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ISOLATION OF L GLUTAMINASE PRODUCING ACTINOMYCETES FROM THIRUVALLUM BRACKISH WATER

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ABSTRACT
Extreme environmental conditions prevalent in the marine ecosystem forces the marine organisms to be the source of novel bioactive compounds. Among the marine microorganisms, Actinomycetes gains importance as they play vital role in the production of useful metabolites. An experimental study was planned with a view to explore the potential of marine actinomycetes present in the brackish water. Isolates with different growth pattern was isolated from the marine sediments of Thiruvallum, Kerala. All the isolates were subjected to rapid plate assay technique for screening L glutaminase activity. Sea water glutamine agar supplemented with 0.018 % phenol red was used for assessing glutaminase activity. Only one isolate among the eight isolates showed significant enzyme activity and KT 05 showed highest zone of clearance. The crude enzyme extracted from the promising isolate KT 05 was purified by salting out with ammonium sulphate followed by sephadex gel filtration.

KEY WORDS: Marine Actinomycetes-Brackish Water - L Glutaminase –Partial Purification.

INTRODUCTION

The marine organisms are distributed in the coastal to offshore regions, from the surface of the water to abyssal depths and considered as a treasure of essentially unimaginable types of microbes (Bungi *et al.,* 2004 and Konig *et al.,* 2006). Marine microorganisms, including bacteria, cyanobacteria, microalgae, and fungi have become an important source of new pharmaceutically active metabolites (Laatsch, 2006, Mayer *et al.,* 2008, Blunt, 2008). Marine actinomycetes are underexplored and exploited source for the discovery of novel bioactive compounds. Among the bioactive compounds, enzymes from marine organisms has gained importance due to their stability, economic production, consistency, ease of process optimization and active than the corresponding enzymes from plant or animals or terrestrial ecosystems (Bull *et al*., 2000). It offers enzymes with wide application potentials and novel biocatalyst with extraordinary properties (Samuel *et al*., 2011).

In recent days, cancer, particularly leukemia is a major global problem and hence search for efficient drug is necessary. As compared to several kinds of treatment, enzyme therapy is equally effective towards tumor cells. As the availability of the anticancerous drugs in the treatment of cancer is often unsatisfactory to the normal cells, there is an increasing demand for search of new anticancerous compounds. In recent years clinical research focused on the involvement of glutaminase and other amino acid degrading enzymes as anticancerous agents. Glutaminase plays a major role as a therapeutic agent as they posses antileukemic properties (Holcenberg *et al.,* 1982, Tapiero *et al.,* 2002). Marine Actinomycetes with maximum activity of L-glutaminase was reported by Balagurunathan *et al*., 2010 and several other researchers (Krishnakumar *et al*., 2011 and Teja *et al*., 2014). Abdallah *et al.* (2013) optimized the conditions for the purification of glutaminase from *Streptomyces avemitilis.* Jey Prakash *et al.*, 2009 reported the increased production of glutaminase by marine bacteria than the terrestrial sources. They also reported that glutaminase production was growth independent.

Chidambara Rajan *et al.,* 2015 isolated glutaminase producing *Streptomyces* from the coastal zone of Kerala. Since it is very difficult to obtain sufficient quantity of L glutaminases from marine microorganisms. not much work has been carried out especially with marine Actinomycetes. these enzymes. Hence an attempt was made to study the marine Actinomycetes from the thiruvallum brakish water as a source of L glutaminase

MATERIALS & METHODS

Collection of Marine sediments

Marine sediment samples were collected from brackish water of Thiruvallam, Kerala at a depth of 6 feet near Punkulam Road. The sediment samples were collected by inserting a polyvinyl corer with a diameter of 10 cm into the sediments. Locations with widely varying characteristics were selected while collecting the samples. The sediment samples were analyzed for its physiochemical and biological properties as per the standard procedures (APHA, 1989 and Cappuccino and Sherman, 2002).

