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Isolation, characterization and identification of bacteria by FAME based analysis for herbicide degradation

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Abstract

Fatty acid profile using Sherlock system is a technique in which unique fatty acid is matched with Sherlock micro-organism library. In the current investigation, the bacterial species degrading 2, 4-Dichlorophenoxy acetic acid (2, 4-D) were isolated from soil and monitored for their ability to degrade herbicide. These species were cultivated on Bushnell Hass Agar (BHA) and Bushnell Hass Broth (BHB) with increasing concentration of 2, 4-Dichlorophenoxy acetic acid (2, 4-D) as a sole carbon source. The growth of organisms and percentage degradation of 2, 4-Dichlorophenoxy acetic acid (2, 4-D) was studied by turbidometric method. In the given study, by using FAME based analysis system, two species were obtained which degrade herbicides and they were confirmed on the basis of fatty acid profile as *Escherichia coli* and *Citrobacter koseri*. Hence from the current investigation one may conclude that, these two species can be used in the field for purpose of bioremediation in near future.

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Keywords: *2,4-Dichloroacetic acid (2,4-D), FAME based analysis, fatty acid profile, herbicides, Sherlock microorganism library, turbidometric method.*

Introduction

Herbicides are the agents, usually chemicals used for killing or inhibiting growth of unwanted plants or weeds. They compete with important crop plant for water, light, nutrients, space and carbon dioxide. Other than they reduce crop quality by contaminating the commodity, interfere with harvesting, serving as hosts for crop diseases or providing shelter for insects to overcome winter and also have some effects on human and animal health. Herbicides provide a convenient, economical, and effective way to manage weeds (Dwight ,1998). An herbicide's mode of action is the biochemical or physical mechanism by which it kills plants. Most herbicides kill plants by disrupting or altering one or more of their metabolic processes. Some disrupt the cellular membranes of plants, allowing cellular content to leak out, but do not directly disrupt other metabolic processes. (Tu, 2001).

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In the current study, we have taken 2, 4-dichlorophenoxy acetic acid (2, 4-D).2, 4-dichlorophenoxy acetic acid (2, 4-D)2, 4-dichlorophenoxy acetic acid, commonly abbreviated as 2, 4 D is one of the most widely applied phenoxy herbicides in many parts of the world. (Wilson *et. al* 1997; Botre *et al*, 2000). 2, 4-D products of can be used at very low application rates as growth regulators by application of aqueous foliar spray 20-40mg 2,4-D/liter on apple trees to reduce premature fruit drop, on potato plants to increase the proportion of medium size tubers or to intensify the tuber skin colour of the red varieties (Bristol *et.al.*, 1982). At low doses, these herbicides act as plant growth regulators and stimulate plant cell growth. However, at high doses, they induce phytotoxic effects. (Naylor, 2002; Vencill, 2001)

Mode of action

2, 4-D is generally applied to the foliage of broadleaf plants or directly to the soil as either a liquid or granular products. Plants absorb 2, 4-D through their roots and leaves within 4-6 rain free

hours after application (Munro *et al*.1992); if rain occurs it will dissolve in the rain water and runoff of the plants and soil before sufficient amount are absorbed by the plants. Following foliar absorption 2, 4-D progress through the plant in the phloem most likely moving with the food materials. If absorbed by roots it moves upward in the transpiration stream . It mimics the effect of auxins or other plant growth regulating hormones and stimulates growth rejuvents old cells. (Mullison, 1987).

