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Partial purification and anti-leukemic activity of L-asparaginase enzyme of the actinomycete strain LA-29 isolated from the estuarine fish, *Mugil cephalus* (Linn.)

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Abstract: *The actinomycete strain LA-29 isolated from the gut contents of the fish, Mugil cephalus of the Vellar estuary showed excellent L-asparaginase activity. The enzyme was purified 18-fold and the final recovery of protein was 1.9%, which exhibited an activity of 13.57 IU/mg protein. The partially purified L-asparaginase inhibited the growth of leukemia cells in male wistar rats. Average survival period of the rats was more in an optimum enzyme dose of 100 units and the survival period was less when the dosages were increased and at the same time the enzyme became less effective when the dosages were decreased. Higher survival of 17.2 days was recorded when 100 units of the enzyme was given in three intermittent doses (50/25/25 units) at the interval of 24* hr. *Analysis of cell components of the strain LA-29 has revealed the wall type - I which is the characteristic of the genus Streptomyces. Further, the morphological, physiological and biochemical features along with the micromorphological results obtained for the strain LA-29 were compared with that of the Streptomyces species found in Bergey's Manual of Determinative Bacteriology and the strain LA-29 has been tentatively identified as Streptomyces canus.*

Key words: *Estuarine fish, Actinomycetes, L-asparaginase, Anti-leukemic activity*
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Introduction

L-asparaginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings (Savitri *et al.*, 2003). It is an enzyme drug of choice used in combination therapy for treating acute lymphoblastic leukemia in children (Schemer and Holcenberg, 1981; Ucar and Carliskan, 2006; Cory and Cory, 2006; Verma *et al.*, 2007). Since 1922, L-asparaginase has been considered as a therapeutic agent against malignant tumors. Haley (1961), Tozuka *et al.* (1997), Arico *et al.* (2005), showed the results using a mouse leukemia cell line. It was further shown that growth of normal cells was not dependent on L-asparagine, making L-asparaginase a potential tumor specific drug.

Since extraction of L-asparaginase from mammalian cells is difficult, microorganisms have proved to be a better alternative for L-asparaginase extraction, thus facilitating its large scale production. Mashburn and Wriston (1964) successfully purified *Escherichia coli* L-asparaginase and demonstrated its tumoricidal activity. Clinical results have shown that L-asparaginase from *E. coli* causes toxicity and immunosuppression in addition to development of resistance (Connors and Jones, 1973), thus necessitating studies to get L-asparaginase from other sources (Gaffar and Shethna, 1975; Sahu *et al.*, 2007). Sources like actinomycetes have been in the hopes of finding out an enzyme with a low molecular weight, high affinity for substrate and low toxicity.

During the course of screening of marine actinomycetes for L-asparaginase an actinomycete strain LA-29 was isolated from the gut contents of the fish, *Mugil cephalus* collected from the Vellar estuary (Lat.11°29'N and Long.79°46'E), situated along the southeast coast of India. This communication is based on that and deals with partial purification and anti-leukemic activity of L-asparaginase enzyme and also identification of the strain LA-29 using chemotaxonomical and conventional methods.

Materials and Methods

Isolation of actinomycetes: *Mugil cephalus* was collected using cast net from the Vellar estuary. Sample was kept in sterile polyethylene bags and transported to the laboratory under ice for microbiological analysis. Before the alimentary tract of the fish was removed, the body surface was wiped with 70% ethanol by using sterile cotton (Sudha *et al.,* 2002). The abdomen was opened aseptically and then the alimentary tract was carefully taken out. The contents of the alimentary tract were squeezed out with forceps. Then, one gram of the squeezed out gut contents were taken and homogenized in a sterile mortar and pestle. The sample was serially diluted (10^{-2} dilution) with filtered and sterilized 50% seawater. One ml of the serially diluted samples were plated (triplicate) in petriplates containing Kuster's Agar medium and incubated at 35°C for seven days. All the colonies that grew on the petriplates were sub-cultured and were maintained in slants. Among them, one strain LA-29 showed good L-asparaginase activity and used for further study.

