Research Article

Seaweed Endophytic Fungi: A Potential Source for Glutaminase Free L-Asparaginase

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Abstract

In the search of glutaminase free L-asparaginase enzyme, 60 endophytic fungal strains were isolated from the inner tissue of different seaweeds and in medicinal plants. In total 25 fungal isolates were isolated from the seaweeds and 35 isolates from the medicinal plants. Through preliminary screening for L-asparaginase production for the 25 and 35 isolates from the seaweeds and medicinal plants respectively, 26 isolates were showed L-asparaginase activity. Among the 26 isolates, 15 isolates from seaweeds and 11 isolates from the medicinal plants were showed L-asparaginase activity. Secondary screening of the L-asparaginase producing isolates for glutaminase free form reveals that only 4 isolates from the seaweeds produced free form of glutaminase and the isolates medicinal plants were failed to produce the free form of glutaminase activity. Appearance of pink zone from the four endophytes was identified to be, *Fusarium sps*, *Alternaria sps*, *Aspergillus sps* and *Colletotrichum sps*.

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Introduction

L-asparaginase (EC 3.5.1.1) is an anticancer enzyme, which has more attention towards the treatment of specific kinds of cancer particularly acute lymphoblastic leukemia, since it was reported from *Escherichia coli* [1]. L-asparaginase was applied in the therapeutics due to significant number of patients with acute lymphoblastic leukemia, especially lymphosarcoma. The neoplastic cells are dependent on an exogenous source of L-asparagine for cell proliferation and survival. The most of the therapeutic applications of L-asparaginase enzyme are Hodgkins disease, treatment of acute lymphoblastic leukemia in children, acute chronic leukemia, acute myelocytic leukemia, acute myelomonocytic leukemia, melanosarcoma treatment and lymphosarcoma [2, 3]. The main role of this enzyme is to catalyze the hydrolysis of L-asparagine into L-aspartic acid and ammonia. Neoplastic cells require L-asparagine amino acid for cell proliferation but it does not synthesize due to lack of asparagine synthetase and it uptakes the asparagine amino acid synthesized from the normal cells and thereby it is proliferating. This enzyme degrades the L-asparagine amino acid of tumor cells leads to cell death, due to inability to synthesize this amino acid. Moreover, L-asparaginase degrades the L-asparagine amino acid in the food materials before baking. This gives rise to free amino acid, which is a precursor of acrylamide in foods. L-asparaginase activity has been found in various biological systems such as animal tissues, plants and microbes including actinomyctes, bacteria, fungi and yeast [4-9]. The major part of enzyme activity has been reported in bacterial species such as *Escherichia coli* [10], *Enterobacter aerogenes* [11], *Serratia marcescens* [12], *Erwina carotovora* [13]. Actinomycetes include *Streptomyces karnatakensis*, *Streptomyces gulbargensis*, *Streptomyces venezuelae* [14]. Yeast includes *Candida utilis* [15], *Saccharomyces cerevisiae* [16] and from fungi *Aspergillus terreus*, *Aspergillus tamari*, *Aspergillus niger* [17], *Pencillium* and *Fusarium sps*. Studies on L-asparaginase production from fungi are limited. Although L-asparaginase

enzyme has been isolated from various sources, shows some adverse side effects due to some allergic responses and anaphylaxis in the patients due to glutaminase associated activity in L-asparaginase [18]. The current research should be focused on finding novel sources of the enzyme should be free from glutaminase activity [19, 20]. The present study deals with the isolation of extracellular glutaminase free L-asparaginase from seaweed and medicinal plant endophytic fungi.

Experimental

Collection of Seaweeds and Medicinal plants

Ten different seaweeds and five different medicinal plants were collected from the coastal area of Mandapam region, Rameshwaram district and from the Botanical garden in Madurai Kamaraj University respectively. Algal samples was picked with hand and washed with the sea water to remove the external residues. Then it was collected in sterilized zip lock bags containing sea water. The medicinal plants were collected in a sterilized zip lock polytene bags were brought to the laboratory for processing.

