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BIOPROSPECTING POTENTIAL OF ENDOPHYTIC BACTERIA ISOLATED FROM INDIGENOUS PLANTS OF AMBALA (HARYANA, INDIA)

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ABSTRACT: In the present endeavour, bioprospecting potentials (antimicrobial activity, antibiotic susceptibility pattern, enzyme activity and dye degradation ability) of endophytic bacteria isolated from different plants were explored. Total of thirty bacterial endophytes were isolated from the leaves of selected plants by using sterilization treatment followed by serial dilution agar plate technique. All the isolates were evaluated for the antimicrobial activity against 7 pathogenic strains including 2 Gram positive bacteria (*Staphylococcus epidermidis* and *Bacillus amyloliquifaciens*) and 2 Gram-negative bacteria (*Escherichia coli* and *Salmonella enterica ser. typhi*) and 3 fungi (*Aspergillus fumigatus*, *Aspergillus* sp. and *Candida albicans*) using agar well diffusion method. Of the 30 bacterial isolates, twenty endophytic bacterial isolates exhibited both antifungal and antibacterial activity. It was observed that 33.33% of isolates exhibited urease activity, 66.66% amylase activity, while 50% esterase activity. Malachite green degradation was observed in 16 (53.33%) endophytic bacterial isolates. The antibiotic susceptibility pattern of endophytic bacterial isolates was evaluated. Five Gram negative bacterial isolates were sensitive to Amikacin, Levofloxacin, Cephotaxime, four isolates sensitive to Aztreonam and three isolates sensitive to Imipenem while four isolates were resistance to Ceftazidime and one was resistant to Imipenem and Aztreonam. Gram positive bacterial isolates were sensitive to Cephalothin, Clindamycin, Erythromycin and Penicillin G and resistant to Oxacillin and Amoxyclav. Further investigations are suggested in order to classify the microorganisms and exploit the potential of the substance produced to inhibit pathogenic microorganisms.

INTRODUCTION: Endophytes are defined as "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects"[1].

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Common endophytes include a variety of bacteria, fungi and actinomycetes, and they can be isolated from wild or cultivated crops of either the monocotyledonous or dicotyledonous plant groups [2]. It is noteworthy that, of the nearly 300,000 plant species that exist on earth, each individual plant is host to one or more endophytes[3]. However, only a handful of plants, mainly grass species, have been completely studied in relation to their endophytic biology [4]. Endophytic bacteria in a single plant host are not restricted to a single species but comprise

several genera and species. The exact mechanism by which bacteria induce protection in the host plants remains unclear, although production of siderophores, metabolites with anti-fungal activity, or competition for nutrients and exclusion from the ecological niche of colonizing microorganisms have been suggested as possible mechanisms [5]. Some endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and induction of resistance to plant pathogens [6].

Therefore a better understanding of endophytic bacteria may help to elucidate their function and potential role more effectively in developing sustainable systems of crop production. Endophytes provide a broad variety bioactive compounds include various secondary metabolite with unique structure, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinines, steroids, terpenoids, tetralones, xanthones, and others [7].

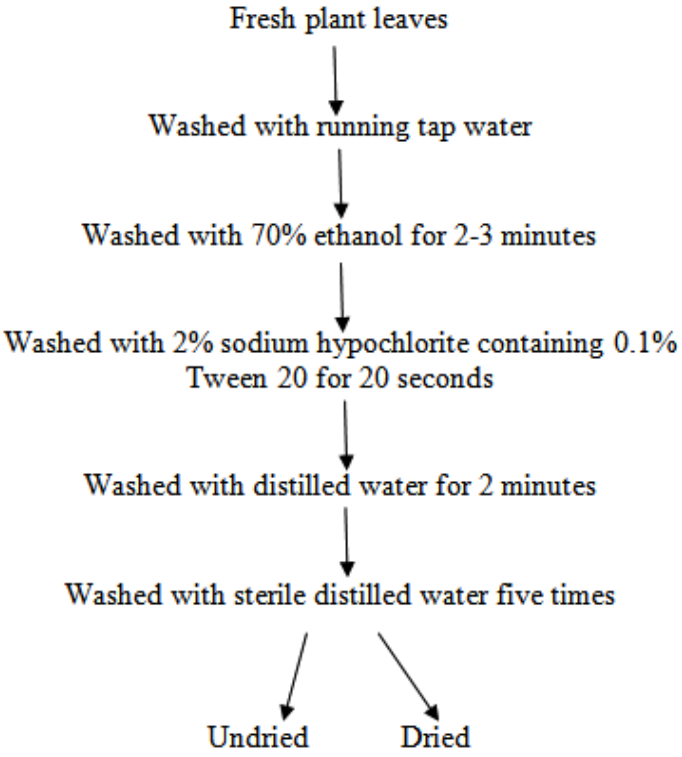
Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents [8]. Many workers have demonstrated that the endophytes isolated from medicinal plants are excellent producers of strong fungicides, bactericidal and cytotoxic metabolites [9].

