Generation and evaluation of nanoparticles of supernatant of *Photorhabdus luminescens* (Thomas and Poinar) against mite and aphid pests of cotton for enhanced efficacy

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Cell-free supernatant of *Photorhabdus luminiscens* was converted to nanoparticles (NPs) using a spray dryer fitted with ultrasonic nozzle. NPs were characterized by both scanning electron microscopy and zeta size analyser, and found to have average particle diameter of 89 nm. While converting to NPs, gum arabica @ 3% was used to eliminate hygroscopic property. Nanoparticulated supernatant exhibited superior pesticidal property against serious sucking pests of cotton, viz. *Tetranychus macfarlanei* and *Aphis gossypii*. On mites, NPs of *P. luminiscens* recorded lower median lethal concentration (LC_{50} : 0.0001 ppm) compared to normal form (8.36×10^2 ppm) within 12 h of exposure. Similarly, on aphids, lower LC_{50} (LC_{50} : 0.0027 ppm) was recorded by NPs compared to normal form (LC_{50} : 2.12×10^3 ppm). High mortality coupled with quick action emphasizes the potential of nanotechnology in enhancing the pathogenicity of a microbial pesticide.

Keywords: Aphid, cotton, mite, nanoparticles, *Photorhabdus luminescens*.

PHOTORHABDUS LUMINESCENS (Thomas and Poinar) is an endosymbiont of entomopathogenic nematodes (EPNs) *Heterorhabditis* spp. belonging to the family Enterobacteriaceae in the gamma subdivision of purple bacteria. It is a non-spore forming, motile, bioluminescent, Gram-negative, facultative anaerobic bacterium producing an array of toxin complexes and enzymes. These extracellular secretions have a wide range of insecticidal actions against both sucking and chewing arthropod pests of agricultural crops. Some of the broad-spectrum toxin complexes include oral toxin complex proteins (T_c), injectable toxin (makes caterpillar floppy 1 – mcf1), binary toxin (Pir AB)[1–5] and several enzymes, viz. chitinase, protease and lipase[6–8]. As a result, bacterial solution of *P. luminiscens* has been employed as topical pesticide against a wide range of insects like mite, aphid, mealybug, etc.[9–12]. Topical application of *P. luminiscens* in the form of spray is advantageous in terms of safety, potency and specificity compared to chemical spray, besides being bio-degradable, providing a large and competitive market. However, to increase the efficacy of this biopesticide comparable to synthetic pesticides, it is quite essential to achieve the quick knockdown of the target pest.

Nanotechnology offers a good opportunity to enhance the efficacy of such bio-molecules by decreasing the particle size, thus increasing the surface-to-mass ratio compared to bulk equivalents. It also aids in developing superior bio-pesticide formulations comparable to chemical pesticides. Surprisingly, few reports of conversion of bio-agents or their by-products are available to enhance the toxicological properties. Hence, in the present study an attempt has been made to improve the efficacy of extracellular secretions of *P. luminiscens* by downsizing the particles through nanotechnological methods.

The endosymbiotic bacterium, *P. luminescens* used in the present study was recovered from the local isolate of entomopathogenic nematode, *Heterorhabditis indica* from the soils of sugarcane cropping ecosystem during 2012–13. Identification of the bacterium was done through genome sequence studies which matched *P. luminescens luminiscens* (accession no. KP224437) to the extent of 85% through blasting at the National Center for Biotechnology Information (NCBI), USA.

A loop full of symbiotic bacteria, *P. luminescens* from NBTA (nutrient bromothymol blue agar) medium was inoculated to sterilized nutrient broth (ca. 150 ml) under aseptic condition and incubated in biochemical oxygen demand (BOD) under ambient condition (28°C and 85–90% relative humidity) for 24–48 h. Stock solution having bacterial concentration of 10^{10} cfu was prepared through serial dilution method and centrifuged at 4000 rpm for 20 min to get a supernatant solution. The cell-free supernatant (CFS) was collected in separate test tubes and dried using a spray dryer fitted with ultrasonic nozzle for the production of nanoparticles (NPs).

