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Reearch Article

IMPACT OF ENDOPHYTIC *RALSTONIA* SP. FROM *ALOE VERA* GEL AND ITS ANTIMICROBIAL ACTIVITY

ASTHA SINHA, RUCHIKA PRIYA, MADHUMATHY, NIMISHA, JABEZ OSBORNE W*

Department of School of Biosciences and Technology, VIT University, Vellore - 632 014, Tamil Nadu, India.
Email: jabezosborne@vit.ac.in

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ABSTRACT

Objective: Isolation of endophytic *Ralstonia sp.* from *Aloe vera* gel and its antimicrobial activity. This study was conducted to determine the significance of endophytic bacteria in *A. vera* gel for the prevention and treatment of various infections caused by bacteria.

Methods: The endophytic bacteria from *A. vera* gel were isolated using Luria-Bertani media. The six isolates obtained were found to be Gram-negative, which was mass multiplied and bacterial secondary metabolites were extracted using various solvents. Antimicrobial activity was detected by agar-well diffusion method and was tested against *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli.* Bacterial strains were characterized using 16S rRNA technique.

Result: Ribosomal database project 10.28 release showed that nearest neighbour for VITNARMJ-3 is *Ralstonia pickettii,* accession no AY741342 (sequence similarity 94.6%). Cut off <98.7% identical to the nearest neighbor are always members of different species.

Conclusion: The bioactive compounds obtained from isolate VITNARMJ3 can be effectively used in food and pharmaceutical industries against various bacterial pathogens.

Keywords: Endophytes, *Aloe vera,* Bioactive compounds, Antimicrobial activity, 16S rRNA sequencing.

INTRODUCTION

Plants have been an important source of medicine for thousands of years [18]. Even today 80% of peoples rely mainly on traditional remedies such as herbs for their medicines. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases [9]. The use of plant extracts, with known antimicrobial properties, can be of great significance in the treatment of various microbial infections [40].

Aloe vera is a plant which belongs to the family Liliaceae and is mostly succulent with a whorl of elongated, pointed leaves [41]. Despite its wide use as a folk remedy over a long time, the biochemical functions have not been systematically investigated [38]. The plant has been presented as a constituent of many phytochemicals, vitamins, nutrients, and anti-nutrients found in foods [27]. The leaves have a high capacity of retaining water also in very warm dry climates and therefore this plant can survive very harsh circumstances where most other vegetation disappears [44]. *A. vera* gel consists of 99.3% water [3]. The remaining 0.7% is of solids with glucose and mannose constituting for a large part. These sugars together with the enzymes and amino acids in the gel give it special property [1].

Endophytes are microorganisms that include bacteria and fungi living within plant tissues without causing any immediate overt negative effects have been found in every plant species examined to date and recognized as the potential sources of novel natural products for exploitation in medicine, agriculture and industry with more bioactive natural products isolated from the microorganisms [6,42,22]. Endophytes are ubiquitous with rich biodiversity, which have been found in every plant species examined to date. It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant is the host to one or more endophytes [42]. Mutualism interaction between endophytes and host plants may result in fitness benefits for both partners [36]. However, only a handful of them have been described, which means the opportunity to find new and targeting natural products from interesting endophytic microorganisms among myriads of plants in different niches and ecosystems is great. Some of the endophytes are the chemical synthesizers in inside the plants [34]. Endophytic bacteria embrace a wide variety of species and genera from nonpathogenic relationships with their hosts: Some beneficial, some neutral, and some detrimental. Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against human pathogens and some of these compounds have been proven useful for novel drug discovery [5].

Studies have been reported on natural products including substance of alkaloids, terpenoids, flavonoids, steroids, etc. from endophytes [19]. Thus by far, they have not been widely explored for therapeutic properties. A single endophyte may be able to produce not one but several bioactive metabolites. As a result, the role of endophytes in the production of novel structures for exploitation in medicine is receiving increased attention [46,12,13]. Endophytes provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones and others. Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents. Antimicrobials in one form or another have been in use for centuries [43].
The present study was focused on the isolation of endophytic bacteria from *A. vera* gel which is capable of showing antibacterial activity that can be used in food and pharmaceutical industries against various bacterial pathogens.

