3.1 Introduction

Nanobiotechnology is interdisciplinary field of nanotechnology and nanoscience having numerous application in the medical field and related area. For instance, drug and gene delivery (Kataoka, et al., 2012), detection of proteins molecules (Vanderwalle et al., 2009), tissue engineering (Gloria, 2013), cancer treatment (Khatri et al., 2014) etc. Nanoencapsulated pesticide formulation is a recent emerging concept for to delivery of pesticides effectively. It has numerous advantages such as enhanced solubility (Rabinow, 2004), permeability (Wang et al., 2007), efficacy and stability in the fields (Bordes et al., 2009). Its eco friendly nature has minimum human exposure reduced phytotoxicity and damage to nontarget organisms (Margulis and Magdasshi, 2012; Peteu et al., 2010). Although nanoencapsulated pesticide is eco-friendly, incomplete knowledge of the encapsulation method and lack of a cost benefit analysis of nanoencapsulation materials restricted their application in agriculture fields. Nanocarriers such as chitosan, PLA and casein were extensively used in nanoencapsulation of protein by internal gelation method (Elzoghby et al., 2013; Grenha et al., 2005; Vila et al., 2002). The advantages using nanocarriers based method were no use of strong chemical and an emulsifying agent which could often toxic to organisms and prevents damage to active ingredients particular to the biological agent (Berger et al.,2004).

Nanopesticides formulation such as PEG-coated nanoparticles loaded with garlic oil control 80% population of the storage pest *T. castaneum* till five months (Yang et al., 2009). Moreover, Nanoemulsion formulation of neem oil has remarkable biocontrol dipteran larvae of *Culex quinquefasciatus* (Anjali et al., 2012). Nanoencapsulation techniques such as chemical conjugation and physical encapsulation have been

demonstrated to incorporate protein into polymer carrier or tissue engineering scaffold (Gopferich et al., 2007 ;Lee and Shin, 2007)**.** However, some drawbacks of these methods are protein denaturation and loss of bioactivity under conditions such as high temperatures, organic solvents, and sonication (Zhu et al. 2000). Ionotropic gelation is one of the well known methods to nonencapsulat therapeutic protein, vaccine and hormone because of its simple and mild procedure of preparation without the application of harmful organic solvents which minimize protein denaturation during encapsulation. (Houa et al., 2012). Chitosan –a polymer of N-acetylglucosamine is used extensively for drug delivery in medical and pharmaceutical applications due to its favorable biological features such as nontoxic, biocompatibility, biodegradability (Mikos et al. 1993; Zhu 2000). Chitosan nanoparticles widely applied to peptides and proteins delivery across mucosal transport, (Fernandez et al.,1999; Saranya et al., 2011). Although chitosan nanoparticles have been extensively explored in medical and pharmaceutical applications, there is no report of to preparation of nanoencapsulated insecticidal Cry protein in chitosan nanoparticles as insecticides delivery systems.

Cotton bollworm (*Helicoverpa armigera*) is a major pest of cotton, tomato, pigeon pea and other crops and found all over of the world. It is reported that loss of $5 million per annum is caused by *H.armigera* alone (Lammers & MacLeod, 2007). The pest has been controlled by chemical pesticides which are responsible for polluting the environment and soil fertility. *Bacillus thuringiensis* is a Gram-positive bacteria which produces different insecticidal proteins known as Cry proteins during sporulation. Cry protein has insecticidal activity against lepidoptera, coleopteran, diptera, hymenoptera, homoptera (Abdullah et al., 2009; Shah et al.,2016). Cry toxin has been employed either transgenic

plant or spore-crystal formulation in the field to control crop pests *Helicoverpa armigera*. Optimization of Bt toxin formulation is a key parameter of commercial production Bt (Brar et al., 2006). Thus, present research work focused on optimization of nanoencapsulation of recombinant Bt toxin Cry1Ac -Cry9Aa in chitosan –TPP nanoparticles and determination of bioefficacy against polyphagous crop pest *Helicoverpa armigera*.

