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Enzyme Production and Antimicrobial Activity of Endophytic Bacteria Isolated from Medicinal Plants

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Abstract

Objectives: Endophytic microorganisms inhabiting the medicinal plants synergistically produce pharmaceutically important metabolites in their host plants. To explore the possibility of identifying bacterial endophytes producing industrially important enzymes if any, leaves of medicinal plants were collected from the campus of Gurunank College, Chennai, Tamil Nadu. **Method:** Six bacterial endophytes were isolated from the leaves of the three traditionally practiced medicinal plants, *Mangifera indica*, *Calotropis gigantea* and *Hibiscus rosa-sinensis* and were screened for antimicrobial and enzyme activity. **Findings:** Endophytic microbial isolates exhibited amylase, protease and cellulase activities in addition to antibacterial and antifungal activities. **Application:** This study implies that further analysis of these microorganisms will provide promising results in the development of new antimicrobial agents and enzymes with potential usage in various industries.

Keywords: Antimicrobial Activity, Endophytic Bacteria, Medicinal Plants, Protease and Amylase

1. Introduction

The survey report of World Health Organization indicated that almost 80% of the world's population especially in developing countries depends on traditional medicines which involve the use of plant extracts for primary health care. Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and/or reduced toxicity[1].

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. India has a rich diversity of medicinal plants and the knowledge on the phytochemical properties of these plants has been put in to practice to improve health status of people and in the preparation of pharmaceutical and nutraceutical products.

Endophytic bacteria are those live symbiotically with the internal plant tissues without any sign of peripheral infection or adverse effect on their host[2]. Plant endophytic bacteria have been widely accepted as an economic resource of vital and novel biomolecules and enzymes having potential application in agriculture, pharmaceutical and food industry[3]. 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, *Mangifera indica*, *Hibiscus rosa-sinensis* and *Calotropis gigantea* that have remarkable medicinal and commercial importance.

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2. Materials and Methods

Three medicinal plants, *Mangifera indica*, *Hibiscus rosa-sinensis* and *Calotropis gigantea* collected from the campus of Gurunanak College, Velachery, Chennai were used for the present study.

2.1 Isolation of Endophytic Bacteria

The leaves of medicinal plants were collected from Gurunanak College campus and cut in to small bits of 5 × 5 mm (length and breadth) with sterile blades in the laminar air flow. These bits were washed with tap water and with sterile water thrice each. Then the leaflets were washed with sterile water twice, allowing the bits to stay for 3 minutes in each wash. The leaf bits were surface sterilized using 70% ethanol for one minute and immersed once in 2% sodium hypochlorite solution for 5 minutes. Finally the samples were rinsed 3 times in sterile distilled water. For further drying the sterilized leaf bits were retained in the laminar air flow on sterile filter paper.

After drying the bits were placed on LB agar plates and incubated overnight at 37°C in an inverted position. On the following day, the endophytic bacteria that had grown out of the leaf bits were taken in an inoculation needle and subcultured in order to get isolated colonies. Single colonies obtained were streaked on Luria Bertani (LB) agar plates for acquiring pure cultures. These pure lines were preserved in glycerol stocks and used for further analysis.

Gram staining was done for all the isolates.

2.2 Antimicrobial Activity

Antimicrobial activity of the isolated endophytes was examined by pipeting 20µl of overnight grown bacterial cultures, in the wells punched in LB agar plates with precultured pathogenic lawns. *E. coli* and *Bacillus subtilis* were the strains used for antibacterial screening, and *Aspergillus niger* was the fungal strain tested for antifungal activity. Overnight culture mixed with equal volume of DMSO/Chloroform was vortexed vigorously for 20 minutes and the supernatant obtained after centrifugation was used for testing the antimicrobial activity.

2.3 Bacteriocin Production

Dimethyl sulphoxide (DMSO) and chloroform were used separately for the extraction of bacteriocin from the strain. Three wells were formed on the LB agar spread plated with the pathogenic isolate of *E. coli*. An aliquot of 10 µl each of C.g.1 culture, C.g.1 bacteriocin+DMSO and DMSO were added to the wells. *Bacillus subtilis* was used as the control.

