

First study on Antimicrobial activities of Endophytes Isolated from Aerial parts of *Mentha Piperita*

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Abstract- Endophytic microbes are considered as an outstanding source of bioactive natural products because there are so many of them occupying millions of unique biological niches growing in different types of environment. Plants infected with endophytes are often healthier than endophytes free ones. These endophytes are the prominent source of secondary metabolites similar to the plant tissue on which these endophytes are grown. The present study illustrates about the isolation and screening of endophytes from aerial parts (leaves and stems) of *Mentha piperita* plant. A total of 4 different bacterial endophytes were isolated from leaves which were marked as L1, L2, L3 and L4 while only 2 different types of bacterial endophytic cultures were isolated from stems of the plant which were marked as S1 and S2. These were categorized and nominated separately as per the colonies growth on LB media. The pure cultures of these bacterial endophytes were isolated and maintained in pure form on LB medium. These bacterial endophytes were further screened for biochemical tests and gram staining in order to confirm the genera of bacteria isolated. There were no viable fungal growth appeared in PDA medium. These bacterial cultures were bacilli, cocci and coco-bacilli. These bacterial cultures were separately inoculated in sterilized LB broth for the production of secondary metabolites from each of the organism. The secondary metabolites were extracted with ethyl acetate followed by drying to yield the crude secondary metabolites. The crude extracts of secondary metabolites were further screened for antimicrobial activity against bacterial and fungal pathogens. The extracts showed significant antibacterial activity against *Leuconostoc mesentroides*, *Bacillus subtilis*, *Bacillus licheniformis* and *Saccharomyces cerevisiae* (yeast).

Index Terms- Bacterial endophytes, aerial parts, *Mentha piperita*, LB media, secondary metabolites.

I. INTRODUCTION

Endophytes are those microorganisms that inhabit interior of plants especially leaves, stems, roots shows no apparent harm to host [1]. Almost all classes of vascular plants and grasses examined to date are found to host endophytic organisms [2]. Different groups of organisms such as fungi, bacteria, actinomycetes and mycoplasma are reported as endophytes of plants [3]. Research of endophytic fungi has a long history and their diversity among plants has been found to be considerably large. Each plant has been reported to harbor one or more endophytes [4, 5]. Recently endophytes are viewed as

outstanding source of secondary metabolites bioactive antimicrobial natural products. These microorganisms received considerable attention in last 20 years when their capacity to protect against insect and pest pathogens was noticed. Endophytes are the prominent source of secondary metabolites which can be potent antimicrobial, antioxidant and anticancer activities. The present study is focused on the isolation and characterization of endophytes from aerial parts of *Mentha piperita* L.

II. MATERIALS AND METHODS

Collection and Identification of the plant parts

The aerial parts of *Mentha piperita* L. viz. leaves and stem were collected and were taxonomically identified by some Taxonomists/Botanists in the form of herbarium.

Surface sterilization of plant tissues and isolation of endophytes

Further the tissues of the plant were soaked in 70% alcohol for few seconds or in 0.5-3.5% sodium hypochlorite for 1-2 minutes followed by rinses in sterile double distilled water before placing it on a LB medium for isolation of endophytic bacteria [6]. Some isolates require months or more time in culture before they sporulate. Even stop sporulating after they have been transferred several times. The LB plates were incubated for about 7-10 days for observation of any growth of bacterial endophytes. For isolation of fungal endophytes surface sterilization of tissue requires 70% ethanol for 1-3 minutes, aqueous sodium hypochlorite (4% available chlorine) for 3-5 minutes again rinse with 70% ethanol 2-10 seconds and final rinse with double distilled water and drying in laminar air flow, also added 50mg/l chloramphenicol within PDA medium to suppress bacterial growth [7]. Sterile knife blade was required to remove outer tissues from sample and to excise inner tissues. The PDA plates were kept for about 5-6 days for observation of growth of any fungal endophytes. All the plates were incubated at 28°C to promote the growth of endophytes and were regularly monitored for any microbial growth. On observing the microbial growth, sub-culturing was done. Each endophytic culture were checked for purity and transferred to freshly prepared PDA plate. Appropriate controls will also be maintained in which no plant tissues were inoculated.

