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Screening an antifungal efficacy of indigenous bacteria from rhizosphere of *Periploca apylla*

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Abstract

The *Periploca aphylla* is an important medicinal plant helps in surmounting constipation and curing swollen joints. Bacterial isolates were screened from rhizosphere of *Periploca apylla* for antifungal efficacy against *Alternara pane, Colletotrichum cocealesx,* and *Rhizocotina solani*, Gogdara Hill-Odigram, Swat Pakistan. Isolates were cultured on nutrient agar and identified through gram staining and biochemical tests. Lipolytic activity, salinity, temperature ranges, antibiotic sensitivity and antifungal efficacy were also studied. The results of cultural and morphological study revealed that all bacterial isolates were gram negative except PaS2. Biochemical analysis showed that all Isolates were positive for Ortho-Nitro Phenyl-ß-D-Galacto-pyranosidase, glucose fermentation, arabinose fermentation, ornithine Decarboxylase, citrate utilization and negative for lysine decarboxylase, H_2S production, urease production and indole production (IND) test. Based on result of cultural and biochemical analysis PaS1 was classified under the genus of Phenylobacterium, PaS2, PaS3 and PaS4 were suspected to be *Bacillus lentus, Entrerobacter aerogene* and *Aeromonas caviae* respectively. All strains were able to tolerate different salt concentration (3%, 1%, 0.5%) except PaS1 which showed growth at 0.5 %, whereas maximum growth for all isolates were observed at 30°C to 37°C.The bacterial isolates sensitive to different concentrations of ampicillin, kanamycin and streptomycin. Antifungal efficacy indicated that all fungal strains were resistant to PaS3 and sensitive to PaS4, whereas only *Alternara panex* were sensitive to PaS1 and PaS2. It is concluded that PaS4, PaS1 and PaS2 can be used as a bioinoculant against fungal pathogens. The findings will be helpful for the production of useful antifungal substances at commercial level.

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Introduction

Rhizosphere is the region of soil where plants secretions are influenced by association of plant's roots and soil microflora (Beutner *et al*., 1990; Blasko *et al*., 1998; Canel *et al*., 2000). The microbial population in rhizosphere is higher than the microbial population in the bulk soil (soil which surrounds the rhizosphere).The bacteria residing in this region are known as rhizobacteria, these bacteria not only protects the plant roots by excreting antibiotic which has toxic effect on the parasites, but may also compete for mineral nutrients that have a limited availability in the rhizosphere. The health of plant is a sign of presence of positive microflora and it indicates that microflora in the rhizosphere soil is higher than the soil without rhizosphere, the residing rhizobacteria are helpful in removing the harmful microorganism by secreting antibiotics which will have impact on plant growth by killing plant pathogens (Shamima *et al*., 2007).

The *Periploca aphylla* belong to the family Asclepiadaceae. It is an erect milky shrub branched and leafless, smooth or juvenile at tips. When leaves are present they are minute about 6 mm long, ovate oblong, opposite, thick, acute and nerveless. The Flowers are consisting of five sepals. The outside portion of corolla is greenish and the inner portion is dark-purple. The stamens are within the corona, filament is short and flat. Follicles are about 7.5 - 10 cm long and 6 mm broad. The *Periploca aphylla* gives flowers and fruits during the month of March to November and is found in Rajputana, Pakistan, Afghanistan, Iran, and Egypt. Fresh juice half tea cup of *Periploca aphylla* is given twice a day to surmount constipation. For curing swollen joints and other body parts the ash of *Periploca aphylla* is used as poultice (Badshah *et al*., 2011). The milky juice of the plant is applied to tumors and swellings while the plant decoction is used as purgative (Murad *et al*., 2011). Roots are used as stimulant and are used for constipation, urticaria and tumor (Mahmood *et al*., 2011). Rhizosphere microbial flora is in close association with plant's roots and both partner (plant and microbial community) affect each other.

