Isolation and Identification of Bacteria from soil contaminated with dyeing and printing effluent

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Abstract: Many small dyeing and printing industries in Kaithun discharge their effluents directly or indirectly into land and water streams.In this present research work bacterial strains were also isolated from dyeing and printing effluent contaminated soil samples and three Bacterial isolates such as *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas aeruginosa* were isolated and identified in contaminated soil of Kaithun region

Keywords: Soil Sample, Dyeing and printing Effluent, Biochemical test.

I. Introduction

Soil is an important natural resource formed as a result of millions of years of activity. It influences the distribution of plant species and provides a habitat for a wide range of microorganisms. Soil plays an important role in growth of plants and trees maintaining the ecosystem with its natural fauna and flora and indirectly sustains the environment. The effluent from the industries contains large number of bacteria and fungi. Some of these microorganisms are beneficial to the plants. These organisms help to breakdown the complex organic matter into the simple form and consequently increase the fertility of the land. But some micro-organisms are pathogenic for the plants. These organisms can cause different diseases to the plants. The industries utilize many toxic substances which are very harmful to the plants and soil microorganisms. Soil degradation from inorganic and organic contaminants is not only ecological risk but simultaneously is a socioeconomic issue; soil becomes poor in physicochemical properties susceptible to erosion, loss of productivity, sustainability and diminished food chain quality. Healthy soil has microorganisms essential for the decay and decomposition of dead organic matter and provides the fertile layer humus essential for plant growth. The health of soil is indicated by diversity of micro flora. Microbial population of these soils showed difference with the micro flora of normal soil. This could be attributed to many facts like availability of organic nutrients, nitrogen and other minerals, increase in acidity due to the dyes specially azo dyes leads to oxygen depletion.

II. MATERIALS AND METHODS

1. Collection of soil sample from experimental site

The soil samples were collected from control site, effluent discharge site and effluent dumping site of dyeing and printing industries at Kaithun region, Kota.

2. Isolation and Identification of bacteria-

This method is based upon the principle that when material containing microorganisms is cultured and each viable microorganism will develop into a colony, hence the number of colonies appearing on the plates represents the number of living organisms present in the sample. The serial dilution agar plate method is one of the commonly used procedures for the isolation and enumeration of bacteria.

Serial dilution and plate count method-

The isolation process of bacterial species was performed by the 'serial dilution method'. At first, the stock solution was prepared with 0.85 % NaCl concentration and then serial dilution blanks were prepared in test tubes and marked sequentially starting from 10^{-1} to 10^{-5} dilution and autoclave sterilized. 1 gm of soil sample was dissolved in 9 ml solution i.e. 10^{-1} dilution. 1 ml from this was then transferred to 9 ml of the 10^{-2} labeled test tube i.e. 10^{-2} dilution, using a fresh sterile pipette; and this was repeated for each succeeding step till 10^{-5}. Nutrient Agar (NA) media was used for the isolation of bacterial strains. From 10^{-3}, 10^{-4}, and 10^{-5} dilution tubes, 0.1 ml of dilution fluid was then spread on sterilized Petri plates in triplicates using the standard spread plate technique, for bacterial strain isolation.

The Nutrient agar plates were then incubated at 37 °C for 24 h. After successful growth of microorganisms, characteristics of each distinct colony, e.g., shapes, color, transparency, etc. were determined. Gram stain was performed to observe the cellular morphology and gram reaction of the bacteria. The number of bacterial colonies in the contaminated soil samples was counted and the density was expressed as Colony Forming Units (CFU) as given below:

$$\text{CFU/ml in original sample} = \frac{\text{Colonies counted}}{(\text{Dilution factor}) \times (\text{volume plated in ml})}$$

Biochemical characterization of the isolated bacteria -

Gram stain is differential technique which is used to identify and classify bacteria into gram-positive and gram-negative. Four different reagents are used in the fixed bacterial smear in the sequence: crystal violet (primary stain), iodine solution (mordant), alcohol (decolorizing agent) and safranin (counter stain). Gram- positive bacteria retain the primary stain and appear dark blue or violet whereas the Gram-negative bacteria lose primary stain and counter stained by safranin and appear red.

