4A.1 Introduction

Microbial bioinsecticides are alternative to chemical insecticides with respect to eco friendly approach for biocontrol of pest in fields. *Bacillus thuringiensis* based bioinsecticides have been proved successfully to control insect pests which feed on different crops. Bt produces different kind of insecticidal toxins which have insecticidal property against different orders of insect lepidoptera, diptera, coleoptera. Cry toxin of Bt causes pores into the epithelial membrane of insect gut and ultimately insect dies due to septmia (de Maagd et al., 2003; Melo, et al., 2016). Hence, it is one of the excellent biocontrol agents in agriculture, forestry and human disease vector. However, δ-endotoxin has main key limitations such as inactivation by UV irradiation, wash out in rain from the target sites, narrow specificity to insects and ineffective against sucking pests etc. (Griego and Spence, 1978; Behle et al.,1997).

Microencapsulation is a technique by which solid particles, gas molecules and liquid droplets were entrapped into a thin film of encapsulating agent. It can extend shelf life and efficacy of active ingredients (Balasubramani, et al., 2015). Although numerous microencapsulating techniques have been employed, suspension cross-linking is the most acceptable technique in pharmaceutical application due to very simple method and low cost requirement. Chitosan is considered to be an excellent encapsulating agent because it exhibits low toxicity, low viscosity and good solubility (Kean and Thanou, 2010).

Response surface methodology (RSM) is a collection of mathematical and statistical modelling technique helpful for improving, developing and optimization of process parameters. The most remarkable applications of RSM are in selection and designing of

experiments, optimizing parameters and their effective levels in multi-response experiments, prediction and verification of model equation and generating response surfaces (Maran et al., 2013a). The main advantage of response surface methodology is to find high efficiency for production of new or improved products as well as process with minimum time and cost. Thus, present work deals with microencapsulation of *Btk HD-1* spore-crystal aggregate (SCA) using chitosan as encapsulating material by suspension cross-linking technology. The encapsulated spore-crystal aggregates are evaluated for encapsulation efficiency.

4A.2 Material and Methods

4A.2.1 Experimental design

Box–Behnken design (BBD) was used with three –factor and three-level in the present study in order to optimise the process parameters for encapsulation of the spore-crystal aggregate in microcapsules. A set of seventeen experiments were generated and randomised using Design Expert® 10.0. software for factors namely concentration of polymer (chitosan) (A), concentration of cross-linker (glutaraldehyde) (B) and active ingredient- spore-crystal aggregate (C). The encapsulation efficiency (R1) was considered as the response variable and the α-level at which every term in the model should be significant was set as 5%.

Encapsulation efficiency for all 17 formulations (Table 4A.2) were determined experimentally. The Design Expert® 10.0 software was used to analyse the experimental results by regression analysis and Analysis of variance (ANOVA) and the coefficients of determination (R^2) were determined. The model was then used to determine the optimum conditions for encapsulation efficiency. Further the validation

of the model was done by conducting experiments with parameters set at optimized levels. The encapsulation efficiency obtained after experiments were compared to the one estimated by the model.

Table 4A.1: Experimental design set with different levels of each independent variable; A: Chitosan (mg/ml); B: Glutaraldehyde (% v/v); C: Spore- crystal aggregate (mg/ml)

	Levels		
Factors	-1	0	1
A	10	20	30
B	0.5	1.0	1.5
C	5	10	15

4A.2.2 Microencapsulation *Btk HD-1* spore-crystal by suspension cross linking

The emulsions were prepared as per experimental design conditions (Table 4A.2). In each run of the experiment, amount of spore-crystal aggregates was used from lyophilized *Btk-HD-1* (~1×10^{15} spores/ ml) and resuspended in 5 ml of chitosan (w/v) (TC242-50G, Himedia. Ltd) as per defined concentration. Then, 35 ml parafin oil (Loba chemicals.), 25 ml petroleum ether and 200 µL span-80 as emulsifying agent were added into prepared formulation mixture. The final mixer was homogenized at 5000 RPM by homogenizer (REMI RQT 127 A) for 5 minutes. Subsequently, 1.8ml of different concentrations of glutaraldehyde were added diluted from commercially available (25% v/v) stock in a drop wise manner. Finally, the emulsion was kept under shaking condition at 30°C for 3h to harden into microcapsules. An aliquot was taken for the determination of encapsulation efficiency.

4A.2.3 Measurements of encapsulation efficiency

The efficiency of the microencapsulation process (E %) of *Btk HD-1* was determined by estimation of protein concentrations before microencapsulation process (A) and the protein concentration left after encapsulation (B). The protein concentration was quantified by Bradford method (1976). The efficiency of the spore –crystal aggregate microencapsulation process was calculated by the following equation:

Efficiency (E %) = (A - B /A) x 100. Where, initial protein concentration (A); uncapsulated protein concentration (B)

4A.3 Results

In this study, three factors, each at three levels (BBD) were employed to investigate the influence of process variable on encapsulation efficiency by suspension cross linking technique and results were given in Table 4A.2. Four models - Linear, interactive, quadratic and cubic models were fitted to experimental data to check whether approximating model would give deceptive or poor results. The different tests namely the sequential model sum of squares and model summary statistics were carried out in this models to show the responses. The adequacy of model summary output (Table 4A.3) suggests that the quadratic model was statistically significant for the current microencapsulation method.

Among all models, the Quadratic model was found to have high predicted R^2 and adjusted R^2 and it also exhibited low p-value than linear, interactive and cubic models. Thus, quadratic model was found to be the most suitable model and it was further justified by analysis of variance (ANOVA).

Table 4A.2: Box -Behnken experiment design matrix and responses

Runs	A Chitosan (mg/ml)	B Glutaraldehyde (% (v/v))	C Spore-crystal (mg/ml)	R1 Encapsulation efficiency (%)
1	10	0.5	10	75.96
2	20	1	10	86.12
3	10	1	15	65.73
4	30	0.5	10	75.39
5	30	1.5	10	89.34
6	20	1	10	85.63
7	10	1	5	60.73
8	10	1.5	10	75.94
9	20	1.5	15	66.56
10	20	1	10	86.33
11	20	0.5	15	84.82
12	20	1.5	5	90.40
13	30	1	15	64.13
14	30	1	5	58.30
15	20	1	10	86.12
16	20	0.5	5	52.57
17	20	1	10	85.75

Table 4A.3: Summary statistics for encapsulation efficiency

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	
Linear	0.7642	< 0.0001	-0.1299	-0.7153	
2FI	0.1715	< 0.0001	0.0897	-1.0651	
Quadratic	< 0.0001	0.0001	0.9612	0.7308	Suggested
Cubic	0.0001		0.9994		Aliased

Table 4A.4: Sequential Model Sum of Squares (Type I)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	97860.92	1	97860.92			
Linear vs Mean	196.23	3	65.41	0.39	0.7642	
2FI vs Linear	835.48	3	278.49	2.05	0.1715	
Quadratic vs 2FI	1321.16	3	440.39	75.97	< 0.0001	Suggested
Cubic vs Quadratic	40.24	3	13.41	159.40	0.0001	Aliased
Residual	0.34	4	0.084			
Total	1.003E+005	17	5897.32			

Table 4A.5: Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	2196.88	9	244.10	2900.75	< 0.0001	
2FI	1361.40	6	226.90	2696.37	< 0.0001	
Quadratic	40.24	3	13.41	159.40	0.0001	Suggested
Cubic	0.000	0				Aliased
Pure Error	0.34	4	0.084			

4A.3.2 Verification of Model

The optimised conditions were validated experimentally and mean value of the triplicate was compared with the predicated value. The encapsulation efficiency was found to be 86.97 ± 7.63 % using the following model optimized parameter setting: 10 mg /ml *Btk HD-1* (SCA), 20 mg/ml chitosan concentration, 1% (v/v) cross linking agent. The encapsulated crystal protein concentration was 1.12 mg/ml under optimized condition.

