Study of L-asparaginase production by *Streptomyces noursei* MTCC 10469, isolated from marine sponge *Callyspongia diffusa*

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Abstract
L-asparaginase is an anti-neoplastic agent used in the chemotherapy of lymphoblastic leukaemia. The present work deals with production of extra-cellular L-asparaginase from marine actinomycetes, using submerged fermentation. Marine actinomycete *Streptomyces* associated with marine sponge *Callyspongia diffusa* was isolated using specific ISP medium. Sponge-associated *Streptomyces* was characterized by conventional methods, and identified as *Streptomyces noursei* MTCC 10469. Production of L-asparaginase by submerged fermentation was carried out using medium Tryptone Glucose Yeast extract (TGY) broth. The enzyme was purified to near homogeneity by ammonium sulphate precipitation, dialysis, gel filtration on Sephadex G-100 column, CM Sephadex C-50 and SDS-PAGE. The enzyme was purified at 98.23 folds, and showed a final specific activity of 78.88 IU/mg, with 2.14% yield. SDS-PAGE of the purified enzyme revealed an apparent molecular weight of 102 kDa for it. The optimum pH, temperature and incubation time of L-asparaginase was found to be 8, 50ºC and 35 min, respectively. The study suggests that marine actinomycetes, particularly *Streptomyces*, may be used as a potential source of L-asparaginase.
***Keywords*:** Marine Actinomycetes; *Streptomyces*; L-asparaginase

INTRODUCTION

Marine microbes represent a potential source for commercially important bioactive compounds. Among marine microorganisms, actinobacteria have gained special attention as the most potent source of antibiotics and other bioactive secondary metabolites (Newman *et al*., 2000). Actinobacteria represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria, including five subclasses and 14 suborders (Stackebrandt, 2000). Among the five subclasses, actinobacteria (bacteria belonging to the order Actinomycetales; commonly called actinomycetes) account for approximately 7,000 of the metabolites reported in the Dictionary of Natural Products. Marine actinomycetes are a prolific source of secondary metabolites and the vast majority of these compounds are derived from the single genus *Streptomyces* (Das *et al*., 2006). *Streptomyces* species are distributed widely in marine and terrestrial habitats (Pathom-aree *et al*., 2006) and are of commercial interest due to their unique capacity to produce novel metabolites. While most of the studies on marine *Streptomyces* have focused on antibiotic production, only few reports have dwelt on their enzymatic potential.

Production of various enzymes, such as protease, lipase, chitinase, and alginate lyases has been reported from marine *Streptomyces* (Dharmaraj, 2010). *Streptomyces* also serve as a good source of L-asparaginase (Hi-Media Laboratories, India), an enzyme which converts L-asparagine to L-aspartic acid and ammonia, and has been used as a chemotherapeutic agent (Fisher and Wray, 2002). L-asparaginase has received increased attention in recent years for its anti-carcinogenic potential (Manna *et al*., 1995). L-asparaginase is an anti-neoplastic agent used in the chemotherapy of lymphoblastic leukaemia (Keating *et*

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al., 1993). The clinical action of this enzyme is attributed to reduction of L-asparagine, so that tumor cells unable to synthesize this amino acid are selectively killed by L-asparagine deprivation. Several terrestrial *Streptomyces* like *S. karnatakensis*, *S. venezualae*, *S. longsporusflavus*, and *S. albidoflavus* are capable of producing detectable amounts of L-asparaginase (Narayana *et al*., 2008). There are limited reports on production of L-asparaginase from marine *Streptomyces* like *S. aurantiacus* (Gupta *et al*., 2007), *Streptomyces* sp. PDK2 and PDK7 (Dhevagi and Poorani, 2006), and *Streptomyces* sp. S3, S4 and K8 (Basha *et al*., 2009). The enzyme is produced throughout the world by both submerged and solid-state cultures. Extra-cellular asparaginases are more advantageous than intracellular ones, since they can be produced abundantly in the culture broth under normal conditions, and purified economically. Considering the above facts, an attempt was made for the first time on the production, purification and characterization of an extra-cellular L-asparaginase, under submerged fermentation from sponge-associated *Streptomyces noursei* MTCC 10469.