Isolation of the Isolates

Enrichment of the sediments was done to improve the population of Actinomycetes as suggested by (Ellaiah and Reddy, 1987). After pretreatment, enumeration was done with the specific medium. Seven different selective media were used to enumerate the Actinomycetes from the

sediment samples. Ken knight 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. The media were supplemented with filter sterilized antibiotics like cycloheximide (50µg/ml) and nalidixic acid (35µg/ml) for selective isolation of Actinomycetes. One ml of the enriched broth was spreaded on medium by using sterile L-rod. Triplicates were maintained and all the plates were incubated at 37°C for 1 month with uninoculated plate as control. All the plates were observed from the second day of incubation. Individual colonies with different cultural characteristics were selected and screened for the production of glutaminase enzyme.

Screening for enzyme activity

The isolates were screened for L-glutaminase activity by plate assay method using sea water glutamine agar (0.5g KCL, 0.50g $MgSO_4$, 1.0g KH_2PO_4, 0.1 g $FeSO_4$, 0.1g $ZnSO_4$, 0.5 g NaCL, 10.0 g L glutamine in one litre of water) spiked with 0.018 percent phenol red (Imada *et al*., 1973, Gulati *et al.*,1997). The pH of the medium was adjusted to 6.8, in which glutamine acts as a carbon source and phenol red acts a pH indicator to indicate the changes in pH.

Crude enzyme extract was obtained by transferring actively growing isolate (KT05) to a sea water glutamine broth at room temperature in a shaker at 100ppm. After 72 hours of incubation, the broth was centrifuged to get cell free supernatant, which was considered as crude enzyme extract. About 0.5 ml of the crude enzyme extract was added to 0.5 ml of 0.04M glutamine and 0.50ml of distilled water. To this mixture 0.5 ml of 0.1M phosphate buffer (pH-8) was added and incubated for 30 min at 37^{o}C. The enzyme reaction was stopped by adding 0.50ml of 1.5M trichloroacetic acid was added. Then 0.25 ml of above mixture was taken and added to 9.25 ml of distilled water. 0.5ml Nessler's reagent the absorbance was measured at 450nm in a visible spectrophotometer using ammonium sulfate as standard. The enzyme yield was expressed as units/ml (U/ml) (Katikala *et al.,* 2009). Further purification of L glutaminase was done by salting out method using ammonium sulphate precipitation and filtration.

The crude enzyme prepared was brought to 45% saturation, centrifuged at 4200rpm for 10 min at 4^{o}C. The supernatant was subjected to 85% saturation with ammonium sulphate at pH 8.4 and centrifuged as mentioned earlier. Then the precipitate collected after each of the 45 %, 85 % saturation steps were dissolved in 1 Mm phosphate buffer (pH 8) and dialyzed (Sabu, 2003). The dialyzed samples were dissolved in 0.05 M Tris HCl (pH 8.4) buffer and loaded in a preequilibrated column with 0.05 M Tris HCL Sephadex G50 and eluted with 0.05 M Tris HCL buffer containing 0.1 M KCl. Five ml of the fractions at an interval of 30 minutes were collected and assayed for glutaminase activity. Fractions which showed maximum activity was freeze dried and used for cytotoxic studies (Gaffar and Shethna, 1975).

Characterization of the Isolates

Individual colonies with different morphological characteristics were selected and sub cultured for further identification. The cultural, biochemical characteristics of the pure cultures were studied in accordance with the guidelines developed by the ISP (Shirling and Gottlieb, 1969) and Bergeys manual of determinative bacteriology (Bergey, 2000). The carbon and nitrogen substrate utilization were also studied according to the procdedure described by Pridham and Gottlieb, 1948.

RESULTS

The collected sediment samples are dark sandy soil, had alkaline pH of 7.54 with high EC value 5.89 dSm^{-1}. Organic carbon content of the sediments was 1.41 and 0.38 % (Table 1).