Partial purification: For partial purification of L-asparaginase, the crude cell free extract of enzyme was subjected to centrifugation at 1,00,000 rpm for 60 min in the refrigerated centrifuge. The supernatant and the pellet suspended in known volume of

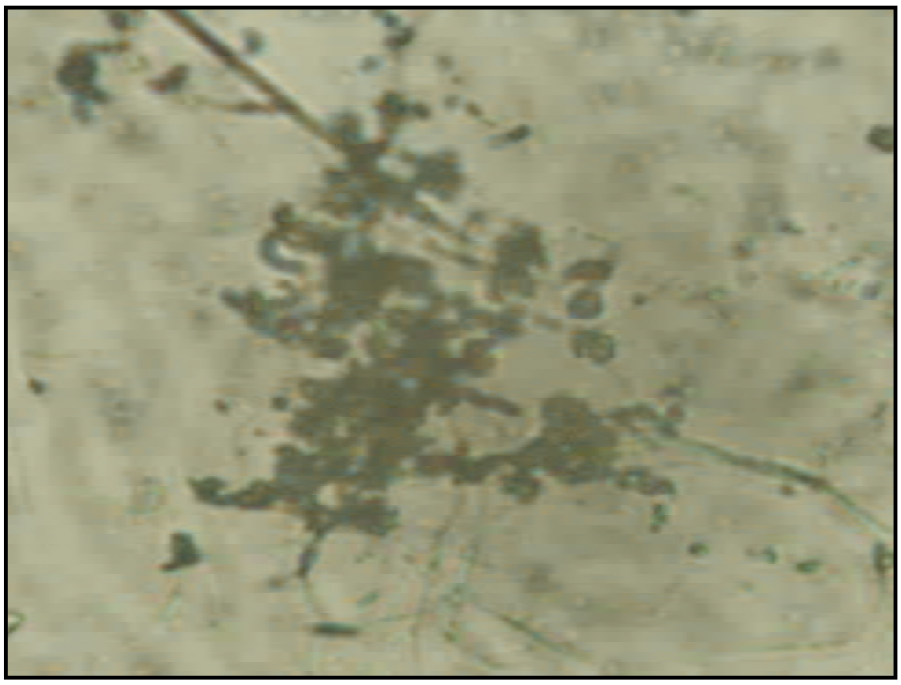

Fig. 1: Strain LA 29 - Spiral spore chain (400X)

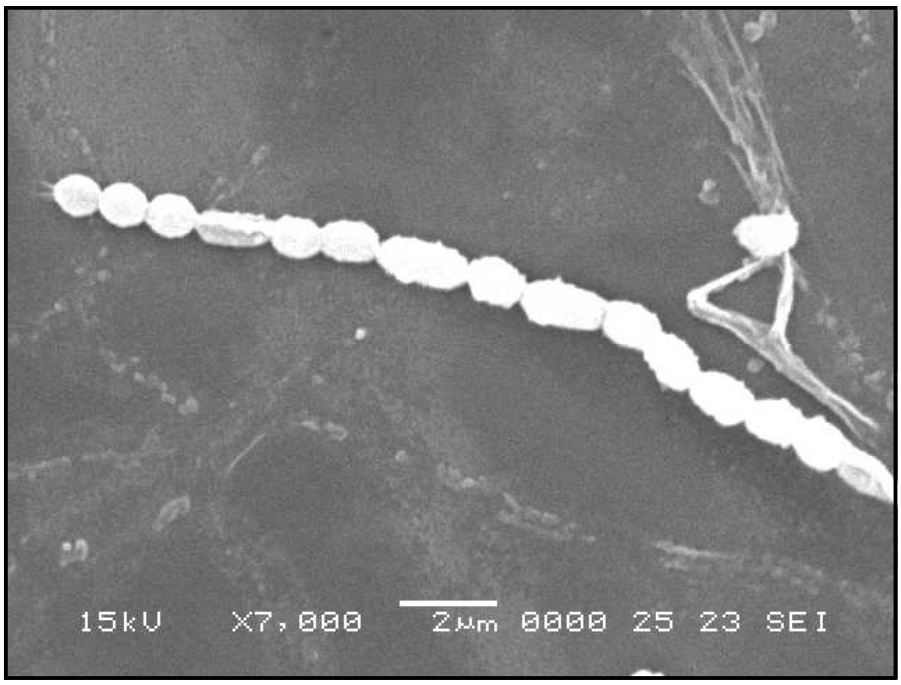

Fig. 2: Strain LA 29 - Spores with spiny surface (7,000X)

