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# Ability to select PGPR strains to remediate organophosphate pesticides commonly used in agriculture

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Abstract:

Pesticide fate in the environment is affected by microbial activity. Some pesticide is readily degraded by microorganism. Pesticide is degraded in the environment principally by the action of microorganism, a process term biodegradation. Pesticides are substances that are meant to control pests or weeds.Plant growth promoting rhizobacteria are the soil bacteria inhabiting around/on the root surface and are directly or indirectly involved in promoting plant growth and development via pro-duction and secretion of various regulatory chemicals in the vicinity of rhizosphere.The aim of the study is to isolate the PGPR strain from soil for the degradation of pesticide. PGPR strain isolated from rhizosphere and checked for their viability against the pesticide at various concentrations ranging between 10 to 100 ppm. Inoculation of these bacteria competitively colonizes the roots of the plant and can act as biofertilizers and/or antagonists (biopesticides) or simultaneously both. . PGPR are diverse, complex, and important assemblages in the biosphere, they are considered as a group of beneficial free-living soil bacteria for sustainable agriculture and environment. Along with this, they are also involved suppressing the root pathogenicity.

Keywords: Pesticide, rhizobacteria, PGPR, Biodegradation, Biofertilizer, Pathogenecity

## 1. INTRODUCTION

In general, a pesticide is a chemical or biological agent (such as a virus, bacterium, antimicrobial, or disinfectant) that deters, incapacitates, kills, or otherwise discourages pests. Target pests can include insects, plant pathogens, weeds, mollusks, birds, mammals, fish, nematodes (roundworms), and microbes that destroy property, cause nuisance, or spread disease, or are disease vectors. Although pesticides have benefits, some also have drawbacks, such as potential toxicity to humans and other species

Pesticide is widely used in different parts of the world to control insect pests and to enhance crop production. However, these pesticides are found to have deleterious effects due to their non-degradable nature and residual effect on non-target organisms. Some microorganisms can develop resistance against these pesticides and use them as the carbon source.

In modern agriculture, large Number of Pesticides are being used to improve crop production but their excessive and unreasonable use causes stress and yield loss in addition to deterioration in soil health. Some micro-organism Develop resistance after long term exposure to agrochemical and can successfully is used for bioremediation of pesticide.

Pesticides have become the part and parcel of modern day agriculture. The absence of pesticides will jeopardize the health of plants, animals and humans. Pesticides are not only an agricultural commodity but find use in non-agricultural regions. But the very nature of the pesticides to kill renders them harmful for the humans and other living beings. Their extensive use has contaminated our soil, water and food, thus risking our wellbeing. Many persistent pesticides and their degradation products penetrate into the plant tissues or stay in the water and soil thus appearing in our food chain. The pesticide residues are magnified during food pro-cessing, thus making even the processed foods, storage house of harmful chemicals. Taking into account the rampant use of pesticides which has lead to the contamination of various strata, continuous monitoring of environmental and food samples is of almost importance.

People have contradictory ideas about the meaning of pesticides. The dictionary defines pesticide as a sub- stance for destroying harmful insects. The scientists are of the opinion that pesticides are chemical or biological substances that are designed to kill or retard the growth of pests

interfering with the growth of crops, shrubs, trees, timber and other vegetation desired by humans. The term pesticide includes substances intended for use as plant growth regulators, defoliants, desiccants or agents for thinning fruit or preventing the premature fall of fruit. The substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport also come under the category of pesticides.

Pesticides are broadly classified into two groups

A) Chemical pesticides and

B) Bio-pesticides

A) Chemical pesticides are conventionally synthetic materials that directly kill or inactivate the pest. They are classified according to the type of organisms they act against as for example 1) insecticides, 2) herbicides, 3) fungicides, 4) rodenticide, 5) nematicides.

Insecticides include organophosphates, organ chlorines and botanical insecticides.

Herbicides are used to destroy other weeds that interfere with production of the desired crop. Based on their structure they are grouped into chlorophenoxy compounds (e.g.: 2,4-D, 2, 4,5-T), dinotrophenols like 2-methyl-4,6-dinitrophenol (DNOC), bipyridyl compounds like paraquot, carbamate herbicides, substituted urea, triazines and amide herbicides like alanine derivatives.

Fungicides include a number of structurally different chemicals like cap tan, folpet, pentachlorophenolziram, nambam etc. Fungicides containing mercury are known to cause nerve disorders.