3.2 Materials and Methods

3.2.1 Materials

Chitosan (CS) from shrimp cell was purchased from Himedia (TC242-50G). Pentasodium tripolyphosphate (TPP) was purchased from chemcruz (Santa Cruz Biotech.inc). Plasmid pET28a (+) -*cry1Ac-cry9Aa* encoding lepidopteran specific Bt toxin was constructed in lab. *cry1Ac-cry9Aa* construct prepared by domain-I swapping between *cry1Ac* and *cry9Aa* gene. His-tag purified Cry1Ac-Cry9Aa (pI~ 6.1, Mw ~74 kDa) toxin from *E.coli* (DE3) plysS was employed for the preparation of nanoencapsulated toxin.

3.2.2 Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared based on the chitosan –TPP .(Kawashima et al. 1985). The method is based on electrostatic interactions between the amine group of chitosan and the negatively charged group of TPP as a polyanion. During this process involving a chemical reaction, chitosan undergoes ionotropic gelation and precipitates to form spherical particles that are distinguised by opalescence of solution and presence of a bluish tint (Figure 3.1). Chitosan was dissolved in double distilled water containing 0.1N acetic acid to a concentration of 0.6% (w/v) as a stock solution. The isoelectric point of

recombinant Cry1Ac-Cry9Aa protein and pK_{α} of chitosan were approx 6.1 and 6.5 respectively. The pH of the chitosan solution was adjusted to pH 5.5 by NaOH for maximum entrapment of Cry1Ac-Cry9Aa toxin.TPP with the concentration of 0.5% (w/v) in DDW was prepared as the stock.

3.2.3 Optimization of chitosan and TPP concentrations

To optimize the particle size and the entrapment efficiency, various chitosan concentrations (0.2%, 0.3%, and 0.4% (w/v)) were prepared from the stock solution. The concentrated TPP solution (0.5% (w/v)) was used to avoid chitosan / Cry1Ac-Cry9Aa mixture. From stock solution, different volumes of TPP solution (Table 3.1) was added dropwise (10-15 µL per 10 s interval) to 10 ml of each chitosan concentration (containing 10 mg lyophilized Cry1Ac-Cry9Aa toxin) with stirring (about 700 rpm) and particular care taken to avoid foam formation. In addition to the applied volumes of TPP, Table 3.1 represents the final concentrations of the added TPP (% w/v). After 10 min of stirring, the nanoparticles were collected by centrifugation at 25,000 × g, 25°C for 30 min in 500-µl glycerol bed. The supernatants were separated to estimate the entrapment efficiency (%). The pellets of the nanoparticles in glycerol were suspended in 1 ml of distilled water for further study.

3.2.4 Entrapment efficiency of Cry1Ac-Cry9Aa Bt toxin nanoencapsulated in chitosan nanoparticles

Entrapment efficiency of recombinant toxin Cry1Ac-Cry9Aa loaded nanoparticles was detected by the Lowry method (1976) by taking the amount of free hybrid toxin in the clear supernatant. The Cry1Ac-Cry9Aa entrapment efficiency was calculated using the following equation. Entrapment efficiency (%) = [(Total insecticidal protein used in formulation −Free amount of protein) / Total protein used in formulation] × 100.

3.2.5 Particle size and zeta potential of toxin loaded chitosan nanoparticles

The size of toxin loaded nanoparticles and size distribution (polydispersity index (PDI)) were measured by Zeta Sizer (Malvern Instruments, Worcestershire, UK) based on the dynamic light scattering (DLS) technique. The mean particle size was approximated as the z-average diameter and the width of the distribution as the PDI. DLS measurements were performed at 25°C with a detection angle of 90°. For calculation of zeta potential, each diluted nanoparticles solution was put in a universal folded capillary cuvette equipped with the platinum electrode to measurement. All measurements were preformed in triplicate and the results were reported as mean ± SD.

