AJES
ASIAN JOURNAL OF ENVIRONMENTAL SCIENCE
VOLUME 11 | ISSUE 2 | DECEMBER, 2016 | 144-151 | e ISSN-0976-8947 | Visit us : *www.researchjournal.co.in*
DOI: 10.15740/HAS/AJES/11.2/144-151
RESEARCH ARTICLE

Isolation of actinomycetes from marine sediments of Muttukadu

P. DHEVAGI AND V. KARTHIGA

Article Chronicle :
***Received* :**
13.09.2016;
***Revised* :**
09.11.2016;
***Accepted* :**
19.11.2016

ABSTRACT : Among the marine organisms, Actinomycetes had gained special attention due to its major role in recycling organic matter, production of wide varieties of bioactive compounds like enzymes, antibiotics etc. In the present study Actinomycetes producing L-asparaginase activity were isolated from marine sediments of Muttukadu. Nineteen isolates were obtained and among 19, three isolates *viz.*, MK1, MK15 and MK 16 showed maximum asparaginase activity. The enzyme extracted from the isolates was partially purified by salting out with ammonium sulphate precipitation, followed by sephadexG200 filtration. The isolate MK 16 showed high specific activity of 65 IU/mg.

HOW TO CITE THIS ARTICLE : Dhevagi, P. and Karthiga, V. (2016).Isolation of actinomycetes from marine sediments of Muttukadu. *Asian J. Environ. Sci.*, **11**(2): 144-151, **DOI: 10.15740/HAS/AJES/11.2/144-151.**

Key Words :
Marine ecosystem, Actinomycetes, Therapeutic enzymes

Author for correspondence **:**
P. DHEVAGI
Department of Environmental Sciences, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA
Email : devagisivaraj@gmail.com

See end of the article for **Coopted authors'**

Micro-organisms from extreme environments provides a good source of new secondary metabolites due to the fact that they are adapted to marine habitat and hypersaline lakes and hence as source of different compounds with modified structures and properties. Among the organisms of extreme environments, marine Actinomycetes gained special attention as they are responsible for the production of more than half of the known bioactive secondary metabolites. These micro-organisms produce extracellular enzymes which are alkalophilic or acidophilic with different immunological properties and contains high proportion of acidic or basic amino acids, hence shows good stability in the environment especially during the synthesis of compounds (Arjith Das *et al.*, 2012 and Lekshmi *et al.*, 2014).

L-Asparaginase is widely used in the treatment of certain cancers (Ebrahiminezhad *et al.*, 2011). It hydrolyses the aminoacid asparagine to aspartic acid and ammonia. The anti lymphoblastic leukemia (ALL) cells are not capable of synthesing L-asparagines and depend on external source for their multiplication. L-asparaginase exploits this property to kill these cells. After the isolation of L-asparaginase from *E.coli* similar to guinea pig serum, there has been considerable interest on other sources also. Although many organisms have the potential for the enzyme production (Alapati and Muvva, 2012, Prakasham *et al.*, 2010; Thenmozhi *et al.*, 2011 and Narta *et al.*, 2011), *E.coli, Erwinia* sp. were highly exploited as drugs due to its high substrate affinity (Verma *et al.*, 2007). But at the same time due to its immunological responses there is a need to explore newer asparaginases with new immunological properties.

Poorani *et al.* (2009) isolated the tumour inhibiting L-asparaginase from marine actinomycetes. The efficacy of various culture conditions of actinomycetes on L-glutaminase production was studied by Krishnakumar *et al.* (2011) and anti-leukemic

agent was reported by Ramya *et al*. (2012). Although some work has been done on marine actinomycetes not much work has been done with marine sediment microflora of the present marine environment. Hence in the current study, an attempt was made to isolate the actinomycetes from Muttukadu Estuary in order to explore the new sources of L-asparaginase.

EXPERIMENTAL METHODOLOGY

Collection of marine sediments :

The sediment samples were collected from three locations [(North zone (S_9), Central zone (S_{10}), South zone (S_{11})] at an interval of 200 m between each location from the mouth of the Muttukadu backwater (lat.12° 46'N and long. 80° 18'E) upto the boat house. Care was taken while selecting the point of collection in such a way that the point of collection had widely varied characteristics as possible. Samples were collected by inserting a polyvinyl corer (10 cm dia.) into the sediments. The sediment samples collected were analyzed for physico - chemical (APHA, 1989) and biological properties (Cappuccino and Sherman, 2002).

