academicJournals

Vol. 7(31), pp. 4015-4021, 2 August, 2013
DOI: 10.5897/AJMR12.2184
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Efficient production of l-asparaginase by marine *Streptomyces* sp. isolated from Bay of Bengal, India

Palaniappan Sivasankar*, Shanmugam Sugesh, Packiyaraj Vijayanand, Kannan Sivakumar, Shanmugam Vijayalakshmi, Thangavel Balasubramanian and Packium Mayavu

Faculty of Marine Sciences, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai - 608 502, Tamil Nadu, India.

Accepted 28 July, 2013

Marine actinobacteria isolated from Bay of Bengal and evaluated for the l-asparaginase production capability. A total of 75 actinobacterial strains were isolated and screened for antibacterial activity. Among them, 10 actinobacterial strains showed significant antibacterial activity. Those strains were identified based on phenotypic and chemotaxonomic characteristics and screened for l-asparaginase production. During screening process, *Streptomyces* sp. (SS7) showed large pink colored zone (10 mm) around the colony. The strain was further studied for production and characterization of l-asparaginase enzyme. The preferable culture conditions for l-asparaginase were pH 8.5, temperature 40°C, maltose (22 IU) and soya bean meal (25.7 IU) as best carbon and nitrogen sources, respectively. Different substrates were used to optimize the l-asparaginase production. Among them, mangrove leaf extract-I showed maximum yield of 14.5 IU, the enzyme showed 18 fold during sephadex G-100 column purification. These results show that the great potential of *Streptomyces* sp. (SS7) for the production of l-asparaginase from cheaper source could lead to economic feasibility. The l-asparaginase from *Streptomyces* sp. (SS7) may be a new active source for natural anticancer agent with potential significance for therapeutic applications.

Key words: Bay of Bengal, marine actinobacteria, asparaginase.

INTRODUCTION

Pharmaceutical industries are in need for new class of antibiotics from cheaper and safer sources due to alarming occurrence of multiple-drug resistant pathogens. Recently, marine ecosystems are recognized as potent source for novel bioactive compounds (Fenical, 1997; Porkish et al., 2002). Marine microorganisms are taxonomically diverse, genetically unique and largely unexplored. Bioactive compounds from marine microorganisms have typical features because of its varied environmental conditions (pH, temperature, salinity, pressure, etc). Among marine microorganisms, actionbacteria are recognized as most promising prokaryote for novel bioactive metabolite production. More than 50% of the reported bioactive metabolites are discovered from actinobacteria (Schumacher et al., 2003; Charan et al., 2004; Mitchell et al., 2004; Bredholt et al., 2008; Basavaraj et al., 2010; Radhakrishnan et al., 2011). In particular, genus *Streptomyces* contributes more than 85% of the total antibiotic production. Streptomycetes are distributed in both marine and terrestrial environments. It has unique features to produce novel bioactive metabolites such as antibiotics (Maskey et al., 2003; Kwon et al., 2006), enzymes (Amena et al., 2010), antitumor compounds (Mitchell et al., 2004; Stritzke et al., 2004),

*Corresponding author. E-mail: Sivasankar.ps@gmail.com, Cmrdtn@gmail.com. Tel: +919944376973, +91 4144-243223-Ext-235. Fax: +91 4144-243223.

immunosuppressive agents (Mann, 2001). The identification of actinobacteria is always a tricky task for the taxonomist, because bacteriologist considered as bacteria and mycologist considered as fungi, in recent era it is accepted as bacteria and they are closely related to true bacteria as shown by their size, chemical composition and biochemical activities (Waksman, 1959).