Table 4A.6: Analysis of variance and statistical parameters of the model for encapsulation efficiency

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2352.88	9	261.43	45.10	< 0.0001	significant
A-A chitosan	9.68	1	9.68	1.67	0.2373	
B-Glutaraldehyde	140.28	1	140.28	24.20	0.0017	
C-spore-crystal	46.27	1	46.27	7.98	0.0256	
AB	48.79	1	48.79	8.42	0.0229	
AC	0.17	1	0.17	0.030	0.8680	
BC	786.52	1	786.52	135.68	< 0.0001	
A^2	348.58	1	348.58	60.13	0.0001	
B^2	21.62	1	21.62	3.73	0.0947	
C^2	905.99	1	905.99	156.29	< 0.0001	
Residual	40.58	7	5.80			
Lack of Fit	40.24	3	13.41	159.40		
Pure Error	0.34	4	0.084			
Cor Total	2393.45	16				

4A.4 Discussion

The regression analysis and ANOVA were used to test the adequacy and fitness of the developed model which was shown in Table 4A.6. The ANOVA was done to know the effect of independent (factors) on dependent (response) variable by predicting the linear, interactive, cubic and quadartic relationships (Maran et al., 2015). The p value of the model was less than 0.05 ($p < 0.5$) which indicated that the model fitness was significant. The high F-value and low probability value indicated justification of a high degree of quadratic model adequacy. The goodness of fit of the model was evaluated by predicted determination of co-efficient (R^2_{pre}) and adjusted determination of co-efficient

(R^2_{Adj}), which has been given in (Table 4A.3). The calculated R^2_{pre} (0.73) and R^2_{Adj} (0.96) are also high and suggest a high correlation between the predicated and observed values. The goodness of predicted response value is measured by R^2_{pre}. The R^2_{pre} and R^2_{Adj} were approximately within 0.20 of each other to be in reasonable agreement (Maran et al., 2013b; Balasubramani et al., 2015). Sequential Model Sum of Squares (Type I) (Table 4A.4) suggested that quadratic model fitted the current microencapsulation process. The lack of fit tests (Table 4A.5) however, was significant which can be explained by the absence of significant but unknown factor governing the encapsulation efficiency. Overall the model was useful in predicting the optimum levels for the factors considered as shown by the improvement in encapsulation efficiency to significant levels. Further studies focus to efficacy of *Btk HD-1*formulation at lab and field level.

4A.5 References

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4B.1 Introduction

Biopesticides based on the entomopathogenic bacterium *Bacillus thuringiensis* are most accepted agent to control lepidopteran, coleopteran, and dipteran insect pests. *Bacillus thuringiensis* is Gram-positive, aerobic, endospore forming bacterium commonly present in water, agricultural soil, marine sediments and insect cadavers, etc (Baig and Mehnaz, 2010; Patel et.al., 2013). *Bacillus thuringiensis* produces a different type of such as insecticidal δ-endotoxins, vegetative insecticidal protein (VIP), β-exotoxin, Cyt toxin, chitinase, phospholipase and various protease (Estruch et al., 1996; Chen et al., 2007; Akiba et al., 2009; Liu et al., 2010). Bt strains produce different Cry toxins in their sporulating stage of the growth cycle. These toxins interact to receptors present in insect gut and forms pores in the membrane and ultimately kill the larvae of lepidopteran and dipteran (Frankenhuyzen et al., 2009; Shah et al., 2016).

The success of using *B.thuringiensis* as an effective biocontrol agent solely depends upon the development of proper formulation. The desirable properties of the efficient microbial formulation are good residual activity, relentless storage stability, ease in the mixing, handling and apply and also economical to farmers (Hadapad et al., 2011). Several commercial formulations of Bt are available ranging from the pellet, granular and suspension concentrate particularly of *Bacillus thuringiensis* var. *kurstaki* has been produced in many countries of the world (Sansinenea ,2012; Brar et al., 2006). Many formulations are successful in the field condition. However, the major limitation of using δ-endotoxins and spores as an insecticidal agent against crop pests is their lack of persistence due to prolonge exposure to UV radiation. Inactivation of Bt toxins caused due

to the destruction of tryptophan, histidine residues and oxidation of amino acids by free radicals generated upon UV radiation (Pozsgay et al.,1987).

Natural polymer such as starch, carboxymethylcellulose and sodium alginate based encapsulation of Bt spores-crystals have used to increase the photostability of the formulation (McGuire et al.,1996; Elcin et al.,1995). Also, emulsification is considered relatively easy and reliable method for microcapsule production (Vemmer & Patel 2013; Tripathi & Giri 2014). The microcapsule prepared by alginate matrix has shown contribution in the enhancement of cell viability and improved storage ability of encapsulated probiotic bacteria (Krasaekoopt et al., 2004).Currently, chitosan polymer used extensively in drugs and medicine delivery in form of nanoencapsulation with the sustain-released mechanism. The advantages of using chitosan are nontoxic, biocompatibility, biodegradability (Mikos et al., 1993; Zhu et al., 2000. *H.armigera (Hubner)* is a kind of polyphagous pests feed on different economical important crops cultivated in Asia, America, and Australia. *Amsacta albistriga* (Arctiidae) is one of the devastating pests found in fields of south Asian countries India and China. It feeds on ground nut, castor, cotton, cowpea etc. and causes severe loss of crops.

The aim of present work was to study the microencapsulation process of a freeze-dried (lyophilized) spore-crystals aggregate of *Bacillus thuringiensis krustaki* HD-1. The method employed based on the suspension cross-linking (Nayak et al., 2009) and focused on making suitable diameter microcapsules (~17 µm) with photostability of spore and δ-endotoxin under extreme UV radiation. The efficacy of prepared *Btk* HD-1 formulation evaluated at laboratory level against *H .armigera* and *A.albistiga* pests followed by *C.*

cajan grown plants at pot level in sun light exposed condition and in the field infested with *H .armigera* pest.

4B.2. Material and Methods

4B.2.1 Enrichment of *Bacillus thuringiensis krustaki* HD-1

Standard strain *Btk-HD1* obtained from BGSC, Ohio, USA. Cells were grown in sporulation medium namely GYS (in gram%: 0.1 g Glucose; 0.2 g Yeast extract powder; 0.2 g $NH_4(SO_4)_2$; 0.006 g $MnSO_4$; 0.04 g $MgSO_47H_2O$; 0.008 g $CaCl_2$; 0.5 g K_2HPO_4) at 30 °C in orbit Incubator shaker at 180 RPM for sporulation (48-72 h). Sporulated culture of *Btk- HD1* was lyophilized by the lactose-acetone based method as defined by Dulmage et al., (1970).

4B.2.2 Preparation of chitosan microspheres of *Btk HD-1*

Formulation prepared by modified method of Nayak et al., (2009) in which 50 mg (~1×10^{15} spores / ml) lyophilized spore-crystal aggregate (SCA) of *Btk-HD-1* was resuspended in 5 ml of an aqueous solution of 1 %, 1.5 % and 2 % chitosan (w/v) (TC242-50G, Himedia. Ltd) subsequently mixed in 35 ml partafin oil (Loba chemicals.) containing 200 µL span-80 as emulsifying agent. Further, 25 ml petroleum ether was added to the same mixture. The final mixer was homogenized at 5000 RPM by a homogenizer (REMI RQT 127 A) for 5 min. Subsequently, 1.8 ml of 1% Glutaraldehyde (25% v/v) added by dropwise in the prepared emulsion and kept shaking at 30 °C for 3h in order to harden microcapsules. An aliquot was taken for the determination of encapsulation efficiency. The

solution of a prepared batch of formulation washed three times with ether and stored at 4°C to further study.