On the basis of above justification, the present endeavour was to study the bioprospecting potential of endophytic bacteria isolated from indigenous plants of Ambala, Haryana, India.

MATERIALS AND METHOD:

Sample collection: For the isolation of endophytic bacteria, healthy and fresh leaves of different plants like Tomato, Aloevera, Chilli, Radish, Cauliflower, Cabbage, Arjun, Pomegranate, Grass, Carrot, Coriander, Guava, Stevia, Mint, Garlic, Peas, Giloy, Turmeric, Neem, Rose were collected from different areas of Ambala, Haryana. Each sample was tagged and placed in separate polythene bags and transported aseptically to the laboratory and processed within 24 hours of collection. Fresh plant materials were used for isolation to reduce the chance of contamination [10].

Sample Pre-treatment: For the pre-treatment of leaf samples and isolation of endophytic bacteria all the leaf samples were excised and subjected to a surface sterilization procedure described by Strobel [4], Arunachalam and Gayathri [11]. The procedure for sample pre-treatment is shown below;



Isolation of endophytic bacteria: For the isolation of endophytic bacteria pre-treated dried leaf sample were crushed with sterile distilled water using sterile mortar and pestle. About 1mL of crushed sample was serially diluted up to 10^{-5} dilutions. Nutrient agar medium supplemented with antifungal antibiotic (Ketoconazole) to suppress fungal growth was prepared and used for the isolation of endophytic bacteria, about 0.1 mL of aliquot from 10^{-2} to 10^{-5} dilutions were taken and spread on nutrient agar medium using sterile cotton swab. All the plates were incubated in incubator for 24hr at 37^{o}C. In case of wet samples, leaves were cut into small pieces by using sterile knife. Leaves were placed on NA plates supplemented with antibiotic (Ketoconazole) and incubated in incubator for 24hours at 37^{o}C for bacterial growth. The plates were observed for appearance of colonies and number of colonies produced on each plate. Highly sterile conditions were maintained for isolation of endophytic bacteria [10 -11].

Purification and maintenance of endophytic bacteria: Bacteria were purified by streak plate method on NA and incubated at 37^0C for 24hrs and transferred to NA slants and then maintained in refrigerator at 4^0C till further analysis [12].

Screening:

1. Screening for Antibacterial and antifungal activity
2. Screening for Enzymatic activity
3. Screening for Dye degradation
4. Screening for Antibiotic susceptibility pattern

1. Screening for antibacterial and antifungal activity:

a. **Test microorganisms:** A total of seven human pathogenic strains such as three test fungi *Aspergillus fumigatus*, *Aspergillus* sp.(molds), *Candida albicans* (yeasts) and four test bacteria *Escherichia coli, Salmonella enterica ser. typhi, Staphylococcus epidermidis, Bacillus amyloliquifaciens* were procured from IMTECH, MTCC, Chandigarh. All the test fungal and bacterial strains were maintained in their respective media (Czapek dox broth for *Aspergillus* sp., *Aspergillus fumigatus*; Potato dextrose broth for *Candida albicans*; Nutrient broth for *Bacillus amylo-liquifaciens*, *Staphylococcus epidermidis*, *Escherichia coli*, Trypticase soy broth for *Salmonella enterica ser. typhi*) for further study

TABLE 1: A LIST OF BACTERIAL STRAINS USED IN PRESENT STUDY

Test Strains	MTCC No.
Gram negative Bacteria	
Escherichia coli	723
Salmonella enterica ser. Typhi	3216
Gram positive Bacteria	
Staphylococcus epidermidis	435
Bacillus amyloliquifaciens	1488
Fungal strains	
Aspergillus fumigatus	4163
Aspergillus sp.	1344
Candida albicans	3017

b. **Standardisation of tested microorganisms:** The test microorganisms were standardised by using 0.5 McFarland standards. McFarland Standards was used as reference to adjust the density of microbial suspensions so that their number would be within a given range. 0.5 McFarland gives approximate cell density of 1.5 x 10^8 CFU/ml, having absorbance of 0.132 at wavelength of 600 nm. For preparation of the 0.5 McFarland standard, 0.05mL of barium chloride ($BaCl_2$) (1.17% w/v $BaCl_2.2H_2O$) was added to 9.95 ml of 0.18M H_2SO_4 (1.0% w/v) with constant stirring. The McFarland standard tube was tightly sealed to prevent loss by evaporation and stored for up to 6 months. To aid comparison, the test and standard were compared against a white background with a contrasting black line or by measuring the absorbance with that of the standard [13].

c. **Production and evaluation of antimicrobial metabolite from endophytic bacteria:** Nutrient broth was used for antimicrobial metabolite production from endophytic bacteria, 500mL Erlenmeyer's flasks each containing 200 mL Nutrient broth autoclaved at 121^{o}C and 15psi for 15 minutes and inoculated with endophytic bacterial isolate grown on NA. The inoculated flasks were incubated at 37^{o}C for 2-3 days under stationary condition. Then centrifuged at 10000 rpm for 10 min. Antimicrobial activity of culture supernatant (100µL/well) and broth (100µL/well) was tested by agar well diffusion method using test microorganism. The antibiotic Ketoconazole was used as negative control.