Rhodenticides are designed to kill rodents, mice, squirrels, gophers and other small animals. They vary from highly toxic one with the ability to kill an organism with one-time dose or less toxic ones requiring repeated ingestion over a period of time.

Nematicides act against nematodes like *Meloidogyne incognita, Criconemellaxenoplax*etc.

B) Bio-pesticides are pesticides derived from natural sources like animals, plants, bacteria, and certain minerals. For example, canola oil and baking soda have pesticidal applications and are considered Bio-pesticides. Bio- pesticides fall into three major classes.

(1) Microbial pesticides consist of microorganisms like bacteria, fungi, viruses or protozoa as the active ingredients. They can control many different kinds of pests, although each with separate active ingredient that is relatively specific for its target pest(s). 2) Plant Incorporated-protectants

(PIPs) are pesticidal substances that are produced by genetically modified plants for example: introduction of BT toxin gene in the cotton plants. 3) Biochemical pesticides are naturally occurring substances that control pests by non-toxic mechanisms (for e.g. in-sect sex pheromones that interfere with mating as well as various scented plant extracts). Bio-pesticides are environmentally safe and non toxic to plants and animals. However, their use is limited due to

1) Less social awareness,
2) Comparatively lower crop yields,
3) need for frequent applications,
4) Less worked research area

On the contrary, application of chemical pesticides has proved to be economically beneficial and hence their use has increased globally especially after the advent of "Green Revolution". The productivity of crop has been increased by use of suitable pesticide. They protect the crop from disease causing organisms, from plant pathogens and also from vector borne diseases. Another important advantage is reduction in cost of labor.

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Even though pesticides play significant role in agriculture they are the most important environmental pollutants. This is due to their wide spread presence in water, soil, atmosphere and agricultural products. Currently it poses major threat not only to living organisms but also to environment specially ground and surface water. Synthetic pesticides affect the growth of plants. Chemical compounds in the pesticides are not biodegradable. This causes their sedimentation near plant roots making the supply of essential NPK inefficient. This inefficiency hinders growth of crops and their resistance to other harmful microbes. Pesticides percolate into the soil and get mixed with ground water. This causes draining of pesticides into the nearby stream or lake. This in turn adversely disturbs the aquatic eco system. Soil is another important component for plant growth. Pesticides hamper the fertility of soil by inhibiting the storage of nitro- gen and other essentials in soil. Light and toxic com- pounds are suspended in air by pesticide spray. This causes air borne diseases and nasal infections. Besides all the environmental hazards; pesticides pose serious risk to mankind. Health hazards caused by some of the pesticides are summarized in Different pesticides have different acceptable residual levels and these are set up by World

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Health Organization (WHO), European Community (EU), FAO (Food and Agricultural Organization) of UN, US environmental protection agency (EPA) and the US National Institute for Occupational Safety and Health (NIOSH) . The Toxicity of pesticides, made it essential to have accurate and reliable methods of monitoring their levels for safety purposes.

**2. Rhizosphere**

The narrow zone of soil directly surrounding the root system is referred to as rhizosphere, while the term'rhizobacteria' implies a group of rhizosphere bacteria competing colonizing the root environment. In addition to providing the mechanical support and facilitating

Water and nutrient uptake, plant roots also synthesize, Accumulate, and secrete a diverse array of compounds. These compounds secreted by plant roots act as chemical attractants for a vast number of heterogeneous, diverse and actively metabolizing soil microbial communities.

The chemicals which are secreted by roots into the soils are generally called as root exudates. The exudation of a wide range of chemical compounds modifies the chemical and physical properties of the soil and thus, regulates the structure of soil microbial community in the immediate vicinity of root surface. In fact, some of the exudates act as repellants against microorganisms while others act as attractants to lodge the microbes. The composition

Of these exudates is dependent upon the physiological status and species of plants and microorganisms. Moreover, these exudates also promote the plant-beneficial symbiotic interactions and inhibit the growth of the competing plant Species. Also, microbial

Activity in the rhizosphere affects rooting patterns and the supply of available nutrients to plants, thereby modifying the quality and quantity of root exudates. A fraction of these

Plant derived small organic molecules is further metabolized by microorganisms in the vicinity as carbon and nitrogen Sources, and some microbe-oriented molecules are subsequently

Retaken up by plants for growth and development. Indeed, carbon fluxes are critical determinants of rhizosphere function. It is reported that approximately5–21% of photo-synthetically fixed carbon is transported to the rhizosphere through root exudation. Thus, the rhizosphere can be defined as any volume of soil specifically influenced by plant roots and/

Or in association with roots hairs, and plant-produced materials. Largely, three separate but interacting components are recognized in the rhizosphere: the rhizosphere (soil), the rhizoplane, and the root itself. Of these, the rhizosphere is the zone of soil influenced by roots through the release of substrates that affect microbial activity. The rhizoplane,

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on the other hand, is the root surface including the strongly adhering soil particles while the root itself is a component of the system, because many micro-organisms also colonize the root tissues .Microbial colonization of the rhizoplane and/or root tissues is known as root colonization, whereas the colonization of the adjacent volume of soil under the influence of the root is known as rhizosphere colonization.

