Research Paper Biology

A Study on Pesticide Degradation by Pseudomonasputida

KEERTHI YEKOJI M.sc Micro Biology Research associate JK Agri genetics. Hyderabad

ABSTRACT

Pesticide is a chemical or biological agent which is used to kill or destroy crop damages. Because of using pesticides we are facing some disadvantages like pesticides consists toxins and carcinogen, these causes environmental pollution. Pollutes effects on soil and water and also predominantly effecting the health of human beings. Furthermore we cannot reduce the pesticides usage, so as to protect the crop, it is better to degrade them rather than reduction.

The powerful tool discovered in the recent past was Pseudomonasputida , it was found to degrade pesticides especially chlorophyrifos used frequently in paddy, chilli and cotton fields.

KEYWORDS

Role Of Bacteria In Degradation:

Microbial degradation of chlorpyrifos results in higher concentration of 3,5,6-trichloro-2-pyridinol, a major metabolite of chlorpyrifos was produced (Robertson et al., 1998). In alkaline soils, chlorpyrifos is hydrolysed readily to 3,5,6-tricholoro-2-pyridinol which is further degraded by microbial activity. The Pseudomonas species had a capacity to degrade p-nitro phenol very effectively but could not degrade methyl parathion. Pseudomonas putida was able to degrade both methyl parathion and p-nitro phenol.

Material and Methods

Materials Required:

1. Soil sample 2. Test tubes 3. Micropipettes and tips 4. Conical flasks 5. Petri plates 6.Beaker 7. Measuring cylinder 8. Spatulas 9. Inoculation loop and L-rod 10.Spirit lamp 11. Aluminium foil 12. No absorbent cotton 13. Electronic weighing balance 14. Laminar Air-Flow chamber 15. Autoclave 16. Centrifuge 17. Eppendroff tubes 18. Distilled water 19. Agarose gel electrophoresis kit 20. PCR 21. PCR eppendroffs 22. Water bath 23. Incubator 24. Colorimeter 25. Cuvettes 26. Tissue paper

Equipment Required:

1. Hot air oven
2. Auto clave
3. Laminar air flow
4. Incubator
5. Centrifuge
6. Polymerase chain reaction
7. Agarose gel electrophoresis
8. Colorimeter
9. UV-VIS trans illuminator

COMPOSITION OF NUTRIENT AGAR MEDIUM:

Table : Composition of nutrient agar medium

S.no	Ingredients	Per Liter
1	Peptone	5 Grams
2	Beef Extract	3 Grams
3	Sodium Chloride	5 Grams
4	Agar	15 Grams
5	Distilled Water	1000 ml
6	pH	7.0

28gms of nutrient agar is dissolved in the 1000ml of distilled water in a conical flask and cover the mouth of the conical flask with the non-absorbent cotton. Autoclaved the conical flask which contains nutrient agar solution and cooled at room temperature and poured into Petri plates.

COMPOSITION OF NUTRIENT BROTH:

Table

S.no	Components	Volume
1	Peptic digest of animal tissue	5gms
2	Sodium chloride	5gms
3	Beef Extract	1.5gms
4	Yeast extract	1.5gms
5	pH	7.2
6	Distilled water	1000ml

13gms of nutrient broth dissolve in 1000ml of distilled water and mix well. Cover the mouth of the conical flask with non-absorbent cotton plug and autoclave the solution.

Composition of M.S.M medium: (Minimal salt medium)

Composition of MSM media-

K2HPO4 - 0.06gms
KH2PO4 - 0.02gms
MgSO4.7H20 - 0.008gms
NaCl - 0.002gms
NH4NO3 - 0.06gms

Reagents:

1. T.E Buffer 2.ST Buffer 3.SN Buffer 4.Lysis Buffer 5.3M Sodium acetate 6.Isoproponal 7.70% Ethanol

COMPOSITION OF T.E BUFFER:

Table

S.no	Ingredients	Grams per 100ML
1	Tris	100mM
2	EDTA	10mM

10ml of tris and 2ml of EDTA was dissolved in the 100ml of distilled water, EDTA pH was adjusted to 8 by NaOH and total volume was made up to 100ml.

COMPOSITION OF ST BUFFER:

Table

S.no	Ingredients	Grams per liter
1	SDS	500gms
2	Triton X	100ml

500gms of SDS was dissolved in warm water of 500ml, add 100ml of Triton X solution, final volume was made up to 1000 ml by using distilled water.

COMPOSITION OF SN BUFFER:

Table

S.no	Ingredients	Grams per liter
1	SDS	50gms

2	1M NaCl	58gms

50gms of SDS was dissolved in 100ml of distilled water, to that 58gms of 1M NaCl was added, after completely dissolving the SDS, total volume was made up to 1000ml by using distilled water.

