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Isolation Screening and Identification of Bacterial Endophytes from Medicinal Plants as a potential source of L-Asparaginase Enzyme

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ABSTRACT

L-Asparaginase is an enzyme (EC3.5.1.1) used as chemotherapeutic agent for the treatment of Acute Lymphoblastic Leukemia (ALL). The commercial L-Asparaginase now available is of bacterial origin and since it shows many side effects, search for alternative sources of this enzyme is highly necessary, owing to its high therapeutic significance. In the present study an attempt has been made to isolate bacterial endophytes, with L-Asparaginase potentiality, from medicinal plants. From 16 different medicinal plants 127 bacterial endophytes have been isolated, among which 51 endophytes were capable of producing L-Asparaginase enzyme. Two isolates that showed excellent enzyme activity as 1.436 U/ml and 1.827 U/ml were identified, based on biochemical tests and 16s rRNA gene sequencing, as *Acinetobacter baumannii* and *Bacillus subtilis* which were isolated from the medicinal plants, *Annona muricata* and *Averrhoa carambola* respectively. The phylogenetic tree for the two isolates were constructed based on the 16s rRNA blast results and the 16s rRNA gene sequences were deposited in gene bank and accession numbers were received.

KEY WORDS: *Acinetobacter baumannii*, Acute Lymphoblastic Leukemia (ALL), *Bacillus subtilis*, Endophytes, L-Asparaginase.

1. INTRODUCTION

L-Asparaginase is an enzyme being used as therapeutic drug against Acute Lymphoblastic leukemia (ALL) in children. This enzyme, which catalyzes the hydrolysis of L-Asparagine into L-Aspartic acid and ammonia, helps in the clearance of L-Asparagine in the blood, thereby reducing the availability of this amino acid to leukemic cells for their survival. Due of its efficacy in selective killing of leukemic cells, it is also considered as a targeted therapy (Rizzari, 2013). But since the drug in present use bears many side effects (Barba, 2017; Shrivastava, 2016), researchers are in continuous search of new sources for this enzyme. The non-immunological side effects of this enzyme can be mitigated by the discovery of new sources for L-Asparaginase enzyme and its characterization (Ali, 2016). This enzyme also finds a place in industrial application because of its role in effective reduction of acrylamide levels in fried foods (Zyzak, 2003).

Microorganisms are considered as good source of many bioactive compounds because of their 'easy to handle' nature. Presence of L-Asparaginase in microbes has been established by Mashburn and coworkers in 1963 in *Escherichia coli* (Mashburn & Wriston 1963). Other microorganisms like *Erwinia aroideae* (Peterson, & Ciegler, 1969), *Streptomyces griseus* (DeJong, 1972) and *Bacillus licheniformis* (Golden & Bernlohr, 1985) were also found as good sources of L-Asparaginase enzyme in various studies. Recently there are reports on endophyte derived L-Asparaginase. *Staphylococuus capititis* isolated from the plant *Mentha spicata* (Paglla, 2013) and *Talaromyces pinophilus* obtained from *Curcuma amada* (Krishnapura & Belur, 2015) were found as potential sources of this important enzyme. Abhini and Zuhara had previously reported the production of L-Asparaginase by four fungal endophytes isolated from three different medicinal plants (Abhini & Zuhara, 2016).

2. MATERIALS & METHODS

Isolation of endophytes: Sixteen different plants with known medicinal property (Prajapati, 2006) were selected for the isolation of bacterial endophytes and their fresh leaves were taken as sample for this study. Surface sterilization is an important step in the isolation of endophytes and it was carried out by the method put forward by Petrini (1984). The collected samples were washed under running tap water and immersed in 70% of ethyl alcohol for 30 seconds, followed by dipping in 2% sodium hypochlorite solution for 2 min. The sample was again immersed in 70% ethyl alcohol for 30 seconds and then drained in sterile blotting paper. Washing with sterile distilled water was repeated for 3-4 times. These surface sterilized leaves were then dried under Laminar Air Flow unit, cut into small pieces and injured using sterile scalpel and seeded on to Nutrient Agar (NA) Plates. Plates were incubated at 37^0C for 2 days and endophytes were isolated.

Effectiveness of the surface sterilization has been checked by culturing an aliquot of 0.1 ml water from final rinse on nutrient agar plates and observed for growth of microbes (Cao, 2004).