buffer were used for the enzyme assays. The crude extract was treated with protamine sulphate (1 mg protamine sulphate per 10 mg of protein) and centrifuged at 27,000 rpm for 10 min to remove the nucleic acids and then the supernatant was collected. The supernatant obtained in the above step was brought to 45% saturation by slowly mixing ammonium sulphate (pH 8.5) with gentle agitation and allowed to stand for 24 hr at 4°C in the cold room. The pH of supernatant was maintained at 8.5 by the addition of ammonium hydroxide. After the equilibration, the precipitate was removed by centrifugation (10,000 rpm at 4°C for 20 min) and the supernatant was once again brought to 95% saturation with ammonium sulphate and allowed to stand for 30 min at 4°C. This precipitate was again centrifuged at 10,000 rpm at 4° C for 20 min and the precipitate obtained was dissolved in 10 ml of 0.5M Tris-HCl buffer (pH 8.5) and then protein content was estimated (Lowry *et al.*, 1951). A 10 cm pretreated dialysis bag (manufactured by HIMEDIA Co.) was

Table - 1: L-asparaginase activity of the strain LA-29 during the partial purification process

Purification steps	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Fold purification	Yield (%)
Crude extract	446	951	2.13	-	100.00
Ammonium sulphate precipitation	115	795	6.91	2.90	33.89
DEAE-cellulose chromatography	35	317	9.05	9.70	8.50
Sephadex G-200 column chromatography	7	95	13.57	18.01	1.90

Table - 2: Anti-leukemic activity of L-asparaginase enzyme obtained from the strain LA-29

Dose	Number of injection	Days of survival of each rat	Average survival period (days)
Control	-	6/4/7/3/6	5.2
75 units	1	12/14/11/13/10	12.0
100 units	1	16/12/7/15/17	13.4
150 units	1	12/15/13/11/14	13.0
200 units	1	14/8/12/10/14	11.6
Intermittent units (50/25/25 units each after 24 hr)	3	18/16/20/17/15	17.2

Table - 3: Enzyme dose required to arrest leukemic in mice

Microorganism	Dose (units)	Reference
Mycobacterium tuberculosis	140	Connors and Jones (1973)
Escherichia coli	500	Roberts and Burk (1969)
Azotobactor vinelandii	200	Gaffar and Shethna (1975)
Vibrio sp	60	Selvakumar *et al*. (1991)
Strain LA-29	100	Present study

taken and activated by rinsing in doubled distilled water. One end of the dialysis bag was tightly tied and the precipitate recovered was taken inside the bag. The other end of the dialysis bag was tightly tied to prevent the leakage. After that, the dialysis bag was suspended in a beaker containing 0.5M Tris-HCl buffer (pH 8.5) for 24 hr and then it was transferred to 5% sucrose solution so that the excess water was removed and absorbed by sucrose solution. After the dialysis, the volume was measured and analyzed for proteins and stored in deep freezer.

The dialyzed enzyme solution obtained from the previous step was loaded into a DEAE-cellulose column (10 cm-GENEI), pre-equilibrated with 1mM Tris-HCl buffer (pH 8.5) and 1mM potassium chloride (pH 8.5). After loading the column, it was washed with the same buffer. The enzyme was eluted from the column using a linear gradient of 0.5M KCl and the fractions were collected using a microfractionator (Bio-Red). The collected fractions were then assayed for the proteins and enzymes. The fractions that showed higher activity in the previous steps were pooled and concentrated using sucrose solution. The concentrated fractions were loaded onto a pre-equilibrated (0.5M Tris-HCl buffer) Sephadex G-200 column (2.6 x 100 cm-GENEI). The enzyme was eluted with the same buffer and the fractions were collected. Fractions containing enzyme activity were pooled, concentrated and used in the study of anti-leukemic activity.

Anti-leukemic study: Male wistar rats were used for anti-leukemic study. Groups of five rats were taken for each experiment and all the experiments were terminated after three weeks of initiation of the experiment. The leukemia cells were maintained by serial intravenous transplantation of 30-40 million cells once in 5 to 6 days. The cell lines were supplied by the Chittaranjan Cancer Institute, Kolkata. The enzyme preparation obtained from Sephadex G-200 fraction was used for the present study. After 24 hr of cancer implantation (about 1-3 x 10^7 leukemia cells from the cancer bearing rats were injected intravenously), L-asparaginase enzyme was administered by the same route in a single dose and in 3 intermittent doses with an interval of 24 hr between each administration. Groups of five rats per experiment were observed daily and the days of survival recorded. Appropriate controls were maintained.

