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Formulation of Bioinsecticide using *Pseudomonas fluorescens* to study the mortality rate against *Aedes aegypti*

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ABSTRACT

Pseudomonas fluorescens, a Gram-negativerod-shaped bacterial species, which was highly involved as a Plant Growth Promoting Rhizobacterium, had the ability of acting as a good source of biopesticide and bioinsecticidefor its ability of eradicating the nuisance pests such as mealy bugs, leaf-rolling caterpillar, house flies, mosquito larvae etc., as they were very much trouble causing by spreading out various type health ailments to humansand affecting the growth quality of the plants. The present study dealt with the experiment of talc-based formulation of an effective bioinsecticide using P. flourescens as the main source and carrier materials such as wheat bran and maize bran are involved for formulating the product. Various basic studies such as serial dilution, bacterial growth curve, biochemical tests etc., were done to highlight the characteristics of P. fluorescens. The shelf-life testing of the product was also done and the presence of bacterial colonies improved the quality of the prepared bioinsecticide.FTIR characterization studies was carried to find out the functional groups ofthe both the samples and various groups like ethers, halogen bromide, carbon monosulfide, carbon iodide and fingerprint region were also present.

Keywords:Pseudomonas fluorescens, Bioinsecticide, Aedes aegypti, talc, wheat bran, maize bran.

1. INTRODUCTION

The ecological and environmental risks of using the chemical-based pest control or insect control agents are major threats to both flora and fauna on world basis. One of the most effective solutions to the mentioned problems is represented by using of bacterium *Pseudomonas fluorescens* which is well known as plant growth promoter and plant protectant against a wide range of plant pathogens in the rhizosphere area. *Pseudomonas fluorescens*is have a broad-spectrum antagonistic activity on plant pathogen, such as antibiosis, siderophores production and nutrition or site competition. For example: *Azorhizobium caulinodans* in rice, *Burkholderiapickettii* in Maize, Pseudomonas *fluorescens* and *Pseudomonas putida*, *Bacillus spp*. in Citrus plants and *Streptomyces* in Wheat are some of the examples [16].Talc in powdered form, often combined with corn starch, is used as baby powder. Wheat bran (WB) is a by-product of the milling of wheat grain and represents a unique reserve of nutrients due to the

amount and quality of its proteins, its content of minerals and B complex vitamins, as well as of dietary fibre [24]. The maize kernel is formed from four leading structures, the bran (pericarp), endosperm, germ and tip cap [6].Carboxy Methyl Cellulose has been widely used in pharmaceuticals (drug delivery, antimicrobial) and biomedical (wound dressing) applications. It is non-toxic to humans, water-soluble and it is also abundant in nature. *Aedes aegypti* is the invasive mosquito that has caused the most human casualties worldwide, initially as the vector of devastating yellow fever epidemics [5]. The only way of decreasing the incidence of this disease is the eradication of *A. Aegypti*[2],[12].The current study shows how *Pseudomonas fluorescens* in acting as a bioinsecticide to eradicate the population of *Aedes aegypti.*

2. MATERIALS AND METHOD:

A.SOURCE OF THE BACTERIAL ORGANISM:

Pseudomonas *fluorescens* was collected from the Microbial Type Culture Collection and Gene Bank (MTCC). The accession number of the culture type is6035. The culture type was an active slant maintained in 10% glycerol stock.

B. SUBCULTURING THE ORGANISM:

Agar and broth medium:

The organism was sub cultured in both agar and broth medium. The mother culture was streaked in the agar medium using a sterile inoculating loop. Then the plates were incubated at 37˚C for 24 hours. The broth was sub cultured by inoculating 100μl of the stock culture and incubating it at 37˚C for 24 hours. Then it was stored at 4˚C for further use.

C.SERIAL DILUTION:

Serial dilutions were made up to 10^{-7} dilutions, spread on LB agar plates and incubated at 37˚C for 24 hours. The colonies were counted and colony forming units of the organism were calculated. Colony count (CFUs) on an agar plate = total dilution of tube (used to make plate for colony count) X volumeplated[18].