4B.2.3 Microcapsules diameter optimization

For optimization of capsules diameters, each system of the formulation prepared with 1 %, 1.5 % and 2 % chitosan were homogenized by stirring speed 2000, 3000, 4000, 5000 RPM. SEM image of formulation was used to measurement of diameter of capsules by Image J program. The average of fifty microcapsules diameter was counted and reported.

4B.2.4 Morphology analysis of microcapsules by SEM (Scanning Electron Microscopy)

An aliquot od microencapsulated spore-crystal of *Btk HD-1* has centrifuged 10,000 RPM for 10 Min. The supernatant was discarded, and the pellet was resuspended in surfactant with conc. 0.1 % Tween-20 to the prevention of agglomeration of microcapsules. The sample was prepared by carbon coated brass grid by dropping small on the grid and extra solution was absorbed with blotting paper, and the sample was observed by scanning electron microscope model JSM-5610LV.

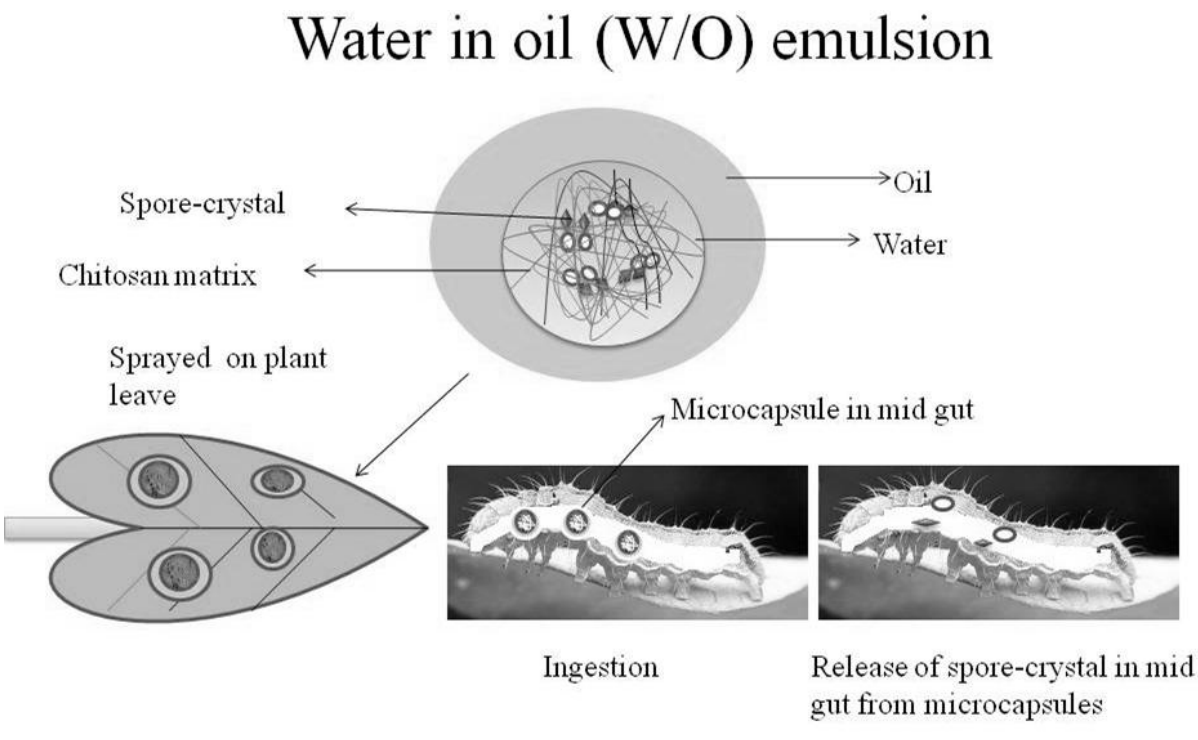

Figure 4B.1: Schematic presentation of biocontrol of lepidopteron insect larva by chitosan Microcapsule

4B.2.5 TEM analysis of encapsulated Cry proteins

The sample prepared for transmission electron microscopy (TEM) by placing a drop of formulated *Btk-HD1* spore-crystal on carbon coated copper grid allowing the solvent to evaporate. 100 µL of microencapsulated SCA diluted to 1 ml and 5 µL of the diluted solution was placed on a copper grid and stained with 2 % (w/v) phosphotungstic acid and kept 10 min at room temperature to dry subsequently performed TEM. The qualitative characterization of nanoparticles carried out by Philips –Tecnai (Philip electron, optics, Holland) instrument operated at 200 kV.

4B.2.6 Insect bioassay of microencapsulated *Btk-HD1* against lepidopteran pests

To estimate the LC_{50} value of formulation, the bioassay performed against lepidopteran pests *Helicoverpa armigara* and *Amsacta albistriga* by leaf disc method (Figure 4B.1). For *H.armigera,* four concentrations in the range of 0.017-0.140 µg/ml of encapsulated *Btk-HD1* were assessed for the insecticidal activity. Twenty-four larvae of the second instar were used for each concentration. The larval mortality was scored every 48 h till a week. For *Amsacta albistriga,* 0.03-0.42 ng/ml microencapsulated *Btk-HD1* taken to the determination of larvicidal activity. Ten larva in triplicate were used for each dose of formulated and unformulated *Btk-HD1*. Unformulated *Btk-HD1* spore-crystal mixtures were also evaluated to compare LC_{50}. Control bioassays was performed with 0.1 % Tween-80 and empty chitosan microcapsules. The bioassay experiment was carried out at 28 ± 2 °C and 60 to 70 % humidity. The LC_{50} values were calculated from mortality as per probit analysis (Finney, 1971).

4B.2.7 Release analysis of microencapsulated spores

To determine release time, microencapsulated *Btk HD-1* spore-crystal was resuspended in 5 mL of phosphate buffer saline (10 mM, pH –7.4) and kept in shaking condition at 180 RPM on 37 °C. An aliquot was taken each 6 h interval and release spores were counted by spread plate technique.

4B.2.8 Effect of UV irradiation on Bt formulation

The UV radiation protection property of chitosan based microencapsulation of spore viability and insecticidal activity were evaluated by exposing microspheres to short range

UV –C (254 nm) and medium range UV –B (365 nm) irradiation with Entella lamp model UVGL-25 4W. The radiation energy was measured in Joule unit (J = watts / exposure time(s)). The radiation 0.4 ± 0.1 mW was set by radiation meter before exposure the sample. Sample (5 ml) of microencapsulated spore-crystal aggregates of known spore and protein concentration were kept in triplicate in glass petri plates of 5 cm diameter used for irradiation exposure. Also, control samples containing plates were covered with a layer of aluminum foil. The lost of water by evaporation by heat is replenished by sterile distilled water to maintain volume of sample

4B.2.9 Effect of UV-C (254nm) and UV-B (365 nm) irradiation to Bt formulation

The irradiation selected for UV-C effect Bt formulation in range of 0.021-0.21 J. Free and microencapsulated *Btk HD-1* spore-crystal were subjected to UV-C exposure of 0.02, 0.04, 0.06, 0.08,0.1, 0.12,0.14, 0.16,0.18 and 0.2 J. UV-B irradiation was applied to unformulated and formulated *Btk HD-1* in submerged condition with rate of 1.14 J/ h. An aliquot of irritated samples was taken every 12 h interval. Spore survival of UV-C and UV-B exposed were evaluated to samples by released up to 50-60 h followed by serial dilution and spread in N-agar plate and incubated at 30 ℃. Control kept with covered with aluminum foil and given the same condition. Spore viability was evaluated as follow:

$$V_{spores}(\%) = (Spores_{irradiated} / spores_{initial}) \times 100$$

Where, $spores_{irradiated}$ was the count of irradiated spores (unformulated and formulated) and $spores_{initial}$ the initial count of spores (unformulated and formulated). All spore survival

experiments were performed with three replications and followed by ANOVA analysis by XLSTAT program.