Taxonomic investigation: The genus level identification was made for the strain LA-29 using cell wall composition analysis and micromorphological studies (Lechevalier and Lechevalier, 1970). Characterization of the strain LA-29 was made by the following methods described by Shirling and Gottlieb (1966) using the

Table - 4: Comparison of phenotypic properties of the strain LA-29 with *Streptomyces canus*

Characters studied	Strain LA-29	*S. canus*
Aerial mycelium	Grey	Grey
Reverse side pigment	Pinkish in colour	Pinkish in colour
Spore chain	Spiral	Spiral
Spore surface	Spiny	Spiny
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Manitol	+	+
Fructose	+	+
Rhamnose	-	+
Sucrose	+	+
Raffinose	±	±
Utilization of sole nitrogen sources		
L-asparagine	+	+
L-hydroxyproline	+	-
L-histidine	-	-
L-phenylalanine	-	-
Biochemical properties		
Cellulose degradation	-	-
Hydrogen sulphide production	-	-
Melanin production	-	-
Gelatin liquefaction	+	-
Nitrate reduction	+	+
Starch hydrolysis	+	-
Milk coagulation	+	+
Growth in the presence of 0.1% phenol	-	-
Optimum temperature	37°C	35-38°C
Optimum pH range	7	7-8
NaCl requirement	0-2%	0 -2 %

standard yeast extract-malt extract agar (ISP medium 2). The species level identification of the strain was based on the keys of Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Results and Discussion

L-asparaginase activity of the strain LA-29 measured in each step of purification is given in Table 1. The crude extract gave 446 mg of protein with a total activity of 951 IU and a total yield of 100%. Specific activity and purification gradually increased in all the purification steps but total protein, total activity and yield decreased proportionally. In the final step, the enzyme showed the specific activity of 13.57 IU/mg protein with approximately 18-fold purity. The final recovery of protein was 1.9% though the specific activity of the enzyme in crude cell free extract was considerably less than that of *E. coli* L-asparaginase (3.59 IU/mg) (Roberts *et al.*, 1968).

Anti-leukemic activity of L-asparaginase enzyme obtained from the strain LA-29 is given in Table 2. All the rats which received 100 units of enzyme in intermittent doses, survived for an average period of 17.2 days. Average survival period of the rats was more in the enzyme dose of 100 units. Survival period became less when the dosage was increased and at the same time the enzyme became less effective when the dosage was decreased. Even the 200 units of the enzyme from the strain LA-29 did not protect the experimental rats upto 15 days when given in a single dose. However, the treated animals survived better than the control animals.

The data obtained from the present study shows that L-asparaginase of the strain LA-29 is effective in inhibiting the growth of leukemia cells in rats. All the rats survived for an average period of 17.2 days, when 100 units of the enzymes were given in three intermittent doses (50/25/25 units) each after 24 hr. This is in conformity with the successful regime of intermittent doses of several L-asparaginases on the anti-leukemic activity (Gaffar and Shethna, 1975; Selvakumar *et al.*, 1991; Asselin, 1999). The reduction in the survival period of the rats with large doses of the enzyme protein could be due to the toxicity of the enzyme preparation (Peterson and Giegler, 1969a; Gaffar and Shethna, 1975; Selvakumar *et al.*, 1991).

L-asparaginase from different sources was found to vary greatly with respect to their anti-leukemic properties. The L-asparaginase from *E. coli* (Mashburn and Wriston, 1967; Roberts and Burk, 1969), *Erwinia carotovora* (North *et al*., 1969), *A. vinelandii* (Gaffar and Shethna, 1975) and *Vibrio* sp. (Selvakumar *et al*., 1991) are known to suppress the leukemia cell growth. But L-asparaginase from other microbial sources *viz., Bacillus coagulous* (Law and Wriston, 1971) and *Saccharomyces cervisae* (Broome, 1965) was, however, not inhibiting the growth of leukemia cells.

