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Study of L-Asparaginase from Marine Fungi and its Potential as an Anti-cancer Agent: A Preliminary Study using Chorioallantoic Membrane Assay

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ABSTRACT

Introduction: L-asparaginase is a therapeutic enzyme that is used in combination therapy for mainly Acute Lymphocytic Leukemia (ALL) and other cancers. It hydrolyzes L-Asparagine required by neoplastic cells into L-aspartate and ammonia which leads to the death of tumor cells. But commercially available L-asparaginases show fatal side effects because of its prokaryotic source of origin, and associated glutaminase and urease co-activities.

Aims: Screening of marine fungi for L- asparaginase activity and study of its potential as an anti – cancer agent.

Methodology: This study was designed to isolate fungi from marine water and soil samples collected from regions of Maharashtra and Goa, that are capable of producing L-asparaginase enzymes that are free of urease and glutaminase activity, and further study the potential of anticancer activity of these eucaryotic enzymes using Chorioallantoic membrane (CAM) assay.

Result: Among all twelve marine fungi isolated, RF2 (identified as *Aspergillusaculeatus (Genbank accession no.ON023823)*) from Mandvi beach, Ratnagiri MH demonstrated high levels of L-asparaginase activity within 48-96 hrs, while lacking glutaminase and urease activity.

Conclusion: The Chorioallantoic membrane (CAM) assay revealed that this fungal isolate could be a promising source of eucaryotic L-asparaginase to treat cancer with lower side effects.

Key Words: Anti-cancer, Chorioallantoic membrane assay, Eucaryotic, glutaminase, L-asparaginase, Urease

INTRODUCTION

Oceanic waters are prime sources for a wide variety of microorganisms as they contribute a unique habitat for microbial growth. Marine microbes are considered as important entities in marine environments due to their role in biogeochemical processes. In accordance with the characteristics of marine habitats, enzymes that are produced by marine life like bacteria, fungi, algae and sponges, exhibit unique functional and stereochemical properties.[1] L-asparaginase is considered as one of the most important groups of enzymes with commercial applications with 40 percent of the total enzyme sales globally.[2] L-Asparaginases are amidohydrolases that bring about the hydrolysis of the amino acid L-asparagine (Asn)to L-aspartate (Asp) and ammonia.[3,4]

In normal cells, Asparagine synthetase enzyme, produced intracellularly, is responsible for de novo synthesis of asparagine. However, neoplastic cells are dependent on extracellular Asparagine as they fail to induce synthesis of asparagine synthetase.[5,6] L-asparaginaseis a tetramer protein which causes selective death of this asparagine dependent tumor cells by utilizing free plasma asparagine that led to apoptosis and G1 phase cell cycle arrest.[2,7] L-Asparaginase is produced in microbes, plants and animals but not in humans. Clinically, asparaginase formulations have been prepared from two bacterial sources *Escherichia coli* and *Erwinia chrysanthemi*.[4,8] Being from a prokaryotic source, L-Asparaginase therapy is associated with side effects such as pancreatitis, hyperglycemia, coagulation abnormality, thrombosis, liver dysfunction, anaphylaxis, and cerebral dysfunction, etc.[8,9] Research suggests some people develop neutralizing antibodies (an anti-asparaginase antibody)[10] Besides these, commercially available L-Asparaginase (L-ASNase) exhibit high glutaminase activity and low substrate specificity that

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may lead to liver dysfunction, pancreatitis, leukopenia, coagulation abnormalities leading to intracranial thrombosis or hemorrhage and neurological seizures as the presence of glutaminase eliminates the necessary glutamine essential for growth of normal cells and produces toxic ammonia as by product. Few also possess urease activity leading to production of ammonia which has similar effects .[2,5,11]

Obtaining L-ASNase from fungi shows promise as the probability of developing an immune response against eukaryotic source of L-ASNase would be reduced. Further, growing, maintaining and downstreaming of fungal asparaginase is comparatively simpler than from bacterial sources. Literature suggests fungi of marine origin have been reported as a promising source of L-asparaginase.[12] According to WHO, cancer is among four non communicable diseases that accounts for 80% of all deaths worldwide. Hence it is crucial to search for enzymes or compounds that can help in treating cancer. It is the major cause of disease characterized by genetic alteration of the normal cells.[13,14] Therefore, the current study aims to isolate fungi from marine environments that can produce asparaginase enzyme with high stability, but at the same time lack the ability to produce glutaminase and urease enzymes. The objective of this study also includes checking the anticancer activity of L-Asn produced, using the Chorioallantoic membrane assay.

