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Challenges in Biochemical Engineering and Biotechnology for Sustainable Environment

Biocompatible Chitosan Nanoparticles Incorporated Pesticidal Protein Beauvericin (Csnp-Bv) Preparation for the Improved Pesticidal Activity Against Major Groundnut Defoliator *Spodoptera Litura* (Fab.) (Lepidoptera; Noctuidae)

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Abstract : Nanotechnology is generally seen as a new and fast emerging field that involves the manufacture, processing, and application of structures, devices and systems by controlling the shape and size at the nanometer scale .Their application in agriculture is relatively recent compared with their use in drug delivery and pharmaceuticals. Smart delivery of nutrients, pesticides, herbicides, biocontrol agents and sensors for the diagnosis of plant diseases are some of the emerging topics of nanotechnology for agriculture. In the present study, chitosan nanoparticles incorporated insecticidal protein beauvericin (CSNp-BV) was evaluated against improved pesticidal activity against *Spodoptera litura* .CSNp-BV was prepared by ionic gelation method and the nanospheres formed were characterized using scanning electron microscopy which showed a particle size of nanosphere in the range of 160-230nm. Loading efficiency and entrapment efficiency was found to be 82 and 85%. The *in vitro* drug release profile was studied by continuous dialysis method. Cumulative release reached almost 91% after 24 hours. Pesticidal activity revealed all the life stages were susceptible to the CSNp-BV formulation and maximum mortality was recorded in early larval instars. CSNp-BV treatment revealed reduced pupal and adult emergence.

Key words: chitosan nanoparticles, beauvericin, pesticidal activity, *Spodoptera litura.*

Introduction & Experimental

All over the world, the conventional practice of using chemical pesticides and other agrochemicals is either waning or being banned due to their toxic effects on human beings and live stock, residual toxicity, pest outbreaks and adverse impact on beneficial non-target organism.[1,2] In the current WTO regime, it is absolutely necessary to limit the use of chemical pesticides to remain in the world market and sustain the competition. Among the safe and effective alternatives considered, entomopathogens such as bacteria, virus, fungi, protozoa and nematodes even otherwise play a major role in the natural regulation of pest population and therefore, offer opportunities for gainful employment as augmentative bio-control agents. Their relative specificity to target pest, safety to non-target beneficial organisms and their ability to cause epizootics make them attractive alternatives for sustainable pest management.[3,4,5] The edible oil economy of India primarily depends on groundnut (*Arachis hypogea* L.)Cultivation.[6,7] The army worm *Spodoptera litura* (Lepidoptera; Noctuidae) is

the deadliest of all the 360 species of groundnut pests. The early instars of *S. litura* feed on the leaves, flowers and pods of groundnut and reduce the production whereas, the late larval stages feed on pods and also other parts.[8] The control of the pest using chemical pesticides has failed as the pest develops resistance to them. Biopesticides based on fungi and toxic pesticidal compounds they produce, are highly effective against major lepidopteron pests including *S. litura*. Destruxin, a cyclodepsipeptide from *Metarhizium anisopliae* , beauvericin, aurenicin and enniatin complex from *Beauveria bassiana* and *Peacilomyces fumosoroseus* and baassiamide and dipicolinic acid from *Verticillium lecanii*[9] have all proved to be effective against lepidopteron defoliators and sucking pests , have established the pesticidal effects of a cyclodepsipeptide from the culture filtrate of *Nomuraea rileyi* itself against the third instar larvae of soybean defoliator, *Anticarsia gemmatalis*.

Nanotechnology has captured the imagination of researchers, manufacturers, and even the general population in recent years. Nanotechnology is generally seen as a new and fast emerging field that involves the manufacture, processing, and application of structures, devices and systems by controlling the shape and size at the nanometer scale[10,11].Their application in agriculture is relatively recent compared with their use in drug delivery and pharmaceuticals. Smart delivery of nutrients, pesticides, herbicides, biocontrol agents and sensors for the diagnosis of plant diseases are some of the emerging topics of nanotechnology for agriculture. In the form of nanoparticulated biopesticide, beauvericin (BV) a cyclodepsipeptide produced by entomopathogenic fungi *Beauveria bassiana* is expected to be very active and deadly against *Spodoptera litura* with enhanced levels of safety, free from environmental concerns and producing chemical-free groundnut which is used both as food and oil crop. However, the mode of action will be different. With *M.anisopliae* biopesticide, the time from exposure to morbidity/paralysis and death of the target insect may be between 3 and 14 days depending on species and size. Understanding the fundamental differences in the mode of action of nanopesticide based biopesticide is important since there is no involvement of cuticle penetration, germ tube formation, hyphae multiplication and spore formation on the surface of cadavers as applicable to the fungal conidia[12].The present study deals with preparation of beauvericin incorporated with chitosan nanoparticles against larval instars of major groundnut defoliator *Spodoptera litura*.