Comparison of anti-leukemic property of L-asparaginase enzyme produced by the strain LA-29 was made with that of other microorganisms and it has revealed that the enzyme produced by *Vibrio* sp alone requires lesser dose (60 units) for the activity than the strain LA-29 which requires 100 units. Enzyme produced by the other microbes *viz. Mycobacterium tuberculosis*, *E. coli* and *A. vinelandii* require higher doses to arrest leukemia in mice (Table 3).

Thus the present study clearly indicates that the L-asparaginase produced by the strain LA-29 is highly active against the growth of leukemia cells when compared to that of *E. coli*, *M. tuberculosis* and *A. vinelandii.* It is also evident from the present study and other reports that higher enzyme concentrations have negative impact on the host organism as the L-asparaginase itself becomes toxic and kills the ordinary cells, thereby leading to early death. Hence, it is highly essential to find out the optimum concentration and also the effect of intermittent doses of the enzyme in controlling the growth of leukemia cells.

The strain LA-29 possesses LL-Diaminopimelic (LL-DAP) and the strain tested contains glycine in its cell wall. Presence of LL-DAP along with glycine indicates the cell wall chemotype – I. The strain with chemotype - I does not have characteristic pattern of sugars (Lechevalier and Lechevalier, 1970). The species belonging to the wall type I are *Streptomyces*, *Streptoverticillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elyptrosporangium*, *Microellobosporia*, *Sporichthya* and *Intrasporangium* (Lechevalier and Lechevalier, 1970). The micromorphological observations of the strain LA-29 reveal that it belongs to the genus *Streptomyces*.

The strain LA-29 was also studied extensively for its cultural, morphological, biochemical and physiological characteristics. The strain showed good growth in Kuster's Agar medium. The aerial mass and reverse side pigments were gray and pink in colour respectively. The spore chain was spiral and spiny (Figs. 1, 2). The strain LA-29 did not degrade the cellulose but it hydrolyzed the starch. It did not produce the melanin pigment and hydrogen sulphide. It liquefied the gelatin and reduced the nitrate. It coagulated the milk and its growth has not occurred in the presence of 0.1% phenol. The strain also strongly utilized the carbon sources *viz.,* arabinose, xylose, inositol, manitol, fructose, sucrose, glucose and did not utilize rhamnose and raffinose. It utilized the nitrogen sources *viz.*, L-asparagine and L-hydroxyproline and did not utilize L-histidine and L-phenylalanine.The strain LA-29 showed good growth in the presence of 0-2% of NaCl. It also showed good growth at 37°C and pH 7 and hence this temperature and pH can be considered as the optimum range for this strain. The phenotypic characters of the strain LA-29 were compared with that of the *Streptomyces* species (Table 4) found in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The strain failed to utilize rhamnose as carbon source while it used L-hydroxyproline as nitrogen source. It liquefied gelatin and hydrolyzed starch when compared to the species *Streptomyces canus*. Except these, all the other characters conform to that of the species *S. canus.* Hence, the strain has been tentatively identified as *S. canus.*

From the study, it is concluded that the L-asparaginase produced by *S. canus* (LA-29) is good in controlling the growth of leukemia cells when compared to L-asparaginase produced by other microbial species. However, the exact concentration, doses and the periodicity of administering the doses should be ascertained before using the L-asparaginase of the actinomycete for controlling the growth of leukemia cells in animals and human beings.

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References

Arico, M., V. Conter, M.G. Valsecchi, C. Rizzari, M.F. Boccaratte, E. Barls, C. Messine, G. De Rossi, L. Lo Nigro, A. Pession, F. Loocatelli, C. Micalizzi and G. Basso: Treatment reduction in highly selected standard risk childhood acute lymphoblastic leukaemia. *Haematologica*, **90**, 186-1191 (2005).

Asselin, B.: The three asparaginases comparative pharmacology and optimal use in childhood leukaemia. *Adv. Exp. Med. Biol.*, **457**, 621-629 (1999).

Broome, J. D.: Antilymphoma activity of L-asparaginase *in-vivo*. Clearance rates of enzymes preparation from guinea pig serum and yeast in relation to their effect on tumor growth. *J. Natt. Cancer Inst*., **35**, 967-974 (1965).

Buchanan, R.E. and N.E. Gibbons: Bergey's manual of Determinative Bacteriology (8th Edn.). The Williams and Wilkins Co., Baltimore. pp. 747-842 (1974).

Connors, T.A. and M. Jones: Recent results in cancer research. Springer-Verlag, Berlin, 33, 181-183 (1973).

