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Efficacy of *Photorhabdus* as a Promising Entomopathogenic Bacteria in the Eco-Friendly Biocontrol of White Grub Larvae

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Abstract: White grubs are a major polyphagous pest that imposes damage upon several plant species, mostly by feeding their roots. White grub larvae are one of the hazardous pests found in sugarcane. This problem causes a substantial drop in sugarcane crop productivity every year in India. In this research, white grub larvae were subjected to biocontrol using developed formulations of *Photorhabdus* bacteria, symbiotically associated with entomopathogenic nematodes under laboratory conditions. The *Photorhabdus* bacteria isolated from entomopathogenic nematode- *Heterorhabditis indica* have insecticidal activity towards insect pests by exerting an array of toxic effects. The main insecticidal activity, i.e., chitinase enzyme production by these bacteria, was associated with mortality of insect pests. In the present investigation, chitinase production by *Photorhabdus* bacteria was determined by DEAE column chromatography. The various bacterial formulations of *Photorhabdus* were examined for their insecticidal activity against the white grub larvae under laboratory conditions. Mortality of the white grub larvae was observed after 24–48 hours of exposure. The formulations of *Photorhabdus* showed statistically significant effects on mortality of larvae. The percent mortality of larvae after treatment with formulation 3 was highly significant compared to those treated with formulation 1 and 2. Formulation 3 expressed a significantly lower LD50 value, i.e., 121.31 CFU/mL, over formulations 1 and 2, i.e., 127.34 & 133.56 CFU/mL, respectively. Formulation 3 showed greater efficacy in killing white grub larvae at lower concentrations. The formulation 3 of *Photorhabdus* bacteria has great potential to kill white grub larvae under laboratory conditions and requires further evaluation for its promising use as a biocontrol agent by pot and field studies.

Introduction

Sugarcane is the main cash crop in India and Maharashtra state has established a supreme position in the Indian sugar industry. Infestations of insect pests hamper crop productivity. The control strategies for white grub infestation in India mostly rely on the repeated use of conventional chemical pesticides at high dosages. The necessity for developing integrated pest management (IPM) solutions to control pests has increased in recent years due to concerns over human safety, the release of chemical toxins into the environment, and the low performance of prescribed chemical pesticides (Gangadhara, 2019). Due to the harmful effects of chemicals on human health and the environment, there is a rising public response to organically produced food.

There are many biological control options available; nevertheless, it is important to note that these alternatives also have some limitations. Entomopathogenic nematodes (EPNs) from the *Heterorhabditis* and *Steinernema* genera are found in soil across various regions. These nematodes

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have several characteristics that make them suitable for employment as agents for biological pest control (Rajagopal and Bhatnagar, 2002). Associations exist between entomopathogenic nematodes - *Heterorhabditis, Steinernema*, and the symbiotic Proteobacteria *Photorhabdus* and *Xenorhabdus* (Akhurst, 1982). The symbiotic bacteria residing in the gut of EPNs (George, 1977) may aid in the progression of the nematodes inside the host organism by killing insect pests (Ogier, 2020). The secreted enzymes by *P. luminescens* convert the insect tissue utilised by bacteria and nematodes for their growth (Kaya, 1993). The nematode undergoes several generations in the insect host, leaving the insect cadaver to search for the next insect host (Gerdes, 2015). Due to their notable reproductive capacity, EPNs can locate the hosts and respond to pest-level fluctuations (Shrestha, 2010; Lawrence, 2012). These EPNs do not have adverse effects on human beings and are considered environmentally safe. They can be easily produced and applied in the field by using conventional practices (Kajuga, 2018). But there are limitations in commercial production and application of EPN in the field due to which wide use of EPN is not much practised.

Root feeding insects are the most damaging agricultural pests on the planet. A promising approach to control root pests is using symbiotic bacteria associated with entomopathogenic nematodes. In particular, studies on *Photorhabdus sp.* aim to depict symbiotic association of EPN-bacteria and pathogenicity towards pest insect hosts. This has been achieved by finding pathogenicity factors such as toxin complexes, the pathogenic and mutualistic phase of bacteria associated with nematode (Salazar-Gutiérrez, 2017).

Nevertheless, *P. luminescens* could be cultured anoxically as the association is not obligatory for entomopathogenic nematodes and symbiotic bacteria. *P. luminescens* kills its insect host by secreting toxins into the insect's haemocoel. *P. luminescens* bacteria secrete multiple pore-forming toxins; these toxins penetrate the plasma membrane of cells to exert their effects. These toxins can either generate perforating pores, which dissipate key electrochemical gradients or act as a puncturing mechanism to translocate a cytotoxic molecule into the cytoplasm (Roderer, 2019). Tripartite toxin complexes (Tc toxins) are high molecular weight protein complexes exhibiting insecticidal properties. These complexes were initially discovered in the bacterium *P. luminescens* which is associated with nematodes (Liu, 2019). The Tc complex, responsible for oral toxicity, and the Mcf toxin, which causes a decrease in insect body turgor and, ultimately, death, are two examples of the categorised toxins that can be found in pathogenic islands (Cabral and Cherqui, 2004).