Marine actinobacteria have diverse range of enzyme activities and are capable of catalyzing various biochemical reactions. Recently, it was proved that marine actinobacteria are the good source for production of l-asparaginase (l-asparagine aminohydrolase EC 3.5.1.1), which converts l-asparagine to l-aspartic acid. It is an effective anti-neoplastic agent, used in the treatment of acute lymphoblastic leukemia (Narta et al., 2007). Several types of microorganisms were producing l-asparaginase with novel chemical properties. The enzyme asparaginase from *Escherichia coli* and *Erwinia chrysanthemi* are currently in clinical trials (Dhevagi and Poorani, 2006). The bacterial source l-asparaginase showed harmful side effects (Duval et al., 2002). So we need safer enzyme from cheaper sources. Recently, *Steptomyeces noursei* MTCC 10469 isolated from sponge proven to be the best source for the production of l-asparaginase (Dharmaraj, 2011). Hence, the present study is aimed to find out a potent l-asparaginase producing actinobacteria from sediment samples of Bay of Bengal, India.

MATERIALS AND METHODS

Sample collection and pretreatment

The marine sediment sample was collected from Bay of Bengal, India (11° 42' 23.15" N; 79° 46' 57.97" E) using Peterson-grab and transferred to a sterile polythene bag and brought to the laboratory for analysis. 1 g of sediment was transferred to a conical flask containing 100 ml of sterile starch casein broth (Shirling and Gottlieb, 1966) for the pre-enrichment of sample and incubated at 28°C for 7 days in static condition with intermittent shaking (full strength sterile sea water was used throughout the process).

Isolation of actinobacterial population

A loopful of inoculum from the pre-enriched starch casein broth was streaked on starch casein agar (SCNA) medium (starch 1.0%, casein 0.03%, KNO_3 0.2%, NaCl 0.2%, K_2HPO_4 0.2%, $MgSO_4.7H_2O$ 0.005%, $CaCO_3$ 0.002%, $FeSO_4$. $7H_2O$ 0.001% and agar 1.8%; supplemented with Nystatin 50 µg/ml and Nalidixic acid 20 µg/ml). The plates were incubated at 28°C for seven days. Single discrete colonies were isolated and maintained in SCNA agar slants for further studies.

Antibacterial activity screening

The isolated actinobacterial strains were screened for its antibacterial activity by cross streak technique. Human pathogens(*Vibrio cholerae*, *Salmonella typhi*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Vibrio alginolyticus*) are obtained from The Rajah Muthiah Medical College Hospital, Chidambaram. The actinobacterial strains streaked on modified nutrient glucose agar (glucose 5 g, peptone 5 g, beef extract 3 g, Nacl 5 g, agar 15 g, 50% sea water 1 L). After a weak the test pathogens were streaked at right angles to the original streak of the actinobacteria. The inhibition zone was recorded after 24 to 48 h of incubation.

Phenotypic and chemotaxonomic characteristics

The isolated actinobacterial strains were identified by following the guidelines of International *Streptomyces* Project (Shirling and Gottlieb, 1966). The morphological and cultural characteristics such as aerial mass color, substrate mycelium, melanoid pigments and spore chain morphology were studied (Nanomura, 1974). Carbohydrate utilization was analyzed by the method of Shirling and Gottlieb (1966). The cell wall was purified and analyzed by using the method Lechevalier and Lechevalier (1970). The whole-cell chemical composition was also analyzed (Becker et al., 1965).

Screening for l-asparaginase production

All the actinobacterial strains were screened for l-asparaginase production. The isolated strains were inoculated in Glycerol asparagine agar (Glycerol 1.0%, l-asparagine 0.1%, K_2HPO_4 0.1%, Agar 1.5%, Trace salt solution 0.1 ml) supplemented with 0.3 ml of 2.5% phenol red dye at pH 6.5. The formation of pink colored zone around the colony shows the positive result for l-asparaginase production.

Optimization of l-asparaginase production

Based on initial screening, the isolate SS7 was selected for optimization studies. The physio-chemical parameters like temperature (25 to 55°C), pH (5 to 10), carbon sources (fructose, sucrose, maltose, mannitol and lactose) and nitrogen sources (peptone, beef extract, yeast extract, soya bean meal and ammonium sulphate) were selected and optimized for the submerged fermentation.