Health effect

Acute and chronic exposure to 2, 4-D causes inhalation, ingestion, eye or skin contact and absorption through the skin, low blood pressure, etc. exposing to large amount of 2,4-D develop extreme stiffness of the extremities, lethargy,stupor, coma, severe dilation. It is also an excitant and a depressant of the central nervous system. (Hathway *et al.* 1991)

Biodegradation of 2, 4- D

Microbial degradation of 2, 4- D has been subject for the extensive studies and more recent studies has elucidated the kinetic of degradation of 2,4 D (Igbinosa, *et al*. 2007). It is considered to be the major route in the breakdown of 2, 4-D in the soil. Some microorganisms are capable of using 2, 4-D as their sole carbon source (IPCS,1989). Hemmett and Faust (1969) conclude that the size of the microbial population, the concentration of 2, 4- D and the ratio of the two factors determine 2,4-D degradation rates. Soil conditions that enhance microbial population that is warm and moist facilitate 2, 4-D degradation.(Foster & McKercher 1973). Six species of microorganisms were isolated from the soil previously treated with herbicides. These were *Flavobacterium peregrinum, Pseudomonas fluorescence, Arthrobacter globiformis, Brevibacterium* species, *Streptomyces viridochromogenes*, and an unidentified *streptomyces* species. *Flavobacterium* was the most active organism in the degrading the 2, 4,-D.

The present paper highlights on the isolation and screening of bacteria which can degrade herbicide from the soil as well as its identification by FAME based analysis. For more than 15 years a substantial portion of the pharmaceutical industry has relied on the MIDI Sherlock Microbial Identification System for the identification in their microbial testing laboratories as well as it is used in many clinical, environmental and biodefence laboratory. The Sherlock System identifies microorganisms based on fully automated gas chromatographic (GC) analysis of extracted microbial fatty acid methyl esters (FAME). More than 300 fatty acid and related compounds are found in bacteria. This system show both qualitative differences (genus level) and quantitative differences (species level).(Craig kunitsky., 2007)

Materials and method

1] Isolation and Screening

Soil sample were collected from College of Agriculture Maharajbagh, Nagpur and serially dilution from 10^{-1} to 10^{-6} was performed. For isolation of hydrocarbon degrading bacteria 0.1ml of soil sample from each dilution was inoculated to the Bushnell Hass Agar ($MgSO_4$ 0.02%, $CaCl_2$0.0002%, K_2HPO_4 0.1%, KH_2PO_4 0.1%, NH_4NO_3 0.15% ,$FeCl_3$ 0.0005%, Agar 2% pH 7.0-7.2) by spread plate techniques. Similarly for isolating Pseudomonas species process was repeated in Pseudomonas isolation agar. Incubate all the plates at 37^0c for 24 hrs; and that pure culture slants were prepared for each bacterial isolates. Now, isolated bacteria were inoculated to the Bushnell Hass agar (BHA) with 5ul of herbicide (2, 4-D) by centre streaking and incubate the plates at 37^0c for 24 hours to 48 hours. Similar process was repeated in the presence of 10ul of 2, 4-D. That bacteria were able to grow even in the presence of 10µl of 2, 4- D were selected for further studies.

2] Characterization

Gram staining, capsule staining, endospore staining methods and biochemical tests like catalase production test, urease production, gelatinase production, hydrogen sulphide production, fermentation of carbohydrates, and IMViC test were performed according to standard procedure to categorize all the bacteria isolates depending on their morphological and biochemical characteristics.

3] Study of growth of bacterial isolates in different amounts of 2, 4-D

Broth cultures were prepared by inoculating the bacteria in 5ml of Nutrient Broth and incubate at 37^0c for 24 hours. Then 5ml Bushnell Hass Broth (BHB) was taken in 18 test tubes in three sets. 2,4-D was added to each set with increasing amount

starting from 0.5µl, 0.6µl, 0.7µl, 0.8µl, 0.9µl to 1.0µl and add 0.1ml broth culture to each tube expect for one set consider as a control. Incubate all the tubes at 37^0c for 24 hours. Take optical density of broth of each tube with respect to their corresponding controls at 610 nm using UV/Vis spectrophotometer.

Graph was plotted of optical density against amount of 2, 4-D added for all bacterial isolates.

