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L-Asparaginase from Marine Actinomycetes of Thoothukudi Coastal Ecosystem

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A B S T R A C T

Marine Actinomycetes are a valuable resource for novel bioactive compounds. L asparaginase, a bioactive compound obtained from marine Actinomycetes is gaining momentum as an anticancerous agent because of its stability in varied environments. The present study aims at the isolation of actinomycetes from the marine sediment of Thoothukudi coast and evaluates their potential for L asparaginase. Grab and core samplers were used for collecting the marine sediment samples. Out of 21 recommended media, seventeen media were used for the enumeration of Actinomycetes. Rapid plate assay with phenol red as indicator was used for quick screening of L asparaginase positive cultures. Among seventeen different media, starch casein agar media, sea water complex media and Actinomycetes isolation agar were found to be suitable for isolating Actinomycetes. Nearly hundred isolates were obtained and only six isolates showed L asparaginase activity. The isolate DS8 showed more activity compared to other isolates. Starch casein agar with 0.015 % phenol red was highly suitable for rapid assay of asparaginase activity. The final purified enzyme obtained from sephadex G200 showed a specific activity of 45.18 IU/mg of protein. The present study concludes that marine sediments of Thoothukudi harbouring Actinomycetes with therapeutic enzymes could be exploited as a source of L asparaginase.

Introduction

During recent years, the rate of discovery of new compounds from terrestrial Actinomycetes has decreased and it is crucial that new groups of Actinomycetes from under exploited habitats be explored as sources of novel bioactive secondary metabolites. Marine actinomycetes are the most economically and biotechnologically valuable prokaryotes and are best known for their ability to produce bioactive compounds which are relatively more stable and active than the corresponding enzymes from plants and animals (Lam, 2006, Bull *et al.*, 2000). They provide a good source of new secondary metabolites due to the fact that they are adapted to a marine habitat. Like bacteria, actinomycetes are also a good source of L- asparaginase (Savithri and

Azmi, 2003). Marine bacteria producing L asparaginase enzyme were isolated by many workers (Meena *et al.*, 2015; Bhargavi and Jayamadhuri, 2016; Dhevagi and Poorani, 2006). Not only the qualitative study, quantitative study was also being done many workers (Selvam and Vishnupriya, 2013.). In one of their study *Streptomyces acrimycini* with a total activity of 1510 U/ml with a specific activity of 10.79 U/mg of protein was reported.

Isolation and characterization study of Actinomycetes reveal that the marine Actinomycetes from coastal environment are the potent source of novel antibiotics and can be useful in the discovery of novel species (Valli *et al.*, 2012). Thus, there is enormous scope for investigations that explore the probabilities of deriving new products from potential marine actinomycetes. Although some work has been done on marine actinomycetes not much work has been done with the microflora of coastal areas of Thoothukudi, which is a unique habitat and hence there is a potential for isolates with novel therapeutic activity. Hence, the present investigation was designed for the isolation of marine actinomycetes suitable for therapeutic applications from the coastal zone of Thoothukudi in Tamil Nadu

Materials and Methods

Sample Collection

Marine sediment samples were collected from six different locations viz., Threspuram (TS), Meenavar Kuppam (MK), Beach road near Roach Park (RP), Harbor camp (HC), Deep sea sediments (D1, D2) from Thoothukudi district of Tamil Nadu. Depending upon the location either Grab sampler or Core sampler was used for collecting the samples. Alcohol rinsed Peterson Grab sampler (RISC, 1999) was used for collecting deep sea sediments samples (D1 and D2) and core sampling method was used in the case of TS and MK. The samples RP and HC were collected from the rhizosphere region of mangrove plants (Günter *et al.*, 2009). The depth of sample collection varied from 1 feet to 20 feet. Subsamples of minimum three was taken and mixed as composite samples.

The sediments were transferred to sterile polyethylene bags before transported to the laboratory and were analyzed for its physical, chemical (APHA, 1989) and biological properties (Cappuccino and Sherman, 2004).

Enrichment

Pretreatments are essential before enumerating Actinomycetes from marine sediments. In the present investigation two methods of pretreatments were followed as a preliminary step. In the first method, one gram of sediment was transferred to a conical flask containing 100 ml of sterile starch casein broth and incubated at 37° C for 14 days in an incubator cum shaker. In the second method the sediment samples were dried and heated at 80°C for 2 hours and then inoculated into starch casein broth and incubated.