Maintenance of Endophytes for Identification and Future Use

The purified endophytic isolates were transferred separately to LB/PDA slants and broths depending on the case for bacterial and fungal endophytes respectively and accessioned accordingly depending upon the plant parts from which they have been isolated. Finally all the purified endophytes were maintained at 4°C till further used. Different biochemical tests were done for identification of bacterial and fungal endophytes. The bacterial isolates were tested for their morphological and biochemical characteristics (catalase enzyme activity). Gram stains were performed to determine the characteristics of the cell wall, cell shape and the arrangement of cells. The morphology of the endophytic bacterial strains was observed on slides under a microscope. For staining, 15 µL of a bacterial culture that is grown in nutrient broth overnight at room temperature with shaking at 150 rpm were heat-fixed onto a slide and then stained. The fungal slides if isolated were stained with lactophenol. The structures were observed using a photomicroscope. The samples were then compared to other samples reported in the literature [8-10].

Production of Secondary metabolites

LB broth was prepared and autoclaved. Endophytic bacterial cultures were inoculated in the broth medium separately within the flasks. These flasks were labeled as L1, L2, L3, L4, S1 and S2. Flasks were then incubated at 28°C for 96 h in incubator shaker at 120 rpm. Further, each of the broth culture was centrifuged at 10,000 rpm to produce the supernatant/filtrate. The extraction of the supernatant/filtrate with different solvents (Chloroform: Ethyl acetate) in 1:1 ratio and left for 15-30 minutes. The organic phase of each of the extracts were collected and kept for drying at 37°C. Further, the dried secondary metabolites were kept in sterilized vials for future use.

Determination of antimicrobial activity of the secondary metabolites

Culture Media

Soyabean casein digest agar/broth and Sabouraud's dextrose agar/broth of Hi Media Pvt. Bombay, India were used for antibacterial and antifungal activities respectively.

Inoculum of pathogenic microbes

The pathogenic microbes viz. *Leuconostoc mesentroides*, *Bacillus subtilis*, *Bacillus licheniformis* and *Saccharomyces cerevisiae* (yeast) were inoculated into Soyabean casein digest broth and Sabouraud's dextrose broth followed by incubation at 35°C for 48 h and suspension was checked to provide approximately, 2×10^5 CFU/ml.

Determination of diameter of zone of inhibition by well diffusion method

The agar well diffusion method was modified [11]. Soyabean Casein Digest agar medium (SCDM) was used for bacterial cultures. The culture medium was inoculated with the pathogenic bacteria separately suspended in nutrient broth. A total of 8 mm diameter wells were punched into the agar and filled with metabolite extracts and solvent blanks. Standard antibiotic (Erythromycin and Fluconazole, 1 mg/ml) were simultaneously used as the positive controls. The plates were incubated at 37°C for 18 h for bacterial growth and 28°C for 48 h for fungal growth. The antibacterial and antifungal activities were evaluated by measuring the diameter of zone of inhibition observed.

Determination of Minimum Inhibitory Concentration (MIC)

MIC values of potent antimicrobial metabolites were determined by the method adopted with some modifications [12, 13]. Metabolites extracted prepared in highest concentration (200 µg/ml) in sterile distilled water and were serially diluted with N-saline (0.85 % NaCl) and similar quantity of bacterial/fungal suspension were added to respective test tubes and incubated for 48 h. The inhibition of turbidity appeared in the minimum dose at which total growth of bacteria/fungus gets killed is known as minimum lethal concentration (MLC) while little turbidity appeared in the minimum amount of dose of plant extract which inhibits the growth of bacteria/fungus is known as Minimum inhibitory Concentration (MIC).

