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Isolation and Characterization of Endophytes from *Sorghum Vulgare* and their Effect on Pathogen

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Abstract: *Endophytic bacteria were isolated from the stalks and leaves of jowar (Sorghum vulgare).Four isolates, representing the differentcolony morphology types, were obtained after growing them on nutrient agar and yeast maltose agar. Gram staining and biochemical tests were performed to characterize them. These isolates were also used for in vitro inhibition tests against Curvularia lunata (causative agent of curvularia leaf spot in maize and leaf blight in jowar) and Pseudomonas syringae (a causative agent of chocolate spot in maize).*

Keywords: Gram's staining, Anti-Bacterial, Anti-fungal activity and Biochemical test

I. INTRODUCTION

An **endophyte** is an endosymbiont, often a bacterium or fungus, that lives within a plant for at least part of its life without causing apparent disease. Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects**".** While the symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens. Both fungi and bacteria are the most common microbes existing as endophytes. It would seem that other microbial forms most certainly exist in plants as endophytes, but no evidence for them has yet been presented e.g. mycoplasmas, and archebacteria.[1] The most frequently isolated endophytes are the fungi. It turns out that the vast majority of plants have not been studied for their endophytes. Endophytes are ubiquitous and have been found in all the species of plants studied to date. It is noteworthy that of nearly 300,000 plant species that exist on the earth, each individual is host to endophytes. It is estimated that there is as many as 1 million different endophyte species; however only handful of them are described. Although known since long time, their importance become evident only more recently when it was shown that they play specific roles as for instance, protecting the host-plants against insects and diseases ,it also improve the ability of these plants to tolerate abiotic stresses such as drought, as well as improve their resistance to insect and mammalian herbivores. It has been found also that some endophytic microorganisms can produce valuable pharmaceutical substances of biotechnological interest.[2]

Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture, and industry. It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes. Only a few these plants have ever been completely studied relative to their endophytic biology.[3] Consequently, the opportunity to find new and interesting endophytic microorganisms among myriads of plants in different settings and ecosystems is great. The intent of this review is to provide insights into the presence of endophytes in nature, the products that they make, and how some of these organisms are beginning to show some potential for human use. The majority of the report discusses the rationale, methods, and examples of a plethora of endophytes isolated and studied in the authors' laboratory over the course of many years.[4] This review, however, also includes some specific examples that illustrate the work of others in this emerging field of bioprospecting the microbes of the world's rainforests.

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II. MATERIAL AND METHODS

MATERIALS:

For Isolation:

- Nutrient Agar
  Peptone – 5 g, Beef extract/ Yeast extract – 3 g, Agar – 15 g, NaCl – 5 g, Distilled water – 1 L, pH – 6.8
- Yeast Maltose Agar
  Yeast extract – 4 g, Maltose extract – 10 g, Glucose – 4 g, Agar – 20 g, Distilled Water – 1 L, pH – 7.3.
- Potato Dextrose Agar
  Potato infusion – 300 g, Dextrose – 20 g, Agar – 20g, Distilled water – 1L.

Gram's staining:

- Hucker's crystal violet:

a. Hucker's ammonium oxalate
   Ammonium oxalate – 0.8 g, Distilled water – 80ml

b. Crystal violet staining solution
   Alcohol (95%) – 20ml, Crystal violet - 1 g, Mix a and b

- Lugol's iodine
  Iodine crystals – 1 g, Potassium Iodine – 3 g, Distilled Iodine – 300ml
- Safranine
  Safranine – 1 g, Alcohol(95%) – 10ml, Distilled Iodine – 100ml
- Gram's Alcohol
  Alcohol(95%) – 98 ml, Acetone – 2 ml, Safranine – 1 g, Alcohol(95%) – 10 ml
  Distilled iodine – 100 ml

III. CHEMICALS FOR BIOCHEMICAL CHARACTERIZATION

Catalase test

- Luria agar and 3% Hydrogen peroxide

Iodole test

- 1% Tryptone water, Xylene – 0.3 ml and Kovac's reagent

Methyl red test

- Glucose phosphate broth and Methyl red

Starch hydrolysis

- Starch agar

Casein hydrolysis

- Casein agar

METHOD

ISOLATION OF ENDOPHYTES:

- For maize and jowar, endophytic populations were collected from the pith tissue of stalks and the leaves.
- Individual plants were severed aseptically 3 cm above the soil level, and the stalks were stripped of leaves, put into plastic bags, and kept on ice until further processing.
- In the laboratory, the stalks were wiped with 70% ethanol.
- Plant leaves and stems were surface sterilized for 10 s with 2% sodium hypochlorite containing 0.1% Tween 20.

