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Isolation and Characterization of Endophytes from Recalcitrant Plant Species

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Endophytes play crucial role in the life of the plants. Endophytes present in recalcitrant plants are of great value because such plant which harbor endophytes are more efficient in having antimicrobial activity, higher rates of nitrogen fixation and production of various useful biochemical products. In the present study, isolation of endophytes from recalcitrant plants was done followed by characterization. A total of seven bacterial endophytes and seven fungal endophytes were obtained. The endophytic bacterial isolate EBML showed good antibacterial property against *E.coli* NAIMCC 008483 and *Bacillus subtilis NAIMCC* and endophytic fungal isolate EFMR obtained showed good antibacterial property against and *E. coli* NAIMCC 008483*, Bacillus subtilis* NAIMCC 01233 and *Klebsiella* NAIMCC 00702. The endophytic isolates also showed good cellulase production in the range of 0.10-0.19 mg/ml/min in case of bacterial endophytes and in the range 0.19 – 0.21 mg/ml/min in case of fungal endophytes. pH characterization was found to be optimum at 7 for cellulase production for bacterial and fungal isolates. On the basis of 16srDNA and 28srDNA sequencing bacterial endophytic isolates were identified as *Bacillus* sp. and *Trichoderma* sp. respectively.

Key words: Endophytes; Recalcitrant plant; Cellulases; Antimicrobial activity.

Endophytes are known to impact on the life of plants in many ways. They shape plant communities (Clay, 1988) and manifest strong effects on the community structure and diversity of associated host organisms. They interact genetically and biochemically with their host and a greater understanding of these mechanisms will bring great potential for practical exploitation. Their capacity for the biosynthesis of new compounds, such as phytohormones, or enzymes that can be utilized for biodegradation purposes and phytoremediation, has generated lots of interest, as well as the possibility of new, sustainable biocontrol strategies for fighting pathogenic bacteria and diseases. They increase host shoot and/or root biomass, which may be brought about by induction of plant hormones or biosynthesis of plant hormones by the fungi (Tudzynski and Sharon, 2002). Endophytic isolates of *Fusarium oxysporum* and a *Cryptosporiopsis sp*. have been reported to confer disease resistance against virulent pathogens in barley (*Hordeum vulgare*) and larch (*Larix decidua*), respectively (Schulz *et al*., 1999). Also the resistance was found to be correlated with increased concentrations of phenolic metabolites. Endophytes provide certain benefits to the host plant by giving the resistance to certain pathogens or helping them in obtaining certain nutrients that cannot be produced by the plants but can be produced by the endophytes.

An endophytic fungus isolated from *Taxus baccata* bark displayed considerable antimicrobial activity. The fungus was identified

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as *Fusarium solani* based on morphological and molecular characteristics. The fungal metabolite showed activity against both bacterial and fungal pathogens. The presence of alkaloids and other chemicals synthesized by the endophytes made the plant more resistant to nematodes, insect herbivores and livestock (Schardl *et al*., 2004)

It has also been demonstrated that the endophytes isolated from medicinal plants are excellent producers of strong fungicidal, bactericidal and cytotoxic metabolites (Radu and Kqueen, 2002). Over 50 antibiotic substances have been detected in *Fusarium* spp. and *Trichoderma* spp. alone, an endophytic fungus having antimicrobial properties against *Bacillus subtilis, Staphylococcus aureus, Mycobacterium smegmatis, M. phlei, E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans, Fusarium oxysporum* and *F. semitectum* (Gogai *et al.*, 2008). Occasionally, these substances or products are the same as those produced by the respective host plants, thus giving us the idea that endophytic fungi can serve as an alternative source of important plant secondary metabolites. Endophytes produce certain antibiotics that can be used to treat human diseases to great extent, because they are natural and not chemicals and are found to show better effect than chemical drugs. *Colletotrichum* which is present as endophyte present in *Aertemisia annua* produces certain metabolites that act as fungistatic for certain human pathogenic fungus and hence can be explored for treatment of fungus related diseases (Guo *et al.*, 2008).

