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ENZYME PRODUCTION FROM ENDOPHYTIC BACTERIA ISOLATED FROM MEDICINAL PLANTS

***Radha Palaniswamy, Dhanyasri S**
Dr. N.G.P. Arts and Science College, Dr. NGP Kalapatti, Coimbatore-641048, Tamil Nadu, India.
Email: palaniswamyradha@gmail.com

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Abstract:

Endophytic microorganisms inhabiting the medicinal plants synergistically produce pharmaceutically important metabolites in their host plants. To explore the possibility of identifying bacterial endophytes which are capable of producing industrially important enzymes from leaves and roots of medicinal plants was considered in the current study. Many bacterial endophytes were isolated from the leaves of the three traditionally practiced medicinal plants namely, *Catharathus roseus, Murraya koenigii* and *Nerium oleander* and were screened for enzyme activity. Endophytic microbial isolates exhibited amylase, protease, cellulase and pectinase activities. The study implies that further analysis of these microorganisms will provide promising results in the development of new enzymes with potential usage in various industries.

Key words: Endophytic bacteria, amylase, pectinase, protease and pectinase.

1. Introduction:

World is encompassing accelerated loss of wild medicinal plant species; one third of the medicinal plant species are threatened with extinction from over harvesting and natural anthropogenic habitat destruction (Chen *et al.*, 2016). To circumvent this, many biological systems like bacteria, fungi, yeast, cyanobacteria, actinomycetes and plants have been used (Ganesh Babu *et al.*, 2013). (Atanasof *et al.*, 2015). Therefore, it is important to find alternative approaches to produce the medicinal plant-derived biologically active compounds from endangered plant species to meet the medical demand. This can be achieved by exploiting the ability of endophytic bacteria

residing in plants to produce the same or similar bioactive compounds as their hosts (Rai *et al.,* 2017; Ramirez-Puebla *et al.,* 2016).

Endophytes are an endosymbiotic group of microorganisms that colonize inter and/or intracellular locations of plants. They are ubiquitous in nature and exhibit complex interactions with their hosts, which involve mutualism, antagonism and rarely parasitism (Miliute *et al.,* 2015). Endophytic bacteria seem to be distributed in most plant species and have been isolated from roots, leaves and stems and a few from flowers, fruits and seeds (Lodewyckx *et al*., 2002).

The extensive range of bioactive molecules generated by plants perhaps evolved as a chemical defense against predation or infection making them a suitable source of medication which has been put in to practice in the preparation of pharmaceutical and nutraceutical products (Palaniswamy and padma, 2017). In addition to the production of usual secondary metabolites of plant importance, bacterial endophytes have revealed the ability to inhibit disease development in plants. With an aim of exploring the biodiversity of endophytic strains for novel metabolites that would lead to the identification of new drugs for effective treatment of diseases, the present study was carried out on three medicinal plants, *Catharanthusroseus , Murrayakoenigii , Nerium oleander* that have remarkable medicinal and commercial importance.

Enzymes from Endophytic Bacteria:

Many endophytes are members of common soil bacterial genera, such as Pseudomonas, Burkholderia and Bacillus (Lodewyckx *et al*., 2002) which are well known for their secondary metabolic products including antibiotics, anticancer compounds, volatile organic compounds, antifungal and antiviral agents (Ryan *et al.,*2008). The importance of the microorganisms in enzyme production is due its high production capability, low cost and susceptibility to genetic manipulation. Actually, the enzymes of microbial origin have high biotechnological interest such as in the processing of foods, manufacturing of detergents, textiles, pharmaceutical products, medical therapy and in molecular biology (Pilnik and Rombouts, 1985; Murugesan and Palaniswamy, 2018). Hence, the above 3 plants have been used a source for the production of enzymes from the endophytic bacteria present in them.

2. Materials and Methods:

Sample collection: For the isolation of endophytic bacteria, healthy leaves and roots of *C. roseus, M.koenigii* and *N.oleander*were collected from saplings grown in pot cultures at the college campus, Coimbatore. Each plant sample was tagged and placed in separate polythene bags and were brought to the laboratory and processed within 24 hours of collection. Fresh plant materials were used for isolation of endophytic bacteria to reduce the chance of contamination. Identification of the plant specimens were done at Botanical Survey of India, TNAU campus, Coimbatore as *Catharanthusroseus* (Voucher Number:BSI/SRC/ 5/23/2018/Tech./2257), *Murraya koenigii* (Voucher Number: BSI/SRC/5/23/ 2018/Tech./2256) and *Nerium oleander* (Voucher Number: BSI/SRC/5/23/ 2018/ Tech. /2258).