2.4 Enzyme Production by Endophytes

Enzyme screening was done by inoculating the endophytic cultures into wells formed on quarter-strength LB agar medium with 1% substrate (Table 1). For protease, cellulase, amylase and lipase, the substrates namely casein, carboxy methyl cellulose, starch and tributerin were used as substrates respectively. Enzyme activity was confirmed by flooding the plates with congo red for cellulase, iodine reagent for amylase and Coomassie Brilliant Blue for protease. Development of clearance zones was checked for lipase. Selected strains were inoculated in suitable production media to quantitate enzyme activity spectrophotometrically (Beckman) using enzyme assays.

The methods followed for the estimation of protease[5], amylase[6] and cellulase[7] were according to those reported earlier.

3. Results and Discussion

3.1 Isolation of Endophytes

Endophytic bacteria were obtained and purified from the edges of the surface sterilized leaf bits placed on nutrient agar plates incubated overnight. Endophytic bacteria emerging from the peripheral regions of leaf bits are shown in Figure 1.

Three isolates from *Calotropis gigantea*, two from *Hibiscus rosa-sinensis* and a single bacterial strain from *Mangifera indica* were named as C.g.1, C.g.2, C.g.3, H.r.1, H.r.2 and M.i. respectively (Figure 2). These pure cultures were then preserved in glycerol stocks for further analysis.

Table 1. Culture media used for the production of enzymes (for 100 ml)

AMYLASE	CELLULASE	PROTEASE
Maize powder : 6 g	Yeast extract: 4 g	Sucrose : 600 mg
Sucrose : 2 g	Bagasse : 4 g	Na_2HPO_4 : 60 mg
$CaCO_3$: 1 g	Sucrose : 4 g	NaH_2PO4 : 60 mg
NaH_2PO_4 : 100 mg	CMC : 2 g	Peptone : 500 mg
Termamyl : 1 mg	K_2HPO_4 : 300 mg	
	KH_2PO_4 : 500 mg	

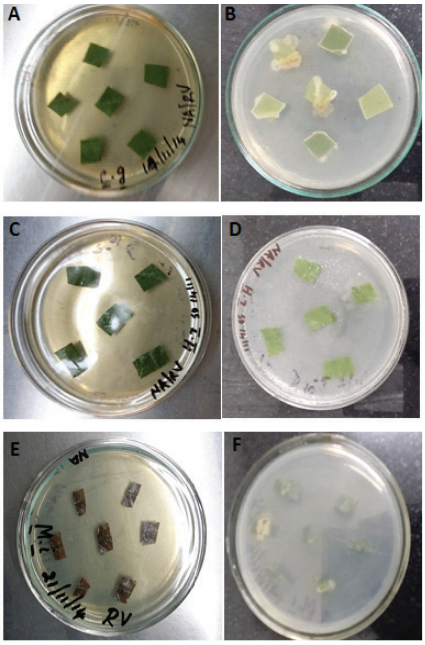

Figure 1. (a), (b) Leaf bits of *Calotropis gigantea* before and after incubation. (c), (d) Leaf bits of *Hibiscus rosa-sinensis* before and after incubation. (e), (f) Leaf bits of *Mangifera indica* before and after incubation.

3.2 Gram Staining

All except C.g.2 appeared purple after the conventional Gram staining procedure, indicating that they are Gram positive. Even though all the isolates were rod shaped, each strain showed different colony morphology (Figure 3).

Plants constitute vast and diverse niches for endophytic organisms. Endophytes secrete an array of biomolecules that may help in the synthesis of pharmaceutical products in the medicinal plants from which they are isolated[8]. The plant growth promoting effect of diazotrophic endophyte from *Mangifera indica* in well demonstrated[9]. Endophytes from the medicinal plant *Tridax procumbens* has a great involvement in the wound healing property of the plant, indicating the contribution of microorganisms in the medicinal properties of the residing plant[10].