Downsizing of any material mainly follows two approaches, viz. top-down and bottom-up, both of which use physical, chemical and biological methods to produce the NPs[13]. CFS was converted to nano-sized particles by physical method using spray dryer (Labultima, LU-222 advanced, Labultima, Mumbai; vertical co-current type with an evaporation rate of 1000 ml/h fitted with an ultrasonic nozzle).

CFS of the bacterium was mixed with gum arabica (3%) as a binder to eliminate the hygroscopic property of the material by hand-stirring. Later, the thoroughly mixed solution was sprayed using a spray dryer fitted with an ultra sonic nozzle to convert into NPs by setting suitable parameters (Table 1).

The CFS solution was first broken into small droplets using ultrasonic assisted atomizer. These fine droplets

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were directed into a hot air-assisted vacuum chamber. Through continuous heat and mass transfer in the chamber, moisture evaporated from the particles and dry powder was collected in the cyclone chamber, which was later removed through gravitational effect.

The morphological features of nano supernatant were studied using scanning electron microscope (SEM; EVO 10, Carl Zeiss Microscopy, Germany). Fine particles were placed on a double-sided graphite tape fixed to stubs. A thin layer of gold–palladium (Ag–Pd) was coated over the nano supernatant for 45 sec to make the samples conductive using sputter coater (Quorum technologies, OM-SC7620, United Kingdom). SEM of the samples was performed at an accelerating voltage of 5–20 kV.

The bio-efficacy of nano CFS of *P. luminiscens* was evaluated against cotton mite following concentration – mortality response method (median lethal concentration – mLC_{50}). Cotton mites were exposed to 10 different concentrations of NPs of CFS ranging from 0.0001 to 10^5 ppm on an inert surface such as borosilicate glass petri dish (9 cm × 1.5 cm, diameter × height) in order to avoid the influence of other factors on toxicological properties of *P. luminiscens*. NPs solution (ca 2 ml) was pipetted out into a liquid reservoir fitted in the potter tower. The solution was sprayed on the inner surface of a petri dish placed on a pneumatic spray table under high pressure. Similar procedure was followed to cover the inner surface of the lid as well. After a few minutes, 10 second instar mites were released on the treated plate, covered with the lid and tightly packed with the help of parafilm. The solution with only distilled water spray served as control. In another set of experiments, different concentrations of normal CFS of *P. luminiscens* equivalent to NPs of CFS were treated on an inert surface following the same methodology. In both the experiments, each treatment was replicated four times and each replication consisted of 10 mites. Observations on nymphal mortality were recorded at 12 and 24 h after treatment. Data obtained were subjected to probit analysis to find mLC_{50}.

Similar procedure was followed to evaluate the bio-efficacy of NPs of CFS in comparison to normal CFS of *P. luminiscens*. In this study, second instar nymphs of the aphid were subjected to evaluation. The data obtained were subjected to probit analysis to find mLC_{50}.

The particle size characterization of nano supernatant by zeta potential revealed an average particle diameter of 89 mm. Figure 1 shows three peaks corresponding to a particle size below 100 mm, with average sizes of nano supernatant at 89, 90 and 93 nm respectively, in three counts. Thus, the nanosuspension of supernatant meets the quality criteria.

Table 1. Defining parameters for downsizing cell free supernatant by spray dryer with ultrasonic nozzle

Parameter	Value
Inlet temperature	120°C
Outlet temperature	80°C
Feed-flow rate	1 ml min^{-1}
Aspiration rate	40 $Nm^3.h^{-1}$
Inlet high temperature	180°C
Outlet high temperature	90°C
Cool temperature	60°C
D-block on	1 sec
D-block off	90 sec
Cycle time	225 min

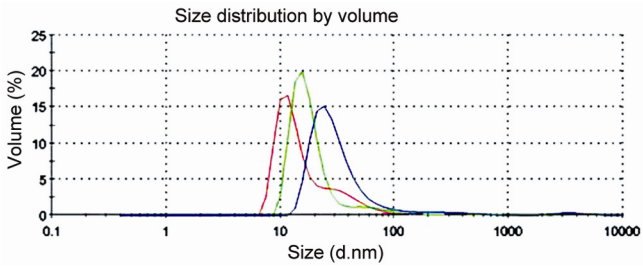

Figure 1. Distribution of particle size of nano supernatant using a Zetasizer (three counts).