Gram positive - bacteria that were appeared as purple

Gram negative - bacteria that were appered as pink.

Indole production Test -

Some bacteria have tryptophanase enzyme which helps in oxidizing tryptophan (an essential amino acid) and form indole, pyruvic acid and ammonia. Kovac's reagent (dimethylaminobenzaldehyde) is used for detecting the formation of indole during the reaction and confirmed by the presence of cherry-red reagent layer at the top.

$$\text{Tryptophan} \xrightarrow{\text{Tryptophanase}} \text{Indole} + \text{pyruvic acid} + NH_3$$

$$\text{Indole} + \text{Kovac's reagent} \xrightarrow{HCl} \text{Rosindole} + H_2O$$

(Butanol) (Cherry red compound)

Methyl Red-Voges Proskauer Test -

The two major types of facultative anaerobic enteric bacteria, one that produce large amount of acid and other those produce the neutral end products are used to differentiate by the methyl red (MR) and the voges-proskauer (VP) test. The production of large amount of acids such as formic, actic, acetic and succinic acid as end product from glucose is detected by the addition of methyl red which act as pH indicator in the medium. The medium remain red and pH remains below 4.4 is the positive test. If the organic acids produced during the glucose fermentation were enzymatically converted to nonacidic end products such as acetoin and ethanol, the methyl red will turn yellow due to the elevation of pH above 6.0 is the negative test.

MR Test Positive - bright red color

Negative - yellow color

VP Test Positive - pink color, becoming crimson in 30 minutes

Negative - yellow or colorless

Citrate utilization Test

Some microorganisms produce an enzyme citrase that break down citrate. On the basis of the ability to utilize citrate as a sole carbon source the citrate test is used to differentiate among enteric bacteria. Citrate is break down to oxaloacetic acid and acetic acid which later on by the enzymatically converted to pyruvic acid and carbon dioxide. Sodium citrate in the medium (Simmon's citrate agar) is the only source of carbon and energy. During metabolism of citric acid the CO_2 is formed which combines with sodium and water to form sodium carbonate, which is an alkaline product and changes of the medium from green to blue. This is a positive test. Bromothymol blue is used as an indicator which is green in color at pH 6.8 and below (acidic) and turns blue at pH 7.6 and higher (alkaline).

$$\text{Citric acid} \xrightarrow{\text{Citrase}} \text{Oxaloactic acid} + \text{acetic acid} \xrightarrow{\text{Enzymes}} \text{pyruvic acid} + CO_2$$

$$CO_2 + 2Na^+ + H_2O \longrightarrow Na_2CO_3 \text{ (alkaline pH blue color) (Produced during citric acid metabolism)}$$

Positive - blue color and streak of growth

Negative - green color and no growth

Catalase Test -

Microorganisms during aerobic respiration in the presence of oxygen produce hydrogen peroxide (H_2O_2) which is lethal to the cell. The enzyme catalase present in some microorganisms breaks down hydrogen peroxide to water and oxygen and helps them in their survival. Release of free oxygen gas bubbles is a positive catalase test.