Surface sterilization : The roots and leaves samples of 3 different plants were rinsed with autoclaveddistilled water, disinfected with hydrogenperoxide for 2 minutes and rinsed for5 minutes with 70% ethanol followed by 3% Hypochlorite + Tween 20 (0.1%) andfinally rinsed with autoclaved distilledwater.

Isolation of endophytic bacteria: The surface sterilized leaves samples were macerated using mortar and pestle. From this 1ml was taken and serial dilution was done following which it was spread on the nutrient agar plates and kept for incubation at 37°C for 24 hrs. A total of 12 colonies were isolated from the 3 different medicinal plants and depending upon the population and different morphological appearance of the colonies 7 isolates were chosenand further sub-cultured to isolate pure colonies. Therefore, totally 7 colonies (2 from root and 5 from leaves)table.1 were taken for further study as described below.

Table 1. Isolates obtanined from 3 different medicinal plants.

Plant sample	Leaf	Root
C.roseus	3 isolates	1 isolate
M.koenigii	1 isolate	1 isolate
N.oleander	1 isolate	-

Identification of Bacteria:

Morphological Test: The selected strains were morphologically characterized in order to determine the morphology of the bacterial cells based on observable characteristics such as cell shape, colony color and

texture. This was determined by the classical gram staining method as described by (Cappuccino and Sherman, 1992).

Biochemical Test: Series of biochemical tests like Indole test, Methyl red, Voges-Proskauer (VP), Citrate utilization, H_2S production, starch hydrolysis, urease production and nitrate reduction was done.

Indole test: The Indole test is performed by inoculating bacterium with tryptophan broth. The Indole produced during the reaction is detected by Kovac's reagent, which produce a cherry red color.

Methyl red test: The methyl red test is employed to detect the ability of the organisms to oxidized glucose with the production of high concentration of acids. It is used to differentiate the species.

Voges - Proskauer(VP) test:VP test determine the capability of microorganisms to ferment carbohydrate with the production of non-acidic or neutral end products such as acetyle methyl carbinol or its reduction product 2,3 butylene glycol.

Citrate utilization test: In the absence of fermentable glucose or lactose some microorganisms are capable of utilizing citrate as a carbon source and enable conversion of citrate to oxaloacetic acid and acetate. These products generate CO_2 combines with sodium and water to form sodium carbonate which changes the boromothymol blue to deep prussion blue.

Carbohydrate fermentation: Nutrient broth ingredients are added to the medium for the support of the growth of all organisms. A specific carbohydrate, that serves as the substrate for determining the organism's fermentative capabilities which result in evolution of the gases which will be visible as a bubble in the inverted vial.

H_2S production test: Some bacteria liberate sulphurthat is used as final hydrogen acceptor during anaerobic respiration, leading to the formatting conditions are present and Hydrogen Sulphide can be detected by the test system.

Starch hydrolysis test: Iodine reagent complexes with starch medium to form a blue-black color in the culture medium. Clear zone surrounding colonies is indicative of their ability to digest the starch in the medium due to the presence of alpha-amylase.

Urease production: Urease broth is a differential medium that tests the ability of an organism to produce an exoenzyme, called urease that hydrolyzes urea to ammonia and carbon dioxide.

Nitrate reduction test: Heavy inoculum of test organism is incubated in nitrate broth. After 4 hrs incubation, the broth is tested for reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by adding sulfanilic acid reagent and α-naphthylamine.

MALDI-TOF-MS (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) Analysis: The organisms were further confirmed using MALDI-TOF-MS analysis. During MALDI-TOF analysis, the m/z ratio of an ion is measuredby determining the time required for it to travelthe lengthof the flight tube. A fewTOFanalyzersincorporate anion mirror at the rear end of the flight tube, which serves to reflect back ions through the flight tube to a detector. Thus, the ion mirror not only increases the length of the flight tube, it also corrects small differences in energy among ions (Singhal, 2015).

Enzyme Production from Endophytes: All bacterial isolates were screened for production of extracellular enzymes namely amylase, protease, cellulase, and pectinase using simple plate assay.

Amylase enzyme: The isolates were spot inoculated on the plate containing nutrient agar supplemented with starch as substrate, which is previously sterilized, maintained at pH 6.0. After incubation, the cultures were treated with Gram's iodine, to check for the formation of clear halos around the colony.