III. RESULTS AND DISCUSSION

The present investigation reveals the isolation of 4 different pure cultures of bacterial endophytes from leaves named as L1, L2, L3 and L4 and 2 different pure bacterial endophytic cultures from stems of *Mentha piperita*, named as S1 and S2. The results are shown in **Figures 1-4**. These bacterial endophytes were further screened for biochemical tests and gram staining (**Figure 5 & Table 1**) in order to confirm the biochemical characterization of the isolated bacterial colonies. These bacterial cultures were determined as bacilli, cocci and coco-bacilli as gram positive and gram negative colonies. There were no viable fungal growth appeared in PDA medium. This is the first kind of study reported for the isolation of bacterial endophytes and production of secondary metabolites from leaves and stems of *Mentha piperita* L. The secondary metabolites (**Figure 6**) were extracted from each of the endophytic bacterial strains and antimicrobial activity was evaluated against *Leuconostoc mesentroides*, *Bacillus subtilis*, *Bacillus licheniformis* and *Saccharomyces cerevisiae* (yeast). The crude dried extract of each of the secondary metabolite was dissolved in DMSO to obtain the concentration viz. 200 µg/ml. Further, the antimicrobial activity was determined against the concerned pathogens. It was found that, secondary metabolites from S2 and L4 had potent antibacterial activity against *Leuconostoc mesentroides* while, S1, L1, L2 and L3 had no activity against the concerned pathogen. The secondary metabolites from L3 had potent antibacterial activity against *Bacillus subtilis* while no other endophytic metabolite was found antibacterial against the same organism. The metabolites from S1, S2, L3 and L4 showed potency against *Bacillus licheniformis* while no antibacterial activity of L1 and L2 was recorded against the same organism. Dominant antibacterial activity of L2, L3 and L4 was found against *Saccharomyces cerevisiae* (yeast) followed by slight antibacterial activity of S1 while no antibacterial activity was reported of L1 and S2 against the same organism (**Figure 7 & Table 2**). Further MIC and MLC values were determined of the potent extracts by serial dilution technique (**Table 3**). The endophytic ascomycetes group fungal cultures were isolated and checked for the effects on the growth of peppermint [14, 15].

IV. CONCLUSION

The present study determines the four different endophytic bacterial strains from leaves viz. gram positive, Bacilli, non-

motile, spore forming (L1), gram positive, Bacilli, non motile, non spore forming (L2), Gram positive, Bacilli, non motile, non spore forming (L3) and gram positive, Bacilli, non motile, non spore forming (L4) while two different bacterial endophytic bacterial strains were isolated from stems viz. Gram negative, Cocci, motile, non spore forming (S1) and Gram negative, Cocco-bacilli, motile, non spore forming (S2). This endophytes bacterial strain illustrates the bacterial endophytic diversity in the

aerial parts of *Mentha piperita* plant. However, no fungal endophytes were isolated in the present study. The present investigation will help to explore endophytes as per the variation of species, climate and regions variability in *Mentha piperita* L. These endophytes produce antibacterial compounds which have potent antibacterial activities. Further, these compounds should be investigated and determined.



Figure 1: Leaves and stem twigs of *Mentha piperita*



Figure 2 (a): Surface sterilized stem of *Mentha piperita* on LB and PDA plates

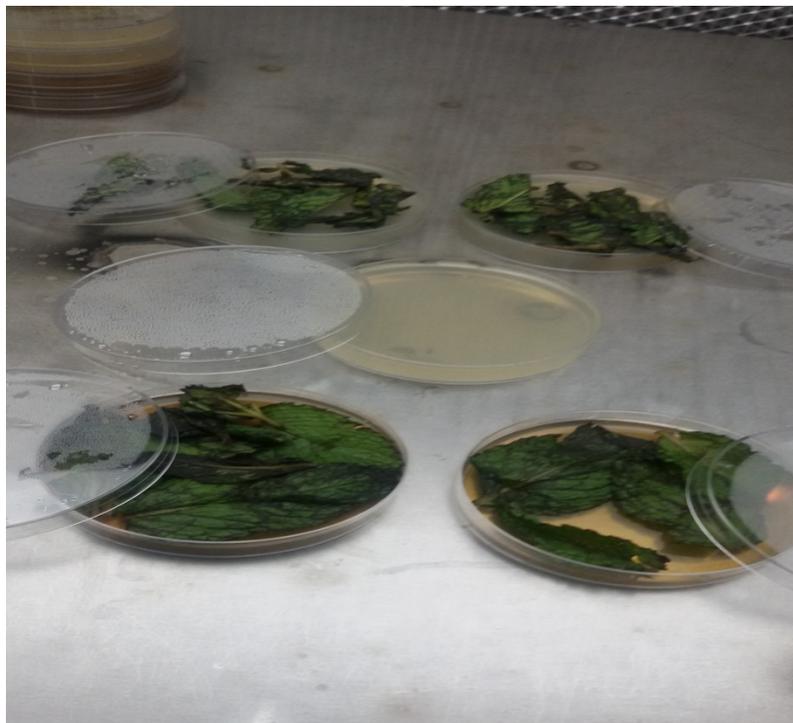


Figure 2 (b): Surface sterilized stem of *Mentha piperita* on LB and PDA plates



Figure 3 (a): Bacterial endophytes growth in stems of *Mentha piperita* on LB plates