METHODS

Sample collection and surface sterilization

A. vera plant was collected from VIT University. The leaves were surface sterilized using sterile distilled water, followed by 70%

ethanol (v/v) for 2 minutes and washed again with distilled water, disinfected by immersing in sodium hypochlorite solution (200 ppm) for 5 minutes, and rinsed with distilled water [20]. Plant samples were subsequently rinsed thrice in sterile de-ionized water, to remove the contaminants [39,47].

Isolation of endophytic bacteria from *A. vera* gel

In sterile condition a cut was made in the plant and a loopful of gel was streaked onto Luria-Bertani (LB) media and was incubated at room temperature for 24 hrs. Further the gel was also inoculated in LB broth and was incubated for 24 hrs. From the inoculated samples 50 µl was transferred into plates containing LB media. After incubation, the endophytic bacterial colonies appeared [33,11,8].

Phenotypic and genotypic characterization of effective isolate

According to Bergey's Manual various biochemical tests were performed: Gram staining, endospore stains and catalase test were carried out with the effective isolate VITNARMJ3 [47,14].

Growth kinetics studies

For growth kinetics studies 50 ml of media was prepared in 250 ml conical flask and autoclaved at 121°C and 15 lbs pressure for 15 minutes this was inoculated with bacterial culture and incubated at 37°C for 24 hrs in orbital shaker at 120 rpm. 1.5 ml of this culture was inoculated in 150 ml of LB broth, and optical density readings were obtained at 600 nm every 2 hrs interval till stationary phase was achieved [11].

Mass multiplication

For mass multiplication studies, 350 ml of media was prepared in 500 ml flask and autoclaved at 121°C and 15 lbs pressure for 15 minutes, 6.5 ml of inoculum (24 hrs old) was inoculated onto this media by using sterilized loop and incubated again at 37°C for 6 days and the flask was kept in orbital shaker at 120 rpm [35].

Solvent extraction

After mass multiplication, the culture was centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected, and an equal volume of hexane and chloroform were added, respectively. Solvent and culture broth were separated using a separating funnel by shaking vigorously shaking for 15 minutes. The solvent extracts were concentrated and dissolved in 1 ml hexane and chloroform respectively [21].

Antibacterial assay

Antibacterial activity was assessed by agar-well diffusion method against *Pseudomonas aeruginosa* MTCC No. 10462*, Staphylococcus aureus* and *Escherichia coli* MTCC No. 9721 [31]. A 100 µl aliquot of the obtained solvent extract (hexane and chloroform respectively) was transferred into wells on agar plates previously spread with selected pathogens. The plates were incubated at room temperature for 24 hrs to observe the zone of inhibition [26].

Molecular characterization using 16S rRNA sequencing

Bacterial strains were characterized using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTAC GACTT-3') DNA was extracted from cells and the 16S rRNA sequence was determined by the fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on an ABI13730XL capillary DNA sequencer (ABI Prism 310 genetic Analyzer, Tokyo, Japan). The aligned sequences were computed using ClustalW softwares and sequence homologies were determined using BLASTn search to create an evolutionary distance matrix [37].

RESULTS

Isolation

Based on different cellular and morphological characteristics six different bacterial isolates were obtained and maintained on LB media.

Phenotypic and genotypic characterization of effective isolate

Based on visual identification and colony morphology, the isolates were differentiated. All the isolates were circular in shape and opaque in nature with a smooth surface. Of six isolates, five were off white in color having an entire margin from colony morphology studies the most effective isolate VITNARMJ3 was examined for further studies. According to Bergey's manual staining and biochemical tests were performed [2]. Based on colony morphology and biochemical characterization the strain was found to be Gram-negative, rods and hence was identified as *Bacillus sp.*

Growth kinetics studies

Absorbance was measured at 600 nm for every 2 hrs interval. It was observed that on 10 hrs stationary phase was achieved for VITNARJ3 (Fig. 1) [11].