D.BACTERIAL GROWTH CURVE:

100 ml of the overnight culture of Pseudomonas *fluorescens* was taken and from that 10ml of the culture was transferred to the 90ml of fresh LB broth and mixed well. From this 4ml of the culture was added into a sterile test tube labelled as "0". The "0" test tube was considered as blank i.e., is the 0th level of growth curve point. Then the absorbance was calculated at 600nm in UV Spectrophotometer.Incubate the conical flask at 37˚C at 150rpm for 1 hour. Next, 4 ml of culture was transferred to the 2nd hour test tube and absorbance was calculated and the culture flask was incubated as mentioned earlier.Likewise, up to 4 hours, the growth curve readings were noted and plotted down as a graph.

E. BIOCHEMICAL TESTS:

Biochemical tests were carried out to highlight the characteristics of *P. fluorescens*. The tests carried out were Methyl red test by Shanmugaraj e*t al* [22], Voges-Proskauer test byAnokhe*et al* [8], Catalase test by Khatoon *et al* [8], Citrate test by (Method of Simmons, 1926) and Motility test by Green *et al*,[7].

F. FORMULATION OF BIOCONTROL AGENT:

The formulation of biocontrol agent was carried out on the procedure followed by Commare*et al*, [3] *and* for this *Pseudomonas fluorescens* strain sub cultured from the active slant was used for the formulation.

- **Source of raw materials and chemicals:**

The talc – based formulation of the biocontrol agent was prepared using the individual bacterial strain, but the source of the carrier was purely natural raw materials such as wheat bran and maize bran. There were 2 types of formulation prepared using wheat and maize bran.The main source of chemicals were Talc and Carboxy Methyl cellulose.

- **Preparation of the biocontrol agent:**

2 types of formulation were carried out on the basis of raw materials sourced and they were:

Formulation 1 :150g of Talc, 50g of Wheat bran and2g Carboxy Methyl Cellulose (CMC)

Formulation 2:150g of Talc, 50g of Maize bran and 2g of Carboxy Methyl Cellulose (CMC) [19].

- **Mixing and Storage:**

Before the formulation of biocontrol agent, the chemicals and raw materials were sterilized separately in autoclavable bags at 15psi for 20 minutes. Then they were spread on separate metal trays or aluminium foil sheet at sterile conditions wherein the 20ml of fresh broth culture of the *Pseudomonas fluorescens* was added on the talc contents and they were mixed with hands by wearing sterile gloves. Once the formulation has been done, the talc mixtures were shade dried for 40% at

sterile conditions for 24 hours and packed separately in a clean sealable polythene bag and stored at room temperature for 7 – 10 days.

G.ASSESSING THE SHELF LIFE OF THE PRODUCT:

After a week, the shelf life of the product was assessed by serial dilution technique. For this, 1g of talc formulation was mixed with 10ml of sterile water and 10^{-3} dilutions were carried out. Then 1ml of 10^{-3} dilution was taken in a micropipette, poured into a sterile petri dish and swirled well until the water touched every space of the dish. Then 20 ml of the autoclaved and cooled LB agar was poured above and allowed to incubate for a period of 24hrs at 37°C.

H. COLLECTION OF MOSQUITO LARVAE (*Aedes aegypti*):

For testing the formulated product, mosquito larvae were collected. Around 24 mosquito larvae were dispensed in a glass tumbler containing 100ml tap water.

- **Feeding and testing the mortality:**

The mosquito larvae were in the 2nd stage of their life cycle and they were fed with yeast powder at an interval of 24 hours for 2 days before they enter into the next stage of their life cycle.After the larvae was collected, it was fed for 2 days with yeast and on the 3rd day, 1g of talc biocontrol from each formulation were taken, separately mixed in tap water and added in drops inside the tumbler. The process was repeated at an interval of once every 24 hrs. On the 4th day, 1.5 grams of each formulation were mixed and added at an interval of 24 hrs once and 2g+2g were added on the 5th day for 24 hrs once and mortality was calculated.

$$\text{Mortality \%} = \frac{\text{No. of dead larvae}}{\text{No. of alive larvae}} \times 100$$

I. FT-IR CHARACTERIZATION STUDY:

The technique is based upon the identification of functional groups within molecules where such groups vibrate (either through stretching or bending in various ways) when irradiated with specific wavelengths of light. These vibrations and their intensity (% transmission) are plotted against the frequency of light (cm^{-1}) to which the sample is exposed to produce an FTIR spectrum. Portions of the FTIR spectrum are unique to the compound under test (this is called the fingerprint region) [4].