Enrichment and isolation of actinomycetes:

Systematic screening was done for the isolation of actinomycetes from marine sediments which require pretreatment as the first step. One gram of sediment samples were transferred to a conical flask containing 100 ml of sterile starch casein broth for pre-enrichment of the samples and incubated at 37° C for 14 days in an incubator cum shaker (Ellaiah and Reddy, 1987). Out of 21 recommended media, seven media were used to determine the efficacy for isolation, growth and activity of actinomycetes from the marine sediment samples. Loopful of inocula from the pre-enriched broth was streaked on seven different media and the plates were incubated at 37° C for 7 days. After incubation, single colonies on the plates were sub-cultured for further studies.

Screening for L-asparaginase activity :

The isolates were screened for L-asparaginase enzyme activity by plate assay with SCA spiked with 0.05 per cent phenol red dye (Gulati *et al*., 1997). The plates were inoculated with 72 h old culture of the isolates and the isolates showing zone of pink coloration were selected as positive cultures. The diameter of the colonies and the diameter of total clear hydrolytic halos including the colonies were determined for all the nineteen isolates. The strains that yielded higher halos were selected as potential micro-organisms for L-asparaginase production.

Identification of actinomycetes:

Purified cultures were subjected to morphological and biochemical characterization to identify the organisms, using standard procedure described in the International Journal of Systematic Bacteriology (Shirling and Gottlieb, 1969).

Partial purification of L-asparaginase :

Asparaginase was partially purified from the crude enzyme. For crude enzyme preparation, 24 hours old actively growing isolate was transferred to SCA broth containing asparagine (0.1%) and grown at room temperature at 100 rpm. After 72 hours of incubation, the broth was centrifuged to get cell free crude enzyme extract. The assay mixture containing 0.25 ml of crude enzyme extraction, 1.25 ml of 0.2M borate buffer (pH 8), followed by 0.5 ml of 0.04 M L-asparagine in borate buffer was added and incubated at 35° C for 30 min. The reaction was stopped by the addition of 0.5 ml of 15 per cent TCA and the assay mixture was subjected to centrifugation at 4,000 rpm for 20 min. After the centrifugation, the supernatant (1 ml) was mixed with 4 ml of sterilized ammonia free distilled water. To this 0.5 ml Nessler's reagent was added and the colour intensity read in a spectrophotometer at 450 nm and expressed in International units (IU) per mg of protein (Benny and Ayyakkannu, 1992). Further purification of L-asparaginase was done by salting out method using ammonium sulphate precipitation and filtration.

Salting out with ammonium suphate and dialysis :

The crude enzyme prepared was brought to 45 per cent saturation with ammonium sulphate at pH 8.4 and kept overnight in cold room. After that the supernatant was subjected to centrifugation at 4200g for 10 min at 4°C. The collected supernatant was brought to 85 per cent saturation and the same procedure was repeated before separating the precipitates. The precipitate collected after the saturation steps were dissolved in 1 Mm Tris HCl buffer and dialyzed using a pre treated dialysis bag overnight at 4°C. After dialysis, the samples were used for protein estimation and enzyme assay (Benny and Ayyakannu, 1992).

The dialyzed samples were dissolved in 0.05 M Tris HCl (pH 8.4) buffer and loaded on to a pre equilibrated column with 0.05 m Tris HCl Sephdex G 50. It was eluted with 0.05 M tris HCl buffer containing 0.1 M KCl. Fractions were collected at the flow rate of 5ml/30 min and L asparaginase was assayed by procedure described earlier. Fractions showing high activity were pooled and freeze dried for further use (Gaffar and Shethna, 1975).

EXPERIMENTAL FINDINGS AND DISCUSSION

Samples collected from Muttukadu were fine mud with coarse sand in nature showed slightly alkaline pH with varied EC values (Table 1).

The collected sample had very low bacterial and fungal population. The bacterial population varied from 30 to 360 per g of sediment sample and fungal population varied from 20 to 84 per g of sediment. The entire sample recorded very low actinomycetes population (14 to 100 / g of sediment). Based on this observation enrichment was carried out before enumerating the actinomycetes population.