The sequence of genes that encode chitinase, a component of Tc toxin, is found within the intricate toxin loci (Liu, 2019). After ingestion, these toxins induce major damage to the epithelial cells of insect larvae. Cytoplasmic vesicles undergo "blebbing", which releases the epithelial cells of gut lumen followed by the complete destruction of the gut lining (Busby, 2012). The efficacy of *X. nematophilia* was studied in initial experiments for pest control by applying a bacterial suspension (Mahar, 2004). Experiments comparing pathogenicity caused by the nematode and bacteria or only by bacteria illustrate that the virulence of the nematode was mainly due to associated bacteria (Cabral and Cherqui, 2004).

The main goal of this study was to find a new approach, an alternative to using chemical pesticides. In the present investigation, we aimed to develop commercially suitable, stable formulations of biocontrol agents with adequate shelf-life. This study aimed to examine the efficacy of developed formulations of symbiotic *Photorhabdus* bacteria, which can combat the insects independently of their nematodes and are used as a biocontrol agent against white grub larvae under laboratory conditions.

Materials & Methods

Isolation of symbiotic bacteria from Entomopathogenic nematodes

Heterorhabditis indica nematode was isolated by insect bait method from soil samples collected from the sugarcane fields, maintained on *Corcyra* larvae (Rice moth larvae) and white grub larvae by white trap method. Infective juveniles (IJs) captured in a white trap were subjected to a two-hour soak in a thiomersal solution (0.1% w/v) containing streptomycin, followed by three washing in sterile Ringers' solution. Fifty infective juveniles were seeded onto a piece of Whatman filter paper (90 mm) in a petri dish with Corcyra larvae (Dunphy and Webster, 1985) to maintain EPNs. Alternatively, using a micropipette, 100 IJs were applied to the white grub larvae (McMullen II and Stock, 2014). After 48 hours, the insects were sterilized by spraying with 1% v/v sodium hypochlorite for 2-3 minutes, followed by washing three times with sterile distilled water. Insects were kept for drying and haemolymph (George, 1977) was streaked on a nutrient agar plate containing 2,3,5 triphenyl tetrazolium chloride (0.04 g/L) and bromothymol blue (0.025 g/L) (Woodring and Kaya,

1988). Plates were incubated at 28°C for 2-3 days, and the greenish-coloured luminescent colonies were picked and transferred in Luria Burtani broth and incubated for 72 hours at 120 rpm on a rotary shaker at 28^0C.

Molecular Identification of Isolated Bacteria by 16s rRNA gene sequencing

Isolates were sequenced for molecular identification at the National Centre for Microbial Resource (NCMR) sequencing laboratory at the National Centre for Cell Science in Pune, India. A standard phenol/chloroform extraction procedure was used for isolating genomic DNA. (Sambrook, 1989), the 16S rRNA gene was amplified by polymerase chain reaction (PCR) with the universal primers 16 F27 (5'-CCA GAG TTT GAT CMT GGC TCAG-3') and 16 R1492 (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). Denaturation is at 94 degrees Celsius for 30 seconds, reannealing at 55 degrees Celsius for 30 seconds, and extension is at 72 degrees Celsius for 90 seconds. The reaction products were visualised under ultraviolet light after being resolved in 1% agarose gels containing 2% ethidium bromide. The 16S rRNA gene was amplified in a polymerase chain reaction (PCR), and the resulting PCR product was purified using PEG-NaCl precipitation before being sequenced using an ABI 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Sequencing was performed from both ends using extra internal primers to ensure that each site was read at least twice. The Lasergene package was used for assembly, and the EzBioCloud database was consulted for identification.

Chitinase enzyme purification

For the purification of chitinase enzyme from the extracellular secretion of bacteria, the Luria Burtani (LB) broth was inoculated with *Photorhabdus* bacteria and incubated for 3-4 days at 28^0C on the rotary shaker. The culture was centrifuged at 10,000 rpm for 30 mins at 4 degrees Celsius. The supernatant was filtered (0.2 µm membrane filter) and the filtrate was concentrated using centrifugal devices purchased from PALL (Macrosep MWCO 30 KDa) by 10 times (Ray, 2019) (Akeed, 2020). The retentate was further purified by ion exchange – DEAE column chromatography (Gangwar, 2016).