3.2.6 Morphological analysis of chitosan-TPP nanoparticles

Morphological analysis of hybrid Bt toxin Cry1Ac-Cry9Aa loaded and free chitosan nanoparticles were carried out by a Transmission Electron Microscope (TEM) (Philips–Tecnai 20 (Philip electron optics, Holland) instrument. Nanoparticles solution of 2 to 5 uL of was placed on a copper grid and stained with 2 % (w/v) phosphotungstic acid and kept 10 min for dry subsequently performed TEM.

3.2.7 Insect bioassay of nanoencapsulated Cry1Ac-Cry9Aa toxin against *Helicoverpa armigera*

Laboratory reared insect larvae of lepidopteran insect pest *Helicoverpa armigera* were used. Nanoencapsulated and free recombinant insecticidal protein Cry1Ac-Cry9Aa was subject to performed insect bioassay. The bioassay was carried out in control environment condition (12h, light: night paradigm). Toxin doses were prepared in the range from 0.1- 10 ng per cm^2 leaf area of cabbage leaf. Twenty-four insect larvae employed for each dose. A

second instar larva of *H .armigera* was released to each dose. Insect larva were fed with 0.1% Tween -80 kept as a negative control (Dulmage, 1981). Mortality was calculated after a week. Probit analysis was performed by SPSS software version.17.00 (Chicago, IL) to the determination of LC_{50} value of nanoparticles incorporated recombinant toxin.

3.2.8 Stability study of nanoparticles of chitosan-TPP- Cry1Ac-Cry9Aa

Free Cry1Ac-Cry9Aa toxin and prepared chitosan-TPP nanoparticles of Cry1Ac-Cry9Aa toxins were stored at room temperature (20- 22°C). An equal amount of free and encapsulated Cry1Ac-Cry9Aa toxins were taken each two days interval and examined its larvicidal activity against *H.armigera* pest. Lab reared twenty larvae in triplicate were tested for single replicate. Mortality was observed every 24h till a week.

3.3 Results

3.3.1 Optimization entrapment efficiency of Cry1Ac- Cry 9Aa insecticidal protein

The recombinant Bt toxin was entrapped successfully in a matrix of chitosan-TPP nanoparticles by ionic gelation. Encapsulation efficiency of Cry1Ac-Cry9Aa toxin in nanoparticles was directly related to cross linking agent in solution. It was observed that increase in the concentration of cross linking agent resulted in increasing in encapsulation efficiency. The lowest entrapment exhibited 28.73 ± 0.93% of chitosan/TPP ratio 0.2/0.04 while highest entrapment 42.28 ±0.53% occurred when chitosan/TPP ratio was 0.4/0.095.