The plants provide nutrients to the microbial community in the rhizosphere while the microbes provide protection against plant pathogens including pathogenic bacteria and fungi. The use of antibiotics is globally increasing which lead to resistivity against many drugs demanding for production of new antibacterial drugs with improved quality. Therefore this study was planned to isolate and characterize bacterial community associated with *Peroplca aphylla* rhizosphere may be helpful in identification and isolation of new antimicrobial and antifungal drugs of medical and agricultural importance.

Materials and methods

The valley of Swat, the Switzerland of Pakistan, is famous for its scenic beauty, snowcapped peaks of hills, glaciers and waterfalls, water springs, streams and rivulets, lakes and dark forests. The altitude varies from 25000 feet to 7500 feet. The total area of Swat is 5337 square Km with a population of about 12, 50,000. There are several mountain peaks ranging from 4500 to over 6000 meters above sea level, mostly covered with everlasting snow (Swat valley)

Collection of soil samples

The soil samples were collected from different plants of *Periploca aphylla* with different age groups of the same species by uprooting the plant in different heights in Gogdara hill Swat. The plants were shaken to remove the unwanted soil particles. The soil particles adhered to the roots was collected with sterile spatula and transferred to a sterilized polythene bags and brought to laboratory for further studies.

Preparation of soil samples and Isolation of rhizobacteria

Nutrient agar medium was used for culturing of bacteria. Media was prepared and autoclaved at 121˚C for 15 minutes and was allowed to cool down for some time. The media was poured into Petri plates and were kept on plane surface to uniformly solidify the media. For preparation of soil sample and serial dilutions, five gram of soil was added to 100 ml of distilled water and shacked well for one minute so that the particle were dissolved completely.

This suspension was called master sample. Nine milliliter of distal water was taken in five autoclaved test tubes labeled as 1 to 5. From the master sample 100 ml solution was added to the first tube with the help of micropipette and was shaken well. Then100 ml of solution from test tube one was added to the second test tube and after proper mixing same process was repeated for all five test tubes. A previously reported standard method "soil dilution plate method" was followed (Warcup, 1950). Five gram of soil sample was suspended in 100ml of double distilled water to make microbial suspensions (10^1 to 10^5). Dilution of 10^3, 10^4 and 10^5 were used to isolate bacteria. 100 micro liter of microbial suspension of each concentration were added to sterile Petri plates (triplicate of each dilution) containing of sterile nutrient agar media. Two percent clotrim (Clotrimazole Topical Solution USP 1% w/v) was added to the media before pouring into Petri plates for preventing fungal growth. The Petri plates were then incubated at 28 + 20 ˚C in dark. The plates were visually observed for a period of three days. The soil sample from each dilution was inoculated on culture media plates and was uniformly distributed using a sterilized spreader. The plates were labeled according the dilution from 10^1 to 10^5. The Petri plates were incubated for 24 to 48 hours at 36˚C to get considerable bacterial colonies.

Purification of bacterial colonies

The petri plates with mix microbial colonies on media were said to be the parent/master culture plates. These colonies were of different colors and morphology. The colonies were counted to be 4 in number. From the parent/master culture these different colonies were purified by inoculating the single/isolated colonies using sterilized inoculating loop on different Petri plates having nutrient agar media and subsequently incubate for 48 hours at 36°C to get considerable growth. After 48 hours of incubation the same colonies were observed on separate Petri plates. The same process was repeated 3 to 4 times for all morphologically different colonies to get pure culture of all possible bacterial colonies.

Identification of bacterial strains

Gram staining was used to discriminate between Gram positive and Gram negative based on their cell wall chemical and physical properties. For the conformation of these isolates different biochemical tests Ortho-NitroPhenyl-ß-DGalactopyranosidase (ONPG) test, Glucose fermentation (GLU) test, Arabinose fermentation (ARA) test, Ornithine Decarboxylase (ODC) test, Citrate utilization (CIT) test, Decarboxylase (LDC) test, H_2S production (H_2S) test, Urease production (URE) test and Indole production (IND) test were performed.

Effects of different conditions on growth of isolated bacterial strains

Effect of temperature and salinity

To study the growth of isolates on different temperatures all the four strains were incubated on different temperatures (10°C, 27°C, 30°C, 37°C, and 60°C) for 24 hours using nutrient agar media. For the determination of NaCl tolerance of isolated strain, nutrient agar media was prepared in three different flasks with different concentrations of NaCl (0.5 %, 1% and 3%).