Natural substrates for l-asparaginase production

Different natural sources were used for l-asparaginase production (castor seed cake, mangrove leaf I - *Avicennia marina*, mangrove leaf II - *Rhizophora mucronata*, sea weed I - *Sargassum tenerrimum*, sea weed II - *Caulerpa scalpelliformis*, gastropod muscle - *Babylonia spirata* and green muscle - *Perna viridis*). Spore suspension was prepared from 14 day old culture by adding 10 ml of sterile distilled water containing 0.01% of Tween 80 (Lingappa and Babu, 2005). The prepared inoculum used for mass production. The selected sources (10 g) mashed well and dissolved in 100 ml of sea water. And the contents were boiled for 10 min, cooled and filtered through cheese cloth. The extracts were used as substrate for l-asparaginase production (devoid of l-asparagine).

Assay for l-asparaginase activity

L-asparaginase enzyme assay was performed by a colorimetric method (Wriston and Yellin, 1973). Using UV-visible spectrophotometer by estimating the ammonia produced during l-asparagine catalysis using Nessler's reagent. Reaction mixture consisting of 0.5 ml of 0.08 mM l-asparagine, 1.0 ml of 0.05 m borate buffer (pH 7.5) and 0.5 ml of enzyme solution. The reaction

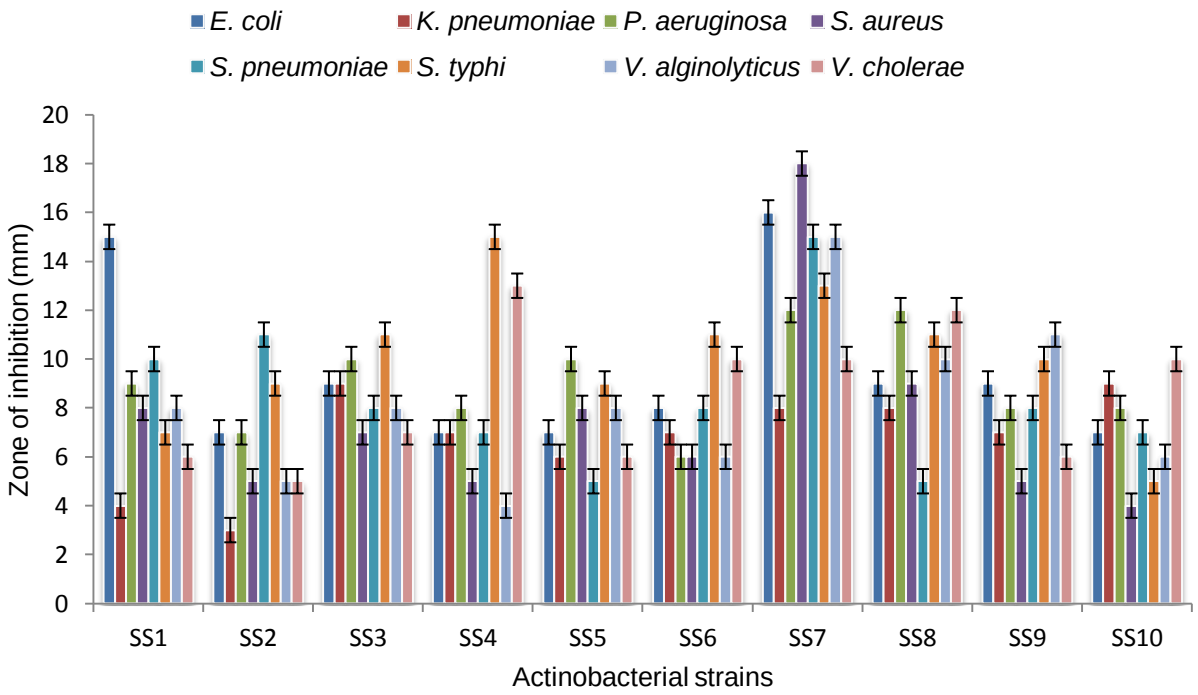

Figure 1. Significant antibacterial activity of the isolated actinobacterial strains against human pathogen.

was terminated by the addition of 0.5 ml of trichloroacetic acid (15%) solution after 30 min of incubation. The liberated ammonia was coupled with Nessler's reagent and was quantitatively determined using a calibration curve from standard solutions of ammonia. One unit of the l-asparaginase (IU) is defined as that amount of enzyme capable of producing 1 µmol of ammonia per minute at assay conditions.