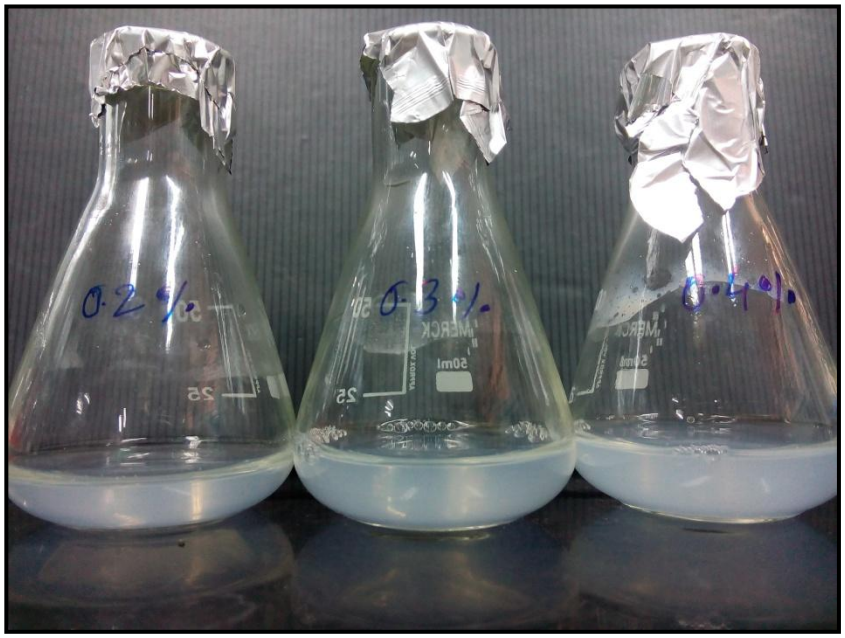

Figure 3.1: Bluish tint nanoparticles solution of 0.2 %, 0.3% and 0.4 % (w/v) chitosan

3.3.2 Size analysis of hybrid Cry toxin loaded chitosan nanoparticles

Nanobiopesticide formulation of hybrid Cry toxin was prepared by chitosan-TPP nanoparticles. To optimize size of nanoparticles, it was observed that size of nanoparticles increase with a corresponding decrease in chitosan: TPP ratio. The size of nanoparticles prepared from the different volume of TPP shown in Table 3.1. The diameter (as Z average) of nanoparticles prepared with chitosan/TPP ratio 0.3/0.06 was around ~177 nm measured by DLS technique (Figure 3.2 a). Neonate larva of target pest can be capable of engulfing nanoparticles of size 177 nm.

3.3.3 Zeta potential and polydispersion index analysis

The Nps were positively charged with a zeta potential found to the 37.29 to 19.90 mv of from different batches. It is observed that zeta potential decrease relatively with an increase

in TPP concentration in the system as well as an increase in entrapment of protein in nanoparticles (Table 3.1).

3.3.4 TEM analysis

The morphology characteristics of Cry1Ac-Cry9Aa toxin loaded nanoparticles were confirmed by TEM. The unloaded chitosan nanoparticles appeared smooth and spherical in shape while Bt hybrid toxin loaded particles showed a symmetrical, rough and spherical morphology (Figure 3.2: c, d). Immobilization of toxin on the surface of nanoparticles resulted in variation in nanoparticles morphology. The size of Nps was found in range of 170-180 nm and no aggregation of nanoparticles as revealed in TEM image.