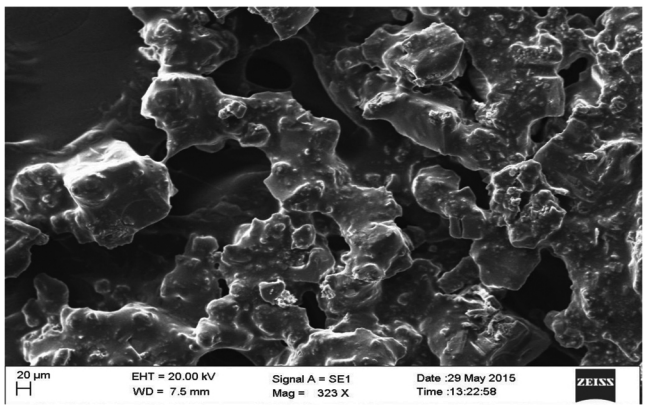

Figure 2. SEM image of normal supernatant of *Photorhabdus luminescens*.

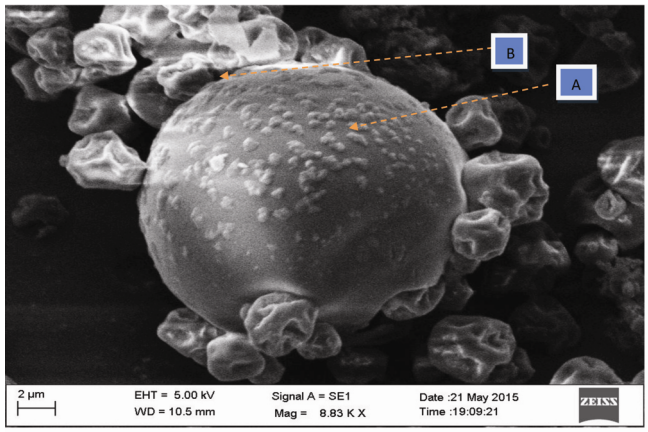

Figure 3. SEM image of nano supernatant of *P. luminescens*. (A) Nano deposits of supernatant; (B) Gum arabica particles.

Table 2. Median lethal concentration of normal and nano supernatant of *Photorhabdus luminescens* against *Tetranychus macfarlanei* on inert surface

Time (h)	χ^2	Regression equation	LC_{50} (ppm)	Fiducial limits LL–UL	LC_{99} (ppm)	Fiducial limit LL–UL	R^2
Median lethal concentration of normal supernatant							
12	0.68	$Y = 0.24x + 4.29$	8.36×10^2	1.73×10^2–4.02×10^3	3.08×10^{12}	3.07×10^9–3.16×10^{15}	0.98
24	5.21	$Y = 0.34x + 4.90$	1.07	0.25–4.50	1.72×10^8	1.51×10^6–4.84×10^{10}	0.92
Median lethal concentration mortality of nano supernatant							
12	1.40	$Y = 0.29x + 6.12$	0.0001	1.88×10^{-5}–0.0006	3.93×10^4	4.15×10^2–3.72×10^6	0.95
24	1.43	$Y = 0.33x + 6.37$	7.61×10^{-4}	2.20×10^{-5}–0.0002	1.06×10^3	3.2×10^1–3.53×10^4	0.96

*LL, Lower limit; UL, Upper limit. **n = 40 second instar nymphs.

Table 3. Median Lethal concentration of normal and nano supernatant of *P. luminescens* against *Aphis gossypii* under spray and release method on inert surface

Time (h)	χ^2	Regression equation	LC_{50} (ppm)	Fiducial limits LL–UL	LC_{99} (ppm)	Fiducial limit LL–UL	R^2
Median lethal concentration of normal supernatant of *P. luminescens*							
12	0.60	$Y = 0.22x + 4.24$	2.12×10^3	3.29×10^2–1.35×10^4	6.47×10^{13}	1.14×10^{10}–3.66×10^{17}	0.98
24	1.75	$Y = 0.28x + 5.27$	0.12	0.023–0.69	2.64×10^7	3.52×10^5–1.98×10^9	0.94
Median lethal concentration mortality of nano supernatant of *P. luminescens*							
12	1.58	$Y = 0.32x + 5.74$	0.0027	0.00056–0.013	4.41×10^5	5.34×10^3–3.64×10^7	0.96
24	1.08	$Y = 0.23x + 5.94$	0.00018	3.21×10^{-5}–0.0021	2.24×10^5	1.76×10^3–2.85×10^7	0.99

n = 40 second instar nymphs.