Extraction and Purification

Beauvericin was isolated and purified according to the method with minor modification. A litre of Czapek dox broth broth was prepared in 2 litre conical flasks and inoculated with 10 ml of Beauveria *bassiana* spore suspension (1.0 x 10^8spores/ml) and incubated at 28°C on a rotary shaker at 150 rpm for seven days. After the incubation period, the broth was filtered through sterile cheese cloth and the collected filtrate was extracted with double the volume of ethyl acetate and concentrated in a rotator evaporator to leave the crude residues containing beauvericin and subjected to successive cations-anions exchange chromatography and individual fractions will be pooled, lyophilized and used for further studies.

Preparation of Chitosan Nanoparticles

0.75 %w/v of chitosan (analytical grade) was dissolved in 1.5 %v/v acetic acid solution. Sodium tripolyphosphate solution was prepared in distilled water in concentrations at 0.75.%w/v and added drop wise with a syringe to chitosan solution while stirring, followed by sonication for 20 min. The resulting suspension was centrifuged at 15000 rpm for 10 min. The pellets obtained was re-suspended in deionised water by sonication, centrifuged and dried at room temperature (about 25^0C). Beauvericin-loaded chitosan nanoparticles were formed spontaneously upon drop wise addition of 12 ml of 0.4 % aqueous sodium tripolyphosphate solution to 20 ml of 0.35 %w/v chitosan solution containing 2 – 5 mg/ml of the beauvericin under magnetic stirring, followed by sonication. The suspension thus obtained were freeze dried. The synthesized particles were characterized by electron microscopic studies

Incorporation of Beauvericin with Chitosan Nanoparticles (CSNP-BV)

Incorporation of beauvericin in nanoparticles was carried out by modified method[13]. In this method, beauvericin with the final concentration of 0.05mg dissolved in 50 ml of chitosan nanoparticle solution under magnetic stirring for 6 hrs at 25°C and the suspension were freeze dried, used for further analysis.

Determination of Loading Efficiency

The loading capacity of nanoparticles was determined by first separating the nanoparticles formed from the aqueous medium by ultracentrifugation at 15000 rpm for 30 min. The amount of free beauvericin in the supernatant was measured by UV spectrophotometry at 257nm .Beauvericin entrapped in the nanoparticles was calculated by the following formula

Entrapment efficiency (%) = (Tp – Tf) x100/Tp where Tp is the total beauvericin used to prepare the nanoparticles and Tf is the free beauvericin in the supernatant.

Evaluation of *IN VITRO* Release

Beauvericin incorporated chitosan nanoparticles after separation by ultracentrifugation, was re-dispersed in 5mL 0.2 mol/L phosphate buffer solution (pH 7.4), placed in a dialysis membrane bag, tied and immersed in 150mLof PBS in a 250ml beaker. The entire system was stirred continuously at 37 °C with a magnetic stirrer. At pre-determined time intervals, 5mL of the release medium was removed and replaced with 5mL of fresh PBS solution. The amount of beauvericin in the release medium will be evaluated by UV spectrophotometry at 257nm.

Screening of Pesticidal Activity Against *Spodoptera litura*

Pest Collection and Maintenance

The egg masses and larvae instars were collected from groundnut fields and maintained in the laboratory in a plastic container. Ten instars of respective larval stages were transferred to the plastic containers provided with moist filter paper on the surface and fresh groundnut leaves as feed.

Bioassay with Larvae

Chitosan nanoparticles incorporated beauvericin preparation was dissolved in distilled water at concentrations of 1.0, 0.1, 0.01 and 0.001 g/ml. Fresh groundnut leaves were sprayed with respective concentration using hand held atomizers and sprayed leaves allowed to shade dry and will be placed in s plastic container (34 mm x 21 mm) provided with moist cotton swap covered with tissue paper at the bottom of the container to provide humidity. The containers were covered with meshed lid to provide aeration to the larvae. The containers were incubated at 28 ± 0.5^{0}C in an incubator (Remi BOD incubator, Mumbai). Daily observation on larval mortality was recorded for a period of 4 days. After 96 hours of the treatment, all the surviving larvae from each treatment was transferred to another container of the same size for further development. The total larval and pupal durations, adult longevity and the adult emergence were recorded.