DEAE column chromatography:

All stock solutions and buffers needed for column chromatography were filtered (with 0.2 µm syringe filters) and autoclaved. The retentate obtained by centrifugal device was used for further purification by column chromatography. Purification was carried out by AKTA start system (GE Healthcare) by using the DEAE SF column (Bowen and Ensign, 1998) according to the instruction of the manufacturer (Li et al., 2004). Column was washed with equilibration buffer (100 mM Tris HCL) and bound proteins eluted with 0.1M NaOH, 1mL protein peak fractions exhibiting chitinase activity were collected and the amount of protein was evaluated by the Folin Lowry method (Gangwar, 2016).

Infectivity assay of developed formulations of *Photorhabdus luminescence* against white grub larvae

The identified *P. luminescens* culture was used to prepare three different formulations, and its lethal concentration against white grub larvae was studied in a laboratory. For the infectivity assay white grub larvae were collected from an infested sugarcane field. The larvae of second-instar white grub were surface-sterilized with alcohol (70%) (Mathur, 2019). The individual larvae were kept in each sterile petri plate in triplicates. A bacterial culture incubated for 72 hours at 28 degrees Celsius was quantitated in the colony-forming unit by plating bacterial culture from each serial dilution. Three different formulations of bacteria using stock ($1x10^8$ CFU/mL) were prepared by varying concentrations of additives, surfactant and sugar. The desiccant agent and surfactants were used in the formulation to protect the bacteria from shearing and to achieve uniform spreading at the site of application, respectively. The three formulations were applied by spraying on white grub larvae (Bussaman, 2012). The dead insects show a brick-red colour. The untreated control larvae were treated with sterile distilled water. The experiment was independently repeated thrice. LD50 was calculated as a measure of bacterial virulence.

Statistical analysis

The minimum lethal dose (LD50) of *Photorhabdus* bacteria was estimated from larval mortality data subjected to probit analysis. Kaplan-Meier survival data analysis was used to calculate the median lethal exposure times. The log-rank test was used to examine the differences between the survival times. In order to compare the whole curves of each group, the chi-square value (X^2) is calculated using the log-rank test results (Rich, 2010).

Results

Isolation of *Photorhabdus luminescence* from Entomopathogenic nematode

The isolated blueish-green colonies from haemolymph were purified and subcultured on nutrient agar containing bromothymol blue, trehalose and Luria Burtani agar plate (Figure 1, 2, 3). The isolated colonies were identified by 16s rRNA gene sequencing.

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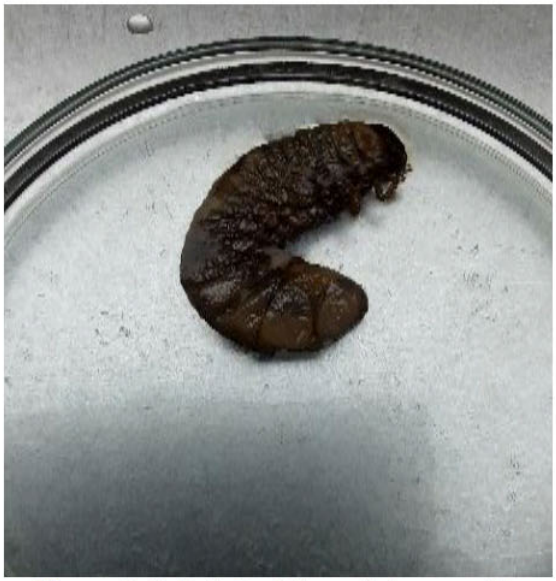