4] Comparison of growth activities of bacterial isolates in the presence and absence of 2, 4 D

Take 50 ml of BHB in 3 conical flasks and sterilized. Add 1ml broth culture in each flask except the control. Take optical density with respective to control at 610 nm using spectrophotometer starting from the time of inoculation and continuing with the interval of 1 hour. About 7 readings were taken in all. Similar procedure was followed in the presence of 5ul and 10ul of 2, 4-D in each conical flask and reading was recorded. The growth activity was compared by plotting graph of optical density against time in hours for each bacterial isolates in the absence as well as in the presence of lowest and highest amount of 2,4-D.

5] Determination of presence of 2, 4-D biodegradation

Take 50ml BHB in 5 conical flasks with one blank, two as control (I0 and I24) and other two as experimental and sterilized. Add 5ul 2, 4-D in each conical flask except blank and 1 ml culture broth was inoculated in only two experimental conical flask and incubate it at 37^oC at 24 hours. Take O.D just after inoculation of 5µl of 2, 4-D for control I0 at 284nm using spectrophotometer with respect to blank containing only BHB, after filtering and centrifuging them at 10,000 for 10 min and take reading. After incubation, the remaining flask were filtered and centrifuged and optical density was taken at 284 nm with blank. Percentage of biodegradation was determined through calculation. Similar procedure was repeated for 10µl of 2, 4-D.

6] Bacterial identification by fame based analysis.

For identification of aerobic bacteria isolated from environmental sample, bacterial cultures were inoculated by four ways streaking on Trypticase Soy Broth Agar (TSBA) (Casein peptone 1.5%, soya peptone 0.5%, Sodium chloride 0.5%, Agar 1.5%) and incubated at 28^0C for 24 hours.

6.1 **Sample processing** – The five steps to prepare GC ready extracts are as follows-

Harvesting- A 4mm loop is used to harvest about 40mg of bacterial cells from the third quadrant (second or first quadrant if slow growing) of the quadrant streaked plate. The cells are placed in a clean culture tube.

Saponification-1.0ml of Reagent 1(45g sodium hydroxide,150 ml methanol, 150 distilled water) is added to each tube containing cells. The tubes are securely sealed with Teflon lined caps, vortexed briefly and heated in a water bath at 100^0 c for 5 minute; the tubes are vigorously vortexed for 5-10 seconds and returned to the water bath for further 25 minute heating.

Methylation-The cooled tubes are uncapped, 2.0ml of reagent 2 (325ml certified 6N hydrochloric acid, 275ml methyl alcohol) is added. The tubes are capped are briefly vortexes. After vortexing, the tubes are heated for 10 minute at 80^0 c (This step is critical in time and temperature.)

Extraction-Addition of 1.25ml of reagent 3 (200ml hexane, 200 ml methyl tertiary butyl ether) to the cooled tubes is followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes at 6 rpm. The tubes are uncapped and aqueous (lower) phase is repeated out and discarded.

Base Wash-About 3.0ml of reagent 4 (10.8g sodium hydroxide, 900ml distilled water) is added to the organic phase remaining in the tubes. The tubes are recapped and tumbled for 5 minutes at 6 rpm. Following uncapping, About 2/3 of the organic phase is pipette into a GC vial which is capped and ready for analysis.

6.2 Run the calibration mixed and samples into the Sherlock MIDI by rapid method.

Results and discussion

1) Isolation & Screening:-

Bacterial cultures were obtained after incubation of Pseudomonas isolation agar and Bushnell Haas agar (BHA) containing plates inoculate with serially diluted soil sample. After incubation number of colonies appeared on pseudomonas isolation agar but all were single type. Out of them 1 colony was chosen from 10^{-5} dilution plate for preparing pure culture of *Pseudomonas* species, has been represented by culture A. Similarly on

Bushnell Haas Agar (BHA) three different type's of hydrocarbon degrading bacterial colonies were found to be appeared. Two were grown on 10^{-5} dilution plate and the third one from 10^{-3} dilution plate. Then pure cultures were prepared which are represented as culture B, culture C, and culture D respectively. On inoculation of these isolated bacteria in Bushnell Haas agar (BHA) with 5µl and 10 µl of 2, 4-D similar growth pattern was obtained after incubation period of 24 hours and 48 hours.

