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EFFECT OF CRUDE OIL CONTAMINATED SOIL ON *PHASEOLUS AUREUS* AND ITS BIOAUGMENTATION BY *PSEUDOMONAS AERUGINOSA* CH23

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ABSTRACT

Petroleum hydrocarbons are the most obvious pollutants in both terrestrial and aquatic realm. Its seepage in agriculture field can create toxic conditions and harm valuable crops. In the present study, effect of Bioaugmentation/ Bioremediation of such crude oil contaminated soil on mung beans is studied by using an indigenous isolate identified as Psedomonas aeruginosa CH23. A pot study was carried out to study effect of augmented (amended) crude oil contaminated soil on mung beans (Phaseolous aureus) along with controls such as un-augmented contaminated soil and normal soil. The artificially crude oil contaminated soil (5%) was amended with test organism (appx. 5.2x10^{7}CFU/gm of soil). Various parameters like number of seeds germinated, height, wet weight of plants and chlorophyll a and b content of the leaves were studied after every 5 days till 30 days. The pot study confirmed that the organism Pseudomonas aeruginosa CH 23 is not only effective in degradation of crude oil in laboratory shake flask condition but could also compete with indigenous soil micro-organisms in in-situ bio-degradation as well in decreasing the phytotoxicity of crude oil.

KEY WORDS

Bioaugmentation, Bioremediation, Amendment, Crude oil, Phytotoxicity.

INTRODUCTION:

Industrial development, population growth, urbanization and a disregard for the environmental consequences of releasing chemicals into the environment all contributed to the modern pollution situation. Over 2 billion tons of petroleum is produced annually worldwide. Spillages of oil have become a common occurrence. Depending on the site location, the level of oil contaminants in the soil may be as high as 10% (w/w) [1]. Large quantities of hazardous substances are carelessly disposed of in the environment and are thus creating enormous pollution problems in soils and waters around the world [2]. Petroleum compounds are considered to be recalcitrant to microbial degradation and persist in ecosystems because of their hydrophobic nature and low volatility and thus they pose a significant threat to the environment [3]. Protective and preventive measures need to be taken into account to avoid spillage into the environment [4]

Accidental spills caused by pipeline leakages and ruptures and accidents during transport have been reported. The environmental consequence of soil pollution causes adverse effect on the soil microflora and reduces soil fertility [5]. Contamination of soil results in damage of crop growth; depending on the degree of contamination, the soil may remain unsuitable for plant growth for months or several years. Contamination of soil with petroleum products has been a major cause for concern. Damages due to soil

contamination may be extensive and its effect may be long term. Crude oil is not a systemic killer; it kills plants cells on contact. Contamination by crude oil can kill the roots, and this prevents the plant from taking up water and other nutrients. It can also disrupt plant and water relationship in soil [6]. Petroleum derived diesel consist of 75% saturated hydrocarbons primarily paraffin and 25% aromatic hydrocarbons. Regardless of this complexity, it can be degraded by a number of soil microorganisms. Crude oil is phyto-toxic to plants even at low concentrations. At levels below this phytotoxic level, the development of plants grown in crude oil contaminated soil differs greatly from plants grown in normal soil conditions [7, 8].

Various petroleum products are common soil contaminants and often contain potentially hazardous chemicals, particularly the polycyclic aromatic hydrocarbons [9]. The accumulations of PAHs in soil are due to many anthropogenic sources such as coking plants, solid fuel domestic heating, aircraft exhaust, car exhausts and forest fires [10]. Amongst petroleum pollutants spent oil contains heavy metals and polycyclic aromatic hydrocarbons and chemical additives including amines, phenols, benzenes, Ca, Zn, Pb, Ba, Mn, P and S which are dangerous to living organisms [11]. The spent lubricant, otherwise called waste engine oil, is usually obtained after servicing and subsequent draining from automobile and generator engines. Pollution from even this spent engine oil is one of the environmental problems and is more widespread than crude oil pollution [12]. The concentration of PAHs in lubricating oil increases with time of usage and those with two and three rings accumulate rapidly in oil to very high levels [13, 14].

Petroleum can create unsatisfactory conditions for plant growth through a number of processes; (a) oil could displace air from soil pore spaces, (b) an increase in the demand for oxygen brought about by activity of oil-decomposing microorganisms [15], and (c) petroleum hydrocarbon creates hydrophobic environment, which limits water absorption to plant roots [16].