Isolation of endophytes from seaweeds and medicinal plants

The algal thalli and medicinal plants were washed in sea water and running tap water to remove external residues and cut into small segments approximately 0.5cm^2. Segments were surface sterilized with 70% ethanol for 1 min followed by immersion in sterile artificial sea water for seaweeds and Milli-Q water for medicinal plants for 1 min. Sterilized algal tissues were placed on the isolation medium [21]. Sterilized medicinal plant samples were placed on the potato dextrose agar plates containing potato infusion 200g/L; dextrose 20g/L; agar 15g/L and streptomycin sulfate 250 mg/L.

Incubation for fungal growth

The sterilized segments from each algae and medicinal plant leaves were placed on isolation medium and potato dextrose agar (PDA) medium amended with the antibiotic, streptomycin sulfate 250 mg/L. Four segments were placed on each isolation agar and potato dextrose agar medium contained in petri plates and incubated in a light chamber for 2 weeks at 28°C [22]. The Petri dishes were periodically monitored for the growth of fungi from the host tissues. Fungi grown on medicinal plants were separated on PDA medium and fungi grown on algal tissues were initially cultured on isolation medium and after three to four subcultures it has been transferred to PDA medium.

Screening of glutaminase free L-asparaginase strains

Fungal isolates obtained from the seaweeds and medicinal plants were screened for glutaminase free L-asparaginase production on plate assay. To identify Glutaminase free L-asparaginase strains, initially the fungi were screened for L-asparaginase production on Czapek Dox's agar plate's [23]. The medium initial pH 6.2 was supplemented with 0.009% phenol red as indicator. For further screening, L-asparaginase producing strains were screened for glutaminase free L-asparaginase strains on Czapek Dox's agar containing L-glutamine instead of L-aspargine.

Morphological observation of positive isolates

For observing fungal morphology, fungal spores were taken with the fungal inoculation loop from the 5 days old culture grown on PDA plates. Spores were mounted in lactophenol cotton blue staining solution (0.01% w/v) and observed with LX 400 Research microscope (Labomed).

Result and Discussion

Isolation of fungal endophytes from Medicinal plants

Five medicinal plants were collected from the Botanical garden, Madurai Kamaraj University (8°04'46.15'N

77°33'05.45'E) and send to the Department of Plant science, Madurai Kamaraj University. The identified medicinal plants were given in Table 1. Medicinal plant sampling was carried out at the month of September when the temperature was very lower, where the fungal diversity will be higher. Totally 35 fungus isolates were obtained from these medicinal plants. Maximum of 12 fungal isolates were obtained from *Justicia jenderusa* followed by 9 fungal isolates obtained from *Costus spictus* than other medicinal plants.

Isolation of fungal endophytes from seaweeds

In the littoral zone of the Mandapam coastal line, (9°56'13.49'N 78°00'43.08'E) of Rameshwaram district, six species of red algae, three species of green algae, and one species of brown algae were collected and send to Centre for Advanced studies in Botany, University of Madras, guindy campus for taxonomical identification. Identified algal species were shown in Table 2. Algal sampling was carried out at the month of November when the water temperature was very lower than in the summer. Totally 25 fungal strains were isolated from the 10 seaweeds. Comparing the seaweeds, maximum fungal isolates was obtained from the brown algae *Sargassum wightii* followed by the red algae *Gracilaria follifera* than other seaweeds.

Screening of glutaminase free L-asparaginase fungal isolates

Total of 60 fungal isolates from seaweed and medicinal plants were screened for glutaminase free L-asparaginase production. Initially all the fungal isolates were examined for L-asparaginase production. 26 isolates were showed l-asparaginase production, indicating the presence of pink zone formation around the colonies. Among these, 15 isolates from seaweeds and 11 isolates from the medicinal plants were showed L-asparaginase production. Based on the results obtained from the L-asparaginase production, positive isolates were selected to identify the glutaminase free asparaginase producers. Results were showed that, out of 15 L-asparaginase producing isolates from seaweeds, 4 isolates revealed the free form of glutaminase free asparaginase (**Figure 1, Table 2**). Glutaminase free resulted 4 isolates were isolated from *Gracilaria follifera*, *Amphiroa coralline sps*, *Sargassum wightii* and *Amphiroa anceps* respectively. L-asparaginase producing isolates of medicinal plants were failed to produce glutaminase free form L-asparaginase (Table 1).