3.3 Antimicrobial Activity

3.3.1 Antibacterial Screening

On testing with the pathogenic strain of *E. coli*, endophytic culture C.g.1 isolated from *Calotropis gigantea*

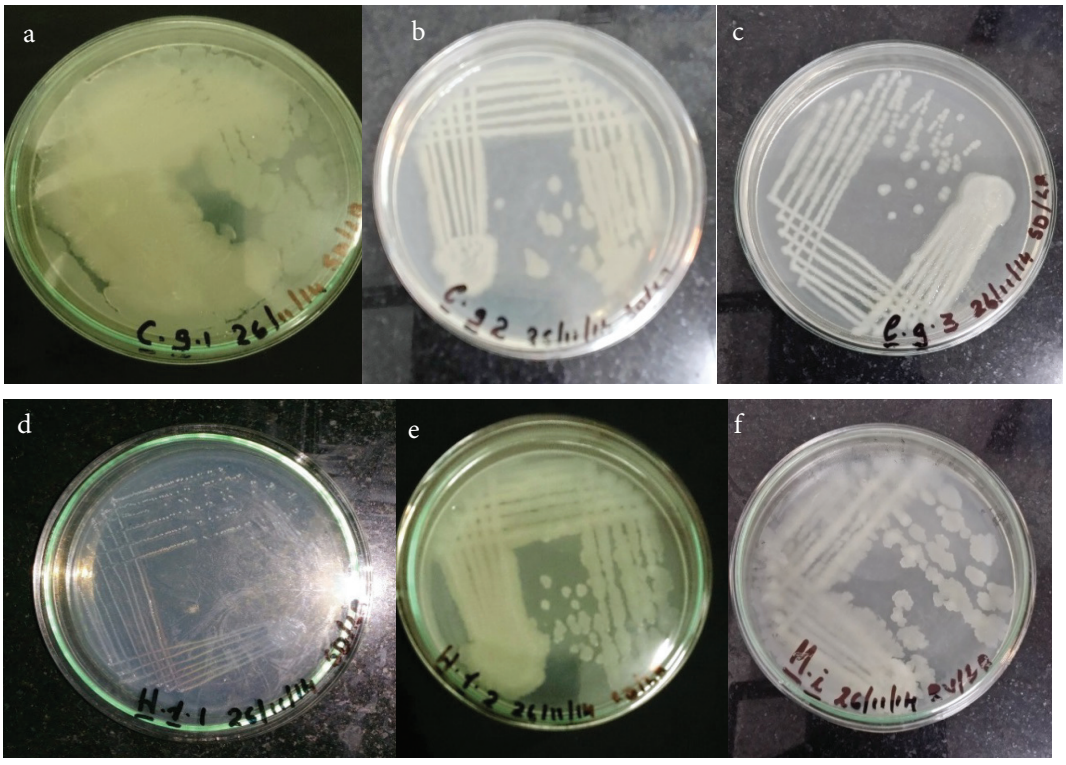

Figure 2. Pure colonies of bacterial isolates on LB agar plates. (a) C.g.1. (b) C.g.2. (c) C.g.3. (d) H.r.1. (e) H.r.2. (f) M.i.