COMPOSITION OF LYSIS BUFFER:
Table no

S.no	Ingredients	Grams per liter
1	SDS	50gms
2	Triton X	100gms
3	NaCl	2 M
4	EDTA	10 mM

COMPOSITION OF 1% AGAROSE:
Table no

S.no	Ingredients	Grams per liter
1	Agarose	10gms
2	TAE Buffer	20ml
3	Ethidium Bromide	125µl

10gms of Agarose was dissolved and 20ml of TAE Buffer and made upto 1000ml with distilled water. The contents were heated until clear solution appears,cooled top 40c,125µl of Ethidium bromide was added and poured into gelcaste.

COMPOSITION OF TANK BUFFER:
Table no

S.no	Ingredients	Per liter
1	Tris Base	242gms
2	EDTA	0.5M
3	Glacial Acetic Acid	57.1

COMPOSITION OF GEL LOADING DYE:
Table no

S.no	Ingredients	Per liter
1	Bromophenol Blue	0.25%
2	Xylene Cyanol	0.25%
3	Glucose or Sucrose	30% or 40%

Serial dilution Technique
The serial dilution is a technique where the number of bacteria present in the soil sample decreases as the dilution rate increases. The purpose of dilution is to obtain pure isolated colonies (Chellapandi and Himanshu,2008).

Procedure
- A series of sterilized test tubes capped with cotton plugs were taken and marked as 10^{-1}, 10^{-2},10^{-3},10^{-4} up to 10^{-8}.
- 1g of enriched soil sample was inoculated into 10ml of the molecular grade water containing test tube marked as 10^{-1} so that the dilution is brought about by 1:10ratio (10 fold dilution).
- 1ml of the diluted sample was taken from test tube of 1:10 dilution 10^{-1} and transferred into a teast tube containing 9 ml of molecular grade water marked as 10^{-2} so the dilution is now 1:100.
- The dilution were carried upto 10^{-8}.
- For each transfer the dilution increased bu 10 fold, that is 1:10, 1:100, 1:1000, 1:10000, 1:100000 and so on. 0.5 ml was taken from the 3 following dilutions (10^{-4},10^{-5},10^{-6}) and inoculated into the nutrient agar plates.

Gram Staining
Gram staining technique is a differential staining method used to differentiate bacterial species into Gram-positive and Gram-negative on the basis of physical and chemical properties of their cell walls. Gram-negative cells accept the counter stain safranin and appear pink to red in colour whereas Gram-positive cells retain the crystal violet stain and appear blue to purple.

Material and Reagents
- 24 hour old culture
- Wash bottle droppers
- Inoculating loops
- Glass slides
- Blotting papers
- Bunsen burner
- Microscope

Crystal Violet (Hucker's)
Solution A:
- Crystal Violet (90% dye Content) 2gms
- Ethyl Alcohol(95%) 20ml

Solution B
- Ammonium Oxalate 0.8gms
- Distilled water 80ml

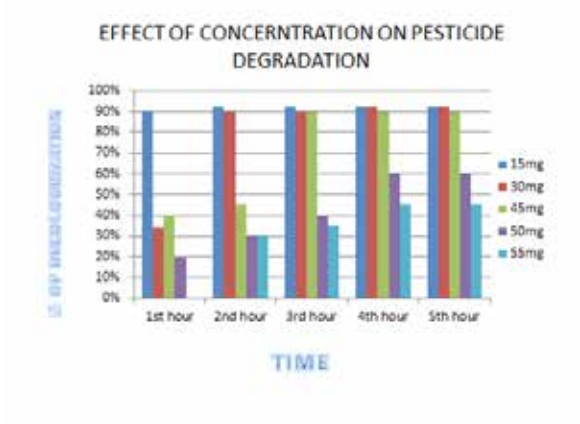
Dissolve the crystal violet in ethyl alcohol and the ammonium oxalate in distilled water, mix solution A and B.

Grams Iodine = Iodine 1gram + Potassium Iodide 2 grams +Distilled water 300ml
- Ethyl Alcohol (95%)
- Safranin (2.55 solution in 95% ethyl alcohol) 10ml
- Distilled water 100ml

Procedure for gram staining
- Isolated bacterial culture was inoculated, prepared a smear and air dried.
- The dry slide was passed through a Bunsen burner flame for 2 to 3 minutes to heat fix the smear.
- The side was placed on a staining rack and the smear was flooded with primary stain crystal violet for 1 minute.
- After a minute of exposure the dye was washed gently in tap water and dried with a paper towel.
- The smear was then flooded with gram's iodine for 1 minute which serves as a mordant.
- The slide was again rinsed with tap water and drained carefully.
- Then the bacterial smear was subjected to decolonization step for 10 seconds by adding 20 drops of 95%ethanol.
- After the smear is decolorized it washed in water without any delay and drained carefully.
- The smear was finally treated with few drops of counter stain safranin for 60 seconds, washed with water and blot dried.
- The slide was observed under the microscope (Olympus Inverted Microscope) with 10X objective.
- Later with an oil immersion 100X objective and recorded the results. The biochemical tests and other microbial basis tests may take much more time and the results procured from these process may really create ambiguity. Hence in the present steady instead of Bergious manual based biochemical tests, PCR based 16s rRNA amplification was used.