TABLE. 1. PhysicoChemical analysis of the sediment samples collected from Thiruvallum

Sample No	Depth & method	pH	EC (dSm^{-1})	OC (%)	N (%)	P (%)	K (%)	Bacteria	Fungi	Actino mycetes
KTS12	6 feet, manual	8.45	7.54	1.41	0.39	0.03	0.006	44	4	11
KTS13	6 feet, manual	8.06	5.89	0.38	0.42	0.02	0.001	38	12	16

The sediments had very low bacterial and fungal population. The samples recorded very low Actinomycetes population (11 to 16 / g of sediment). Enrichment was done to increase the Actinomycetes population

TABLE. 2. Screening of different media for enumeration of marine actinomycetes

S.No	Media used	Growth pattern	Morphology
1	Kenknight agar (Cappucino and Sherman, 2002)	Moderate	Few numbers of colonies without any pigmentation
2	Sea water Glutamine Agar (Parsons *et al*. 1984),	Very Good	Larger, well defined and white colored colonies
3	Sea water complex agar (SWC) (Parsons *et al*.,1984)	Good	White well defined small colonies
4	Actinomycetes isolation agar(AIA) (Shirling and Gottlieb, 1966)	Good	White well defined small colonies with cottony growth.
5	Modified M_9 media (MM9) (Dharmaraj, 2011)	Moderate	Bright White colonies
6	Nutrient agar (NA) (Cappucino and Sherman, 2002)	Poor	White colonies

7	Modified Nutrient Agar (MMA) (Rathna kala and Chandrika, 1993)	Moderate	Few colonies with creamy centre
8	Starch casein agar (SCA) (Shirling and Gottlieb, 1966)	Good	Well defined larger dirty white, fibrous margins.

After sterilization, the medium was supplemented with antibiotics *viz*., cycloheximide (50 µg/ml) and nalidixic acid (35 µg/ml), in order to retard the growth of fungi and bacteria populations (Fig.1). The plates were incubated for 30 days and populations were counted from second day onwards. Isolates showing different morphological pattern were selected for further use. Three isolates from location KTS 12 and five samples from KTS 13 was selected and maintained for further use (Table 4).

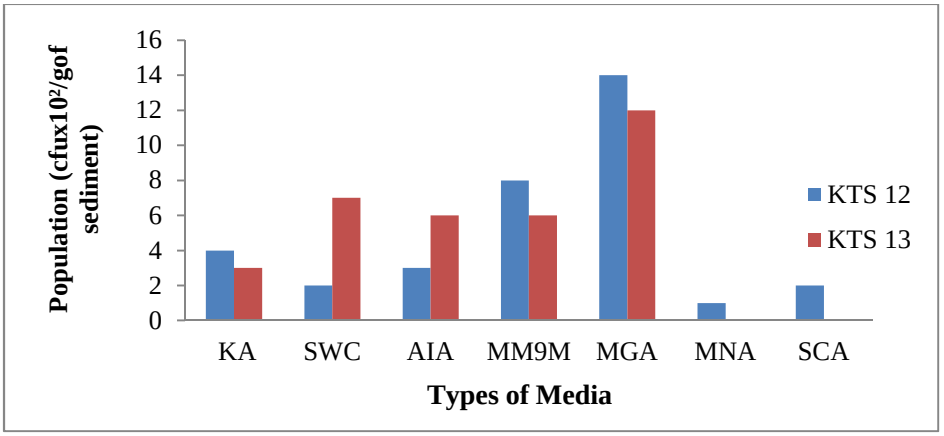

FIGURE 1. Screening Different Media for Enumeration of Actinomycetes

Screening for L-glutaminase activity

The screening for glutaminase enzyme activity was done with plate assay method. It was done with sea water glutamine medium containing phenol red. The concentration of 0.018% of dye was added to the sea water glutamine agar medium, autoclaved and plates were prepared, with control (without dye and without glutamine). The plates were then inoculated with 72 hr old cultures of the isolates for rapid screening of glutaminase. The diameter of the colonies and the clear zone were determined. The glutaminase positive isolates yielded pink coloration due to increase in pH of the filtrates. Isolates which had enzyme activity in rapid plate assay were used for further study (Table 3).