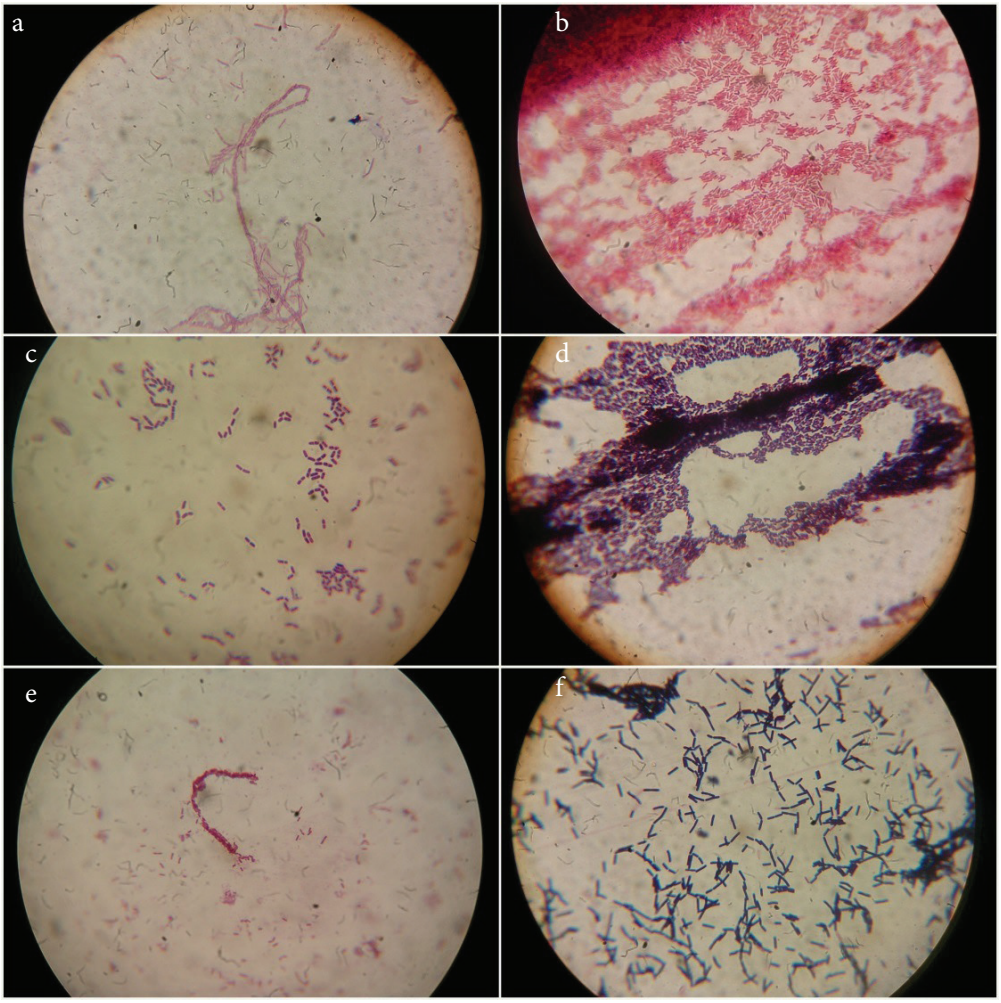

Figure 3. Gram staining. (a) C.g.1. (b) C.g.2. (c) C.g.3. (d) M.i. (e) H.r.1. (f) H.r.2.

exhibited a visible zone of inhibition confirming its antibacterial potential against *E. coli* (Figure 4). M.i strain obtained from *Mangifera indica* also had inhibitory effect on *E. coli*.

3.3.1.1 Effect of C.g.1 and its Bacteriocin on E. coli

No inhibitory activity was observed either for DMSO or for the crude bacteriocin of C.g.1 extracted with DMSO. However, when the culture of C.g.1 was used as such, the growth of *E. coli* was affected and the zone of inhibition was observed However, when the beneficial bacterium, *Bacillus subtilis* was used, none of the endophytes used showed any inhibition (Figure 5(a)). Eleven bacterial endophytes from *Hygrophila spinosa* were identified11 and tested against six human pathogens. The authors conclude

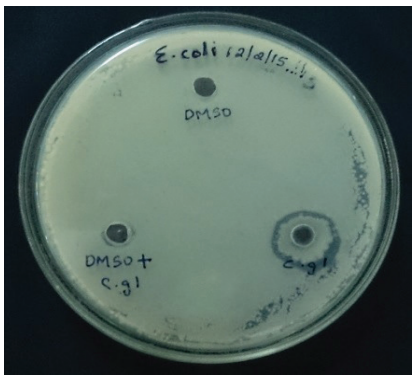

Figure 4. Antibacterial activity of the isolate C.g.1 with and without the solvent.

that they had a broad spectrum of antibacterial activity inhibiting both Gram positive and Gram negative bacteria. In our study, the Gram positive bacterium, *B. subtilis* and Gram negative bacterium, *E. coli* were included to test the antibacterial activity of the strains namely M.i, C.g1 and H.r.1. The endophytes M.i., and C.g1 showed inhibition towards *E. coli* and not against B. subtilis indicating that the endophytes live in symbiosis with beneficial organisms.