Bioaugmentation is defined as the addition of pollutant-degrading microorganisms (natural / exotic/ acclimatized / genetically engineered) to augment the bio-degradative capacity of indigenous microbial populations. Sometimes microorganisms from the remediation site are collected, separately cultured, and returned to the site as a means of rapidly increasing the microorganism population at the site. Usually an attempt is made to isolate and accelerate the growth of the population of natural microorganisms that preferentially feed on the contaminants at the site. In some situations, different microorganisms may be added at different stages of the remediation process because the contaminants change in abundance as the degradation proceeds. Hence the aim of augmentation is to improve the fertility status of such soils and to enhance the rate of oil degradation thus improving the crop production.

The present study reported in this chapter has been undertaken with three main objectives: (1) to determine the extent of damage caused by crude oil pollution to the foliar parts of Mung beans (*Phaseolus aureus)* and its germination, (2) to ascertain the remedial effect of *Pseudomonas aeruginosa* CH23 on the germination and growth pattern of the crop and (3) to determine how far this plant can serve as an indicator of oil pollution.

2. MATERIALS AND METHOD

2.1 Test organism

The organism *Pseudomonas aeruginosa* CH 23 isolated indigenously from soil near oil well from where the crude oil under test is obtained and is used to augment crude oil contaminated soils in pots.

The isolated bacteria was initially acclimatized for its oil degrading efficiency by growing in the Bushnell and Haas Medium (Hi media 350 M) with increasing concentration of crude oil as sole source of carbon. Further it was screened for oil degradation efficiency by an initial rapid Screening test using dye 1,6 DCPIP. [17]

2.2Identification of Isolate:

The most efficient degrading bacteria was selected out of 15 isolates which was screened and identified by 16SrRNA method and was named as *Pseudomonas aeruginosa* CH23.

2.3 Assessment of Hydrocarbon degradation Efficiency:

This was done by growing the bacteria on different petroleum fractions as well as monocyclic aromatic hydrocarbons and measuring the efficiency in terms of turbidity [18] as well as Whole cell protein by Folin Lowry's method. [19]

2.4 Degradation of PAHs:

The PAH degradation efficiency of *Pseudomonas aeruginosa* CH23 was also assessed by using Naphthalene sprayed BHM medium and observing clear

zone of utilization. [20] Finally, this most efficient isolate was selected for Bio-augmentation Pot study.

2.5 STUDY OF BIOAUGMENTATION

2.5.1Determination of Colony Forming Units (C.F.U/gm of soil)

Initially before augmentation of soil, the viability of the test organism was determined by standard plate count (S.P.C). The colony count was performed for sterile soil, un-augmented soil as well as for augmented soil after various time periods.

Pot study

To determine the capability of *Pseudomonas aeruginosa* CH23 for determining the efficacy to support the seed germination and plant growth in crude oil contaminated soil, 100 gm. of soil was dispensed in small plastic pots, mixed with 5% crude oil and then amended with the test organism (appx. $5.2x10^7$ CFU/gm soil). Addition of crude oil and the test organism was then made in such a way that different treatment periods culminated on the same day. Ten Mung *(Phaseolus aureus)* beans were then planted per pot. The results were noted after 5, 10, 15 and 30 days in terms of number of seeds germinated, height, wet weight of plants and the chlorophyll (a and b) content of the leaves **[21].** In all the cases the final readings are taken as the mean of at least three sets.

2.5.2 Study of growth parameters affected by Bio-augmentation and Pot study.

To study the growth parameters affected by Bio-augmentation, the results were noted after 5, 10, 15 and 30 days in terms of

- Number of seeds germinated
- Height of plants.
- Wet weight of plants
- The chlorophyll (a and b) content of the leaves [21]. In all the cases the final readings are taken as the mean of at least three sets.