4B.2.10 Insect bioassay of UV-B exposed *Btk HD-1* spore-crystal formulation

Microencapsulated and free *Btk HD-1* SCA of exposed at 259.20 J with protein concentration 0.112 µg / ml and 1.124 µg/ml was employed for insect bioassay analysis by leaf disc method. 250 µL of same was dried on 5 cm^2 fresh cabbage leaf. The leaf was further cut into 1cm^2 pieces and fed to each second instar larvae of devastating pest *H.armigera*. Twenty-four larva in triplicate for each dose were employed for insecticidal activity of UV-B exposed samples of *Btk HD-1* and control which was covered with aluminum foil also evaluated for insect bioassay. Mortality was recorded till seven days. Probit analysis was carried out as per described by (Finney, 1971).

4B.2.11 Efficacy and persistence of formulation on sun-exposed potted *C .cajan* plant

The experiment was set up with as described by Navon et al.,(1987). A pot grown with six healthy *C. cajan* plants and six pot for a treatment selected for the single dose-response experiment. Four treatments such as negative control, 0.035 µg/ml, 0.105 µg/ml and 0.210 µg/ml of microencapsulated *Btk HD-1* spore-crystal in chitosan microspheres were sprayed by hand held spray bottle to wet entire plant. All the treated plants were kept outdoor (i.e. in the sun) throughout the study. Ten second instar larva of *H. armigera* was infested artificially to each pot and six pot were used for the single treatment. The number of surviving larvae on each plant was enumerated on the 2nd, 4th and 6th day after infestation

and the corrected mortalities were calculated according to Abbott's formula (Abbott, 1925).

4B.2.12 Experimental design and field level efficacy of formulation

The field level evaluation of *Btk HD-1* formulation was carried out on pigeon pea plant (*C. cajan)*. The experiment was set up as a RBD (Randomized block design) with total 25 blocks, representing five treatments with five replications. The plot size was 4.50 X 3.60 m (gross) and 3.60 X 1.80 m (net) with a spacing 90 X 45 cm. The treatment included three doses of W/O chitosan microspheres *Btk HD-1* SCA emulsion with 35 µg L^{-1} (Treatment-1), 105 µg L^{-1} (Treatment -2) and 210 µg L^{-1} (Treatment - 3) in Tween -80 (0.1% v/v) surfactant and chemical insecticide Indoxacarb (14.5%, SC) used as Treatment – 4 and sprayed as recommend dose 60 g of a. i. / hector to compare efficacy with *Btk HD-1* biopesticide. Tween -80 (0.1% v/v) in water used as Treatment – 5. The sprays were carried out by Knapsack sprayer at evening time. The pod borer (*H. armigera*) larva were counted randomly selected ten plants per plot before spray and after sprayed on third, sixth and ninth day. The percentage of reduction in population was calculated using the Henderson and Tilton equation (1955). The final assessment was compared across treatments and days by two-way ANOVA followed by post hock test LSD as described by Sharma & Arora (2010).

4B.3. Results

4B.3.1 Effect of stirring speed (RPM) on the size of microcapsules

The microcapsules were prepared by using different stirring speed (2000, 3000, 5000 RPM) with different chitosan concentration (1%, 1.5%, 2%) to check the effect of both on microcapsule diameter. The mean diameter of capsules was prepared on 2000 RPM 13.03 ± 5.12, 17.68 ± 8.79 and 29.00 ± 6.88 µm corresponding to 1%, 1.5% and 2% chitosan. While on 3000 RPM, the diameter was reduced to 12.53 ± 5.83, 15.534 ± 6.58 and 26.20 ± 8.79 µm of 1%, 1.5% and 2% chitosan compared to 2000 RPM. Further, smaller diameter obtained when formulation stirring with speed of 4000 RPM which were 8.27 ± 3.42, 14.62 ± 1.60, 22.15 ± 4.96 µm. At last, the expected size of capsules with coated polymer achieved when stirring speed was 5000 RPM, which were 6.32 ± 3.0, 10.82 ± 3.18 and 17.69 ± 3.56 µm by using 1%, 1.5% and 2% chitosan solution respectively (Figure 4B.2). The capsules formed with 2% chitosan polymer on 5000 RPM were analyzed for stability against UV radiation to microencapsulated spore-crystal.

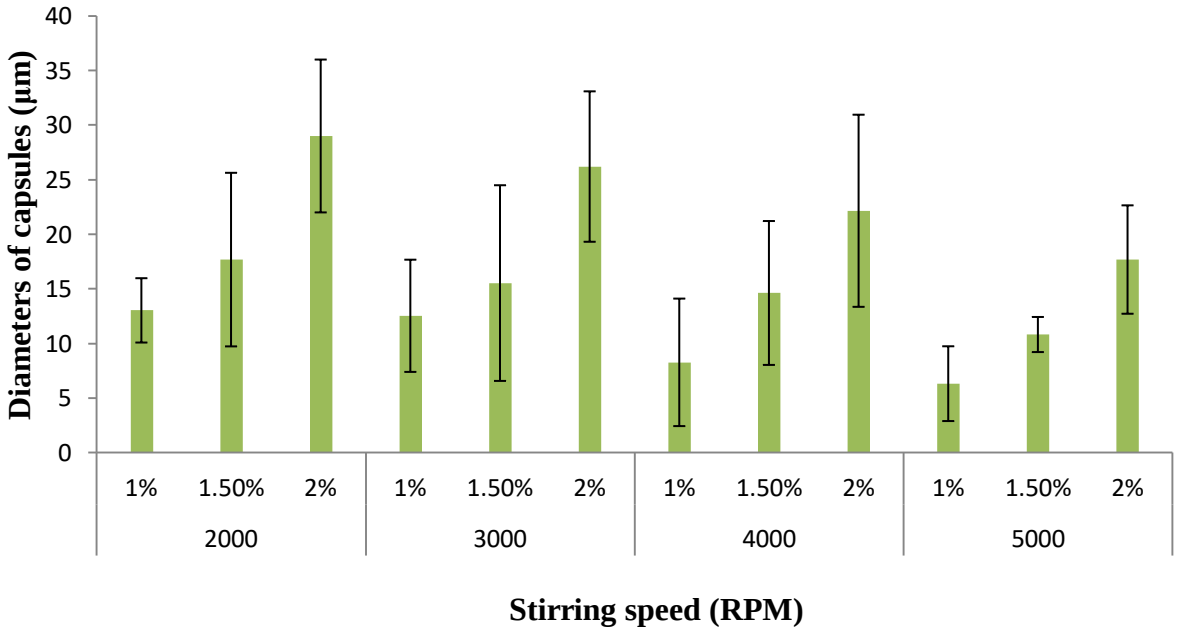

Figure 4B.2: Relation between capsule diameter and stirring speed (RPM)

4B. 3.2 Morphological analysis of microcapsules

The prepared W/O emulsion formulation of encapsulated *Btk HD-1* subjected to scanning electron microscopy analysis. It was observed that prepared capsules were spherical in shape, with rough integrity and presence of pores on the surface. The size of capsules revealed around 10 -15 μm (Figure 4B.3a). TEM analysis of chitosan microspheres prepared by W/O emulsion method was verified by the shape of insecticidal crystal proteins in embedded chitosan matrix. The bypyramidal shape of Cry1A protoxin was revealed with capsule size around 1.5 - 2.0 μm diameter in TEM image. As well as, microencapsulated Cry2 δ-endotoxin by cuboidal shape observed with capsule size approx 1 μm (Figure 4B.3b). The oil surrounding crystal toxin could act as UV screen and retain insecticidal activity even strong exposure of UV radiation.