Partial purification of l-asparaginase

The crude enzyme was treated with ammonium sulphate at different saturation levels (up to 80% saturation level) under continuous stirring. The precipitate was collected by centrifugation at 10,000 rpm for 10 min (4°C). The obtained enzyme precipitate was dialyzed against 1 M phosphate buffer (pH 8.5) for 24 h at 4°C with stirring and the buffer was changed intermittently. The precipitate was applied in the sephadex G-100 column which pre-equilibrated with 1 M phosphate buffer, pH 8.5. The protein elution was done with same buffer at a flow rate of 5 ml/min. The fractions were collected manually and assayed for asparaginase activity.

RESULTS

Isolation and screening

Totally, 75 strains were isolated from marine sediment samples, out of that, only 24 strains showed antagonistic activities against the bacterial pathogens. Among these, 10 actinobacterial strains were showed significant antagonistic activities towards dreadful human bacterial pathogens (Figure 1).

The 10 actinobacterial strains were screened for l-asparaginase production by using glycerol asparaginase agar. Based on the zone formation ability, the strains were separated into three categories: high producers (+++), medium producers (++), weak producers (+) and (-) negatives. Among 10, the strain SS7 showed large pink colored zone around the colony (Figure 2). The strain SS7 was selected and identified using phenotypic and chemotaxonomic characteristics (Table 1). Based on these results, the strain SS7 was identified as *Streptomyces* sp.

pH and temperature

The optimum pH for enzyme production was determined by adjusting the initial pH ranging from 5 to 10 in different sets. The yield of l-asparaginase was increased with increase in pH of the medium up to 8.5 and thereafter the production was decreased. The maximum (11 IU) enzyme production was observed in pH 8.5 (Figure 3). The optimum temperature for enzyme production was determined by incubating the production medium in different temperature ranging from 25 to 55°C. The highest yield (11.9 IU) was found at 40°C (Figure 4).

Carbon and nitrogen sources

Different carbon sources (fructose, sucrose, maltose, mannitol and lactose) and nitrogen sources (peptone,