MATERIALS AND METHODS

Fine Chemicals were purchased from Loba Chemie and Himedia, Ammonium Sulfate was purchased from Merck. The media Urea broth base and all media components were procured from Himedia laboratories including L-asparagine monohydrate and L-glutamine. Fertilized eggs were purchased from Central Poultry development organization, Goregaon, Mumbai.

Enrichment, Isolation and qualitative screening of Asparaginase producers:

Different water and soil samples were collected from various beaches of Maharashtra, and Goa (Table-1). The water and soil samples were collected in bottles and plastic bags respectively from 2-3 inches below the surface. Collected samples were stored at 4°C and inoculated for enrichment into 100 mL sterile Modified glucose Czapek's Dox (MCD) broth containing 1% L-asparagine as nitrogen source, 2.0 g/L glucose, 1.52 g/L KH_2PO_4, 0.52 g/L KCl, 0.52 g/L $MgSO_4.7H_2O$, 20.0 g/L NaCl, traces of $CuSO_4.7H_2O$; $ZnSO_4.7H_2O$; $FeSO_4.7H_2O$, pH-6.0, 18.0 g/L agar within 72 hrs. The flasks were incubated at ambient temperature for 10 days. [14,15] After two enrichments, a loopful of broth was streaked on sterile MCD plate supplemented with 2% agar and 0.009% phenol red dye for isolation and qualitative screening of L-asparaginase producer.[2,10,12,16,17]

Screening for L-asparaginase producer free of glutaminase and urease activity:

The medium contained 1% Glutamine instead of 1% Asparagine in MCD broth. Due to presence of phenol red dye, the plates showing the pink zone around growth were considered to be positive for glutaminase production.[13,14,18,19,20] For urease enzyme detection, urea broth was inoculated with a loopful of isolates and tubes incubated at ambient temperature till 7th day. The positive culture shows a color change from yellow to pink. Whereas isolate not producing urease shows no color change.

Screening for isolate producing high L- asparaginase activity:

L-asparaginase activity of crude enzymes was assayed by performing Nesslerization. A standard plot using Ammonium sulfate was performed. The standard graph of absorbance at 470 nm VS Conc. of $(NH_4)_2SO_4$ in mM was plotted. The enzyme activity of all isolates was then calculated and compared. For enzyme activity, 0.5 ml of crude enzyme supernatant was mixed with 1.0 ml of 0.1M Sodium borate buffer (pH 8.5) and 0.5 ml substrate 40mM of L-Asn. After 10 mins of incubation at 37°C, 0.5 ml of 15% TCA reagent was added to stop the reaction. 0.2 ml of supernatant obtained from centrifuging above solution was diluted with 4.3 ml of distilled water. 0.5 ml Nessler's reagent was added and after 10 mins O.D. was measured at 470 nm. [2,13,20,21,22,23,24,25]

After the concentration of ammonium sulfate was converted into micrograms/mL; the enzyme activity was calculated using a formula: -

$$Enzyme\ activity(\mu g/ml/min) = \frac{amount\ of\ product(\mu g) \times volume\ of\ enzyme(ml)}{Time(min)}$$

Where, Volume of enzyme = 0.5 mL, Time = 10 minutes

The culture showing high L-asparaginase activity within a week, no glutaminase, and no urease production was considered as best isolates and was chosen for identification and further application testing.