MATERIALS AND METHODS

Isolation and characterisation of *Streptomyces*: In present study, marine actinomycete *Streptomyces* was isolated from marine sponge *Callyspongia diffusa*, as described by Ridley (1884), collected at a depth of 5-10 m off Kovalam coast of Kerala, India. The sponge extract was obtained by squeezing the sponges gently with a glass stick. Aliquots (1 ml) of each sponge extract were diluted with sterilized seawater. A quantity of one millilitre of the dilutions were mixed with 20 ml of sterile glycerol-asparagine (ISP-5) agar medium, and incubated at room temperature (28 ± 2 °C) for seven days. Rifampicin (2.5 μg/ml) and amphotericin B (75 μg/ml) were added to ISP-5 medium to inhibit bacterial and fungal contamination, respectively. The isolated culture was maintained as slant culture at 28±2ºC (Dharmaraj and Sumantha, 2009; Dharmaraj *et al*., 2009a, Dharmaraj and Dhevendaran, 2010). The strain was characterised by acid-fast staining and Gram staining techniques. The isolate was also studied by employing various parameters detailed below.

Pigmentation of mycelia and spore morphology: The culture was grown on a Petri dish containing casein-starch-peptone-yeast extract (CSPY) agar medium, with a cover slip inserted at an angle of 45º. The cover slip was removed after seven days of incubation, air dried and observed under the scanning electron microscope (Locci, 1989).

Utilisation of carbon sources: Various carbon sources, namely glucose, xylose, arabinose, rhamnose, fructose, galactose, raffinose, salicin, mannitol, inositol, and sucrose were added at a concentration of 1% each, to 10 ml of basal mineral salt medium, and incubated at 28ºC for seven days.

Sodium chloride tolerance:. Sodium chloride at varying concentrations (1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, and 10 percent) were added to 5 ml of the basal medium, and incubated at 28 °C for seven days. The biomass thus obtained was separated from the broth, dried and weighed. The weight of the biomass was expressed in grams.

Physiological and biochemical characteristics: These were studied according to procedures described by Buchanan and Gibbons (1974).

Screening of L-asparaginase production by rapid plate assay: The isolate was screened for L-asparaginase production using a method in which modified M9 medium (composition for 1 l: 6.0 g $Na_2HPO_4 \cdot 2H_2O$; 3.0 g KH_2PO_4; 0.5 g NaCl; 5.0 g L-asparagine; 0.5 g $MgSO_4.7H_2O$; 0.014 g $CaCl_2.2H_2O$; 2.0% w/v glucose; and 20.0 g agar) incorporated with a pH indicator (phenol red) was used (Gulati *et al*., 1997). L-asparaginase activity was identified by formation of pink zone around colonies.

Crude enzyme production by submerged fermentation: 100 ml of Tryptone Glucose Yeast extract (TGY) broth (production media, pH 7.0), comprising of 0.1 g glucose, 0.1 g K_2HPO_4, 0.5 g yeast extract, 0.5 g tryptone, and water to 100 ml, contained in a 250 ml Erlenmeyer flask, was inoculated separately with the isolate, and incubated at 28ºC in a shaker-incubator oscillating at 200 rpm for 24 h. At the end of the fermentation period, the crude enzyme was prepared by centrifugation at 10,000 rpm for 20 min. The cell-free supernatant was taken as the crude enzyme (Peterson and Ciegler, 1969). All the experiments were performed independently in triplicates.

Assay of L-asparaginase: To determine the enzyme

activity, 5 ml of the culture broth was withdrawn aseptically from the flasks, at an interval of 24 h. The broth was filtered using Whatman filter paper No.1, and then centrifuged at 9,000 g for 8 min (Ding *et al*., 2003). The supernatant thus obtained was used as crude extract for L-asparaginase assay (Imada *et al*., 1973). The enzyme activity was expressed in International Unit (IU); one IU being the amount of enzyme which liberates 1 μM of ammonia per ml per min (μM/ml/min).