d. **Screening of endophytic bacteria for antimicrobial activity by agar well diffusion method:** In the agar well diffusion method, plates containing the media according to the test organism were inoculated with standardized test organism and spread with sterile swabs. Wells of 7mm were made with sterile cork borer into agar inoculated plates. 100µL of test metabolite from each of the bacterial isolate was poured into a well of the inoculated plates. The plates thus prepared were left at room temperature

for ten minutes allowing the diffusion of the filtrate and broth into the agar. After incubation for 24 hrs at 37^0 C for bacteria and at 25^0C for 3-5days for fungi, the plates were observed. If antibacterial and antifungal activity was present on the plates, it was indicated by an inhibition zone surrounding the well containing the bacterial metabolite.

The zone of inhibition was measured and expressed in millimetres. Antibacterial and antifungal activity was recorded if the zone of inhibition was greater than 8 mm [14].

2. **Screening for enzymatic activity:**

   a. **Determination of urease activity:** Bacterial isolates were inoculated onto the urea agar slants and incubated at 37^oC for 24 hrs. After incubation slants were observed for change in reddish pink colour indicating positive urease activity [15].

   b. **Determination of amylolytic activity:** The isolates were streak on the plate containing nutrient agar (NA) supplemented with 0.2% starch as substrate, pH 6.0 which is previously sterilized. After incubation the culture were treated with Gram's iodine, which allow the formation of clear halos around the colony [2]**.**

   c. **Determination of esterase activity:** The medium containing peptone 10.0g/l, NaCl 5.0g/l, $CaCl_2.2H_2O$ 0.1g/l, agar 18.0g/l, pH 7.4 was prepared for determining esterase activity. To the sterilized culture medium, previously sterilized Tween 80 was added in a final concentration of 1% (v/v). Endophytic bacterial isolates were inoculated on medium. The precipitation of ester compound around the colony indicates the presence of esterase enzyme [2].

3. **Screening for dye degradation:** Endophytic bacterial isolates were spot inoculated onto screening medium supplemented with malachite green (0.01%) and incubated at 37^oC for 1 to 2 days. Clear zone around the bacterial spot indicated dye degradation [10, 16].

4. **Identification of endophytic bacteria:** For the identification of endophytic bacterial isolates the methods described by Cruickshank *et al* [17] was followed which includes morphological, cultural, biochemical tests.

5. **Antibiotic susceptibility pattern of endophytic bacteria:** Antibiotic susceptibility pattern of those endophytic bacterial isolates which showed the best antimicrobial activity and enzymatic activity was determined by Kirby-Bauer disc diffusion method[15] (Hi media Laboratories Pvt. Ltd. Mumbai, India). Broth cultures of endophytic bacteria was prepared using Nutrient broth and adjusted to 0.5 Mcfarland standards. All the cultures were inoculated into nutrient agar plates using sterile cotton swab. Standard antibiotic discs, Penicillin G (10 units), Cephalothin (30µg), Oxacillin (1µg), Clindamycin (2µg), Erythromycin (15µg), and Amoxyclav (30µg) for Gram positive bacteria and for Gram negative bacteria resistance was assessed against cephotaxime (30µg), Levofloxacin (5µg), Aztreonam (30µg), Imipenem (10µg), Amikacin (30µg), Ceftazidime (30µg) were placed and incubated at 37^0C for 24 hr. After incubation, antibiotic susceptibility pattern was determined by measuring the zone of inhibition.

RESULTS AND DISSCUSION:

Isolation of endophytic bacteria: Endophytic bacteria were isolated from different plants. The isolates were differentiated on the basis of their colony morphologies; two-three distinct colonies were observed and selected from each sample. A total of thirty bacterial isolates were finally selected, purified and maintained for further analysis.

Antimicrobial activity of bacterial isolates: The agar well diffusion method was used to assess the antimicrobial activity of thirty isolated bacterial cultures against 7 pathogenic strains including 4 bacterial and 3 fungal strains. Out of 30 isolates tested, some isolates were found to exhibit antimicrobial activity against indicator strains as shown in **Table 2**. The isolate N_1 showed activity against five tested pathogenic strains whereas isolate N_4 exhibited activity against 4 tested strains

out of 7 and N_{30} showed activity against 3 tested strains. Ten isolates did not show any activity against any of the tested bacterial and fungal strains as shown in **Fig. 1**.