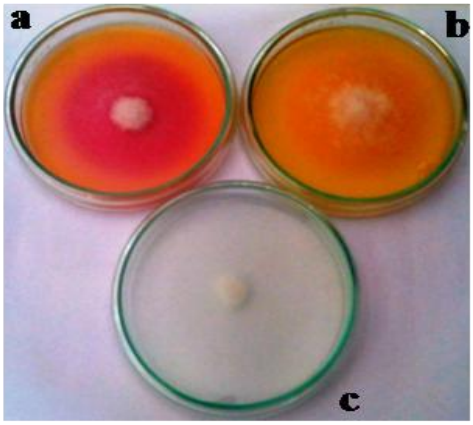

Figure 1 Plate assay. L-asparaginase production, Pink color zone indicates activity (a), Glutaminase free form (b) and without $NaNo_3$ and indicator (c).

Table 1 L-asparaginase and Glutaminase free L-asparaginase activity of endophytic fungi isolated from the Medicinal plants

Medicinal Plant host	Fungal isolates	L-asparaginase	Glutaminase free L-asparaginase
Justicia jenderusa	MEJJ 01	+	-
	MEJJ 02	-	-
	MEJJ 03	+	-
	MEJJ 04	-	-
	MEJJ 05	-	-
	MEJJ 06	-	-
	MEJJ 07	+	-
	MEJJ 08	-	-
	MEJJ 09	-	-
	MEJJ 10	-	-
	MEJJ 11	+	-
	MEJJ 12	+	-
Pleurostylis opposita	MEPO 13	-	-
	MEPO 14	-	-
	MEPO 15	-	-
	MEPO 16	-	-
	MEPO 17	+	-
	MEPO 18	-	-
Morinda citrifolia	MEMC 19	-	-
	MEMC 20	+	-
	MEMC 21	-	-
	MEMC 22	-	-
	MEMC 23	-	-
Sauropus androgynensis	MESA 24	-	-
	MESA 25	+	-
	MESA 26	+	-
Costus spictus	MECS 27	-	-
	MECS 27	+	-
	MECS 27	-	-
	MECS 27	-	-
	MECS 27	-	-
	MECS 27	-	-
	MECS 27	-	-
	MECS 27	+	-
	MECS 27	-	-

Conclusion

Fungi have been widely studied for the isolation of bioactive molecules for its potential application in therapeutics. Several endophytic fungal strains were isolated from the medicinal plants and in seaweeds to confirm the presence of glutaminase free L-asparaginase enzyme. Observations from the result reveals that fungal endophytes isolated from the seaweed have the ability to produce glutaminase free L-asparaginase. This is the first report that the fungal endophytes isolated from the seaweeds to produce glutaminase free L-asparaginase.

Table 2 L-asparaginase and Glutaminase free L-asparaginase activity of endophytic fungi isolated from Seaweeds

Algal host	Fungal isolates	L-asparaginase	Glutaminase free L-asparaginase
Caulerpa racemosa var occidentalis	SECR 01	+	-
	SECR 02	-	-
	SECR 03	+	-
Caulerpa scalfeliformis	SECS 04	+	-
	SECS 05	+	-
Gracilaria corticata	SEGC 06	+	-
Gracilaria follifera	SEGF 07	+	-
	SEGF 08	+	-
	SEGF 09	+	+
	SEGF 10	-	-
Amphiroa coralline sps	SEAC 11	-	-
	SEAC 12	-	-
	SEAC 13	+	+
Grateloupia lithophila	SEGL 14	-	-
Sargassum wightii	SESW 15	-	-
	SESW 16	-	-
	SESW 17	+	+
	SESW 18	-	-
	SESW 19	+	-
Hypnea valantiae	-	-	-
Chaetomorpha antenina	SECA 20	+	-
	SECA 21	-	-
	SECA 22	+	-
Amphiroa anceps	SEAA 23	-	-
	SEAA 24	+	-
	SEAA 25	+	+

+ Presence; – Absence

Morphological observation of positive isolates

Three positive fungal isolates showed maximum appearance of pink zone with free form of glutaminase free L-asparaginase was selected for spore morphological identification. Morphological features of spore and conidia were observed using 400 X microscope. The identified organisms were belonging to the following genera, *Fusarium sps* (Figure 2), *Alternaria sps, Aspergillus sps* and *Colletotrichum sps*.