Purification of L-asparaginase: The purification was carried out using crude enzyme extract (Distasio *et al*., 1982). The enzyme was purified by the following steps at 4ºC, unless otherwise mentioned. Finely powdered ammonium sulfate was added to the crude extract. The L-asparaginase activity was associated with the fraction precipitated at 80% saturation. The precipitate was collected by centrifugation at 9,000 g for 15 min, dissolved in 1 M Tris-HCl buffer and dialyzed against the same buffer. The dialyzed fraction was applied to a Sephadex G-100 column (100 × 1.5 cm), that was pre-equilibrated with 0.05M Tris-HCl buffer (pH 8.4). The protein elution was done with the 0.05 M Tris-HCl buffer (pH 8.4), containing 1M KCl at a flow rate of 5 ml per 30 min. The active fractions were pooled, dialyzed and concentrated. The concentrated enzyme solution was applied to the column of CM Sephadex C-50 that was pre-equilibrated with 50mM Tris-HCl buffer (pH 8.4). It was eluted with 50mM Tris-HCl buffer (pH 8.4), containing 0.1M KCl at a flow rate of 5 ml per 30 min. The active fractions at each purification steps were collected, dialyzed and concentrated, and L-asparaginase was assayed by the direct Nesslerization method (Basha *et al*., 2009). Protein estimation was done with Folin-Phenol reagent using BSA (Hi-Media Laboratories, India) as a standard (Lowry *et al*., 1951). SDS-PAGE was performed according to the method of Laemmli (1970), with a 10% separating gel and 5% stacking gel containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250, and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5.

Enzyme characterization

Effect of pH, temperature and incubation time: Activity of L-asparaginase was evaluated at different levels of pH, temperature and incubation time. Purified enzyme was incubated with 0.05 M buffers of pH 4-10 under assay conditions, and the amount of ammonia liberated was determined. The buffers used were potassium phosphate (pH 4.0-7.0), Tris-HCl (pH 8.0-9.0), and glycine-NaOH (pH 10). Pre-incubation was carried out for 60 min, and then the residual activity was measured. Optimum temperature of activity for the enzyme was determined by incubating the assay mixture at temperatures ranging from 20 to 100ºC. Thermostability studies were carried out by pre-incubating the enzyme at different temperatures for 60 min. The effect of the incubation time on L-asparaginase activity was studied in the range of 5 to 45 min (El-Bessoumy *et al*., 2004). The enzyme characterization experiments were performed in triplicates.

RESULTS

Isolation and characterisation of *Streptomyces*: In the present study, sponge-associated *Streptomyces* was isolated and characterized. The isolated strain was slowly growing, chalky, folded and aerobic. Mycelial colour pattern of the strain was of white series. The

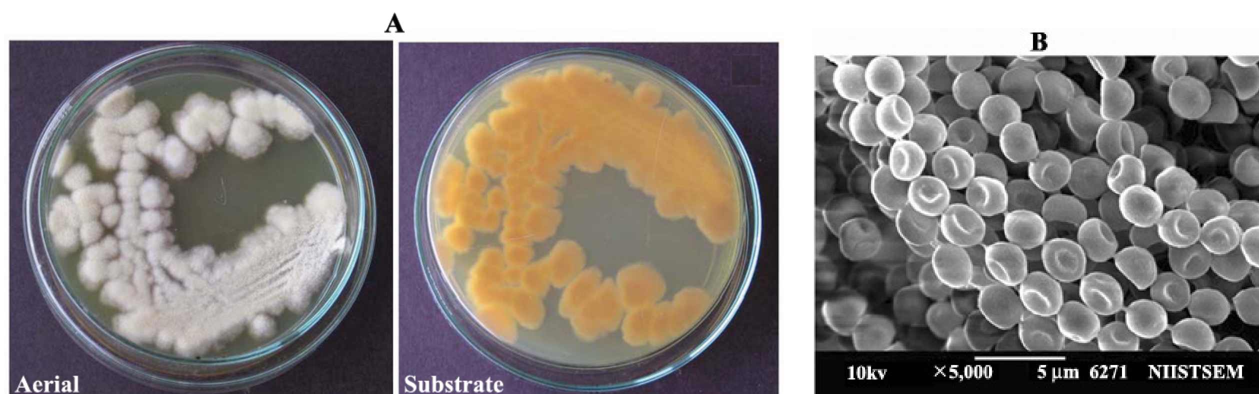

Figure 1. A: The mycelial colouration and B: scanning electron micrograph of *Streptomyces noursei* MTCC 10469.

aerial mycelium was white, and the vegetative mycelia bore orange coloration in ISP-5 media (Fig. 1A). The strain was acid-fast negative, and was found to be Gram-positive. The scanning electron microscope results showed the spore morphology as having a smooth surface and rectiflexibiles (RF) hyphae (Fig. 1B). The physiological results state that the strain was able to grow in the range of 25 to 37ºC, and at pH 8 to 10. Nutritional characteristics of the strain were studied, using criteria like carbon utilization and sodium chloride tolerance. The strain grew well in media containing glucose, galactose, mannitol, and fructose; but it did not assimilate arabinose, raffinose, salicin, xylose, sucrose, rhamnose, and inositol. The strain showed effective growth up to 6% concentration of sodium chloride, and above 6% the growth diminished. Biochemical characterisation showed that the strain was able to hydrolyze starch and casein, but could not produce hydrogen sulphide. The strain was unable to liquefy gelatin, and showed negative citrate utilization. The microorganism exhibited negative urease and catalase activity.