Screening of Endophytes for L-Asparaginase production: Qualitative assay (Gulati, 1997): Modified M-9 agar media (L-Asparagine-10g/l, KH_2PO_4-3g/l, Na_2HPO_4-6g/l, NaCl-0.5g/l, $MgSO_4.7H_2O$-0.12g/l, $CaCl_2.2H_2O$-0.001g/l and Agar-20g/l) supplemented with the pH indicator, phenol red (0.009%) was used for screening purpose. Endophytes were inoculated into the media and after incubation for 48 hrs. At a temperature of 37^0C, the diameter of the pink zone developed around the colony was measured.

Quantitative assay: (Nesslerization method) (Imada, 1973): The reaction was performed by adding 0.5ml of the supernatant prepared from the endohytic bacterial suspension into 0.04 M L-Asparagine and 0.05 M tris HCl buffer pH 8.6 and incubated at 37^0C for 30 minutes. 0.5 ml of 1.5M Trichloroacetic Acid (TCA) was added to stop the reaction. Blanks were maintained by adding the supernatant after the addition of TCA. The ammonia released was determined by adding 0.2 ml of Nessler's reagent in to tubes containing 0.1 ml of reaction mixture and 3.75 ml of distilled water and incubated at room temperature for 10 minutes and absorbance was read at 450 nm. The enzyme activity was calculated. One unit of L-Asparaginase enzyme is the amount of enzyme which liberates 1 µmol of ammonia in 1 minute at 37^0C.

Estimation of protein (Lowry, 1994): The protein estimation was performed through Lowry's method and specific activity of the enzyme was calculated.

Identification of Endophytes: Biochemical Tests (Aneja, 2003): Biochemical tests were performed to identify the bacterial endophyte and based on the results organisms were identified (Boone, 2001).

16s rRNA gene Sequencing: 16s rRNA gene sequencing was performed for identifying the two selected potent producers of the enzyme. Based on the gene sequences, phylogenetic trees were constructed using neighbor joining method in Mega 7 software. The sequences were deposited in gene bank and accession numbers were obtained.

3. RESULTS & DISCUSSION

In the present study 16 different medicinal plants were selected for the isolation of endophytes. The bacteria emerged from injured plant tissues were considered as endophytes as the control plates vouched for 100% efficacy of surface sterilization. Out of the127 bacterial endophytes isolated, 51 showed L-Asparaginase activity and they were categorized as excellent, good, average and poor producers of enzyme based on the qualitative enzyme assay (Table.1). The remaining 76 isolates were non producers of L-Asparaginase enzyme.

Table.1. L-Asparaginase activity of bacterial endophytes isolated from medicinal plants

Medicinal Plants	Total	No. of isolates showing enzyme activity				Total	Negative
	No. of isolates	Excellent	Good	Average	Poor	positive	Isolates
		*(>3.5)	*(2.5- 3.5)	*(1.5- 2.5)	*(< 1.5)		
Adathoda vasica	5	0	1	0	0	1	4
Annona muricata	5	1	1	0	0	2	3
Averrhoa bilimbi	12	0	2	1	1	4	8
Averrhoa carambola	11	1	3	2	1	7	4
Catahranthus roseus	7	0	2	1	0	3	4
Centella asiatica	15	0	5	3	1	9	6
Curcuma longa	6	0	1	1	1	3	3
Garcinia cambogia	8	0	1	1	1	3	5
Gloriosa superba	7	0	1	0	1	2	5
Hibiscus rosa sinensis	7	0	0	2	0	2	5
Manihot esculenta	8	0	0	2	0	2	6
Momardica charantia	5	0	1	0	1	2	3
Ocimum sanctum	6	0	1	0	2	3	3
Phyllanthus niruri	7	0	1	1	0	2	5
Physalis angulate	9	0	1	1	0	2	7
Theobroma cacao	9	0	1	1	2	4	5
	127	2	22	16	11	51	76

* Diameter of the pink zone around the colony in centimeters

The two bacterial isolates showing excellent activity (pink zone area of more than 3.5cm) were considered as Sample 1 (S1) and Sample 2 (S2); endophyte of *Annona muricata* and *Averrhoa carambola* respectively were selected for further study (fig.1.).