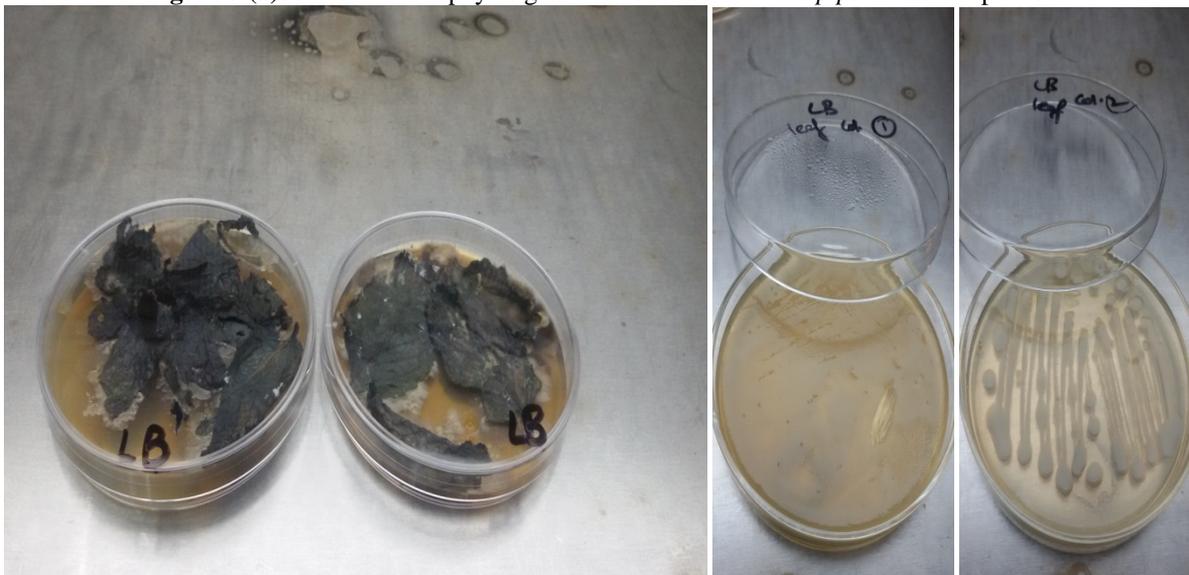


Figure 3 (b): Bacterial endophytes growth in leaves of *Mentha piperita* on LB plates

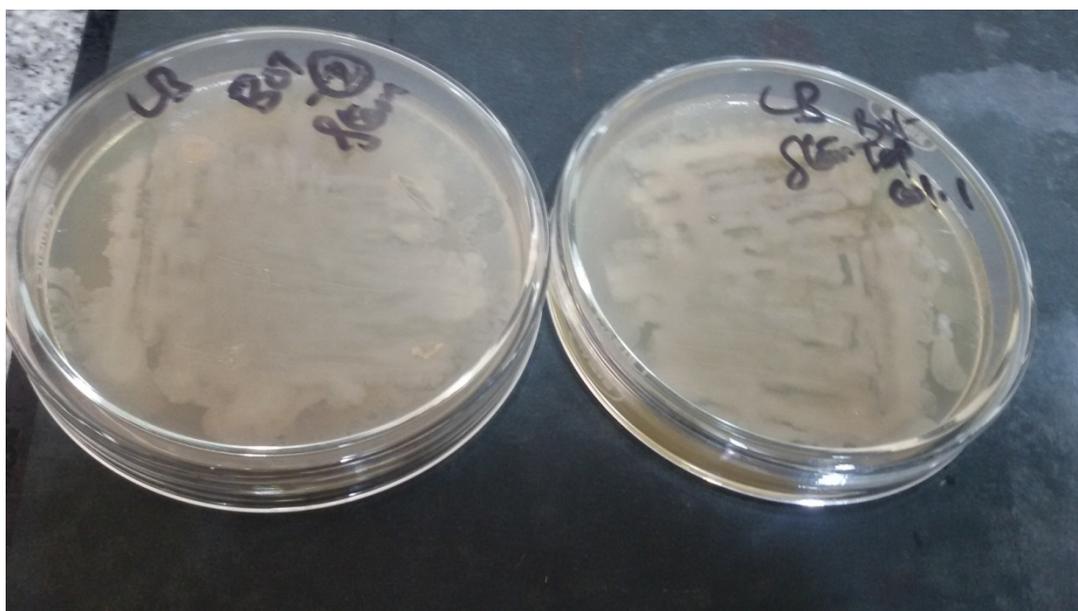


Figure 4 (a): Bacterial endophytic pure cultures isolated from stem of *Mentha piperita* on LB agar

