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Multi-Trait Activity of *Enterobacter* sp. Strain MHR_4 towards Fluorene Degradation as well as in Plant Growth Promotion

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Abstract

Polycyclic Aromatic Hydrocarbons (PAH) are ubiquitous contaminants in environments, are generated as a by-product of incomplete combustion of organic substances. PAH continuously increases and accumulate in surrounding finally affect the environment as well as crop production. Thus, appropriate treatment is required to reduce the concentration and toxicity of these substances. Bioremediation, an effective method, uses the ability of an organism to reduce the concentration of PAH to an acceptable level. This study investigated the ability of PAHs (fluorene) degrading as well plant growth promoting activity by three bacterial strains (MHR_4, MHR_2 and MB_2) isolated from crude oil polluted soil near fuel filling stations from Haldwani and Bhowali in Uttarakhand region. These strains showed considerable growth over fluorene, as the sole carbon source with 100-500 ppm concentration in Mineral Salt Medium (MSM) agar plates after 24 h. Although, all the strains have potential towards plant growth promoting activity. Comparative study of fluorene degradation was found prominent, 81.2% in strain MHR_4 followed by strain MB_2, 55% and strain MHR_2, 37.5%, likewise, the efficiencies of various PGP activities in strain MHR_4 were detected quite high. This was convincing enough to investigate the strain MHR_4 for their molecular identification and to check their efficiency in pot trail experiment. Therefore, the efficient strain MHR_4 was identified on the basis of 16S rDNA sequencing and confirmed as *Enterobacter* sp. The multi traits strain effectively to remove fluorene both from the oil contaminated environment as well as to eliminate the chemical fertilizer by providing diverse PGP activity, is a novel achievement and suggests that *Enterobacter* sp. MHR_4 have extremely useful for a biotechnological process involving fluorene degradation and plant growth promotion. The unique nature of fluorene degradation as well as plant growth promotion activity has not yet been studied in the same bacterial strain to our knowledge.

Keywords: PAH; Fluorene Degradation; MHR_4 Strain; Phosphate Solubilization; IAA Production

Introduction

Polycyclic Aromatic Hydrocarbons (PAH) are highly persistent and recalcitrant nature of contaminants, widely distributed in environment, and spreading through creosote, petroleum products, coke products, spills or leaks during the extraction, transport and storage of crude oil as well as the activity of oil refineries and petrochemical industries [9,15]. The contamination of soil with these aromatic compounds is of particular environmental concern as they exhibit carcinogenic and mutagenic properties and also effect unhelpfully on physiological and biochemical characteristics in different plant species and their germination [18,28,29]. PAHs are organic compound composed of two or more fused benzene rings. They are found in fossils fuel and results from incomplete combustion of organic compounds and another form of pyrolysis and pyrosynthesis [12]. Fluorene is a nonalternate PAH composed of two benzene rings between which is tucked a five member ring. It is a typical by-product of coal-conversion and energy-related industries and is commonly found in vehicle exhaust emissions, crude oils, motor oils, coal and oil combustion, waste incineration, and industrial effluents [25]. Unlike many of the lower molecular weight PAHs such as naphthalene, phenanthrene, and anthracene, degraders are not as readily isolated from the different environment. However, a wide range of bacteria spanning both Gram positive and Gram negative genera including *Pseudomonas, Sphinghomonas, Burkholderia, Brevibacterium, Micrococcus, Arthrobacter,* and *Terrabacter* have been reported [36]. Microorganisms are ubiquitous in nature and make up the biodiversity on earth. They perform several important processes in the cycling of nutrients and degradation of various compounds globally. Knowledge of such microbial activities helps mankind to find various strategies to utilize agricultural natural resources in long-term sustainable manner. Microorganisms are not only utilized for resource generation but also can be exploited in environmental cleanup [13]. Now a day, there is increased interest to detoxify PAH-contaminated sites.

Bioremediation which is based on microbial transformation and degradation is one of the most promising methods applied in the field of environmental biotechnology for cleanup of contaminated environments [33]. However, the success of bioremediation projects has been limited by the scarcity of microorganisms capable of degrading a broad range of PAHs. Addition of Plant Growth Promoting (PGP) increased the organic pollutant (PAH) removal probably by enhancing plants germination and survival in soils that were heavily contaminated and by stimulating. The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, the plants to grow faster and accumulate more root biomass, supports large active groups of bacteria known as PGP and can stimulate plant growth directly and indirectly [3,27,20,30]. Several bacteria of the genera *Arthrobacter* and *Pseudomonas* have been isolated that utilize fluorene as the only carbon and energy source. On the other hand, there are the bacterial genera that are able to metabolize the fluorene as carbon and energy sources [32]. In this article, we described the degradation of fluorene under aerobic conditions in the liquid medium in addition to analysis of plant growth promoting activity under *in vitro* and pot trail condition. The unique nature of fluorene degradation as well as plant growth promotion activity has not yet been studied in the same bacterial strain to our knowledge.

Materials and Methods

Isolation and Screening of Fluorene Degrading Bacterial Strains

Soil samples were collected from crude oil polluted soil near fuel filling stations from Bhowali (29.3823° N, 79.5196° E) and Haldwani (29.2183° N, 79.5130° E) in Uttarakhand region. Bacterial strains were isolated by enrichment culture techniques using 5 g of petroleum contaminated soil as inoculum in 50 ml Minimal Salt Media (MSM) comprises {$(NH_4)_2SO_4$ 2.5g, Na_2HPO_4 1.0g, $MgSO_4$ 0.5g, $Fe_2(SO_4)$ 0.01g, $CoCl_2$ 0.005g, $CaCl_2$ 0.001g, $MnSO_4$ 0.0001g, KH_2PO_4 0.0005g, and $(NH_4)_6Mo_7O_{24}.4H_20$ 0.0001g per liter distilled water, pH=7)} sterile medium flask incorporated with 100-500 ppm fluorene (fluorene was added after autoclaving). Flasks were incubated at 35°C for 14 days in a shaker. 5 ml aliquot was transferred to 50 ml of a fresh MSM having same fluorene concentration and incubation. Following four successive enrichments, the culture suspension was serially diluted and plated onto the MSM agar plates having same fluorene concentration and incubation. Subsequently for screening, colonies were selected on the basis of forming clear zones in MSM agar plate having same fluorene concentration and incubation. Furthermore, well isolated single colony was picked up from MSM plate and streaking them on nutrient agar medium (Peptone 5.0g, Beef extract 3.0g, Potassium nitrate 5.0g per liter with 2% agar, pH=7.2) and incubate them at 35°C for 3 days. Likewise liquid minimal media supplemented with fluorene, to check the fluorene degradation was used earlier for the isolation of psychrotrophic *Sphingomonas* sp. L-138 and for isolation of *Bacillus* sp. [32,25].