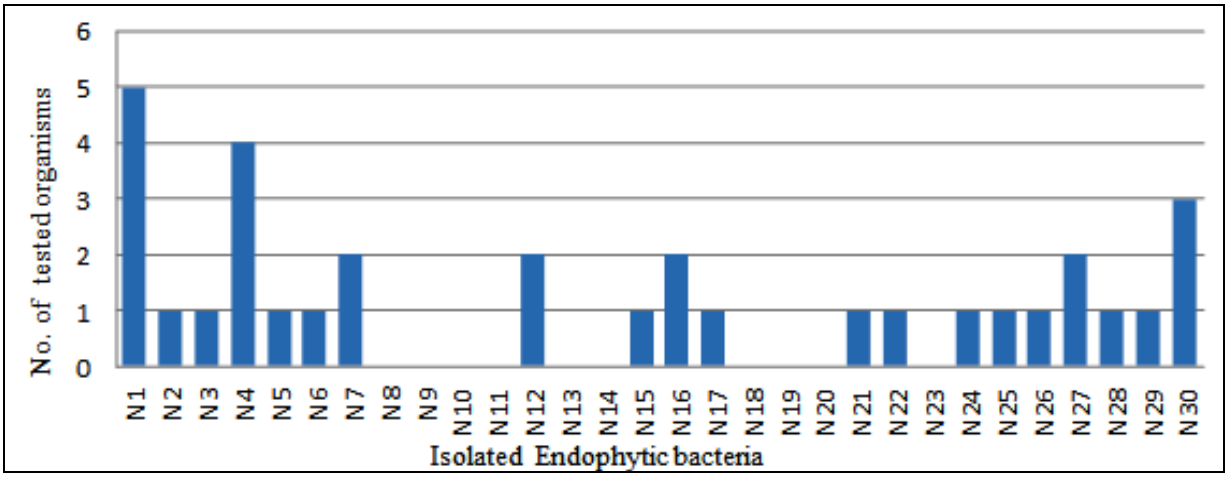
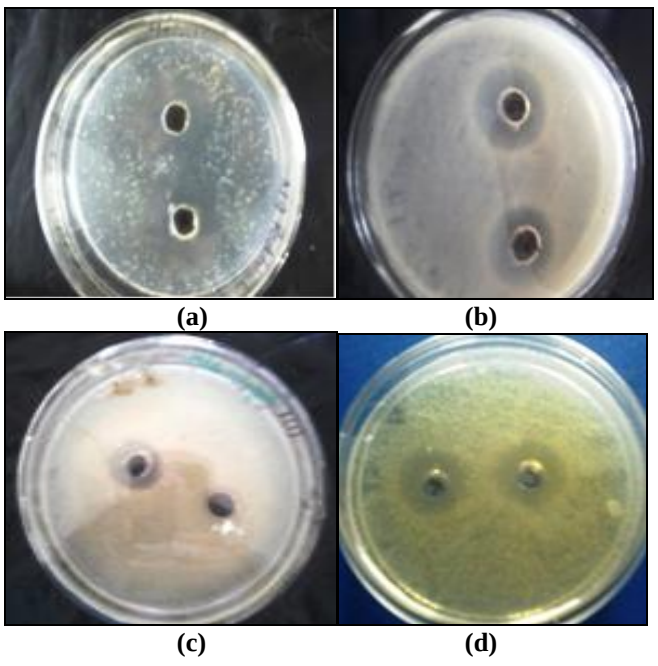


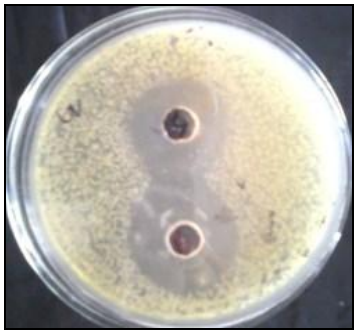
FIG. 1: ANTIMICROBIAL ACTIVITY OF BACTERIAL ISOLATES AGAINST PATHOGENIC STRAINS BY AGAR WELL DIFFUSION METHOD

The spectra of inhibition were different among the isolates selected. Out of the isolates which showed activity against five tested strains, Isolate N_1 showed maximum zone of inhibition against *Aspergillus* sp. of 40mm, followed by *S. typhi* (27mm), *C. albicans* (21mm), *A. fumigatus* (20mm), *S. epidermidis* (20mm) (**Fig. 2**); Isolate N_4 exhibited maximum zone of inhibition against *C. albicans* (27 mm), followed by *A. fumigatus* (23mm), *S. epidermidis* (22mm), and minimum against *Aspergillus* sp. (16mm), and Isolate N30 showed maximum zone of inhibition against *S. typhi* of 30mm, followed by *S. epidermidis* (22mm) and minimum against *Aspergillus* sp. with zone of inhibition 11mm (**Table 2**).