Screening, crude enzyme production and assay of L-asparaginase: Preliminarily screening of L-asparaginase production by strain showed positive result in rapid plate assay. Upon submerged fermentation, the strain exhibited maximal enzyme production at 24 h. The strain produced crude L-asparaginase of 3.310 µM/mg/ml/h ammonia, having specific activity of 0.803 IU/mg in the production medium.

Purification of L-asparaginase: Purification of L-asparaginase was carried out in four steps, as shown in Table 1. Partial purification of L-asparaginase crude extract affected by ammonium sulfate (80%) precipitation showed that most of the enzyme activity was preserved in the precipitate. Total protein decreased from 412 to 224 mg, and specific activity increased from 0.803 to 0.933 IU/mg, at approximately 1.16 folds purity in the ammonium sulfate precipitation step. Further purification of the enzyme by Sephadex G-100 resulted in specific activity of 25.21IU/mg, with approximately 31.4 folds purity and yield of 9.06 percent. The final purification of L-asparaginase was achieved by CM Sephadex C-50 coloumn chromatography, which resulted in specific activity of 78.88 IU/mg, approximately 98.23 folds purity, and yield of 2.14 percent. SDS-PAGE analysis of the purified enzyme showed the apparent molecular weight 102 kDa for L-asparaginase (Fig. 2).

Enzyme characterization:

Effect of pH, temperature and incubation time: The purified enzyme was characterised in terms of optimum pH, temperature, and incubation time, as shown in Figure 3. The activity gradually increased up to pH 8, at which time the maximum activity was observed. At higher pH, the enzyme activity decreased. The maximum enzyme activity was obtained at 50ºC. At higher temperatures, the reaction rate declined sharply. Also in the range of 5 to 45 min incubation time, activity increased with the incubation time. The activity ran at maximum for 35 min and decreased as the time increased.

DISCUSSION

Streptomyces associated with marine sponge *Callyspongia diffusa* was preliminarily characterized morphologically. The strain showed typical morphology of *Streptomycetes* when analyzing shape and spore chains under scanning electron microscope as described earlier by Locci (1989). The nutritional, physiological and biochemical characterization suggest that the strain be classified under *Streptomyces* genus, as reported previously (Dharmaraj and Dhevendaran, 2010; Dharmaraj *et al*., 2009b; Dharmaraj and Sumantha, 2009). The strain was further confirmed and identified as *Streptomyces noursei* MTCC 10469 by Microbial Type Culture Collection and Gene Bank, Institute of Microbial

Table 1. Purification profile of L-asparaginase from *Streptomyces noursei* MTCC 10469.

Purification steps	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification folds	Yield (%)
Crude Extract	331.00	412.00	0.803	1.00	100.00
Ammonium sulfate precipitation	209.00	224.00	0.933	1.16	63.14
Sephadex G-100 filteration	30.00	1.19	25.21	31.39	9.06
CM Sephadex C-50 Chromotography	7.10	0.09	78.88	98.23	2.14

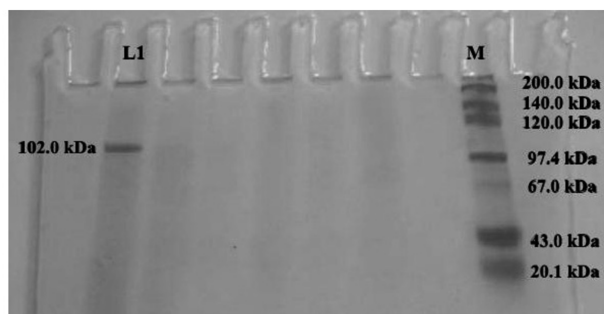

Figure 2. Electrophoretic separation of purified L-asparaginase (L1) and marker (M).