Solvent extraction

Solvent extraction was performed using hexane and chloroform to purify the secondary metabolites of VITNARJ3 broth. The amount of the bioactive component left after air drying was calculated by the formulae mention below. The amount of hexane extract was found to be 0.16 g and chloroform extract was 0.15 g respectfully, which was later dissolved in 2 ml of the respective solvents.

The percentage of secondary metabolites extracted was calculated by the following formulae:

Percentage of extraction = (Weight of the beaker after evaporation of solvent − Weight of initial beaker) × 100

Antibacterial assay

The bioactive component obtained by solvent extraction was tested for its antimicrobial activity against *P. aeruginosa* (MTCC No. 10462*),* *S. aureus* (MTCC No. 3160), *E. coli* (MTCC No. 9721). Zone of inhibition was found to be Fig. 2. Bacterial secondary metabolites in hexane extract, showed inhibitory activity against *S. aureus* with a zone of inhibition of 24 mm and in chloroform extract activity was observed against *Pseudomonas sp*. giving a zone of inhibition of 24.5 mm respectfully.

Identification of bacteria by 16S rRNA sequencing

Bacterial strains were characterized using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTAC GACTT-3') DNA was extracted from the cells and 16S rRNA sequence was determined by the fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on ABI-13730 into L-capillary DNA sequencer (ABI13730XL). Capillary DNA sequencer (ABI Prism 310 Genetic analyzer, Tokyo, Japan). The aligned sequences were computed using ClustalW software and sequence homologies

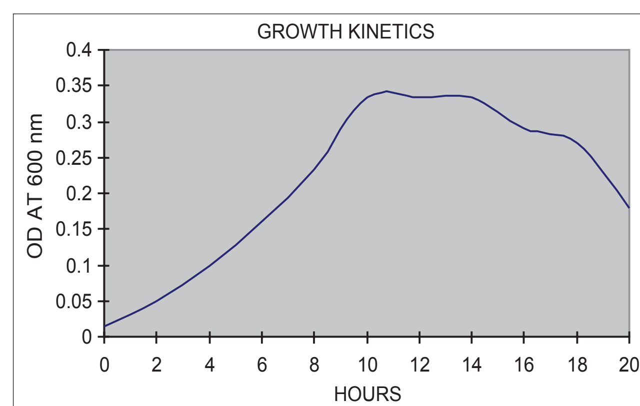

Fig. 1: Growth curve of isolate VITNARMJ3 obtained after growth kinetics studies

were determined using BLAST search to create an evolutionary distance matrix (Fig. 3) [37].

DISCUSSION

Plants derived compounds have been a major source for the discovery of novel drug and have made large contributions to human health. Although significant number of studies have been carried out in endophytic bacteria from plants. This is the first report on the presence of endophytes from *A. vera* gel extracts. *A. vera* has been widely used in skin cares, cosmetics and as nutraceuticals, in various skin conditions such as cuts, burns, and eczema. Microorganisms have been used for discovery of novel drug for the past few decades, but presence of endophytic bacteria in *Aloe barbendosis* was reported by Mamta, *et al.* 2012 [29].

The isolation of endophytic bacteria from *A. vera* was carried out with the idea that unusual endophytes may produce unexploited natural products. In the present study, the impact of endophytic *Ralstonia sp.* from *A. vera* gel and its antimicrobial activity was studies. The plant extracts were prepared according to the method described by Ahmad, *et al.* 1998 [3] with minor modifications. A total of six different Gram-negative bacterial isolates were obtained from *A. vera* gel. Growth kinetics studies were performed to determine the stationary phase as