Several different combinations of media have been suggested for the isolation of Actinomycetes from soil (Waksman, 1961). Out of 21 recommended media, seventeen media (Kuster and Williams, 1964; Paul *et al*., 1991; Shirling and Gottlieb, 1966; Selvakumar, 2011 and Poorani *et al*., 2009) were selected to determine the efficacy for isolation, growth and activity of Actinomycetes from the marine sediment samples. After incubation, growth of the Actinomycetes was observed in all the

seventeen media and evaluated for their suitability to enumerate the Actinomycetes from enriched marine sediments.

Screening for L asparaginase

Screening for L-asparaginase activity was done with rapid plate assay method (Imada 2005). This method was found to be more advantageous as this method is quick and results can be visualized directly from the plates. Screening for enzyme activity was done with starch casein agar media spiked with different concentrations of the phenol red dye. A 2.5% dye was prepared in ethanol and the pH was adjusted to 7.0 using 1 m NaOH and added to the SCA medium. Then the medium was plated along with the control and inoculated with 72 hr old isolates for rapid screening of the enzyme activity.

The diameter of the colonies and the diameter of total clear hydrolytic halos including the colonies were determined. The strains that yielded higher halos were selected as potential strains for L-asparaginase production using asparagine as substrate. Percent of the cleared zone was calculated as per the formula given below.

$$\text{Percent of cleared zone} = \frac{\text{Diameter of the cleared zone - Diameter of the colony} \times 100}{\text{Diameter of colony}}$$

The isolate which showed asparaginase activity were further characterized (Shirling and Gottlieb 1966) before purifying the enzyme.

Partial Purification of L asparaginase

Sterilized starch casein broth (pH 7.0) was inoculated with promising isolates obtained from the plate assay and incubated at 30°C for 3 days in an incubator cum shaker. After incubation, the enzyme was extracted by centrifugation and assayed for Lasparaginase activity. For further purification, the crude enzyme was brought to 45 per cent saturation with ammonium sulfate at pH of 8.4 and kept overnight in a cold room for calibration. Then the supernatant was centrifuged at 4200 rpm, for 10 min at 4° C. The same process was repeated and the supernatant was brought to 85 per cent saturation with ammonium sulfate. The precipitates were collected and dissolved in 1 M Tris HCl buffer and dialyzed using pretreated dialysis tubes. Dialyzed precipitates were dissolved in 0.05 M Tris HCl (pH 8.4) buffer and was loaded onto pre-equilibrated Sephadex G 50 column using 0.05 M Tris HCl (pH 8.4) containing 0.1 M KCl elution buffer. The same process was repeated with Sephadex G 200 column. Fractions were collected at a flow rate of 5ml/30 min from both the columns and assayed for L-asparaginase activity (Narayana *et al*., 2007, Amena *et al*., 2010). All the process was carried out at $4^{o}C$

Results and Discussion

Marine sediment samples collected from Thoothukudi coastal zone were analyzed for physical – chemical and biological properties. Sediment samples collected from Threspuram (TS) were clay with fine organic materials and MK, D1 and D2 sediments were found to be muddy sand types. All the samples showed had an alkaline pH ranging from 8.41 to 8.52 and drastic variation was observed in case of EC (1.96 to 9.36 dSm^{-1}). The organic carbon content of the samples varied from 5.1 to 13.3 %. The collected sample had very low bacterial and fungal population. The bacterial population varied from 30 to 170 x 10^2 per gram of sediment sample and fungal

population varied from 20 to 110 x 10^1 per gram of sediment. The population of Actinomecetes varied from 6 to 32 x 10^1/g of sediment sample. Sample from Meenavar Kuppam and Roach Park recorded highest actinomecetes population 24 x 10^1 and 32 x 10^1/g of sediment respectively. Thoothukudi sediment samples were enriched by 14 days and incubated in SC broth as mentioned earlier. After enrichment, the cultures were isolated in media supplemented with cycloheximide 50 µg/ml and nalidixic acid 35 µg/ml.

Screening different media

After thorough scanning of literatures, seventeen different specific media were screened for the isolation of actinomycetes from enriched marine sediment samples (Table 1). Among the different media used for screening of actinomycetes, starch casein agar media, sea water complex media, actinomycetes isolation agar showed the highest number of colonies as compared to other media (fig. 1). Least population was observed in modified M9 media and Kenknight agar.