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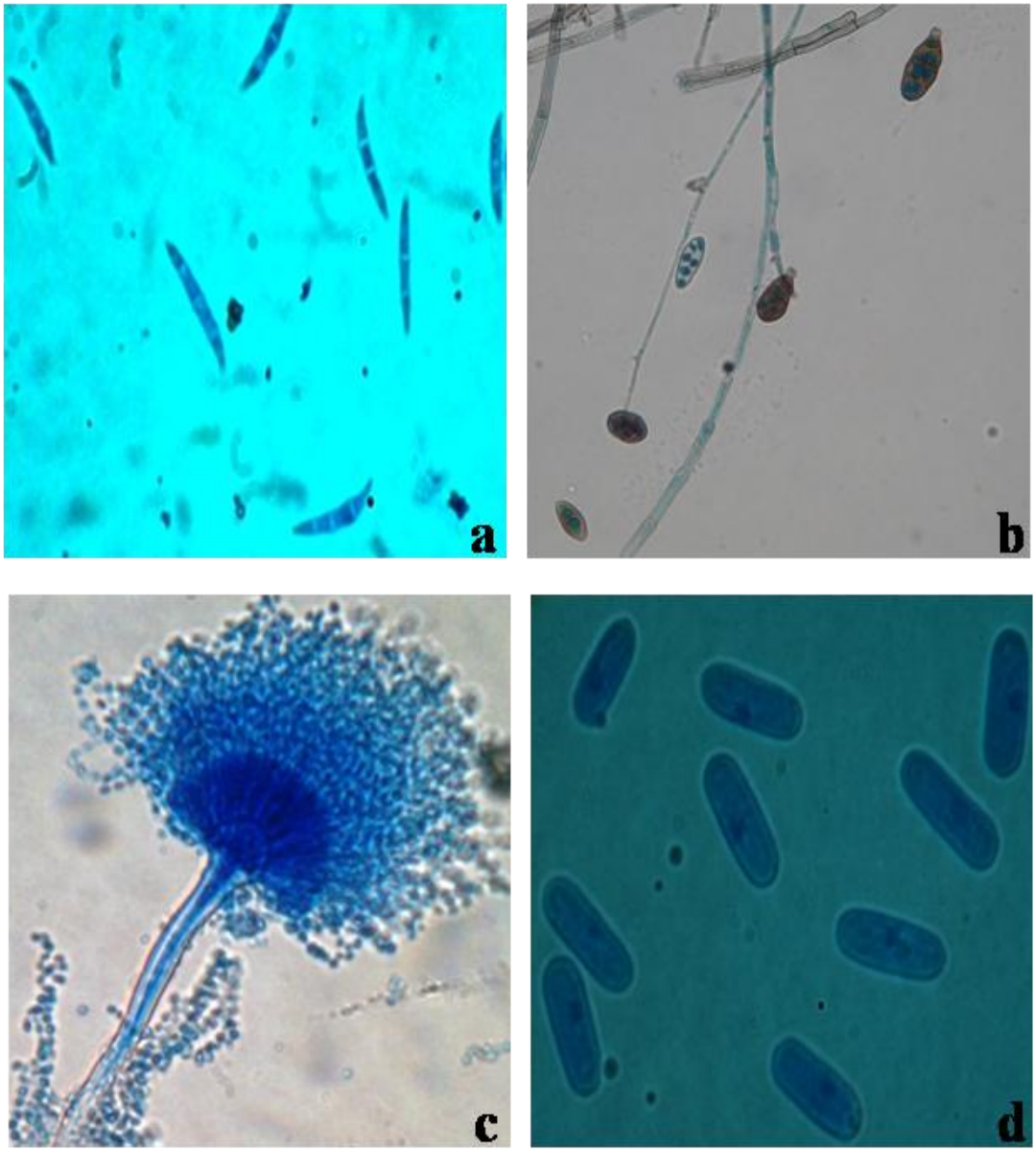

Figure 2 Spore morphology of glutaminase free L-asparaginase strains. *Fusarium sps* (a), *Alternaria sps* (b), *Aspergillus sps* (c) and *Colletotrichum sps* (d).

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