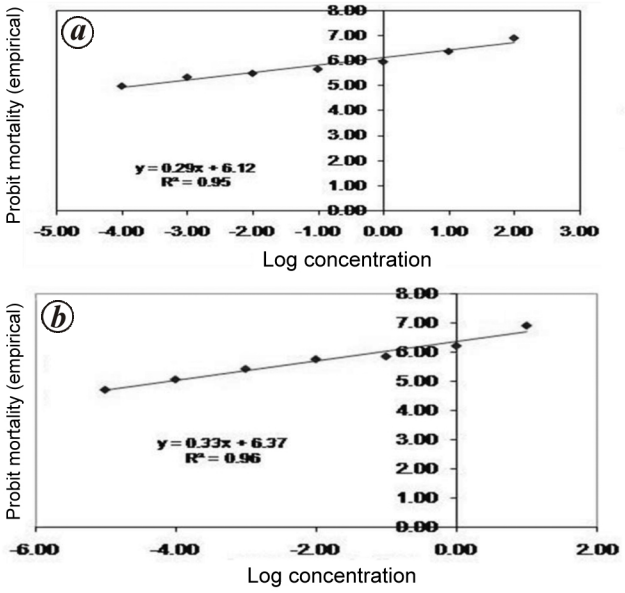

Figure 4. Regression equation of nano supernatant of *Photorhabdus luminescens* against *Tetranychus macfarlanei* at different time intervals: (***a***) 12 h and (***b***) 24 h.

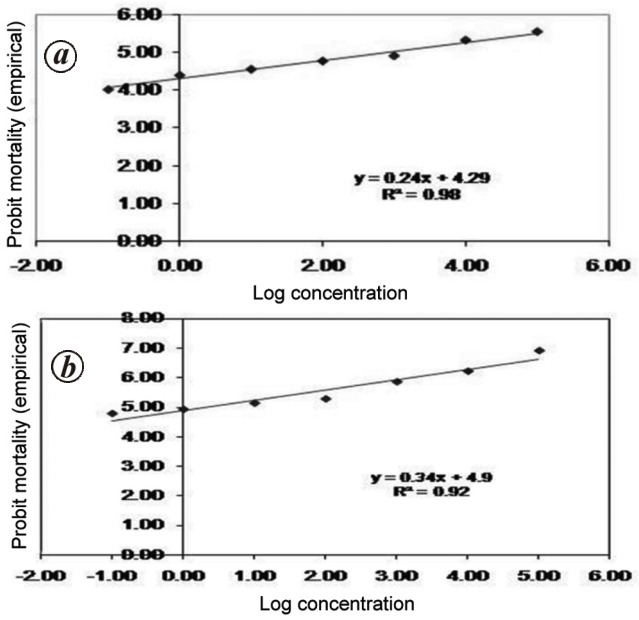

Figure 5. Regression equation of normal supernatant of *P. luminescens* against *T. macfarlanei* at different time intervals: (***a***) 12 h and (***b***) 24 h).

SEM of normal and nano supernatant of *P. luminiscens* can be visualized under magnification ranging from 5 to 20 kV. At 20 kV, SEM image of normal supernatant contained larger diameter particles with irregular shape (Figure 2), whereas at 5 kV SEM image of nano supernatant contained almost spherical-shaped particles with tiny particles of toxin deposited on the bigger spherical particles which discharge on dilution to universal solvent (Figure 3, A). The other irregular particles are of gum arabica (Figure 3, B).