Characterization and Identification of Screened Strains

The selected strains were characterized by colony morphology on nutrient agar, gram staining, and different biochemical analysis was carried out according to Bergey's manual of systematic bacteriology [8]. Other important biochemical properties were performed by using biochemical test kits (KB001 HiIMViC™ Biochemical Test Kit).

Molecularly characterized are done on the basis of 16S rDNA sequencing. Polymerase Chain Reaction (PCR) amplification of the partial 16S rDNA gene region was carried out with the bacterial primer set 27 forward primer- 5' AGA GTT TGA TCA TGG CTC AG 3', 1492 reverse primer- 5' ACC TTG TTA CGA CTT 3'. The templates were purified by Ethanol/EDTA precipitation method. The sequence was then analyzed by Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information database (NCBI, USA). Further, the isolate was identified based on the similarity scores. The phylogenetic tree was constructed using the neighbor-joining method and the phylogenetic data were obtained by aligning the different sequences of the 16S rDNA of closely related strains. The culture information was submitted under NCBI database and accession no. obtained.

Optimization of Growth against Different pH and Temperature

Two set of test tubes containing MSM medium incorporated with 0.5 mg L^{-1} fluorene concentration were prepared. One set of test tubes having different pH5 to 8, were inoculated with 500 µl of the active culture (A_{600} = 0.6) of bacterial cells and incubated them at 35°C. Another set of test tubes having a specific fixed pH 7, were inoculated with 500 µl of the active culture (A_{600} = 0.6) of bacterial cells and incubated them at a different temperature from 25, 35, 40 and 45°C. Growth was monitored by taking OD after 6 days using spectrophotometer at 620 nm.

Degradation Assay and Analytical Procedure

Culture condition was optimized based on growth assay at pH 7 with 1 mg L^{-1} fluorene incubated, incubated at 35°C for 6 days. 1 ml liquate from the assay medium was extract off and acidified to pH 2 with 6 N HCl to inhibit bacterial activity by addition of 2 ml ethyl acetate using a shaker. The organic and water layers from the acidified medium were separated by centrifugation at 10,000 rpm for 10 mins. The water layer was discarded while the organic layer was analyzed with UV-VIS spectrophotometer at 238 nm OD. Percent degradation was calculated following the formula of % degradation = [Abs (b) - Abs (a) /Abs (b)] X 100. To where Abs (b) was the absorbance of the medium before incubation and Abs (a) was the maximum absorbance after the 6th day of the incubation period. The percent degradation of fluorene was also determined earlier by Mukesh, et al. and fluorene utilized by *Bacillus* sp. by Hidayati, et al. [19,7].

Hydrogen Cyanide, Ammonia and Siderophore Production

Production of HCN was assessed on King's B medium containing 4.4g l^{-1} of glycine. Freshly grown broth cultures of all the strains were spread on King's B medium containing glycine. A Whatman filter paper No. 1 soaked in 0.5 % picric acid solution (2 % sodium carbonate) was placed inside the lid of the plate. Plates

were sealed with parafilm and incubated at 10°C for 4-5 days. Development of orange to red color indicates HCN production. Freshly grown bacterial culture was tested for the production of ammonia by inoculating them into peptone water broth tubes and incubated them for 48-72 hours at 35°C. Subsequently, Nessler's reagent was added in each tube, development of brown to yellow color indicates the production of ammonia by isolates. Siderophore production was detected by Chrome Azurol S (CAS) assay. It based on the high affinity of siderophore for ferric iron, releasing the free dye, is complexes and released from the dye. The blue color of the medium is due to the dye complexed with iron. When siderophore is added, it binds the ferric iron, releasing the free dye, which is orange in color. Hence, the presence of siderophore is indicated by a color change from blue to orange.

Phosphate Solubilization and its Quantification

Each bacterial isolate was placed in the center of separate pikovskaya's agar plate and incubated at 35°C for 4 to 5 days. Clearing zone indicates the phosphate solubilization activity of the isolate. Further, phosphate solubilization potential quantified by taking 1 ml of overnight culture of each and inoculated in 100 ml of pikovskaya's broth, and incubated at 35°C for 6 days. The amount of Pi released in the broth was estimated by sampling broth culture after every 24 hours [21]. Each day about 10 ml of broth cultures was centrifuged at 10,000 rpm for 10 min to separate the supernatant from the cell growth and insoluble phosphate. The available phosphate in the supernatant was estimated by phosphomolybdic blue colour method [10]. 1 ml of the culture supernatant was taken in a 50 ml volumetric flask to which 10 ml of chloromolybdic acid was added and mixed thoroughly. The volume was made up to approximately three fourth with distilled water and 0.25 ml chlorostannous acid was added to it. Immediately, the volume was made to 50 ml with distilled water and mixed thoroughly. After 15 min, the blue colour developed was quantified at 610 nm spectrophotometrically. Simultaneously, a standard curve was prepared using various concentration of phosphate solution. The amounts of phosphorus solubilized by the isolates were calculated from the standard curve.

Optimization of Phosphate Solubilization at Different Parameters

Two sets of the conical flask containing pikovskaya's broth were prepared to optimize the phosphate solubilization at different parameters. One set of the conical flask containing constant pH7, with varying temperature 15 to 35°C and second set of conical flasks containing constant temperature 35°C, with varying pH 5 to 9. Subsequently, both the set of the conical flask was inoculated with 500 µl of the active culture (A_{600} = 0.6) of bacterial culture and incubating them for 48 hours. The available phosphate in the supernatant was estimated by the phosphomolybdic blue color method.