(a) **(b)**

(c) **(d)**



(e)

FIG. 2: ANTIMICROBIAL ACTIVITY OF BACTERIAL ISOLATES N_1 AGAINST (a) *C. albicans* (b) *S. epidermidis* (c) *S. typhi* (d) *Aspergillus* sp. (e) *A. fumigatus*

The isolates which showed activity against 2 tested strains were N_7, N_{12}, N_{16} and N_{27}. Isolate N_7 showed maximum zone of inhibition against *B. amyloliquifaciens* and *A. fumigatus* with zone of inhibition of 16mm, but did not show inhibition against all other test strains; Isolate N_{12} showed maximum inhibition against *C. albicans* with zone of inhibition of 31mm, and minimum against *B. amyloliquifaciens* (16mm) but no inhibition against other test strains was observed; Isolate N_{16} showed maximum inhibition against *C. albicans* with zone of inhibition 16mm and minimum against *A. fumigatus* (12mm); N_{27} showed maximum inhibition against *E. coli* with zone of inhibition of 17mm, and minimum against *A. fumigatus* (11mm); and N_2 showed zone of inhibition against *S. epidermidis* (22mm), but failed to show inhibition against all other test strains. N_3 showed activity against *S. epidermidis* (20mm), N_5 showed

activity against *C. albicans* with zone of inhibition 40mm, N_6 showed activity against *C. albicans* with zone of inhibition 28mm; N_{15}, N_{17} showed activity against *B. amyloliquifaciens* with zone of inhibition 18mm,13mm; N_{21} showed activity against *Aspergillus* sp. with zone of inhibition 10mm; N_{22} showed activity against *C. albicans* with zone of inhibition 11mm; N_{24}, N_{26}, N_{28} showed activity against *E. coli* with zone of inhibition 15mm, 12mm, 20mm; N_{25} showed activity against *C. albicans* with zone of inhibition 15mm; N_{29} showed activity against *S. epidermidis* with zone of inhibition 30mm; N_8, N_9, N_{10}, N_{11}, N_{13}, N_{14}, N_{18}, N_{19}, N_{20} showed no activity as shown in **Fig. 3**. The same work was performed by Yang *et al.* [18] he was isolated 72 endophytic bacteria from healthy tomato stems and leaves from field-grown plants, the strain W4 gave strongly inhibitory effect on *Botrytis cinerea* Pers, with the inhibition rate 78% in dual culture assay.

In the present study, isolate obtained from tomato showed strong antibacterial activity against *S. typhi* with zone of inhibition 30mm. In vitro experiments on antifungal activities of the isolates against pathogenic fungi showed that endophytic bacteria also possess the ability to inhibit the growth of several plant pathogenic fungi by the production of diverse microbial metabolites including antibiotics. In the study carried out by Ebrahimia *et al* [19] none of the five endophytic bacterial isolate showed activity against *E. coli* but in our study four out of 30 isolates showed activity against *E. coli.* Other endophytic bacterial filtrates which showed low or lack of antimicrobial activity in the bioassays may have active compounds but probably in smaller amounts and/or the screened filtrates could yield more potent compounds once they had undergone some purification [20]. Also, extracts which showed no antimicrobial activity in these assays may be active against other microbes which were not tested [19].

Enzymatic and Biodegradation activity of bacterial isolates: It was observed that out of the 30 bacterial isolates 10(33.33%) were urease producers, 20 (66.66%) were amylase producers, while 15(50%) were esterase producers and 16 (53.33%) isolates showed positive results for malachite green degradation as shown in **Table 3**. Clear zone around the colony indicates the degradation of dye. In the study carried out by Panchal and Ingle [2] on *Chlorophytum borivilianum* (safed musali) 80% of the isolates were amylase producers and 60% were esterase producers but in the present study 66.66% were amylase and 50% were esterase producers. Amylase positive isolates indicated starch degradation on starch agar plates. Plant tissue store starch as a food source and the endophytes can consume the starch before other new colonizers appear. Clear halo on Tween 80 plates indicated esterase activity of the endophytes.