3 RESULTS AND DISCUSSION

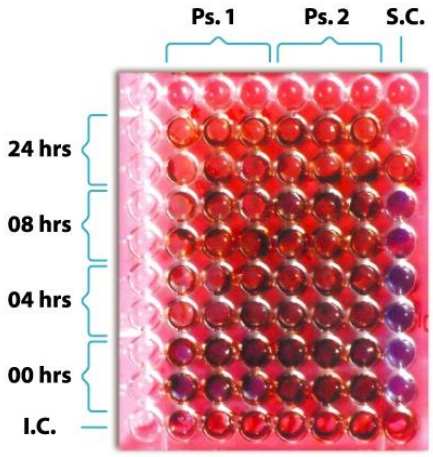
3.1 Rapid confirmative Screening test of the selected isolate: by 2,6- Dichlorophenol Indophenol (2,6-DCPIP) oxidation test

Hydrocarbons are highly reduced substrates requiring an electron acceptor for the initial oxidation step [22]. A rapid miniaturized screening technique using a redox indicator dye 2, 6, Dichlorophenol indophenol (2, 6, DCPIP) was used to assess the potential of most efficient isolates to degrade oil.

The rate and extent of color change of dye from blue (oxidized) to colorless (reduced) with time indicated the potential of the bacteria to degrade crude oil. As seen in the Figure I, complete decolorization of the dye occurred within 8 hours in wells inoculated with two bacterial isolates *Ps 1* and *Ps 2*. Dye containing medium without crude oil but with inoculum (I.C.-Inoculum Control) and medium without crude oil but with inoculum (S.C.-Substrate Control) were kept as controls which showed no decolorization. The faster and complete decolorization of dye 2, 6, DCPIP by isolates Ps 1 and Ps 2 indicates their high potential for crude oil degradation.

Thus, based on growth (assessed in terms of Whole cell protein) and rate of crude oil oxidation (based on 2, 6, DCPIP decolorization), the isolate Ps 1 was. selected for further pot study which was further identified and named as *Pseudomonas aeruginosa CH23*.

Fig I: A Micro titre plate showing time- course decolorization of 2.6, DCPIP in BHM with crude oil by isolates Ps1 and Ps 2. The last vertical row shows Substrate Control (S.C) where no crude oil is added while last horizontal row shows Inoculum Control (I.C) where no inoculum is added. The gradual decolorization of dye is witnessed in each row with time



3.2 a. Bacterial degradation of Petroleum hydrocarbons (Fractions)

Measurement of increase in cell mass in terms of turbidity and whole cell protein directly indicates the utilization of various petroleum fractions as they are added as the sole source of carbon in each individual BHM flask. As shown in Table I.

Table I Degradation (Utilization) pattern of five petroleum fractions along with crude oil by *Pseudomonas aeruginosa* CH23 in terms of Turbidity and Whole Cell Protein content

Carbon source	Turbidity(A_{600})	Whole Cell Protein(µg/ml)
Crude oil	+++	228
Petrol	+	175
Diesel	++	300
Kerosene	+++	300
Lubricant oil	+++	321
Paraffin oil	++++	363

[Where: + indicates poor growth, ++ indicates moderate growth and +++ and ++++ indicates luxuriant growth in terms of turbidity.

Table: II Degradation (Utilization) of various Aromatic Hydrocarbons by *Pseudomonas aeruginosa* CH23, in terms of Turbidity and Whole Cell Protein content

s.no	Volatile hydrocarbon	Turbidity(A_{600})	Whole Cell Protein(µg/ml)
1.	Benzene	+	298
2.	Toluene	-	-
3.	Ethyl-benzene	+	171
4.	Xylene	++	288
5.	Phenol	++	187

[Where: - indicates no growth, + indicates poor growth, ++ indicates moderate growth and +++ and ++++ indicates luxuriant growth in terms of turbidity].

Table III: Determination of C.F.U. in Bio-augmented soil.

INCUBATION TIME	Un-augmented soil	Augmented Soil
05 Days	$0.50x10^2$	$1.40 x10^4$
10 Days	$0.75 x10^3$	$2.05 x10^5$
15 Days	$0.67 x10^4$	$3.03 x10^6$
30 Days	$0.61 x10^5$	$4.45 x10^7$

Table IV: No. of seeds germinated

	INCUBATION TIME			
POT NO.	**5 Days**	**10 Days**	**15 Days**	**30 Days**
A- Fertile soil	8.5	9.0	10.0	10
B- Soil +oil	1.0	2.2	3.0	3.2
C-Soil+ oil+ Inoculum	8.9	9.0	10	10.0
D- Soil +inoculums	3.30.	5.5	6.3	6.6
SEM±	0.288675	0.527046	0.372678	1.870829
CV	10.16949	14.60593	8.90341	46.84873