MATERIALS AND METHODS

Collection of samples and maintenance

Samples of leaves, roots and bark of various recalcitrant species were collected from Kapurthala region of Punjab. Recalciterant species viz Lychee (*Litchi chinesis*) - 9 year old tree, Jackfruit *(Artocarpus lakoocha)*- 14 year old tree, Mango (*Mangifera indica)*- 12 year old tree. Samples were stored at -20°C in Ziploc bags till further use. Sample were surface sterilized using 15% (w/v) hydrogen peroxide solution. Samples were surface sterilized in 70% ethanol for 1 minute, followed by 15 minutes in a solution of 15% H_2O_2, dipped again in for 1 min in 70% ethanol and then rinsed in distilled water (Inga and Odeta, 2011). Samples for isolation of bacterial endophytes were washed under running tap water to remove the soil particles or dirt and then immersed in 70% ethanol for 5 min followed by immersion in solution of sodium hypochlorite for 20 min. After this, the samples were washed in sterilized water to remove the surface sterilization solution and then immersed in 10% sodium hydrogen carbonate solution to disrupt the plant tissues and prevent the fungal growth (Lixiang *et al*., 2005).

Isolation of endophytes

Fungal endophytes were isolated as described by Murray *et al.* (1995). Surface sterilized plant parts were cut into small pieces and crushed and piece was kept on potato dextrose agar media. The plates were incubated at 27°C until the growth was observed. After the growth was observed fungus was point inoculated on fresh potato dextrose agar media and incubated at 27°C. Bacterial endophytes were isolated as described by Downes *et al.* (2001). Surface sterilized plant samples were cut into small pieces and crushed. The ooze from the samples was streaked on nutrient agar media and the sterilized plant piece was also kept in nutrient agar media. It was incubated at 37°C for 24 hours or until the growth was observed. The isolates were purified by pentagonal streaking on nutrient agar media.

Characterization of endophytes

Endophytes isolates were characterized according to their staining properties and on the basis of different biochemical tests. Fungal endophytes were characterized as described by Gilman (1944). Bacterial endophytes were classified as per Bergey's manual of determinative bacteriology (Krieg and Holt, 1994).

To study the morphology of fungal isolates, lactophenol cotton blue staining was done as described by Larone (1993).

Gram staining was done as described by Hucker (1921). Isolated bacterial endophytes were grown on specialized media like *Pseudomonas* Isolation agar (Isenberg and Garcia, 2004) and crystal violet pectate media (for *Erwinia*)(Cuppels and Kelman, 1974).

For the bacteria that showed growth on the specialized media, certain biochemical tests were done to confirm the identity of the isolated bacteria like IMViC test, nitrate reduction test,

catalase test (Cappuccino, J.G and Sherman, 1992).

Enzyme Activity

Endophytic microbial isolates were checked for production of enzymes like cellulase, chitinase and gelatinase. Cellulase activity was checked as per method described by Kim *et al.* (2012). Cellulase assay was done by DNS method (Irfan *et al.* 2012) .

Chitinase assay was done as per described Vaidya *et al*. (2001). Bacterial endophytes were grown on chitin agar media plates and assessed for formation of a clear zone. Gelatinase activity was checked as described by Murray *et al.*(1995).

Antimicrobial property

Antibacterial properties of the endophytic isolate were tested against the bacteria *E. coli* NAIMCC 008483, *Bacillus subtilis* NAIMCC 01233 and *Klebsiella* NAIMCC 00702 as per described by Pavithra *et al.* (2012).

16s rDNA and 28s rDNA sequencing

Endophytes showing maximum antimicrobial activity were identified on the basis of 16SrDNA and 28srDNA sequencing carried out at Samved Biotech, Ahmedabad. The 16s rDNA sequencing was done with the help of 27F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. For 28srDNA sequencing forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16srRNA and 28srRNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database.

RESULTS AND DISCUSSION

Isolation of fungal endophytes

Different endophytes were obtained from recalcitrant plant species *Litchi chinesis*, *Artocarpus lakoocha* and *Mangifera indica* and the growth of fungal endophytes potato. Total seven isolates of fungal endophytes were obtained, two from litchi, two from jackfruit and three from mango. In litchi, the isolates were obtained from Phyllospere region (leaves) and Rhizosphere region, in jackfruit from bark and stem and in case of mango, from leaves, bark and roots. The pure culture of endophytic fungus was obtained by point inoculation into fresh potato dextrose agar. Fungal endophytes obtained were named as shown in Table-1. After the isolation, the fungi were identified according to the colony morphology of the fungi and the morphology was seen under the microscope by staining it with lactophenol cotton blue as per manual of soil fungi Gilman (1944). It was found that the isolate EFLL and EFLR were *Aspergillus nidulans* and the isolate EFML was *Fusarium oxysporum* and in isolates EBJS, EBJB, EBMB and EBMR there was no sporulation so they were categorized as mycelia sterlia according to the morphology seen as per manual of soil fungi (Gilman, 1944).