Figure 1. Infected white grub larvae with *Heterorhabditis indica*

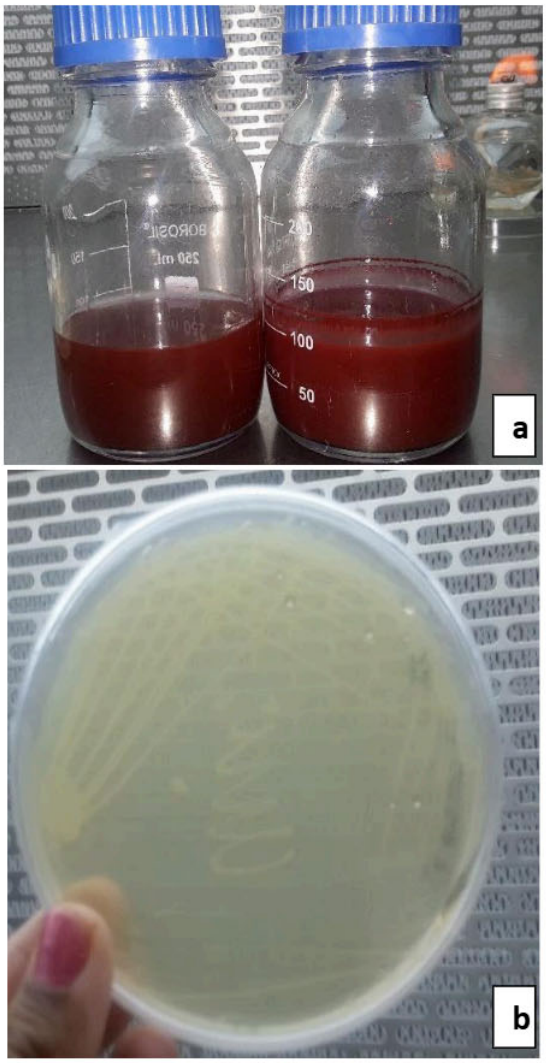

Figure 2. (a & b). Enrichment of bacteria in NBTA medium and Growth on LB plate

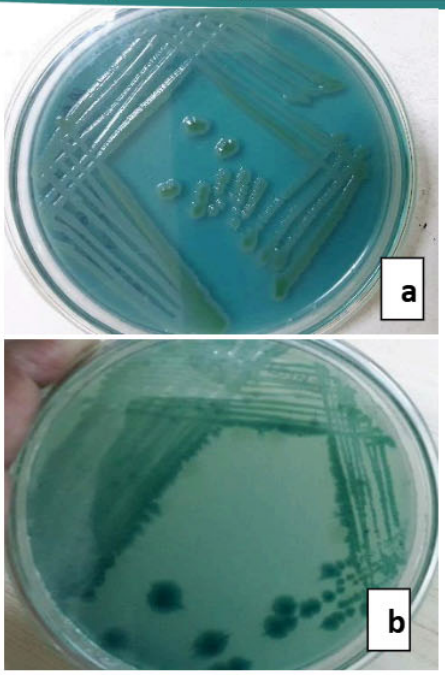

Figure 3. (a & b). Growth of isolated bacteria on Nutrient agar containing bromothymol blue plate.

Identification by 16s rRNA gene sequencing of Isolated bacteria

Molecular identification of bacteria was conducted by the National Centre for Microbial Resources- NCCS, Pune by 16s rRNA sequencing and identified isolated bacteria was *Photorhabdus luminescens*.

DEAE column chromatography

The extracellular chitinase enzyme secreted by *P. luminescence* was concentrated by centrifugal device (30 kD) to yield enzyme concentrate which was purified by DEAE column chromatography. The chromatograph showed the peak of the enzyme in the concentrate when compared with the standard chitinase enzyme chromatograph. The amount of enzyme in the fractions of column chromatography as protein content was confirmed by Folin Lowry method.

Infectivity assay of developed formulations of *P. luminescence* against white grub larvae

The infectivity of formulations of *P. luminescence* against white grub larvae was studied under laboratory conditions. The insecticidal activity was quantified as the percentage of larval mortality by recording the periodic mortality of larvae exposed to formulations of *Photorhabdus* bacteria. The obtained values were subjected to a percent white grub mortality (Table 1 and Figure 6). The white grub larvae mortality was demonstrated when exposed to *Photorhabdus* formulations after 48 hours of exposure. Formulation 3 expressed higher than 50% mortality after 72 hrs. of treatment and was significantly higher than formulations 1 and 2 tested in the study.

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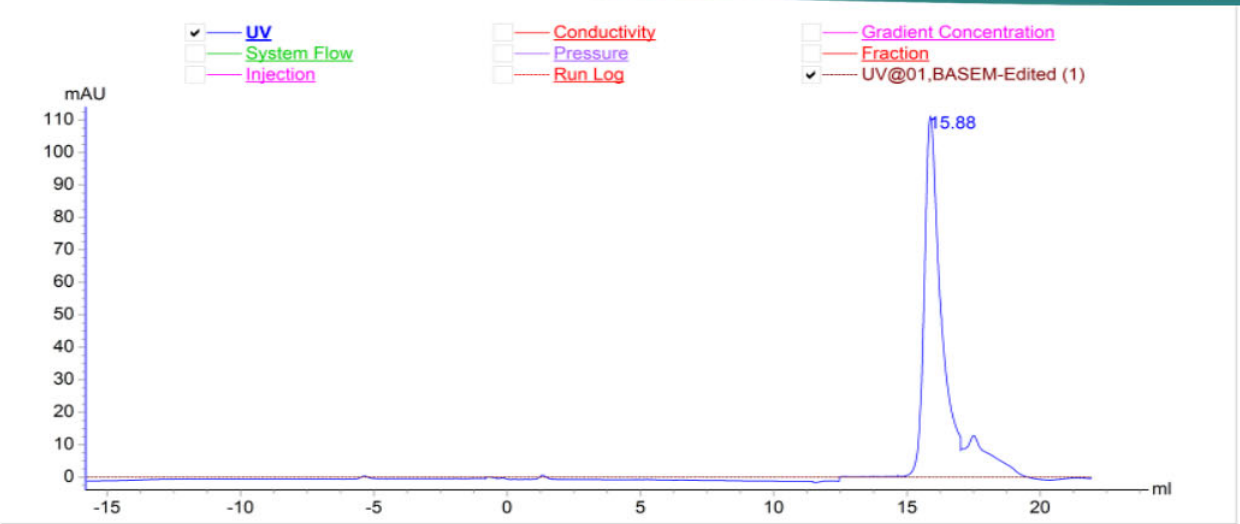

