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Short Communication

GLUTAMINASE FREE L-ASPARAGINASE PRODUCING ENDOPHYTES FROM MANGROVES

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ABSTRACT

Objective: To screen endophytes isolated from mangroves for their potential to produce glutaminase free L-asparaginase.

Methods: Endophytes were isolated from leaves and stems of three mangrove plants using surface sterilization technique followed by inoculation of the plant parts in nutrient media. Bacterial and fungal endophytes isolated were tested for production of glutaminase free L-asparaginase by inoculating them in modified M9 and modified czapek dox media, respectively, supplemented with asparagine/glutamine. L-asparaginase activity in glutaminase free L-asparaginase producers was measured by Nesslerization method.

Results: Six bacterial endophytes and one fungal endophyte were found to produce glutaminase free L-asparaginase. The highest L-asparaginase activity was shown by bacterial endophytes of the mangrove *Sonneratia caseolaris*.

Conclusion: Endophytes isolated in the present study hold the potential to produce glutaminase free L-asparaginase, and they need to be considered further in the search of L-asparaginase with high therapeutic index.

Keywords: L-asparaginase, Anticancer drug, Endophytes, Mangroves.

L-asparaginase is an anticancer drug which has revolutionized the therapy for blood cells related cancers such as acute lymphoblastic leukemia (ALL). The presence of L-asparaginase depletes the levels of serum L-asparagine; it takes asparagine and removes its amine, releasing aspartate and ammonia [1]. Most cells in our body use the enzyme asparagine synthetase to make their own asparagine. Leukemic lymphoblasts and certain other tumor cells lack or have very low levels of asparagine synthetase and hence rely on L-asparagine present in the serum for their proliferation and survival [2].

Bacterial sources have been widely used in the production of L-asparaginase. Eukaryotic microorganisms, such as yeast and filamentous fungi, are also reported to produce L-asparaginase. Although this enzyme was found to be very prominent in treating ALL, its use is limited by serious side effects such as liver dysfunction, pancreatitis, leukopenia, neurological seizures, and coagulation abnormalities due to the accompanying glutaminase activity [3]. Glutaminase activity was found to be observed in the majority of the sources that produce L-asparaginase [4]. On the other hand, the anticancer activity of L-asparaginase has also been attributed by some workers to glutaminase activity, especially in asparagine synthetase positive cells [5]. Based on the studies conducted in a glutaminase-free mutant L-asparaginase (Q59L) [6], it was found that glutaminase-negative variants of L-asparaginase would provide larger therapeutic indices than wild type for asparagine synthetase negative cancers and glutaminase activity of L-asparaginase is necessary for anticancer activity only against cancer cells that express significant asparagine synthetase. Hence, much emphasis was given on the production of glutaminase free L-asparaginase enzyme, and many attempts are in progress to produce the enzyme that does not involve the metabolism of glutamine. Protein level modifications in L-asparaginase sourced from *Erwinia chrysanthemi* [7] and *Escherichia coli* [8] were found to yield enzyme with reduced glutaminase activity. As the enzyme's biochemical and kinetic properties vary with the genetic nature of the microbial strain used [9,10], newer organisms are also being explored to identify an enzyme with less adverse effect. Glutaminase free L-asparaginase from bacterial sources, such as *Pectobacterium carotovorum* MTCC 1428 [11,12], *Bacillus licheniformis* [13], and *Pseudomonas plecoglossicida* RS1 [14], have been reported and their production parameters were optimized.

Endophytes or microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects [15], are an underexplored group of microorganisms. Recent studies have shown that secondary metabolites obtained from endophytic microbes possess antimicrobial, antineoplastic, antioxidant, antidiabetic, immunosuppressive, antithrombotic, anti-inflammatory, and anti-Alzheimer's activity [16]. The present study screened endophytes in mangrove plants for their potential to produce glutaminase free L-asparaginase.

Leaves and stems of three mangrove species *Rhizophora mucronata*, *Excoecaria agallocha,* and *Sonneratia caseolaris* were collected from ayiramthengu mangrove forest in the southwest coast of Kerala. Plants were identified based on the records maintained by Aquaculture Development Authority of Kerala, Ayiramthengu mangrove forest. Plant parts were washed in running tap water and were surface sterilized by dipping in 70% ethanol for 3 minutes, 4% sodium hypochlorite for 3 mintues followed by rinsing in sterile distilled water for 2 mintues. Efficacy of the surface sterilization procedure was validated by imprinting the surface sterilized plant part onto nutrient media and was maintained as a control.

The surface sterilized plant parts were cut into small pieces of about 1 cm and were inoculated onto nutrient agar and potato dextrose agar. The bacterial and fungal endophytes growing out from the cut ends were isolated and subcultured. Pure cultures were prepared from isolates with distinct colony morphology. These isolates were tested for their ability to produce glutaminase free L-asparaginase.

Modified M9 (MM9) broth and modified czapek dox (MCD) agar supplemented with L-asparagine and 0.009% phenol red dye (pH 6.2) were used to screen L-asparaginase production by bacterial and fungal isolates, respectively [17]. To check the glutaminase free

form of L-asparaginase, the asparaginase positive isolates were grown in the same media supplemented with L-glutamine instead of L-asparagine [18]. Isolates showing a change in color of the asparagine-containing medium from yellow to pink but maintaining the yellow color of glutamine containing medium were considered as those producing glutaminase free L-asparaginase.

The glutaminase free L-asparaginase producers were taken for L-asparaginase production in shake flasks. Bacteria were inoculated into 100 ml MM9 broth supplemented with asparagine and were incubated in at 35°C at 150 rpm for 48 h. Fungi were inoculated in 100 ml MCD broth supplemented with asparagine and were incubated at 28°C at 150 rpm for 72 h. The bacterial cultures were centrifuged at 10000 rpm for 10 mintues, and fungal cultures were filtered using Whatman No.1 filter paper to separate the cells. L-asparaginase activity in the supernatants was measured by Nesslerization method [19].