Lipophilic activity of the isolated bacterial strains

The lipophilic activity of the isolates was studied using olive oil through streaking method. 1ml of olive oil was added to 100ml nutrient agar media (1% solution). The culture media was autoclaved and was poured into four Petri plates for streaking. The Petri plates were then incubated for 48 hours at 37°C. After incubation the direction of fungal strains growth towards the bacterial strains were observed. Growth of fungal in both directions was indicated as sensitive bacterial strain and growth limited towards the bacterial strain was indicated as resistant bacteria strain.

Antibiotic sensitivity of the isolated bacterial strains

The antibiotics sensitivity of isolated bacterial strains was determined by different concentrations of Ampicillin, kanamycin and Streptomycin. For this purpose three different stock solutions (100µg/ml, 50µg/ml and 25µg/ml) were prepared and were added to nutrient agar media.

All the isolated strains were streaked on agar plates with different concentration of antibiotic and were incubated for 24 hours at 37°C.

Antifungal activity of Isolated Bacteria

The bacterial strains were grown in nutrient broth and incubated for 24 h in a shaker at 150 rpm. The grown bacterial culture was centrifuged and the supernatant was filtered through 0.22µm Millipore filters. The cell free supernatant was used to determine antifungal activity (Sumathi *et al.,* 2012). Antifungal activity of bacterial isolates was determined by agar plate method Cell free supernatant (67 µl) of each bacterial strain was loaded in autoclaved plates containing sabouraud dextrose agar after autoclaving and allowed to solidify. The plates were inoculated with fungal culture from 7 days old culture of fungus. The petri plates were incubated at 28°C for 7days and reading were recorded.

Results and discussion

The primary objective of the study was to isolate and identify the bacteria present in the rhizosphere of *Pereploca aphylla* caring antifungal ability. The study of rhizosphere of this plant was selected because it grows on high altitude and the discovery of novel medically important antibiotic producing species was expected. Furthermore, the rhizosphere of this plant has not been explored before which could be quite helpful in identification of novel organisms. Production of antibiotic by microorganisms from the rhizosphere of high altitude soil is higher than that of the low altitude; as well these antibiotics are more effective. In future it will be worth to examine other antibiotic producing microorganisms like fungi.

Table 1. Morphological characteristics of isolated colonies.

Bacterial isolates	Shape	Color	Surface
PaS1	Cocci	Off white	Smooth
PaS2	Rod	Yellow	Wrinkled
PaS3	Rod	White	Irregular
PaS4	Cocci	Red	Smooth

PaS1= Periploca aphylla strain 1, PaS2= Periploca aphylla strain 2, PaS3= Periplocaaphylla strain 3, PaS4= Periploca aphylla strain 4.

Table 2. Biochemical characterization of the bacterial isolates.

S. No	Tests	Active ingredients	QTY (Mg/cup)	RXN/Enzyme	PaS1	PaS2	PaS3	PaS4
1	ONPG	2nitrophenyl-ß-Dgalactopyranoside	0.223	ß-galactosidase	+	+	+	+
2	GLU	D-glucose	1.9	Fermentation/ Oxidation	+	+	+	+
3	ARA	L-arabinose	1.9	Fermentation/ Oxidation	+	+	+	+
4	LDC	L-lysine	1.9	Lysine Decarboxylase	-	-	-	-
5	ODC	L-ornithine	1.9	Ornithine Decarboxylase	+	+	+	+
6	CIT	Trisodium citrate	0.756	Citrate utilization	+	+	+	+
7	H2S	Sodium thiosulfate	0.075	H_2S production	-	-	-	-
8	URE	Urea	0.76	Urease	-	-	-	-
9	IND	L-tryptophan	0.19	Indole production	-	-	-	-

Isolation and Morphological characteristics of bacterial isolates

Four different types of colonies were isolated from the rhizosphere soil of *Periploca aphylla* on Nutrient agar media. These colonies were labeled as PaS1, PaS2, PaS3 and PaS4. The colors of these colonies were off white, yellow, white and red, respectively. PaS1 and PaS4 were round in shape, while PaS2 and PaS3 was rod shaped (Table 1 and Fig. 1). Gram staining test indicated that PaS1, PaS2 and PaS4 were Gram negative while PaS3 was Gram positive (Fig. 2).