Identification of Fungal strain:

All the isolates were streaked on sterile Potato Dextrose Agar (PDA) plates, which was composed of 200.00 g/L Potato infusion, 20.0 g/L Dextrose, 15.0 g/L Agar, and 5.6 ± 0.2 pH and incubated at ambient temperature for 7 days.[2,26] For microscopic characteristics, wet mount with lactophenol cotton blue staining was done for the best isolates- RF2. Figure-1 shows the microscopic observation at 45X**.** The best isolate was further identified by performing polymerase chain reaction (PCR) by targeting the 18S ITS small subunit ribosomal

RNA sequence.[7,27,28] The sequencing was performed at SLS Research Pvt. Ltd, Surat, Gujarat.

Chorioallantoic membrane (CAM) assay:

Two days fertilized eggs were taken from the poultry farm and incubated till the 7th day at 37°C (Fig.2). Air sac, embryo position, and the chorioallantoic layer were observed and marked by candling (Fig 3). On the 8th day, 0.5 mL of crude enzyme was loaded into the CAM layer and further incubated at 37°C. The formation of coagulation, low density of vessels or disappearance, and increased air sac was examined/ observed for the next 24-48 hours. Simultaneously, an egg inoculated with 0.5 mL of sterile saline was used as a blank or control and was also incubated at 37°C and observed.[29]

RESULT

Enrichment and Isolation and qualitative screening of Asparaginase producers:

A total of 20 marine water and 20 marine soil samples were collected from different regions of Maharashtra, and Goa. Table-1 shows a list of various sites from where water and soil samples were collected and the number of samples. The collected samples were enriched in sterile Modified glucose Czapek's Dox (MCD) broth at ambient temperature (28±2°C) for 10 days. Except for Alibaug and Goa beach samples, rest all showed turbidity.

After two enrichments, a loopful of broth was streaked on sterile MCD medium containing phenol red dye as an indicator. A total twelve fungal cultures viz. B1, G1, HA1, J1, M1, M2, M3, N1, RF1, RF2, RF3, and RF4 were isolated. Rest isolates obtained were either bacterial or actinomycetes(Table-2).

Screening for L-asparaginase producer lacking glutaminase and urease activity:

On MCD media supplemented with 1% Glutamine, cultures RF2, RF3, and RF4 have shown no glutaminase activity. Other than G1, M1, and RF1 fungal isolates, all showed a negative urease test.

Screening for isolate producing high L- asparaginase activity:

Graph-1 represents Nesslerization assay for standard ammonium sulfate performed. Concentration of ammonium sulfate in mM versus average absorbance at 470 nm was plotted. Enzyme assay was performed using 40mM Asparagine as a substrate for 1 week period. The unknown values were extrapolated on std. Ammonium sulfate graph and conc. of ammonium sulfate in mM were obtained. From these values, the enzyme activity was calculated and a scattered graph of Enzyme activity v/s time in hours was plotted (Graph-2). Fungi isolated from Ratnagiri (RF2) showed maximum L-asparaginase activity within 48-96 hours.

Identification of Fungal strain:

Table-2 shows the results obtained by streaking the fungal isolates sterile Potato Dextrose Agar (PDA) plates to observe their morphological characteristics. Figure-1 shows the microscopic observation at 45X magnification. Our macroscopic and microscopic results indicated that the RF2 can be a species of Aspergillus [Fig 1]. The sequencing with 18S rRNA along with the analysis report confirmed RF2 to be *Aspergillus aculeatus* ***(Genbank accession no.ON023823)***.

Chorioallantoic membrane assay:

The crude enzyme preparation obtained from 72 hrs old RF2 isolate was inoculated on the 8th day into a fertilized egg (Fig 2 and 3). Before inoculation, the chorioallantoic membrane, air sac, and embryo positions were marked by candling. Along with crude enzyme preparation, another egg was inoculated with sterile saline as a control for the experiment. Both the eggs after inoculation were sealed and incubated at 37°C for 24 hours. From Fig.3(A) it can be observed that on the 8th day, the egg was having good capillary growth, and after the 24 hrs of st. saline inoculation, the embryo has still grown and has become thick, the air sac has minimized. When the egg was broken a complete and healthy chick can be seen with body parts developing. While in the case of crude enzyme inoculated egg [Fig.3(B)], the air sac has increased after 24 hrs of inoculation, and blood vessels have completely disappeared. When the egg was broken, the embryo structure was looking abnormal and body parts were not developed, as were in the control one. Chorioallantoic membrane assay was performed, the isolates showed a remarkable anti-angiogenesis activity, protein coagulation and abnormal embryo (Fig.2 and Fig.3).