Production, Quantification and Confirmation of Indole-3-Acetic Acid (IAA)

All the isolates were inoculated into 10 ml of nutrient broth supplemented with 0.5 mg ml^{-1} of tryptophan and incubated at 35°C in shaking incubator for 48 hours. Broth cultures were then centrifuged at 7500 rpm for 10 min. Then 1 ml of the supernatant was taken in another tube and 2 ml of salkowski's reagent was added and incubated at 35°C. Development of dark pink to orange color indicates production of IAA. Furthermore, to quantify the IAA produce in tubes, the absorbance was taken at 530 nm and the concentration of IAA in each bacterial strain was determined and quantified by comparing with a standard curve of IAA [1].

The confirmation of IAA production is executed by means of Thin Layer Chromatography (TLC), for which single bacterial colony was inoculated to 10 ml of nutrient broth containing 1 mg ml^{-1} of tryptophan and incubated at 35°C for 5 days on a shaker. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5 to 3.0 with 1 N HCl and extracted with ethyl acetate. Extracted ethyl acetate fraction was evaporated in a rotatory evaporator at 40°C. The extract was dissolved in 1 ml of methanol and kept at -20°C. In a glass chamber mixture of benzene: n butanol: acetic acid at 4:5:1 proportions were prepared as solvent system for the chromatogram. 1 µl of the extract was spotted and marked at the lower portion of the TLC plate. Similarly, a control spot of IAA (1 mg ml^{-1}) was placed. The plate was placed inside the glass chamber and was covered. Once the solvent front reached the top layer of the plate, the plate was dried naturally. IAA was identified by spraying the plates with salkowski's reagent. Test and control sample were compared by spot size and Rf value.

Optimization of IAA Production at Different Parameters

Three sets of the conical flask containing nutrient broth were prepared to optimize the IAA production at different parameters. One set of conical flask contains constant pH7 and temperature 35°C, with varying concentrations of, tryptophan 1 to 7 mg ml^{-1} The second set of conical flask contains constant temperature 35°C and concentration 2 mg ml^{-1} , pH 5 to 9. The third set of conical flask contains constant concentration, 2 mg ml^{-1} and pH with varying temperature 15 to 40°C. All the set were inoculated with a 500 µl of the active culture (A_{600} = 0.6) of volume of culture and incubate for 48 hours. Subsequently, the cultures were centrifuged at 7500 rpm for 10 min afterward 1 ml of supernatant and 2 ml of Salkowski's reagent was mixed and incubated at 35°C for 25 min. Optical density was taken at 530 nm by using UV spectrophotometer to measure the amount of IAA production in all set of the flask.

Evaluation of Growth Performance to Check Pea under Pot Trials

Gram (*Cicer atrium*) seeds were surface sterilized by exposing to 95% ethanol and immersing in 0.2 % $HgCl_2$ solution for 3 min, followed by five times bathed with sterile distilled water. 1 mL of overnight developed bacterial culture was applied on each seed for 10 min and treated seeds were dried. Seed germination test is executed with sterile non treated dried seeds as control, soaked with non inoculated media for 10 min and the treated dried seeds were sown on soft agar (0.8%) plates under the axenic

condition and incubated them at 35°C for 5 d. The percent seed germination and root length were measured as it was considered the main parameter in determined the effect of IAA. For Pot trial, the sample of soil was collected, air-dried, sieved and three times sterilized repeatedly by autoclaving prior to filling the pots. The sterilized untreated dried seeds (control) and the treated dried seeds was transfer to pots containing sterile soil to a depth of 5 mm. Three seeds were sown in each pot and the experiment was performed in triplicate. The pots were kept in the green house and were observed every day for 25 days. After 25 days the plant were uprooted carefully and the length of root and shoot were measured.

Results

Isolation, Screening and Biochemical Characterization of Bacterial Strains

Isolated strains MHR$_2$, MB$_2$ and MHR$_4$ were successfully isolated from crude oil polluted soil near fuel filling stations by applying enrichment technique. All the isolated strains were gram negative, rod shaped, white color with a smooth surface while the strain MHR$_2$ has a rough surface (Figure 1A, 1B and C). The screening was done on the basis of clear halo zone formation on MSM plates incorporated with fluorene (Figure 1D). All the strains were analyzed for metabolic properties by observing their response to diverse biochemical reactions using specific HiMedia test-kits for carbohydrate and by performing different another test (Table 1).

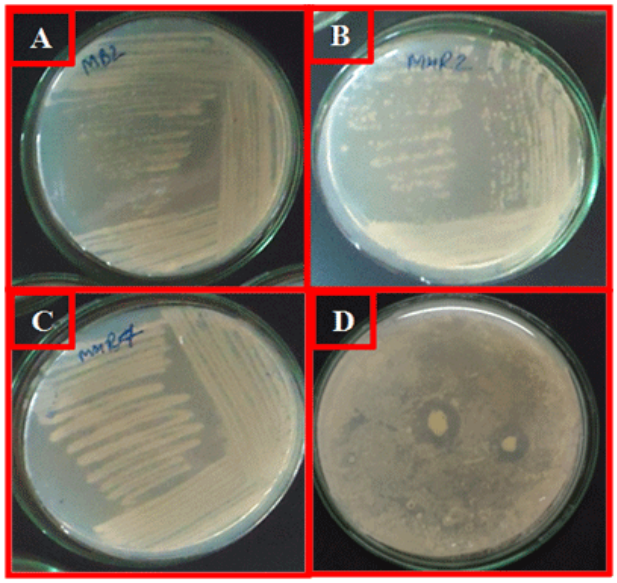

Figure 1: Morphology of isolated bacterial strains, (A) MB$_2$, (B) MHR$_2$, (C) MHR$_4$, (D) Clear zone of fluorene degradation

Optimization of Bacterial Growth against Different pH and Temperature

Optimal growth of all the bacterial strains was found higher at temperature 35°C, followed by 40°C, 30°C, and 25°C with the exception of strain MHR$_4$, it shows higher density almost in all the temperature range from 25°C to 35°C while less in 40°C. So we concluded that MHR$_4$ has more capability of growth in a wide range of temperature in compare to other strains. Similarly, the optimal growth for pH of bacterial strain MHR$_4$ and MB$_2$ were found higher at pH 7 followed by pH 8 and lower at pH 5 with the exception of strain MHR$_2$, it shows almost same optical density in all the PH range but slightly higher at pH 5 and 6. So we concluded that MHR$_4$ and MB$_2$ isolates were preferred to grow optimally at neutral to the slightly alkaline environment. While strain MHR$_2$ prefer slightly acidic to neutral condition (Figure 2). Similar type of optimization of bacterial growth in presence of hydrocarbon was reported [5].