TABLE. 3. Isolates Showing L Glutaminase activity

Sample No	Total no of positive isolates obtained	Glutaminase positive isolates
KT S12	5	-
KT S 13	3	KT 05

The size of the zone was proportional to the glutaminase produced by the isolate. The colony diameter of the isolate KT 05 was 1.08 cm and the zone diameter was 0.90 cm. Enzyme produced was around ±10.54. Simultaneously pH of the crude enzyme extract was also measured and it was 8.44. Glutaminase enzyme was partially purified using ammonium sulphate and sephadex gel filtration. After 45 % and 85% saturation with ammonium sulfate, the pellet was used for the estimation of protein and for L glutaminase enzyme assay. The specific activity of the enzyme was increased, where as the total protein and total activity were decreased proportionaly (Table 4).

TABLE. 4. Partial purification of Glutaminase

Enzyme source	Total Volume (ml)	Total Protein (mg)	Total Activity (IU)	Specific enzyme activity (IU/mg)
Crude enzyme	100	278	1198	4.31
80 % saturation	5.0	5.1	41.4	8.12
Sephadex G 50 filtration	5.0	1.06	12.26	11.56

The different fractions which showed maximum activity was separated and lyophilised for further use (Fig. 2)

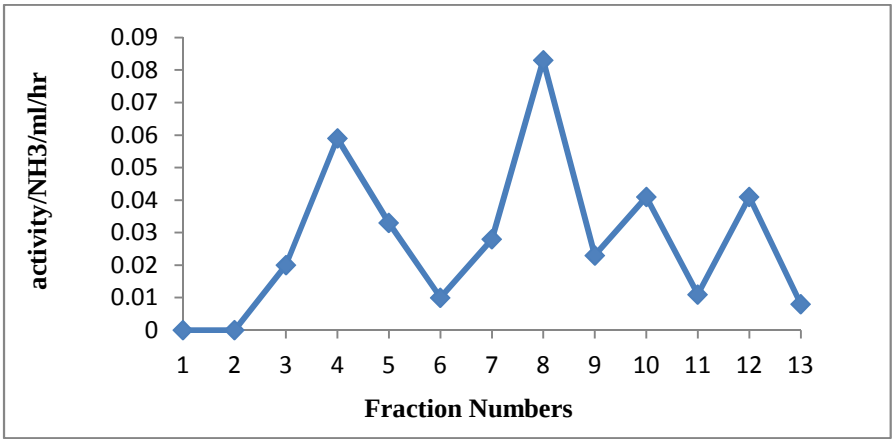

FIGURE 2. Fractions showing Glutaminase Activity

Identification of Actinomycetes

Among the eight isolates, only one isolate showed glutaminase activity (KT05). The morphological, cultural and biochemical characteristics of the KT 05 are presented in table 3. The isolates showed dull white pigmentation with gram positive and negative acid fast reaction without motility.

TABLE. 5. Morphological and Biochemical Characteristics of the isolates from Marine Sediments

Characteristics	KT 05
Morphological Characterization	
Colony color	defined larger dull white, fibrous margins.
Motility	Non motile
Gram staining	Gram Positive
Acid fast	Negative
Biochemical Characterization	
NaCl requirement 5% (w/v)	Required for growth
Optimum temperature	37- 40°C
Optimum pH range	7-8
Catalase activity	Activity present
Oxidase	No activity
Nitrate reduction	Activity present
Methyl red	Positive
Voges Proskeur	Negative
Gelatin utilization	Utilized Gelatin
Starch degradation	Amylase positive
Casein hydrolysis	Hydrolyzed casein
Growth in the presence of 0.1% phenol	No growth
Glucose	Strongly Utilized
Arbinose	Utilized
Sucrose	Not utilized
Mannitol	Utilized
Fructose	Utilized
Inositol	Not Utilized
Xylose	Utilized
Fructose	Utilized
Rhamnose	Not Utilized
Cellulose	Not Utilized
L-asparagine	Utilized
Leucine	Utilized
Tyrosine	Not Utilized
L-phenylalanine	Not Utilized
L-glutamine	Utilized