Positive - production of gas bubbles Negative - no gas bubbles production

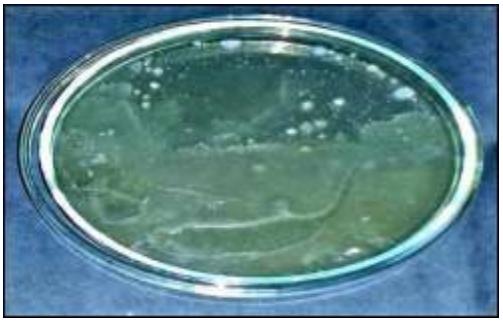
III. OBSERVATION AND RESULTS

Microbial diversity constitutes the most extraordinary reservoir of life in the biosphere that we have only just begun to explore and understand. The present study was aimed to investigate the bacterial population and bacterial diversity of soil. Samples were used for the isolation of bacterial species using serial dilution and plating methods. Serially diluted sample was poured into the nutrient agar medium showed the number of bacterial species. The results revealed that the bacterial density is high in industrial sites as compared to control (Table 1). It could be due to the higher BOD and COD values of the effluents. Bacterial diversity of soil samples were characterized on the basis of morphological examinations of the obtained colonies depending upon their shape, size, color of background, pigment production and types of colony etc. (Table 2). The polluted soil may contain several types of bacterial species. They were identified by Biochemical test includes Indole, Methyl red, Voges-Proskauer test, Citrate, Catalase and gram staining technique. Three Bacterial isolates such as Bacillus subtilis, Bacillus cereus and Pseudomonas aeruginosa were isolated and identified in contaminated soil of Kaithun region (Table 7).

Table 1: Microbiological Analysis of Soil

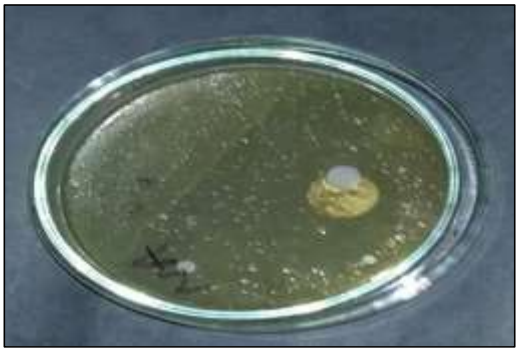
Media Used - nutrient Agar Temperature and Time of incubation - 25 degree centigrade and 48 hours				
Soil Samples from Experimental Sites				
Sample	**Dilutions**	**Dilutions factor**	**Colony count**	**No. of Organisms/ ml**
Effluent discharge site	10^{-4}	10^4	380	19×10^4
	10^{-5}	10^5	320	16×10^5
	10^{-6}	10^6	280	$14X\ 10^6$
Effluent dumping site	10^{-4}	10^4	300	$15X\ 10^4$
	10^{-5}	10^5	260	$13X\ 10^5$
	10^{-6}	10^6	200	10×10^6
Control	10^{-4}	10^4	320	$16\ X10^4$
	10^{-5}	10^5	280	14×10^5
	10^{-6}	10^6	240	12×10^6

PLATE -1



(A) Bacterial population at Site A

(B) Bacterial population at Site B



(C) Bacterial population at Site C

Bacterial Population in Contaminated Soil Sample

Table 2: Morphological Result

No.	Test	Colony Characters on Nutrient Agar Plate Incubated at 25^0 C for 48 hours		
		Soil Samples from Experimental Sites		
		Control Site	Main infected Site Sample	Originate d Site Sample
1	Colony Forms	Punctiform (dot like),Circula r, Rizoid	Punctiform (dot like),Circula r, Rizoid	Punctifor m (dot like), Circular
2	Color of cell	whitish, yellowish, offwhite ,creamy	whitish , yellowish ,offwhite ,creamy	Offwhite ,creamy
3	Color of back ground	pasty, offwhite	pasty, offwhite	pasty, offwhite
4	Pigment Productio n	Negative	Negative	Negative
5	Types of colony	4	4	2

Biochemical tests for identification of isolated *Pseudomonas aeruginosa*

A. ***Indole Test*** - The tube containing SIM agar medium at pH 7.3 were inoculated by isolated *Pseudomonas aeruginosa* bacteria, after 24-48 hr of inoculation on addition of Kovac's reagent, no change in color of media was observed showed negative test for indole (Table-4, Plate-2).

B. ***Methyl Red*** - The tubes containing (MR-VP Broth) at pH 7.3 were inoculated by isolated *Pseudomonas aeruginosa* bacterial culture. After 24-48 hours of incubation on addition of

Methyl red reagent, change in color of the medium was observed (Table-4, Plate-2) and found that red color was not appeared showed negative test for MR.