Table V: Height of plants(cm)

	INCUBATION TIME			
POT NO.	**5 Days**	**10 Days**	**15 Days**	**30 Days**
A- Fertile soil	**3.60**	**7.80**	**12.2**	**17.60**
B- Soil +oil	**1.0**	**2.60**	**7.50**	**10.20**
C-Soil+ oil+ Inoculum	**4.50**	**7.22**	**13.30**	**17.90**
D- Soil +inoculum	**4.00**	**7.25**	**12.2**	**17.50**
SEM±	**0.0872**	**0.15211**	**0.15207**	**0.19149**
CV	**3.552949**	**4.189023**	**2.28953**	**2.098023**

Table VI: Wet- weight of plants(gm)

	INCUBATION TIME			
POT NO.	**5 Days**	**10 Days**	**15 Days**	**30 Days**
A- Fertile soil	**125**	**169**	**200**	**280**
B- Soil +oil	**60**	**100**	**189**	**210**
C-Soil+ oil+ Inoculum	**130**	**160**	**225**	**300**
D- Soil + inoculums	**130**	**155**	**220**	**300**
SEM±	**3.23179**	**2.24227**	**5.78792**	**4.79583**
CV	**4.990447**	**2.640491**	**4.850791**	**3.056715**

Table VII: Chlorophyll content a and b (mg/gm)

POT NO.	**INCUBATION TIME**							
	5 DAYS		**10 DAYS**		**15 DAYS**		**50 DAYS**	
	A	b	A	B	a	b	a	B
A- Fertile soil	0.2	0.05	0.61	0.10	1.11	0.18	1.13	0.23
B- Soil +oil	-	-	0.05	-	0.6	0.02	0.8	0.11
C-Soil+oil+ Inoculum	0.23	0.06	0.62	0.10	1.13	0.17	1.18	0.30
D- Soil + inoculum	0.20	0.08	0.81	0.12	1.234	0.20	1.31	0.36

Biological degradation of pure alkanes and aromatic hydrocarbons by isolated bacterial cultures and mixed bacterial population has been reported [23, 24, 25, 26, 27]. Reports on the utilization of complex hydrocarbon mixtures like crude oil by isolated microbial species are also reported [28, 29 30] reported the degradation of oil sludge by pure strains of *Rhodotorula rubra* and *Pseudomonas aeruginosa.* [29] reported the utilization of crude oil by pure bacterial strains of *Acinetobacter calcoaceticus rAg-1* and Pseudomonas *sp. hl7b*. [31] investigated biodegradation of diesel oil by two psychrotrophic bacteria that were assigned to the genera *Pseudomonas* sp. and *Arthobacter* sp

The results obtained (Table I.) in this investigation indicate that *Pseudomonas aeruginosa CH23* utilized all petroleum hydrocarbons to some extent when they were supplied as the sole source of carbon and energy. When any major oil spill occurs in any ecosystem of the environment, the availability of carbon is dramatically increased hence forth the indigenous population starts the bioremediation process [32].

3.2 b. _Bacterial Degradation of volatile Monocyclic Aromatic Hydrocarbons

Liquid monocyclic aromatic hydrocarbons such as benzene, toluene and phenol are toxic to bacteria when present in liquid phase. However, if they are introduced in vapor phase, good growth can be obtained [33]. Measurement of increase in cell mass in terms of turbidity and whole cell protein directly indicates the utilization of various volatile monocyclic aromatic hydrocarbons as they are added as the sole source of carbon in individual BHM flask. (Table 2)

As shown in Table II, the cell mass (in terms of turbidity) as well as whole cell protein has been found to be sufficiently noteworthy. This indicates that even volatile monocyclic aromatic hydrocarbons are utilized by both bacterial isolate *Pseudomonas aeruginosa CH23*

Many microorganisms have been reported to use various volatile monocyclic petroleum hydrocarbons, (including BTEX) as their sole carbon and energy substrate, despite their extreme insolubility in the aqueous phase. Numerous genera of bacteria are known as good hydrocarbon degraders. They tolerate high concentrations of the hydrocarbons and have a high capability for their degradation. Most of them belong to Pseudomonas*, Sphingomonas, Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Brevibacterium, Xantomonas, Mycobacterium, Rhodococcus* and *Bacillus s*pecies [34]