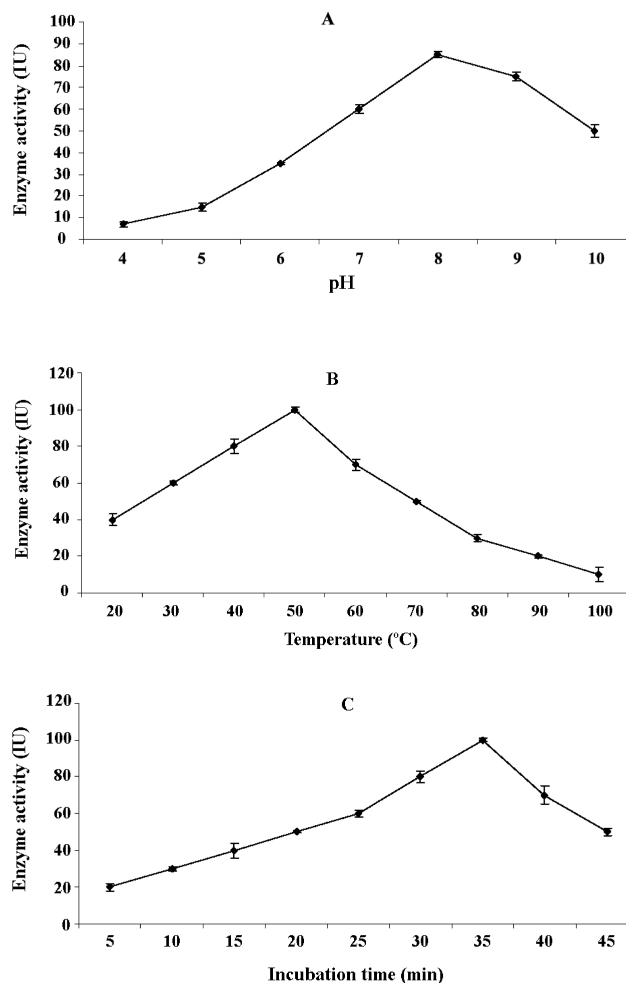

Figure 3. A: Effect of pH, B: Temperature and C: Incubation time on the activity of L-asparaginase.

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Streptomyces noursei MTCC 10469 was screened for L-asparaginase production by plate assay, as well as by submerged fermentation. Similar screening of L-asparaginase production by rapid plate assay were reported in bacterial strains such as *Bacillus circulans* (Prakasham *et al*., 2010), *Streptomyces* sp. PDK7 (Dhevagi and Poorani, 2006), and *Streptomyces* sp. (Basha *et al*., 2009).

Production of L-asparaginase by submerged fermentation yielded crude enzyme with total activity of 331 IU and total protein of 412 mg in the production medium. Crude L-asparaginase production from marine *Streptomyces* sp. PDK7 with total activity of 374.6 IU and total protein of 489.5 mg has been reported by Dhevagi and Poorani (2006). Lower production of crude L-asparaginase from marine *Streptomyces* sp. (S3, S4 and K8) with total activity of 61.53 IU and total protein of 0.16 mg was also reported by Basha *et al*. (2009). Varying amounts of L-asparginase were reported in many genera of marine microorganisms as well (Benny and Kurup, 1991). There are some reports on the production of L-asparaginase from marine sediments (Balakrish Nair *et al*., 1977), as well as estuarine fish's gut (Dhevendaran and Anitha Kumari, 2002; Koshey *et al*., 1997).

Further purification of L-asparaginase enzyme was achieved by ammonium sulfate precipitation, Sephadex G-100 filtration and CM Sephadex C-50 coloumn chromatography. In the final purification step, about 98.23-fold purity for L-asparaginase was obtained. In *Streptomyces albidoflavus*, L-asparaginase enzyme has been purified in CM Sephadex C-50 column up to 99.3-fold (Narayana *et al*., 2008). In another report, 85-fold purified L-asparaginase was obtained from *Streptomyces* sp. PDK2 by the final Sephadex G-200 gel filtration (Dhevagi and Poorani, 2006). Recently, L-asparaginase purity of about 82.12 folds was reported in *S. gulbargensis* (Amena *et al*., 2010).