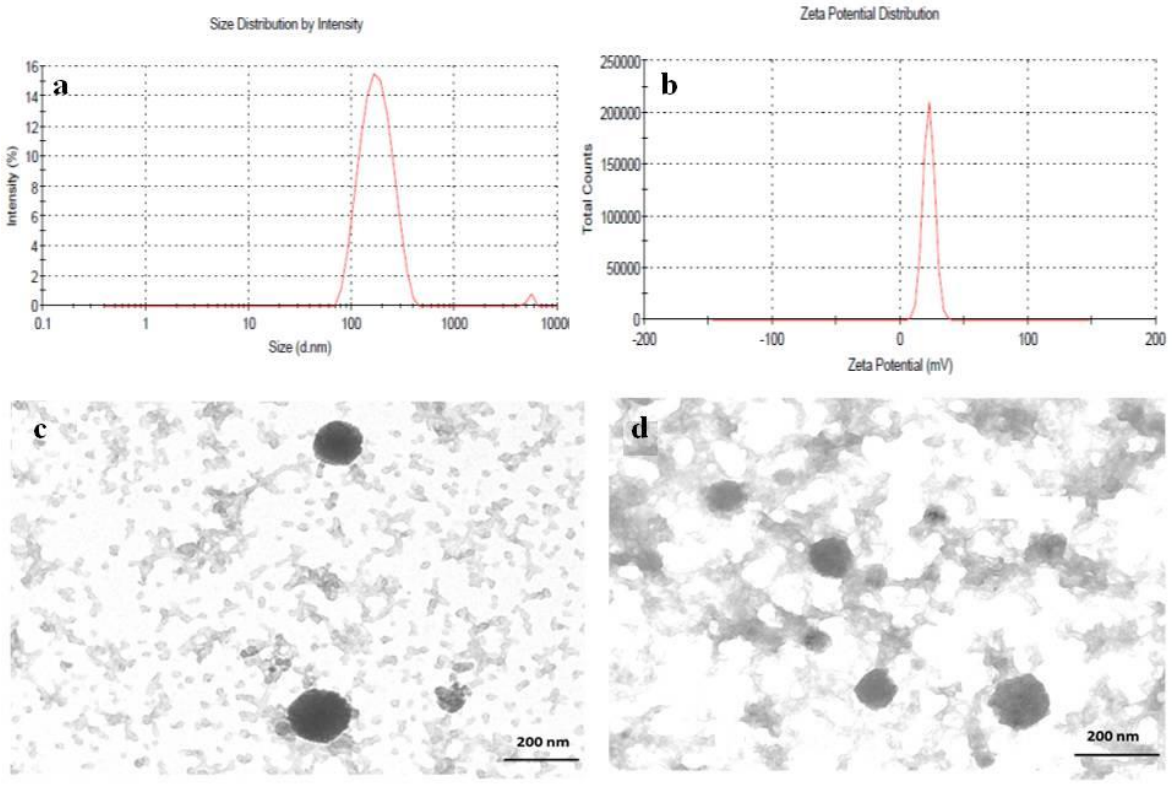

Figure 3.2: In vitro characteristics of Cry1-Cry9Aa toxin loaded chitosan nanoparticles

prepared with chitosan: TPP (0.3/0.06): (a) Particle size distribution, (b) Zeta potential, (c) Transmission electron micrograph of unloaded chitosan nanoparticles, (d) Bt toxin Cry1-Cry9Aa loaded particles.

3.3.5 Insect bioassay analysis

Insect bioassay against larva of *Helicoverpa armigera* showed reduced insecticidal activity after probit analysis. The LC_{50} of nanoparticles encapsulated recombinant protein Cry1Ac-Cry 9Aa was 2.352 ng/cm^2 (95% confidence interval: 1.684 -3.195 ng /cm^2) while free Cry1Ac- Cry9Aa toxin showed 0.725 ng/cm^2 (95% confidence interval : 0.493- 1.620 ng / cm^2).

3.3.6 Stability analysis of Bt toxin loaded nanoparticles

The storage stability of Cry-1Ac-Cry9Aa loaded chitosan nanoparticles was determined by measuring the insecticidal activity to *H.armigera* at room temperature. 90.00 ± 5.00 % of the initial activity of Cry1Ac-Cry9Aa -CSNPs was retained till four days; at the same time, free toxin activity has reduced by 36.66 ± 7.63 %. After 14 days storage at RT, Cry-1Ac-Cry9Aa loaded NPs retained 51.66 ± 5.77 % of its initial insecticidal activity while free Cry1Ac-Cry9Aa toxin completely lost its larvicidal activity after 12 days (Figure.3.2). Storage stability experiment showed that Cry-1Ac-Cry9Aa nanoencapsulation resulted into significantly improved toxin stability against devastating pest *Helicoverpa armigera*.

Table 3.1 Entrapment efficiency, size, Zeta potential (+ mv) and poly dispersion index (PDI < 0.45) analysis of nanoparticles (mean ± S.D.)