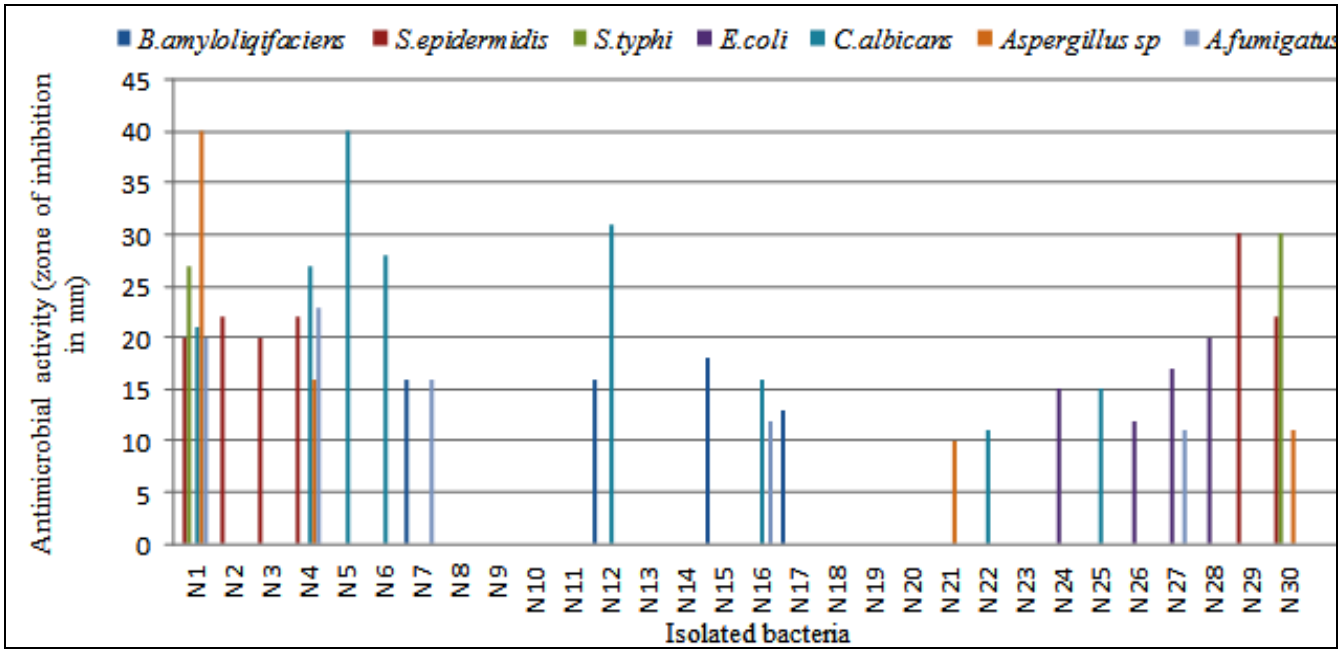


FIG. 3: ANTIMICROBIAL ACTIVITY OF BACTERIAL ISOLATES AGAINST PATHOGENIC STRAINS BY AGAR WELL DIFFUSION METHOD

TABLE 2: ANTIMICROBIAL ACTIVITY OF BACTERIAL ISOLATES

S. No.	Isolate	Tested microorganisms (diameter of zone of inhibition in mm)						
		Gram positive		*Gram negative*		*Yeast*	*Molds*	
		B. amyloliquifaciens	***S. epidermidis***	***S. typhi***	***E. coli***	***C. albicans***	***Aspergillus* sp.**	***A. fumigatus***
1.	N_1	-	20	27	-	21	40	20
2.	N_2	-	22	-	-	-	-	-
3.	N_3	-	20	-	-	-	-	-
4.	N_4	-	22	-	-	27	16	23
5.	N_5	-	-	-	-	40	-	-
6.	N_6	-	-	-	-	28	-	-
7.	N_7	16	-	-	-	-	-	16
8.	N_8	-	-	-	-	-	-	-
9.	N_9	-	-	-	-	-	-	-
10.	N_{10}	-	-	-	-	-	-	-
11.	N_{11}	-	-	-	-	-	-	-
12.	N_{12}	16	-	-	-	31	-	-
13.	N_{13}	-	-	-	-	-	-	-
14.	N_{14}	-	-	-	-	-	-	-
15.	N_{15}	18	-	-	-	-	-	-
16.	N_{16}	-	-	-	-	16	-	12
17.	N_{17}	13	-	-	-	-	-	-
18.	N_{18}	-	-	-	-	-	-	-
19.	N_{19}	-	-	-	-	-	-	-
20.	N_{20}	-	-	-	-	-	-	-
21.	N_{21}	-	-	-	-	-	10	-
22.	N_{22}	-	-	-	-	11	-	-
23.	N_{23}	-	-	-	-	-	-	-
24.	N_{24}	-	-	-	15	-	-	-
25.	N_{25}	-	-	-	-	15	-	-
26.	N_{26}	-	-	-	12	-	-	-
27.	N_{27}	-	-	-	17	-	-	11
28.	N_{28}	-	-	-	20	-	-	-
29.	N_{29}	-	30	-	-	-	-	-
30.	N_{30}	-	22	30	-	-	11	-

Number indicates the diameter of zone of inhibitions in mm, '-' No activity

Esterase breaks down fats into fatty acid and glycerine, but it also brings the reversible reaction. In reversible reaction, esterase enzyme helps the plant in production of saponin which gives a significant medicinal property to the plant [2].

In the study carried out by Gayatri *et al* [10] 20 (55.55%) out of 36 isolates results for malachite dye degradation and in the present study 16 (53.33%) out of 30 isolates were degraded malachite dye so our result somewhat similar to the results to the Gayatri *et al* [10]. In the study carried out by Gayatri *et al* [10] endophytic bacteria from coastal plants showed malachite green degradation. Further the dye degrading endophytes isolated in this study will be a potential candidate for dye degrading enzymes. Moreover, the detection of recalcitrant degrading endophytic bacteria from coastal plants will be a biological marker for monitoring the pollution of coastal ecosystem by recalcitrant molecules. The improvement of phytoremediation of water soluble, volatile organic pollutants by selected endophytic bacteria was experimentally proved by Barac [21].