Results & Discussion

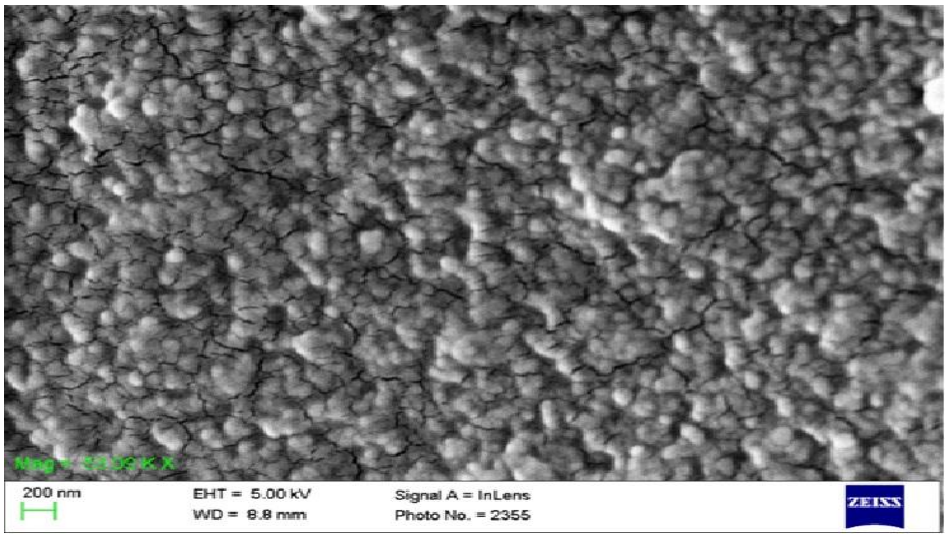

Figure1: Scanning Electron micrograph of chitosan nanoparticles

Beauvericin was isolated from the culture filtrate ethyl acetate extraction followed by successive cation and anion exchange chromatography separation and the final yield of beauvericin was found to be 177.5mg/litre of media. Isolated beauvericin was further functionalized with chitosan nanoparticles. Chitosan nanoparticles were synthesized by ionic gelation method and the synthesized nanoparticles were characterized by scanning electron microscopy which revealed uniform spherical particles with the size of 60-70 nm (Figure 1). Functionalization of beauvericin with the chitosan nanoparticles was carried out by ionic gelation method with

beauvericin. Functionalized beauvericin was further characterized by SEM which revealed spherical particles with the size range 160-230nm of electron dense core shell (Figure 2). The loading efficiency and the entrapment efficiency of the beauvericin on to the chitosan nanoparticles found by the spectrophotometric analysis of the CSNp-BV conjugate suspension. The unbound CS concentration was found by correlating the absorbance of the supernatant after the centrifugation with the standard absorbance concentration ratio. The drug loading and entrapment efficiency was in the range of 82 and 85 % respectively. In vitro control release of beauvericin was studied using 1% PBS. The sample was taken at regular intervals and analysed spectrometrically. The release percentage was calculated using the initial beauvericin concentration and the release at specified time. Beauvericin release was calculated for 24 hours. There was a burst release of drug in the early hours and a total release of about 91% was observed. An initial burst of 32.3% in the first 4 hours can be observed. In the following 6 hours, cumulative release reached 41.8%, in a sustained manner. Cumulative release reached almost 91% at 24 hours and showed an almost released ability of the nanoparticle formulation (Figure 3). The generally sustained and controlled release profile of beauvericin facilitates the application of nanoparticles for the delivery of pesticidal toxins. Pesticidal activity study shows all the larval instars were susceptible more to the CSNp-BV than the free BV.