Many studies indicated that enrichment of samples in selective media paved way for the isolation of new actinomycetes strains from marine sediment samples. Addition of Nalidixic acid in starch casein media enhanced the actinomycetes counts of marine sediments (Takizawa *et al.*, 1993). Balagurunathan (2002) isolated 51 actinomycete strains from marine sediments by heat treatment method. Sivakumar *et al.* (2006) used Nystatin in Kuster's agar for isolation of actinomycetes from mangrove sediments. Hakvag *et al*. (2008) used cyclohexamide and nalidixic acid in the ISP2 medium for the isolation of actinomycetes from sea surface macro layer. Based on the earlier reports and the present observation, enrichment was done to improve the population.

After enrichment, actinomycetes were isolated using selective media. Several different combinations of media

Table 1 : Physico – chemical analysis of the sediment samples

Sample No.	Name of the place	pH	EC (dSm^{-1})	OC (%)	N (%)	P (%)	K (%)
S_9	Muttukadu – Chennai	8.76	1.62	23.7	0.50	0.01	0.01
S_{10}	Muttukadu – Chennai	8.95	1.77	23.3	0.68	0.02	0.02
S_{11}	Mutthukadu – Chennai	8.87	0.60	20.5	0.40	0.03	0.01

Table 2 : Colony diameter and zone of clearance of the isolates

Isolate number	Colony diameter (cm)	Zone diameter (cm)	Per cent of cleared zone
MK 1	1.8	1.4	77.7
MK 2	1.7	0.2	11.8
MK 3	1.7	0.7	41.2
MK 4	2.6	1.1	42.3
MK 5	1.8	0.7	38.9
MK 6	1.7	0.7	41.1
MK 7	2.4	1.3	48.1
MK 8	1.0	0.3	30.0
MK 9	1.9	0.6	31.6
MK 10	1.4	0.6	42.9
MK 11	2.3	1.0	43.5
MK 12	1.5	0.6	40.0
MK 13	1.4	0.5	35.7
MK 14	1.6	0.7	43.8
MK 15	1.6	1.4	87.5
MK 16	1.2	1.1	91.7
-	1.9	0	0
Mean	1.7	0.76	43.99
S.E. $\pm$	0.30	0.08	4.23
C.D. (P=0.05)	0.61	0.34	8.71

have been suggested for the isolation of actinomycetes from soil (Waksman, 1961). Out of 21 recommended media, seven media were used to determine the efficacy for isolation of actinomycetes from the marine sediment samples. Among the different media used for screening of actinomycetes, starch casein agar media (Shirling and Gottlieb, 1966), sea water complex media (Parsons *et al*.,1984), actinomycetes isolation agar (Hi media laboratories) showed more number of colonies as compared to other media (Fig. 1). Different types of morphology were observed and the colony morphology and size was large in case of starch casein agar. Hence, was used for the isolation of actinomycetes from the sediments supplemented with cyclohexamide 50µg /ml and nalidixic acid 30 µg /ml. Loopful of inocula from the pre-enriched broth of starch casein broth was streaked and the plates were incubated at 37° C for 7 days and after incubation, colonies were isolated. From the enrichment cultures, totally nineteen isolate were obtained and used.

Screening for L-asparaginase activity:

The screening for L-asparaginase enzyme activity was done with plate assay method. This method is advantageous as this method is quick and can be visualized directly from the plates (Wade *et al.*, 1971 and Imada *et al.*, 1973). All the isolates were subjected to the screening and only few isolates were used for enzyme purification. Among the isolates those isolates which showed maximum clear zone were selected for further study (Table 2).

Among the nineteen isolates, three isolates (MK 1, MK 15 and MK 16) had maximum enzyme activity in rapid plate assay and the per cent zone of clearance was high incase of MK16.