In the current research, four different bacterial were isolated from rhizosphere soils. Our study is in line with the "Bergey's Manual of Determinative Bacteriology" (9th edition) who classify large number of soil bacteria based on morphological characteristics. Various cultural and morphological characteristics of bacteria have been studied by various researchers (Shubhrasekhar C *et al*., 2013). In the present study, colony shape, size, elevation, margins, opacity, surface and pigmentation were studied and observed in different genera. Further the genra of these strains were confirmed by biochemical tests.

Biochemical identification of bacterial isolates

The biochemical analysis of these strains showed that all the four strains (PaS1, PaS2, PaS3 and PaS4) were positive for Ortho-NitroPhenyl-ß D Galactopyranosidase (ONPG) test, Glucose fermentation (GLU) test, Arabinose fermentation (ARA) test, Ornithine Decarboxylase (ODC) test and Citrate utilization (CIT) test, while they were negative for Lysine Decarboxylase (LDC) test, H_2Sproduction (H_2S) test, Urease production (URE) test and Indole production (IND) test (Table 2).

Table 3. Effect of antibiotic concentration on the isolated bacteria's growth.

Strain Name	Antibiotic Name	100mg/100ml	50mg/100ml	25mg/100ml
PaS31	Streptomycin	_	_	_
	Ampicillin	_	_	_
	Kanamycin	_	_	_
PaS2	Streptomycin	_	_	_
	Ampicillin	_	_	_
	Kanamycin	_	_	_
PaS3	Streptomycin	_	_	_
	Ampicillin	_	_	_
	Kanamycin	_	_	_
PaS4	Streptomycin	_	_	_
	Ampicillin	_	_	_
	Kanamycin	_	_	_

Growth remarks: - = No colony, + = 1-85 colonies, ++ = 85-130 colonies, +++ = 190-250 colonies, ++++ = 250-400 colonies.

Table 4. Effect of salt concentration on the growth of isolated bacteria.

Strain Name	0.5%	1%	3%
PaS1	+	_	_
PaS2	++	+	+
PaS3	++++	+++	+++
PaS4	++++	++	++

Growth remarks: - = No colony, + = 1-85 colonies, ++ = 85-130 colonies, +++ = 190-250 colonies, ++++ = 250-400 colonies.

Based on the biochemical behaviors and morphological features of PaS1 (gram negative *Cocci*), there may be possibility that this bacterial isolate might be classified under the genus *Phenylobacterium,* as this results were compared to characteristics of strain in bergey's manual.

Similar biochemical and morphological features were studied by Musliu *et al.,* 2012. The strains PaS2 and PaS3 had shown similar characteristics to *Bacillus lentus* and *Entrerobacter aerogene,* respectively.

Table 5. Effect of Temperature on the growth isolated bacteria.

Strain Name	10°C	27°C	30°C	37°C	60°C
PaS1	++++	++++	++++	++++	-
PaS2	++++	++++	++++	++++	-
PaS3	+++	+++	++++	++++	-
PaS4	++	++	+++	+++	-

Growth remarks: - = No colony, + = 1-85 colonies, ++ = 85-130 colonies, +++ = 190-250 colonies, ++++ = 250 400 colonies.

Table 6. Effect of olive oil concentration on the growth of isolated bacteria.

S.NO	Strain Name	1% Oil
1	PaS1	_
2	PaS2	++
3	PaS3	++++
4	PaS4	++

Growth remarks: - = No colony, + = 1-85 colonies , ++ = 85-130 colonies, +++ = 190-250, colonies, ++++ = 250-400 colonies.

The morphological and biochemical feature of strain PaS4 was correlated with the study conducted by Roshetko, J. M *et al.*, 2007 and was found to have somewhat similar behaviors like *Aeromonas caviae*.