Figure 4. DEAE chromatograph of Standard chitinase

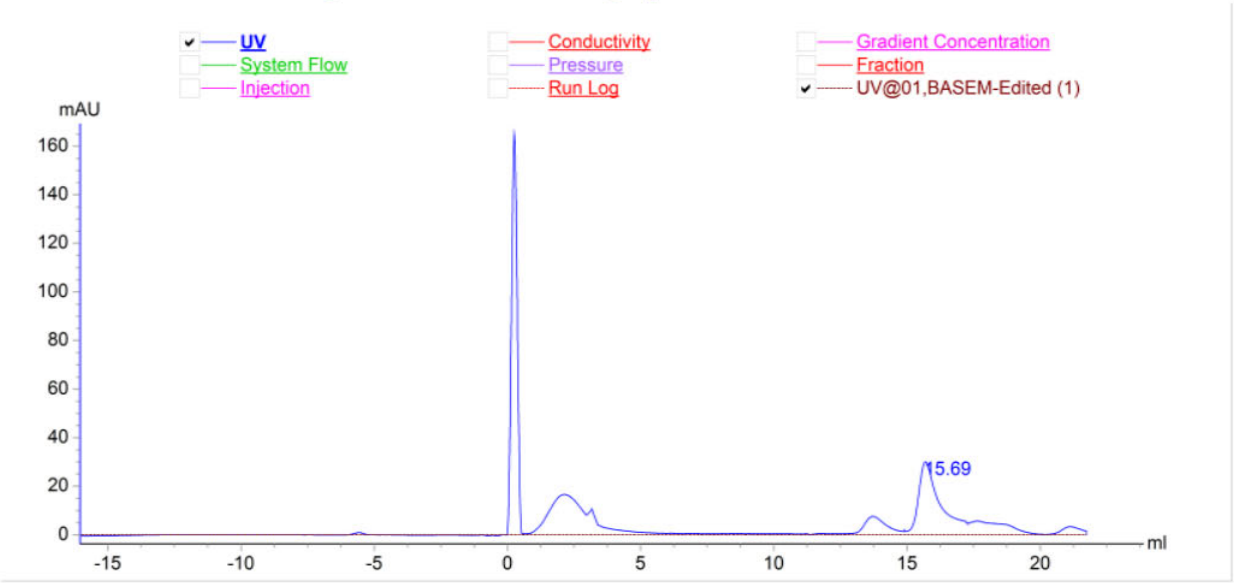

Figure 5. DEAE chromatograph of chitinase purified from supernatant

Table1. Evaluation of percent mortality of white grubs by using different formulations

S.N.	CFU/mL of formulations	Percent grub mortality (at 96 hrs)		
		Formulation 1	Formulation 2	Formulation 3
1	50	10	9	11
2	100	20	19	22
3	150	54	44	56
4	200	78	86	89
5	Untreated Control	0	0	0

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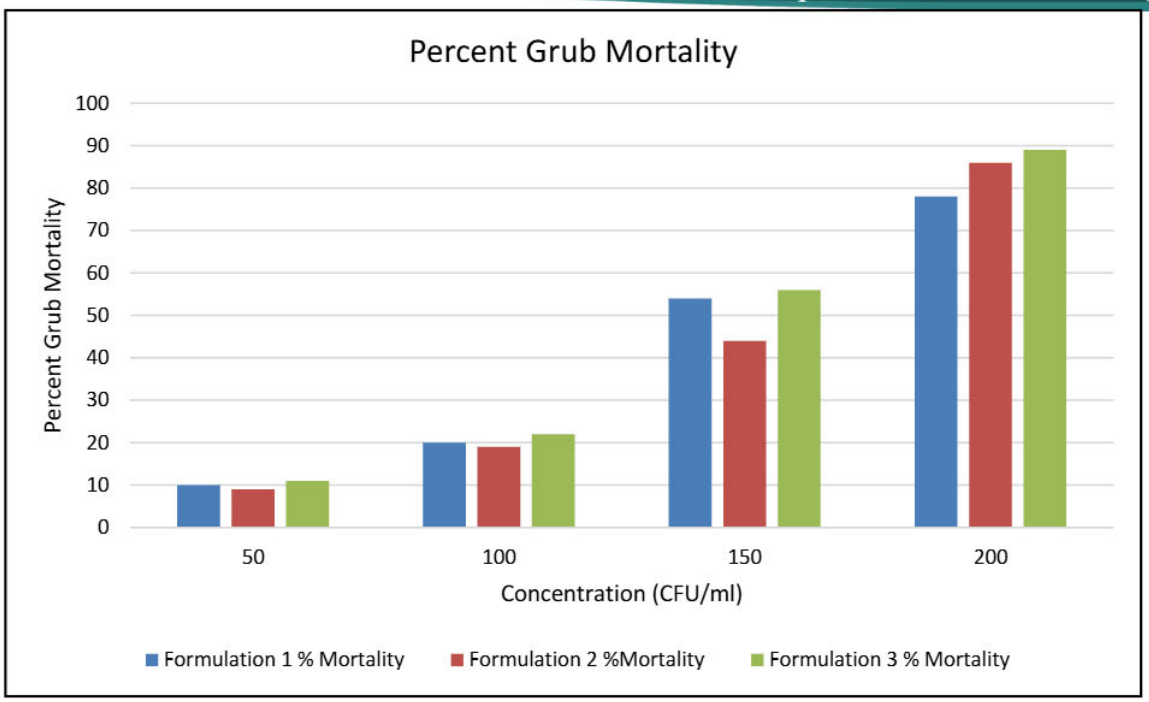