The results obtained in the presences of 10 µl of 2, 4-D has been represented in Table 1. It has been found that culture A was unable to growth in presence of 2,4-D even after 48 hours were as out of other three, Culture B and C was showing good growth just after 24 hours also their growth was fairly good after 48 hours while Culture D was good only growth after 48 hours. Therefore, only culture B and C was selected study and the growth of culture B & C in the presence of 10 µl of 2, 4-D is represented in Table 2 and 3.

Bacterial isolates	Bacterial growth after 24 hours	Bacterial growth after 48 hours
Culture A	-	-
Culture B	++	+++
Culture C	++	+++
Culture D	+	++

Table 1:- Growth of isolated bacteria in BHA with 10 µl of 2, 4-D

No growth -
Poor growth +
Good growth ++
Very good growth +++

Sr. No	Staining techniques	Culture B	Culture C
1	Gram staining	Gram negative	Gram negative
2	Capsule staining	Non capsulated	Non capsulated
3	Endospore staining	Non endosporic	Non endosporic
4	Motality	Motile	motile

Table 2:- Staining techniques for culture B and culture C

Sr.	Biochemical Test	Culture B	Culture C
1	Catalase	+	+
2	Urease	-	-
5	Gelatinase	-	-
6	Hydrogen sulphide production	-	-
7	Fermentation of carbohydrates Glucose Lactose Sucrose	 A+G A+G A+G	 A+G - A+G
8	IMViC Indole test Methyl Red test Voges proskauer test Citrate test	 + + - -	 - + - +

Table 3:- Biochemical tests for culture B and culture C

A=Acid
G=Gas
+ =Producing
- =Not producing

2) Characterization:

The morphology & biochemical character of the two bacterial isolate were identified by performing various staining techniques and biochemical test. The result obtain are represented in Table 2 and 3.

3) Study of growth activity of bacteria of the bacterial isolated in different amount of 2, 4-d:-

The growth of both culture B and C in different amounts of 2, 4-D has been observed by the help of turbidometric method. The optical density obtained at 610 nm of the culture B and C with increasing amount of 2,4-D in BHB after incubation is represented in Table 4 and 5 respectively. From figure 1 it has been found that growth of culture B initially increases with increasing amount of 2,4-D but after 0.9µl it has decreased. Therefore 0.9 is the most optimum amount of 2,4-D in 5ml BHB which show the maximum growth.

From figure 2 it has been found that growth of culture C initially increases upto 0.8ul but after it start decreasing. Therefore 0.8µl of 2, 4-D in BHB media is the amount at which it show maximum growth. Thus their growth is concentration

dependant and at optimum concentration they show maximum growth.

4) Comparison of growth activities of isolated bacteria in absence and presence of 2, 4-d

This study was also done by the turbidometric method. Table 6 and 7 represents O.D. of Culture B and C in the absence of 2, 4-D and in the presence of 5µl and 10 µl of 2,4-D in BHB every hour. The optical density obtained verses time were plotted for culture B and culture C and are represented in figure 3, 4 and 5 respectively and shown in table 8,9,10 and 11. It was found that the bacterial growth increases on adding 2, 4-D and also growth is more in the presence of higher amount of 2,4-D. presence of 10µl of 2,4-D in 50 ml of BHB is represented in figure 6 From this graph it has been found that culture C grows faster as compared to culture B.