Fusarium aqueductum was isolated from a tropical Palm tree by Rodrigues and Samuels (1990). Cother and Gilbert (1994) reported *Fusarium* as the most prevalent endophytic fungus associated with bladder saltbush plant. Also, endophytes like *Colletotrichum gloeosporiodes* and *Cytosphaera mangiferae* have been found in litchi plants (Johnson *et al*., 1992) similarly and *Haplotrichum minitissimum* were reported from jackfruit by Novas and Carmaran (2008).

Isolation of bacterial endophytes

From recalcitrant plants the bacterial endophytes were isolated on nutrient agar media after incubation at 37°C for 24 hours. Total seven isolates of bacterial endophytes were isolated from various parts of three plant species taken. In litchi the isolates were obtained from leaves and roots, in jackfruit from bark and stem and in case of mango, from leaves, bark and roots . The isolated colonies of these endophytic bacteria isolated were purified by pentagonal streaking after incubation of 24 hours. Wide range of colony morphology was shown by the bacterial endophytes like isolates EBJS showed white mucoid colonies and isolate EBMR formed pale yellow pin pointed colonies (Table- 2). Endophytic bacteria of genus *Bacillus, Pseudomonas* and *Corynebacterium* are the most frequently isolated endophyte (Shi *et al.*, 2013). Several *Bacillus* sp. like *B. subtilis* were found to exist as an endophyte in recalcitrant plants like *Eucalyptus urophylla* (Paz *et al*., 2012). Some bacterial endophytes like *Lasiodiplodia theobromae* have been reported in jackfruit plant and *L. theobromae, Neofusicoccum mangiferae, Neofusicoccum parvum* have been reported in mango plants (Huang and Wang, 2011).

Characterization of endophytes

The purified bacterial isolates were subjected to Gram's staining and it was found that all endophytic bacterial isolates were gram negative bacteria except the isolate EBML which was gram positive. In some studies it has been found that gram negative endophytic bacteria are more prominent than gram positive endophytic bacteria (Hung and Annapurna, 2004). Also, the isolate EMBL was found to give positive results for spore staining also, while no sporulation was observed in any other bacterial isolates.

The gram negative bacterial isolates obtained were grown on specialized media like Pseudomonas isolation agar specific for *Pseudomonas* and crystal violet pectate media specific for *Erwinia*. It was found that the isolate EBMR showed growth in pseudomonas isolation agar and the colony produced were pale yellow mucoid which shows that it may belong to genus *Pseudomonas* (Galli *et al.,* 1992). To confirm the bacteria various biochemical tests were performed

Table 1. Endophytic fungal isolates obtained from recalcitrant plant samples

Fungal Isolate	Source	Colony color on surface	Colony color at bottom	Tentative Identification
EFLL	Litchi leaves	Light green	Green	*Aspergillus nidulans*
EFLR	Jackfruit bark	Light green	Green	*Aspergillus nidulans*
EFJB	Jackfruit bark	Brown	Dark brown	Mycelia sterlia
EFJS	Jackfruit stem	Light brown	Dark brown	Mycelia sterlia
EFML	Mango leaves	White	Black at the centre and white on the periphery	*Fusarium oxysporum*
EFMR	Mango roots	Cottony white	White	Mycelia sterlia
EFMB	Mango bark	White	White	Mycelia sterlia

Table 2. Endophytic Bacterial isolates obtained from recalcitrant plant samples

Bacterial Isolate	Source	Form	Size	Surface	Texture	Color
EBLL	Litchi leaves	Circular	Small	Shiny	Moist	White
EBLR	Jackfruit bark	Circular	Small	Slimy	Moist	White
EBJB	Jackfruit bark	Circular	Small pinpointed	Shiny	Moist	Pale yellow
EBJS	Jackfruit stem	Circular	Small	Slimy	Moist	White
EBML	Mango leaves	Circular	Small	Rough	Dry	Off white
EBMR	Mango roots	Circular	Small pinpointed	Rough	Dry	Pale yellow
EBMB	Mango bark	Circular	Small	Slimy	Moist	White