3.3. Bacterial degradation of water insoluble, solid Polyaromatic Hydrocarbons (PAHs) using solid agar plates

Due to volatilization of alkylbenzene, biphenyl and naphthalene at 30° C to 37°C temperatures ordinarily used for bacterial cultivation, these hydrocarbons are often provided separate from the growth medium and they are assumed to be utilized from the vapor phase. Larger PAHs are however nonvolatile and water insoluble at this temperature. Naphthalene dissolves in water at a concentration of 98μM, but Phenanthrene, Anthracene and Naphthalene dissolves at concentration

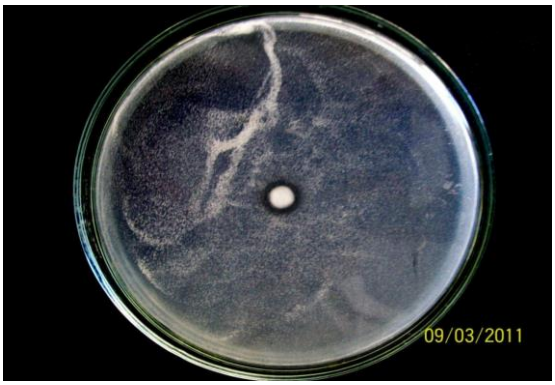
of only 9.0, 0.45 and 0.06μM respectively **[35, 36]**. Therefore, no basal medium in which Phenanthrene and larger PAHs are uniformly dispersed have been developed. If an emulsifier is added in the medium for uniform dispersion of hydrocarbon, bacteria are subject to inhibition and if an agar medium containing a larger amount of a solid hydrocarbon is autoclaved, the melted hydrocarbon forms larger masses on the surface of agar on cooling. Thus, a rapid procedure is devised for the bacterial assimilation of water insoluble, solid hydrocarbons like PAHs on solid media. The organism is inoculated on a plate containing mineral salts agar, an ethereal solution (10%w/v) of the PAH is sprayed on the surface of the plate and then incubated at 30°C for 2 to 3 days. The colonies showing degradation were surrounded by clear zone on the opaque plate.

Growth of PAH- degrading bacteria on mineral salt agar plates:

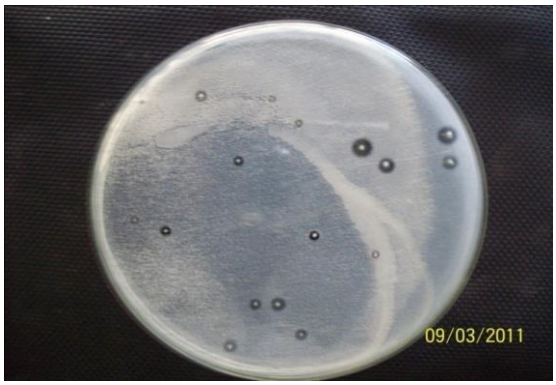
The bacterial strain *Pseudomonas aeruginosa CH23* was incubated for 1 to 2 days on Naphthalene sprayed mineral salt agar plates which grew at the expense of the solid Naphthalene to form colonies which were surrounded with transparent areas of clear zone (Fig II). Solid naphthalene disappeared from the cleared area, because of its degradation.

Figure 2: Bacterial degradation of water insoluble, solid Polyaromatic Hydrocarbons (PAHs) using Napthalene sprayed MSM agar plates

Using paper disc for inoculum



Using tooth picks for inoculum



Alternatively, the bacterial cultures which were also incubated on the plates by another method of using sterile tooth picks for inoculation also showed clear zones around the colonies of *Pseudomonas aeruginosa CH23*, as shown in the (Fig. II).

This study clearly reflects the fact that PAHs are among the compounds recalcitrant to biological degradation, possibly due to their insolubility in aqueous cultural media. However, the same can be achieved and studied by using this spray technique on solid mineral medium which is rapid and useful for the isolation of naphthalene degrading bacteria and their $Naph^-$ mutants and it should also contribute to genetic studies of bacterial degradation. It is also widely applicable for the isolation of bacteria able to degrade other non-volatile, water insoluble biologically inert, and solid compounds such as solid paraffins, wax and larger PAHs such as benz[a]anthracene and benzopyrene [32].