KT 05 isolate required NaCl for growth and can able to tolerate up to 5% concentration. It grows well at ambient temperature with pH a range of 7-8. The isolate is catalase and oxidase negative and reduces nitrate, MR positive reaction with VP negative results. The isolate showed gelatinase and amylase activity which were identified by gelatin liquefaction and starch degradation. The isolate showed good growth in medium with glucose as carbon source, but grows well in mannitol and fructose containing media. Innosital and Rhamnose were not utilized by the isolate. Even though hydrolysed casein and starch, these isolates were not able to hydrolyse cellulose. Sucrose was not a preferred carbon for KT 05. Differences were observed in case of nitrogen utilization. KT 05 utilized Lasparagine and L glutamine, but not utilized L-phenylalanine and L tyrosine (Table .5).

DISCUSSION

The brackish water ecosystem is an unexplored region for bioactive metabolites. It is a well known potent area for

distribution of microbes (Gupta *et al.,* 2007). The sediment obtained from brackish area indicated the presence of Actinomycetes population. Many studies had indicated that enrichment of samples in selective media will increase the new actinomycetes strains (Porterj *et al.,* 1960, Takizawa *et al.,* 1993). In the present study also enrichment of the sediments for 14 days at 37^{o}C was found to be good for isolating Actinomycetes from the marine sediments.

Waksman, 1961 suggested twenty one different media for the isolation of Actinomycetes from soil. Out of the recommended media, sea water glutamine agar medium was selected to determine the efficacy for isolation, growth and enzyme activity of Actinomycetes from the marine sediment samples. From the enrichment cultures a total of 8 different strains were recovered using Minimal Glutamine agar medium, which shows the suitability and sensitivity of the media for the isolation of glutaminase positive isolates. The salts of seawater provide complex ionic sources that make the medium suitable for marine microbial flora and also buffering capacity of the medium (Wellington and Cross, 1983).

The glutaminase activity 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 change in the pH of the medium, as L-glutaminase causes ammonia production during the breakdown of amide bond in glutamine (Mohamed Hemida *et al.,* 2013). Among the eight isolates, KT 05 had significant enzyme activity in plate assay were used for further study. The present study also confirms the results of Wade *et al.,* 1971 and Imada *et al.,* 1973 that plate assay method is advantageous as this method is quick and can be visualized directly from the plates.

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. Differences were observed in the nitrogen and carbon utilization. It was identified by morphological, biochemical, carbon utilization and nitrogen utilization pattern as Streptomyces sp, but needs molecular confirmation.

L-Glutaminase obtained from earlier sources has issues like hypersensitivity and allergic reactions and anaphylaxis due to long-term usage (Ahlke *et al.,* 1997), glutaminase resistance (Worton, *et al.,* 1991), leukemoginicity (Puri *et al.,* 1995), as agents in the treatment of cancer and HIV (Katikala *et al*., 2009) and glutaminase activity (Ramya *et al.,* 2012). So, there is a need for newer glutaminases with new immunological properties. Halophilic Micro- organisms from extreme environments are expected to have halophilic proteins and enzymes with modified structure and different immunological properties, hence L-glutaminase from these halophilic organisms may be used in the treatment for hypersensitive patients (Ebrahimine zhad *et al.,* 2011). The outcome of this project highlighted the potential of marine sediment of Thiruvallum , Kerala where therapeutic enzymes from marine Actinomycetes might be harnessed.

In the current study, an L-glutaminase producing organism was isolated from marine sediment of Thiruvallum, Kerala. The outcome of this project highlighted the potential of marine environment, where therapeutic enzymes from marine Actinomycetes might be harnessed. Even though some hurdles in the extraction of enzymes from Actinomycetes, the current awareness regarding the compounds from biological origin indicates that marine Actinomycetes application to therapeutic industry deserves attention.

RECOMMENDATIONS

In the present study the isolate TK 05 showed maximum Glutaminase activity, which could be utilized commercially after further substantial cytotoxic studies to determine the therapeutic potential. Even though some hurdles in the extraction, there is a possibility for getting glutaminase, which doesn't cause any side effects.

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