Table 7. Antifungal activity of isolated bacteria.

Strain	*Alternara panex*	*Colletotrichum coceales*	*R. solani*
PaS1	+	-	-
PaS2	-	-	-
PaS3	+	-	-
PaS4	+	_	+

Antifungal activity: +, No antifungal activity: -

Effects of different conditions on growth of isolated bacterial strains

Effect of temperature and salinity

Different concentrations of NaCl **(**0.5, 1 and 3%) were tested against the growth of bacterial strains isolated in this study (PaS1, PaS2, PaS3 and PaS4) and all the strains were able to tolerate different concentrations.

The strain 1 was found to be sensitive to 1% and 3% NaCl concentration while it showed growth on 0.5 % NaCl. The results of current study suggest that PaS4 could be classified as halotolerant (willey *et al.,* 2009). The growth of PaS4 on all concentrations of NaCl was greater than that of the PaS1, PaS2 and PaS3 (Table 3). In the present study the effect of temperature on the growth of bacteria was also studied, which revealed variations in growth and morphological traits. Isolated bacterial strains were grown on different temperatures ranges from 10˚C to 60˚C and maximum growth was observed in all isolates at 30˚C and 37˚C. However, no growth was seen at 60˚C (Table 4).

Effect of olive oil on the growth of isolated bacteria

Strains were screened for liohophilic activity on Nutrient agar with 1% olive oil was used for lipase activity of the identified isolates.

The results indicate that PaS2, PaS3 and PaS4 have the ability to degrade lipids while PaS1 was unable to grow. The growth of strain 1 (PaS1) was inhibited by the 1% olive oil, whereas considerable growth was observed in case of strain 2 (PaS2), strain 3 (PaS3) and strain 4 (PaS4) (Table 5).

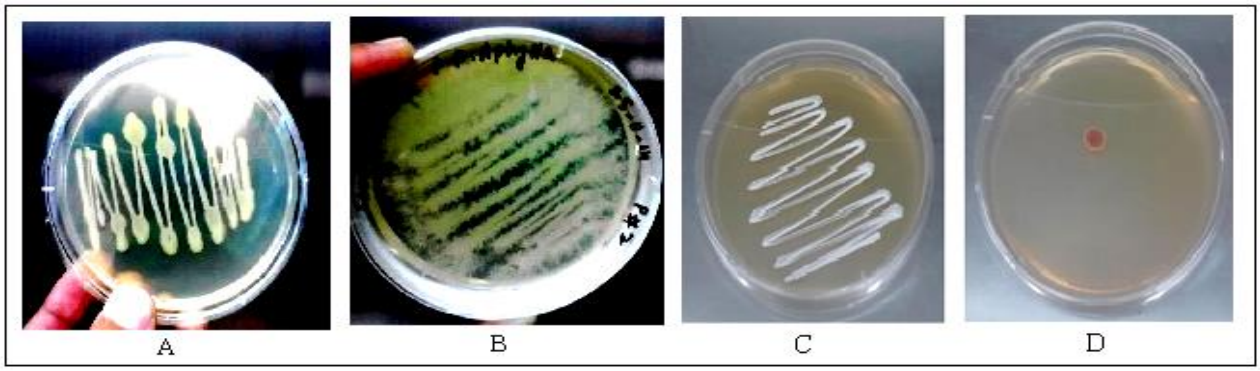

Fig. 1. Pure culture of the bacteria isolated from rhizosphere soil of *Periplocaaphylla* (A: PaS1; B: PaS2; C: PaS3; D: PaS4).