Sr. No.	Amount of 2,4-D added (µl)	Optical density
1	0.5	0.109
2	0.6	0.182
3	0.7	0.199
4	0.8	0.381
5	0.9	0.604
6	1.0	0.383

Table 4:- O.D. obtained at different amount of 2, 4-D for culture B

Sr. No.	Amount of 2,4-D added (µl)	Optical density
1	0.5	0.120
2	0.6	0.209
3	0.7	0.219
4	0.8	0.356
5	0.9	0.162
6	1.0	0.177

Table 5:-O.D.obtained at different amount of 2, 4-D culture C

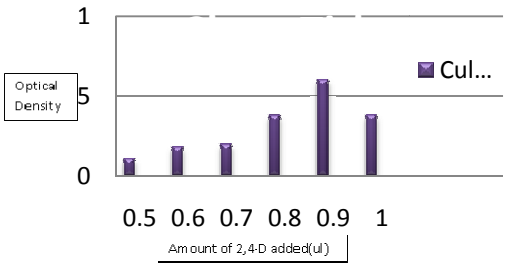

Figure 1:- Graph of growth activity of Culture B in increasing amount 2,4-D

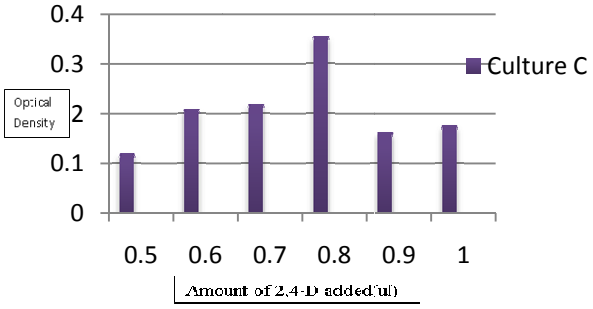

Figure 2:- Graph of growth activity of Culture C in increasing amount 2,4-D

Sr No	Time (hr)	Optical density		
		0 µl	5 µl	10 µl
1	0	0.049	0.057	0.064
2	1	0.054	0.051	0.076
3	2	0.055	0.080	0.089
4	3	0.058	0.068	0.090
5	4	0.058	0.075	0.104
6	5	0.056	0.065	0.110
7	6	0.061	0.079	0.122

Table 6:- O.D. obtained every hour for culture B in 0 µl and 5 µl and 10 µl of 2, 4-D.

Sr. No.	Time (hr)	Optical density		
		0 µl	5 µl	10 µl
1	0	0.059	0.055	0.065
2	1	0.052	0.063	0.075
3	2	0.077	0.056	0.079
4	3	0.070	0.095	0.096
5	4	0.061	0.096	0.128
6	5	0.060	0.090	0.122
7	6	0.065	0.112	0.141

Table: 7:-O.D. obtained every hour for culture C in 0 µl and 5 µl and 10 µl of 2, 4-D.