C. ***Voges Proskauer***- The tubes containing (MR-VP Broth) at pH 7.3 was inoculated by isolated *Pseudomonas aeruginosa* bacterial culture. After 24-48 hours of incubation on addition of Barritt's reagent A and B, change in color of the medium was observed and found that there was no color change was observed it indicate negative test for VP (Table-4, Plate-2).

D. ***Citrate Utilization***- The tube containing (Simmons Citrate Agar) at pH-6.9 were inoculated by isolated *Pseudomonas aeruginosa* bacterial culture. After 24-48 hours of incubation change in the color of the media was observed showed positive test for citrate (Table-4, Plate-2).

E. ***Catalase Test***- Slide containing 2-3 drops of (Trypticase soya broth) at pH-7.3 were inoculated by 24-48 hours isolated *Pseudomonas aeruginosa* bacterial culture. After few seconds on addition of 3% hydrogen peroxide observed. Appearance of bubbles showed positive test for catalase (Table -4, Plate-2).

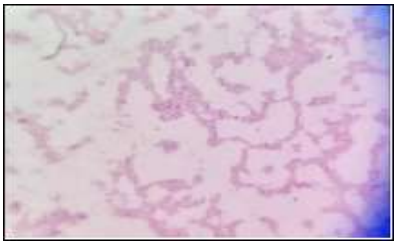
Table: 4 Biochemical Test and Identification of *Pseudomonas aeruginosa*

S. No.	Characteristics	Morphological, Cultural and Biochemical Results (*Pseudomonas aeruginosa)*
1.	Gram Staining	Negative
2.	Shape	Rods
3.	Motility	Motile
4.	Capsule(Capsulated/NonCapsulated)	Non-Capsulated
5.	Spore (Sporing/Non-Sporing)	Non-Sporing
6.	Flagella(Flagellated/Non- Flagellated	Single Flagella
7.	Catalase	Positive (+ve)
8.	Oxidase	Positive (+ve)
9.	MR – Test	Negative (-ve)
10.	VP – Test	Negative (-ve)

11.	Indole	Negative (-ve)
12.	Citrate	Positive (+ve)
13.	H_2S	Negative (-ve)
14.	Glucose Fermentation	Negative (-ve)

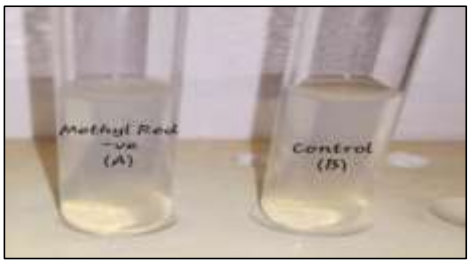
PLATE - 2

(A) Culture Plate of *P. aeruginosa*

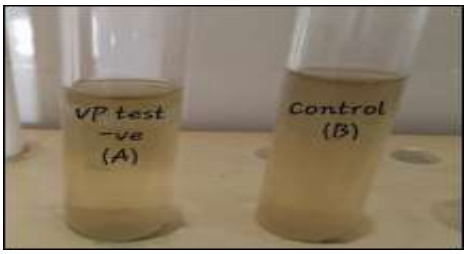


(B) Microscopic View

(C) Indole Test



(D) Methyl Red Test



(E) Voges Proskauer Test

(F) Citrate Test

(G) Catalase Test

Biochemical Reaction by *Pseudomonas aeruginosa*

Biochemical tests for identification of isolated *Bacillus subtilis*

A. ***Indole Test*** - The tube containing SIM agar medium at pH 7.3 were inoculated by *Bacillus subtilis* isolated bacteria, after 24-48 hr of inoculation on addition of Kovac's reagent, no change in color of media was observed showed negative test for indole (Table-5, Plate-3).

B. ***Methyl Red*** - The tubes containing (MR-VP Broth) at pH 7.3 were inoculated by isolated *Bacillus subtilis* bacterial culture. After 24-48 hours of incubation on addition of Methyl red reagent, change in color of the medium was observed, (Table-5, Plate-3) and found that red color was not appeared showed negative test for MR.