The mLC_{50} of nano form of the supernatant against *T. macfarlanei* registered 0.0001 ppm ($y = 0.21x + 5.74$; $\chi^2 = 1.4$; $R^2 = 0.95$) and 7.61×10^{-4} ($y = 0.33x + 6.37$; $\chi^2 = 1.43$; $R^2 = 0.96$) at 12 and 24 h after exposure

respectively (Table 2 and Figure 4 *a* and *b*). This is quite low compared to mLC_{50} of normal supernatant recorded at 12 (8.36×10^2 ppm; $y = 0.24x + 4.29$; $\chi^2 = 0.68$; $R^2 = 0.98$) and 24 h (1.07 ppm; $y = 0.34x + 4.90$; $\chi^2 = 5.21$; $R^2 = 0.92$) after exposure (Table 2 and Figure 5 *a* and *b*). It was noticed that the LC_{50} value reduced drastically as the exposure period increased both in nano and normal forms of supernatant of *P. luminiscens*. Similarly, nano supernatant registered lower mLC_{50} against *Aphis gossypii* at 12 h (0.0027 ppm; $y = 0.32x + 5.74$; $\chi^2 = 1.58$; $R^2 = 0.96$) and 24 h (0.00018 ppm; $Y = 0.23x + 5.94$; $\chi^2 = 1.08$; $R^2 = 0.99$) (Table 3 and Figure 6 *a* and *b*). Whereas normal supernatant registered higher mLC_{50} at 12 h (2.12×10^3 ppm; $y = 0.22x + 4.24$; $\chi^2 = 0.6$; $R^2 = 0.98$) and 24 h (0.12 ppm; $y = 0.28x + 5.27$; $\chi^2 = 1.75$; $R^2 = 0.94$; Table 3 and Figure 7 *a* and *b*)). Here also, the LC_{50} value decreased as the exposure period increased from 12 to 24 h in both the cases. This indicates the enhanced toxicity level of supernatant of *P. luminiscens* when converted into nano size.

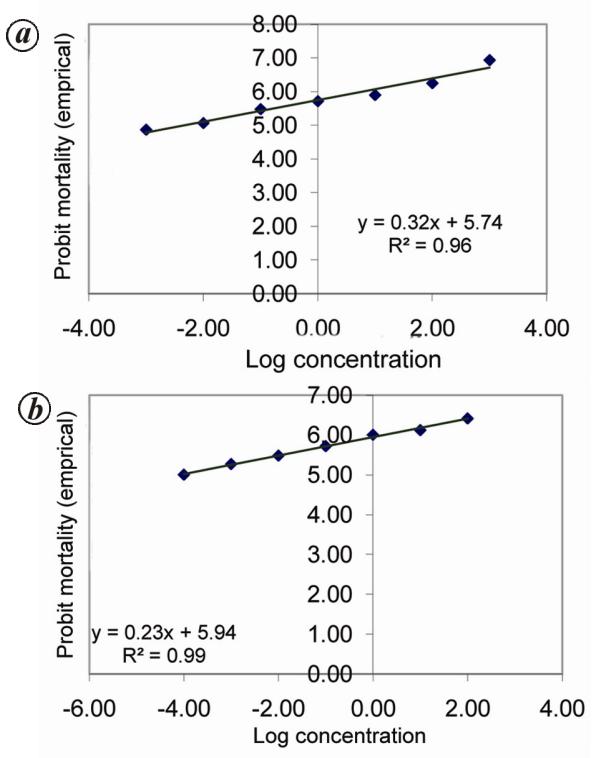

Figure 6. Regression equation of nano supernatant of *P. luminescens* against *Aphis gossypii* at different time intervals: (***a***) 12 h and (***b***) 24 h.

