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Isolation and Screening of L - Asparaginase Producing Marine Actinomycetes from South Indian Coastal Region

K.Selvam[1*], B. Vishnupriya[1]

1. *Dr .N.G.P Arts and Science College, Affiliated to Bharathiar University, Coimbatore - 48, Tamilnadu.*

ABSTRACT

A total of fifty six actinomycete isolates were isolated from the ten marine sediments of south India. Marine environment is a potential source of novel actinomycetes, which are a potent source of antibiotics and novel bioactive compounds. The isolates were identified as actinomycetes by morphological, biochemical and microscopic studies. The isolated actinomycetes were screened for L-asparaginase activity by rapid plate assay method. Based on screening, isolate 1, 2 and 18 were showed large clear pink zone and its diameter (dm) was measured as 9.0, 8.5 and 8.5 cm and the enzyme production has been determined by nesslerization method. The spectrophotometric assay of enzyme activity of the isolates 1, 2 and 18 were found to be 1.92, 1.48 and 1.46 U/ml.

Keywords: L-asparaginase, Marine actinomycetes, Nesslerization, Semi quantitative plate assay

*Corresponding Author Email: selsarat@yahoo.com

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INTRODUCTION

The marine biosphere is one of the earth's richest innumerable habitats. The oceans are highly complex environments and a diverse assemblage in environments with extreme variations in pressure, salinity and temperature[1]. Marine microorganisms encompass a complex and diverse assemblage of microscopic life forms, of which it is estimated that, only 1% has been cultured or identified[2]. Considering the fact that marine environment is saline in nature. It could provide rare and unique microbial products, particularly the enzymes that could be safely used for human therapeutic purpose[3].

Among the microorganisms, marine actinomycetes have attracted great attention since they have developed unique metabolic and physiological capabilities that not only ensure survival in extreme habitats, but also differ the potential to produce compounds with antitumor and other interesting pharmacological activities that would not be observed in terrestrial microorganisms, perhaps because of their close relationships with marine eukaryotic organisms including mammals[4&5].

Bacterial L-asparaginase (L-asparagine amido hydrolase E.C 3.5.1.1) has been therapeutic agent in the treatment of certain human cancers mainly in Acute Lymphoblastic Leukemia (ALL). However, the existing L-asparaginase produces harmful side effects[6&7]. Hence search is ongoing for new bacterial source of L-asparaginase with better properties. Like bacteria, marine actinomycetes have been shown to be a good source for L-asparaginase, because of extreme adaptation within the marine environment[8].

The present investigation deals with isolation, identification and screening of L-asparaginase from marine actinomycetes and to assess their enzyme production by nesslerization method.

MATERIALS AND METHODS

Sample collection from marine environment

The marine sediment samples were collected from different sites of south Indian coastal region of Tamilnadu, Kerala and Pondicherry at a depth of 2 - 3 m. The samples were collected using alcohol rinsed Peterson grab and were transferred to new zip lock bags using sterile spatula[9]. The samples were transported to the laboratory for the isolation of actinomycetes.

Enrichment and Isolation of marine actinomycetes

One gram of sediment was transferred to 100ml of starch casein broth supplemented with fuconazole and incubated at 30^0C for 7 days in shaker[10]. A loopful of inoculum from the starch casein broth was streaked onto the starch casein agar (SCA), supplemented with 50 µg/ml and

incubated at 30^0C for 7 days[11]. Single separated colonies were selected and the subcultures were maintained on starch casein slants at 4^0C until further use.

Identification of actinomycetes

All the isolates were identified as actinomycetes by morphological, physiological biochemical characterization studies[12].

Screening of L-asparaginase producer by plate assay

The strains obtained from the above steps were subjected for rapid screening of L-asparaginase production by plate assay[13]. A minimal M9 medium (KH_2PO_4 2.0g, L-asparagine 6.0 g, $MgSO_4.7H_2O$ 1.0 g, $CaCl_2.2H_2O$ 1.0 g, glucose 3.0 g, Agar 20.0 g, d.H_2O 1000 ml) was used for plate assay. A 2.5 per cent stock solution of phenol red was prepared in ethanol (pH 6.2) and 3.0 ml of this was added to 1000 ml of minimal M9 medium. Point inoculation has been carried out in a petridish containing 20ml of this medium and inoculates at 30^0C for 7 days. After an incubation period, the appearance of pink zone around the colony in the medium indicated L-asparaginase activity.

