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RESEARCH ARTICLE

Isolation and Screening of chitinase producing endophytic bacteria from *Datura metal* L.

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ABSTRACT:
Endophytes are endosymbiont, often a bacterium or fungus that lives within a plant for atleast part of its life cycle without causing apparent disease. Apart from the role of endophytes in host growth, nutrient acquisition, which decrease biotic stresses by enhancing plant resistance to insects, pathogens and herbivores. Replacement of chemical pesticides by alternate control measures is expected to make a significant contribution for the protection of human health. Chitin and chitinolytic enzyme are gaining importance for their biotechnological application, especially the chitinases exploited in agriculture fields to control pests/pathogens. In this view, present study initiated to isolate and screen the chitinolytic endophytic bacteria from *Datura metel* L. The bacterial isolates obtained will be very useful for the production of chitinase which can be employed for the biocontrol of harmful insects and pathogens. This study presents a first time report of chitinase producing endophytic bacteria from *Datura metel* L.

KEYWORDS: Alternate control, Chitinase, Endosymbiont, Harmful insects, Human health

INTRODUCTION:
Datura metel L. has a long history of use as a herbal medicine and its medicinal properties are notable. Bio control compounds from plant origins have no side effects on non target soil microorganism and human beings. Medicinal plants are repository of bioactive compounds containing naturally pesticidal properties. An endophyte can be defined as a microorganism such as fungi and bacteria that expends either the complete tissues of a living plant, typically causing no symptoms of disease[1]. The population density of endophytic bacteria can vary from 10^2 to 10^9 and depends on many factors, including the plant being studied, the part under analysis, the developmental stage of the plant, the plant cultivar (genotype) and the interaction with other organisms, as well as other environmental related factors.

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However, many isolates seem to have beneficial effects on their hosts. These beneficial effects including promoting host growth and promote the resistance mechanism against varius pests/pathogens.

Chitin $(C_8H_{13}O_5N)n$, an insoluble, most abundant polysaccharide composed of linear chains of beta-1,4-N-acetylglucosamine (GlcNAc) residues that are highly cross-linked by hydrogen bonds. Chitin is widely distributed in nature and it is the principle structural component of outer skeleton (50% of the cuticle made up of chitin), foregut, hindgut, midgut lining of peritrophic membrane and also essential for structural integrity of many insects, nematodes[2] as well as component of the cell wall of the most fungi[3]. Chitinase degrade chitin into its monomeric or oligomeric components, it might be speculate that if applied on to the insect, either it enters the gut of insect larvae, it can cause significant damage to the peritrophic membrane structure which will result in the insect not able to feed and consequently leads to death or it disrupts the cuticle which subsequently causes abnormal moulting.

Recently, chitinase has received considerable attention because chitinase might play a role in plant defense systems against chitin-containing pathogens and mosquito control[4]. Chitinases (EC 3.2.1.14) are a set of enzymes that are produced by several bacteria, actinomycetes, fungi, and also by higher plants[5]. Chitinases play a major role in many areas such as the production of single cell protein, growth factor, a biocontrol of fungal pathogens, mosquito control, and to control many important crop pests[6]. Chemical pesticides have been used worldwide for enhance crop production and control plant pests. In contrast, biological control agents received increased attention as safe and environmental friendly alternative to the use of high volume of chemical pesticides[7]. Instead of spraying bacteria as such to control pest incidence, in the recent years, research has focused more on the use of selective metabolite or enzyme which is present in the bacteria, because they are generally considered as target specific, safer and environment friendly. Considering this, the present study has been narrowed on isolation, and screening of chitinase producing endophytic bacteria from the plant *D. metel* L. The presences of chitinolytic bacteria indicate it may have the possible role in biocontrol.

MATERIALS AND METHODS:

Collection of plant sample:

Healthy leaves and roots of *Datura metal* L. were collected from the surroundings of Vels Univerity Campus, Chennai, Tamil Nadu, India (Fig.1). Roots were collected by digging the soil adjacent of the main stem. The roots sample were cutted about 5-8 cm in length and healthy leaves were collected in sterile polythene bags. Samples were brought into the laboratory and isolation were done within in 48 h. The research work was conducted during January to April 2019.

Fig. 1: Leaf and root sample of *D. metal* L.