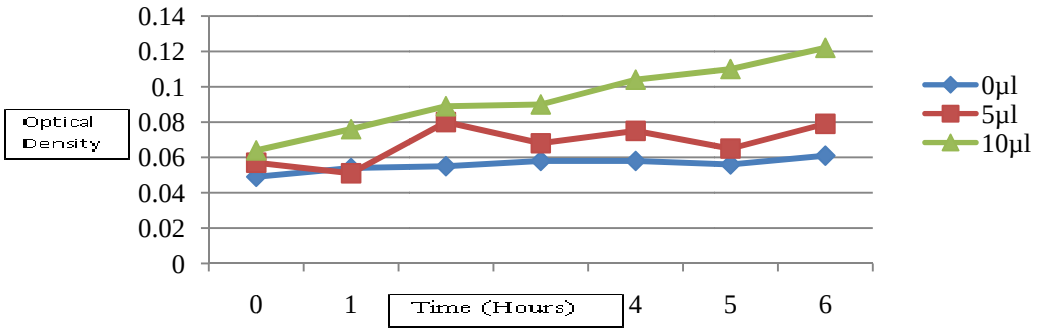

Figure 3:- graph of growth activity culture B in 0 µl, 5 µl and 10 µl of 2,4-D

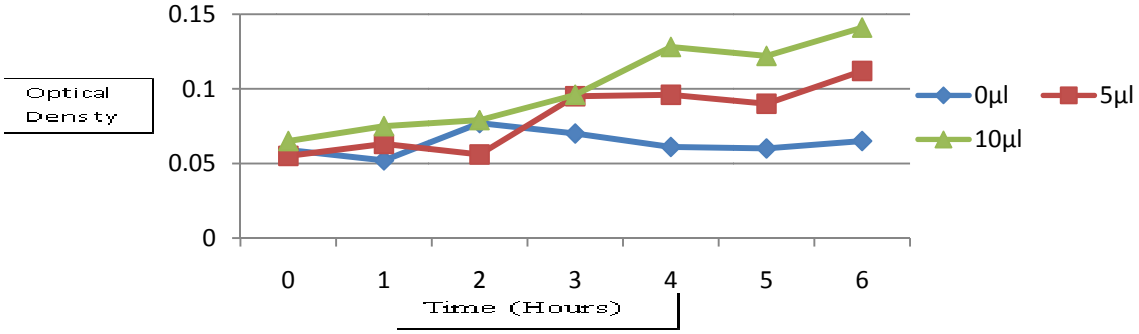

Figure 4:- graph of growth activity culture C in 0 µl 5 µl and 10 µl of 2,4-D.

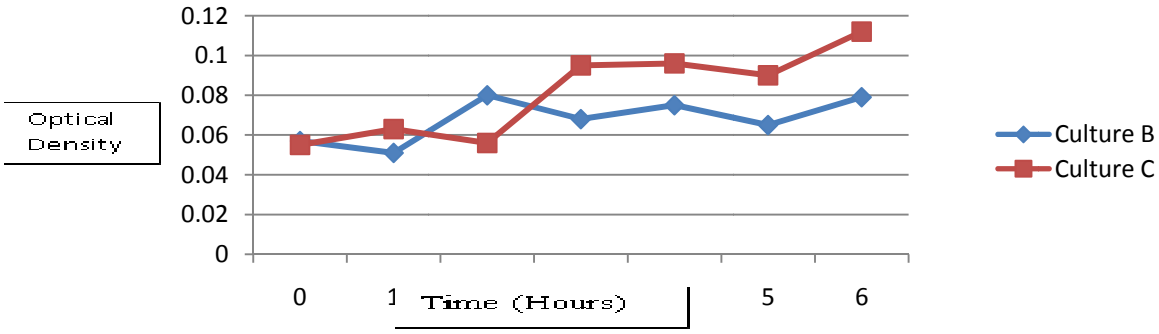

Figure 5:- Graph comparing growth acitivities of culture B and C in 5µl of 2, 4-D

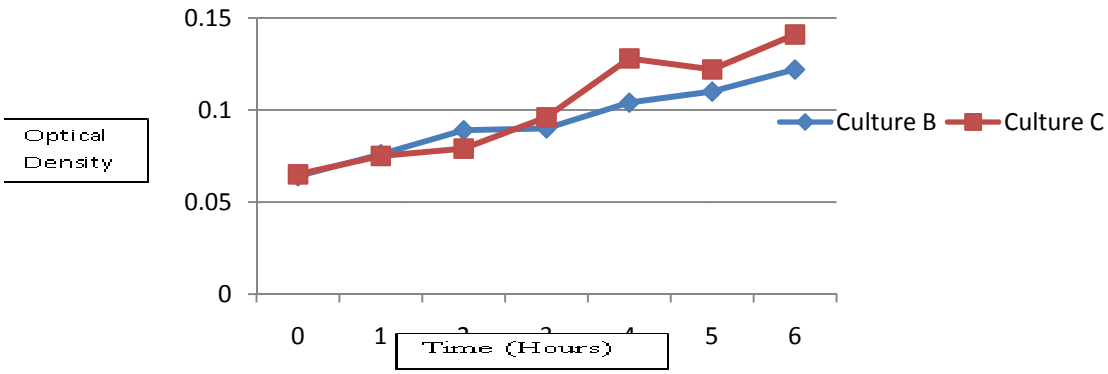

Figure 6:- Graph comparing growth acitivities of culture B and C in 10µl of 2, 4-D

