International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Isolation of L-Asparaginase Producing Endophytic Bacteria from Plants Recommended for Cancer Therapy

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Abstract: ***Endophytic microorganisms are recognized as potential source of novel chemical molecules that might be useful in the treatment of infectious diseases. This study was conducted with the aim to isolate and identify endophytic bacteria from medicinal plants recommended for cancer therapy with a potential to produce L-asparaginase. Five different plants were selected to enhance diversity as Catharanthus roseus, Oscimum sanctum, Alovera, Withania somnifera and Morrayo konengi. Isolation of endophytic bacteria was done on Tryptic soy agar media and characterized on the basis of morphological and biochemical characteristics. A total 45 bacterial endophytes were obtained out of which 25 showed varied levels of L-asparaginase activity.***

Keywords: Endophytes, L- asparaginase, Tryptic soy agar

1. Introduction

In Ayurveda, plants were used as source of different medicines having broad application in treating various diseases. Drug discovery from medicinal plants continues to provide an important source of new drugs. Herbal remedies have been used to cure a variety of disorders or conditions such as diabetes, cardiovascular problems, dermal infirmities and cancer (Shukla *et al*., 2014). Plants recommended for cancer therapy involves, *Catharanthus roseus*, *Oscimum sanctum, Alovera, Withania somnifera, Allivum sativum, Curcuma longa* etc. (Dixit and Ali, 2010). Plant-associated bacteria that live inside plant tissues without causing any harm to plants are defined as endophytic bacteria. Plants are constantly involved in interactions with a wide range of bacteria. These plant associated bacteria colonize the rhizosphere, phyllosphere (epiphytes) and the inside of plant tissues (endophytes). Endophytic bacteria in association with the rhizospheric bacteria exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and resistance to plant pathogens (Mahajan *et al.*, 2014). As a phylogenetic view, endophytic bacteria are between saprophytic bacteria and plant pathogens. Endophytic bacteria behave as biotrophic symbionts either obligate or facultative. The natural therapeutic compounds produced by endophytic bacteria do have several potential applications in pharmaceutical industry.

Endophytes, microorganisms that reside internal tissues of all plant species, are a proven source of novel organic natural molecules, presumed to emphasizing the frontiers of drug discovery. Next to the clinically acknowledged antineoplastic agent, taxol, endophyte research has yielded potential drug, given compounds with antimicrobial, antioxidant, antiviral, antidiabetic, anti-alzheimer's disease and immunosuppressant etc. These evidences arises a hope to combat incurable diseases, drug resistance, other challenges related to human health. The potential of finding new drugs that may be effective candidates for treating newly developing diseases in humans is great.

Research on production of anticancer agents from various medicinal plants was done at a large scale. Oza, *et al*., (2009) carried out production of L- asparaginase, enzyme with antitumor activity from plant *Withania Somnifera*. They utilized different parts of the plant for screening of L-asparaginase enzyme. Less attention was on manufacture of new anticancer medicines or L- asparaginase enzyme from microbial endophytes of same medicinal plants recommended for cancer therapy.

Production of enzymes for therapeutic use is an important facet of today's pharmaceutical industry. L- Asparaginase (L-aspargine amido hydrolase, E.C. 3.5.1.1.) is an antineoplastic agent used in lymphoblastic leukemia (Basker, 2009). Cancer cells differentiate themselves from normal cells, in diminished expression of L- aspargine due to absence of an important enzyme L- Aspargine synthetase (Manna *et al.*, 1995; Swain *et al.*, 1993). Hence, they depend upon circulating plasma pools for L- aspargine.

2. Materials and Methods

A) Selection of plants and isolation of endophytic Bacteria: In the present study, total 5 five different plants viz. *Withania somnifera* (Ashwagandha), *Ocimum sanctum* (Tulsi), *Alovera* (Korfad), *Catharanthus roseus* (Sadaphuli) and *Morrayo konengi* (kadipatta) recommended for cancer therapy and readily available at Parbhani district were selected for the isolation of endophytic bacteria . Different plant samples viz. root, inner bark, leaves, flowers etc. were collected from each mature healthy plant and were immediately processed. The plant samples were initially subjected for surface sterilization as per the methodology given by Costa L.E.O. *et al*., (2012) along with some modifications. The surface sterilized plant parts viz. leaves and flowers were further ground with 6ml 0.9% NaCl solution using sterile pestle and mortar and kept aseptically for 15-20 min. for the release of endophytic bacteria from host tissue. The tissue extract was diluted with 0.9% NaCl solution and plated on tryptic soy agar medium plates. The

plant parts viz. inner bark, roots were cut into pieces with sterile knife to excise inner tissues. The excised inner tissues were further inoculated on tryptic soy agar medium plates. All the plates were incubated at 30^0 C for 3-5 days. After incubation, various colonies were selected showing different morphological and growth characters. The colonies were further maintained as pure culture on nutrient agar slants for further use.