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- To remove the disinfectant, sections were rinsed five times each in two washes of nonsterile deionized distilled water and a wash of sterile water; the sections were dried with sterile paper towels.
- The outer stalk was removed, exposing a cylinder of tissue inside the cork borer.
- Sections of leaves and the stalks of 1cm were taken and inoculated on both nutrient agar and yeast maltose agar petri dishes and incubated.
- Growth on all the petri-plates was observed after 4-5days.

IV. MORPHOLOGICAL CHARACTERIZATION

Gram staining

It is the type of differential staining, which divides bacterial cells in two major groups gram +ve and gram –ve which makes it an essential tool for classification and differentiation of microorganisms. The Gram's reaction is based on differences in chemical composition of bacterial cell wall. Gram +ve have thick peptidoglycan layer which is cross linked and is low in lipid percentage. The crystal violet iodine complex get firmly fixed in the pores of peptidoglycan. After decolorisation with alcohol the lipid are dissolved and CV-I_2 complex is trapped in cell wall network. The counter stain is not taken up as bacteria are already stain and hence they appear violet on other hand the Gram – ve cell are much thinner and surrounded by outer lipid containing layer. The CV-I_2complex does not fixed firmly. As a amount of peptidogylcan is less and network loose. The CV-I_2 complex is washed of during decolorisation, as lipid are soluble in alcohol and pore size increases the Gram –ve bacteria therefore taken up counter staining and appear pink in color.[5]
Grams iodine serves as mordent (substance that increases cells affinity for stain) stain. It does this by binding to the primary stain, thus forming an insoluble complex. The resultant CV-I_2 serves to intensify the color of stain. At this point all cells will appear purple black.

Procedure:

- Prepare smear of given culture on clean glass slide and heat fix it. Cool the slide.
- Stain the smear with primary stain that is crystal violet and let stand for 1 min. Wash with tap water.
- Gently flood the smear with lugol's iodine, mordent and allow to react for 1 min. Wash with tap water.
- Decolorize with 95% ethanol or acetone. Do not over decolorize, add reagent drop till the drop leaving the slide is free of stain. Wash with tap water.
- Counter stain with safranine for 1 and half min and wash with tap water.
- Blot dry the smear, add a drop of immersion oil and observe under oil immersion objective. Note the color of the bacteria.

V. BIOCHEMICAL CHARACTERIZATION

A no of biochemical and miscellaneous test in well defined media and under standard condition are used for identification of microorganisms. The microorganisms are differentiated on the basis of various enzymes metabolic reactions. Presence or absence of certain enzymes, metabolite or end product often gives valuable information in identifying the organism.[6]

Catalase test:

Hydrogen peroxide, a byproduct of oxygen metabolism, is toxic to some type of cell due to its ability to oxidize cell components. Therefore many bacteria produce the enzyme Catalase, which converts hydrogen peroxide to hydrogen and oxygen. Therefore, where a Catalase producing culture is brought in contact with hydrogen peroxide, bubbles of oxygen are released.

Procedure:

- A loop full of culture suspension was streaked on Luria agar plate and incubation was done at 37˚c for 24 hours.

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- 3% hydrogen peroxide was put on a culture grown on a plate.
- A positive catalase test is indicated by effervescence in the form of bubbles.

Indole test:
This test detects the ability of organism to produce indole from tryptophan only by the means of enzyme tryptophanase. Thus the produce accumulate in medium and can be extracted using xylene. Kovac's reagent was used to detect the presence of the xylene layer. The indole reacts with the para di methyl amino benzaldehyde in the presence of acid and alcohol to give a pink colored complex. Pink color of xylene layer therefore indicates a positive test.