EFFECT OF CONCENTRATION ON PESTICIDE DEGRADATION:
PERCENTAGE OF DEGRADATION:

S.NO	CONC. OF PESTICIDE	1ST HOUR	2ND HOUR	3RD HOUR	4TH HOUR	5TH HOUR
1	15mg	90%	92%	92%	92%	92%
2	30mg	34%	90%	90%	92%	92%
3	45mg	40%	45%	90%	90%	90%
4	50mg	20%	30%	40%	60%	60%
5	55mg	0%	30%	35%	45%	45%

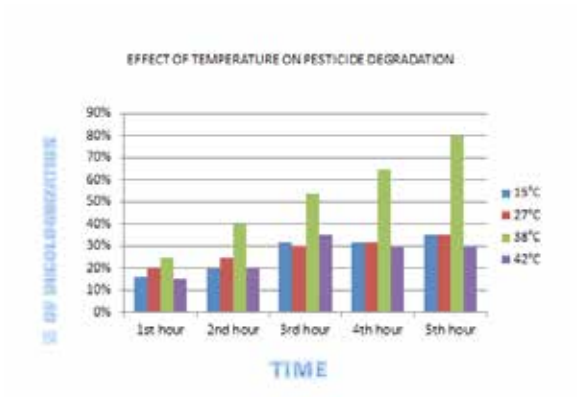


Maximum degradation was observed at 15 mg/lit.

EFFECT OF TEMPERATURE:
After incubation of bacterium to the growth medium with pesticide in separate conical flask at different temperatures such as 15, 27, 38, 42 degrees of Celsius respectively.

EFFECT OF TEMPERATURE ON PESTICIDE DEGRADATION: PERCENTAGE OF DEGRADATION:

S.NO	TEMPERATURE	1ST HOUR	2ND HOUR	3RD HOUR	4TH HOUR	5TH HOUR
1	15˚c	16%	20%	32%	32%	35%
2	27˚c	20%	25%	30%	32%	35%
3	38˚c	25%	40%	54%	65%	80%
4	42˚c	15%	20%	35%	30%	30%



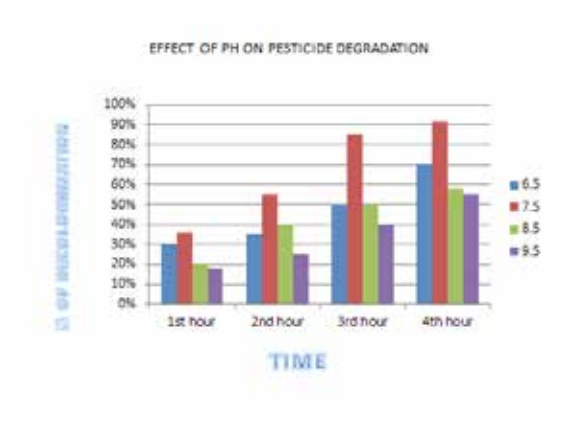
Maximum degradation was observed at 38˚c.

EFFECT OF pH:
After incubation of bacterium to the growth medium with pesticides in separate conical flasks at different pH are incubated such s 6.6, 7.5, 8.5 and 9.5. Maximum degradation was observed at pH 7.5.

EFFECT OF pH ON PESTICIDE DEGRADATION:

S.NO	pH	1ST HOUR	2ND HOUR	3RD HOUR	4TH HOUR
1	6.5%	30%	35%	50%	70%
2	7.5%	36%	55%	85%	92%
3	8.5%	20%	40%	50%	58%
4.	9.5%	18%	25%	40%	55%

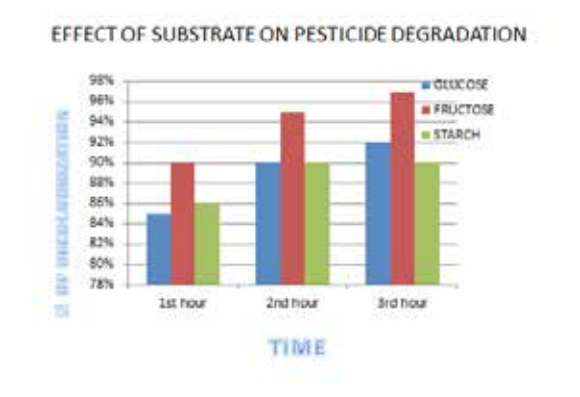
Maximum degradation was observed at 7.5 pH.



EFFECT OF SUBSTRATE:
After incubation of bacteria to the growth medium with pesticide in separate conical flasks at different substrates such as glucose, fructose and starch. Maximum degradation was observed at fructose.

EFFECT OF SUBSTRATE:

S.NO	SUSTRATE	1ST	2ND	3RD
1	GLUCOSE	85%	90%	90%
2	FRUCTOSE	90%	92%	93%
3	STARCH	86%	89%	89%



Maximum degradation was observed using fructose as a substrate after incubation of culture growth medium.

RESULT:
From the above studies it was found that Pseudomonas putida paddy fields of khammam district of Tiruvur village possed the maximum degradation at the concentration of 15 mg/lit, temperature is 38˚c at pH 7.5 by utilizing the Fructose as substrate.