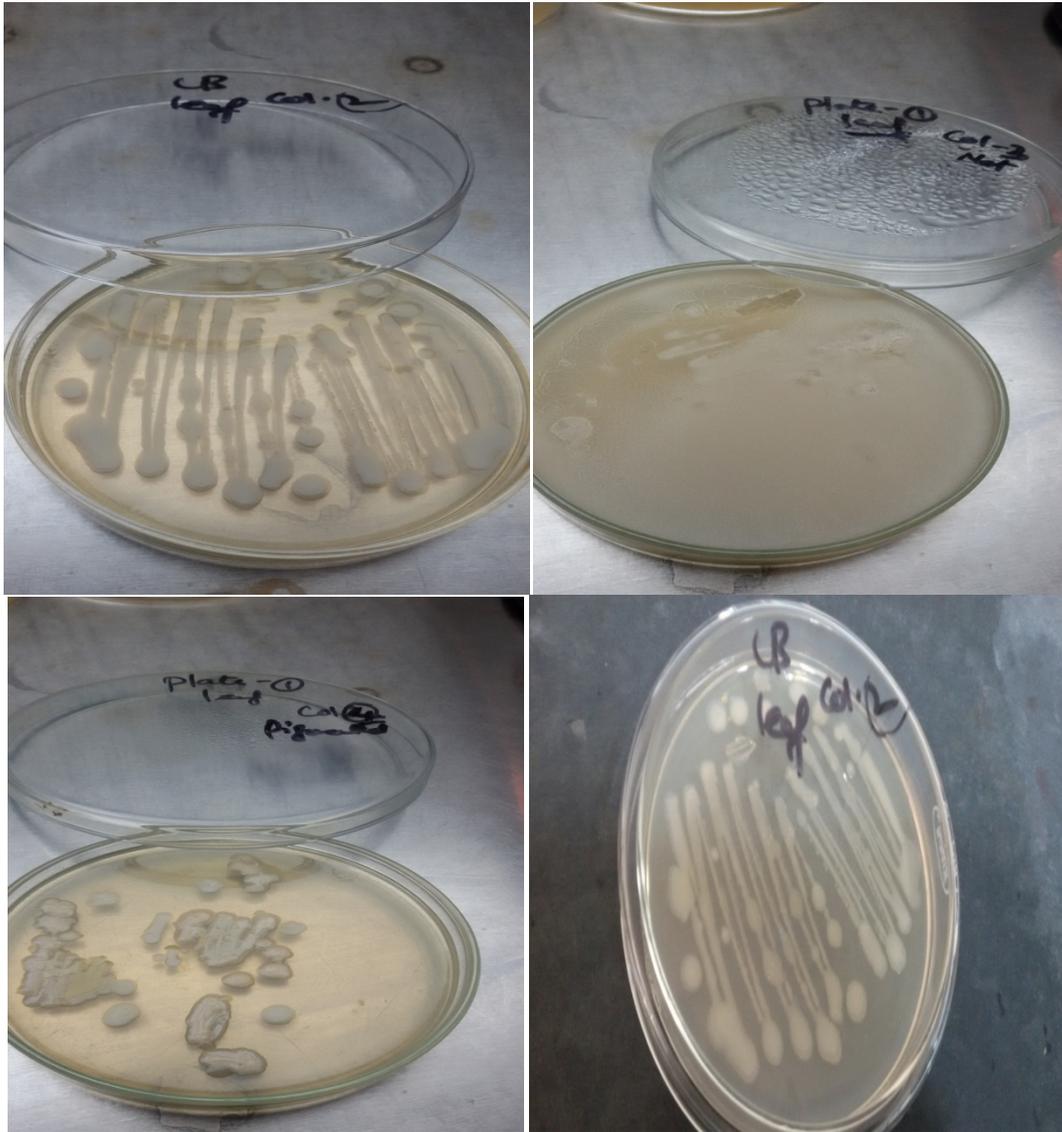


Figure 4 (b): Bacterial endophytic pure cultures isolated from leaves of *Mentha piperita* on LB agar