Table 1: Biochemical test of bacterial strains

S.N.	Biochemical Test	Bacterial Strains		
		MB$_2$	MHR$_2$	MHR$_4$
1.	Oxidase	+ ve	+ ve	-ve
2.	Catalase	+ ve	+ ve	+ ve
3.	Indole	+ ve	+ ve	+ ve
4.	Methyl Red	+ ve	+ ve	+ ve
5.	Voges Proskauer	- ve	- ve	- ve
6.	Citrate Utrilization	+ ve	+ ve	- ve
7.	Dextrose	- ve	- ve	- ve
8.	Lactose	- ve	- ve	-ve
9.	Sucrose	+ ve	+ ve	- ve
10.	Urease	- ve	+ ve	+ ve
11.	Nitrate Reduction	- ve	+ ve	+ ve
12.	Adonitol	+ ve	+ ve	- ve
13.	Arabinose	- ve	- ve	- ve
14.	Sorbitol	- ve	- ve	- ve
15.	Mannitol	- ve	- ve	- ve
16.	Rhamnose	- ve	- ve	- ve
17.	H_2S Production	- ve	- ve	- ve
18.	Gelatin Hydrolysis	- ve	+ve	+ve
19.	Starch Hydrolysis	- ve	- ve	- ve
20.	Casein Hydrolysis	- ve	- ve	- ve
21.	Gram Staining	G-ve	G-ve	G-ve

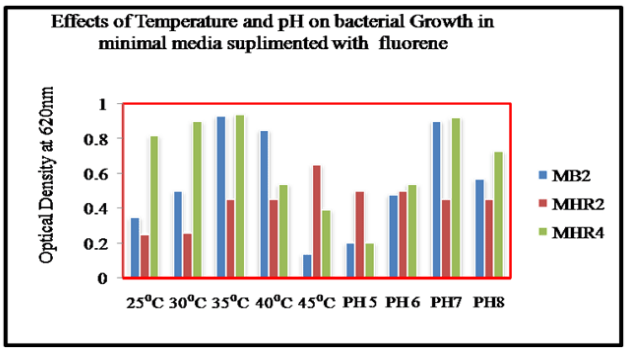

Figure 2: Optimization of Bacterial growth in response to various temperatures and pH in minimal media supplemented with Fluorene

Fluorene Degradation Assay

All the strains were inoculated in the minimal media incorporated with fluorene concentration 0.5 mg L^{-1}, with neutral pH and subsequently incubated at their optimal condition of temperature 35°C for 6 days. Fluorene degradation was determined by the taking optical density at 238 nm and the percent degradation of fluorene was measured. Percent degradation of fluorene was found high (81%) in case of strain MHR_4 followed by strain MB_2 and MHR_2 (55% and 37.5%) respectively (Table 2). The percent fluorene fluorene degradation is also reported by [25].

Table 2: Percent degradation of fluorene

Isolates	OD in 238 nm	% degradation= [Abs (b) - Abs (a)/Abs(b)]X100
MB_2	0.036	[0.08 - 0.036/0.08] X100 = 55%
MHR_2	0.05	[0.08 - 0.050/0.08] X100 = 37.5%
MHR_4	0.015	[0.08 - 0.015/0.08] X100 = 81.2%

OD of control at 238nm = 0.08, (a) = OD of treated sample at 238nm, (b) = OD of control at 238nm

Siderophore, Ammonia and HCN Production

Siderophore productions of all the strains were confirmed by CAS assay. The appearance of a reddish-brown zone on CAS plates suggests the positive result for siderophore production. This low molecular weight iron binding protein is well known to exhibit antagonistic activity against phytopathogenic fungi. The changes in color in the CAS agar plate by the strains recommend the production of siderophores, and the color intensity can be the consequence of siderophore concentration (Figure 4D). Ammonia production of all the strains was found positive, the result of ammonia production is exposed by brown to yellow color. The production of HCN was checked for all the strains. The strain MHR_4 found positive while strain MHR_2 and MB_2 is negative. The results of HCN production revealed a remarkable change in color from yellow to brown against the control (Figure 4A and 4B).

Quantitative Measurement and Optimization of Phosphate Solubilisation

Qualitative phosphate solubilization was observed in pikovskaya's agar plate by halo zone formation around the inoculums (Figure 3C). Afterward, quantitative examine of phosphate solubilization was performed in pikovskaya's broth. The magnitude of soluble phosphate liberated in broth from tri-calcium phosphate solubilization was measured by means of KH_2PO_4 curve at 600nm upon 5 days of growth. Relative account of solubilized phosphate was found high in case of MHR_2 (102-209 µg ml^{-1}) followed by MHR_4 (123-204 µg ml^{-1}) and MB_2 (103-185 µg ml^{-1}). The statistics to reveal a time-dependent augment in the sum of solubilized phosphate (Figure 4A). The data also disclose interesting results for MHR_4 is that the efficiency of phosphate solubilization is better from initial day to the later stage, but in rest strain, the efficiency is better only in the later stage, initially the potential is quite low. Intermittent examination of pH of the culture filtrate showed a notable decrease from pH