Chitosan (w/v)%	TPP (µL)	TPP (w/v) %	Chitosan (%W/V) /TPP (%W/V)	Entrapment efficacy (%)	Size (nm)	Zeta Potential (+ mv)	PDI
0.2	100	0.04	0.2/0.04	28.783±0.93	149.05± 2.01	38.48±1.09	0.263
	120	0.05	0.2/0.05	35.593±0.34	155.0 ± 3.7	22.2 ± 0.87	0.313
0.3	150	0.06	0.3/0.06	30.633±0.41	177.96 ± 6.9	30.92±0.50	0.281
	180	0.07	0.3/0.075	37.570±1.06	233.21±5.0	21.57±0.86	0.381
0.4	200	0.08	0.4/0.08	33.537±1.44	242.82 ± 3.1	28.37±0.68	0.301
	240	0.095	0.4/0.095	42.287±0.53	324.52± 2.6	20.83±0.95	0.413

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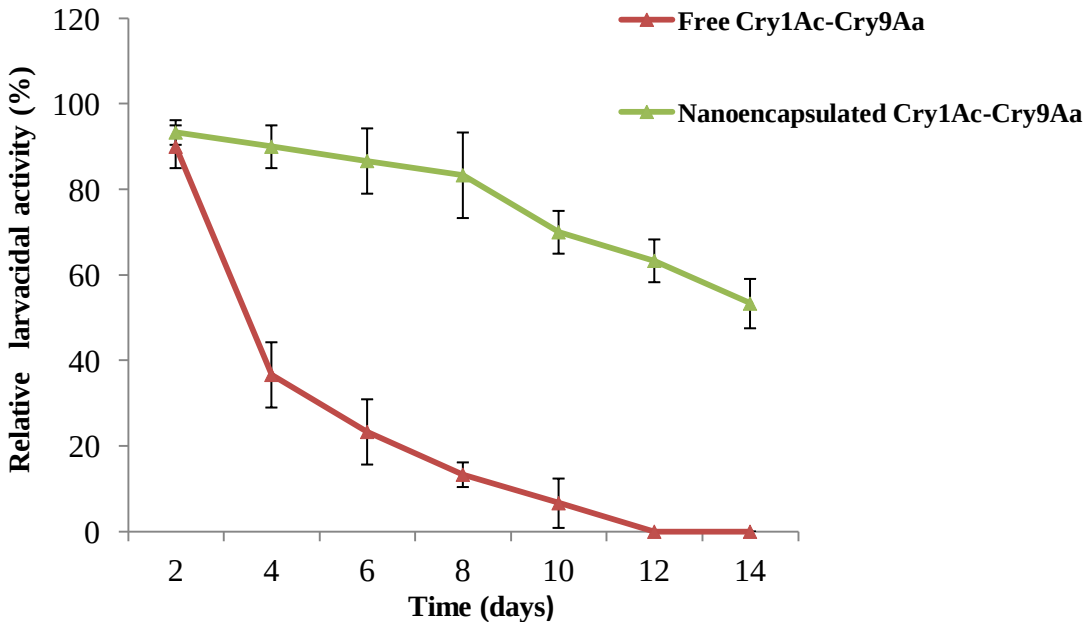

Figure.3.2 Storage stability of free and nanoencapsulated Cry1Ac-Cry9Aa *Bacillus thuringiensis* toxin (mean ± S.D.)

3.4 Discussion

The success of employing *Bacillus thuringiensis* as an effective biopesticide solely depends upon the development of desirable formulation (Couch, 2000; Rowe & Margaritis, 2004). Optimization of formulation is used with important cost effective factor in the commercial production of biopesticides (Rodham et al., 1999). In present work, ionotropic gelation method is used with an anionic cross-linking agent (TPP) is an aqueous solution of chitosan in an acidic environment. The cross-linking structure of nanoparticles of chitosan/TPP is mainly determined by pH dependent reaction between the NH_3^+ group of chitosan and TPP ions (Sun et al., 2011). Bhattarai et al., (2010) demonstrated that alteration in parameters such as drug/polymer ratio, cross-linker concentration and physical conditions could affect the final entrapment efficiency. Nanoencapsulation of Bt toxin Cry1Ac-Cry9Aa in chitosan-TPP nanoparticles, entrapment of toxin obtained in the range of 28.78 ± 0.73% to 42.28 ± 0.53% as increased TPP (cross-linker).

