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Isolation and Biochemical characterization of Endophytic Bacteria from *Punica granatum*

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Abstract
Endophytes are microorganisms which present inner parts of plant tissues, without causing any adverse effect. The purpose of this work is to investigate Biochemical potentials of Endophytic Bacteria from medicinal plant (*Punica granatum*). Endophytic Bacteria isolates are *Clostridium sp, Staphylococcus sp* are identified by Staining and Biochemical examination. Biochemical studies on the endophytic bacteria growth medium in the presence of Starch, Catalase Casein, Glucose, Maltose, Manitol and Sucrose. The enzyme activity studied on the endophytic fungi it shows the activity of amylase, protease, lipase, cellulase and bacteria showed the amylase and protease activity.

Keywords: endophytes, punica *granatum*

Introduction
Endophytes are chemical synthesizer insider of plants in other words; they play a role as a selection system for microbes to produced pharmacologically active substances with low toxicity or no toxicity towards mammalians (Strobel 2003) [9]. Endophytes provide a broad variety of active secondary metabolites with unique structure, including alkaloids, glycosides, benzophyranones, flavonoids, phenolic acids, quinones, steroids, xanthones, terpenoids, tetralones and others (Tan *et.al*.2001) [10].
Endophytes are believed to carry out a resistance mechanism to overcome pathogen invasion by producing secondary metabolism (Tan *et al.,* 2001) [10]. So that, studies suggest a large number of antimicrobial compounds isolated from endophytes, belonging to several structural classes like alkaloids, peptide, steroids, quinines, terpenoids, phenols, and flavonoids (Yun *et al*., 2010) [11]. According to Berdy more than 20,000 bioactive metabolites are of microbial origin. It also believed the endophytes have important roles in plant protection, acting against herbivores, insects and pathogens of the host and may also increase plant resistance to pathogen and biotic and abiotic stress (Ahholm *et. al*.2002) however; association between plants and micro-symbionts has been controversial.

Materials and Methods
Isolation of Endophytic Bacteria
Collected leaves were washed with the running water and they were transfer in the sterile cabinet. The leaves and flowers were followed through the following protocol: 70% alcohol for 1min, sodium hypochlorite 2.5% for 1 min and finally rinse in sterile distilled water for 10 seconds. After the surface sterilization leaves and flowers are cut into small pieces about .5 to 1 cm by using a sterile surgical blade and then transfer into nutrient agar medium. After inoculation petridish were sealed using parafilm^{TM} and incubated at 37 ^{o}c for 48 hours.

Culture Identification
Identification of bacteria
Gram Staining
An important preliminary guide in the identification of unknown bacterial isolate is the application of Gram staining procedure. The advantage of this method is that it provides useful information about the shape and size of the cells and distinguishes between Gram- positive and Gram- negative species. Perform a bacterial smear; saturate the smear with crystal violet for 1 minute. Rinse the slide gently with water. Saturate the smear with iodine for 1 minute. Rinse the slide gently with water. Decolorize with Gram decolorizer (acetone /alcohol) for 3-5 seconds; if you leave the decolorizer on too long, Counterstain with safranin for 1 minute. Rinse the slide gently with water. Observe the slide under the microscope.

Spore staining test
A differential staining technique is used to distinguish between the vegetative cells and the endospores. Malachite green is used to stain the endospores. Endospores resist staining; the malachite green will be forced into the endospores by heating. The primary dye for endospore staining is malachite green. It takes a long time for the spores to stain due to their density, so time acts as the mordant when performing this differential stain; the slide with the bacterium should be soaked in malachite green for at least 30 minutes and then rinsed off with water which acts as the decolorizer. A counterstain to differentiate the vegetative cells is commonly 0.5% safranin. In the end, a proper smear would show the endospore as a green dot within either a red or pink-colored cell.

Motility Test
Thereafter, a loopful of the active inoculum from the colony edge was transferred into a moisture agar slope and incubated until the medium became turbid and was examined using a

hanging drop technique to observe the motility.

Acid fast stain test
It air drying the liquid and heat fixing the cells. The slide is flooded with Carbol Fuchsin, which is then heated to dry and rinsed off in tap water. The slide is then flooded with a 1% solution of hydrochloric acid in isopropyl alcohol (or methanol) to remove the carbol fuchsin, thus removing the stain from cells that are unprotected by a waxy lipid layer. Thereafter, the cells are stained in methylene blue and viewed on a microscope under oil immersion.

Biochemical Characterization of the Bacterial Isolate
Carbohydrate Fermentation Test
The fermentation broth tube (Glucose, Maltose, Sucrose, Lactose, Manitol) with inverted and air tight Durhum's tube were prepared. A loopful of bacteria culture to be characterized was inoculated in to each tube. Then the tubes were incubated at 37^0 c for 24 to 48 hours. After proper incubation, each tube were added few drops of phenol red. The result was observed.

Indole Production Test
The bacterial isolate to be characterized was aseptically inoculated in sterile tryptone broth tube. It was incubated at 37^0 c for 24 to 48 hours. After proper incubation 1ml of Kovac's reagent was added. The result was observed and recorded after 15 minutes.

Citrate Utilization Test
The bacterial isolate to be characterized was aseptically inoculated in Simmon's Citrate agar slant. The tubes were incubated at 37^0 c for 24 to 48 hours. After proper incubation, the result was observed and recorded.

Catalase Test
One or two drops of 10% hydrogen peroxide to the bacteria cultures to be characterized. Then it was observed for the production brisk effervescences, the result were record.