The final purified enzyme was examined using SDSPAGE, which revealed one protein band with molecular weight of 102 kDa. Purified L-asparaginase from *Streptomyces* sp. PDK2, *S. albidoflavus*, and *S. gulbargensis* exhibited molecular weights of 140, 112 and 85 kDa, respectively (Dharmaraj and Dhevendaran, 2010; Dharmaraj *et al*., 2009b; Dharmaraj and Sumantha, 2009). Other strains, such as *V. succinogens* (Distasio *et al*., 1982) and *Chalmydomonas* sp. (Paul, 1982) exhibited molecular weights of 146 and 275 kDa for the final purified L-asparaginase.

Maximum activity of the purified L-asparaginase occurred when it was incubated with an optimum substrate concentration at pH 8. Similar pH value was

obtained for *Streptomyces* sp. PDK2 and *Streptomyces* sp. (S3, S4 and K8) (Basha *et al*., 2010; Dhevagi and Poorani, 2006). A temperature profile showed that the enzyme had a maximum activity at 50ºC. Similar results were recorded for asparaginases from *E. carotovora* (Maladkar *et al*., 1993), *Pseudomonas stutzeri* MB- 405 (Manna *et al*., 1995) and *Staphylococcus* (Sobis and Mikucki, 1991). Incubation of L-asparaginase for different lengths of time showed that the activity reached its maximum at 35 min. L-asparaginase from *Pseudomonas aeruginosa* 50071 showed its maximal activity at 30 min of incubation time (El-Bessoumy *et al*., 2004).

The present study revealed an efficient production of L-asparaginase throughout various purification steps from isolate *Streptomyces noursei* MTCC 10469 under submerged fermentation. There have been many reports on the production of various antibiotics from *Streptomyces noursei* so far, and to the best of our knowledge, this is the first report on the production and purification of L-asparaginase by sponge-associated *Streptomyces noursei* MTCC 10469. Furthermore, high catalytic activity of the enzyme over a wide range of pH and temperature, and its considerable stability, makes it highly favorable for use as a potent anticancer agent, and for other applications in healthcare industry.

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References

Amena S, Vishalakshi N, Prabhakar M, Dayanand A, Lingappa K (2010). Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*. *Brazil J Microbiol*. 41: 173-178.

Balakrish Nair G, Selvakumar N, Chandramohan D, Natarajan R (1977). Distribution and activity of L-asparaginase producing fungi in the marine environment of Porto Novo. *Ind J Mar Sci*. 6: 172-173.

Basha SN, Rekha R, Komala1 M, Ruby S (2009). Production of extracellular anti-leukaemic enzyme L-asparaginase from marine actinomycetes by solidstate and submerged Fermentation: Purification and characterization. *Tropical J Pharmaceutical Res*. 8: 353-360.

Benny PJ, Kurup M (1991). L-asparaginase activity in bacteria from marine sediments and mollusk. *Indian J Mar Sci*. 20: 36-39.

Buchanan RE, Gibbons NE (1974). *Bergey's Manual of Determinative Bacteriology*, 7th Edn., Bergey. Taxon, London. 24: 377-378.

Das S, Lyla PS, Ajmal Khan S (2006). Marine microbial diversity and ecology: importance and future perspectives. *Curr Sci*. 25: 1325-1335.

Dharmaraj S, Ashokkumar B, Dhevendaran K (2009a). Food-grade pigments from *Streptomyces* sp. isolated from the marine sponge *Callyspongia diffusa*. *Food Res Int*. 42: 487-492.

Dharmaraj S, Sumantha A (2009). Bioactive potential of *Streptomyces* isolated from marine sponges. *World J Microbiol Biotechnol*. 25: 1971-1979.

Dharmaraj S, Ashokkumar B, Dhevendaran K (2009b). Fermentative production of pigments from marine actinomycetes. *Iran J Microbiol*. 1: 36-41.

Dharmaraj S (2010). Marine *Streptomyces* as a novel source of bioactive substances. *World J Microbiol Biotechnol*. 26: 2123-2139.

Dharmaraj S, Dhevendaran K (2010). Evaluation of Streptomyces as a probiotic feed for the growth of the ornamental fish Xiphophorus helleri. *Food Tech Biotechnol*. 48: 497-504.

Dhevagi P, Poorani E (2006). Isolation and characterization of L-Asparaginase from marine actinomycetes. *Ind J Biotech*. 5: 514-520.

Dhevendaran K, Anitha Kumari YK (2002). L-asparaginase activity in growing conditions of *Streptomyces* sp. associated with *Therapon jarbua* and *Villorita* cyprinoids of Veli lake, South India. *Ind J Mar Sci*. 39: 155-159.