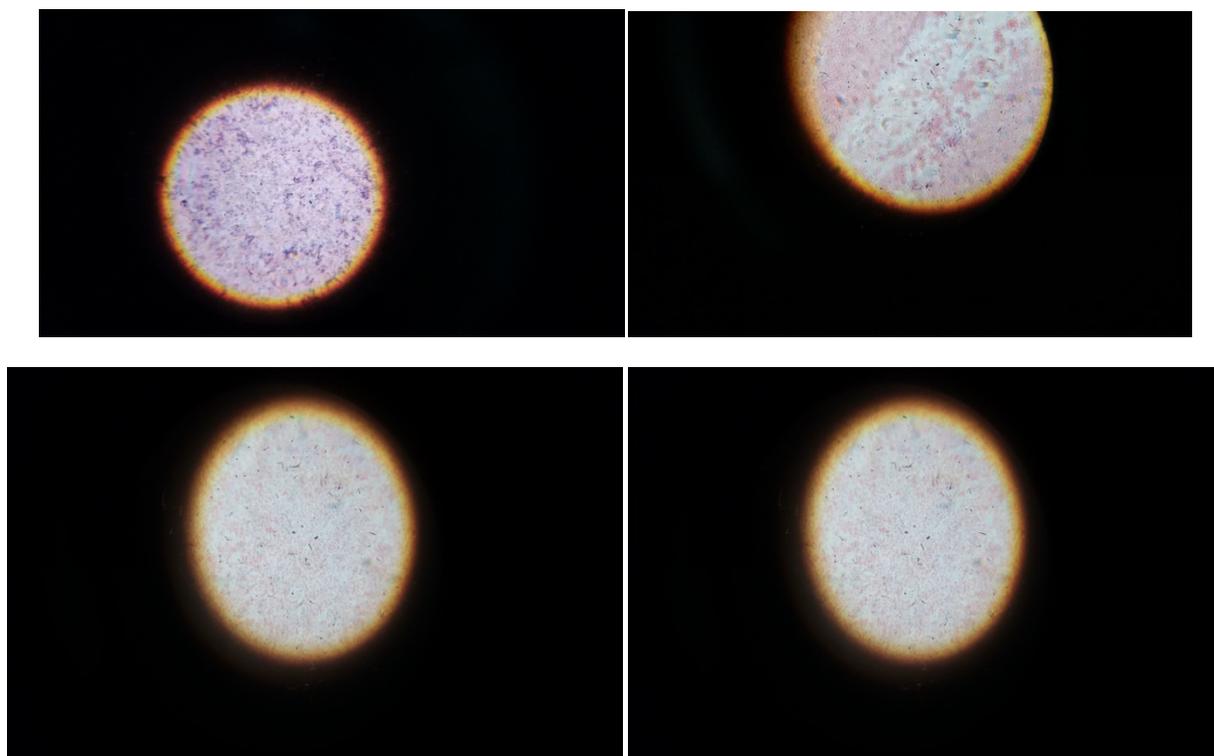


Figure 5: Microscopic gram staining images of the isolated bacterial cultures

Table 1 (a): Morphology and Biochemical Characteristics of Gram positive Bacterial endophytes

Isolate	Gram Staining/Shape/Motility/Spore formation	Sugar Fermentation Test	Amylase test	Indole test	Methyl red test	VP test	H ₂ S production	Citrate test
L1	Gram positive, Bacilli, non-motile, spore forming	+	+	-	+	-	-	-
L2	Gram positive, Bacilli, Non motile, Non spore forming	+	+	-	+	-	-	+
L3	Gram positive, Bacilli, Non motile, Non spore forming	+	+	-	+	-	+	-
L4	Gram positive, Bacilli, Non motile, Non spore forming	+	+	-	+	-	-	-

+, present; -, absent

Table 1 (b): Morphology and Biochemical characteristics of Gram negative Bacterial endophytes

Isolate	Gram Staining/Shape/Motility/Spore formation	Sugar Fermentation Test	Amylase test	Indole test	Methyl red test	VP test	H ₂ S production	Citrate test
S1	Gram negative, Cocci, motile, non spore forming	+	+	+	-	+	+	+
S2	Gram negative, Cocco-Bacilli, motile, Non spore forming	+	+	-	+	+	+	+