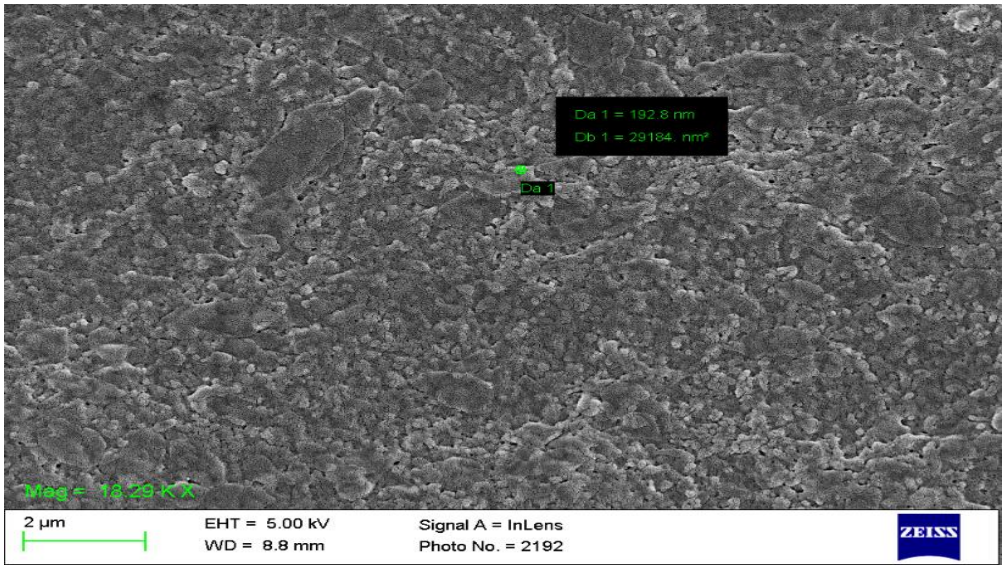

Figure 2.Scanning electron micrograph of chitosan nanoparticles incorporated beauvericin (CsNp-BV).

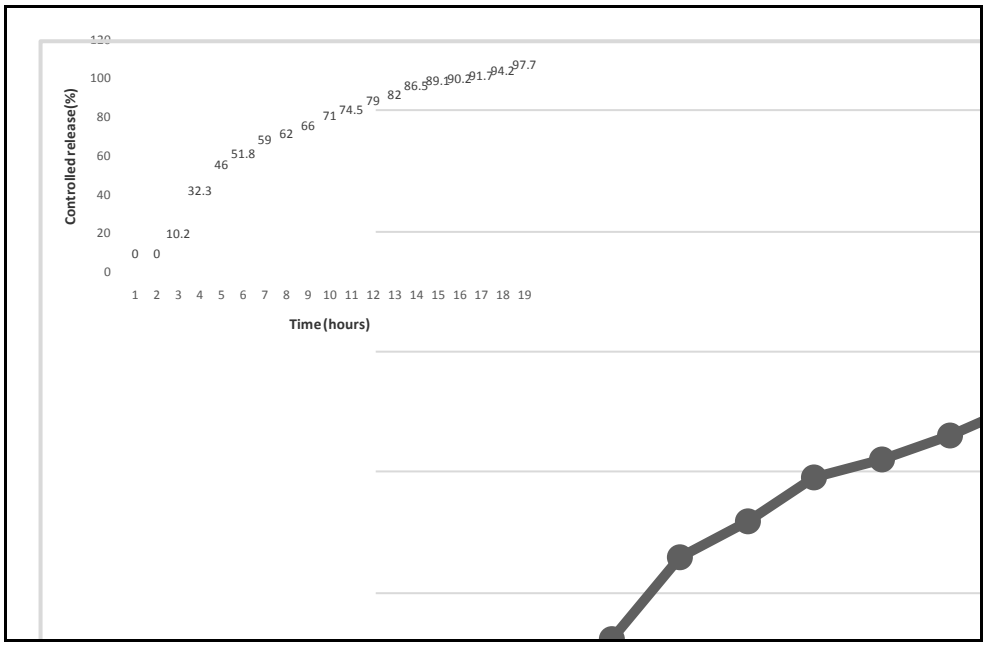

Figure 3.Cumulative release (%) of CSNp-BV

All the stages of *S.litura* were found to susceptible to the CSNp-BV formulation as dose dependent manner. Maximum mortality was recorded in early instars (Table 1).In the case of I^{ST} and II ND instars, complete

mortality was recorded in 1.0, 0.01 and 0.001mg concentration. Mortality rate was found to be decreased in later instars at all the tested concentration. Mortality was not noticed in fourth, fifth and sixth instars at 0.001mg. 1.0, 0.1 and 0.001 mg of CSNp-BV brought about 76.0, 60.0 and 41.2% of mortality to the third instars. Similar mortality was recorded in fourth instars.40.1, 23.5, 7.0 % and 24.0, 11.2, 3.0 % mortality was inferred from fifth and sixth instars at the concentration of 1.0.0.1.0.001mg respectively. Pupal period and the rate of adult emergence was highly reduced in CSNp-BV (Table 2).