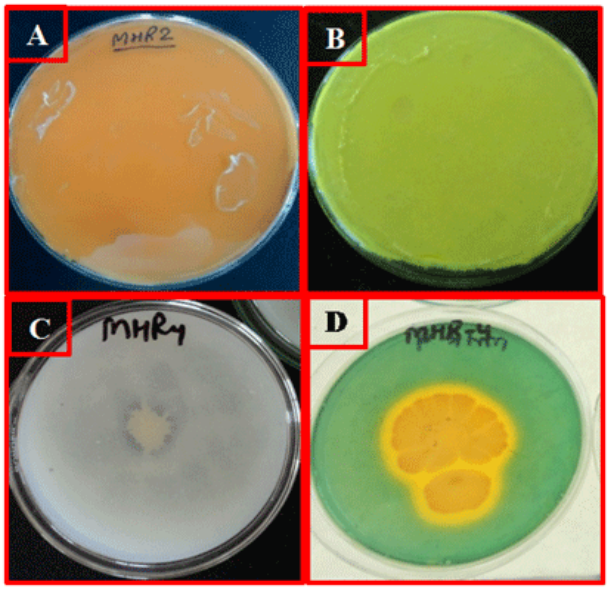

Figure 3: *In vitro* PGP activity of Isolated Strains (A) Positive HCN production (B) Control of HCN production (C) Positive Phosphate Solubilization (D) Positive Siderophore production

7.0 to 4.1 (Figure 4B). Furthermore, these figures also reveal interestingly that the reduction of pH synchronized with the amount of solubilized phosphate.

The optimum temperature of all the strains for phosphate solubilization was obtained at 35°C are almost same (136 µg ml^{-1}), the results are very interesting, all the strains solubilized phosphate as the temperature increases up to attaining an optimum temperature and subsequently, reduction is started after crossing the optimum temperature (Figure 4C). The pH optima of all the strains for phosphate solubilization were found to be 7.0 (132 -137 µg ml^{-1}) (Figure 4D). Statistically the results data set were found to be in reliable limits as shown in Figure 4 A, C and D in which small standard deviation bar indicates more reliable value compare to the larger standard deviation bar.

IAA Production, Confirmation and its Quantification

The extended incubation of all the strains for a period of 5 days demonstrated a few degree of decrease in cell viability (Figure 5A). A clearly demonstrates the production of the significant amount of IAA in LB broth medium. Though, adequate bacterial biomass undergoes in the stationary phase yet its incubation up to 5 days. The filtrate of all the strains at varying time periods confirmed a straight and time-dependent augmentation in IAA formation. IAA production is found high (20.16 µg ml^{-1}) at 5^{th} days in the case of MHR_4 by comparing to the other two strains. Improved production of IAA of strains MHR_2 and MB_2 is found (18.16 µg ml^{-1}) and (13.53 µg ml^{-1}), respectively, were determined by adding 0.5 mg ml^{-1} of tryptophan concentration (Figure 6A) [13]. This result corresponds with the earlier observations indicating IAA production in the stationary phase of culture [34]. Furthermore, the confirmation of IAA was done by TLC method,

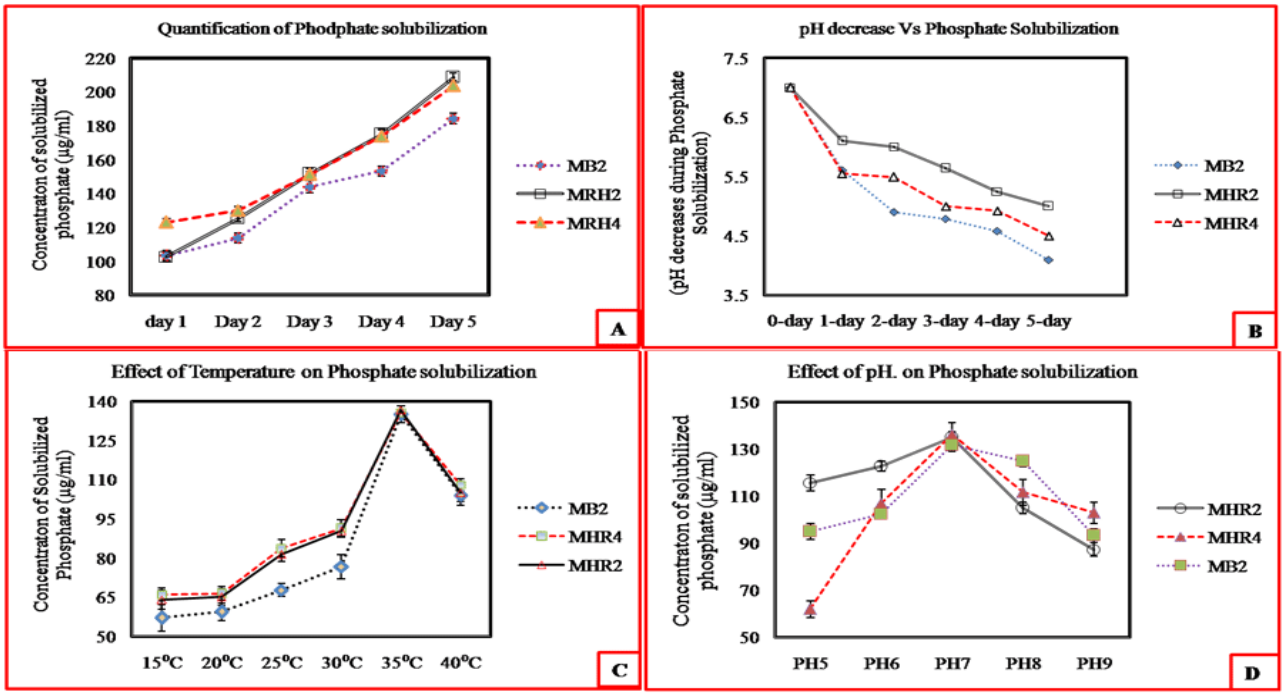

Figure 4: (A) Quantification of Phosphate solubilization (PS), **(B)** Reduction of pH during PS, **(C)** Effect of temperature on PS, **(D)** Effect of pH on PS

in which the culture filtrate of all the strains was used to extract IAA for characterization. The spots of ethyl acetate extract of culture and standard IAA were tested in the solvent mixture of chloroform: ethyl acetate: formic acid (5:3:2). Chromatograms of spots were, sprayed with salkowski reagent that shows almost the same Rf value as obtained by the standard IAA solution (Figure 5B). Thus, it validates the intrinsic aptitude of isolated strains to produce phytohormone. The same TLC findings are in agreement with reports by another scientist [24,35].