Total 8 bacterial endophytes and 8 fungal endophytes were isolated from the different parts of the mangroves under study. They were numbered based on the plant parts from which they were isolated. Seven bacterial endophytes produced L-asparaginase, of which six were glutaminase free L-asparaginase. Out of three L-asparaginase producing fungal endophytes, only one was found to be producing glutaminase free L-asparaginase (Table 1). Phenol red is yellow at acidic pH and turns pink at alkaline pH. The pink in the medium indicates pH alteration originated from ammonia accumulation in the medium as a result of asparaginase/glutaminase production (Figs. 1 and 2).

Shake flask production of L-asparaginase using glutaminase free L-asparaginase strains showed high enzyme activity with bacterial isolates compared to fungal isolate. The highest enzyme activity was shown by bacterial isolates from *S. caseolaris* (Fig. 3).

Table 1: Production of L-asparaginase/glutaminase free L-asparaginase by endophytes

Isolate code	Plant part	L-asparaginase	Glutaminase free L-asparaginase
Bacteria			
RM.S.01	*Rhizophora mucronata*-stem	+	+
RM.S.02	*Rhizophora mucronata*-stem	+	+
RM.L.01	*Rhizophora mucronata*-leaf	+	+
EA.S.02	*Excoecaria agallocha*-stem	+	–
EA.L.01	*Excoecaria agallocha*-leaf	+	+
SC.S.01	*Sonneratia caseolaris*-stem	+	+
SC.L.01	*Sonneratia caseolaris*-leaf	–	–
SC.L.02	*Sonneratia caseolaris*-leaf	+	+
Fungi			
RM.S.01	*Rhizophora mucronata*-stem	+	–
RM.S.02(a)	*Rhizophora mucronata*-stem	–	–
RM.S.02(b)	*Rhizophora mucronata*-stem	+	+
RM.L.01	*Rhizophora mucronata*-leaf	+	–
EA.S.02(a)	*Excoecaria agallocha*-stem	–	–
EA.S.02(b)	*Excoecaria agallocha*-stem	–	–
EA.L.02	*Excoecaria agallocha*-leaf	–	–
SC.S.02	*Sonneratia caseolaris*-stem	–	–

The results of the present study reveal the potential of several endophytes isolated from mangroves to produce glutaminase free L-asparaginase. Fungal endophytes isolated from seaweeds have also been reported to produce glutaminase free L-asparaginase [18]. There is also a report of endophytic fungi isolated from plants of Western Ghats and Rono hills producing glutaminase free L-asparaginase [20]. In our study, bacterial endophytes producing glutaminase free L-asparaginase was more prevalent than fungal endophytes. Further research is needed for optimization of enzyme production, characterization of the isolates, and to study the cytotoxic properties of the enzyme.

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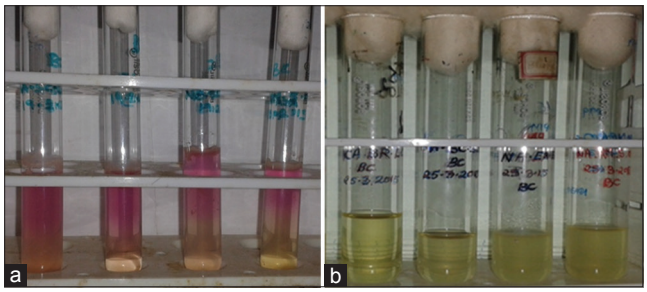

Fig. 1: Bacteria positive for glutaminase free L-asparaginase (a) Modified M9 (MM9) broth supplemented with asparagine (b) MM9 broth supplemented with glutamine

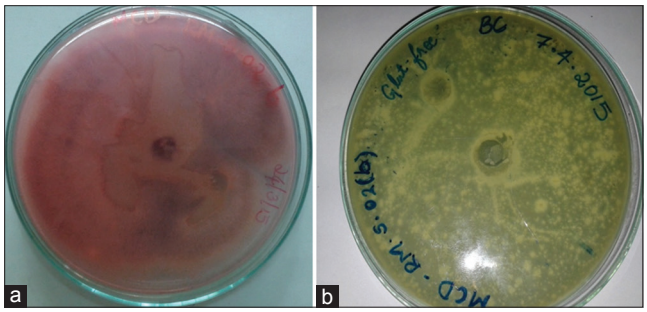

Fig. 2: Fungus positive for glutaminase free L-asparaginase (a) Modified czapek dox (MCD) agar supplemented with asparagine (b) MCD agar supplemented with glutamine

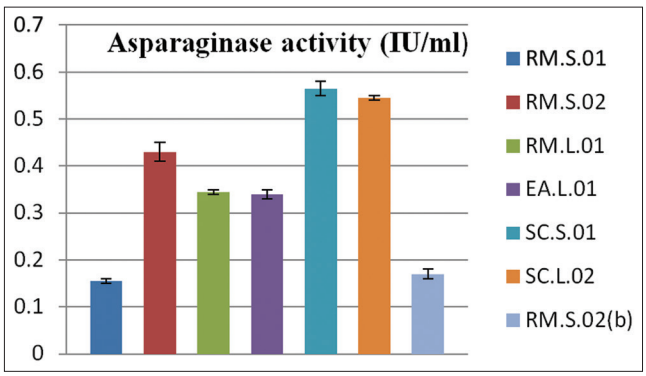

Fig. 3: L-asparaginase activity of bacterial and fungal endophytes positive for glutaminase free L-asparaginase production

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