Figure 6. Percent white grub mortality after treatment with formulations of

Table 2. Minimum lethal concentration of various formulations of *Photorhabdus* bacteria

Formulation	LC 50 (CFU/mL)	Fiducial limits	Probit Equation	X^2 Value
1	133.56	72.40-272.58	y = 2.3744 x - 0.091	4.465
2	127.34	73.52-228.26	y = 2.4156 x - 0.1263	6.726
3	121.31	122.96-216.09	y = 2.4613 x - 0.1408	6.389

During probit analysis Chi-square value and coefficient of determination value, R^2 was 0.98, as the lower confidence limit and upper confidence limit were 0.001, at 95% confidence limit.

Infectivity tests were performed on white grub larvae to identify the most effective formulation that could instantly and efficiently kill the larvae at low concentrations. The LD_{50} was calculated by analysing larval lethality values (Table 2, Figure 7).

The median lethal dose (LD_{50}) is the dosage at which 50% of the larvae population is killed. In present investigation, formulation 3 showed a LD_{50} value of 121.31 CFU/mL, which was significantly lower than the other two formulations (133.56 & 127.34 CFU/mL) (Figs 8, 9 and 10)

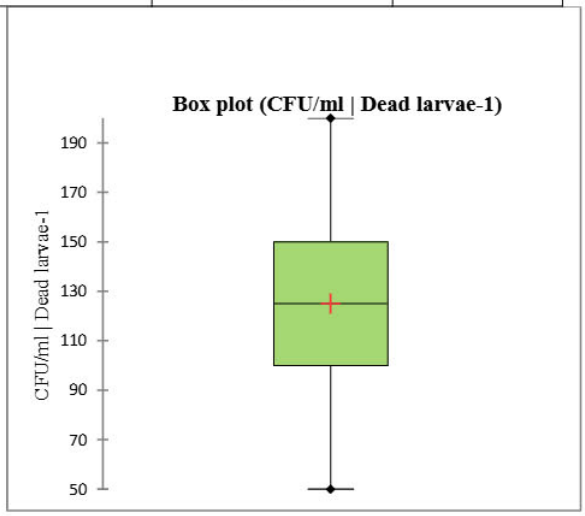

Figure 7. Box Plot – CFU/mL and Dead larvae

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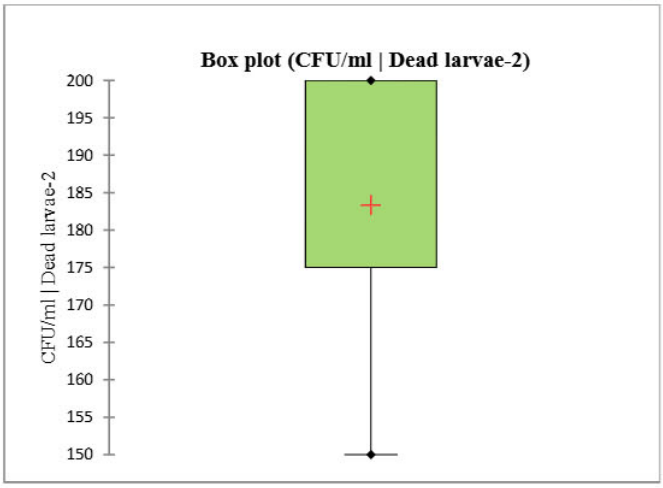

Figure 8. Box Plot – CFU/mL and Dead larvae

Table 3. Log Rank Test of Equality of Survival Distribution Functions

Statistic	Observed value	Critical value	p-value	Alpha
Log-rank	38.520	11.070	<0.0001	0.050
Wilcoxon	32.454	11.070	<0.0001	0.050
Tarone-Ware	37.016	11.070	<0.0001	0.050