Table 3. Biochemical tests of Bacterial Endophytes Isolate

Bacterial Isolate	Gram staining	Cell Shape	Spore Staining	Catalase test	Indole test	Methyl red	Voges Proskauer Test	Utilization of citrate
EBLL	-	Rod	-	+	-	-	+	+
EBLR	-	Rod	-	-	+	+	_	-
EBJB	-	Rod	-	+	-	-	+	+
EBJS	-	Rod	-	+	-	+	_	-
EBML	+	Rod	+	+	+	-	_	-
EBMR	-	Rod	-	-	-	-	-	+
EBMB	-	Rod	-	+	+	+	_	-

like IMViC test and nitrate reduction test. The results obtained (Table 3) shows that the isolated bacteria on the media seem to belong to the genus *Pseudomonas*. It has been reported by Aravind *et al.* (2009) that among the gram negative bacteria *Pseudomonas* sp. was found to be the dominant bacterial genus in *Piper nigrum*. On crystal violet pectate medium the isolate EBLL showed growth and purple colored colonies were which may be of *Erwinia* that were further confirmed by various biochemical tests and the results obtained (Table-3) shows that the bacteria isolate EBLL is of genus *Erwinia* (Amin *et al*., 2010).

Table 4. Enzyme production profile of various endophytic microbial isolates

Microbial Isolate	Gelatinase	Cellulase	Chitinase
EFLL	–	+	–
EFLR	–	+	–
EFJB	–	–	–
EFJS	–	–	–
EFML	–	+	–
EFMR	–	+	–
EFMB	–	–	–
EBLL	+	+	–
EBLR	+	–	–
EBJB	–	–	–
EBJS	+	–	–
EBML	+	+	–
EBMR	+	+	–
EBMB	–	–	–

Enzymatic activity

Gelatinase test

The enzymatic capability of the endophytes suggest their potential to access carbon, nitrogen and phosphorous with a potential to aid host nutrient uptake (Pragathi *et al*., 2013). Endophytes were tested for the presence of gelatinase enzyme that hydrolyzes gelatin. Out of seven bacterial isolates, five isolates EBLL. EBLR, EBJS, EBML and EBMB were found to produce gelatinase enzyme whereas no fungal endophytes was found to produce gelatinase enzyme (Table-4). Endophytes like *Colletotrichum gleosporiodes* and *Penicillin corylophilum* have been reported to produce gelatinase enzyme (Pragathi *et al*., 2013).

Chitinase activity

Endophytes obtained were tested for production of chitinase enzyme by inoculating in chitin agar media. The results showed that neither endophytic bacteria nor endophytic fungi (Table-4) exhibited the property of production of chitinase enzyme. This is in agreement with the findings of Maria *et al*. (2005) who have reported that endophytes are known to produce several enzymes but no chitinase activity has been evident in the endophytes.

Table 5. Cellulase Activity for Microbial Endophytic Isolates

Fungal Endophytic Isolates	Enzymatic activity(mg/ml/min)	Bacterial Endophytic Isolates	Enzymatic activity(mg/ml/min)
EFLL	0.185±0.01	EBLL	0.16±0.02
EFLR	0.19±0.02	EBJS	0.14±0.01
EFML	0.17±0.01	EBML	0.19±0.01
EFMR	0.20 ±0.02		

Table 6. Effect of Temperature on the Cellulase Activity of Endophytic Isolates

Temp. Isolate	27ºC	37ºC	47ºC
EFLL	0.17±0.01	0.14±0.03	0.14±0.01
EFLR	0.18±0.02	0.15±0.01	0.15±0.01
EFML	0.19±0.02	0.12±0.01	0.10±0.01
EFMR	0.21±0.01	0.18±0.02	0.18±0.02
EBLL	0.09±0.02	0.15±0.02	0.11±0.02
EBJS	0.10±0.01	0.14±0.02	0.11±0.03
EBML	0.12±0.01	0.18±0.02	0.15±0.02

Cellulase test

Cellulase test was done to detect the presence of cellulase enzyme in the endophytes. In case of bacterial endophytes isolates EBLL, EBML and EBJS out of seven isolates showed cellulase activity and in fungal isolates also four isolates EBML, EBMR, EBLL, EBLR were found to show cellulase activity (Table- 4). Hydrolytic enzymes play a role in the mechanism by which endophytic bacteria penetrate and persist in the

host. These enzymes help in the spreading of endophytes in the host plants as the cell wall of the host plants contain cellulose (Hung and Annapurna, 2004).