Hence, these three isolates were used for enzyme

Table 3 : Characterisation of the isolates from Muttukadu

Characteristics	Marine Isolates		
	MK 1	MKI5	MKI 6
Gram staining	+	+	+
Acid fast	-	-	-
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	+	+	+
Indole	-	-	-
Citrate	-	-	-
H_2S production	+	+	+
Methyl red	+	+	+
Voges Proskeur	-	-	-
Gelatin utilization	+	+	+
Starch degradation	+	+	+
Casein hydrolysis	+	+	+
Cellulose degradation	-	-	-
Glucose	++	++	++
Sucrose	+	-	+
Mannitol	+	+	+
Fructose	+	+	+
Lactose	+	+	+
L-asparagine	+	+	+
Leucine	+	+	+
Tyrosine	+	+	+
L-phenylalanine	-	-	-

extraction and purification study. The enzyme L-asparaginase was partially purified using ammonium sulphate and sephadex gel filtration. After 45 per cent and 85 per cent saturation with ammonium sulfate, the pellet was used for the estimation of protein and for L-asparaginase enzyme assay.

Identification of Actinomycetes:

The isolates were characterized for it's cultural, morphological and biochemical properties (Table 2 and 3). All the isolates are non-motile, showed gram positive reaction with high sodium requirement. Temperature for growth ranged from 37 to 40^{0}C and grew at 5 per cent NaCl. The isolates were acid fast negative with catalase and oxidase positive reaction. All the three isolates produced dull white colored colonies without pigmentation and showed fast growth. The isolates prefered to grow at neutral pH, reduceed nitrate and showed MR positive reaction. All the isolates showed gelatinase, amylase activity and not utilized tyrosine and cellulose, grew well in media with glucose and moderate growth in the presence of sucrose, mannitol and fructose. The isolates were able to hydrolyse casein and starch, able to liquefy gelatin and showed negative citrate utilization. It was

Table 4 : Purification of L-asparaginase enzyme from marine actinomycetes

Isolate	Purification steps	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)
MK1	Crude extract	468.2	304.3	0.65
	Ammonium sulphate precipitation (45-85 %)	362.7	246.1	0.68
	Sephadex G 50 filtration	2.2	34.8	15.8
	Sephadex G 200 filtration	0.21	9.43	44.9
MK15	Crude extract	408.2	294.3	0.72
	Ammonium sulphate precipitation (45-85 %)	262.7	146.1	0.56
	Sephadex G 50 filtration	1.2	24.8	20.7
	Sephadex G 200 filtration	0.41	15.4	37.6
MK16	Crude extract	349.5	204.5	0.59
	Ammonium sulphate precipitation (45-85 %)	204.7	186.8	0.91
	Sephadex G 50 filtration	1.46	32.26	22.09
	Sephadex G 200 filtration	0.10	6.5	65.0