Isolation of endophytic bacteria:

To isolate the bacterial endophytes, initially the leaf and root samples were washed under running tap water to remove the dust particles and debris. The leaves and roots were cut into segments (0.5 – 1cm). Under in-vitro condition, the samples were surface sterilized using 0.5 % $HgCl_2$ (Mercuric chloride) for 6 minutes and then rinsed with double distilled water for 3 times. Followed, the samples were dried by paper towel drying to remove the excess water. Surface sterilized root and leaf samples were placed in petri dishes containing NA medium. These media plates were sealed using parafilm and incubated at 28-35ºC for 3-7 days. The petri dishes were monitored everyday to check the growth of endophytic baterial colonies from the plant segments.

Preparation of colloidal chitin:

Colloidal chitin was prepared by the method of Roberts and Selitrennikoff[8] with a few modifications and supplemented in the chitin agar medium as a sole carbon source. Acid hydrolysis of chitin was done in conc. HCl by constant stirring using a magnetic stirrer at 4°C (refrigerator) overnight, and thoroughly mixed to obtain a homogenous suspension. This was further transferred through filter paper and washed with distilled water until the colloidal chitin reaches pH 7. Colloidal chitin was collected, freeze dried to powder and stored at 4°C until further use.

Primary screening of chitinase producing bacteria:

Quadrant streak of all the bacterial isolates was carried out in NA plate to isolate the pure bacterial cultures. Single streak inoculation measuring 2 cm length was performed for all the bacterial isolates on NA medium supplemented with colloidal chitin and incubated at room temperature for 2 days. The plates were stained with 0.1% Congo red and distained with 1% NaCl, and the bacterial isolates producing a clear zone of more than 10 mm were selected.

Identification of potent chitinase producing bacteria:

Single colony of primary screened bacterial cultures was inoculated in 50mL of nutrient broth containing colloidal chitin as a substrate. Culture flasks were kept in shaker at 120rpm for 48 hrs. From each flask 1mL of culture broth were added on the wells made on the NA plate supplemented with colloidal chitin and the plates incubated at room temperature for 2 days. The plates were stained with 0.1% Congo red and distained with 1% NaCl, and the bacterial isolates producing a clear zone of more than 15 mm were selected. The screened pure isolates were stored in NA slants added with 1% colloidal chitin at 4 °C to maintain the viability of chitinase producers.

RESULTS AND DISCUSSION:

A total of 18 different bacterial strains from root and 17 different bacterial strains from leaves of *Datura* were isolated. Therefore the bacterial strains were named as DR-1 (*D. metal* L. root) to DR-17 for the bacterial strains isolated from root samples and DL-1 (*D. metal* L. leaf) to DL-17 for the bacterial strains isolated from leaf samples. Cream and white color colony growth was observed around single leaf and root sample of *D. metal* L. (Fig.2). Further, these bacterial colony growth was quadrant streaked in NA plate to isolate the pure bacterial cultures. Similarly, 35 endophytic bacteria have been isolated from four medicinal plants *viz*., *Catharanthus roseus, Ocimum sanctum, Mentha arvensis*, and *Stevia rebaudiana*[9].

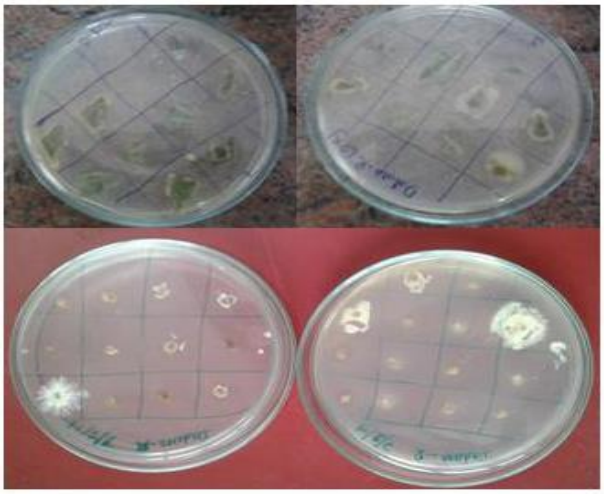

Figure. 2: Isolation of Endophytic Bacteria from leaf and roots of *D. metal* L.