Cellulase production under liquid culture conditions

Cellulase activity was checked of endophytic bacterial isolates and fungal isolates which had given positive result for cellulase on CMC plates. It was found from the results obtained that the fungal isolates had higher cellulase activity than the bacterial isolates. The fungal isolate EFMR showed highest cellulase activity (0.20 mg/ml/min) as compared to other isolates (Table- 5). It was reported by Doolotkeldieva and Bobusheva (2011) that fungi like *Trichoderma*, *Aspergillus* and *Penicillium* have high cellulase activity.

Optimization of culture conditions for cellulase activity

The culture conditions play a very crucial role in the production of various enzymes. Hence

Table 7. Effect of pH on the Cellulase Activity of Microbial Endophytic Isolates

pH Isolate	4	5	6	7	8
EFLL	0.15±0.01	0.17±0.02	0.18±0.01	0.18±0.01	0.15±0.02
EFLR	0.16±0.02	0.18±0.02	0.19±0.01	0.19±0.01	0.15±0.01
EFML	0.12±0.03	0.15±0.01	0.19±0.01	0.20±0.02	0.17±0.01
EFMR	0.17±0.01	0.18±0.03	0.21±0.02	0.21±0.01	0.20±0.01
EBLL	0.10±0.03	0.12±0.02	0.14±0.01	0.16±0.01	0.13±0.02
EBJS	0.09±0.01	0.10±0.01	0.14±0.01	0.15±0.02	0.09±0.02
EBML	0.11±0.01	0.14±0.01	0.16±0.01	0.19±0.01	0.10±0.02

Table 8. Effect of CMC concentration on Cellulase Activity of Endophytic isolates

CMC conc. Isolate	1%	2%	3%
EFLL	0.18±0.01	0.15±0.01	0.13±0.02
EFLR	0.19±0.02	0.15±0.01	0.14±0.01
EFML	0.20±0.01	0.16±0.02	0.13±0.02
EFMR	0.21±0.02	0.17±0.03	0.14±0.01
EBLL	0.16±0.01	0.14±0.02	0.07 ±0.01
EBJS	0.14±0.01	0.10±0.01	0.07±0.02
EBML	0.19±0.02	0.15±0.02	0.09±0.03

Table 9. Antibacterial activity of various endophytic isolates

Target bacteria Isolate	*E. coli* NAIMCC 008483 (Zone of inhibition in mm)	*Bacillus* NAIMCC 01233 (Zone of inhibition in mm)	*Klebsiella* NAIMCC 00702(Zone of inhibition in mm)
EFLL	+ (23)	- (0)	+ (15)
EFLR	+ (25)	- (0)	+ (18)
EFJS	+ (13)	- (0)	+ (15)
EFJB	+ (16)	- (0)	+ (14)
EFMR	+(13)	- (0)	+ (14)
EFML	+ (14)	- (0)	+ (17)
EFMB	+(14)	- (0)	+ (16)
EBLL	- (0)	- (0)	- (0)
EBLR	- (0)	- (0)	- (0)
EBJS	- (0)	- (0)	- (0)
EBJB	- (0)	+(15)	- (0)
EBMR	- (0)	- (0)	- (0)
EBML	- (0)	- (0)	+(10)
EBMB	- (0)	+ (17)	- (0)

the impact of various factors were assessed by varying one variable at a time. Bacterial isolate EBML showed highest cellulase activity (0.18±0.02 mg/ml/min) at 37 ^{0}C with the temperature proving to be suitable for the rest of the bacterial isolates also. In case of fungal endophytes, the isolate 27°C was found to result into maximum enzyme production (Table -6). Similarly Shakoor *et al.* (2013) reported that for bacteria, the maximum cellulase activity occurs at temperature 37°C for *Bacillus megarterium*. However, Verma *et al.* (2012) reported that the best temperature in case of *Bacillus subtilis* for cellulase activity was found to be 45°C. Fagade and Bamigboye (2012) also found that in case of *Bacillus subtilis* the highest cellulase activity was found at 28°C while *Bacillus licheniformis* had the highest production at 40°C. It was reported by Said *et al.,* (2014) that in case of fungi *Chaetomium globosum* and *Trichoderma harzianum* 25-28°C was the optimum for cellulose production whereas for fungus *Alternaria alternata* the optimum temperature for cellulase activity was found to be 35°C.

In case of bacterial isolates, the isolate EBML showed highest cellulase activity at pH 7 (0.19±0.01 mg/ml/min). The pH 7 was also found to be optimum for other bacterial isolates. Fungal isolate EFMR showed highest cellulase activity in the pH range 6 and 7 (Table- 7). Shakoor *et al.,* (2013) also reported that the optimum pH value for showing maximum cellulose activity in case of *Bacillus megaterium* was 7. Similar results were reported by Fagade and Bamigboye (2012) in case of *Bacillus subtilis*. Das *et al.* (2011) also found that the highest cellulase activity obtained in case of pectinolytic bacteria was in the range of pH 6-7 after which it starts decreasing.