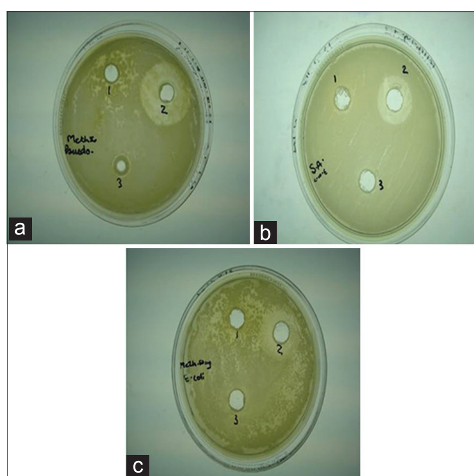

Fig. 2: Agar well diffusion against various pathogens (a) *Pseudomonas aeruginosa* (MTCC No. 10462), (b) *Staphylococcus aureus* (MTCC No. 3160), (c) *Escherichia coli* (MTCC No. 9721)

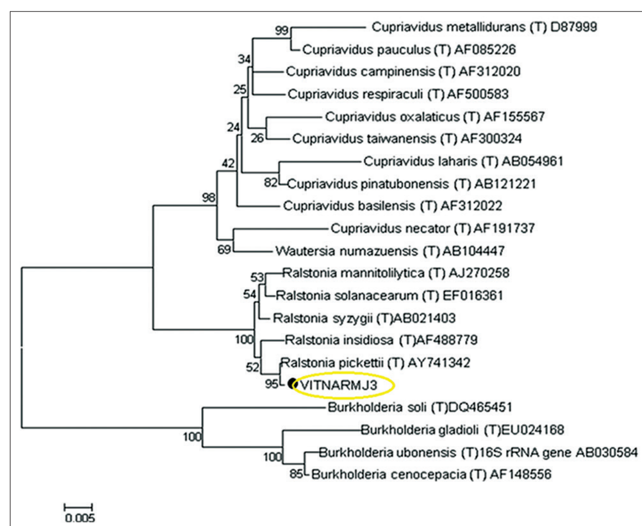

Fig. 3: Phylogenetic tree of effective isolate VITNARMJ3

antimicrobial compounds are secondary metabolites that are produced in stationary phase of growth (Dong, *et al.* 2003 [12]). The bacterial secondary metabolites were obtained using hexane and chloroform as a solvent for better extraction of pigments. These secondary metabolites were extracted by using two solvents: Hexane and chloroform (Kang, *et al.* 2007 [22]).

Antimicrobial assay was performed by agar well diffusion method and the obtained isolate VITNARMJ3 was found to be effective against various pathogens like *P. aeruginosa* (MTCC No. 10462*), S. aureus* (MTCC No. 3160), *E. coli* (MTCC No. 9721). Previously, agar well diffusion method has been routinely used in anti-microbial susceptibility testing. The zone of inhibition around the wells directly indicates the efficiency of the bioactive compound to act against the tested pathogenic bacteria. Bacterial secondary metabolites in hexane extract, showed inhibitory activity against *S. aureus* with a zone of inhibition of 24 mm and in chloroform extract activity was observed against *Pseudomonas sp.* giving a zone of inhibition of 24.5 mm. Agar well diffusion method is a well-known technique for antibiotic analysis of the purified bioactive extracts (Arularasan *et al.*, 2012 [44]; Rahul *et al.*, 2011 [45]).

Endophytes have proven to be rich sources of novel natural compounds with a wide-spectrum of biological activities and a high level of structural diversity. Molecular techniques have been successfully used for identifying endophytic communities in recent studies (Promputtha, *et al.* 2005 [42], Sette, *et al.* 2006 [47], Tedersoo, *et al.* 2006 [51], Morakotkarn, *et al.* 2007 [33]). 16S rRNA analysis identified isolate VITNARMJ3 as closely related species of *Rastonia picketti* having a sequence similarity of 94.6%. Saliou, *et al.* 2010 [38] also reported the existence of *R. picketti* as an endophytic bacterial species obtained from soybean, extending the endophytic bacterial community to *Ralstonia* genus.

Hence, obtained isolate VITNARMJ3 can be effectively used in food and pharmaceutical industries to obtain bioactive compounds for various bacterial pathogens. The study of endophytic bacteria is important, not only for understanding their ecological role in their interaction with plants but also for their possible biotechnological applications.

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