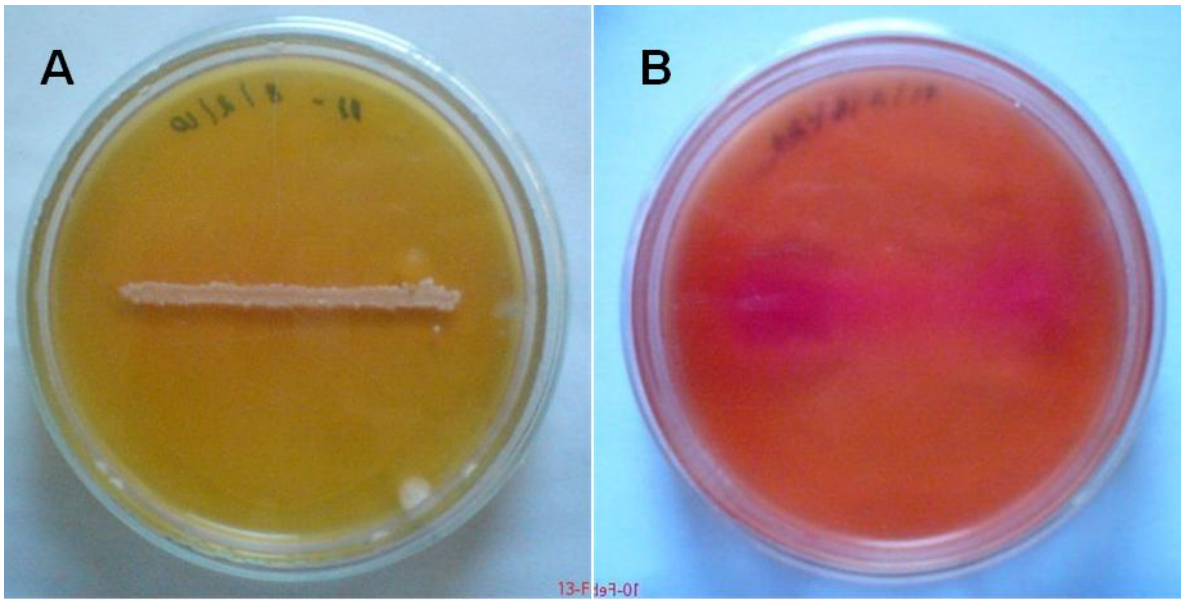

Figure 2. Screening result for l-asparaginase production. A) Negative result, B) positive result.

Table 1. Phenotypic and chemotaxonomic characteristics of the strain SS7.

Characteristic	SS7
Growth on ISP 2	Good
Growth on ISP 4	Good
Growth on ISP 7	Poor
Aerial spore mass colour	Grey
Spore chain	Rectiflexibiles
Spore surface	Smooth
Melaniod pigment	+
Reverse side pigment	Colourless
Growth on sole carbon sources	
D-Glucose	+
L-Arabinose	+
D-Xylose	-
Inositol	-
D-Mannitol	+
D-Fructose	+
Rhamnose	-
Sucrose	-
Raffinose	-

beef extract, yeast extract, soya bean meal and ammonium sulphate) for the production of L-aparaginase. Among all the carbon sources tested, maltose proved to be the best source for the production of enzyme, which yields 22 IU of enzyme (Figure 5). In addition to the organic nitrogen sources examined, soya bean meal proved to be best source for l-asparaginase production, it produced 25.7 IU of enzyme (Figure 6).

Natural substrates for l-asparaginase production

Different sources like plant, algal and animal sources were taken for the enzyme production. Among the three sources, mangrove leaf extract- I (*A. marina*) produced maximum yield (14.5 IU) of enzyme (Figure 7) followed by castor seed cake (13.8 IU) and sea weed-I (*S. tenerrimum*) (10.8 IU). As compared to mangrove leaf substrate, the glycerol asparagine broth yielded only 9.7 IU of l-asparaginase.

Purification

The l-asparaginase was partially purified and analyzed for activity (Table 2). The crude extract yielded 100% recovery of l-asparaginase with 25.9 IU/ mg specific activity. $(NH_4)_2SO_4$ precipitation showed specific activity of 41.14 IU/ mg with 2.1 fold purification. The sephadex G-100 column chromatography showed specific activity of 145 IU/ mg with 18 fold purification.

DISCUSSION

Marine actinobacteria are predominantly screened for its biological significance in the pharmaceutical industries. They produce wide range of bioactive compounds that are unique than the terrestrial ones. Among actinobacterial genera, streptomycetes produce one third of bioactive metabolites such as antibiotics, enzymes, anti-tumor compounds, etc and these compounds are essentially needed for treatment of several dreadful human diseases. In this view, the present study was carried out

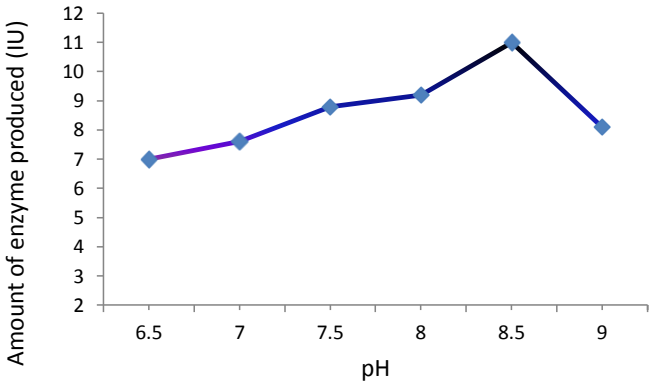

Figure 3. Effect of pH on the production of l-asparaginase.