The substrate concentration was varied in carboxymethyl cellulose broth by using various concentrations of CMC *viz*. 1%, 2% and 3%. The results obtained showed that highest cellulase activity occurred at cellulose concentration of 1% for bacterial endophytes as well as fungal endophytes after which the enzymatic activity starts decreasing (Table -8). The bacterial isolate EBML showed highest cellulose activity at 1% CMC concentration. Similar results were found by Shaikh *et al.* (2013) and Ibrahim *et al.* (2012).

Antimicrobial activity

Endophytes produce certain compounds that do not allow the growth of other bacteria near it thus produces a zone of clearance against those bacteria. Endophytic bacteria and fungi were tested for production of any antibacterial compound against *E. coli* NAIMCC 008483, *Bacillus subtilis* NAIMCC 01233 and *Klebsiella* NAIMCC 00702. It was observed that some endophytic bacteria showed antibacterial property against *Bacillus* and *Klebsiella* (Table-9). The isolate EBMB exhibited a highest antibacterial activity against *Bacillus subtilis* (17 mm) and isolate EBML showed highest antibacterial property against *Klebsiella* (10 mm) compared to other isolates. All the fungal endophytes showed activity against the bacteria *E. coli* and *Klebsiella* whereas no antibacterial property was found against *Bacillus subtilis.* Zone of inhibition was produced against *E.coli* and *Klebsiella*. The isolate EBLR exhibited a highest antibacterial activity against *E. coli* (25mm) and *Bacillus subtilis* (18mm) compared to other isolates (Table 9). It was found that the fungal endophytes showed more antibacterial property than the endophytic bacteria. Idris *et al.* (2013) reported the antibacterial effect of fungi *Cladosporium sp.* and *Aspergillus sp*. and found that these endophytic fungi were effective against the bacteria *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*. Powthong *et al.* (2013) found that the endophytic fungi *Acremonium spp*. and *Fusarium spp*. *Phaeoacremonium spp., Phomopsis spp., Paecilomyces spp.,* and *Cladosporium spp*. isolated from *Sesbania grandiflora* inhibited the growth of *Staphylococcus aureus* and *Bacillus subtilis* thus showing the antibacterial property of endophytic fungi.

Molecular characterization of selected isolates

The rDNA sequence based study was carried out for the isolates EMBL and EFMR, since these were showing antimicrobial activity. For endophytic fungal isolate EFMR 28s rDNA sequencing was done and the sequence obtained was subjected to n-BLAST. The results obtained shows that the isolate EFMR belongs to *Trichoderma* sp. (GenBank Accession Number KJ940977). For endophytic bacteria isolate EBML, 16S rDNA sequencing was done and the sequence obtained was subjected to n-BLAST. The results obtained shows that the isolate EBML belongs to *Bacillus* sp. (Genebank Accession Number KJ914665).

CONCLUSION

In the present study fourteen microbial endophytes were obtained from the recalcitrant plant *Litchi chinesis*, *Artocarpus lakoocha* and *Mangifera indica*. Many of them possessed the ability of producing cellulase and other hydrolytic enzymes while the isolates EBML and EFMR were found to possess antimicrobial activities. Thus the endophytes associated with various plants may act as a very rich source of an array of beneficial metabolites.