4B. 3.3 Insect bioassay analysis of microencapsulated *Btk HD-1*

Chitosan microspheres bearing spore-crystal mixtures have effectively killed the larva of lepidopteran pests *H.armigera* and *A.albistriga*. The LC_{50} of free *Btk HD-1* to *H.armigera* was 0.041μg/ml while encapsulated showed 0.112 μg/ml (Figure 4B.3). Likewise, in case of *A.albistriga*, the LC_{50} of unformulated *Btk HD-1* spore-crystal observed 0.055 ng/ml, and encapsulated 0. 158 ng/ml.

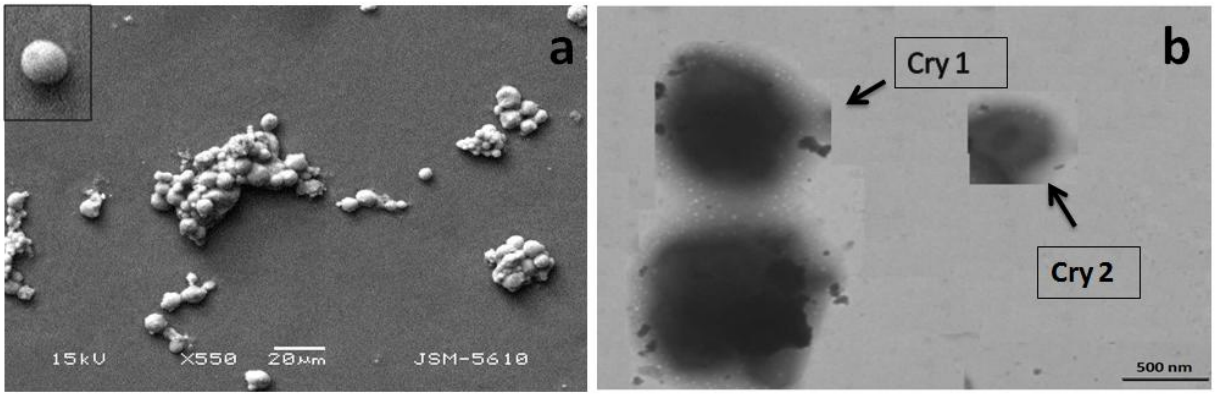

Figure 4B.3: Microscopic analysis of capsules (a) Scanning electron microscopy of capsules observed with spherical shape; (b) Transmission electron microscopy revealed microencapsulated Cry1 and Cry2 toxin.

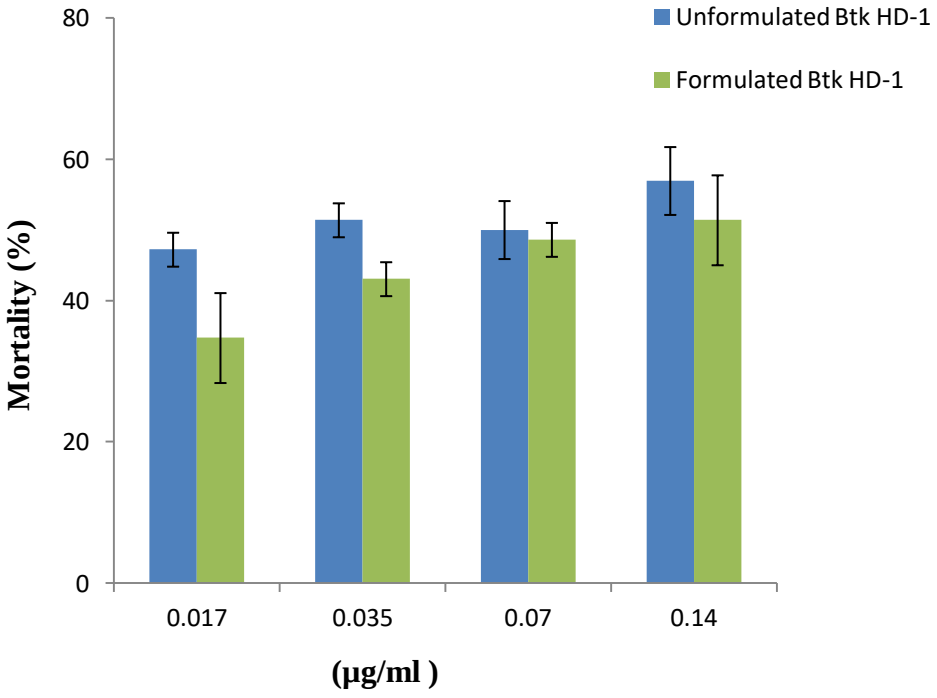

Figure 4B.3: Susceptibility of *H.armigera* larva to *Btk HD-1*

4B. 3.4 Release analysis of microencapsulated *Btk HD-1* spores

Microencapsulated spores were release by dissolution of chitosan polymer at pH- 7.4. It was calculated that average 1.92 $\times 10^{14}$ spore/ml released at around 60 h incubation (Figure 4B.4).

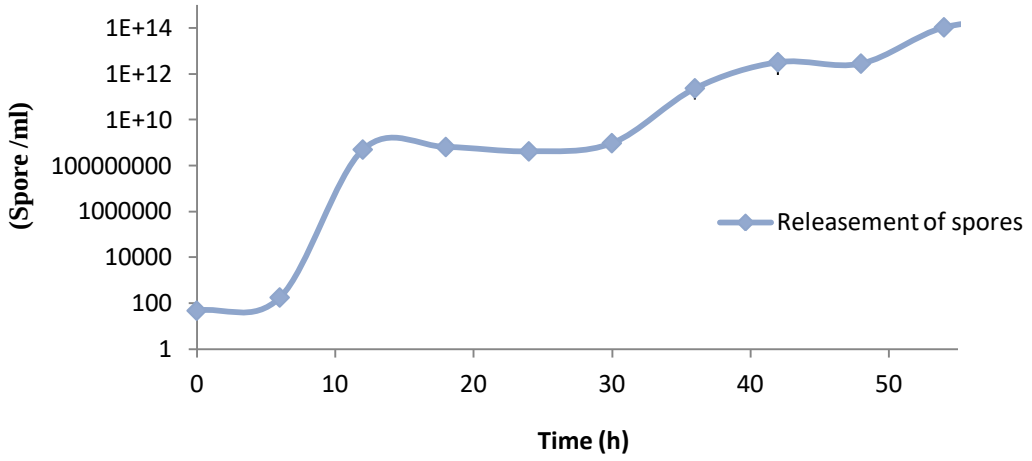

Figure 4B.4: Release of spore from chitosan microcapsules resuspended in phosphate buffer saline (10 mM, pH – 7.4)

4B. 3.5 Spore survival upon exposure to UV-C irradiation

Microencapsulation provided a significant beneficial effect on spore viability in submerge condition. Figure 4B.4 represent the viability of free and microencapsulated spores of *B. thuringiensis krustaki* HD-1. It showed that chitosan polymer trapped spores retained 89.86 ± 8.1 survival ($F=8.90$, $df = 9$, $P < 0.05$) in UV-C (0.2 J) irradiation while free spores observed viability was reduced to 3.11 ± 1.5 % ($F= 30.84$, $df = 9$, $P < 0.01$) at same Joule.

4B. 3.6 Effect of UV-B exposure on spore survival

UV-B irradiation which causes inactivation of Cry proteins. Chitosan polymer microcapsules could protect spore against UV-B irradiation. It was seen that *Btk HD-1* unformulated spore viability was 3.5 ± 0.90 % (F= 82.69, df = 9, P < 0.01) at 43.2 J. On further analysis of spore viability, microencapsulation retained 17.30 ± 7.7 % (F= 39.41, df = 17, P < 0.01) at 311.04 Joule (Figure 4B.5).