DISCUSSION

Modified glucose Czapek's Dox medium is used for the cultivation and maintenance of fungi. The medium contains glucose as Carbon source, $NaNO_3$ as a sole nitrogen source, Sodium chloride for osmotic balance, and other microelements required for growth. The pH of the medium is towards the acidic range (pH 6.0) because of which most of the bacteria were inhibited. Almost all researchers and recent publications have used Modified Czapek's dox medium for enrichment and isolation of L-asparaginase producing fungi.[2,17,30] Vala et al., 2015; Chaw et al., 2015 and Ahmad *et al.*, 2016 have used potato dextrose medium for enrichment. For culture preservation, all researchers have used either MCD or PDA slants.

For screening of L-asparaginase producers rapid plate assays are available, which contains Modified Czapek Dox medium

employed with different acid base indicator dyes like phenol red (PR), Bromothymol blue, Bromocresol purple[31] , Methyl red and Azure-S etc.[32] But PR dye is the most commonly incorporated dye.[8,33,34] The isolates producing L-asparaginase enzymes utilized L-Asn composed in MCD agar and converted L-Asn into L-aspartic acid and ammonia. As ammonia is basic, the color of media around the colony or growth changes from yellow to pink due to the presence of phenol red dye as an indicator of the medium. The important point to consider while screening is not only checking for L-asparaginase but the isolate should also be free from glutaminase and urease activity as they interfere with the therapy and give adverse human health effects, and sometimes can be fatal also. Due to the incorporation of PR dye, the color of medium changes from yellow to pink or red depending upon the L-glutaminase potential of isolate.[33,35,36,37] Similarly for the urease test, the urea broth is composed of phenol red dye. So, when the urease enzyme produced by isolate breakdown urea into ammonia and carbon dioxide the pH of the medium becomes alkaline. Hence, the pink color is observed.[8] The production of L asparaginase has been done on crude substrates like pine apple peels, soybean waste and other agro based wastes.[38]

The Nessler's reagent forms complex with ammonia liberated and hence the color developed is equal to the concentration of ammonia liberated. Usha et al., 2011 have also used the same method and Reagent concentrations and have got good results. Vala et al., 2018 and Ashok et al., 2019 have used 50mM Tris-HCL as a buffer and incubated reaction mixture at RT on 37°C and have got proper results. Till date, mostly fungi identified for production of L-ASNase were found to belong to family *Aspergillus, Penicillium and Fusarium.*[39,40,41,42,43]

To study anticancer activity, generally animal cell lines are used. According to literature Leukemic cell lines, Prostate cancer cell line, Breast cancer cell line, and various lymphoma cell lines have been used to study the anti-neoplastic activity of L-asparaginase.[36,44,45,46] Our work is the first to suggest that we can use chorioallantoic membrane assay also to study the anti-angiogenic activity that is one approach to kill malignant cells. Also, we suggest, by using carcinogenic agents, we can perform CAM assay for checking anti-neoplastic activity also.

CONCLUSION

L-asparaginase (LA or L-ASNase) is an amidohydrolase enzyme (EC 3.5.1.1) that converts amino acid L-asparagine into L-aspartate and ammonia. Unlike normal cells, the tumor cells lack the key asparagine synthetase that is required to convert L-Asp into L-Asn. Hence, tumor cells have to depend on adjacent normal cells for their requirement of L-asparagine. The commercially available L-asparaginase shows severe health effects and sometimes can be fatal for a person due to its prokaryotic source of synthesis, L-glutaminase, and urease activity. For isolation of fungi, marine water and soil samples across various regions of Maharashtra, and Goa were collected. A total of 11 isolates were obtained among which only RF2 obtained from Mandvi beach water, Ratnagiri has shown production of glutaminase and urease free L- asparaginase. The crude enzyme preparation of isolate was also subjected to CAM assay where it showed a remarkable anti-angiogenesis activity.