3. RESULTS AND DISUSSION:

A. SERIAL DILUTION:

Serial dilution was carried out to find the colony forming units of the organism and made up to 10^{-7} dilutions. The colony forming units are given in the following **table I.**

I. Serial dilution from 10^{-1} to 10^{-7}

Dilution	CFU
10^{-1}	Too numerous to count
10^{-2}	Too numerous to count
10^{-3}	124
10^{-4}	83
10^{-5}	45
10^{-6}	19
10^{-7}	7

From the dilutions made up to 10^{-7}, the colonies forming units gradually decreased in their population as shown in **Table 1**.In a study on *B. megaterium*carried out by Andalib*et al*., (2016) five different cell concentrations of bacteria (10×10^5 to 50×10^5cfu/ml) obtained from the appropriate serial dilution (10^5), was applied to the concrete for achieving the optimum concentration of bacteria. This was due to the presence ofa greater number of bacteria (more population) and for the nutrient competition in comparison to the bacterial concentration of 30×10^5cfu/ml.

B. BACTERIAL GROWTH CURVE:

From **Table II**, the bacterial growth curve of the *P. fluorescens* was calculated by the absorbance reading calculated at 600nm and at the end of 4th hour, the rate of absorbance dropped down to 1.126.

II. Bacterial growth curve of *Pseudomonas fluorescens*

Time interval (60 mins)	OD at 600nm
0	0.423
1	0.745
2	1.044
3	1.342
4	1.126

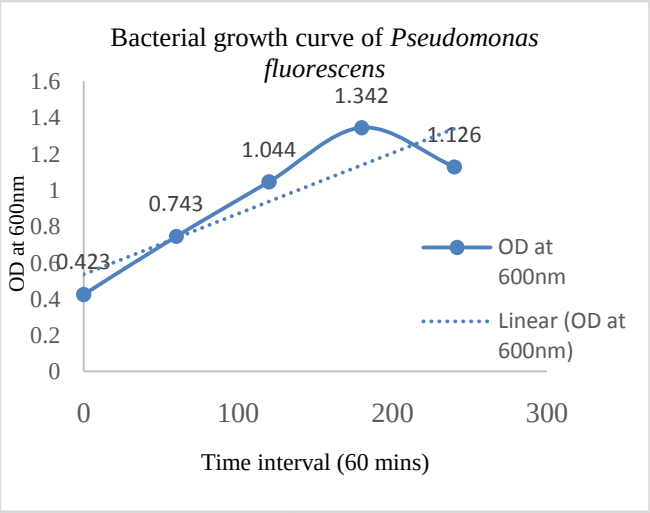

Fig 1: Bacterial growth curve of *P. fluorescens*

Growth studies were carried out by Xie *et al.*, (2020), inoculated camel milk samples were placed in incubators maintained at constant temperatures of 8, 12, 15, 20, 25, 30, 33, 37, 40, 43, and 46°C (±0.1°C). Samples were removed from the incubators at predetermined time intervals according to incubation temperatures. Bacterial growth was examined by enumerating the bacterial cells in each tube and with the present study a clear conclusion was made by observing the doubling time of the cells at the time of incubation and the values as the absorbance values were increasing drastically at every hour.