As far as colony growth pattern was concerned good growth was observed with different types of morphologies in few media. In Seawater Complex agar medium, large sized, white cottony growth was observed. In Actinomycetes Isolation agar medium, small sized, white colored colonies were observed. Large size, white colored colonies with fiber like growth surrounding the colonies were observed in the starch casein agar medium.

The number of isolates from sampling locations TS, MK, RP, HC, D1 and D2 were 12, 13, 28, 15 and 16 respectively. A total of 108 individual colonies with different macroscopic characteristics, colony size and texture were selected and all these isolates were subjected to rapid plate assay method for testing the L asparaginase activity. Among the isolates which had enzyme activity in rapid plate assay were used for further study. Among six positive isolates and DS 8 isolated from deep sea using a grab sampler was subjected to purification of L asparaginase.

Screening for L-asparaginase activity

The cultures showing positive results for L asparaginse were screened for different phenol red concentration (0.001 to 0.019 %) to fix the minimum dye concentration for effective and quick isolation. The isolates TS 3, DS7 and DS 8 produced larger zone and among the three isolates DS 8 showed highest zone (20.7mm) and significantly different from other isolates. Hence this isolate was characterized and used for enzyme extraction and purification study.

The characteristic of the promising isolate DS8 was presented in table 2 and 3. The isolate showed grey colored growth without any motility. The staining reaction of the isolate was gram positive and acid fast negative. It has hydrolyzed casein and starch, but not utilized cellulose. In addition, it preferred glucose as carbon source, but also grow in mannital and fructose containing media. Sucrose was not utilized and differences were observed in the case of nitrogen utilization. DS 8 strongly utilizes L asparagine and L Phenylalanine was not utilized.

Partial purification of l-asparaginase

The crude enzyme extract was assayed for L asparaginase activity after estimating the protein content. Results obtained in the purification of L asparaginase were given in Tabl4. The crude enzyme extract had a total

activity of 368.3 U/ml with specific activity of 0.86 u/mg. The crude enzyme extract when subjected to purification using ammonium sulfate precipitation, the total activity was 242.8U/ml with a specific activity of 0.88 U/mg. When the precipitate passed through sephadex G 50 column, the specific activity was increased (20.08 U/mg) and it was around 45.18 U/mg when the precipitate was passed through sephdex G 200 column. One IU is 1mM of ammonia per minute per ml at 37^{o}C (1µm/minute/ml)

Enrichment of sediment samples

Marine sediment samples collected from Thoothukudi coastal zone were analyzed for physical – chemical and biological properties. The entire sample recorded very low actinomycetes population (6 to 32 x 10^{1}/gram of sediment) and this observation necessiated to carry out enrichment before enumerating the actinomycetes population.

Many studies had indicated that enrichment of samples in selective media led to the isolation of new actinomycetes strains from marine sediment samples. In addition, for selective development of actinomycete colonies, mixture of antibiotics was tested for its selective efficiency (Porterj *et al.*, 1960).

Heat treatment method is also often used for the pre treatment of marine sediments, prior to actinomycete isolation, to reduce the numbers of gram-negative bacteria commonly found in marine samples. Nalidixic acid was used in Starch casein medium to enhance actinomycetes counts of marine sediments (Takizawa, 1993). The effectiveness of addition of NA to the isolation medium on actinomycete counts was compared with conventional heat pre treatment. In general, counts obtained from an isolation medium supplemented with NA were similar, both with and without heat pre treatment, but were significantly higher than counts from preheat treatment alone.

The present study reveals that incubating the sample for 14 days at 37^{o}C followed by plating on media supplemented with cycloheximide 50 µg/ml and nalidixic acid 35 µg/ml was found to be good for isolating Actinomycetes cultures from marine sediments. This was in line with the findings of many researchers (Imada 1973).

Screening different media

Glucose asparagine agar, Green and Meyer's agar and Kusters Agar were found to be the suitable media for isolation of actinomycetes from air dried sediment samples (Kuster and Williams, 1964). Nystatin was used in Kuster's agar for isolation of actinomycetes from mangrove sediments (Meena *et al*., 2015). Cyclohexamide and nalidixic acid was used in the ISP2 medium for the isolation of actinomycetes from sea surface macro layer (Hakvag *et al.,* 2008).