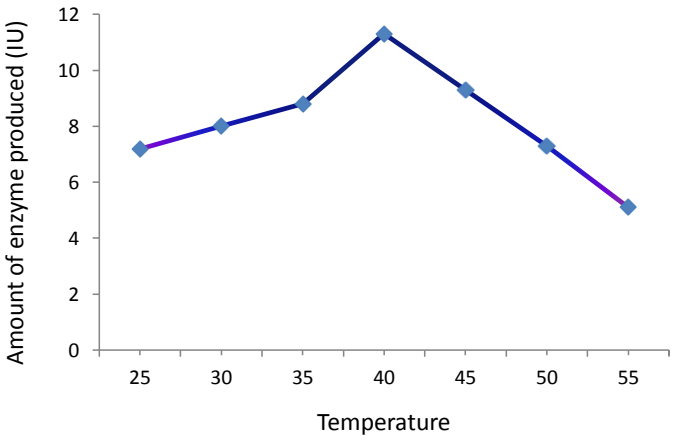

Figure 4. Effect of temperature on the production of l-asparaginase.

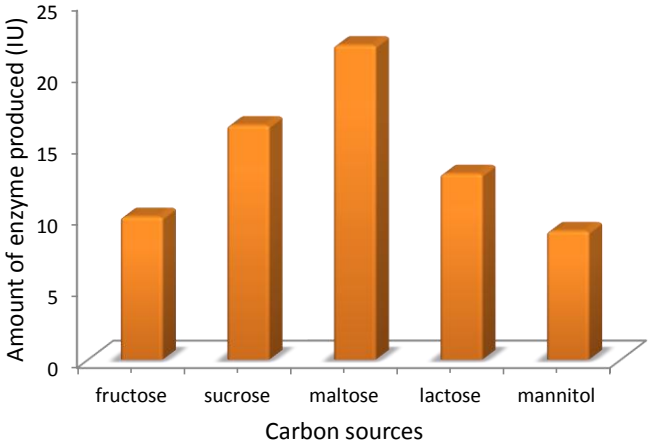

Figure 5. Effect of carbon sources on the production of l-asparaginase.

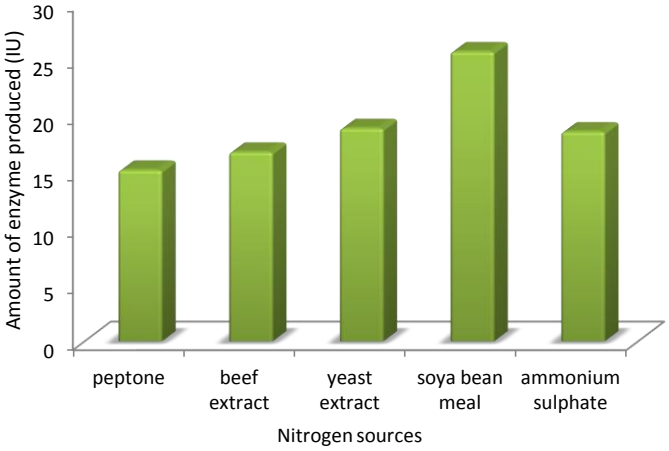

Figure 6. Effect of nitrogen sources on the production of l-asparaginase.