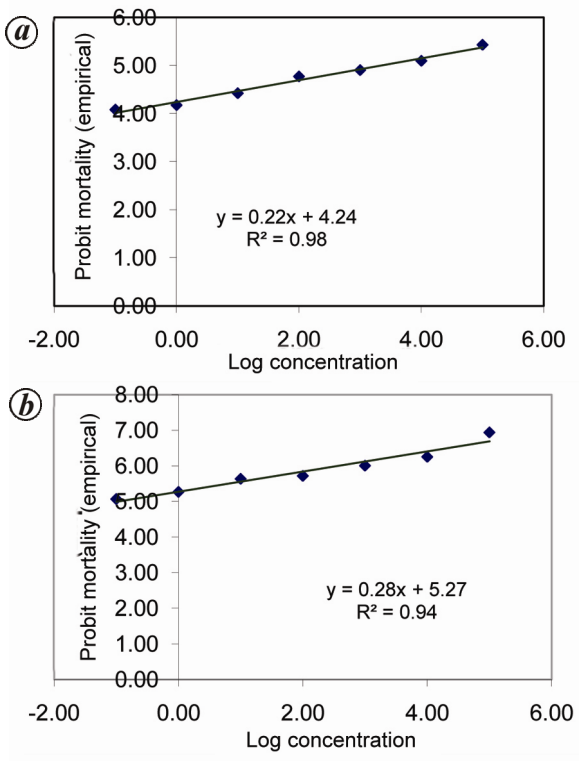

Figure 7. Regression equation of normal supernatant of *P. luminescens* of *Aphis gossypii* at different time intervals: (***a***) 12 h and (***b***) 24 h.

The endosymbiont, *P. luminiscens* produces an array of putative insecticidal toxins which can be categorized into three main classes. Members of the first class include toxins which are orally toxic to caterpillars[1], the second class includes 'makes caterpillars floppy (mcf)' which is effective upon injection[2], and the third being '*Photorhabdus* insect related (Pir AB)' which includes binary toxins[4,5]. Apart from these modes of entry, reports on strong contact mode of action are also available against soft-bodied insects like mealy bugs[9], aphids and thrips[12], and non-insect arthropods like mites[11]. The direct application of extracellular secretion of *P. luminiscens* on these insects resulted in cuticular lysis leading to red-coloured luminescent cadavers.

The present study is a report on the generation and evaluation of NPs of the supernatant of *P. luminescens* using a spray dryer fitted with an ultrasonic nozzle. Nymphal bioassays recorded quicker and higher knock down with nano supernatant even at lower doses, over normal supernatant. The quicker mortality of target insect can be attributed to higher penetration power of the particle, which acts as a tiny carrier of the toxin complex and knocks down the insect immediately and turns the cadaver into slight dark red colour. Similar attempts have been made on *Bacillus thuringiensis*, a different entomopathogenic bacteria[14]. The results revealed the enhanced insecticidal property of the bacterium when converted into NPs.

The increase in insecticidal activity was attained in the present study using nanotechnology, which has the power to revolutionize agriculture, including pest management in near future. Research on NPs and insect control should be geared towards the introduction of faster and eco-friendly pesticides in the future.

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Detection of harmful adulterants in milk supplied to Delhi, India

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Milk adulteration is a serious problem in developing countries. It cheats the consumers and poses a serious threat to their health. The present qualitative analysis was undertaken to study the presence of adulterants in milk supplied to Delhi and adjoining regions (Faridabad, Gurgaon and Noida). A comparative analysis was carried out for the extent of different adulterants present in both packaged and locally available milk samples. Seventy five milk samples were tested for the presence of neutralizers, skimmed milk powder, urea, detergent and ammonium sulphate. Most milk samples collected from Delhi and adjoining regions tested positive for neutralizers and skimmed milk powder. In addition, some samples also tested positive for detergent, urea and ammonium sulphate. Considerable number of unpackaged milk samples showed presence of ammonium sulphate and detergents compared to packaged ones. Surprisingly, urea was present only in packaged samples.

Keywords: Adulteration, ammonium sulphate, detergent, neutralizer, skimmed milk powder, urea.

MILK is a rich source of nutrients required for proper growth and maintenance of body[1]. These nutrients are in readily assimilable form and can be easily absorbed. Milk and milk products form a significant part of our diet and a substantial amount of our food expenditures goes on milk and other dairy products. India is the largest milk producer in the world with an output of 160 million tonnes (MT) recorded in 2015–16 (ref. 2). At the same time, it remains the largest milk consumer as well. The consumption of milk far outweighs its production in India. According to the National Dairy Development Board (NDDB), estimated demand for milk in India would be 200 MT by 2021–22 (ref. 2). To overcome the growing demand, adulteration of milk has become more and more prevalent in India. The opaque and fluid nature of milk makes it highly vulnerable to adulteration, further affecting the dairy products.

Milk adulteration is a significant problem in all developing countries and third world nations[3–8]. There have been reports of adulteration from all parts of India[9–15]. Recently, a report indicated that 25% of the milk samples tested in Maharashtra (India) did not comply with the

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