Protease enzyme: The isolates were spot inoculated on the plate containing nutrient agar supplemented with gelatin as substrate. After incubation the culture were treated with Bromo Cresol Green, to check for the formation of clear halos around the colony.

Cellulase Enzyme: Carboxymethyl cellulose (CMC) agar plates were used. The cultures were spot inoculated and the plates were incubated for 24 hours at room temperature. After 24hrs incubation, the plates were flooded with 10% NaCl for 10 min. Clear zone around the reddish background indicates the production of cellulose by the bacteria.

Pectinase enzyme: For pectinase activity the isolates were spot inoculated on the plate containing Pectinase screening agar medium. After incubation the culture were treated potassium iodine-iodide solution, to check for clear halos around the colony.

3. Results:

Isolation of endophytic bacteria: Seven morphologically different bacterial isolates were isolated on nutrient agar plate supplemented with bavistin (30µg/ml) (Bavistin is a broad spectrum systemic fungicide containing effective against a wide range of pathogenic fungi) from leaf and roots samples after 2-4 days of incubation at 37°C. The isolates were names as EB1,EB 2,EB 3,EB 4,EB 5,EB 6 and EB 7 (Endophytic Bacteria). Colony characters were studied and Gram staining was performed for all the isolates and screened for the enzyme production among which 4 isolates showed positive result in the screening of enzymes like protease, amylase, cellulose and pectinase.

Bacterial identification and characterization:

Morphological test and Biochemical Test: The following morphological and biochemical tests were carried out to confirm the species which is elaborately shown in the Table 2. Based on the growth of the colonies and populations of growth colonies from the 7 isolates it has been shortlisted to 4 isolates as shown in the table below.

Table 2: Morphological and Biochemical Test.

S.No	Gram staining	Motility	Indole	Methyl red	VP	Citr-ate use	Carbohydrate fermentation	Urease	H2S production	Species name
EB 2	N	P	N	P	N	P	N	N	N	*B.flexus*
EB 4	P	P	N	P	N	P	N	N	N	*B.cereus*
EB 5	N	P	N	P	P	P	N	P	N	*B.cereus*
EB 7	N	P	N	P	P	P	N	N	N	*S.warneri*

Screening for production of extracellular enzymes: Isolates EB2,EB4, EB5, EB7 showed activity for amylase , protease , cellulase and pectinase enzyme production after treating with Gram's iodine ,CBB (CoomassieBrillinat Blue), Congo red and potassium iodine iodide which exhibited clear zones as shown in the figures below. This proved that all the 4 isolates could produce the 4 enzymes namely, amylase, protease, cellulose and pectinase. As shown in the fig.1 the isolates were able to produce amylase enzyme which is confirmed by clear halo zones.