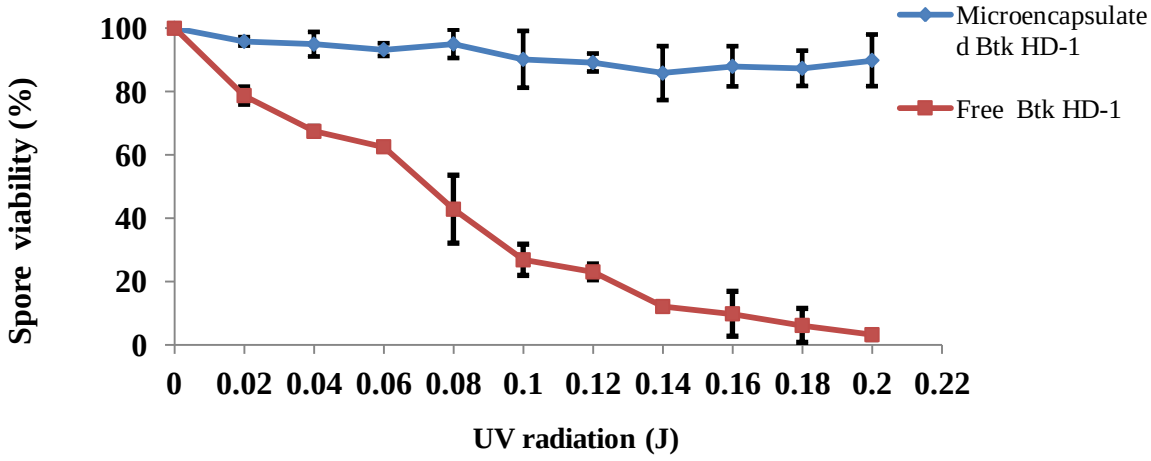

Figure 4B.4: *Btk HD-1* spore viability of free and microencapsulated with 2% chitosan after UV-C irradiation

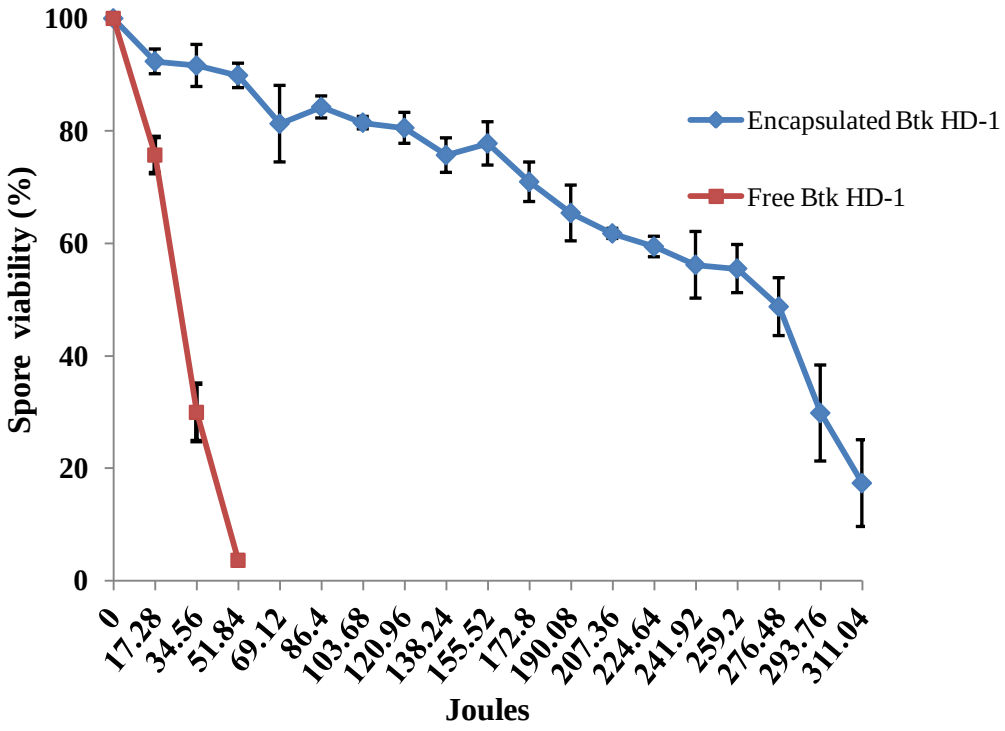

Figure 4B.5: Effect of UV-B irradiation on spore viability of free and microencapsulated *Btk HD-1*

4B.3.7 Insect bioassay analysis of UV-B exposed *Btk HD-1*

The bioassay results revealed that extremely UV-B irradiated spore-crystal of *Btk HD-1* formulation killed insect larvae of *H.armigera.* It was found that 259.2 J irradiated unencapsulated *Btk HD-1* has 5.55 ± 2.4 % larvicidal activity while aluminum foil covered control showed 13.88 ± 6.36 %. In the case of microencapsulated spore-crystal of *Btk HD-1*, insecticidal activity found 47.22 ± 2.4 % of irradiated and aluminum foil covered control showed 62.5 ± 4.16 % larvicidal activity. (Figure 4B.5).

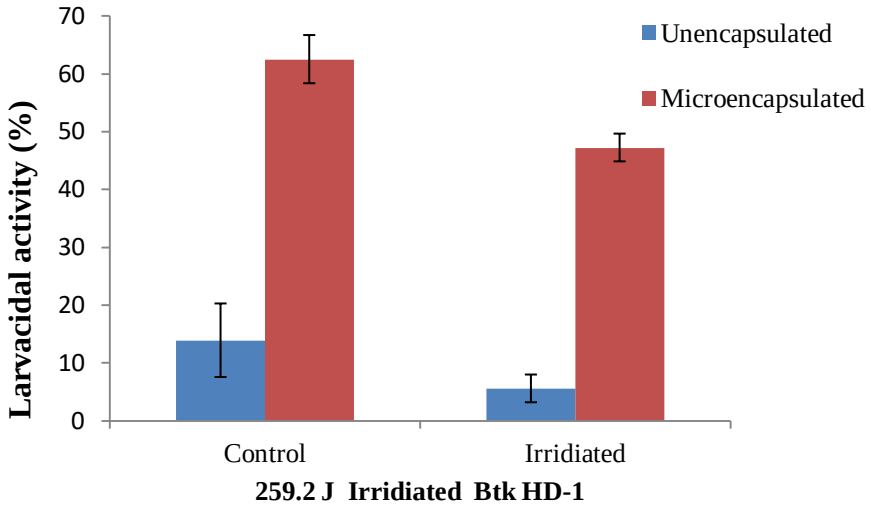

Figure 4B.5: Insecticidal activity of 259.2 J irradiated free and encapsulated SCA against *H.armigera* larva

4B.3.8 Efficacy of formulation on sunlight exposed *C. cajan* pots

The W/O emulsion based formulation of Bt spore-crystal was found effective against the devastating insect larva *H. armigera* at pot level. The bioassay was performed with *Cajanus cajan* plant. It was observed that insect larvae caused extensive damage to leaves of untreated pots (-ve control; Figure 4B.6 b). On sixth day observation, reduction in damage of leaves was observed with 0.035 µg/ml, 0.105 µg/ml, 0.210 µg/ml respectively of formulated Bt spore-crystal (Figure 4B.6 c, d & e**).** Moreover, blackish dead insect larva of *H.armigera* were also observed (Figure 4B.6 c & f). The pot bioassay analysis results found that plant treated with 0.210 µg/ml dose gave around 60 % control population of larva on the fourth day (Table 4B.1). and around 95 % control of larval population exhibited at the end of the sixth day. The LC_{50} of formulated *Btk HD-1* calculated to be

126.93 µg/ml after six days. The chitosan based formulation of *Btk HD-1* spore-crystal provided significant protection to *C.cajan* plant against devastating pest *Helicoverpa armigera*.