3.3.2 Antifungal Screening

Endophytic culture M.i. and C.g.1 showed a remarkable control over *Aspergillus niger* (Figure 5(c) and (d)). Author[12] isolated 78 bacterial endophytes from the aerial parts and roots of eight medicinal plants. Out of seventy eight isolates, ten inhibited the growth of some of the fungal pathogens namely *Aspergillus niger*, *A. avamori*, *Trichoderma koningii* and *Fusarium oxysporum*. Similar to their observation, in our study, the endophytic bacterial strains isolated from the medicinal plants, *Mangifera indica* and *Calotropis gigantea* showed remarkable inhibitory zones against the fungal pathogen *Aspergillus niger*. Author[13] isolated plant growth promoting endophytic bacteria from rice plants and recorded their antagonistic effects against fungal pathogens, *Rhizoctonia solani* and *Fusarium oxysporum*.

Chloroform-ethanol extract of endophytic bacteria isolated from Vinca rosea exhibited potential antimicrobial activity against human pathogenic bacteria[14]. When antibacterial and anifungal effects of the methanolic leaf extracts of five medicinal plants were analyzed, highest antibacterial activity was recorded in *Zizyphus mauritiana* leaf extract against the plant pathogen, *Xanthomonas axonopodis pv. malvacearum*[15]. In the present study, chloroform was also not an effective solvent for extraction of antibacterial protein, as it inhibited the growth of the tested *E. coli* strain, which infers the degradation of antibacterial protein by the solvent used for its extraction.

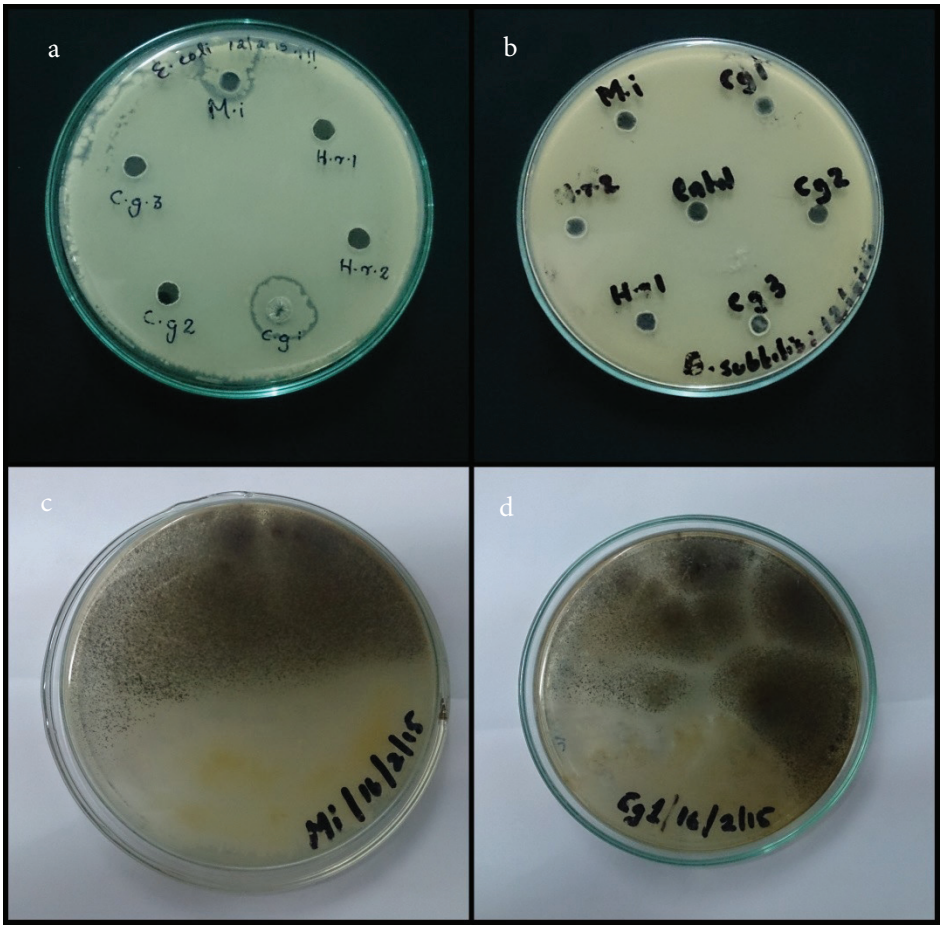

Figure 5. (a), (b) Antibacterial screening with *E.coli* and *B.subtilis*. (c), (d) Antifungal screening with *Aspergillus niger*.