In the present study 17 different media were used for screening. The number of days of incubation also played a major role in the development of the colonies. As the duration of incubation increases the numbers of colonies were also increased to some extent. In few media significant increase was observed and in many media the result was not at a significant level. Based on the above study it was inferred that Sea Water Complex agar, starch casein agar and Actinomycetes isolation agar were highly suitable for isolating marine Actinomycetes. Hence, these media were used for the isolation of actinomycetes from the sediments supplemented with cyclohexamide 50µg /ml and nalidixic acid 30 µg /ml. Isolates obtained from the selected media were purified and maintained.

Table.1 Screening of different media for enumeration of marine actinomycetes

S.No	Media used	Growth pattern	Morphology
1	Kenknight agar (14)	Moderate	White colonies
2	Sea water complex agar (SWC) (15)	Very Good	Larger, well defined and white colored colonies
3	Actinomycetes isolation agar (AIA) (16)	Very Good	White well defined small colonies with cottony growth.
4	Modified M_9 media (MM9) (17)	Moderate	Bright White colonies
5	Nutrient agar (NA) (12)	Poor	White colonies
6	Modified Nutrient Agar (MMA)(16)	Moderate	Few colonies with creamy centre
7	Starch casein agar (SCA) (16)	Very Good	Well defined larger dirty white, fibrous margins.
8	Starch Nitrate Agar Medium (SNA) (12)	Moderate	Grey, powdery colonies
9	Tryptone Yeast Extract Broth (ISP 1) (16)	Moderate	Pellicle formation
10	Yeast Extract Malt Extract Medium (ISP 2) (16)	Good	Ash colored colonies
11	Oat Meal Agar Medium (ISP 3) (16)	Moderate	Dull white thick colonies
12	Inorganic Salt Starch Agar Medium (ISP- 4) (16)	Good	Light brown colonies
13	Glycerol-Asparagine Agar medium (ISP 5) (16)	Good	Dark colored colonies
14	Peptone Yeast Extract agar medium (ISP-6) (16)	Poor	Thin colonies with pigment
15	Tyrosine Agar Medium(ISP -7) (16)	Good	Thick grey colored colonies
16	Potato Dextrose Agar medium (12)	Good	Convex surface with mycelial growth
17	Carbon Utilisation Agar (ISP 9) (16)	Moderate	Yellowish surface with little mycelial growth

Table.2 Morphological and Biochemical characteristics of the Promising Isolate DS8

Characteristics	Results
Colony color	Grey
Gram staining	+
Acid fast	-
NaCl requirement 5% (w/v)	+
Optimum temperature	37- 40 °C
Optimum pH range	7-8
Catalase activity	-
Oxidase	-
Nitrate reduction	+
Methyl red	+
Voges Proskeur	-
Gelatin utilization	+
Starch degradation	+
Casein hydrolysis	+

Table.3 Nutritional Characteristics of the Promising Isolate DS8

Characteristics	Results
Cellulose degradation	-
Glucose	++
Arbinose	+
Sucrose	-
Mannitol	+
Inositol	+
Xylose	+
Fructose	+
Rhamnose	+
L-asparagine	++
Leucine	++
Tyrosine	++
L-phenylalanine	-

(+) -- Indicates positive, (-) -- Indicates negative, (++) -- Indicates strongly positive

Table.4 Purification of L-asparaginase enzyme from promising Isolate DS8 actinomycetes

Purification Steps	Total protein (mg)	Total Activity (IU)	Specific Activity (IU)
Crude extract	429.5	368.3	0.86
Ammonium Sulphate precipitation 45-85 %	274.2	242.8	0.88
Sephadex G 50 filtration	1.06	21.29	20.08
Sephadex G 200 filtration	0.16	7.20	45.18

IU: Amount of enzyme which catalyses the formation of 1µmol of ammonia per min under the conditions of the assay