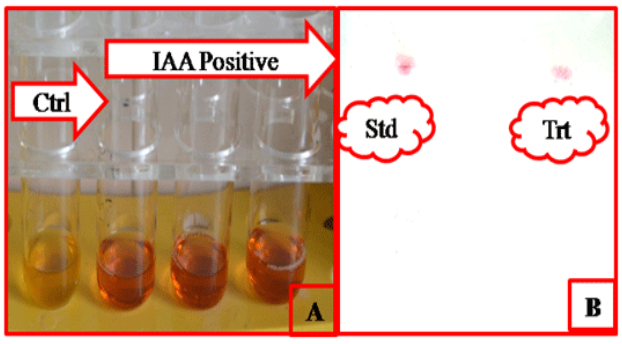

Figure 5: (A) Comparative detection of IAA production, (B) IAA confirmation by TLC method

The optimum temperature for IAA production (36.75, 35.83 and 31.7 µg ml^{-1}) of the strains, MRH$_4$, MB$_2$, and MRH$_2$, respectively, were attaining at 30°C and 35°C. The range of IAA production is quite consistent at the temperature series from 25°C to 35°C and slightly depleted as the temperature fluctuates to higher and lower temperature (Figure 6B). While in case of optimum pH for IAA production (approx 36 µg ml^{-1}) of all the strains were gains at pH 7. The range of IAA production is dramatical changes as the series of pH increases or decreases from pH 7 to pH 9 or to pH5 (Figure 6D). Subsequently, the optimum tryptophan concentrations for IAA production of all the strains were attained at 5 mg ml^{-1}. But the concentration of IAA production were attained high for MRH$_2$ (62.7 µg ml^{-1}) followed by MRH$_4$ (57.26 µg ml^{-1}) and MB$_2$ (50.3 µg ml^{-1}). The range of IAA production is dramatical increases as the conc. of tryptophan increases from 1 mg ml^{-1} to 5 mg ml^{-1} and similarly, noticeably decreases as the conc. of tryptophan increases from 5 mg ml^{-1} to 7 mg ml^{-1} kewise, increase IAA production also observe from 25°C to 35°C and slightly depleted as the temperature fluctuates to higher and lower temperature (Figure 6C). The results also depicted that at lower concentration of tryptophan IAA production is more prominent in the strain MHR$_4$ compare to the rest. The effect of different parameters towards IAA production points out that all the strains were worked best at neutral pH with a range of temperature of 25°C to 35°C and tryptophan concentration 5 mg ml^{-1}. The same optimization findings are in agreement with reports by another scientist [35]. Statistically the results data set were found to be in reliable limits as shown in Figure 6 A, B, C and D in which small standard deviation bar indicates more reliable value compare to the larger standard deviation bar.

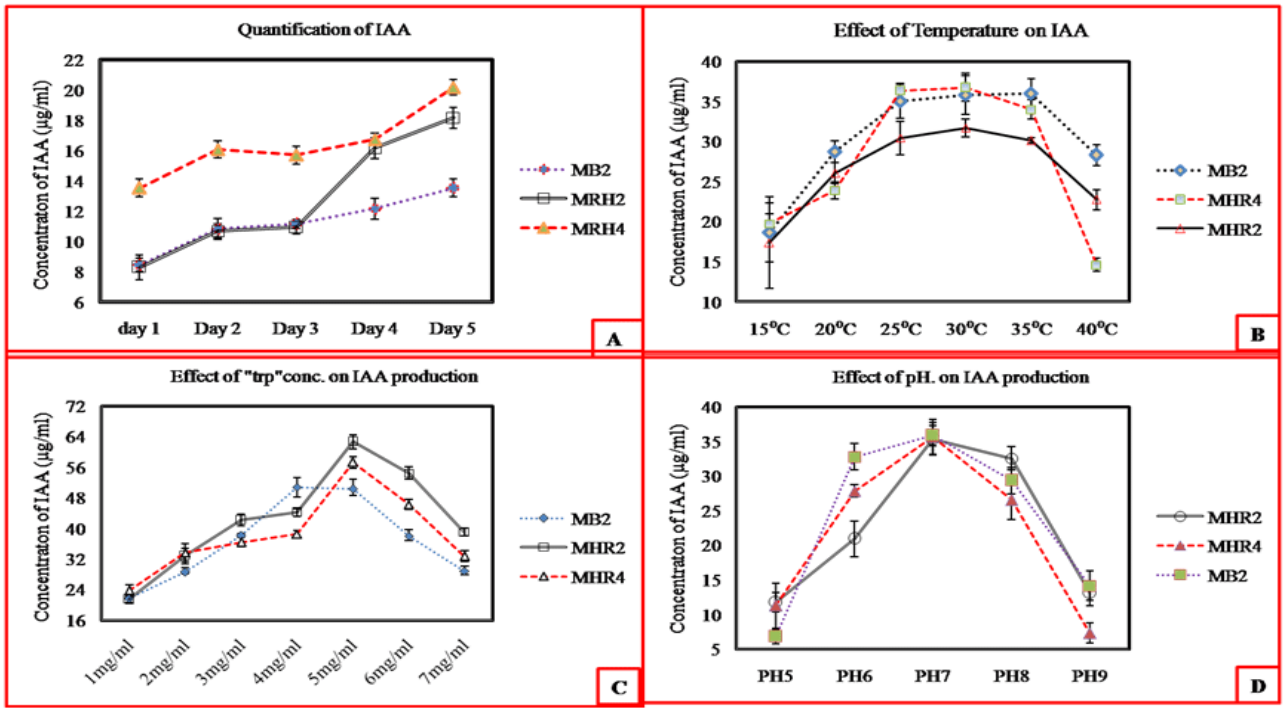

Figure 6: (A) Quantification of IAA production, **(B)** Effect of temperature on IAA production, **(C)** Effect of tryptophan conc. on IAA production, **(D)** Effect of pH on IAA production

Molecular Characterization

Pleasantly, all the strains were responded differently towards the entire biochemical test, but the results of MHR$_4$ strain is more pretty towards all aspect, as this stain has high potential in fluorene biodegradation as well as in plant growth promoting activity, especially in IAA production and HCN production which influences the plant growth both directly and indirectly. Directly by enhancing the root and shoot development in response to plant growth hormone IAA and indirectly by producing HCN, which act as antibiotic thereby reducing the plant pathogen from the rhizospheric soil, this behavior compelled for their molecular identification. So, the strain MHR$_4$ subjected to 16S rDNA sequencing and was analyzed using different bioinformatics tools. The partial sequence data of MHR$_4$ are analyzed by BLAST search that showed unambiguous similarity (99%) with *Enterobacter* sp. and submitted to NCBI database under accession number MG205158. Further, the phylogenetic tree was constructed using MEGA4 software by neighbor-joining method and the resulted phylogenetic data were examined after sequences alignment of the 16S rDNA of closely related strains (Figure 7).