Table 1. Cumulative mortality (%) of *S.litura* treated with free beauvericin (F-BV) and chitosan nanoparticles incorporated beauvericin (CSNp-BV)

S.No	Treatment	Concentration (mg)	Cumulative mortality (%)					
			I	II	III	IV	V	VI
1	F-BV	1.0	67.0	52.1	40.1	27.2	0.0	0.0
		0.1	43.0	32.0	17.0	9.1	0.0	0.0
		0.01	21.0	10.0	0.0	0.0	0.0	0.0
		0.001	0.0	0.0	0.0	0.0	0.0	0.0
2	CSNp-BV	1.0	100.0	100.0	76.0	75.0	40.1	24.0
		0.1	100.0	100.0	60.0	59.4	23.5	11.2
		0.01	100.0	100.0	41.2	40.0	7.0	3.0
		0.001	100.0	100.0	0.0	0.0	0.0	0.0

Table 2.Effect of free beauvericin (F-BV) and chitosan nanoparticles incorporated beauvericin (CSNp-BV) on pupal and adult emergence

S.No	Treatment	Concentration (mg)	PP (Days)	AE (%)
1	F-BV	1.0	2.0	61.0
		0.1	2.1	70.0
		0.01	2.4	81.5
		0.001	4.0	92.0
		Control	4.0	100.0
2	CSNp-BV	1.0	0.5	42.1
		0.1	1.2	50.5
		0.01	2.0	59.0
		0.001	2.4	63.0
		Control	4.0	100.0

PP-Pupal period
AE-Adult Emergence

Unlike the chemicals, biotechnology has a great potential in the development of eco-friendly biopesticidal agents. Among potential biopesticides, fungi hold great promise both as organisms/conidia pesticidal metabolites to selectively control the insect pests. The Indian biopesticide market is highly fragmented with numerous low cost producers having minimum resources and low level technologies. Eight of the 15 registered and commercialized biopesticides in India which includes *M. aniso*pliae, are fungus based especially in the form of conidia. Unfortunately, despite the perceived initial advantages, there has not been a significant uptake of these and other microbial biopesticides in India. Until recently, biopesticide market had occupied only 2.85% of total pesticide market worth of Rs. 2,700 crores. So numerous of Indian groundnut farmers continue to use toxic chemicals as effective means of combating *Spodoptera litura* the devastative pest, to be a successful venture with enhanced acceptability and potent activity, the entomopathogenic fungus (*B.bassiana*) needs to be packaged in technologically advanced formulation with value addition and specifications of dosage and duration of storage. Since this fungus produces toxic cyclodepsipeptides that are lethal to *S.litura*, it makes greater economic and business sense to make use of these fungal metabolites rather than the fungus itself. Even otherwise, by definition of the FAO**.**

In very recent times, nanotechnology has burst upon the scene as *sine qua* nm for diverse applications even in totally unrelated areas of consumer and industrial use. Some pundits suggest that nano boasts bigger than steam and penicillin in the historical continuum of scientific and technological achievements. Among the

identified ten nanotechnology applications, an international panel of experts has identified agricultural pesticide topping the list[14]. In the case of chemical pesticides, often more than one applications of a pesticide are required to have the desired effect. The formulation of a pesticide must thus be to meet the demands of efficacy and suitability to the mode of application and minimizing the use and damage to the environment. Nano-encapsulated chemical pesticides meet these demands due to the trace amounts of the pesticide used with greater efficacy over a given period of time interval, and in that the design enables them to resist the severe environmental processes that act to eliminate conventionally applied pesticides i.e., leaching, evaporation and photolytic, hydrolytic and microbial degradation. Functionalized polymeric nanoparticles encapsulating the pesticidal metabolites (Beauvericin) now proposed are thought to be superior to nanopesticde due to their stability, target specificity, biodegradability and enhanced environmental safety. Harnessing the benefits of such nanobiopesticide is in India's interest, keeping in line with the much emphasized sustainable and value added use of native bioresources. Pesticides having emulsions of nanoparticles have already hit the market in the USA. It is only in the fitness of things that efficient nano sized versions of biopesticidal metabolite (beauvericin) molecules with chitosan nanoparticles are launched as they combine stability, target specificity and toxicity as also envisaged in nanopesticides.

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