**3. Plant growth promoting rhizobacteria**

The plant growth promoting rhizobacteria (PGPR), are characterized by the following inherent distinctiveness's

(i) They must be proficient to colonize the root surface

(ii) they must survive, multiply and compete with other Micro biota, at least for the time needed to express their plant growth promotion/protection activities, and

(iii) They must promote plant growth.

About 2–5% of rhizobacteria, when reintroduced by plant inoculation in a soil containing competitive micro flora, exert a beneficial effect on plant growth and are termed as plant growth promotingrhizobacteria. soil bacterial species burgeoning in plantrhizosphere which grow in, on, or around plant tissues stimulate plant growth by a plethora of mechanisms are collectively known as PGPR (plant growth promotingrhizobacteria).Alternatively, classified PGPR based on their functional activities as

(i) Biofertilizers (increasing the availability of nutrients to plant),

(ii) Phytostimulators(plant growth promotion, generally through phytohormones),

(iii) Rhizoremediators (degrading organic pollutants) and

(iv) Biopesticides (controlling diseases, mainly by the production

Of antibiotics and antifungal metabolites)

Furthermore, in most studied cases, a singlePGPR will often reveal multiple modes of action including biological control. Furthermore PGPR associations range in the degree of bacterial proximity to the root and intimacy of association. In general, these can be separated into extracellular (ePGPR), existing in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex, and intracellular (iPGPR), which exist inside root cells, generally in specialized nodular structures. Some examples of ePGPR are like, Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromo bacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas and Serratiaetc. . Similarly, some examples of the

iPGPR are Allorhizobium, Azorhizobium, Bradyrhizobium, and Mesorhizobiumand Rhizobium of the family Rhizobiaceae. Most of rhizobacteria belonging to this group are Gram-negative rods with a lower proportion being Gram-positive rods, cocci or pleomorphic. Moreover, numerous actinomycetes are also one of the major components of rhizosphere microbial communities displaying marvelous plant growth beneficial traits. Among them, Micromonosporasp. Streptomycesspp. Streptosporangiumsp., and Thermobifidasp. , which have shown an enormous potential as biocontrol agents against different root fungal pathogens.

Some microorganisms develop resistance after a long-term exposure to agrochemicals and can successfully be used for bioremediation of pesticide contaminated soils. Some microbes perform efficiently in the presence of specific pesticides by using them as source of nutrients and energy *Pesticide degrading bacteria for degraded lands.* Microorganisms degrade these pesticides and use them as a carbon source. Their ability to degrade pesticide is an important phenomenon through which these chemicals are eliminated from the environment and control the environmental pollution. These bacterial strains can be used in modern agriculture as inoculants under stress conditions such as heavy metal stress, herbicide stress; insecticides stress fungicides stress, and salinity stress. Therefore, these microbes are essential components recycling process and important to maintain soil fertility. These microbes also have other plant growth promoting traits in addition to pesticide degradation and can be used to enhance the remediation process in addition to plant growth promotion. The use of pesticide tolerant plant growth promoting rhizobacteria may help to degrade pesticides which are being used injudiciously by the vegetable growers in the country so, the present study was conducted to isolate and characterize the pesticide tolerant rhizobacteria for multiple plant growth promoting traits.

Generally, plant growth promoting rhizobacteria facilitate the plant growth directly by either assisting in resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of bio-control agents. Various studies have documented the increased health and productivity of different plant species by the application of plant growth promoting rhizobacteria under both normal and stressed conditions. The plant-beneficial rhizobacteria may decrease the global dependence on hazardous agricultural chemicals which destabilize the agro-eco-system

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## 2. Material and Methods

### 2.1 Collection of soil samples

Rhizosphere soil sample were collected from agriculture field. The soil which is adhering to roots of a plant and near to root is collected.