B) Screening of endophytic bacteria for L-asparaginase production- Screening procedure was based on the principle where the pH indicator phenol red is incorporated in medium containing asparagines (as sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing L-asparaginase. For screening Petriplates and M 9 medium supplemented with 2.5% phenol red was prepared and autoclaved. Media was poured into sterile petriplates under sterile conditions in laminar air flow. Media was allowed to solidify. After solidification the purified cultures were streaked on the solidified plates. Plates were incubated at 37°C for 48 hours. Plates were observed for formation of pink zone around colonies. Screening for maximum L-asparaginase was done by primary screening technique. The primary screening was done by streak plate method on M-9 medium, shows production of pink zone around L-asparaginase producing bacterial colony. (Gulati *et al*., 1997).

3. Results

A) Selection of plants and isolation of endophytic Bacteria

Plants with ethnomedicinal history, as selected in this study were, *Withania somnifera* (Ashwagandha), *Ocimum sanctum* (Tulsi), *Alovera* (Korfad), *Catharanthus roseus* (Sadaphuli) and *Morrayo konengi* (kadipatta) were available everywhere and also with variable medicinal properties along with their application in cancer therapy. A total 45 endophytic bacteria were isolated in this study from the above medicinal plants by adopting methods of Costa L.E.O. *et al*., (2012).

B) Screening of endophytic bacteria with potential of L-asparaginase production:

Main aim of present study was to determine the prevalence, properties, persistence and type of endophytic bacteria with L-asparaginase activity in selected plants recommended for cancer therapy .The isolated bacterial endophytes were evaluated for their ability to produce L-asparaginase, an anticancer enzyme supported anticancer properties of plants. Out of isolated 45 bacterial endophytes, 25 isolates showed L-asparaginase activity. The bacterial endophyte obtained from Kadipatta leaves showed high potency for L-asparaginase production.

Microbial endophytes with L-asparaginase activity

Isolated bacterial endophyte

Table 1: Isolation and screening of L-asparaginase producing endophytic bacteria from different medicinal plants

Sr. No.	*Name of plant*	*Total no of isolates*				*Total*	*No. of isolates showing L-asparaginase*	*Total (%)*
		Root	*stem*	*Leaves*	*Fruit/flower*			
1.	Withania somnifera	04	02	03	01	10	08	80
2.	Ocimum sanctum	03	01	04	-	08	04	50
3.	Alovera	02	05	-	-	07	05	71.42
4.	Catharanthus roseus	03	02	02	03	10	05	50
5.	Morrayo konengi	05	01	03	01	10	03	30
	Total	17	11	12	05	45	25	

C) Characterization of endophytic bacterial isolates

Morphological characterization of L-asparaginase producing strains were done by maintaining pure Cultures of bacteria and observing its colony characteristics on nutrient agar plate. Biochemical characterization was done by preferred convential methods using different cultural media. The probable endophytic bacterial species with L-asparaginase activity which isolated from plants were *Bacillus* sp, *Pseudomonas* sp and *Serratia* sp, *Streptococcus* sp , *Enterobacter sp* and *Micrococcus* sp. Majority of bacterial strains isolated were belong to *Bacillus sp*.

Table 2: Morphology and cultural characteristics of L-asparaginase producing endophytic bacteria [AG- Acid and gas production, A-acid production, + [Positive], _ [negative]