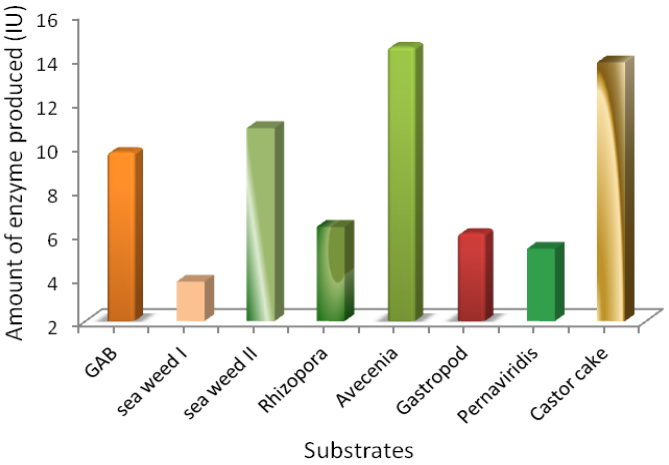

Figure 7. Effect of natural substrates on the production of l-asparaginase

to identify novel actinobacterial population and its potentials from Bay of Bengal, India. Out of 75 isolates, only 11 actinobacterial strains were showed considerable antibacterial activities and all the strains were identified using chemotaxonomical analysis. Similarly, Balagurunathan (1992) isolated 51 actinobacterial strains from Vellar estuary, Bay of Bengal; out of the eight strains were showed significant antagonistic activities towards dreadful bacterial and fungal pathogens. Cwala et al. (2004) isolated four species of Actinomycetes from aquatic environment of Eastern Cape Province of South Africa and it was showed antagonistic activities against gram positive and gram negative microbial forms. The halophilic actinobacterial strain *Actinopolyspora* sp. from east coast of India, showed broad spectrum of antimicrobial activities (Kokare et al., 2004). Suthindhiran and Kannabiran (2008) were explored 50 potent Streptomycetes from Bay of Bengal of the Puducherry coast India. Ramesh and Mathivanan (2009) isolated from 208 actinobacterial strains, in that 111 strains were showed both antibacterial and antifungal activities, from Bay of Bengal. Recently, researchers paid much attention

Table 2. Partial purification of l-asparaginase enzyme produced by strain SS7.

Step	Volume (ml)	Protein (mg)	Activity (IU)	Specific activity (IU)	Purification fold	Recovery (%)
Crude extract	10.00	136.00	3,520.00	25.90	-	100.00
$(NH_4)_2SO_4$ precipitation	7.00	43.00	1,760.00	41.10	2.10	81.60
Sephadex G-100	2.00	2.60	1,200.00	145.00	18.00	36.00

on l-asparaginase, because of its potential for treatment of anti-leukemia. During the tumor cell formation, the enzyme l-asparaginase which converts l-asparagine into l- aspartic acid and ammonia, so that tumor cells were selectively killed due to deficiency of this amino acid. Marine actinomycetes were proven to excellent source for the production of l-asparaginase (Gupta et al., 2007). Out of eleven strains, only four strains were showed acceptable l-asparaginase production. Among the four isolate, *Streptomyces* sp. strain SS7 was proved to be an exceptional l-asparaginase production.