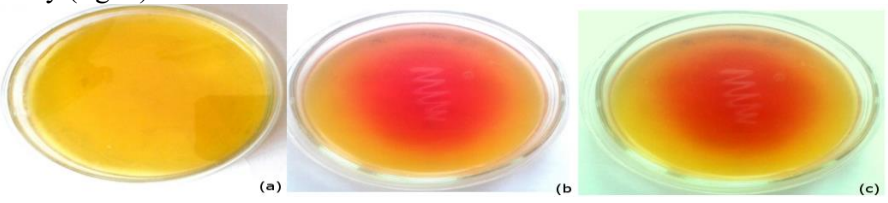

Figure.1. Plates showing control (a) and test results of S1 (b) and S2 (c)

The quantitative enzyme activity of two endophytes, selected based on their qualitative assay, when measured through Nesslerization method; they showed activity of 1.436 U/ml (S1) and 1.827 U/ml (S2). Based on biochemical test results and 16s rRNA gene sequencing, these two organisms that showed high activity were identified. The nucleotide blast result of 16s rRNA gene sequencing revealed that S1 shows 100% identity to *Acinetobacter baumannii strain Canada BC5* and S2 shows 100% identity to *Bacillus subtilis strain* H-1.

The quantitative measurement of enzyme activity of *Acinetobacter baumannii* isolated from the medicinal plant *Annona muricata* and *Bacillus subtilis* isolated from *Averrhoa carambola* is presented in table.2. The agarose gel photograph of DNA and PCR products used for 16s rRNA gene sequencing is shown in fig.2.

Table.2. Enzyme activity and specific activity of selected endophytes

Sl. No.	Medicinal Plant	Sample	Pink zone (cm)	Enzyme Activity (U/ml)	Specific Activity (U/mg)
1	*Annona muricata*	S1	3.6	1.436	2.48
2	*Averrhoa carambola*	S2	4.1	1.827	2.88

Figure.2. Agarose gel photograph showing DNA and PCR product used for 16s rRNA sequencing

The phylogenetic tree was constructed using the sequences obtained from 16s rRNA gene sequence nucleotide blast result (Fig.3). The bootstrap values of the phylogenetic tree indicate confidence limits of the phylogenies based on percentages of 1000 replications. The 16s rRNA gene sequences were deposited in gene bank and accession numbers were obtained (Table.3).

Table.3. The accession numbers of published sequences in public database of National Centre for Biotechnology Information (NCBI)

Sl. No.	Medicinal Plant	Sample	Strain	Accession No.
1	*Annona muricata*	S1	*Acinetobacter baumannii* ZAS1	KX186685
2	*Averrhoa carambola*	S2	*Bacillus subtilis* ZAS2	KX431145

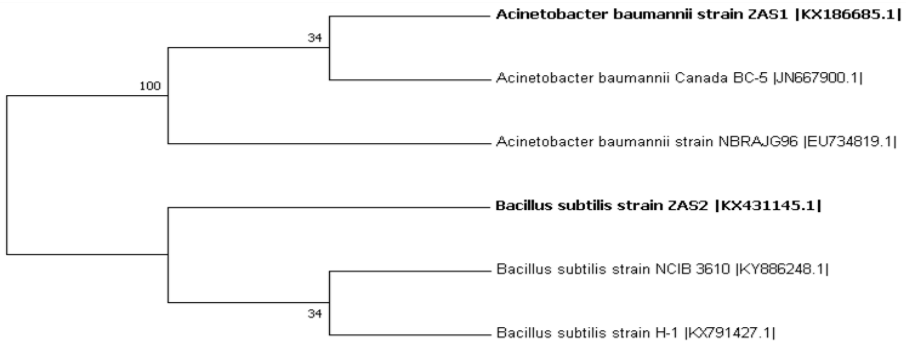

Figure.3. Neighbor-joining tree for bacterial endophytes showing the positions of two potent L-Asparaginase producers

There are several reports on the production of L-Asparaginase by nonendophytic bacterial isolates (Peterson & Ciegler 1969; DeJong, 1972; Golden & Bernlohr 1985). But this study has revealed L-Asparaginase production by endophytic bacteria derived from medicinal plants. The finding is supported by some previous studies (Paglla, 2013; Krishnapura & Belur 2015; Bhagat, 2016) also. L-Asparaginase production by endophytes like endophytic *Serratia marcescens* (0.8579 U/ml), endophytic *Bacillus methylotrophicus* (0.8379 U/ml) (War Nongkhlaw & Joshi 2015) and endophytic *Pseudomonas oryzihabitans (*2.1 U/ml) have also been reported recently (Bhagat, 2016).

4. CONCLUSION

The present study establishes the L-Asparaginase enzyme production of bacterial endophytes *Acinetobacter baumannii ZAS1* and *Bacillus subtilis ZAS2* isolated from medicinal plants *Annona muricata* and *Averrhoa carambola* respectively. Since these plants are reported to have anti-cancerous properties, it can be attributed to

their lodging of these L-Asparaginase producing bacterial endophytes. Further extension of the study involving media optimization, enzyme purification and characterization may throw more light to the potential of this enzyme as a therapeutic agent.

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