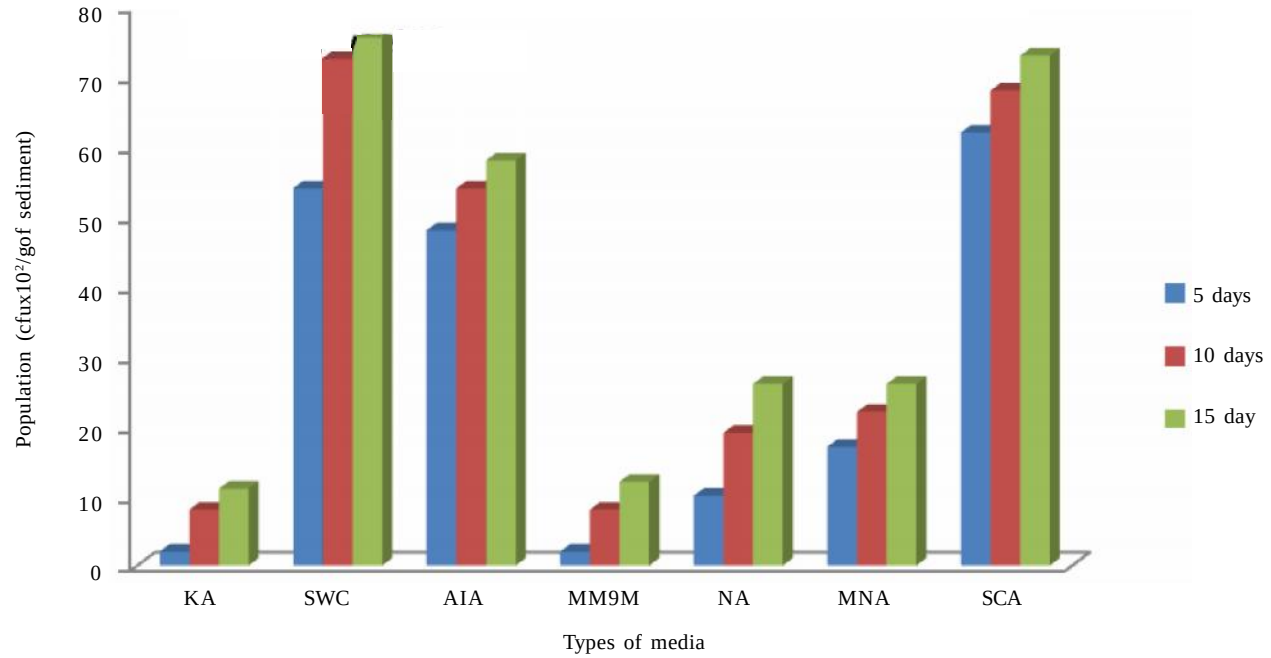

Fig. 1 : Screening different media for enumeration of actinomycetes

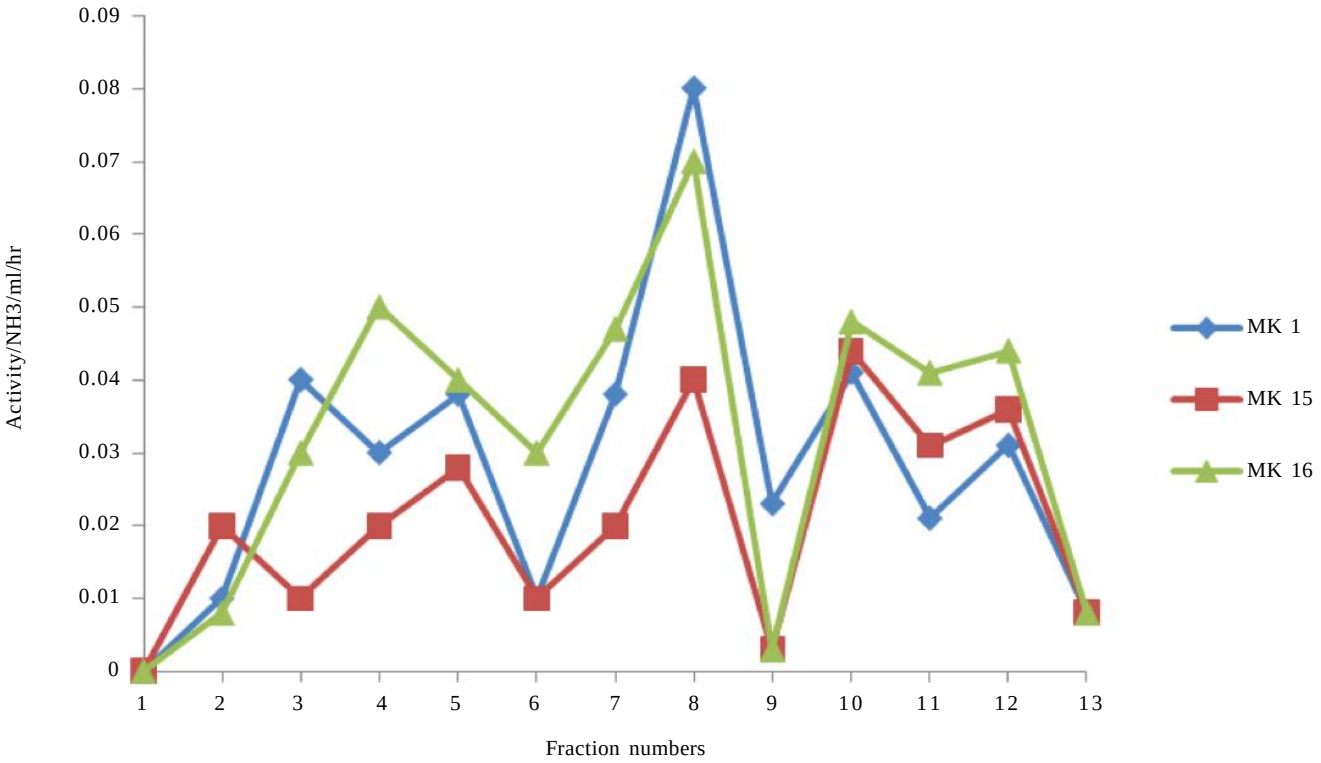

Fig. 2 : L-asparaginase from Marine Actinomycetes

identified by slide culture, morphological and biochemical characteristics and in comparison with the keys given in International Streptomyces Project as *Streptomyces* sp (Bergey, 2000). Its presence in the marine environment falls in line with the findings of Holt *et al.*, 1994 who reported the presence of actinomycetes with enzyme activity in the marine sediments.