### 2.2 Isolation and Identification of microbes

Collected soil sample were air dried for 4h, and isolation was done using serial dilution technique. Selective N-free manitol agar for azatobacter, trichoderma and rhizobium selective media was used for isolation of the strains. 1ml of soil suspension was taken with the help of sterilized pipette and poured on the Petri plate seeded with selective medium. The plates were incubated at $37^0$C for azatobacter and rhizobium for 5 days. Appearance of colonies was recorded and individual colonies selected and mainted as a pure culture for further study.

The isolated colonies were sub-cultured and identified by using morphological and biochemical tests. The various tests were performed for *Azotobacter* like, catalase activity, motility test, oxidize activity, citrate test, indole test, methyl red test, Voges–Proskauer test, triple sugar ion test, nitrate reduction test.

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### 2.3 Biochemical characterization of isolate

1. **Colony morphology**

For the selected bacterial isolates, colony morphology was observed by growing them on agar plates. Morphological characters viz. size, shape, surface, opacity, texture, elevation and pigmentation were determined by visual observation as well as microscopy.

2. **Gram staining**

The Gram staining technique was used for differentiation between gram positive and gram negative bacterial strain. A drop of sterile distilled water was placed on a neat and clean glass slide, and a single isolate colony of 24hr old culture was mixed in it. The smear was made by spreading the culture. This smear was air dried and fixed by rapidly passing the slide three times over the flame. It was then inundated with crystal violet for 2 min and then washed off

with distilled water. Then grams iodine solution was added o the smear and the glass slide was left for 1 min. This step was followed by the application of decolorizing agent ethanol a single wash for few seconds. Ethanol was immediately washed with distilled water and the smear was covered with safaranine for 30 seconds. The slide was washed with distilled Water; air dried and was observed under the microscope.

**3. Motility test**

Motility of the organisms was observed with hanging drop method. A suspension in sterile distilled water was made for each bacterial isolate and glass slide with pit was used to hang the drop of bacterial suspension using cover slip. Motility of the organism was observed under the microscope.

**4. Indole test**

The test medium was prepared by dissolving 2g peptone and 0.5g NaCl in 100ml distilled water, pH adjusted at 7.4. The medium was poured in test tube and autoclaved at $121^0$C 15 psi for 20 min. The bacterial isolate was inoculated in the medium and incubated at $37^0$C for overnight. Next day 500 microliter of Kovacs reagent was added and shaken gently. The development of red coiour in the upper layer showed the positive test.

**5. Methyl Red test**

The medium was prepared by dissolving 5 g peptone and 5 g $K_2HPO_4$ in 1LDistilled water, pH adjusted at 7.6 and poured in test tubes. The medium was sterilized by autoclaving at $121^C$, 15 psi for 20 minutes. Then 0.25 ml glucose solution was added in 5 ml medium in each tube. The above media were inoculated with respective isolates and incubated at 370C for 48 hours. Then 5 drops of methyl red (0.1 g methyl red and 300 mL ethanol in 100 mL distilled water) were added and mixed. The bright red color indicated the positive test, otherwise the test is negative.

**6. Voges-Proskauer (V.P.) Test**

The medium for V. P. test was prepared by dissolving 0.5g peptone, 0.5g dipotassium Hydrogen phosphate in 100 mL distilled water, pH adjusted at 7.6 and the 0.5g of dextrose was added. The 5 mL medium was added to a test tube and autoclaved at $121^0$C, 15 psi for 20 minutes. The medium was inoculated with respective bacterial isolate and incubated at $37^0$C for overnight. Next day, 1 mL 40%KOH and 3 mL 5% _-naphthol were added. The tubes were aerated for 30 minutes and the appearance of red color indicates positive test.

**7. Citrate Utilization**

The Simmons's citrate medium was prepared [NaCl 500mg, $MgSO_4$ 200mg,$NH_4H_2PO_4$ 100mg, $K_2HPO_4$ 100mg, trisodium citrate 500mg and agar 2g, 0.2% Bromothymol blue 4 ml, poured in test tube (5ml each), autoclaved], inoculated with Respective bacterial isolate and incubated at $37^0$C overnight. The Change of color of medium from green to blue indicates positive result.

**2.4 Pesticide tolerance**

The sensitivity of rhizobacterial strains to pesticide was studied quantitatively in medium without agar at two levels of pesticide (100, and 250 mg L-1) as carbon source. The glucose was used as carbon source in case of control. The medium was prepared, poured in glass tubes and inoculated with 1 ml of respective rhizobacterial strains having uniform cell density. These tubes were incubated at 28 ± 2 °C for 72h. The growth of bacterial strains at highest concentration of pesticide was considered as the maximum tolerance level (MTL) and then measures the OD at 600nm.