In the reports on the antibacterial activity of *Acalypha indica* L. against three human pathogens, the inhibitory activity exhibited by the solvent extract of the leaves may also be attributed to the biomolecules secreted from endophytic bacteria of the host[16,17]. The culture filtrate of the endophytic fungus *Nigrospora* sp. caused mortality of nematodes from 80 to 100%[18].

The endophytic fungus, *Colletotrichum gloeosporioides* isolated from *Pumeria acutifolia* has been shown to produce taxol that has antitumour activity[19].

Similar to our studies, 20 have shown antibacterial effects of endophytes from Cassia fistula against two Gram negative bacterial pathogens, two Gram positive bacterial pathogens and three fungal pathogens. These studies including the present study substantiate the antibacterial potential of the endophytic bacteria of medicinal plants.

Screening for the production of hydrolytic enzymes:

All the endophytic isolates were tested for the production of extracellular enzymes. Table 2, shows the positive strains for different enzymes. Most of the strains exhibited reasonable enzyme activity for amylase, cellulase and protease on LB agar plates amended with 1% substrate (Figure 6). No lipase activity was observed in any of the six bacterial endophytes tested. Among the isolated bacterial strains, M.i., H.r.2 and C.g.3 showed inhibition zone when tested against the substrates for cellulase, protease and amylase (Table 3).

Table 2. Bacterial endophytes positive for various hydrolytic enzymes

Enzyme	Positive Bacterial Strains
Amylase	C.g.1, C.g.3, H.r.2, M.i.
Cellulase	C.g.1, C.g.2, C.g.3, H.r.1, H.r.2, M.i.
Protease	C.g.1, C.g.3, H.r.1, H.r.2, M.i.

Table 3. Evaluation of extracellular hydrolytic enzyme activity by endophytic bacteria

Sl.no.	Host	Strain no.	CMC (mm)	Casein (mm)	Starch (mm)
1.	*M.indica*	M.i.	25	35	29
2.	*H.rosa-sinensis*	H.r.1	18	-ve	-ve
		H.r.2	22	25	25
3.	*C.gigantea*	C.g.1	28	-ve	28
		C.g.2	-ve	14	-ve
		C.g.3	20	14	19

3.4 Enzyme Assay

3.4.1 Amylase Assay

Termamyl (Novozymes Corp) was used as the positive control that had 20,000 units/ml. The optical density was measured at 660 nm and the activity is calculated by the equation,

Activity = OD (Blank-Test) * 1 *(Dilution Factor)
OD of blank 10

Even though all the specified bacterial strains displayed visible clear haloes in the screening assays, the activity observed in the quantitative assays was very less (Table 4). And also the activity followed a negative correlation with time. The accuracy of pipetting was ensured with the control values.

3.4.2 Protease Assay

Method was followed for protease assay. Protease (Genencor India) was used as the positive control that had 1600 units/ml. Calculation was done using the formula,

Activity = (Average test- blank) (0.33) (Dilution factor)

M.i. showed a mild protease activity on the first day but it declined with time, while the enzyme activity of H.r 2 exhibited an increase with time (Table 5). C.g.1 showed an increase on day two but declined on day three. The other isolates had only a negligible activity.

3.4.3 Cellulase Assay

None of the isolates positive in the plate assay showed any considerable activity during the quantitative assay.

Research on the potential of using microorganisms as biotechnological sources of industrially important enzymes stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms[20,21]. The enzymatic activity of the endophytes of *Jacaranda decurrens* and showed large clearance zones on nutrient agar plates supplemented with starch (for amylase), casein (for protease) and tween[20] (for lipase). Different endophytic bacteria from *Plectranthus tenuiflorus* exhibit considerable amylase, lipase, protease and cellulose activities[22]. However, in the present study, these enzymes have been estimated in very low quantities. These enzymes may act in harmonious orchestration with other pathogenesis related proteins upon invasion of pathogens in to the plant tissue.