Pot trial experiment

The growth performances of gram (*Cicer atrium*) plants were evaluated in MHR$_4$ strain treated and untreated conditions. In seeds germination test, the percentage seed germination of treated seeds was found high (80.66) as compared with control (50.66) (Figure 8C and 8D). Maximum effect on root length was also observed in germinated seed compare with control seed, the strain also shows the greater length of root and shoot as well as their fresh weight and dry weight in the harvested plant compare with control (Figure 8A and 8B).

Discussions

Bacterial strain MHR$_4$ and MHR$_2$ were isolated from Haldwani soil sample while MB$_2$ from Bhowali soil sample (Figure 1A, 1B and 1C). All the cultures were screened on the basis of fluorene degradation, and some biochemical characteristics were measure according to Bergey's manual of systematic bacteriology [8,16] (Figure 1D). Other important biochemical properties were performed by using biochemical test kits (KB001 HiIMViCTM Biochemical Test Kit). All are gram negative bacteria and also have capabilities to survive in stress condition like fluorene contamination by degrading them as for their energy source but the degradation activity is healthier (81%) in case of strain MHR$_4$ compare to others (Table 2). Likewise, effort was earlier determined [16]. In his study, the test isolate utilized fluorene (3mg L) as the sole carbon source and showed a maximum degradation of 97%. The optimal growth results also depicted that strain MHR$_4$ have the best capability to survive in a wide range of temperature in fluorene contaminated environment compare to others two (Figure 2).

Owing to the fact that these strains are having both fluorene biodegradation as well as plant growth promotion, they can act as an efficient candidate for environmental benefits. Bacterial plant growth promotion is a well familiar and multifaceted phenomenon and is often achieved by the performance of numerous plant growth promoting traits exhibited by the associated bacterium [6]. Among the PGP traits, IAA production by the bacterial

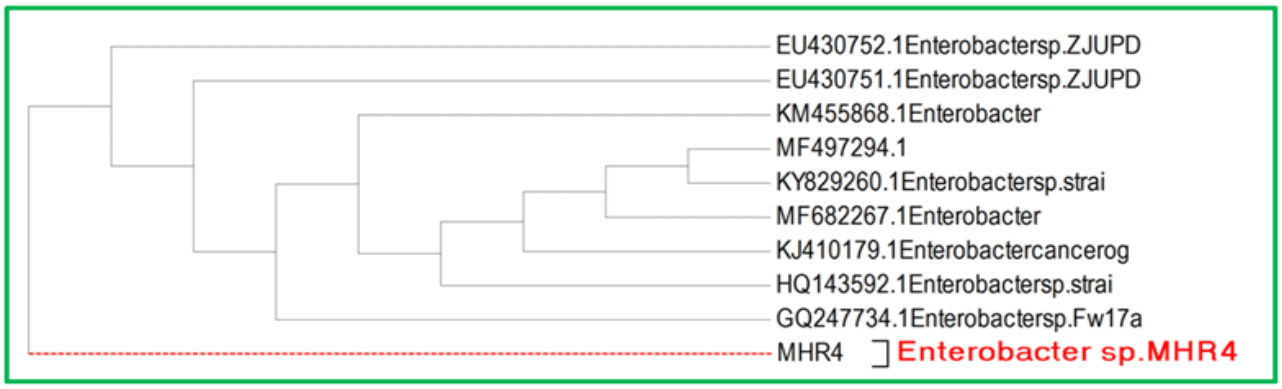

Figure 7: Phylogenetic tree for the 16S rDNA sequences of the bacterial strains constructed using MEGA4 software by neighbor-joining method

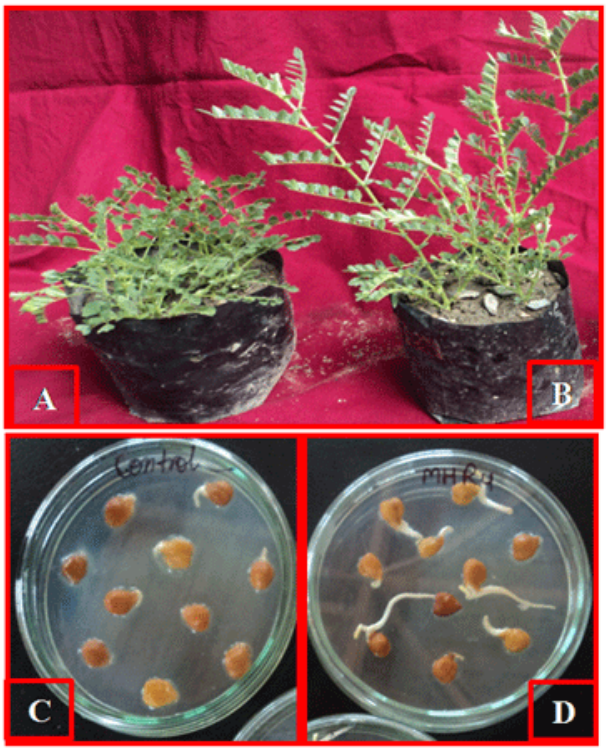

Figure 8: Pot trails and seed germination in presence (B and D) and absence (A and C) of bacterial strains MHR$_4$.