Identification of endophytic bacteria: Out of 30 isolates only 6 isolates which showed the best antimicrobial, enzymatic and dye degradation

activity were selected for further identification. For the morphological and biochemical characterisation of bacterial isolates, different tests were performed.

The morphological tests namely gram's staining, endospore staining and biochemical tests such as catalase test, indole acetic acid production test, MR test, VP test and citrate utilization test were performed for identification of endophytic bacteria (**Fig. 4**). Endophytic bacterial isolates namely N_1, N_2, N_3, N_4, N_{30}, were found to be Gram negative and N_{12} were found to be Gram positive; isolate N_2 N_4, N_{12}, N_{30} were endospore forming and N_1, N_3 were non endospore forming; N_2, N_4, N_{12} , N_{30} catalase positive and N_1, N_3 were catalase negative; isolate N_1, N_2, N_3, N_4 were found to be indole positive and N_{12}, N_{30} were Indole negative demonstrate the inability of bacteria to decompose the amino acid tryptophan to indole; Isolate N_2, N_4, N_{30} were MR positive and all others were MR negative; isolate N_2, N_{12}, N_{30} were VP positive and all others were VP negative; isolate N_{12}, N_{30} were citrate positive and others were negative (**Table 4**).

TABLE 3: ENZYMATIC AND BIODEGRADATION ACTIVITY OF BACTERIAL ISOLATES

S. No	Isolate	Urease	Amylase	Esterase	Malachite green
1.	N_1	-	+	+	+
2.	N_2	+	+	-	+
3.	N_3	+	+	+	+
4.	N_4	+	+	+	+
5.	N_5	+	+	+	+
6.	N_6	-	+	+	-
7.	N_7	-	+	-	+
8.	N_8	+	+	-	-
9.	N_9	-	+	-	+
10.	N_{10}	-	+	-	+
11.	N_{11}	+	+	+	+
12.	N_{12}	+	+	+	+
13.	N_{13}	-	+	-	-
14.	N_{14}	-	+	-	+
15.	N_{15}	-	+	+	+
16.	N_{16}	-	-	+	+
17.	N_{17}	+	+	-	-
18.	N_{18}	-	-	-	-
19.	N_{19}	-	-	-	-
20.	N_{20}	-	-	-	-
21.	N_{21}	-	-	-	-
22.	N_{22}	-	-	-	-
23.	N_{23}	-	+	+	-
24.	N_{24}	-	-	+	-
25.	N_{25}	-	+	-	+
26.	N_{26}	-	-	-	-
27.	N_{27}	+	-	+	-
28.	N_{28}	-	+	+	-
29.	N_{29}	-	-	+	+
30.	N_{30}	+	+	+	+

'+' Presence '-' Absence

On the basis of these test N_2 were partially identified as *Escherichia* sp., N_4 as *Proteus* sp., N_{12} was partially identified as *Bacillus* sp., N_{30} was partially identified as *Salmonella* sp., N_1 and N_3 were not identified. Hung and Anapurna [6] were identified endophytic bacteria on the basis of morphological and physiological test including Gram staining, capsule staining, cellulose, pectinase, Motility test, indole acetic acid production, motility test, fluorescent pigmentation test and BIOLOG system identification test. Isolation of endophytic *Staphylococcus* was reported by Carrim *et al* [22]. Muzzamal *et al* [3] identified various bacterial species belonging to the genera *Bacillus, Pseudomonas, Serratia, Stenotrophomonas and Micromonospora*.

TABLE 4: MORPHOLOGICAL, STAINING AND BIOCHEMICAL CHARACTERIZATION OF SELECTED BACTERIAL ISOLATES

Isolate	Morphological characteristics	Cultural characteristics	Gram's staining	Endospore	Catalase	Indole acetic acid test	Methyl red test	Voges-proskauer test	Citrate utilization test
N_1	Pink, rods, single	Colonies irregular, punctiform, smooth, moist, translucent, flat and curled	-ve	-	-	+	-	-	-
N_2	Pink, cocci, chain	Colonies Round, light yellow, smooth, dry, opaque, flat and entire	-ve	+	+	+	+	+	-
N_3	Pink , rods, minute, single	Colonies irregular, yellow, smooth, moist, cloudy, flat and wavy	-ve	-	-	+	-	-	-
N_4	pink, rods, single	Colonies round ,yellow, smooth, moist, opaque, flat and wavy	-ve	+	+	+	+	-	-
N_{12}	purple, cocobacillary, minute	Colonies pink, puntciform, smooth, moist, translucent, flat and entire	+ve	+	+	-	-	+	+
N_{30}	pink, irregular	Colonies round, yellow, smooth, dry, opaque, flat and entire	-ve	+	+	-	+	+	+

(a) **(b)** **(c)** **(d)**

(e)