3. 4 Determination of Colony Forming Units (CFU)

As shown in Table III, the cell count revealed that the CFU increased by more than three times in 30 days of incubation period in case of augmented soil. While in case of un-augmented soil which was kept as control, the CFU was found to cease after just 10 days of incubation, due to the increased phyto-toxicity of crude oil in the soil. Hence, the increase in CFU in the augmented soil was believed to be due to the decreased phyto- toxicity of crude oil in the soil and at the expense of crude oil.

Some researchers have reported that inoculation had positive, marginal or no effects on oil biodegradation rates [37, 38]. The results strongly indicate that the environmental conditions including physical and chemical conditions of the contaminated sites play a crucial role in the degradation even though additional degrader has been introduced into the contaminated site.

3.2 Study of growth parameters affected by Bio-augmentation

3.2.1. Number of seeds germinated

As observed from Table IV, germination of seeds was severely retarded in case of contaminated, untreated soil as compared to the uncontaminated as well as treated soil and contaminated, treated soil. About 80% of germination was observed in case of treated soil as compared to just 30% as seen in case of untreated soil. Even the onset of leaf formation was found to be absent or delayed in case of untreated contaminated soil. The probable reason for this could be the displacement of water with oil. It prevents the plant from taking up water and other nutrients. It can also disrupt plant and water relationship in soil [39, 8].

3.2.2 Height of plants

The height of the plants was also found to be severely affected in case of contaminated, un-augmented soil as compared to the other two soils kept as control. While in case of augmented soil, the length of the plants was found to be as good as normal uncontaminated soil after 30 days of incubation. (Table V). This can be clearly witnessed in the photographs taken after various incubation periods. Fig III.

Fig III: Efficacy of *Pseudomonas aeruginosa* CH23 for promoting growth of Mung Beans (*Phaseolus aureus*) in crude oil contaminated soil observed for the incubation time of 5, 10, 15 and 30 days.Where: POT A: Fertile Soil, POT B: Soil + Oil, POT C: Soil + oil+ Inoculum and POT D: Soil + inoculum.

3.2.3 Wet weight of plants

Similarly, the wet weight of all the plants of Mung beans was found to be less in case of untreated, contaminated soil as compared to the other three pots kept as control (Table VI).

According to [40] some volatile fraction with less than 3 rings are found in spent oil. These compounds are known to have severe inhibitory impact on germination of several plant species. Another group of chemical compounds which are found abundantly in spent oil are polycyclic aromatic hydrocarbons (PAHs) and has shown to have indirect secondary effects including disruption on plant – water – air relationships [41] and effect on soil microorganisms [42]

3.2.3.1. Chlorophyll content (a and b)

Plant productivity is a unique process that depends greatly on the amount of chlorophyll present in the chloroplast. The quantity of chlorophyll per unit area is an indication of photosynthetic capacity and productivity of a plant. [43, 44, 45]

The chlorophyll a and b content was measured in case of treated, contaminated soil along with the other three soils kept as control. Both chlorophyll (a and b) contents were found to be increasing with incubation time in normal uncontaminated soil and treated contaminated

soil, whereas it was found to be noticeably decreasing in case of untreated contaminated soil as shown in Table VII.

4 CONCLUSIONS

Determination of the potential success of Bio-augmentation requires an understanding of the bioavailability of the pollutant, the survival and activity of the added microorganism(s) or its genetic material, and the general environmental conditions that control soil bioremediation rates [46].

The indigenously isolated strain *Pseudomonas aeruginosa* CH 23 was first isolated, identified, and then assessed for its petroleum hydrocarbon degradation efficiency The pot study carried out in order to measure the effect of *Pseudomonas aeruginosa* CH 23 augmentation on the germination and growth pattern of Mung beans (*Phaseolus aureus)* cultivated on crude oil contaminated soil confirmed that the organism *Pseudomonas aeruginosa* CH 23 is not only effective in degradation of crude oil in laboratory shake flask condition, but could also competes with indigenous soil micro- organisms in in-situ bio-degradation as well in decreasing the phytotoxicity of crude oil. Moreover, the parameters of growth pattern of beans in uncontaminated, un-augmented soil and un-contaminated, augmented soils kept as control were almost similar, confirming that there was no adverse effect of *Pseudomonas aeruginosa* CH 23 on soil. This proves that *Pseudomonas aeruginosa* CH 23 can serve as a promising potential agent for the Bioremediation of crude oil contaminated soil.

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