strain has a cascading effect on the plant development due to its ability to influence root growth as well as shoot development, which in turn affects the nutrient uptake and ultimately the plant productivity (Figure 5A, 6A, 8A and 8B). These trends coincided with the previous reports indicating IAA formation in the stationary stage of culture [24,34,35]. The confirmations of IAA with TLC findings are agreements with previous reports [24] (Figure 5B). Solubilisation of insoluble phosphorous compounds in the rhizosphere by microorganisms is another important means of achieving plant growth promotion (Figure 4A and 4B). The production of organic acid, phosphatase enzyme and its role in phosphate solubilization is well known [1,6]. From the data obtained for pH stability studies of phosphate solubilization, it was contingent that phosphate solubilization was some extent more pronounced in the pH range from pH 7.0 in all the strains. Phosphate solubilization showed a decline in activity as pH increases or decreases much below pH 7.0 suggesting that it is of neutral phosphate solubilizer (Figure 4D). A similar type of the result of phosphate solubilization activity at different pH was reported [11]. Phosphate solubilization statistics showed turn up in activity sporadically as temperature increases from 15°C to 35°C, but decline abruptly at a temperature above 35°C, suggesting that optimum temperature of all strains are 35°C (Figure 4C). The similar results of phosphate solubilization activity on different temperature were supported [23]. We strongly believe that the phosphate solubilization aptitude of the bacterial strains could have played an imperative role in the observed plant growth promotion. The availability of iron for microbial assimilation in environments such as the rhizosphere is extremely limiting. Since it is the 4th most abundant element in the earth's crust it is largely required by all living organisms for direct microbial assimilation. This is too low to support the growth of microorganisms, which generally need concentrations approaching 10^{-6} M for normal growth. Therefore, to stay alive in such surroundings, organisms produce iron-binding ligands (siderophores), which can attach the ferric iron and make it accessible to the host microorganisms (Figure 3D). The responsibility of such iron-chelating siderophores in plant growth promotion is well-known [4]. Bacterial antagonism towards phytopathogenic fungi is known to be mediated by a variety of compounds of microbial origin, viz., bacteriocins, HCN, enzymes, toxic substances, volatiles, and others. So, the HCN production activity by strain MHR$_4$ depicted it as an efficient candidate in against of pathogenic fungi while other strains do not contribute the quality (Figure 3B). Nevertheless, all these unique behavior and metabolic potential of strain MHR$_4$ compel for their molecular identification. Further, the phylogenetic tree was constructed using MEGA4 software by neighbor-joining method. Based on the 16S rDNA gene sequence and BLAST search revealed that strain MHR$_4$ has 99% similarity with *Enterobacter* sp. and the sequence submitted to NCBI database under accession number MG205158 (Figure 7).

Although, the evaluation of the effect on plant growth parameter of these IAA producing strain MHR$_4$ was further studied in seed germination and pot experiment (Figure 2A and 2B). The data are obtained from seed germination and pot experiments demonstrated a positive effect on root and shoot elongations and their respective weight compares with control (Figure 2C). This indicates that the strain MHR$_4$ can improve the plant growth development and thus considered as an effective PGPR. Similar type of results was reported [2,31]. By observing these data we conclude that the strain MHR$_4$ has more positive effect on root and shoot elongation and biodegradation as compared to control. The results also indicate that strain MHR$_4$ has potential to improve the plant growth development, by providing different mechanism to the plant like plants assimilate iron from bacterial siderophores by means of different mechanisms, for instance, chelate and release of iron, the direct uptake of siderophore-Fe complexes, or by a ligand exchange reaction., likewise HCN, is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens, suppress the pathogenic population in rhizospheric environment of the plant as well as it also involved in geochemical processes in the substrate (e.g., chelation of metals), indirectly increasing the availability of phosphate [26,22]. Ammonia released by diazotrophs is one of the most important traits of PGPR's which benefits the crop [14]. So these strains were considered as an effective bioinoculant. A similar type of results was reported [2,17,31](Figure 9).

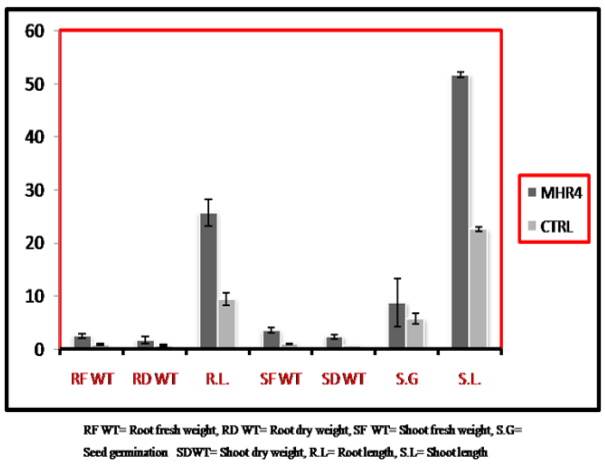

Figure 9: Comparative Growth parameter and seed germination in presence of MHR$_4$ and control.

Conclusion

Many bacteria strains present in petroleum contaminated region have been shown to possess multi-trait activity of fluorene biodegradation as well as plant growth promoting activity, due to which we can collectively designate as multi-trait plant growth promoting bacteria (MT-PGPB). PGPB have several characteristics which help them to promote plant growth and their yield. They have some characteristics like the ability to produce phytohormones, for root and shoot development, some enzyme/acid production to make the availability of solubilized phosphate that easily takes up by plant, HCN, and siderophore production that also act directly or indirectly on plant growth by fighting against phytopathogenic microorganisms or providing ferrous availability. Furthermore, the biodegradation potential of fluorene or PAH as well provides nutrient to the microbial flora that make the soil more porous and ultimately increases their water holding capacity. So, in this context, a bacterial isolate *Enterobacter* sp. MHR$_4$ along with two others were isolated and screened on the multi-trait propose of Fluorene biodegradation activity and PGP activity. On the basis of their best result in both the direction *Enterobacter* sp. MHR$_4$ was chosen for molecular identification and to evaluate the effect on plant growth, seed germination test and pot trail experiments of gram seed were executed and growth index was measured. Hence, the present isolate can act as potential candidates for the development of bio-inoculants for crop plants as well as best fluorene degradation.

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