C.BIOCHEMICAL TESTS:

III. Biochemical tests

Characteristics	Results
Catalase	Positive
Methyl Red	Negative
Voges - Proskauer	Negative
Citrate utilization	Positive
Motility	Negative

In the biochemical studies carried out on *P. Fluorescens*by Lalithambika *et al*, [9] showed **positive results** for Catalase, Oxidase, Citrate, TSI, Nitrate reduction, Gelatine hydrolysis, Casein hydrolysis and showed the **negative result** for Indole, MRVP and starch hydrolysis. A study reported by Bharathi *et al*, [23] stated that biochemical characterization for colony 2 and 3, Gram negative rod-shaped bacteria it is negative for Carbohydrate fermentation, Triple sugar iron, Indole production, VP and Urease test and positive results for MR, citrate and H2S test it shows the colony 2 & 3 indicates the *Salmonella sp.*

D.TALC – BASED FORMULATION:

The *Pseudomonas fluorescens* bioinsecticide was formulated according to Commare *et al*, [3] and stored up to a week in sterile polythene Ziplock bags for further use.

A study reported by Niranjana *et al.*, (2009) on Pseudomonas species, formulations of biocontrol agents were prepared on two carrier materials – talcum powder and sodium alginate. Kandan *et al.*, (2002) reported that for the studies of controlling the sheath blight disease using pseudomonads species, a talc-based powder formulation containing either single strain or mixture of strains containing chitin was tested against sheath blight and leaf folder incidence under greenhouse as well as in field conditions. From the above-mentioned studies, the present study was also carried out by formulating the biocontrol agent with the use of talcum powder as the chief source of composition to control the mosquito larvae (*Aedes aegypti*).

E. ASSESSING THE SHELF–LIFE OF FORMULATED BIOINSECTICIDE:

From the shelf-life studies carried out on the formulated biocontrol agent, both the plates showed the presence of *P. fluorescens* growth after incubation of 24 hours with an appearance of white bacterial colonies on the middle and the sides as mentioned in Figure 2.

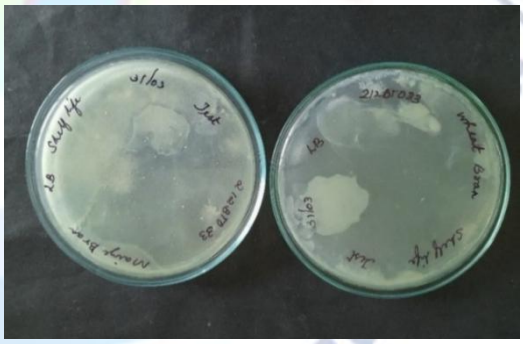

Fig 2: Presence of bacterial growth

The shelf - life studies carried out by Zafar*et al.*, (2000) *Bacillus thuringiensis*biopesticide formulation prepared by Liu *et al*, [11] and were stored at room temperature in light protected air tight containers.

F.APPLICATION OF THE BIOCONTROL AGENT:

The biocontrol agent was applied to the *Aedes aegypti* larvae at various intervals for 3 days from at an interval of 24 hours and the mortality % was calculated.After 24 hours of exposure, larval mortality was recorded. Bioassay was repeated three times using new solutions and different groups of larvae byAbbott *et al*, [1].

The application was carried out and the tabular column**IV**is given below:

IV. Mortality rate observation

Day	Application (Wheat bran + maize bran)	Time duration	No. of alive larvae	No. of dead larvae (mortality)
1	---	---	24	0
2	---	---	24	0
3	1g + 1g	24 hrs once	22	2
4	1.5g +1.5g	24 hrsonce	19	5
5	2g + 2g	24 hrs once	14	10

V. Mortality percentage

Day	Alive	Dead	Mortality %
1	24	0	0%
2	24	0	0%
3	22	2	9%
4	19	5	26.3%
5	14	10	71.4%

At the end of 5th day, 71.4% mortality rate was observed from the study after the application of the biocontrol agent given in the **Table V.**

A study reported on the mortality rate of *L.lepidophora*by Kumbhar*et al.*, (2019), response of eight various formulations of biopesticides were tested for determining their bio efficacy against grubs of *L. lepidophora* under field condition. The clumps mortality observed at 15th,30th,45th and 60th days interval after the treatment. The overall performance of the various formulations of the biopesticides against the white grub under field condition revealed that the treatment with *M. anisopliae*-talc based formulation registered significantly less 6.49, 7.36 and 9.20 % clumps mortality.

From the above-mentioned studies compared with the present study, the mortality rate was comparatively less at the initial stage and at the end of the time, the mortality rate was successful by the death of the larvae having 71.4%.