Isolated endophytic bacteria were screened on colloidal-chitin agar medium. Chitinase production was determined by zones of hydrolysis produced after 96 h of incubation at 37°C. The result of this investigation revealed thirty-five pure bacterial isolates from the leaf and root (Table. 1 and 2) were found to produce clear zone (>10 mm) when incubated in chitin-containing media and further stained with Congo red (Fig. 3 and 4). Clear zone surrounding the colony indicates chitinase activity to break down chitin compound in medium. The screened isolates were further narrowed down to choose the best producers of chitinase based on the zone formation (>15 mm). One isolate from root (DL-2) best chitinase producer, was chosen and stained with Congo red (Fig. 5)

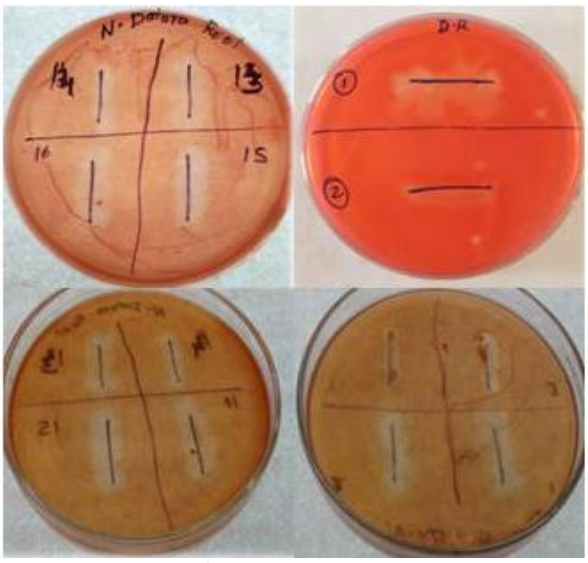

Figure. 3: Screening of isolates with congo red stain-DR-1 to DR-18

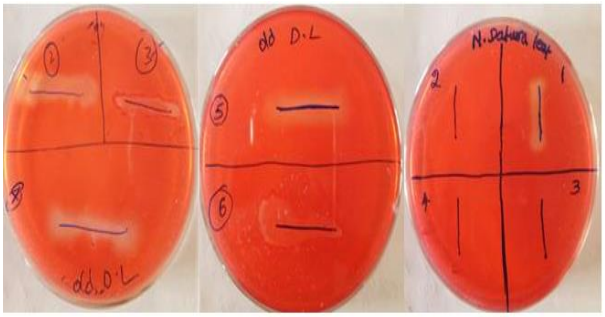

Figure.4: Screening of isolates with Congo red stain-DL-1 to DL-17

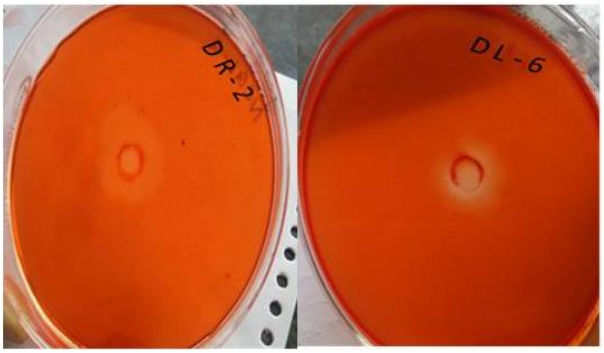

Figure. 5: Sreening of potent chitinolytic bactria with Congo red stain

DR-2 produced the high chitinolytic zone formatiom (>10mm).
DL-6 produced less chitinolytic zone formation (<10mm)

Table 1: Screening of potent chitinase producers from roots of *D. metal* L.

S.NO	Bacterial strains	Chitinase production
1.	DR-1	++
2.	DR-2	+
3.	DR-3	+
4.	DR-4	+
5.	DR-5	++
6.	DR-6	+
7.	DR-7	+
8.	DR-8	+
9.	DR-9	+
10.	DR-10	+
11.	DR-11	+
12.	DR-12	+
13.	DR-13	+
14.	DR-14	+
15.	DR-15	++
16.	DR-16	++
17.	DR-17	++
18.	DR-18	+

++ - indicates clear zone (>10 mm), + - indicates clear zone (<5 mm) and -- indicates no zone

Table 2: Screening of potent chitinase producers from leaves of *D. metal* L.