Ding CH, Diang ZQ, Li XT, Li LQ, Kusakabe I (2003). High activity xylanase production by *Streptomyces olivaceoviridis* E-86. *World J Microbiol Biotechnol*. 20: 7-10.

Distasio JA, Salazar AM, Nadji M, Durden DL (1982). Glutaminase-free asparaginase from *Vibrio succinogenes*: an antilymphoma enzyme lacking hepatotoxicity. *Int J Cancer*. 30: 343-347.

El-Bessoumy AA, Sarhan M, Mansour J (2004). Production, isolation, and purification of L-Asparaginase from *Pseudomonas aeruginosa* 50071 using solid-state fermentation. *J Biochem Mol Biol*. 37: 387-393.

Fisher SH, Wray Jr LV (2002). *Bacillus subtilis* 168 contains two differentially regulated genes encoding L-asparaginase. *J Bacteriol*. 184: 2148-2154.

Gulati R, Saxena RK, Gupta R (1997). A rapid plate assay for screening L-asparaginase producing microorganisms. *Lett Appl Microbiol*. 24: 23-26.

Gupta N, Mishra S, Basak UC (2007). Occurrence of *Streptomyces aurantiacus* in mangroves of Bhitarkanika. *Malaysian J Microbiol*. 3: 7-14.

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

Keating MJ, Holmes R, Lerner SH (1993). L-asparaginase and PEG asparaginase past, present and future. *Leuk. Lympmoma*. 10: 153-157.

Koshey A, Dhevendaran K, Georgekutty MI, Natarajan P (1997). L-asparaginase in *Streptomyces plicatus* isolated from the alimentary canal of the fish, *Gerres filmentosus* (Cuvier). *J Mar Biotechnol*. 5: 181-185.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London). 227: 680-685.

Locci R (1989). Streptomycetes and Related Genera. In: *Bergey's Manual of Syatematic Bacteriology*, Williams ST, Sharpe ME and Holt JG (Eds.), Williams and Wilkins, Baltimore. PP. 2451-2493.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951). Protein measurement by Folin phenol reagent. *J Biol Chem*. 193: 266-275.

Maladkar NK, Singh VK, Naik SR (1993). Fermentative production and isolation of L-asparaginase from *Erwinia cartovora* EC-113. *Hindustan Antibiot Bull*. 35: 77-86.

Manna S, Sinha A, Sadhukan R, Chakrabarty SL (1995). Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri* MB-405. *Curr Microbiol*. 30: 291-298.

Narayana KJP, Kumar KG, Vijayalakshmi M (2008). L-asparaginase production by *Streptomyces albidoflavus*. *Ind J Microbiol*. 48: 331-336.

Newman DJ, Cragg GM, Snader KM (2000). The influence of natural products upon drugs discovery. *Nat Prod Rep*. 17: 215-234.

Pathom-aree W, Stach JEM, Ward AC, Horikoshi K, Bull AT, Goodfellow M (2006). Diversity of actinomycetes isolated from Challenger Deep sediment (10, 898 m) from the Mariana Trench. *Extremophiles*. 10: 181-189.

Paul JH (1982). Isolation and characterization of a *Chlamydomonas* L-asparaginase. *Biochem J*. 203: 109-115.

Peterson RE, Ciegler A (1969). L-asparaginase production by *Erwinia aroideae*. *J Appl Microbiol*. 18: 64-67.

Prakasham RS, Hymavathi M, Subba Rao Ch, Arepalli SK, Venkateswara Rao J, Kavin Kennady P, Nasaruddin K, Vijayakumar JB, Sarma PN (2010). Evaluation of antineoplastic activity of extracellular asparaginase produced by isolated *Bacillus circulans*. *Appl Biochem Biotechnol*. 160: 72-80.

Ridley SO (1884). *Spongiida*. Report on the zoological collection made in the Indo-Pacific Ocean during the Voyage of H.M.S. 'Alert' 1881-2. 366-482, 582-635. British Museum (Natural History), London.

Sobis M, Mikucki J (1991). *Staphylococcal* L-asparaginase: Enzyme kinetics. *Acta Microbiol Pol*. 40: 143-152.

Stackebrandt SP (2000). *The prokaryotes: an evolving electronic resource for the microbiological community*. Springer, New York.