Cory, J.G. and A.H. Cory: Critical roles of glutamine as nitrogen donors, in purine and pyrimidine nucleotide synthesis: Asparaginase treatment in childhood acute lymphoblastic leukaemia. *In Vivo,* **20**, 587-589 (2006).

Gaffar, S. A. and Y. I. Shethna: Partial purification and anti-tumor activity of L-asparaginase from *Azotobacter vinelandii*. *Curr. Sci.*, **40**, 725-729 (1975).

Haley, E. E.: The requirement for L-asparagine of mouse leukemia cells L5178Y in culture. *Cancer Res*., **21**, 532-541 (1961).

Law, A. S. and J. C. Wriston: Purification and properties of *Bacillus coagulaus* L-asparaginase. *Arch. Biochem. Biophys.,* **147**, 744-752 (1971).

Lechevalier, M. P. and H. Lechevalier: Chemical composition as a criterion

in the classification of aerobic actinomycetes. *Int. J. System. Bacteriol.*, **20**, 435-443 (1970).

Lowry, O., N. J. Rosenbrough, A. L. Farr and R. J. Randall: Measurement with the folin phenol reagent. *J. Biol. Chem.,* **193**, 265-267 (1951).

Mashburn, L. T. and J. C. Wriston: Tumor inhibitory effect of L-asparaginase from *E. coli. Arch. Biochem.,* **105**, 450-458 (1964).

Mashburn, L. T. and J. C. Wriston: Tumor inhibitory effect of L-asparaginase from *E. coli. Arch. Biochem. Biophys.,* **105**, 451-452 (1967).

Nonomura, H.: Key for classification and identification of 458 species of the *Streptomycetes* included in ISP. *J. Ferment. Technol.*, **52**, 78-92 (1974).

North, A.C.T., H.E. Wade and K.A. Cammack: Physico-chemical studies of L-asparaginase from *Erwinia cerotovora. Nature,* **224**, 594-595 (1969).

Peterson, R. E. and A. Giegler: L-asparaginase production by *Erwinia aroideae. Appl. Microbiol.,* **18**, 64-67 (1969a).

Roberts, J. and B. Burk: L-asparaginase synthesis by *E. coli. Biotechnol. Bioeng.,* **11**, 1211-1225 (1969).

Roberts, J., G. Burson and J.M. Hill: New procedure for the purification of L-asparaginase with high yield from *E. coli. J. Bacteriol.*, **95**, 2117-2123 (1968).

Sahu, M.K., K. Sivakumar, E. Poorani, T. Thangaradjou and L. Kannan: Studies on L-asparaginase enzyme of actinomycetes isolated from estuarine fishes. *J. Environ. Biol.*, **28**, 465-474 (2007).

Savitri, K., N. Asthana and W. Azmi: Microbial L-asparaginase: A potent antitumor enzyme. *Indian J. Biotechnol.*, **2**, 184-194 (2003).

Schemer, G. and J. S. Holcenberg: Enzymes as drugs (*Eds.*: J. S. Holcenberg and J. Roberts). Wiley Inter Science, New York. pp. 455-473 (1981).

Selvakumar, N., V. Kumar and R. Natarajan: Partial purification, characterization and anti-tumor properties of L-asparaginase (antileukemic agent) from a marine *Vibrio. In*: Bioactive compounds from marine organisms with emphasis on the Indian ocean (*Ed.*: Mary-Frances Thompson). Rachakonda Sarojini and Bombay and Calcutta. pp. 289-300 (1991).

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

Sudha, K., Nirmala Thampuran and P. K. Surendran: Prevalence of *Vibrio* species in fish from pelagic and demersal habitats. *Fish. Tech.*, **39**, 150-154 (2002).

Tozuka, M., K. Yamauchi, H. Hidaka, T. Nakabayashi, N. Okumura and T. Katasujamca: Characterization of hypertriglyceridemia induced by L-asparaginase therapy for acute lymphoblastic leukaemia and malignant lymphoma. *Ann. Clin. Lab. Sci.*, **27**, 351-357 (1997).

Ucar, C. and U. Carliskan: Successful treatment of acute lymphoblastic leukaemia with L-asparaginase induced intracranial haemorrhage to activated recombinant factor VIIa in a child. *Pediatr. Hematol. Oncol.,* **23**, 339-345 (2006).

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