+, present; -, absent

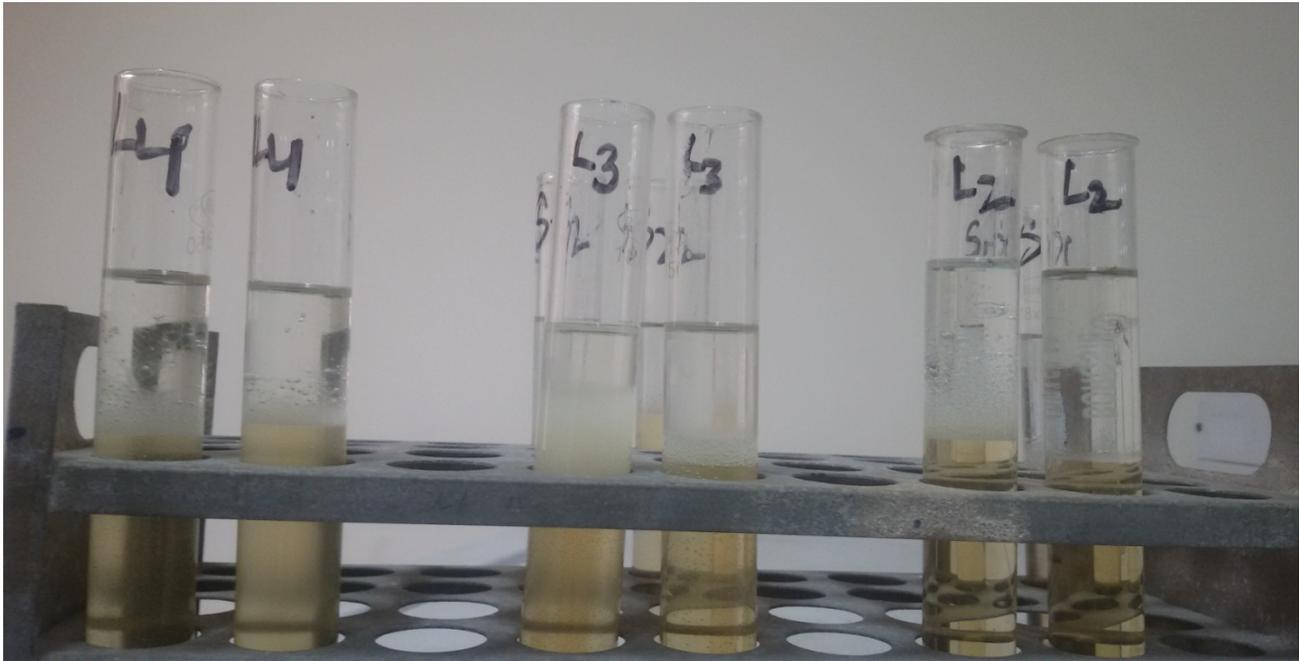
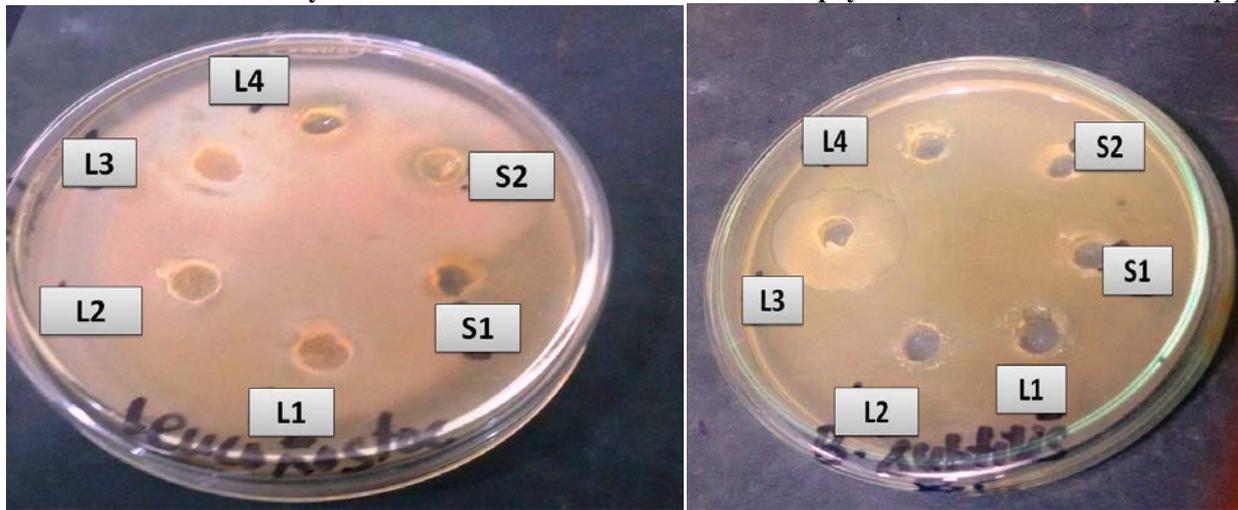
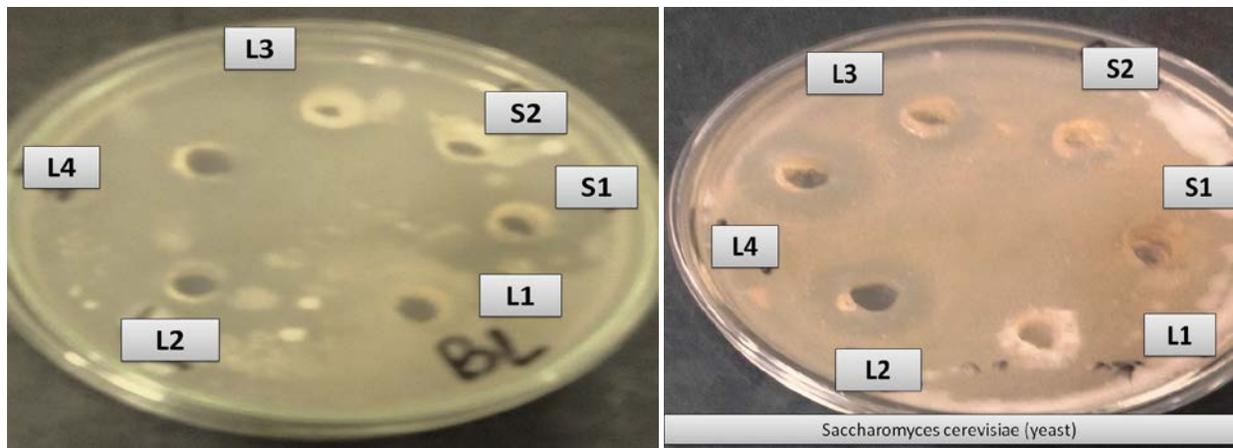