G.FTIR CHARACTERIZATION STUDY OFBIOINSECTICIDE SAMPLES:

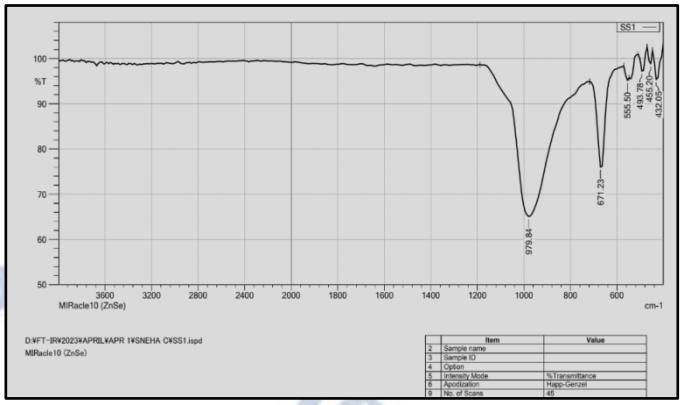

Fig 3: FT-IR analysis of Wheat bran test sample 1(S1)

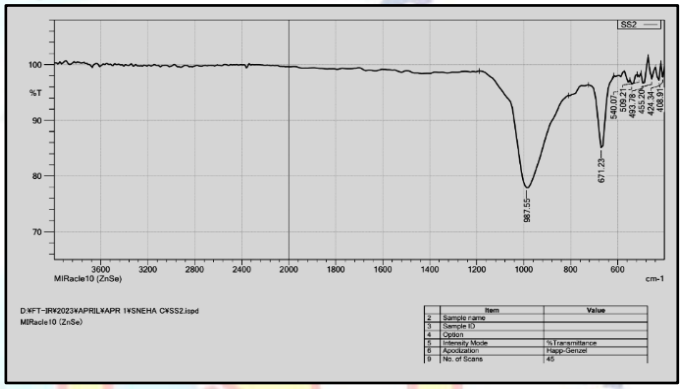

Fig 4: FT-IR analysis of Maize bran test sample 2 (S2)

The present study reveals that at the frequency range of **979.4cm^{-1} and 987.5 cm^{-1}**indicates the presence of **C-O-C groups** which confirms the **presence of ether**. The frequency range **671.23 cm^{-1}** in both the samples S1 and S2 is identified by the presence of **Carbon monosulfide groups** in it. The range **555.50 cm^{-1}** in S1 indicates the **carbon bromide group (halogen groups).** The peak values **540.07 cm^{-1}and 509.2cm^{-1}** shows the **Carbon bromide stretching vibration** which confirms the **presence of aliphatic amine group** in it. The values **493.78cm^{-1}**in both the samples contain **Carbon-iodide (halogen groups)** present in it. The frequency range of **455.20 cm^{-1}, 432.34 cm^{-1} and 432.30 cm^{-1}** reveals the confirmation of **fingerprint region** present in them. They are the spectra which consists of bending vibrations within the molecule and bands of higher length.The present study of FT-IR characterization of both the samples is equivalently compared to the above performed research studies and it is understood that **Carbon monosulfide groups is present in both the samples S1 and S2 and it is assumed that C-S groups were a causative group of the death of the larvae.** In a study reported by Lewis *et al*, [10]. Carbon disulphide is

an absolute insecticidal fumigant and it can cause death in them when they ingest the formulation.

4. SUMMARY AND CONCLUSION

Pseudomonas fluorescens bioinsecticide formulation was used to study the mortality rate against *Aedes* larvae with increasing concentration of bioinsecticide formulation. FTIR characterization studies revealed the functional groups as carbon sulfide present in the sample which was responsible for the death of the larvae as it was extremely lethal for the larvae to survive after the application of the bioinsecticide and it proved to cause the mortality rate.Thus, the conclusion is stated by the way of formulating an effective bioinsecticide against the *Aedes aegypti* larvae using the strain of *Pseudomonas fluorescens*.

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Conflict of interest statement

Authors declare that they do not have any conflict of interest.

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