Table 8:-O.D. obtained for control and cultures in presence of 5 µl of 2, 4-D

Sr. No.	Sample	Incubation period	Optical density
1	Control I_0	0 hour	0.291
2	Control I_{24}	24 hour	0.223
3	Culture B	24 hour	0.198
4	Culture C	24 hour	0.179

Table 9:- Percentage of degradation 5 µl of 2, 4 -D

Sr.No.	Sample	Amount of 2,4-D remaining (ppm)	Aount of 2,4-D lost(ppm)	Percentage of degradation (%)
1	Control I_{24}	76.63	-	-
2	Culture B	68.04	8.59	11.21%
3	Culture C	61.51	15.12	19.73%

Table 10:- O.D. obtained for control and culture in presence of 10 µl of 2,4-D

Sr.No.	Sample	Incubation period	Optical density
1	Control I_0	0 hour	0.309
2	Control I_{24}	24 hour	0.224
3	Culture B	24 hour	0.178
4	Culture C	24 hour	0.158

Table 11:- Percentage of degradation 10 µl of 2,4-D

Sr.No.	Sample	Amount of 2,4-D remaining (ppm)	Aount of 2,4-D lost(ppm)	Percentage of degradation (%)
1	Control I_{24}	72.49	-	-
2	Culture B	57.60	14.89	20.54%
3	Culture C	51.13	21.36	29.47%

5) Determination of percentage of 2,4-dbiodegradation:

The percentage of 2, 4-D biodegradation by Culture B and Culture C was measured by the help of turbidometric method.

CALCULATION:

The biodegradation percentage of 2, 4-Dis calculated by following way.

For 5µl 2, 4-D

The O.D. of control I_0 at 0 hour obtained is 0.291 considering it has 100 ppm then control I_{24} with O.D of 0.223 contains = 0.223 x 100/0.291 =76.63 ppm of 2,4-D

Culture B after 24 hours with O.D. of 0.198 contains =0.198 x 76.63/0.223 =68.04 ppm of 2, 4-D

Culture C after 24 hours with O.D. 0.179 contains=0.179 x 76.63/0.223 =61.51 ppm of 2, 4-D

The amount of 2,4-D degraded by culture B =76.63-68.04=8.59ppm

The amount of 2,4-D degraded by culture C =76.63-61.51=15.12ppm

The biodegradation % of 2,4-D by culture B=8.59x 100/76.63 =11.21 %

The biodegradation % of 2,4-D by culture C =15.12x 100/76.63=19.73%

For 10µl 2, 4-D

The O.D. of control I_0 at 0 hour obtained is 0.309 considering it has 100 ppm then Control I_{24} after with O.D of 0.224 contains =0.224x 100/0.309 = 72.49ppm of 2,4-D

Culture B after 24 hour with O.D. of 0.178 contains=0.178x72.49/0.224 =56.60ppm of 2, 4-D

Culture C after 24 hours with O.D. 0.158 contains=0.158x72.49/0.224 = 51.13 ppm OD 2,4D

The amount of 2, 4-D degraded by culture B =72.49-57.60=14.89pp

The amount of 2, 4-D degraded by culture C =72.49-51.13=21.26ppm

The biodegradation % of 2, 4-D by culture B=14.89x100/72.49=20.24%

The biodegradation % of 2, 4-D by culture C=21.36x 100/72.49=29.47%

From the calculation it has been found that Culture B degrade 11.21% 2,4-D in the presence of 5ul and 20.54% in the presence of 10ul of 2,4-D in BHB media. Whereas Culture C degrade 19.73% 2,4-D in the presence of 5ul and 29.47% in the presence 10ul of 2,4-D in BHB media. Thus the percentage of biodegradation is more at higher amount of 2,4-D in case of both Culture B and Culture C. also the percentage of biodegradation of 2,4-D by culture c is more compared to culture B.