Partial purification :

Purification of L-asparaginase was achieved by using 85 per cent ammonium sulphate saturation followed by sephadex G 200. The specific activity of the purified enzyme increased from 0.65 to 44.9 IU/mg , 0.56 to 37.6 IU /mg and 0.59 to 65.00 IU /mg form MK1,MK15 and MK16, respectively. The different fractions which showed maximum activity was separated and lyophilised for further use (Fig. 2). Among the three isolates , MK 16 showed maximum activity than MK 1 and MK 15 was stored for cytotoxic studies (Table 4). Sindhwad and Desai, 2015 optimised the purification of L-asparaginase from marine isolate. There is an immense demand for new source of L-asparaginase with better therapeutic properties to prevent or reduce the side effects. The present study reveals that the marine isolate MK 16 was identified as a promising candidate for L-asparaginase production and it showed significant enzyme production compared to other isolates. Further studies are needed for harnessing full potential of the organism.

Acknowledgement :

The authors are thankful to the University Grants Commission, Government of India for their financial support.

Coopted Authors' :

V. KARTHIGA, Department of Environmental Sciences, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA

REFERENCES

Alapati, K. and Muvva V. (2012). A study on L-asparaginase of Nocardia lecis MK-VL 113. *Scientific World J.*, http:// *www.hindawi.com/journals/tswj/2012/160 434/*

APHA (1989). Standard methods for estimation of water and wastewater, 16th Edn.*American Public Health Association*. Washington DC., U.S.A.

Arjit Das, E., Mahdi, B., Prashanthi, K., Sandeep, S. and Sourav, B. (2012). Enzymatic screening and random amplified

polymorphic DNA finger printing of soil Streptomyces isolated from Wayanad District in Kerala. India. *J. Bio. Sci*., **12**: 43-50.

Balagurunathan, R. (2002). Diversity of actinomycetes and their high value metabolites. *In: UNU-UNESCO International training course on mangrove biodiversity.* (ed.) A. N. Subramanian. CAS in Marine Biology, Annamalai University, India, 72-86.

Benny, A. and Ayyakkannu (1992). L asparaginase activity of benthic marine bacteria and of the muricid *Gasteropod chicoreus* ramosus, East coast of India, Phuket Mar. Biol. Spec. Publ. No. **11** : 137- 140.

Bergey (2000). Manual of determinative bacteriology. Actinomycetales, 9th Ed.

Cappuccino, J.G. and Sherman, N. (2002). Microbiology: A laboratory manual, 6 th addition, Pearson education (Singapore) Pvt. Ltd, Indian branch, 482 FIE Patparganj, New Delhi, : 143-185.

Ebrahiminezhad, A., Rasoul-Amini, S. and Ghasemi, Y. (2011). L-Asparaginase production by moderate Halophilic bacteria isolated from Maharloo Salt Lake. *Indian J. Microbiol*., **51** : 307-311

Ellaiah, P. and Reddy, A.P.C. (1987). Isolation of *actinomycetes* from marine sediments off Vishakapatnam East coast of India. *Indian J. Mar. Sci.*, **16** : 134-135.

Gaffar, S.A. and Shethna, Y.I. (1975). Partial purification and antitumour activity of l asparaginase from *Azotobacter* vinelandi. *Curr. Sci*., **44** : 727- 729.

Gulati, R., Saxena, R.K. and Gupta, R. (1997). A rapid screening for L-asparaginase producing micro-organisms. *Letters Appl. Microbiol.*; **24** : 23-26.

Hakvag, S., Fjarvik, E. and Josefsen, K.D. (2008). Characterization of *Streptomyces* spp. Isolated from the sea surface microlayer in the Trondheim Fjord, Norway. *Mar. Drugs*, **6** : 620-635.