Table 4B.1: Insecticidal effect of formulated *Btk HD-1* against larvae of *Helicoverpa armigera*

Treatment (µg/ml)	Average No* of larvae survive / pot and % $^{\alpha}$ mortality after indicated days					
	Second day		Fourth day		Sixth day	
	No*	% $^{\alpha}$	No*	% $^{\alpha}$	No*	% $^{\alpha}$
0.035	8.50	8.60	7.00	22.22	2.16	73.00
0.105	8.00	13.98	5.16	42.16	1.33	83.38
0.210	7.00	24.73	3.66	59.33	0.33	95.88
- ve control	9.30	____	9.00	____	8.00	____

No*= Average No* of larvae survive per pot of *C.cajan*

% $^{\alpha}$ = % $^{\alpha}$ mortality after indicated days (Calculated as per Abbott, 1925)

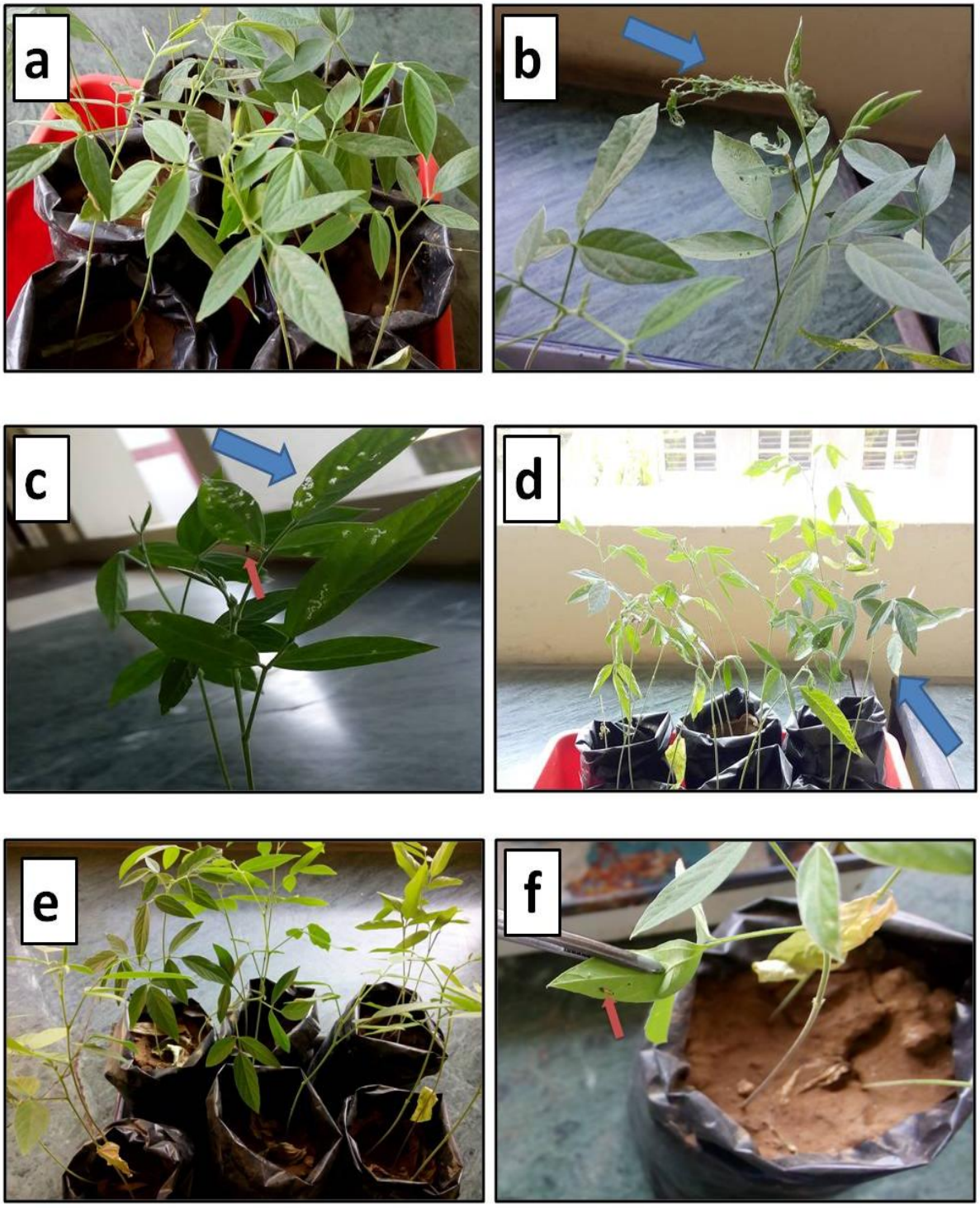

Figure 4B.6 : Pot level larvicidal activity of microencapsulated *Btk HD-1* spore-crystal : Untreated -ve control plants before (a) and after sixth day (b); plants treated with 0.035

µg/ml encapsulated Bt toxin observed less damage of leaf after sixth day and dead insect larva (c) *C.cajan* treated with 0.105 µg/ml formulated toxin on sixth day (d); Plants treated 0.210 µg/ml encapsulated Bt toxin after sixth day (e); dead insect larvae arround leave (f).

4B.3.9 Bioefficacy of *Btk HD-1* formulation on pigeon pea (*C.cajan*) field

W/O based emulsion formulation of *Btk HD-1* was sprayed on well grown, pod bearing *C.cajan* plants (Figure 4B.8 a & b). *H .armigera* population was found in average of 6.3 to 9.7 larva in each plot before spray. But, ninth day after sprayed, all doses of *Btk HD-1* formulation were found to be significant in larval population reduction over Tween-80 sprayed control. Treatments with a significant reduction ($P < 0.01$) and across the day ($P < 0.001$) were exhibited in the reduction of population of larva *H.armigera* with 44.66 to 79.05 % on ninth day after spray (Table 4 B.2). Also, reduction in the larval population treated with Treatment- 2 and 3 by W/O emulsion *Btk HD-1* formulation was significant in reduction of larval population by chemical pesticide Indoxacarb (14.5 % SC).

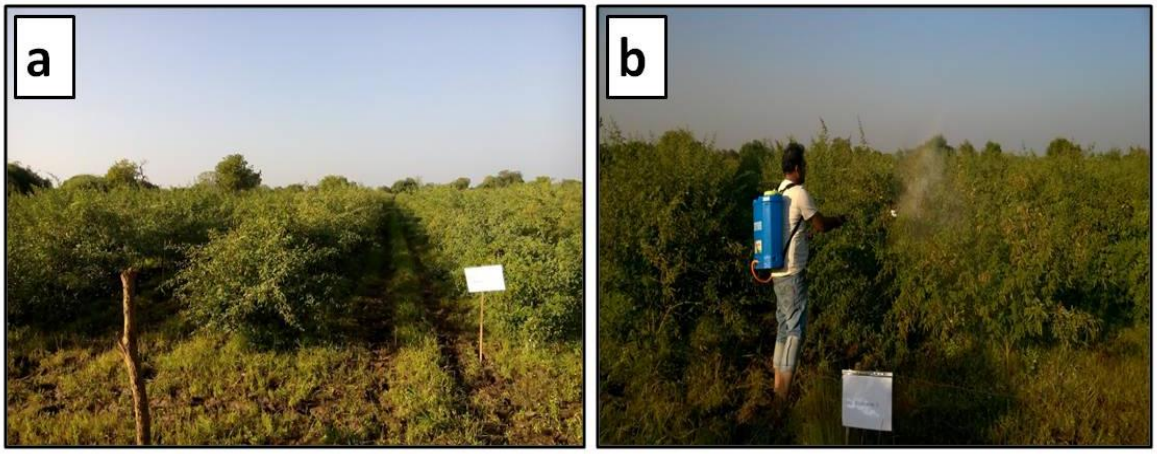

Figure 4B.8: (a) *Cajanus cajan* grown field (b) Foliar spray of formulation with Knapsack sprayer on plants