Strain no.	Grams nature	Motility	Biochemical characters													
			IMVIC I	MR	C	VP	Sugar utilization Glu	lacsucrose	mannitol		H_2S pro^n	Catalase	Oxidase	Urease	Nitraté	Probable sp.
1	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
2	Gram positive	Non-motile	-	+	-	-	A	A	A	A	-	-	-	-	-	Streptococcus sp
3	Gram negative	motile	-	-	+	+	AG	AG	AG	A	-	+	-	-	+	Enterobacter sp
4	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
5	Gram négative	motile	+	-	+	+	A	-	_	_	-	+	+	+	+	Pseudom-onas sp
6	Gram négative	motile	+	-	+	+	A	-	_	_	-	+	+	+	+	Pseudomonas sp
7	Gram positive	Non-motile	-	-	-	+	-	-	-	A	-	-	-	+	+	Micrococcus sp
8	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
9	Gram négative	motile	-	-	+	+	A	_	A	A	-	+	-	_	+	Serratiasp.
10	Gram négative	motile	+	+	_	_	A	-	A	A	-	+	-	_	+	Serratiasp.
11	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
12	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
13	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
14	Gram negative	motile	+	-	+	+	A	-	_	_	-	+	+	+	+	Pseudomonas sp
15	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
16	Gram positive	Non-motile	-	+	-	-	A	A	A	A	-	-	-	-	-	Streptococcus sp
17	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
18	Gram negative	motile	+	-	+	+	A	-	_	_	-	+	+	+	+	Pseudomonas sp
19	Gram négative	motile	-	-	+	+	A	_	A	A	-	+	-	_	+	Serratiasp.
21	Gram négative	motile	-	-	+	+	A	-	A	A	-	+	-	_	+	Serratiasp.
22	Gram negative	motile	+	-	+	+	A	-	_	_	-	+	+	+	+	Pseudomonas sp
23	Gram positive	motile	-	+	+	-	AG	-	AG	A	+	+	+	+	+	Bacillus sp
24	Gram positive	Non-motile	-	-	-	+	-	-	-	A	-	-	-	+	+	Micrococcus sp
25	Gram negative	motile	+	-	+	+	A	-	_	_	+	-	+	+	+	Pseudomonas sp

4. Discussion

Isolation and characterzation of endophytic bacteria was performed from different plants recommended for cancer therapy. As these were candidates for study, since the medicinal uses to which the plant may have been selected relates more to its population of endophytes than to its biochemistry itself. Significant variations in the populations of indigenous endophytes have been reported. These variations are attributed to plant source, plant age, tissue type, time of sampling and environment. Generally bacterial populations are larger in roots and decrease in the stems and leaves (Strobel and Daisy 2003).

Although plants selected in this study have been studied previously for their anticancer properties, but , these was not evaluated for their endophytic bacteria with potential of L-asparaginase activity. Maximum research was done on isolation of bacterial endophytes from medicinal plants with antibacterial activity, then studies on production of anticancer agents from plants (Sakarkar *et al*., 2011). There was also extraction of anticancer enzyme L-asparaginase from *Withania somnifera* (Oza *et al*., 2009) but scarcity of work observed in isolating bacterial endophytes from anticancer medicinal plants with a potential to produce L-asparaginase.

Different species of endophytic fungi were also get isolated fom Thai medicinal plants with L-aspaaginase actifvity by Theatana *et al*.,(2007). Hung *et al*.,(2004) also perform the isolation and characterization of endophytic bacteria using 16s rRNA sequencing. The study of endophytic microorganisms is impotant to comparehend their interaction with their host plants. Endophytic microorganisms may have biotechnological applications. Bacterial endophytes also support therapeutic properties of plants. National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity (Shoeb,2005). Commercially 92 anticancer drugs available Worldwide out of which 60% are of natural origin (Cragg *et al*., 1997).

Much attention has been focused on microbial L-asparaginase because of their use as therapeutic use in treatment of certain types of human cancer. Mostly L-asparaginase isolated from E.coli and Erwinia carotovora have been used in the treatment of acute lymphoblastic leukemia (Dhavegi and Poorani, 2006). The major disadvantage of using L-asparaginase as drug is its hypersensitivity reactions. According to such complications recent research focused on decreasing immune reactivity of available L-asparaginase either by modifying it or by discovering a new L-asparaginase that serologically different but have similar therapeutic effects. The latter approach may require screening of microbial endophytes from medicinal plants recommended for cancer therapywhich were until not get attended.

5. Conclusion

Since, different microbial sources of L-asparaginase are available today but they have undesirable side effects so the need of discovering new bacterial sources of this enzyme still exists.Data exhibited in this study suggests that

examined anticancer medicinal plants are good source to search endophytic bacteria emphasizing the potential of L-asparaginase activity can be used in pharmaceutical industry to devlop novel anticancer drugs .

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