Fig.1 Screening different media for Actinomycetes isolation

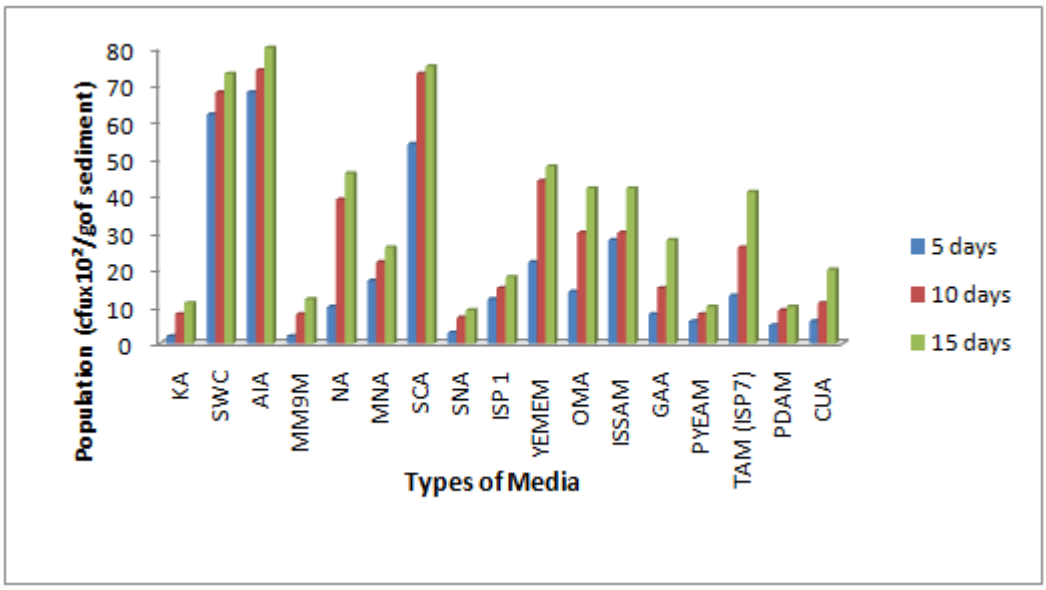


Fig.2 Standardization of Phenol red concentration

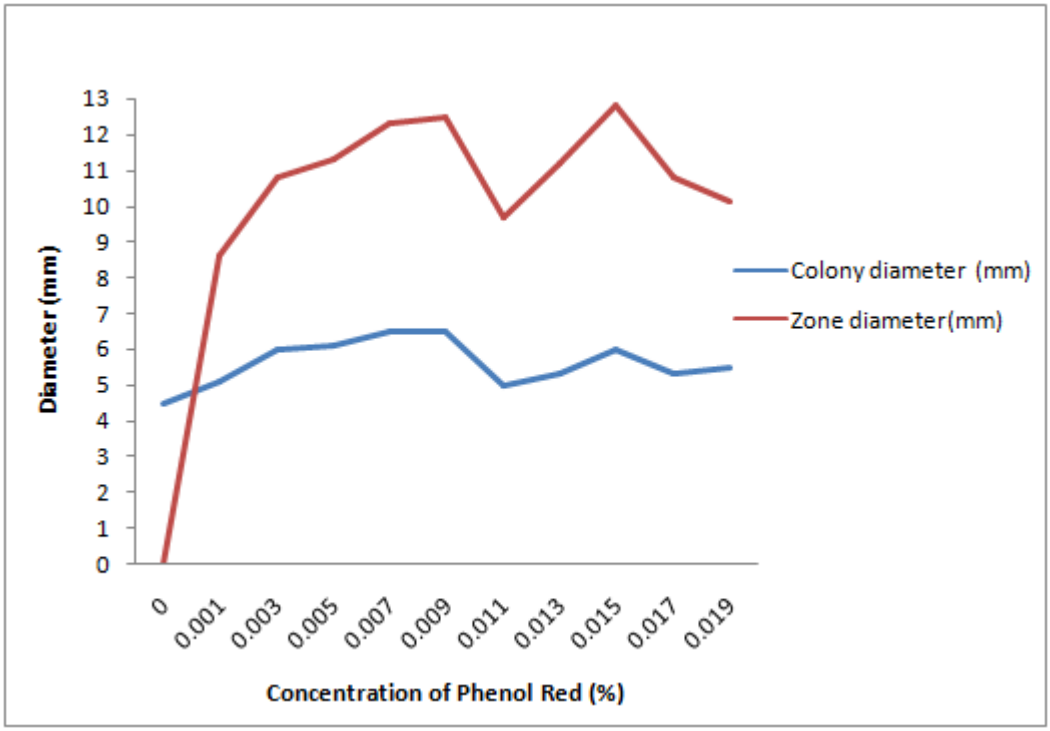
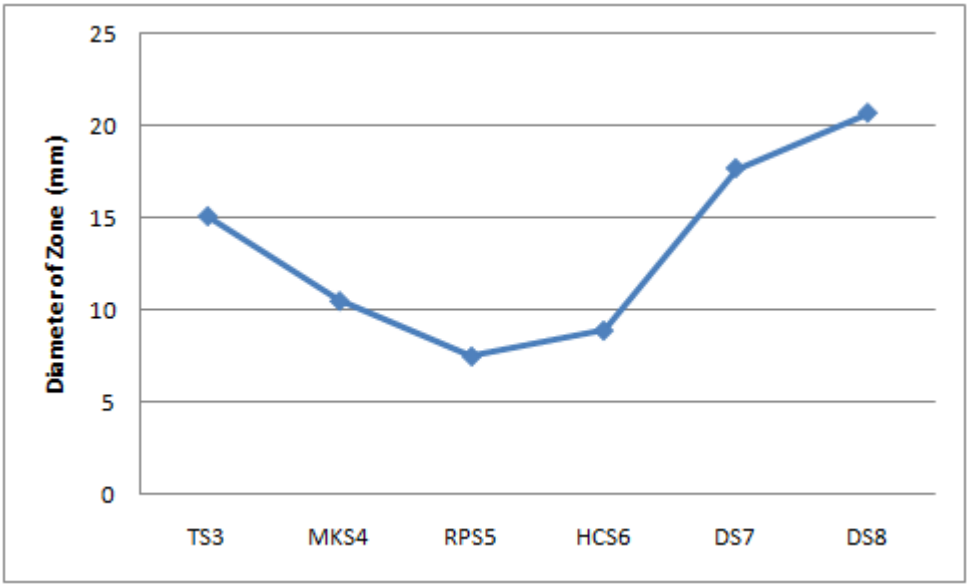