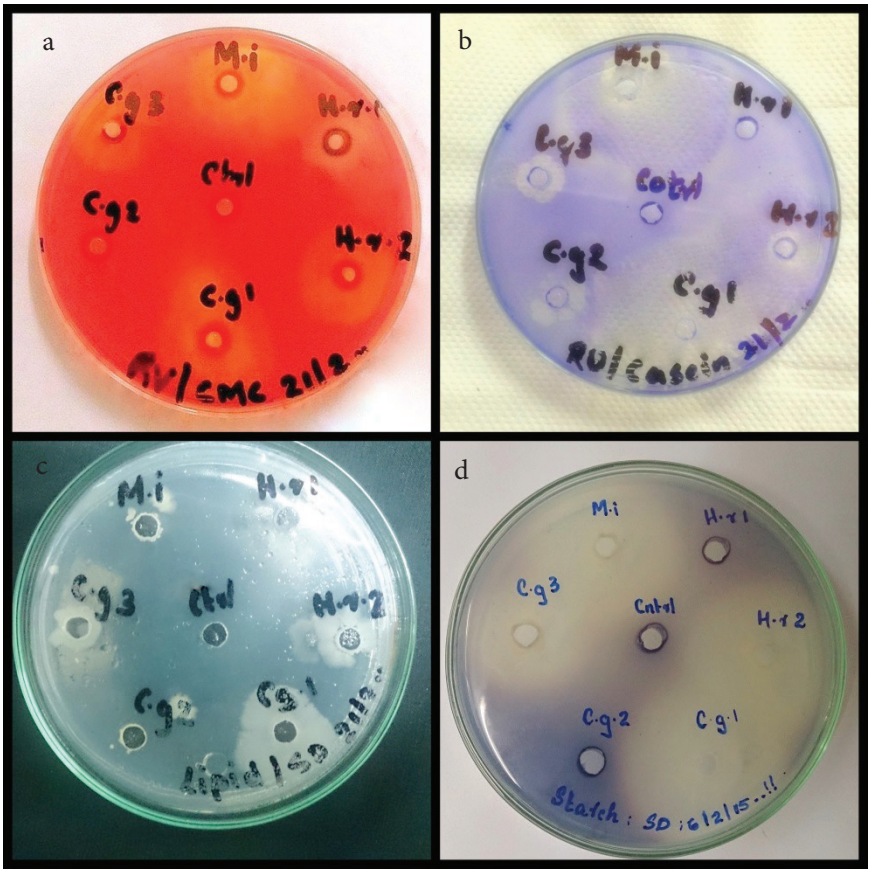

Figure 6. (a) Cellulase screening with congo red staining. (b) Protease confirmed with Coomassie Brilliant Blue. (c) Lipase screening. (d) Amylase with iodine reagent.

Table 4. Amylase activity in the culture supernatants of the endophytes

Sample	Activity at different time intervals		
	24 hours	48 hours	72 hours
C.g. 1	0.094 ± 0.003	0.093 ± 0.003	0.090 ± 0.003
C.g. 3	0.096 ± 0.003	0.097 ± 0.001	0.094 ± 0.001
H.r 2	0.097 ± 0.002	0.097 ± 0.002	0.083 ± 0.002
M.i.	0.095 ± 0.002	0.093 ± 0.002	0.072 ± 0.004

Table 5. Protease production by the endophytic bacteria

Sample	Activity at different time intervals		
	24 hours	48 hours	72 hours
C.g. 1	0.093 ± 0.006	0.577 ± 0.010	0.315 ± 0.013
C.g. 2	0.030 ± 0.006	0.016 ± 0.004	0.016 ± 0.005
C.g. 3	0.024 ± 0.006	0.004 ± 0.003	0.024 ± 0.007
H.r 2	0.223 ± 0.014	0.474 ± 0.057	0.576 ± 0.003
M.i.	0.621 ± 0.025	0.178 ± 0.023	0.160 ± 0.017

4. Conclusion

Bacteria residing in three of the medicinal plants showed meager activity of hydrolytic enzymes and reasonable ability in controlling a pathogenic bacterium and a pathogenic fungus. Further investigation would provide us an insight of the potential use of these endophytic bacterial isolates.

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