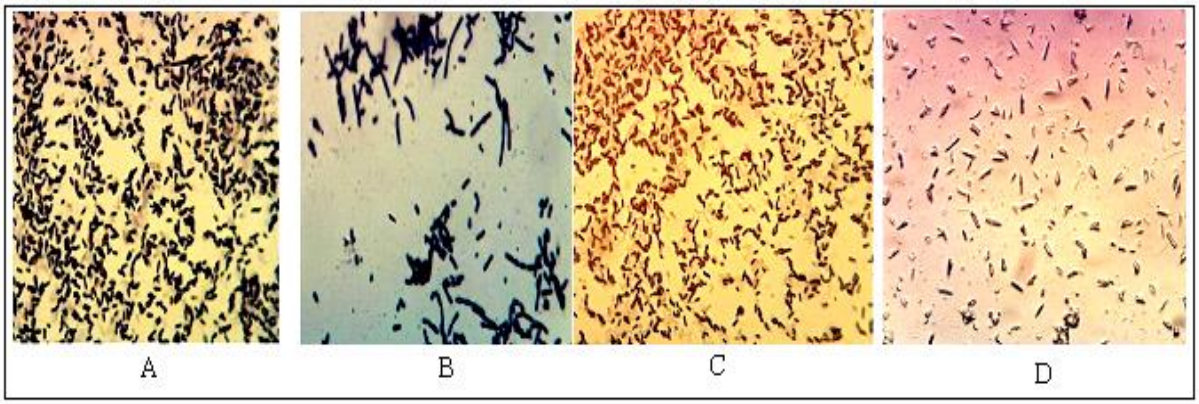

Fig. 2. Gram reaction of isolated bacteria (A: PaS1; B: PaS2; C: PaS3; D: PaS4).

Antibiotic sensitivity of isolated bacteria

The four bacterial strains isolated in this study were found highly sensitive to different concentrations (25mg, 50mg, and 100 mg per 100 ml) of Ampicillin, Kanamycin and streptomycin. All these strains were screened for antibiotic sensitivity against different concentrations of streptomycin, ampicillin, and kanamycin and were found to be sensitive to the entire tested antibiotic (Table 6). It has been previously stated that production of antibiotics by soil microorganisms can be affected by many factors including temperature, Carbon and Nitrogen source (Nazir, T *et al.,* 2006) However, the current strains were isolated from soil samples of high altitude having colder environment which might be one of the reasons for their sensitivity against these antibiotics.

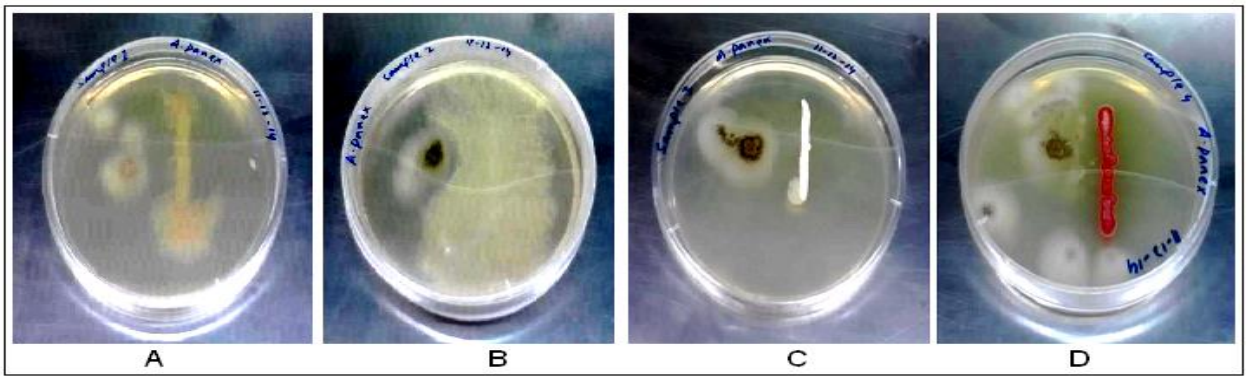

Fig. 3. Antifungal activity of bacterial isolates against *Alternara panex* (A: PaS1; B: PaS2; C: PaS3; D: PaS4).

Antifungal activity of isolated bacterial strains

The results demonstrated that the PaS3 had no antifungal activity, whereas PaS4 had strong antifungal activity against all the fungal species included, *Alternara pane, Colletotrichum cocealesx,* and *R. solani*. PaS1 and PaS2 had antifungal activity only against *Alternara panex* while showed no antifungal activity against the other species like *Colletotrichum coceales* and *R. solani* (Table 7 and Fig. 3, 4, 5).