Holt, J.G., Krieg, N.R. and Sneath, P.H. (1994). Group 19. Regular, nonsporing Gram-positive rods. In: Hensyl WR (ed), editor. Bergey's manual of determinative bacteriology. 9th ed. Baltimore: Williams & Wilkins Inc; pp. 565–70.

Imada, A., Igarasi, S., Nakahama, K. and Isono, M. (1973). Asparaginase and glutaminase activities of microorganisms. *J. Gen. Microbiol*., **76** : 85–89.

Krishnakumar, S., Alexis Rajan, R. and Ravikumar, S. (2011). Extracellular production of L-Glutaminase by alkalophilic Streptomyces sp.- SBU1 isolated from Cape Comorin coast, *Indian J. Geo Mar. Sci.*, **40** (5) : 717-721.

Lekshmi, M., Ayona Jayadev and Navami, S.S. (2014). Isolation and screening of Actinomycetes from marine samples for enzyme production. *Internat. J. Sci. Eng. Res*., **5**(10): 199-204.

Narta, U., Roy, S., Kanwar, S.S. and Azmi, W. (2011). Improved production of L-asparaginase by *Bacillus brevis* cultivated in the presence of oxygen-vectors. *Biores. Technol*, **102**:2083-2085

Parsons, T.R., Maita, Y. and Lalli, C.M. (1984). A manual of chemical and Biological methods for seawater analysis. Pergamon Press (I st Edition), 157-161.

Poorani, E., Dhevagi, P. and Saseetharan, M.K. (2009). L-asparaginase production by newly isolated marine Streptomyces sp. Strain EPD 27: Molecular identification. *Internat. J. Int. Biol*., **7** (3) :150-155.

Prakasham, R.S., Hymavathi, M., Rao Arepalli, S.K., Rao V.J. and Kennady, P.K.. (2010). Evaluation of antineoplastic activity of extracellular asparaginase produced by isolated Bacillus circulans. *Appl. Biochem. Biotechnol.*, **160** :72-80.

Ramya, L.N., Doble, M., Rekha, V.P. and Pulicherla, K.K.. (2012). L-Asparaginase as potent anti-leukemic agent and its significance of having reduced glutaminase side activity for better treatment of acute lymphoblastic leukemia. *Appl. Biochem. Biotechnol.*, **167** : 2144-2159.

Shirling, E.B. and Gottlieb, D. (1966). Methods for characterization of *Streptomycetes* species. *Internat. J. System. Bacteriol*., **16**: 313 - 340.

Shirling, E.B. and Gottlieb, D. (1969). Cooperative description of type cultures of *Streptomyces*. IV Species description from the second, third and fourth studied. *Interntl. J. Systematic Evol. Microbiol*., **19**: 391-512.

Sindhwd, P. and Desai, K. (2015). Media optimization, Isolation and purification of L asparaginase from marine isolate. *Asian Pac.J.Health Sci*., **293** : 72- 82.

Sivakumar, K., Sabu, M.K. and Manivel, P.R. (2006). Optimum conditions for L-glutaminase production by actinomycete strain isolated from estuarine fish, Chanas chanas. *Indian J. Experimental Biol*., **44** : 256-258.

Takizawa, M., Colwell, R.R.and Hill, R.T. (1993). Isolation and diversity of actinomycetes in the Chesapeake Bay. *Appl. Environ. Microbiol*., **59** *:* 997–1002.

Thenmozhi, C., Sankar, R., Karuppiah, V. and Sampathkumar, P. (2011). L-asparaginase production by mangrove derived *Bacillus cereus* MAB5: Optimization by response surface methodology. *Asian Pac. J. Trop. Med*., **4** : 486-491

Verma, N., Kumar, K., Kaur, G. and Anand, S. (2007). LAsparaginase: A promising chemotherapeutic agent. *Crit. Rev. Biotechnol.*, **27** : 45-62.

Wade, H.E., Robinson, H.K. and Phillips, B.W. (1971). Asparaginase and glutaminase activities of bacteria. *J. Gen. Microbiol.,* **69** : 299-312.

Waksman, S.A. (1961). The Actinomycetes: Classification, identification and descriptions of genera and species. The Williams and Wilkins Company, *Baltimore.*, **2** : 61-292.