Mattu et al., (2013) demonstrated that entrapment efficiency of BSA protein (pI- 4.7, Mw- 66 kDa) increases as pH of solution increase; while Cry1-Cry9Aa hybrid toxin has the pI value 6.1. Thus, maximum encapsulation possible at below its pI (i.e. pH ~ 5.5). At pH-5.5, chitosan is positively charged polymer (below its pKa - 6.5) and Bt toxin Cry1Ac-Cry9Aa could also positively charged (pKa - 6.1). At very low pH, ionotropic cross-linking interaction is the only path of neutralization of fully protonated chitosan by TPP ions. Entrapment efficiency of Cry1Ac-Cry9Aa toxin increased corresponding to increase in TPP concentration and reached to 42.28% when chitosan/TPP ratio is 0.4/0.095. It is due to increased number of interacting ions of TPP along with high polymer concentration that caused protein trapping in a matrix of chitosan-TPP.

Consequently, increasing encapsulation efficiency as well as enhancement in size of nanoparticles obtained which supported by results of Alsarra et al., (2001) and Wang et al., (2008).

Nanoparticles aggregation occurred under certain conditions such as the higher amount of cross-linker and inadequate RPM during homogenization. Lopez et al., (2005) demonstrated that in ionic gelation method, where nanoparticles once formed may turn into aggregates in slightly basic pH due to pKa of chitosan near to neutral pH. The pH of prepared Cry1Ac-Cry9Aa loaded nanoparticles was between 5.8 to 6.0 in all the tested TPP- chitosan concentrations which below pKa of chitosan. Moreover, increase in TPP concentration may form nanoparticles aggregates in cross-linking, as observed increase in TPP volume . Agglomeration of nanoparticles of might be prevented by using high RPM or even by sonication but such treatment may result in depletion in the insecticidal activity of Cry1Ac-Cry9Aa loaded NPs. Thus, could not be employed.

The protein loaded nanoparticles must be in a hold ionically active state. The zeta potential of Cry1Ac-Cry9Aa loaded chitosan nanoparticles was decreased from 38.48 to 20.83 mv as toxin content of nanoparticles increased. It is due to a reduction in $-NH_3^+$ groups of the chitosan upon protein loading. Also, negative groups on the surface of Cry1Ac-Cry9Aa were counteracted with $–NH_3^+$ groups of chitosan during the cross-linking process of nanoencapsulation. Furthermore, polyanionic TPP would counteract with positively charged $–NH_3^+$ groups on the surface of Cry1Ac-Cry9Aa and compact the toxin both inside and on the surface of particles. Zeta potential about 20.83 mv considered relatively stable (Grenha et al., 2005). Gan et al.,(2005) and Rampino et al., (2013) reported that ionic interaction between chitosan and protein polypeptide would result in

changes in the shape of nanoparticles. Similarly, the morphological change of toxin loaded particles was observed by TEM analysis. Furthermore, the size measurement by DLS technique supported by TEM results.

Cry1Ac-Cry9Aa being a protein, its characteristics such as 3D structure, conformation (Bekale et al., 2015), folding and unfolding; electrostatic interaction with polymer (Bindhu & Abraham,. 2003), particle size (Lundqvis et al., 2004), and particle surface (Hong et al., 2004) might influence efficacy of nanoencapsulated toxin Cry1Ac-Cry9Aa to *H.armigera*. Garcia. et al., (2011) demonstrated the efficacy of formulated Bt toxin reduced because of factors like a mass of polymer per capsule and kind of insect larva. Similarly, Cote et al., (2001) reported *Btk HD-1* formulation by biogel resulted in 12% reduction in efficacy to *Choristoneura rosaceana* larva. The present work described nanoencapsulated Bt toxin in chitosan nanoparticles to the control of devastating pest *Helicoverpa armigera.*

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