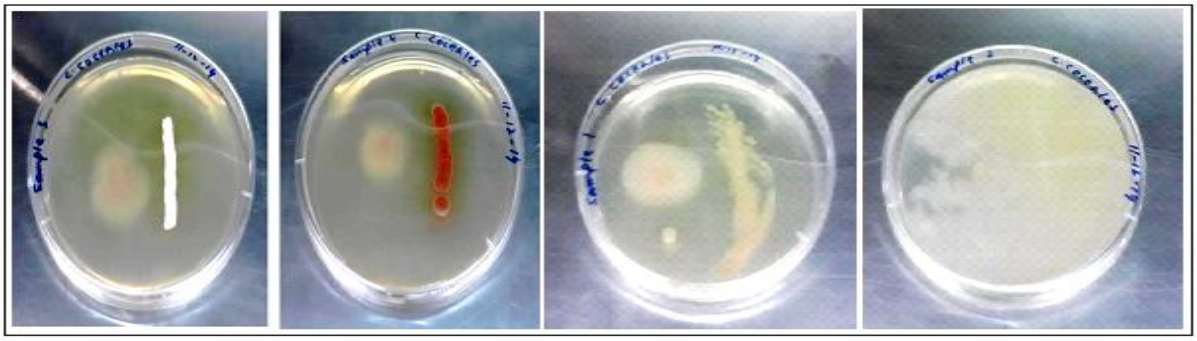

Fig. 4. Antifungal activity of bacterial isolates against *Colletotrichum coceales* (A: PaS1; B: PaS2; C: PaS3; D: PaS4).

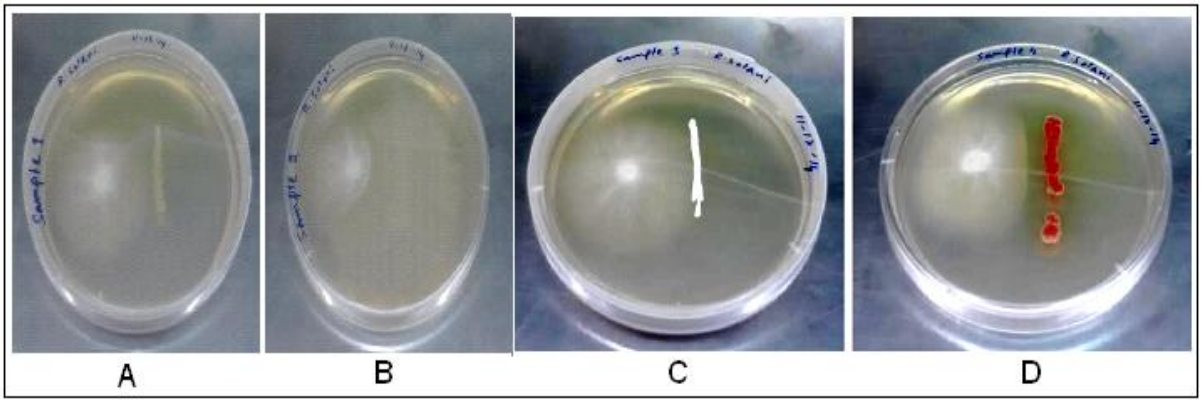

Fig. 5. Antifungal activity of bacterial isolates against *R. solani* (A: PaS1; B: PaS2; C: PaS3; D: PaS4).

Conclusion

The rhizosphere of *Periploca aphylla* was studied for bacterial strains. Four strains of bacteria were isolated from the soil samples consisted of three gram negative and one gram positive bacteria. The isolated strains i.e. PaS1, PaS2, PaS3 and PaS3 based on morphological and biochemical characteristics belong to genus of *Phenylobacterium*, *Bacillus lentus, Entrerobacter aerogene* and *Aeromonas caviae* respectively. These strains were found sensitive to number of antibiotics and were found to have the potential antifungal activity. These findings can be extended towards the production of useful antifungal substances at commercial level. During this study it was observed that these bacterial strains showed optimum growth in saline environments, showing that they tolerate high pH conditions and optimum temperature ranges.

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