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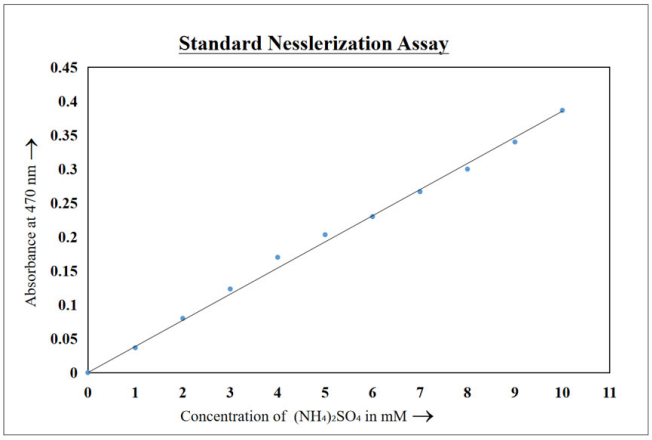
Table 1: List of sites of soil and water samples collected

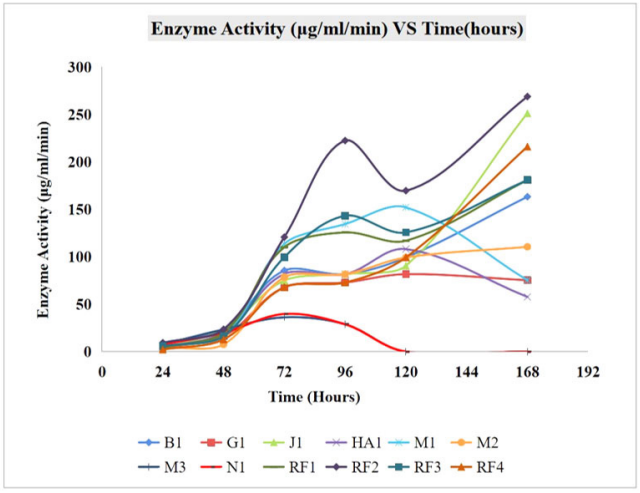
Sr.no.	Site of Sample Collection	Nature of sample (no.)
1.	Marine Drive beach	Water (1) and Soil (1)
2.	Girgaon Chawpatty	Water (1) and Soil (1)
3.	Haji Ali	Water (1) and Soil (1)
4.	Bandstand beach	Water (1) and Soil (1)
5.	Varli sealink	Water (1) and Soil (1)
6.	Juhu beach	Water (1) and Soil (1)
7.	Mahul creek	Water (1) and Soil (1)
8.	Marve beach	Water (1) and Soil (1)
9.	Aksa beach	Water (1) and Soil (1)
10.	Vasai creek	Water (1) and Soil (1)
11.	Alibaug beach	Water (2) and Soil (2)
12.	Nagaon beach	Water (2) and Soil (2)
13.	Akshi beach	Water (1) and Soil (1)
14.	Goa beach	Water (1) and Soil (1)
15.	Mandvi beach	Water (2) and Soil (2)
16	Purngadh Fort	Water (2) and Soil (2)

Table 2: Fungi Colony Characteristics on PDA plates

Sr No.	Name of Isolate	Site of sample	Nature of sample	Colonial Characteristics on PDA plate		
				Mycelium color	Spores color	Pigment production
1	B1	Bandstand beach	Water	White	Pale yellow	Dark pink
2	G1	Girgaon Chawpatty	Water	White	Green	-
3	HA1	Haji ali	Water	White	Green	Yellowish
4	J1	Juhu beach	Water	White	White	Brown
5	M1	Marve beach	Water	White	White	Dark red
6	M2	Mahul Creek	Water	White	White	-
7	M3	Mahul Creek	Soil	Green	Black	-
8	N1	Nagaon beach	Water	White	Dark green	-
9	RF1	Purngad beach	Water	White	Black	Gray
10	RF2	Mandvi beach	Water	White	Brown	Yellowish
J1	RF3	Mandvi beach	Water	White	Dark green	Red
12	RF4	Mandvi beach	Water	White	Green	Yellowish