Figure 6: Extraction of secondary metabolites from each of the bacterial endophytic strain isolated from *Mentha piperita*



(A) (B)



(C)

(D)

Figure 7: Antimicrobial Activity of the secondary metabolites extracted and purified from different endophytic bacterial cultures of leaves and stems of *Mentha piperita*

Table 2: Antimicrobial activity of secondary metabolites (200 µg/ml) against the selected pathogens

Endophytic metabolites of isolated endophytes	Diameter of zone of inhibition (mm)			
	LM	BS	BL	SC
L1	NA	NA	NA	NA
L2	NA	NA	NA	35.0
L3	NA	35	25.0	27.0
L4	14.0	NA	27.0	40.0
S1	NA	NA	12.0	NA
S2	15.0	NA	12.0	15.0

*LM, *Leuconostoc mesentroides*, BS, *Bacillus subtilis*, BL, *Bacillus licheniformis*, SC, *Saccharomyces cerevisiae*. NA, no activity

Table 3: MICs and MLCs of the potent metabolites against the selected pathogens

Endophytic metabolites of isolated endophytes	MIC		MLC	
	MIC	MLC	MIC	MLC
Leuconostoc mesentroides	30 (L4)	40 (L4)	30 (S2)	
	20 (S2)		75 (L3)	
Bacillus licheniformis	50 (L3)		30 (L3)	50 (L3)
			45 (L4)	75 (L4)
			55 (S1)	85 (S1)
			55 (S1)	85 (S1)
Saccharomyces cerevisiae	10 (L4)	20 (L4)		
	25 (L3)	45 (L3)		
	30 (L3)	50 (L3)		
	30 (L4)	40 (L4)		

*LM, *Leuconostoc mesentroides*, BS, *Bacillus subtilis*, BL, *Bacillus licheniformis*, SC, *Saccharomyces cerevisiae*. NA, no activity

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