Fig.3 Zone of Clearance in Starch Casein Agar with phenol red



Screening for L-asparaginase activity

The plate assay used for screening asparaginase activity was found to be more advantageous as this method is quick and results can be visualized directly from the plates. The plates were inoculated with 72 hr old culture of the isolates and the isolates which showed the zone of pink coloration indicated the production of L asparaginase. The change in color of the media may be due to the liberated ammonia from L asparainase accompanied by an increase in pH of the culture filtrates. This study with different concentration of the dye reveals that as the concentration of the dye increases the visibility of the zone also increases and the dye didn't inhibit the growth of the test organisms. Since the zone of clearance was high at 0.015 % phenol red, the starch casein agar was supplemented with 0.015 % phenol red was used for plating (fig 2). This was higher than the concentration used by the earlier workers (Valli *et al*., 2012; Gulati *et al*., 1997)

After standardizing the phenol red concentration for rapid plate assay, all the six isolates was tested for the enzyme activity (fig 3). As reported by earlier workers (De Jong, 1972), the plate assay indicates the positive cultures without consuming much time for screening. The positive isolates were characterized (Table 3) and it was identified by growth on agar surface, physiological and enzymatic properties, carbon utilization and nitrogen utilization as *Streptomyces* sp, but needs molecular confirmation.

Partial purification of L asparaginase

The isolate DS 8 obtained from Thoothukudi was used for purification of enzymes from crude extract. After 45 % saturation with ammonium sulfate, the pellet was used for the estimation of protein and for L asparaginase enzyme assay. It was observed that the 45% ammonium sulfate purified sample showed a specific activity of 0.88 IU/mg, and in the final purification step the enzyme showed a specific activity of 45.18 IU / mg of protein. The culture filtrate of atinomycetes isolated from the marine sediments with L-asparaginase activity was reported earlier by many workers (Distasio

et al., 1976; Manna *et al.,* 1995; Sivakumar *et al.,* 2006) and the present study also confirms that. Thus there is enough scope for new source of L-asparaginase with better therapeutic properties, which may help to prevent or reduce the side effects reported earlier. Hence, the focus on L-asparaginase from marine actinomycets may lead to exploration of new source for novel anticancer enzyme.

In conclusion, studies have shown that marine actinobacteria were found to be a good source for therapeutic L-asparaginase. However these studies have been completed only upon the isolation of marine actinobacteria from marine environment for screening of L-asparaginase potentials. Thus further substantial research is needed to explore the potential of marine actinomycetes. Despite some of the potential hindrances to the extraction of enzymes from actinomycetes, the current awareness regarding the compounds from biological origin indicates that marine actinomycete application to therapeutic industry deserves attention.

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