S.NO	Bacterial strains	Chitinase production
1.	DL-1	++
2.	DL-2	++
3.	DL-3	+
4.	DL-4	++
5.	DL-5	++
6.	DL-6	++
7.	DL-7	--
8.	DL-8	--
9.	DL-9	--
10.	DL-10	--
11.	DL-11	--
12.	DL-12	--
13.	DL-13	--
14.	DL-14	--
15.	DL-15	--
16	DL-16	--
17.	DL-17	--

++ - indicates clear zone (>10 mm), -- indicates no zone

Biopesticides is an alternative approach to control the damage caused by the insect pest/pathogens. Due to high toxicity of chemical pesticide to non target organisms such as human beings, animals and beneficial insects, the use of chemical pesticides is being replaced by environment friendly biopesticides. The growing importance of environment and health drives the search for safer products than chemical pesticides. Increased concern over the impact of chemicals on the environment has resulted in increased interest in biocontrol strategies. Potential use of naturally occurring bacteria, actinomycetes and fungi as replacement for chemical pesticides have been addressed in many studies[10].

Endophytic bacteria are able to lessen or prevent the deleterious effects of certain pathogenic organisms. The beneficial effects of bacterial endophytes on their host plant appear to occur through similar mechanisms as described for rhizosphere-associated bacteria. In our earlier study, the chitinase enzyme was isolated and characterized from the tea soil bacteria *P. fluorescens* MP-13[11]. These mechanisms have been reviewed in great detail by Kloepper[12] or, more recently, by Gray and Smith[13,14]. Diseases of fungal, bacterial, viral origin and in some instances even damage caused by insects and nematodes can be reduced following prior inoculation with endophytes[15]. Instead of using endophytic bacteria as a biocontrol, the use of selective metabolites like chitinase which are produced by antagonistic microorganisms is advantageous[16]. Also, chitinase like antibacterial proteins/peptides was isolated from seeds of millets and used as potential alternatives for the development of novel antibacterial agents[17].

Chitinase act as both contact and systemic toxic component to kill the insects[18] and hence this study will help in developing bio control agent besides opening a new avenue in formulating chitinase based biopesticide. Microbes are potential source of insecticidal enzyme like chitinase. The production of inexpensive chitinase has received attention as potential biocontrol molecule for control of many pests, it is an emerging field of research and it has been evaluated for limited number of pests/pathogens[19,20,21]. Research on bacteria-mediated insect control has indicated that bacterial chitinases has activity at alkaline pH may degrade the chitin which is present in gut lining of insects, chitin metabolism is considered to be an excellent target for selective pest/pathogen control[20,18]. This affects insect digestion and is directly inhibitory to insect growth and development and leads to insect death. Because of their specific activity towards chitin, they are considered to be highly selective, being for instance non-toxic to higher vertebrates. For this reason, chitinase enzyme has been long deemed promising candidates as biopesticides[22].

In continuation with the isolation of endophytic bacteria from *D. metal* L., further investigation focused to screen their chitinolytic activity using Congo red stain incorporated colloidal chitin media. Similarly, Karthika *et al.*,[8] isolated 35 morphologically different microorganisms and screened for their chitinolytic activity in colloidal chitin incorporated media through zone assay using Congo red stain. Similar study also carried out by Saima *et al.*,[23], 58 bacterial isolates were screened for chitinolytic activity and on the basis of chitin hydrolysis zone 6 isolates were selected for chitinase production in broth media. Another study reveals that chitinolytic bacteria, especially endophytes with growth promoting mechanisms, could be better biocontrol agents in the suppression of fungal pathogens[21]. Apart from biocontrol agents, endophytic

bacteria was succesfully isolated from *Cosmos caudatus* Kunth. leaves, to determine the capability of producing flavonoids, and to test their potency as anticancer and antimicrobial[24].

So far no attempt was made on the screening of chitinolytic endophytic bacteria from *D. metal* L. Hence the present study was focused to screen the potent chitinolytic bacteria. Results show that among 35 bacterial endophytes, DR-2 showed high chitinolytic activity. Our study concludes that screened potent chitinolytic bacteria will be used effectively for chitinase isolation. In future, biochemical and moleular identification of potent chitinolytic bacteria will be done and chitinase was isolated, purified and characterized and used as a biocontrol.

CONCLUSION:

Increased concern over the negative impact of chemicals on the environment and consumer health resulted in non-chemical mean of insect/pathogen control using biotechnological approach which attracts the entire scenario. One of the challenges confronting researchers worldwide is the development of new and sustainable ways of protecting crops from pests/pathogen. Nowadays the focus is to identify the molecule/enzyme from the bacterial source and use it as safer biocontrol measures. Chitinolytic enzymes and their genes have gained attention in recent years because of the importance of chitin and its metabolic enzymes in insect growth and development. Hence this study will help in developing new strategies in crop management besides opening a new avenue in formulating chitinase based biopesticide.

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