FIG. 4:(a) Dark pink layer showing the presence of indole positive test (b) Cherry red colour indicates positive methyl red test. (c) Indicates the VP test (d) blue colour indicates the citrate utilizataion (e) shows the catalase activity of different microbes

Antibiotic susceptibility pattern:

(i) Antibiotic susceptibility pattern of isolated gram negative bacterial isolates: Antibiotic susceptibility pattern of selected endophytic bacterial was observed by using Kirby-Bauer disc diffusion method. The results for antibiotic susceptibility pattern of gram negative bacterial strains are shown in **Table 5**. Isolate N_1 and N_4 showed resistance against Imipenem. Imipenem exhibited activity against N_2 (20mm), N_3 (17mm) and N_{30} (25mm). Ceftazidime showed activity against N_1 (20mm) and no activity was recorded against N_2, N_3, N_4, N_{30}.

Cephotaxime showed activity against N_1 (30mm), N_2 (13mm), N_3 (25mm), N_4 (20mm), N_{30} (30mm). Results showed that Levofloxacin be active against N_1 (35mm), N_2(30mm), N_3 (32mm), N_4 (30mm) and N_{30} (33mm). Aztreonam showed its partial effectiveness against N_1 (18mm), N_3 (15mm), N_4 (20mm), N_{30} (10mm). Amikacin showed activity against N_1 (37mm), N_2 (37mm), N_3 (32mm), N_4 (30mm), N_{30} (35mm). The antibiotic resistance of isolated endophytic bacteria was assessed using antibiotic discs (Hi media Laboratories Pvt. Ltd. Mumbai, India) on nutrient agar plates against Levofloxacin, Aztreonam, Amikacin, Ceftazidime, Imipenem, Cephotaxime. In the present study all the six isolates were sensitive to Amikacin (30 µg) (Table 5) but in the study carried by Arunachalam *et al* [11] 13 out of 20 isolates were sensitive to Amikacin (30 µg). This may vary from strain to strain or type of strain.

TABLE 5: ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF GRAM NEGATIVE ENDOPHYTIC BACTERIA

S. No.	Antibiotics	Symbol	Concentration	Selected endophytic bacterial isolates (ZOI in mm)				
				N_1	N_2	N_3	N_4	N_{30}
1.	Imipenem	I	10 µg	R	20	17	R	25
2.	Ceftazidime	Ca	30 µg	20	R	R	R	R
3.	Cephotaxime	Ce	30 µg	30	13	25	20	30
4.	Levofloxacin	Le	5 µg	35	30	32	30	33
5.	Aztreonam	Ao	30 µg	18	R	15	20	10
6	Amikacin	Ak	30 µg	37	37	32	30	35

'R'- Resistance; 'ZOI'- Zone of inhibition

(ii) Antibiotic susceptibility pattern of isolated Gram positive bacterial Isolates: Antibiotic susceptibility pattern of selected endophytic bacterial was observed by using Kirby-Bauer disc diffusion method the result as shown in **Table 6**. Isolate N_{12} was found to be sensitive against oxacillin. Cephalothin showed its effectiveness against N_{12} with diameter of inhibition zone of 16mm. Clindamycin showed activity against N_{12} with zone diameter 12mm. Erythromycin showed activity against N_{12} (10mm). Penicillin G showed inhibitory activity against N_{12} with Inhibition zone 18mm. the results for antibiotic susceptibility pattern of Gram positive bacterial strains are shown in Table 6. The antibiotic resistance of isolated endophytic bacteria was assessed using antibiotic discs(Hi media Laboratories Pvt. Ltd. Mumbai, India) on nutrient agar plates against Oxacillin, Cephalothin, Clindamycin, Amoxyclav, Erythromycin, Penicillin G. Similar work was performed by Gayatri *et al*. [10] using standard antibiotics streptomycin, vancomycin, bacitracin and trimethoprim. In that case most of the isolates were sensitive to all antibiotics except bacitracin. In the present study our isolate N_{12} were resistant to Oxacillin (1 µg) but contrary to present work done by Jalgaonwala [15] the isolate were sensitive to Oxacillin (5µg).

TABLE 6: ANTIBIOTIC SUSCEPTIBILITY PATTERN OF GRAM POSITIVE BACTERIAL ISOLATES

S. No.	Antibiotics	Symbol	Concentration	Isolate (N_{12}) (ZOI)
1.	Oxacillin	Ox	1 µg	R
2.	Cephalothin	Ch	30 µg	16
3.	Clindamycin	Cd	2 µg	12
4.	Amoxyclav	Ac	30 µg	R
5	Erythromycin	E	15 µg	10
6.	Penicillin G	P	10 units	18

'R'- Resistance; 'ZOI'- Zone of inhibition

CONCLUSION: In conclusion, endophytic microorganisms are a very promising source for production of bioactive compounds. They are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas. The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek, study, and spend time

isolating micro floral components. This may facilitate the product discovery processes. Further investigations are suggested in order to classify the microorganisms and exploit the potential of the substance produced to inhibit pathogenic microorganisms.

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