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**2.5Viability of isolate**

Viability of isolates is measured using haemocytometer.Pipette tryphan blue with suspension of isolate at the age of the cover-slip and allows running under the cover slip
Visualize the haemocytometer grid under the microscope, then count viable and dead cell in one and more large corner squares and record cell counts.

**2.6 Pesticide degradation assay**

In this experiment used pesticides with concentrations tested in the range: 10, 20, 40 and 80 ppm.In which first prepares the nutrient agar medium 100ml for each isolate. Then autoclaved at $121^0$C, 15 psi for 20 min. after autoclaving the media adds above concentration of pesticide in the medium and poured in to Petri plates for solidification. Then make the 4 borer in each Petri plate using cork borer and add the different concentration of pesticide in it. Incubate these plates for 48 hrs at room temperature for the appearance of clear zone.

## 2.7 Growth at different pH level

For pH optimization, the rhizobacterial strains were grown in nutrient broth with three pH levels. The medium with different pH levels was prepared, poured in glass tube and inoculated with respective Rhizo-bacterial strain. These tubes were inoculated at $28^0$C for 72hr and optical density was measured at 600 nm after 3 days.

## Result and Discussion

Total two bacterial isolates were isolated by serial dilution and plating methods from rhizosphere soils.


Azatobacter culture


Rhizobium culture

**Fig1: Pure culture of isolate**

Colony morphology:

The morphological characters have shown in TABLE 2. The morphological properties has been found as cocci shape, curled surface, opaque, mucous texture and raised elevation in azotobacter. Rhizobium shows Rod shape, entire surface, opaque, mucoid texture and convex elevation. Morphological characters vary in each microbe which has isolated from soil.

| Name of isolate | Azotobacter | Rhizobium |
|---|---|---|
| Shape | Cocci | Rod |
| Surface | Curled | Entire |
| Opacity | Opaque | Opaque |
| Texture | Mucoid | Mucoid |
| Elevation | Raised | Convex |

Table 2

Biochemical characterization:

General biochemical features of bacterial isolate is shown in TABLE 3 and FIG 2

The bacterial isolate were characterized by their biochemical properties (indole test, methyl red test, voges- proskaures test, citrate utilization test) using standard method.

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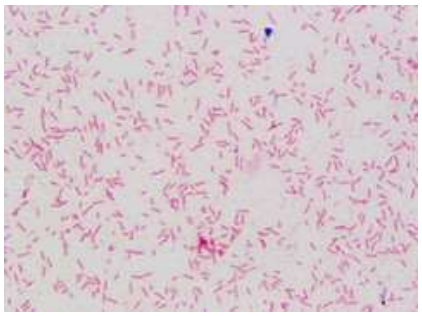
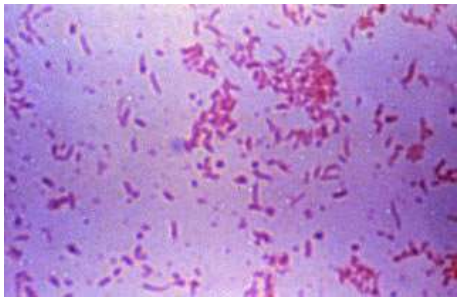
| Name of test/isolate | Azatobacter | Rhizobium |
|---|---|---|
| | | |
| Gram staining | Gram negative | Gram negative |
| Indole test | + | - |
| Methyl-Red test | + | - |
| V.P. test | + | - |
| Citrate utilization | + | - |
| Motility test | Motile | Non motile |

Table 3

Gram staining:



Azotobacter staining



Rhizobium staining

Indole test:


Positive indole test Negative indole test

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Methyl red test:

Positive methyl red test

negative methyl red test

Voges-proskauer test:

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Positive V. P. test

negative V. P. test

Citrate utilization test

Positive citrate utilization test negative citrate utilization test

**Fig2: Biochemical characterization of isolate**

Azotobacter is gram negative bacteria and all positive biochemical tests whereas rhizobium is also gram negative in nature and all negative biochemical tests is observed.

Pesticide tolerance:

Plant growth promoting rhizobacteria showed significance tolerance to pesticides studied, indicating their ability to survive under high pesticide stress conditions.

