrate reduction	+	+	+
Methyl red	+	+	+
Voges Proskeur	-	-	-
Gelatin utilization	+	+	+
Starch degradation	+	+	+
Casein hydrolysis	+	+	+
Cellulose degradation	-	-	-
Utilization of Carbon Sources			
Glucose	++	++	++
Galactose	+	+	++
Arabnoase	-	-	-
Sucrose	+	+	+
Xylose	-	-	-
Inosital	+	-	-
Mannitol	+	±	+
Fructose	-	-	+
Raffinose	+	+	+
Rhamnose	+	±	±
Utilization of Nitrogen Sources			
Glutamine	+	+	++
Asparagine	+	+	++
Leucine	+	+	+
Glycine	+	+	+
Tyrosine	+	+	+
Phenylalanine	-	-	-
Histidine	+	+	+

(++) -- Indicates strongly positive , (+) -- Indicates positive, (-) -- Indicates negative,



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