Procedure:

- A loop full of culture suspension was inoculated in 1% tryptone water.
- The tube was incubated at 37˚c for24 hours.
- After incubation, xylene 0.3ml was added to the tube and the contents were mixed properly by shaking to extract indole.
- Kovac's reagent was added and the color of the xylene layer was observed.

Methyl red test:
Methyl red test detects acid production in glucose phosphate broth using methyl red as an pH indicator. From a given fermentable sugar, some bacteria produce greater amount of acid via the mixed acid fermentation pathway. The acid are not further metabolized and accumulated in the medium. In the buffered glucose medium, sufficient final is thus produced to be detected by methyl red indicator, which turns red under acidic condition indicating a positive test, while a yellow color indicates a negative test.

Procedure:

- A loop full of culture suspension was inoculated in GPB (glucose phosphate broth).
- The tube was incubated at 37˚c for 24 hours.
- After incubation methyl red was added to the medium and the tube was observed for color.

Starch hydrolysis:
Starch is a polysaccharide which is degraded by enzyme amylase. The principle behind this is that starch containing agar when come in contact with iodine turns blue. Clear zone around the growth was observed when bacteria having the ability to degrade starch, i.e having enzyme amylase was inoculated.

Procedure:

- Starch agar is prepared and poured in plate and allowed to set.
- Colony is picked from the slant and added in 0.8% saline.
- The nichrome wire loop is straightened and sterile in flame. Dip in culture solution.
- Stab inoculated is done on starch agar plate and incubation is done at 37˚c for 24 hours.
- After incubation the plate is flooded with Gram's iodine. Starch plate turns blue and the one which show positive result is seen by a clear zone around bacterial growth.

Casein hydrolysis:
Bacteria need protein as their source of essential amino acid. Protein are large molecules that cannot be brought directly into the bacterial cell. They are needed to be degraded into their component part.
Casein is a milk protein caseinase, is an enzyme that will break down casein. Casein is broken down into amino acid, which is the final end product. When a casein positive organism is streaked on to a casein agar plate, it will degrade the milk, creating a clear zone around the line of growth, giving a positive reaction.

Procedure:

- Casein agar is prepared and poured in plate and allowed to set.

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- A loop full of culture suspension was streaked on the surface of casein agar plate.
- The plate was incubated at 37˚c for 48 hours.
- Casein agar is checked for growth by surface clear zone.

VI. RESULT AND DISCUSSION

Anti-microbial activity of the Endophytes

1) For Anti-Bacterial Activity[7]
   - Culture suspensions of the pathogen and the endophytes were prepared.
   - The pathogen (*Pseudomonas syringae*) was spread using spread plate technique on nutrient agar petri dishes.
   - Using a sterile cork borer, a hole was made at the centre of the nutrient agar plate.
   - The endophyte suspension was made inoculated in the hole.
   - The plates were incubated at 37^0 C for 24 hours and then observed for the anti-bacterial activity.
2) For Anti-Fungal Activity[8]
   - Culture suspensions of the pathogen and the endophytes were prepared.
   - Using a sterile cork borer, 1 hole at the centre and 3 holes at the corners were made in Potato Dextrose Agar petri dishes.
   - The pathogen (*Curvularia lunata*) was inoculated in the central hole and the endophytic suspensions were inoculated in the 3 holes at the corner.
   - The plates were incubated at 25^0 C and observed for the anti-fungal activity.

OBSERVATION

Growth of endophyte

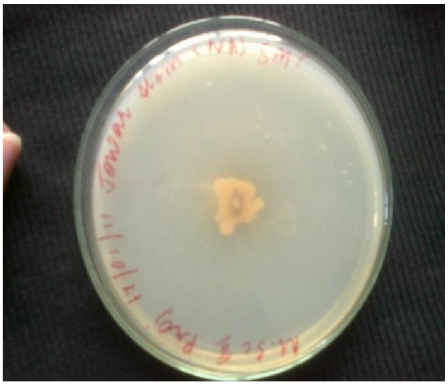

Fig1: Isolate 1 – Jowar stem (NA)