Table 4B.2: Population reduction of *H.armigera* on *C.cajan* plant by foliar spray of insecticides

Treatments	% decrease in larval population of *H. armigera**		
	Day 3	Day 6	Day 9
Btk HD-1 SCA emulsion (35 µg L^{-1})	10.324^{ab}	20.12^{d}	44.666^{f}
Btk HD-1 SCA emulsion (105 µg L^{-1})	12.448^{bc}	29.37^{e}	63.496^{g}
Btk HD-1 SCA emulsion (210 µg L^{-1})	20.438^{d}	46.574^{f}	79.058^{h}
Indoxacarb (14.5%, SC)	19.566^{cd}	31.512^{e}	61.414^{g}
Control (Tween-80)	4.024^{a}	5.392^{ab}	18.12^{cd}

*****value calculated as per Henderson and Tilton equation (1955) following mean of five replication; There was a statistically significant difference observed in treatments ($P < 0.01$) and days ($P < 0.001$). Different superscript shows statistical significant difference ($P < 0.05$)

4B.4. Discussion

Insecticidal Cry toxins and spores based Dipel (*Btk HD-1*) formulation of *Bacillus thuringiensis* strain proved an excellent biocontrol agent with controlling more than 150 lepidopteron pests (Glare & O'Callagham, 2000). However, it remains challenging to

persist their larvicidal activity in the field condition. The polymer coated (encapsulation) surrounding to spore and crystal has been proved to successful strategy as per previously employed polymer acacia gum, sodium alginate, gelatin and paraffin were obtained by Maldonado-Blanco et al.,(2002). Chitosan based encapsulation of different bio molecules such as polypeptides, proteins and DNA have extensively used in pharmaceutical and biotechnological applications. The type of encapsulation method, process parameters during preparation and kind of application are played a key role to be the success of formulation at commercial level.

The experiment results showed, the higher stirring speed (RPM) provided the sufficient energy to chitosan solution to be dispersed as droplets or coaserve in the oil (or suspension) phase. Consequently, microcapsules were smaller in size and decrease in standard deviation achieved. The size of chitosan capsules decreased corresponding to the stirring rate is increased which is compared to results obtained by Nayak et al,.(2009). Brag et al., (2006) suggested that capsule size (< 50 uM) is adequate for the control of pests in the field level. Chitosan microspheres with the size of ~17 uM could effectively control the population of *Helicoverpa armigera* and *A.albistriga.* Moreover, surface morphology of capsule could play role in accomplishment in toxin action by allowing entry of alkaline juice of insect mid gut into the capsule, solubilized crystal proteins and diffusion of the toxin. Likewise, Nayak et al,.(2009) demonstrated that an active ingredient (zidovudine) diffused through pores from the surface of microcapsules. The cadaver of *H.armigera* around treated plant assured stickiness of capsules to plant leaves and longer

persistent activity of *Btk HD-1*. Reduction of sunlight based degradation of crystal protein due to the formation of a protective film on the surface of leaves.

Encapsulation of microbial pesticides has been extensively reported with respect to UV radiation tolerance. However, protection against UV radiation mainly depends on conditions and type of irradiation. Hadapad et al., (2011) reported *B. sphaericus* Neide ISPC-8 microencapsulated by sodium alginate (15 % w/v) had 60 % spore survival at 6 h exposure of UV-B light (Philips Ltd, 288 – 320 nm, 20W fluorescent lamp at 12.5 cm distance) while in current reported results, spore viability was comparatively higher (90 %) at same time in strong UV-B irradiation. The insecticidal protein and spore both were coated in same formulation condition. However, the variation in spore viability and insecticidal activity obtained due to spore and crystal own property. Setlow (2001) reported that DPA protein of spores could provide protection against UV-B irradiation while in the case of Cry proteins, UV tolerance is associated with non-covalently bound mobile chromophore. Thus, insecticidal activity of Cry toxin lost after 24 h exposed to full solar radiation (Puztai et al.,1991). In present work, the insecticidal activity was observed even after prolong exposure to UV-B would account to the together crystal and spore in chitosan matrix which supported to result of Griego and Spench (1978). Garcia et al., (2011) results microencapsulation by sodium alginate based *Btk HD-1* formulation significant activity after 237 J of UV-B exposure against *Spodopetra frugiperda* which supported present chitosan based formulation. In current study, the high temperature (50-60 °C) during exposure duration (~180 h) could be play role in variation in toxicity of control and irritated crystal protein of free and formulated *Btk HD-1*. 259.2 J irradiation is

equivalent to more than 365 days of solar radiation of open sky in Gujarat, India (Purohit and Purohit., 2010)

Since the toxicity of crystal protein in chitosan based *Btk HD-1* formulation retained at 259.2 J irradiation, its bioefficacy was determined on *C.cajan* plant at pot level and field level. The efficacyof bioinsecticides varies and depend on the type of formulation. Ladurner et al., (2011) reported different formulation prepared by *Btk* (EG2348) stain against *Tuta absoluta* (Tomato leaf miner), the suspension concentrate proved to be more effective than the wettable powder. Rosa Garcia (2006) reported that 30g Kg^{-1} spray dried formulation of Bt strain GM-34 could effectively controlled stalkborer larva in sugarcan plant at lab scale. However, significant control was not observed in the field. Generally, insecticidal activity of microencapsulation of δ-endotoxins is similar or lower than unformulated toxins. Toxicity depends on various factors such as type of polymer being used, mass of polymer per capsules, and activity of δ-endotoxins too (Garcia et al., 2011). The difference in performance of bioefficacy could be expected in an aerial application where the density and size of droplets are major parameters (Brar, et al., 2006). As well as, waxiness of leaf surface, food quality, consumption of treated foliage by insect, insect feeding behavior associated with ambient temperature of feeding zone and wind (Frankenhuyzen 1990, Wirth et al.,1991; Meade et al.,1993).

The major goal of pest management is to reduce the time between infection and death of larvae in order to reduce plant damage. At field level, It was expected that formulation behavior in the field would be different from that in the laboratory level. In the

present study, both the higher concentration of *Btk HD-1* formulation (105 µg L^{-1} and 210 µg L^{-1}) provided remarkable higher pest control compared to commercial chemical insecticide. Thus, the most competitive formulation dose from field test was formed to be the 210 µg L^{-1} because 79 % population of *Helicoverpa armigera* controlled in less than two weeks at the field level. The population reduction observed in untreated control at ninth day was compared to third and sixth days. The primary reason might be is the tendency for late instar larvae to wander as they search for a place to pupate (Coyle et al., 2000). Previous report of 1% and 2% petroleum oil based Bt formulation sprayed on cotton plant controlled 52.06 % and 70.82 % population of *Helicoverpa spp.* larvae respectively at fifth day after treatment (Menash et al.,2005) also supported to current field study result. Das et al., (2015) reported that Indoxacarb (14.5 %) controlled 31.5 % and 88.4 % population of *H. armigera* larva after fifth and tenth days after. In the present study, bioinsecticide prepared with microencapsulated *Btk HD-1* spore-crystal could control larval population comparatively to Indoxacarb chemical insecticide. Furthermore, Indoxacarb could control larva population of *Helicoverpa armigera* superiorly than other chemical pesticides like Fibronil, Emamectin benzoate, Novaluron and Thiamethoxam. Thus, emulsion based formulation of *Btk HD-1* could be employed instead of above chemical insecticides to control of *H.armigera* in the field.

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