C. ***Voges Proskauer***- The tubes containing (MR-VP Broth) at pH 7.3 was inoculated by isolated *Bacillus subtilis* bacterial culture. After 24-48 hours of incubation on addition of Barritt's reagent A and B, change in color of the medium was observed and find that color change was observed showed positive test for VP (Table-5, Plate-3).

D. ***Citrate Utilization***- The tube containing (Simmons Citrate Agar) at pH-6.9 were inoculated by isolated *Bacillus subtilis* bacterial culture. After 24-48 hours of incubation change in the color (blue) of the media was observed. Blue color indicated positive reaction (Table-5, Plate-3).

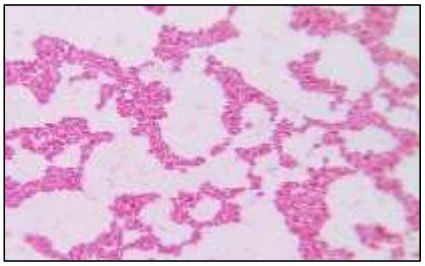
E. **Catalase Test**- Slide containing 2-3 drops of (Trypticase soya broth) at pH-7.3 were inoculated by 24-48 hours isolated *Bacillus subtilis* bacterial culture. After few seconds on addition of 3% hydrogen peroxide observe the change on slide. Appearance of bubbles showed positive test for catalase (Table-5, Plate-3)

Table: 5 Biochemical Test and Identification of *Bacillus subtilis*

S. No.	Characteristics	Morphological, Cultural and Biochemical Results (*Bacillus subtilis*)
1.	Gram Staining	Positive
2.	Shape	Rods
3.	Motility	Motile
4.	Capsule(Capsulated/NonCapsulated)	Non-Capsulated
5.	Spore (Sporing/Non-Sporing)	Non-Sporing
6.	Flagella(Flagellated/Non- Flagellated	Flagellated
7.	Catalase	Positive (+ve)
8.	Oxidase	Variable
9.	MR – Test	Negative (-ve)
10.	VP – Test	Positive (+ve)
11.	Indole	Negative (-ve)
12.	Citrate	Positive (+ve)
13.	H_2S	Negative (-ve)
14.	Glucose Fermentation	Positive (+ve)

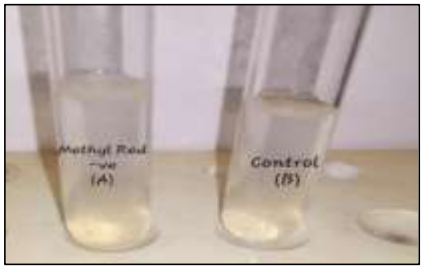
PLATE - 3

(A) Culture Plate of *B. Subtilis*

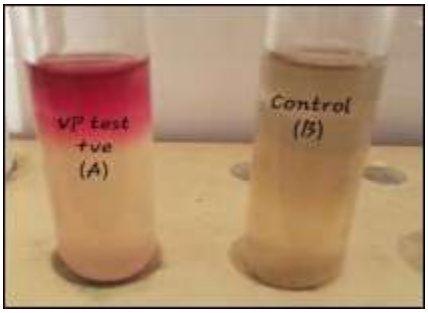


(B) Microscopic View

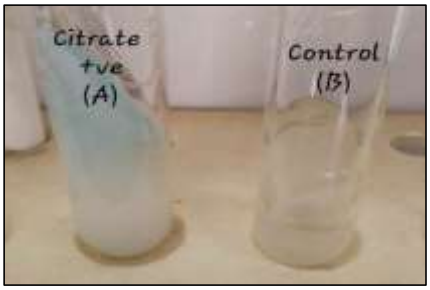
(C) Indole Test



(D) Methyl Red Test



(E) Voges Proskauer Test



(F) Citrate Test

Biochemical Reaction by *Bacillus subtilis*

Biochemical tests for identifcation of isolated *Bacillus cereus*

A. ***Indole Test*** - The tube containing SIM agar medium at pH 7.3 were inoculated by *Bacillus cereus* isolated bacteria, after 24-48 hr of inoculation on addition of Kovac's reagent, no change in color of media was observed showed negative test for indole (Table-6, Plate-4).