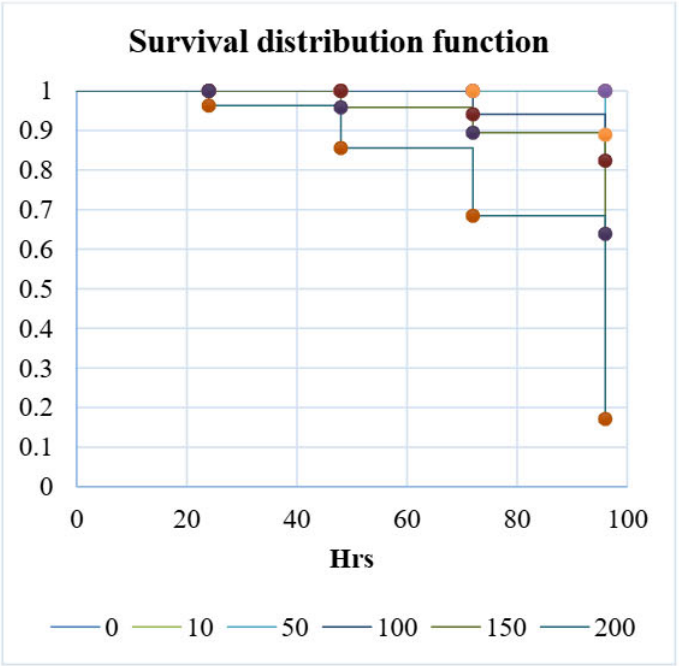

Figure 9. Kaplan- Meier survival curves of formulations against white grub larvae

All formulations were considerably different from the control group when compared to the survival curves ($P < 0.0001$). The survival analysis investigation was conducted with triplicates. The X-axis represents time in hours, while the Y-axis represents the percentage of white grub larvae that survived.

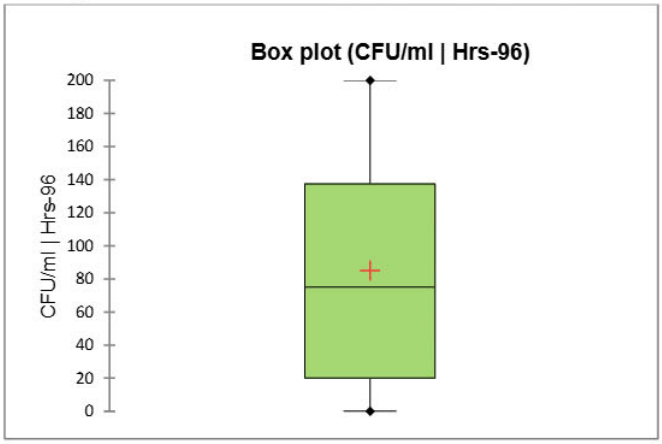

Figure 10. Box Plot – CFU/mL and survival time of larvae

Discussion

In the present investigation, *P. Luminescence* bacteria were isolated from the haemolymph of white grub larvae infested by entomopathogenic nematodes. The *P. luminescence* bacteria exhibited significant extracellular chitinase activity, which plays an important role in the mortality of insects by the dissolution of chitin present in the insect cell wall. The chitinase activity in bacterial extracellular secretion was confirmed by DEAE column chromatography. It confirms the reports of Chen et al. (1996) that the symbiotic bacteria associated with entomopathogenic nematode exhibited both exochitinase and endochitinase activity. Dominelli et al. (2022) had reported anti-fungal activity of *Photorhabdus* against *Fusarium* and demonstrated that chitin-degrading activity of *Photorhabdus* was associated with chitin binding protein and the chitinase enzyme. *P. luminescens* establishes colonisation on the fungal hyphae, subsequently leading to chitin binding protein (CBP) binding to chitin in the cell wall of phytopathogenic *F. graminearum*. This interaction facilitates the degradation of fungal cell walls by chitinase, ultimately preventing infection of plants by the fungus. Selcuk Hazir (2016) conducted a study on the effects of cell-free supernatants (10% v/v) derived from the bacteria *Xenorhabdus* and *Photorhabdus*. The results revealed that these supernatants exhibited inhibitory properties against the germination and growth of various fungal and oomycete phytopathogens. The filtrate had the metabolites that would inhibit the growth of phytopathogens (Hazir,

2016). Present research findings are in line with these previous studies. Thus, in the present investigation, we established that the insecticidal toxins secreted by *P. luminescens* have chitinase activity, which plays a central role in biocontrol activity against insect pests.

The bioefficacy of three different formulations of *Photorhabdus* bacteria was tested against white grub larvae under laboratory conditions. It was found that the mortality of the larvae occurred 24-48 hours after treatment with formulation 3 of *Photorhabdus* compared to formulations 1 and 2. Statistical analysis shows that the formulations of *Photorhabdus* have a highly significant effect on the mortality of larvae. Percent mortality after treatment with formulation 3 was significantly higher than after treatment with Formulations 1 and 2. Formulation 3 expressed significantly lower LD50 value, i.e., 121.31 CFU/mL over formulation 1 and 2 i.e., 127.34 & 133.56 CFU/mL respectively. The present study's finding proposes the biocontrol of white grub larvae using the developed formulation of *Photorhabdus* bacteria.