6) Bacterial identification by fame based analysis:

Two herbicides 2, 4-D degrading bacteria that are culture B and culture C were identified by FAME based analysis using gas chromatography (GC). The extract was prepared according to the procedure and then was run in GC. The growth of culture B and C obtained on Trypticase Soya Broth agar (TSBA). The technique is used by Sherlock system to present result is based on similarities index (SI). The SI numerical value which express the fatty acid composition of unknown compare with mean fatty acids composition of the strain used to create library entry listed as if match. The SI is not "probably" or percentage but an expression of the relative distance of the unknown sample from the population mean. An exact match between fatty acid profile of the unknown and the mean of the library entry will result in SI of 1.0000. As each fatty acid varies from the mean percentage, the SI will decrease in proportion to the cumulative variances between the composition of the unknown and the library entry. Sample with a SI of 0.500 or higher and with a separation of 0.100 between the first and second choice are considered good library comparisons. If the SI is between 0.300 and 0.500 and well separated from the second choice (>0.100 separation), it may be a good match, but an atypical strain (it would fall still very far away from the mean on the normal distribution curve).Values lower than 0.300 suggest that the organism is not a species in the library, but the software will still indicate the most closely related species, which can be useful when a new species is encountered.

The FAME based analysis of bacterial isolates were showed that culture B is *Escherichia coli* with SIM index 0.825 and culture C is *Citrobacter koseri* with SIM index 0.813.

Conclusion

Bacterial species were isolated and monitored for their ability of herbicide (2,4-D)degradation, isolated from land of college of Agriculture Maharajbagh, Nagpur. All bacterial isolates were cultivated in solid media (Bushnell Haas agar) and in liquid media (Bushnell Haas Broth) with 2,4-D as a sole source of carbon. 2 out of 4 bacteria were found to have the ability to utilize 2, 4-D rapidly. Bacterial species capable of degrading 2, 4-D were characterized for their morphological and biochemical feature to the first step of identification. They were further identified as *Escherichia coli* and *Citrobacter koseri* by their fatty acid profile using Sherlock (software use for FAME analysis) in which their unique fatty acid profile was match with the Sherlock microorganisms' library. Increasing amount of 2, 4-D and percentage of 2, 4-D degradation in bacterial growth was studied by turbidomatric method and also compare the growth activities in absence and presence of 2, 4-D by the same method.

The aim of current work is to isolate bacteria that are able to degrade herbicide (2,4-D) has been successfully achieve. Thus it can be concluded that *Escherichia coli* and *Citrobacter koseri* are able to degrade 2, 4-D. their growth concentration dependent and at optimum concentration they show maximum activity. Also *Citrobacter koseri* is better than *Escherichia coli* in herbicide degradation and its growth activity. Thus they can be used in the field for bioremediation. However, in order to increase the feasibility the bacterial isolates as commercial strains there is need of further studies

on the biodegradation pathway and the byproduct produce after it. The byproduct should be less toxic otherwise it can cause more damage to environment as compare to earlier. The oxygen, nutrient, optimum temperature range, salinity and physical state of the soil and other inhibitory factors that can affect their growth in the presence of 2,4-D should also be studied. It is essential to identify the enzyme that is responsible for biodegrading ability and the gene responsible for secreting that enzyme. Other factors such as mutation can also affect their biodegradation capability.

If *Escherichia coli* and *Citrobacter koseri* meets every required standards then they can be applied in bioremediation processes on an industrial scale.

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