B. ***Methyl Red*** - The tubes containing (MR-VP Broth) at pH 7.3 were inoculated by isolated *Bacillus cereus* bacterial culture. After 24-48 hours of incubation on addition of Methyl red reagent, change in color of the medium was observed, (Table-6, Plate-4) and found that red color was not appeared showed negative test for MR.

C. ***Voges Proskauer***- The tubes containing (MR-VP Broth) at pH 7.3 was inoculated by isolated *Bacillus cereus* bacterial culture. After 24-48 hours of incubation on addition of Barritt's reagent A and B, change in color of the medium was observed showed positive test for VP (Table-6, Plate-4).

D. ***Citrate Utilization***- The tube containing (Simmons Citrate Agar) at pH-6.9 were inoculated by isolated *Bacillus cereus* bacterial culture. After 24-48 hours of incubation change in the color of the media was observed indicate positive test for citrate (Table-6, Plate-4).

E. ***Catalase Test***- Slide containing 2-3 drops of (Trypticase soya broth) at pH-7.3 were inoculated by 24-48 hours isolated *Bacillus cereus* bacterial culture. After few seconds on addition of 3% hydrogen peroxide observe the change on slide. Appearance of bubbles showed positive test for catalase (Table-6, Plate-4).

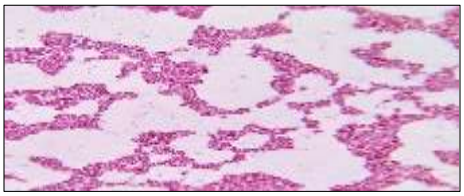
Table: 6 Biochemical Test and Identification of

Bacillus cereus

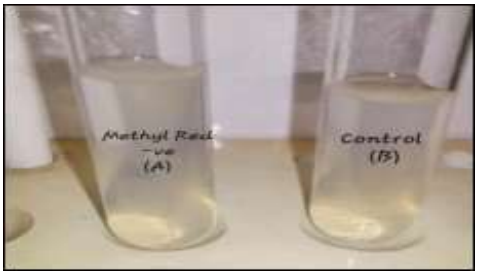
S. No.	Characteristics	Morphological, Cultural and Biochemical Results (*Bacillus cereus)*
1.	Gram Staining	Positive
2.	Shape	Rods
3.	Motility	Motile
4.	Capsule(Capsulated/NonCapsulated)	Non-Capsulated
5.	Spore (Sporing/Non-Sporing)	Sporing
6.	Flagella(Flagellated/Non- Flagellated	Flagellated
7.	Catalase	Positive (+ve)
8.	Oxidase	Negative (-ve)
9.	MR – Test	Negative (-ve)
10.	VP – Test	Positive (+ve)
11.	Indole	Negative (-ve)
12.	Citrate	Positive (+ve)
13.	H_2S	Negative (-ve)
14.	Glucose Fermentation	Positive (+ve)

PLATE - 4

(A) Culture Plate of *B. Cereus*



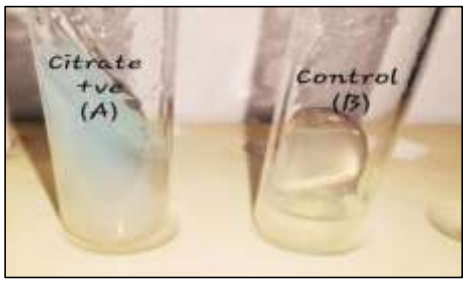
(B) Microscopic View



(C) Methyl Red Test

(D) Indole Test

(E) Voges Proskauer Test



(F) Citrate Test

(G) Catalase Test

Biochemical Reaction by *Bacillus cereus*

Table 7: Morphological, Cultural and Biochemical Characteristics of Isolated Soil Bacteria