The death of white grub larvae after applying the formulation of *Photorhabdus* bacteria revealed that bacteria could penetrate the host without nematodes. Dudney et al. (1997) reported the cotton leaves treated with *X. nematophila* bacterial cells and their metabolites were effective against fire ants and beetroot army worms. These findings are comparable with the results obtained in this study. The bacterial cells can gain entry into a host by directly entering through the cuticle or by use of the spiracle, which serves as an alternative route of entry apart from the mouth and anus, both of which are exposed to the external environment. Elawad et al. (1999) reported on the potential of *X. nematophila* and *P. luminescence* cells to kill beet army worm pupae. This research proves that the spiracle, which serves as the sole organ of pupae, provides an avenue for ingressing these bacteria into the haemocoel, the internal body cavity. The precise mechanism of these bacteria entering the haemocoel has not yet been documented. As reported by Bowen et al. (1998), applying of toxic secretions has been found to have a lethal effect on insect pests driven by bacteria.

Bussaman et al. (2012) analysed the efficacy of a cell-free supernatant, a crude extract, and a complete cell suspension of a culture of *X. stokiae* isolate PB09 on mushroom mites' mortality and reproductive capacity. After 4 days of treatment with a whole cell solution of *X. stokiae* isolate PB09, the mite's death rate and reproductive capacity were both observed to be 81.66 percent reduced. Additionally, the average number of eggs produced per female mite decreased to 192.67. The *X. stokiae* whole cell suspension is as effective as cell-free supernatant i.e., 89.00% after 3 days of treatment & 41.33 eggs/ female (Bussaman, 2012). Shrestha et al. (2010) reported insecticidal activity of *P. temperata* and *X. nematophila* was found in culture broths and aqueous extract of both bacterial culture broth against red flour beetle.

Similarly, Mahar et al. (2004) reported the ability of *X. nematophila* and *P. luminescence* to enter the locust nymph's haemocoel devoid of the nematode vector. Mahar et al. (2004) demonstrated that the direct application of formulations of symbiotic bacteria to plants can control locusts. Similarly, Mohan et al. (2003) applied a concentration of 10^8 CFU/mL on plant foliage within 24 hours examined the pathogenicity of *P. luminescence* bacteria against cabbage butterfly and observed significant larval mortality. David et al. (2017) discovered that the extracellular release of *P. luminescence* exhibited significant pathogenicity towards *G. mellonella* and *S. frugiperda* larvae. This effect was found at doses ranging from 1 to 10 colony-forming units (CFU) per larva, resulting in mortality of insects within 48 to 72 hours after injection. Palmieri et al. (2019) indicated two strains of *Photorhabdus* sp. and their metabolites against *Phytophthora* in the laboratory and field experiments on papaya plants. Both *Photorhabdus* strains inhibited *Phytophthora sp.* growth by over 62% at 120 hours in vitro. These findings showed the potential of bacterial broth compared to the cell-free cultures in managing the oomycete (Palmieri, 2019).

White grubs are highly polyphagous and the most destructive soil pests that damage a wide variety of crops. In the present investigation, we evaluated the efficacy of *Photorhabdus* formulations in the mortality of the white grub larvae. The results revealed the potential of developed formulations of *P. luminescens* as a biocontrol agent against white grub larvae under laboratory conditions.

Conclusion

The *Photorhabdus* bacterial formulation utilised in the current study demonstrated significant insecticidal efficacy towards white grub larvae. Among the formulations, formulation 3 showed 89% mortality at 96 hours with a minimum lethal dose (LD_{50}) of 121.31 CFU/mL. Formulation of *Photorhabdus* bacteria as a biocontrol agent is one of the best alternatives to chemical insecticides. Due to their diverse array of insecticidal toxins, they exhibit enhanced adaptability for field applications in the biocontrol of an extensive range

of insect pests. The experimental results demonstrated a higher level of efficacy against the white grub larvae, suggesting its potential for further evaluation in terms of biocontrol effectiveness through pot and field studies. In order to use these bacteria in field application, it would be necessary to find out techniques for their ease of mass production, application method, and persistence in the environment to fulfil their potential as a biocontrol agent. The *Photorhabdus*, as a microbial biocontrol agent, is an integrated approach that will certainly provide significant and selective insect control. *Photorhabdus* bacterial formulations hold the key to sustainable agriculture through the effective and successful control of white grub infestation, which is an extremely destructive and highly polyphagous soil pest.

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Conflict of interest

No conflict of interest.

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