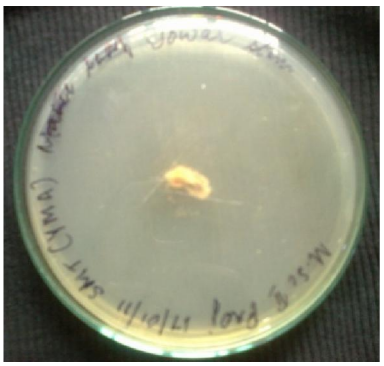

Fig2:Isolate 2 – Jowar stem (YMA)

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Fig3: Isolate 3 – Jowar leaf (NA)

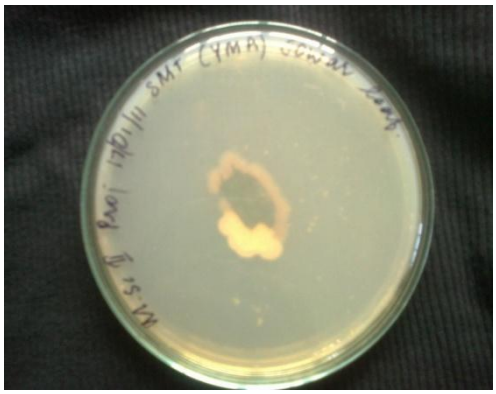

Fig4: Isolate 4 – Jowar Leaf (YMA)

Biochemical test

Indole test

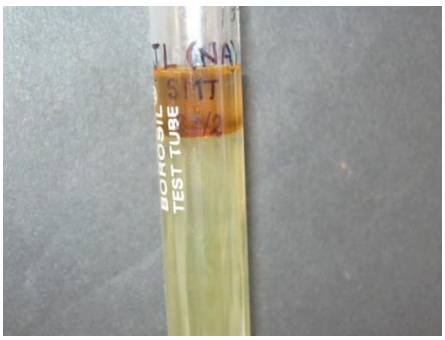

Fig1: Indole test (negative for isolate1-4)

Casein test

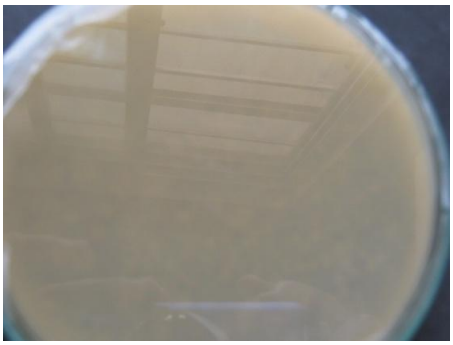

Fig2: Casein test(positive for isolate 1-4)

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Methyl red test

Fig3:Metyl red test(positive for Isolate1,2 & 4)

Fig4:Methyle red test(negative for isolate3)

Starch hydrolysis test

Fig5: Starch hydrolysis(positive for isolate2-4)

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Fig6: Starch hydrolysis (negative for isolate1)

RESULTS

Isolate 1 – Jowar Stem (NA)
Isolate 2 – Jowar Stem (YMA)
Isolate 3 – Jowar Leaf (NA)
Isolate 4 – Jowar Leaf (YMA)

FOR GRAM'S STAINING:

Isolate 1 – Gram Positive Coccobacilli
Isolate 2 – Gram Positive Coccus
Isolate 3 – Gram Positive Coccobacilli
Isolate 4 – Gram Positive Coccobacilli

FOR BIOCHEMICAL TESTS:

	CATALASE	INDOLE	METHYL RED	STARCH HYDROLYSIS	CASEIN HYDROLYSIS
Isolate1	Negative	Negative	Positive	Negative	Positive
Isolate2	Positive	Negative	Positive	Positive	Positive
Isolate3	Positive	Negative	Negative	Positive	Positive
Isolate4	Positive	Negative	Positive	Positive	Positive

VII. CONCLUSION

The results here described showed that endophytes can be isolated from jowar stems as well as leaves. Endophytes commonly thrive in plant tissues, where certain microorganisms reveal tissue-specificity distribution. A total of 4 strains were isolated. Growth was observed after 6 days on the nutrient agar and yeast maltose agar.

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