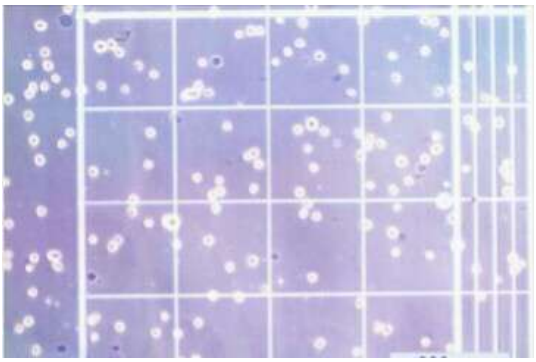
The pesticide had a negative impact on growth of most isolate. Azotobacter isolates showed high optical density at high pesticide level as compare to rhizobium isolate and their growth decreased with increasing concentration of pesticide in case of rhozobium.

| Strain | Control | 100mg/ml | 250mg/ml |
|---|---|---|---|
| Azotobacter | 0.63 | 0.65 | 0.66 |
| Rhizobium | 0.58 | 0.62 | 0.61 |

Table

Viability of isolate:

Live cell count+ dead cell count/Live cell count = %viability



Azatobcter (250mg/L)

The viable cell count for each of the 16 squares were 45, 50, 52, 55

The dead cell count for each of the 16 square were 12, 15, 14, 17

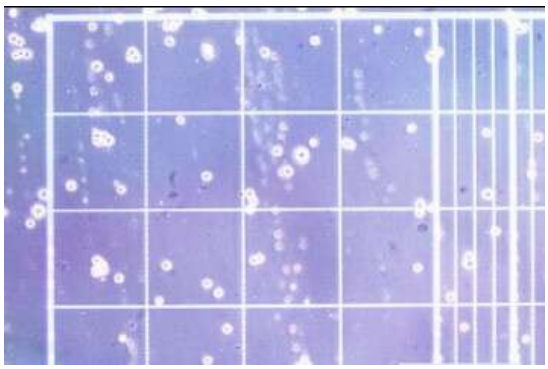
Average viable cell count= 45+50+52+55/4 = 50.5

No of viable cells/ml = $50.5\times10^4\times1$ = 5, 05000 live cells/ml

Dead cell count= 12+13+11+10/4 = 11.5

No of dead cell/ml = $11.5\times10^4\times1$ = 115000 dead cells/ ml

% of viability= 505000+115000/505000× 100 = 81.45%



Rhizobium (250mg/L)

The viable cell count for each of the 16 squares were 18, 15, 17, 19

The dead cell count for each of the 16 square were 8, 10, 7, 8

Average viable cell count = 18+15+17+19 /4 = 17.25

No of viable cells/ml = $17.25\times10^4\times1$ = 172500

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Dead cell count = 8+10+7+8/4 = 8.25

No of dead cells/ml = $8.25\times10^4\times1$ = 82500

% of viability = 172500+82500/172500×100 = 52.2%

Pesticide degradation assay:

This assay shows the zone of inhibition around the well. The different concentration of pesticide is added in different well.

The better result is obtained when the mixture of azatobacter and rhizobium suspension were added in the well and the zone of inhibition is clear and maximum

Growth at different pH level:

Rhizobium show maximum optical density at neutral pH. The optical density was decreased with decreasing pH level from neutral to acidic as shown in TABLE 4.

| Strain | 5 | 7 | 9 |
|---|---|---|---|
| Azatobacter | 0.21 | 0.45 | 0.41 |
| Rhizobium | 0.24 | 0.44 | 0.42 |

**Summary and conclusion:**

PGPR are known for their plant growth promotion capabilities. We have checked the capacity of agriculture field isolates to tolerate pesticide compounds. The result of present study show the isolated PGPR strains are capable to degrade the different concentration of pesticide and use it as carbon source and energy.Azatobacter and rhizobium isolate showed significance tolerance, indicating their ability to survive under high pesticide stress condition. In this study Azatobacter show better zone of inhibition as compare to rhizobium isolate but when the culture of both are mixed and used for the degradation the clear zone of inhibition is observed.Azotobacter is more tolerant to pesticide as compare to rhizobium isolate they use the pesticide as carbon source. Morphological characteristics vary in both the isolate that is shape, texture, elevation, opacity, etc.The bacterial isolate were characterized by their biochemical properties using standard method. Azotobacter shows all positive IMVIC test whereas Rhizobium show all negative IMVIC test. The viability of isolate is calculated that is 81.45% in azotobacter and 52.2% in rhizobium hence this indicates azotobacter is more tolerant to pesticide as compare to rhizobium.At neutral pH both the isolate having greater density and growth.

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