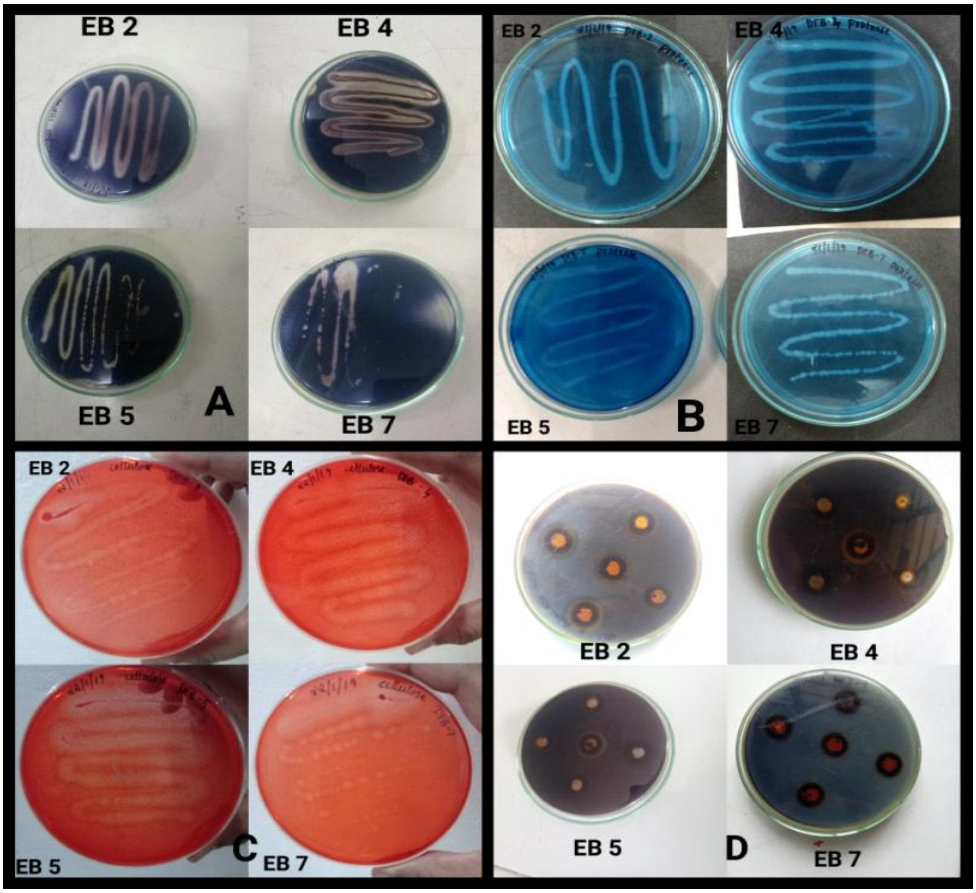

Figure 1. (a) Amylase with iodine reagent (b) Protease confirmed with Coomassie Brilliant Blue. (c) Cellulase screening with congo red staining.(d) pectinase screening with potassium iodide iodine solution.

4. Discussion: Microorganisms are the most important sources for enzyme production (Palaniswamy and Ragunathan 2018; Falch 1991). In this present study the isolates *B.flexus, B.cereus, S.warneri* were able to produce the extracellular enzymes namely Protease, amylase, pectinase and cellulase. Macroscopic characteristics of the colony such as such as the shape, size, color, surface texture morphological the elevation,

form and margin, were recorded by using the criteria described by Harley and Prescott (2007). After carrying out the preliminary morphological analysis further biochemical analysis was carried out so as to authenticate the identification of bacteria. Bacteria were identified and classified largely on the basis of their reactions in a series of biochemical tests like IMVC test, carbohydrate fermentation, sulphur productions (Fawole and Oso, 2004). MALDI-TOF MS was used for identifying the bacterial species which is being isolated from the three medicinal plants and it was identified as *B.flexus, B.cereus* and *S.warneri***.** Another study provides similar information using MALDI-TOF MS from *Azadirachta indica* plant sample where *Bacillus subtilis* and *Staphylococcus aureus* (Jaskolla, 2009) were identified. Selection of the right organism plays a key role in high yield of desirable enzymes.

The present study concordance with another study where the highest proteolytic activity was recorded in bacteria *L.fermentum* from leaves of *Vinca rosea (*Azevedo *et al.,* 2000). Another study proves the protease production was recorded in *bacillus* species from soil samples (Murugesan and Palaniswamy 2018). The amylase activity was done where the nine bacterial isolates showed a clear halo around the colony on nutrient agar supplemented with 0.2% starch as substrate after treating with gram's iodine (Hankin and Anagnostakis 1975)(Pundir *et al*.,2014). Another study proves the pectinase activity was observed in Pomelo peels as alternative substrate for extracellular pectinase production (Ibrahim, 2013). Kashyap *et al* in 2000 isolated pectinase producing bacteria from soil sample where the organism *Bacillus subtilis,* showed higher activity in the pectinase production. For cellulase activity it was observed with a clear halo zone in nutrient agar supplemented with 1% carboxy methyl cellulase as a substrate which is isolated from *Hibiscus rosasinensis* plant sample (Meddeb–Mouelhi 2014). This proves that the Bacteria residing in three of the medicinal plants showed meager activity of hydrolytic enzymes.

5. Conclusion: Due to the swift dwindling of rich endangered species of medicinal plants globally, awareness due to the fact that the herbal drugs are expensive and cannot meet the demands of the world requirement, microbes play a paramount role to replace plant sources. As an alternative to these problems, endophyte area potential source of therapeutic agents. Earlier investigation supports that the metabolites produced by endophytes in culture is a potential source of bioactive agent. Considering all above mentioned aspects, the

focus of the present study is on reiterating the importance of isolation of endophytic bacteria for their natural products which has a wide spectrum of applications.

Conflict of interest: Authors would like to declare that they have no conflict of interest.

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Corresponding Author:

Radha Palaniswamy*,

Dr. N.G.P. Arts and Science College, Dr. NGP Kalapatti, Coimbatore-641048, Tamil Nadu, India.

Email: palaniswamyradha@gmail.com