Casein Hydrolysis Test
The bacterial isolate to be characterized was aseptically inoculated in skim Milk agar. The plates were incubated at 37^0 c for 24 to 48 hours. After proper incubation a zone formed. The result was observed and recorded.

Starch Hydrolysis
The bacterial isolate to be characterized was aseptically inoculated at the center of the starch agar plates as a single line streak. The plates were incubated at 37^0 c for 24 to 48 hours. After incubation the plates were flooded with iodine solution. After discarding the excess iodine solution. The result was observed and recorded.

Determination of enzymatic activity of bacteria
The following enzyme productions were analyzed: amylase, lipase, and protease. The enzymatic activities were performed by initially growing the isolates in nutrient broth for 24 h at 30ºC. After that, isolates were inoculated on the specific culture media for each enzyme to be investigated. The culture was incubated at 30º C during 48h.

Determination of amylolytic activity
The isolated bacteria were inoculated in starch agar medium with 0.2% of soluble starch (g^{-1}) pH 6.0. After incubation, the culture was treated with lugol solution (aqueous solution of iodine and potassium iodide).were poured in to colonies. Amylase production was detected as a transparent halo around the colony.

Determination of proteolytic activity
Proteolytic activities were analyzed using the media of Frazier's gelatin agar (FGA). Isolated bacteria were inoculated and incubate at 30ºC for 72 h. Plates were covered with Frazier's revealers (Smibert and Krieg, 1994). The presence of clear halos around the bacterial growth was observed.

Results
Endophytic Bacteria
Two different morphological bacterial species (*Clostridium sp, staphylococcus sp.*) were isolated from the leaves and flowers of *Punica granatum*. These species identified through the differential staining method and biochemical characterization.

Morphological identification
The bacterial species are identified through the test of gram staining, spore staining, acid fast staining and the biochemical studies.

Gram Staining
The result of Gram staining showed those violet colour colonies. Therefore violet stain was indicating that the test bacterium was positive.

Spore Staining
In the test of spore staining, the test bacterial colonies are treated with staining and careful examination under the microscope. Clostridium sp. indicate that the green ellipses within the cells. In other hand *staphylococcus sp*. revealed that pink colour appears therefore it was indicating the vegetative cell.

Enzyme activity of bacteria
The bacterial enzyme activity studied on the bacterial species (*Clostridium sp, staphylococcus sp.*)Which shows the presence of amylase, protease.

Table 1: Biochemicl characterizarion of endophytic bacteria *Punica granatum*

Characters	Inference	
	Clostridium sp.	*Staphylococcus sp.*
Starch	+	+
Citrate	_	_
Catalase	+	+
Casein	+	+
Glucose	+	+
Maltose	+	+
Lactose	_	_
Manitol	_	+
Sucrose	+	+

Discussion

Tens of thousands of natural products have been described, but in a world where we are not even closed to documenting all the extent species, there are almost certainly many more thousands of compounds waiting to be discovered. Likewise, Plant endophytes are takes place a major role to producing bioactive products which are potential applications in agriculture, medicine and food industry. In the past two decades, these bioactive compounds are useful as antimicrobial and insecticidal activity. Therefore, this work is mainly concentrated to separate the secondary metabolites of endophytes and to analyse its activities.

Endophytes can also be beneficial to their host by producing a range of natural products that could be harnessed for potential use in medicine, agriculture or industry. In addition, it has been shown that they have the potential to remove soil contaminants by enhancing phytoremediation and may play a role in soil fertility through phosphate solubilisation and nitrogen fixation. There is increasing interest in developing the potential biotechnological applications of endophytes for improving phytoremediation and the sustainable production of nonfood crops for biomass and biofuel production. (Robert P. Ryan *et al.*, 2007) [7]

The present investigation, Endophytes are isolated from plant (punica granatum) by following standardize protocols. Total of two different bacterial sp. (*Clostridium sp*, *staphylococcus sp.*)

The enzyme activity studied on the endophytic fungi (*Aspergillus* sp and Rhizophus sp.). It indicates the activity of enzyme such as amylase, protease, lipase, cellulase. The bacterial enzyme activity studied on the bacterial species (Clostridium sp, *staphylococcus sp.*)Which shows the presence of amylase, protease. Fungal enzymes are gaining importance in agriculture, industry and human health, as they are often more stable (at high temperature and extreme pH) than the enzymes derived from plants and animals. Fungal enzymes are used in manufacturing food, beverages, confectioneries, textile and leather and help simplifying the processing of raw materials. Wood-inhabiting marine fungi serve as a potential source of exo enzymes (e.g. Rohrmann and Molitoris, 1992; Raghu kumar *et al.*, 1994, 1999; Pointing *et al.*, 1998, 1999) [8, 5, 6, 4]. Kumaresan and Suryanarayanan (2002) [3] studied the extracellular enzyme production by the foliar endophytic fungi of *Rhizophora apiculata* and demonstrated their involvement in litter degradation after senescence. All the endophytes tested in our study showed cellulase activity similar to that of leaf inhabiting salt marsh fungi (Gessner, 1979). The high lipase activity suggests their ability to use fats as energy source. Gessner (1979) also demonstrated lipase activity in 20 higher marine fungi from salt marshes. Pisano *et al.*, (1964) Protease activity was seen in some endophytes of current study. None of the fungi showed lactase activity although lactase activity is seen in many marine fungi (Raghukumar *et al.*, 1994, 1999; Kumaresan and Suryanarayanan, 2002; Bucher *et al.*, 2004) [3, 1].

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