Spectrophotometric assay of L-asparaginase enzyme

L-asparaginase activity was measured by Spectrophotometric assay method[14]. One International unit (IU) of L-asparaginase is the amount of enzyme needed to liberate one µmol of ammonia in one minute and protein concentration was measured[15].

Identification of marine actinomycetes

The best strains were microscopically identified by viewing their morphology through microscope[11].

RESULTS AND DISCUSSION

In the present study, fifty six marine actinomycetes were isolated from the marine sediments and they were enriched by starch casein broth for 7 days. Enrichment of the actinomycetes culture were made by sterile starch casein broth and incubated at 30^0 C for 14 days in an incubator cum shaker[16]. All the fifty six isolates were identified as actinomycetes by morphological, physiological and biochemical characterization studies which is presented in table 1.

Plate assay

The L-asparaginase positive colonies were identified by formation of pink zone around the colonies of the medium. It indicates deamination with release of ammonia. Zone radius of the L-asparaginase producing actinomycetes was measured and represented in table 2. Among them Isolate 1, 2 and 18 produced large clear pink zone around it and they were illustrated in plate 1.

Table 1 : General characteristic features of marine actinomycetes

Isolates	IS-1	IS-2	IS-3	IS-4	IS-5	IS-6	IS-7	IS-8	IS-9	IS-10	IS-11	IS-12	IS-13	IS-14	IS-15	IS-16	IS-17	IS-18	IS-19	IS-20
Morphological characteristics																				
Colony Colour	W	G	W	C	B	W	C	C	C	C	G	B	B	W	W	G	G	W	C	B
Colony Shape	C	C	C	R	C	R	R	R	C	C	R	R	C	C	R	C	R	R	C	C
Pigment	-	-	-	-	DB	-	-	-	DB	-	-	-	-	Y	-	-	-	-	-	Y
Physiological Characteristics Growth at																				
25°C	+	+	+	+	++	++	+	-	+	+	+	++	-	-	+	++	-	+	+	-
37°C	++	++	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	++	-	+
45°C	+	+	+	+	+	+	+	-	++	-	+	+	-	+	-	+	-	+	++	+
55°C	-	-	+	+	+	_	-	-	+	-	-	+	-	+	-	-	-	-	+	-
Growth in presence of NaCl																				
2.5%	+	+	+	-	+	+	++	++	+	+	-	+	-	++	+	+	+	+	-	-
5.0%	+	+	+	+	+	+	++	+	++	+	+	+	+	++	++	++	++	++	++	+
7.5%	+	+	-	++	-	+	+	+	+	+	+	_	-	-	-	-	-		-	+
10%	++	++	-	+	-	-	-	-	-	-	++	_	-	-	-	-	-	-	-	-
Biochemical characteristics																				
Gram Staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red Test	-ve`	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve
Voges-proskauer Test	-ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve
Caesinase Test	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve
Cellulase Test	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve
Deaminase Test	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
Sugar Fermentation	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
Nitrate Reduction Test	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
Gelatine Test	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve

IS - Isolate

Isolates	IS-21	IS-22	IS-23	IS-24	IS-25	IS-26	IS-27	IS-28	IS-29	IS-30	IS-31	IS-32	IS-33	IS-34	IS-35	IS-36	IS-37	IS-38	IS-39	IS-40
Morphological characteristics																				
Colony Colour	G	G	W	C	W	W	C	W	C	W	G	B	W	W	G	C	G	W	B	W
Colony Shape	R	R	C	R	R	R	C	R	R	R	C	C	R	R	R	C	C	R	C	R
Pigment	Y	-	-	-	DB	-	-	-	DB	-	-	-	-	-	-	-	DB	-	-	Y
Physiological Characteristics Growth at																				
25°C	+	+	-	+	+	++	+	-	+	-	+	+	-	++	-	+	-	+	++	+
37°C	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	++	-	+
45°C	-	+	+	-	+	-	+	+	+	-	+	+	-	+	-	+	-	+	++	+
55°C	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+	+
Growth in presence of NaCl																				
2.5%	++	++	+	++	-	+	+	++	+	+	-	++	-	++	+	+	+	+	-	-
5.0%	++	+	+	+	-	+	+	++	+	+	+	+	+	+	++	++	++	++	+	+
7.5%	+	-	-	+	-	+	-	++	+	+	-	+	-	+	-	+	+	-	-	-
10%	-	-	-	+	-	-	-	-	-	-	+	_	-	-	-	-	-	-	-	-
Biochemical characteristics																				
Gram Staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red Test	-ve`	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve
Voges-proskauer Test	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
Caesinase Test	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve
Cellulase Test	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve
Deaminase Test	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
Sugar Fermentation	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve
Nitrate Reduction Test	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
Gelatine Test	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve

Isolates	IS-41	IS-42	IS-43	IS-44	IS-45	IS-46	IS-47	IS-48	IS-49	IS-50	IS-51	IS-52	IS-53	IS-54	IS-55	IS-56
Morphological Characteristics																
Colony Colour	G	G	C	C	B	C	C	W	W	G	G	W	B	C	C	W
Colony Shape	R	C	R	C	R	C	C	C	R	R	C	R	R	C	R	C
Pigment	-	-	-	R	-	-	-	-	R	-	-	-	-	Y	-	DB
Physiological Characteristics Growth at																
25°C	++	-	++	-	+	+	+	-	+	+	++	+	++	++	++	+
37°C	+	-	++	-	+	+	++	+	+	+	+	+	+	+	+	+
45°C	-	+	+	+	+	+	+	-	+	-	+	++	-	+	-	-
55°C	-	-	+	+	-	_	-	-	+	-	-	-	-	+	-	-
Growth in presence of NaCl																
2.5%	++	+	+	++	++	+	+	++	-	++	-	-	++	++	+	+
5.0%	-	+	-	++	+	+	++	+	++	++	+	+	+	+	+	++
7.5%	-	+	-	++	-	+	+	+	+	+	+	_	-	-	++	++
10%	-	++	-	+	-	-	-	-	-	-	++	_	-	-	-	-
Biochemical Characteristics																
Gram Staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red Test	+ve`	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve
Voges-proskauer Test	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
Caesinase Test	+ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
Cellulase Test	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve
Deaminase Test	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
Sugar Fermentation	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve
Nitrate Reduction Test	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Gelatine Test	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve

Colony color : W -White; G - Grey; C - Cream; B – Black, Colony Shape : C - Cocci ; R – Rod, Pigment : R - Red; Y - Yellow; DB - Dark Brown.

The microscopic characterization of L-asparaginase producing isolates (1, 2 and 18) were presented in plate 2. In the previous report, L - asparaginase producing colonies were selected on the basis of pink zone formation around the colonies of the medium[17, 10].

Isolate 01 Isolate 02 Isolate 18

Plate 1. Screening of L-asparaginase producing actinomycetes

Table 2. Zone radius of L-asparaginase producing actinomycetes

Isolates	Zone radius (cm)
Is - 1	9.0
Is - 2	8.5
Is - 6	5.0
Is - 14	2.8
Is - 15	5.5
Is - 18	8.5
Is - 24	4.5
Is - 31	2.0
Is - 51	1.5

Spectrophotometric assay of L-asparaginase enzyme

Spectrophotometric assay has been carried out for the isolates 1, 2 and 18. By the addition of 0.2ml of nessler's reagent into the culture filtrate, sudden appearance of orange colour obtained and optical density (OD) was taken at 450nm. The enzyme activity (U) was found to be 1.92, 1.48 and 1.46U/ml and the protein concentration was 0.265, 0.203 and 0.201mg/ml. Figure 2 showed the microscopic identification of isolates 1, 2 and 18 which are the efficient producers of L- asparaginase. The previous study investigated the production of L-asparaginase from *Aspergillus terreus;* the production was estimated as 1.20 U/ml[18]. in this investigation *Vibrio succinogenes*, yielded about 2.5 U/ml of the enzyme[19]. Similarly, L-asparaginase production by *Streptomyces noursei* MTCC 10469, isolated from marine sponge *callyspongia diffusa* was performed[20].

Isolate 01 **Isolate 02** **Isolate 18**

Plate 2. Microscopic Characterization of the marine actinomycetes

CONCLUSION

The present work was completed by the screening of L-asparaginase producing marine actinomycetes. The actinomycetes were morphologically and biochemically characterized. Then the efficient producers of marine actinomycete isolates (1, 2 and 18) were microscopically identified and genetic identification (16SrRNA) will be carried out. Purification and anti-cancer activity will be performed in future with the isolates 1, 2 and 18. The statement allows us to conclude that L-asparaginase is one of the most efficient enzyme to treat cancer.

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