REFERENCES

1. Clay, K. Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *J. Ecol.*, 1988; **69**: 10–16.
2. Tudzynski, B., Sharon, A. Biosynthesis, biological role and application of fungal phytohormones. In: *The mycota- Industrial applications* (Osiewacz, H.D., ed.). Berlin: Springer- Verlag, 2002; pp 183–212.
3. Schulz, B., Rommert, A.K., Dammann, U., Aust, H.J., Strack, D. The endophyte-host interaction: a balanced antagonism. *Mycol. Res*., 1999; **10**: 1275–1283 (1999)
4. Schardl, C.L., Leuchtmann, A., Spiering, M.J. Symbioses of grasses with seed borne fungal endophytes. *Ann Rev Plant Biol*, 2004; **55**: 315–340.
5. Radu, S., Kqueen, C.Y. Preliminary screening of endophytic fungi from medicinal plants in Malaysia for antimicrobial and antitumour activity. *Malay. J. Med. Sci*., 2002; **9**: 23–33.
6. Gogai, D.K., Hari, P., Boruah, D., Saikia, R., Bora, T.C. Optimization of process parameters for improved production of bioactive metabolite by a novel endophytic fungi *Fusarium sp.*, DF2 isolated from *Taxus wallichiana* of North East India. *World J. Microbiol. Biotechnol*., 2008; **24**: 79-87.
7. Guo, B., Wang, K., Sun, X., Tang, K. Bioactive natural products from endophytes. *Appl. Biochem. Microbiol.*, 2008; **44**: 136-142.
8. Inga, M., Odeta, B. IAA production and other plant growth promoting traits of endophytic bacteria from apple tree. *Biologija*., 2011; **57**: 98–102
9. Lixiang, C., Zhiqi, Q., Jianlan, Y., Hongming, T., Shining, Z.: Isolation and characterization of endophytic streptomycete antagonists of *Fusarium* wilt pathogen from surfacesterilized banana roots. *FEMS Microbiol. Lett*. 2005; **247**: 147-152.
10. Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, R.J. Manual of clinical microbiology, 6th edn., Washington D.C: American Society for Microbiology,1995
11. Downes, F.P., Ito, K. Compendium of Methods for the Microbiological Examination of Foods, 4th Ed., Washington D.C.: American Public Health Association, 2001
12. Gilman, J.C. A manual of soil fungi. 2nd edn, New Delhi: Oxford and IBH Publishing Co.,1944
13. Krieg, N.R., Holt, J.G. Bergey's Manual of Determinative Bacteriology, 9th edition. Baltimore: Williams and Wilkins, 1994.
14. Larone, D.H. Medically Important Fungi- A Guide to Identification, 2nd edn. Washington D.C.: American Society for Microbiology, 1993
15. Hucker, G.J. A modification and new application of the Gram stain. *J. Bac*. 1921; **6**: 395-397.
16. Isenberg, H.D., Garcia, L.S. Clinical microbiology procedures handbook, 2nd edn. Washington, D.C.: American Society for Microbiology, 2004
17. Cuppels, D., Kelman, A. Evaluation of selective media for isolation of soft- rot bacteria from soil and plant tissue. *Phytopathol*. 1974; **64**: 468-475.
18. Cappuccino, J.G and Sherman, N. Microbiology: A Laboratory Manual, California: Cummings publisher, 1992
19. Kim, Y.K., Lee, S.C., Cho, Y.Y., Oh, H.J., Ko, Y.K. Isolation of cellulolytic *Bacillus subtilis* strains from agricultural environments. *ISRN Microbiol*. 2012 doi: 10.5402/2012/650563
20. Irfan, M., Safdar, A., Syed, Q., Nadeem, M. Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. *Turk. J. Biochem.* 2012; **37**: 287–293.
21. Vaidya, R.J., Sham, I.M., Vyas, P.R., Chhatpar, H.S. Production of chitinase and its optimization from a novel isolate *Alcaligenes xylosoxydans*: potential antifungal biocontrol. *World J. Microbiol. Biotechnol*. 2001; **17**: 62-69.
22. Pavithra, N., Sathish, L., Ananda, K. Antimicrobial and enzyme activity of endophytic fungi isolated from Tulsi. *J. Pharma. Biomed. Sci*. 2012; **16**: 1-6.
23. Rodrigues, K.F., Samuels, G.J. Preliminary study of endophytic fungi in a tropical palm. *Mycol. Res*. 1990; **94**: 827-830.
24. Cother, E.J., Gilbert, R.L. The endophytic mycoflora of bladder saltbush (*Atriplex vesicaria*Hew. Ex Benth.) and its possible role in the plants periodic decline. *Proceed. Linn. Soc. New South Wales*. 1994; **114**: 149-169.