Graph 1: Standard Ammonium sulfate (mM) VS Abs at 470nm



Graph 2: Enzyme activity (µg/mL/min) VS Time (hours)

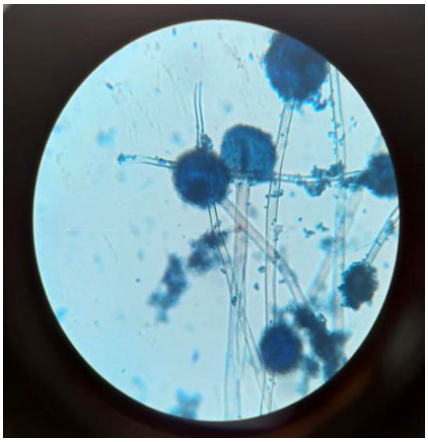


Figure 1: Result of fungal wet mounting.
RF2 showing non-septate hyphae, sporangiospore suspected to be of *Aspergillus sp.*

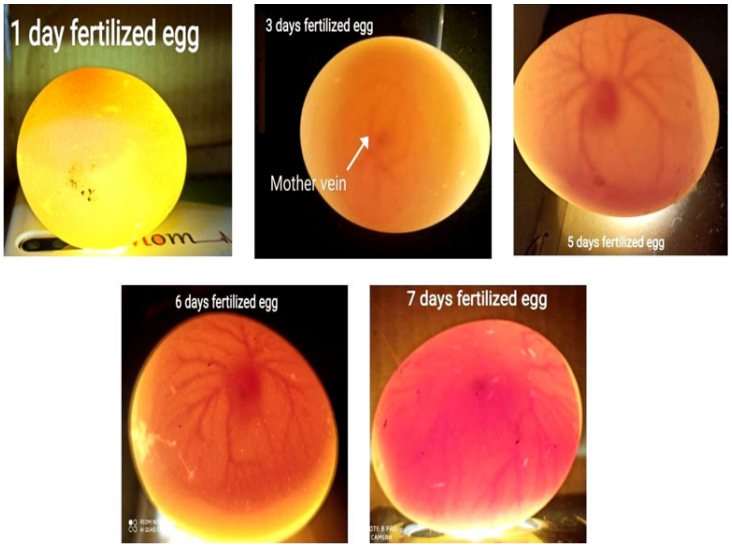


Figure 2: Development of fertilized egg from 1st to 7th day.

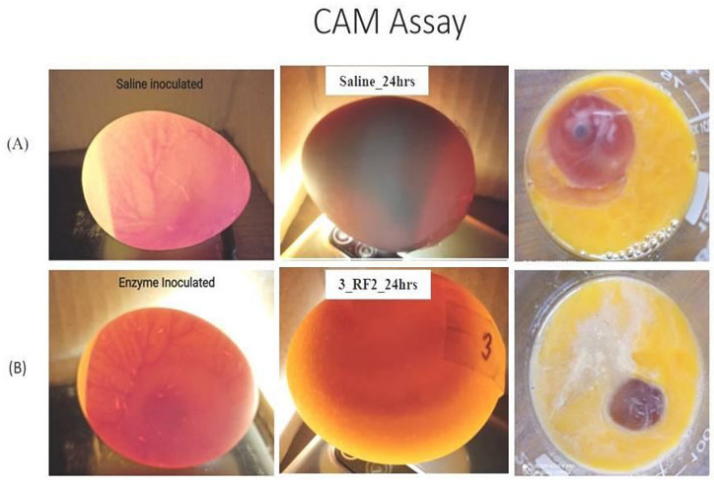


Figure 3: Results of Chorioallantoic membrane (CAM) assay- (A) Egg inoculated with St. Saline as blank/control, and (B) Egg inoculated with Crude enzyme preparation of 72 hrs old RF2 culture isolate.