Organisms	Morphological, Cultural and Biochemical characteristics of soil bacteria							
	Gram stain	Shape	Growth on Agar	Indole production	MR reaction	VP reaction	Citrate test	Catalase test
Pseudomonas aeruginosa	-	Rod	Adundant thin, white medium turns green	-	-	-	+	+
Bacillus subtilis	+	Rod	Rough, abundant, waxy growth	-	-	+	+	+
Bacillus cereus	+	Rod	Abundant, opaque, white waxy growth	-	-	+	+	+

IV. DISCUSSION

Soil formation is the result of the combined action of weathering and colonization of geologic material by micro flora. Soil microorganisms are important because they affect the soil's physical, chemical and biological properties where several common groups of bacteria are especially important to ensure the health of the soil (Egger, 2010). The numbers and species of microbes in soil is depended on environmental conditions like nutrient availability, soil texture, presence of moisture in soil and type of vegetation cover, and other environmental conditions (Brakstad *et. al*. 2015). Bacteria are the largest group of soil microbes, both in total number and in diversity. In this present research work bacterial strains were isolated from dye effluent and bacterial diversity of contaminated soil samples were characterized on the basis of biochemical and morphological examinations of the obtained colonies depending upon their shape, size, color, color of background, pigment production and types of colony etc. The polluted soil may contain several types of bacterial species. They were identified by Biochemical test includes Indole, Methyl red, Voges-Proskauer test, Citrate, Catalase and gram staining technique. Three Bacterial isolates such as *Bacillus subtilis*, *Bacillus cereus* and

Pseudomonas aeruginosa were isolated and identified in contaminated soil of Kaithun region and effluent treated soil sample from Pot experiment. Sarnaik *et. al., (*1995) in a study of soil samples from dye house highlighted low crop yield from the soil irrigated with effluent, owing to the presence of excess soluble salts effluent that is percolated and absorbed by the soil. Also, reduced number of*Pseudomonas* was reported. The results obtained in this study are in agreement with the Sarnaik *et. al., (*1995) as Bacillus species dominate in the soil. Present results revealed presence of enzyme catalase and citrate in bacterial isolates. Our finding supports begum *et. al* (2017) who reported some bacteria isolated from waste dumping sites in Dhaka city for the presence of enzymes such as protease, oxidase, catalase, coagulase.

Biochemical characteristics of the soil bacterial isolates were studied using different tests like Indole, Methyl red and Voges Proskauer test, Citrate utilization, Urease test, Catalase test, Nitrate reduction test, production of Hydrogen Sulphide as described by Aneja, (1996) for *soil samples*. All the three isolated bacterium showed positive test for Catalase activity. Indra Gandhi *et. al*. (2014) worked on textile effluent and dye contaminated soil and isolated *Alcaligenes spp, Bacillus subtilis, B.pumilus, B.cereus, B.megaterium, B.licheniformis, B.alvei, B.macerans, B.maxima, E.aerogens, E.coli, Klebsiella pneumoniae, Micrococcus spp, Lactobacillus spp, Pseudomonas florescence, P.putida, Streptococcus spp, Staphylococcus spp, S. aureus* and *Serratia spp* and identified by using staining and biochemical test. Our findings similar to the (Arun Prasad and Bhaskara Rao 2010) dye decolorizing isolates, *Bacillus* sp., *Klebsiella* sp., *Salmonella* sp., and *Pseudomonas* sp. were isolated from the textile effluent samples collected from Elampillai, Tamil Nadu.

V. CONCLUSION

During the present research work bacteria like *Pseudomonas aeroginosa*, *Bacillus subtilis* and *Bacillus cereus* were isolated from the soil polluted by dyeing and printing effluent. As Bacteria's is a tiny and lower most component of any food chain. But these tiny members have their own importance, without bacteria we can't imagine any food chain. But these important members can survive in good quality of soil, in place of it they return fertility of soil by maintaining NPK contain of soil.

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