25. Johnson, G.I., Mead, A.J., Cooke, A.W., Dean, R.J. Mango stem and rot pathogens – fruit infection by endophytic colonization of inflorescence and pedicel. Ann. App. Biol. 1992; **120**: 225- 234.
26. Novas, M.V., Carmaran, C.C. Studies on diversity of foliar fungal endophytes of naturalized trees from Argentina with a description of *Haplotrichum minutissimum* sp. *Func. Ecol. Plants*. 2008; **203**: 610-616.
27. Shi, Y.W., Zhangb, X., Louc, K.: Isolation, characterization, and insecticidal activity of an endophyte of drunken horse grass, *Achnatherum inebrians*. *J. Insect Sci*. 2013; **13**: 1-12.
28. Paz, C. P., Santin, R.C.M., Guimaraes, A.M., Rosa, O.P.P., Dias, A.C.F., Quecine, M.C., Azevedo, J.L., Matsumura, A.T.S. Eucalyptus growth promotion by endophytic *Bacillus* spp. *Genet. Mol. Res*. 2012; **11**: 3711-3720.
29. Huang, C.L., Wang, Y.Z. New records of endophytic bacteria associated with the Araucariaceae in Taiwan. *Collect. Res.* 2011; **24**: 87-95.
30. Hung, P.Q., Annapurna, K. Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). *Omonrice*. 2004; **12**: 92-101
31. Galli, E., Silver, S., Witholt, B.: *Pseudomonas*: Molecular Biology and Biotechnology, Washington D.C.: American Society for Microbiology, 1992
32. Aravind, R., Kumar, A., Eapen, S.J. Tracking of endophytic bacteria in different parts of black pepper (*Piper nigrum* L.): root, stem and leaves. *Lett. Appl. Microbiol*. 2009; **48**: 58-64.
33. Amin, N.M., Bunawan, H., Redzuan, R.A., Jaganath, I.B. *Erwinia mallotivora* sp., a new pathogen of Papaya (*Carica papaya*) in Peninsular Malaysia. *Inter. J. Mol. Sci*. 2010; **12**: 39-45.
34. Pragathi, D., Vijaya, T., Mouli, K., Anitha, D. Diversity of fungal endophytes and their bioactive metabolites from endemic plants of Tirumala hills- Seshachalam biosphere reserve. *Afr. J. Biotechnol*. 2013; **12**: 4317-4323.
35. Maria, G. L., Sridhar, K.R., Raviraja, N.S. Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. *J. Agric. Technol*. 2005; **1**: 67-80.
36. Doolotkeldieva, T.D., Bobusheva, S.T. Screening of Wild-Type Fungal Isolates for Cellulolytic activity. *J. Microbiol. Insight*. 2011; **4**: 1 – 10.
37. Shakoor, S., Aftab, S., Rehman, A. Characterization of cellulose degrading bacterium, *Bacillus megaterium* S3, isolated from indigenous environment. *Pak. J. Zool*. 2013; **45**: 1655-1662.
38. Verma, V., Verma, A., Kushwaha, A. Isolation and production of cellulase enzyme from bacteria isolated from agricultural fields in district Hardoi, Uttar Pradesh, India. *Adv. Appl. Sci. Res*. 2012; **3**: 171-174.
39. Fagade, O.E., Bamigboye, O.O. Effect of cultural conditions on the cellulase activity of bacteria species isolated from degrading corn. *Arch. Appl. Sci. Res*. 2012; **4**: 2540- 2545.
40. Said, A.H., Saleem, A., Maghraby, T.A., Hussein, M.A.: Cellulase activity of some phytopathogenic fungi isolated from diseased leaves of broad bean. *Bot. Inter. J. Curr. Micro. Appl. Sci*. 2014; **3**: 883-900.
41. Das, B., Chakraborty, A., Ghosh, S., Chakrabarti, K. Studies on the effect of pH and carbon sources on enzyme activities of some pectinolytic bacteria isolated from jute retting water. *Turk. J. Biol*. 2011; **35**: 671-678.
42. Shaikh, N.M., Patel, A.A., Mehta, S.A., Patel, N.D. Isolation and screening of cellulolytic bacteria inhabiting different environment and optimization of cellulase production. *Univ. J. Environ. Res. Technol*. 2013; **3**: 39-49.
43. Ibrahim, A.D., Mukhtar, S.I., Ibrahim, M.N., Oke, M.A., Ajijolakewu, A.K. *Adansonia digitata* (Baobab) fruit pulp as substrate for *Bacillus* Endoglucanase production. *Res. Biotechnol*. 2012; **3**: 1-7.
44. Idris, A., Altahir, I., Idris, E. Antibacterial activity of endophytic fungi extracts from the medicinal plant *Kigelia africana*. *Egypt. Acad. J. Biol. Sci*. 2013; **5**: 1-9.
45. Powthong, P., Jantrapanukorn, B., Thongmee, A., Suntornthiticharoen, P. Screening of antimicrobial activities of the endophytic fungi isolated from *Sesbania grandiflora* (L.) *Pers. J. Agric. Sci*. 2013; **15**: 1513-1522.