Dhevagi and Poorani (2006) isolated actinobacterial forms from Parangipettai and Cochin coastal areas, among that, isolate PDK7 and PDK8 were showed potential l-asparaginase activity. Basha et al. (2009) isolated 10 actinobacterial strains in that three isolate S3, S4 and K8 were showed production of extracellular anti-leukemic enzyme l-asparaginase. Poorni et al. (2009) reported 34 species of Actinomycetes in that *Streptomyces* sp. strain EPD27 produced most potent biocatalyst l-asparaginase. In the same way, *S. noursei* MTCC 10469, which isolated from marine sponge *C. diffusa* showed the production of L-asparaginase (Dharmaraj, 2011). pH of the medium strongly affects many enzyme processes and transport of various components across the cell membrane (Moon and Parulekar, 1991). The maximum (11 IU) enzyme production was observed in pH 8.5. The temperature is one of the most critical parameters controlled in any bioprocess (Chi and Zhao, 2003). The highest yield (11.9 IU) was found at 40°C. Narayana et al. (2009) studied the production of l-asparaginase in *S. albidoflavus* and he reported the optimum pH for l-asparaginase was 7.5 and temperature was 35°C. Amena et al. (2010) reported optimum pH 8.5 and temperature 40°C for the production of L-asparaginase, in *S. gulbargensis.* Nutrient elements such as carbon and nitrogen compounds can affect the growth of several microbial life. In carbon sources, maltose produce higher amount of enzyme production (22 IU) and nitrogen sources of soya bean meal (25.7 IU) proved best source for l-asparaginase production. Enhanced production of l-asparaginase in *S. albidoflavus* was observed in maltose as a sole carbon source and in nitrogen source yeast extract showed higher amount of production (Narayana et al., 2009). Maltose (0.5%) and l-asparagine (0.5%) proved to be the best carbon and nitrogen sources for the production of l-asparaginase in *S. gulbargensis* (Amena et al., 2010).

Each and every organism has its own requirements for maximum enzyme production. Hence, medium optimazation was carried out to maintain a balance between the various medium components, thus minimizing the amount of component utilization at the end of fermentation (Adinarayana and Ellaiah, 2002). An important factor to be monitored while developing a production medium is the cost effectiveness of the medium. In this aspect, we tried different natural sources. Among the three sources, mangrove leaf extract- I (*A. marina*) produced maximum (14.5 IU) yield of enzyme, followed by castor seed cake (13.8 IU) and in sea weed-I (*S. tenerrimum*) (10.8 IU). When compared to mangrove leaf substrate, the glycerol asparagine broth yielded only 9.7 IU of l-asparaginase. Several studies has showed the mangrove leafs contains varied nutrient dynamics (Davis et al., 2003; Lin and Wang, 2001; Ahmed et al., 2010). Similarly, Kumar et al. (2011) studied nutrient dynamics of fresh dried leafs of *A. marina*, it contain high amount of nitrogen, followed by phosphorous and potassium. The *Streptomyces* sp. strain SS7 may metabolize leaf extracts and it can uptake organic (phenolic compounds, proteins, organic acids, etc.) and inorganic (Mg, Ca, K, etc.) compounds. These organic and inorganic minerals can highly influence the growth of actinobacterial populations. However, among three sources, mangrove leaf extract-I (*A. marina*) produced higher amount of l-asparaginase production. The crude extract yielded 100% of l-asparaginase with 25.9 IU/mg specific activity. In ammonium sulphate, specific activity of the enzyme showed 41.14 IU/mg with 2.1 pure fold. Sephadox G-100 column showed a specific activity of 145 IU/mg with 18 pure fold.

Devagi and Poorani (2009) reported 85 folds purification with 2.18% recoveries in sephadex G-100 column from *Streptomyces* sp. PDK2. l-asparaginase from a sponge isolate *S. noursei* MTCC 10649 has been showed 98.23 folds purification with 2.14 yields in sephadox G-50 column chromatography (Dharmaraj, 2011). The *Streptomyces* sp. strain SS7 produced higher l-asparaginase activities with low-cost substrate mangrove leaf extract-1 (*A. marina*). To the best of our knowledge, this is the first report on the utilization of mangrove leaf extracts as a best substrate/medium for the production of l-aspaginase enzyme. With this study, we are concluding that the use of mangrove leaves is good source for actinobacterial l-asparaginase production. A cost effective, economically feasible technique leads a threshold to exploit marine actinobacterial resources to benefit common people to compete against leukemic disorders.

ACKNOWLEDGEMENTS

Authors are obliged to our Annamalai University authorities for providing permission and necessary support to carry out this study. Corresponding author is highly grateful to Dr. M. Kalaiselvam, reader in Microbiology, Faculty of Marine Sciences, Centre of Advanced Study in Marine Biology, Annamalai University for his laboratorial assistance throughout this study.

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