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CLONING, EXPRESSION AND BIOASSAY OF CRY 2AX PROTEIN WITH AND WITHOUT TAG IN *ESCHERICHIA COLI* AGAINST *HELICOVERPA ARMIGERA* AND *SPODOPTERA LITURA*

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Abstract– Proteins are highly unstable and loose biological activity if not frozen under -4 °C to 20 °C. Naturally, every biological system has its own protective mechanism to maintain the biological function of each protein such as chaperones. In case of recombinant protein synthesis, no such mechanism has been observed. In this experiment a synthetic gene Cry 2Ax from *Bacillus thuringiensis* (*Bt*) was isolated and transformed into *Escherichia coli* strain with and without tag (His tag and Fusion tag) to know the expression pattern. pET 28a is used as a vector plasmid. The experimental results revealed that the tagged recombinant yielded more protein (1.5 to 2.0 µg / µl) compared with non tagged system (0.6 to 0.8 µg/µl). It shows the positive role of fusion tags on protection and expression (three fold) of the recombinant protein.

INTRODUCTION

Scientific growth leads to shuffling of gene from one biological system to another to fulfil the human needs like recombinant insulin synthesis to Human Genome Project. Recombination has been shown its importance in wide range of applications like pharmaceutical, agriculture, animals and food industry. Among them, *Bt* toxin to control insect pests in cotton is a popular and dynamic practice, recent studies focusing on feasibility of *Bt* toxin for other crop pest and nematodes.

The underlying mechanism of *Bt* toxin is accumulation of cry proteins in crystalline form during sporulation phase (Kumar and Nambawale 2002). These proteins are toxic towards larva of different orders of insect pests (Lepidoptera, Diptera, Coleoptera, Hymenoptera and Homoptera) with different efficacies (Hofte and Whiteley, 1989). *Bt* toxins are specific to a limited number of insect species with no toxicity against humans or other organisms (Bravo *et al.,* 2011). It needs alkaline pH (>9.5) for solubilisation (Saraswathy *et al*., 2008). The safety of *Bt* formulations for humans, beneficial animals and plants explains the replacement of chemical pesticides in many countries with these environmentally friendly pest control agents and technologies (Mario and Alejandra, 2008).

The success of *Bt* as bioinsecticide came with the development of *Bt*-crops that express the *cry* gene resulting in crops that resist insect attack including borers that were difficult to control with topical *Bt*-formulations leading to the commercial release of *Bt*-crops in 1995 (Sanahuja *et al*., 2011). This feature makes *Bt* as the most important biopesticide on the world market (CAB2010 and Guerra *et al.,* 2001).

Bt cry genes have three domains for its expression and activity. Domain I is involved in the membrane hydrophobicness, capable of forming pore in the midgut of the insect, domain II - determine the insecticidal specificity of the toxin, and domain III is involved in varied function like structural stability, ion channel dating, binding to Brush Border, Membrane Vesicles and insecticidal specificity (Bravo, 1997 and Crickmore, 2000). For the past few decades several research group studied these toxins by rDNA technology with special emphasis on separation and expression of these genes in *E. coli* strain (Tony M Johnson *et al.,* 1996). The current study was aimed to compare the intactness of the protein and modulation of expression pattern of Cry 2Ax gene with and

without tag. The function ability of the protein was estimated by the toxicity against *Helicoverpa armigera* and *Spodoptera litura*.

MATERIALS AND METHODS

All the experiments were carried out in the *Bt* lab of Department of Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Bt, E. coli strains and PET 28a vector

Bt (4Q7 - acrystallierous) harbouring recombinant plasmid pHT with chimeric cry 2Ax gene used in this study was obtained from *Bt*-Biotechnology laboratory, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore. *E. coli* strain DH5a and BL 21 (DE 3), PET 28a + plasmid vector were purchased and maintained as per the manufacturer instruction (Novogen USA)

Media and Growth condition

Luria – Bertani (LB) broth (Miller1972) was used to culture *Bt* and *E. coli* strains as per the standard protocol. *E. coli* harbouring PET 28a and their derivatives were mass cultured with kanamycin marker as per the manufacture instruction (100µg/ml).

DNA isolation

Recombinant *Bt* strain ((4Q7) harbouring 2Ax gene in pHT vector was mass multiplied and isolated genomic DNA using Genei kit (http// :www.bangaloregenei.com) as per instructions given by the manufacturer (Merck India http// :www.merckserono.in). The quantity and quality of DNA was analyzed through agarose gel electrophoresis (Sambrook *et al*., 1989) with known quantity of λ/*Hind* III as reference.

Selection of Gene, Primers and Vector

The 1.9 Kb Cry 2Ax genes were amplified by gene specific primers with genomic DNA of *Bt* clone as templates. The primers were designed with two pairs of restriction site *viz*., Eco R I and Hind III to exclude the fusion tag and NCO I and Hind III to retain the fusion tags. Two set of forward primers were designed, one with Eco R 1(5′ GCTCTAGATATTTAAGGAGGAATTTTATATGAA 3′) and another with NCo 1 (5′CCATGGGTAATGTATTGAATAGTGGAA3′) site as indicated by underline. Since Hind III is common for both type of clone single reverse primer (5′ AC<u>AAGCTT</u>AGTTAATAAAGTGGTGGAAGATTA 3′) was designed with Hind III site as per the underline.

Amplification and purification of PCR product

PCR was accomplished in an Eppendorf thermal cycler in 25 µl reaction volume containing 30 ng of total genomic DNA of *Bt*, 2.5µl of 10X PCR buffer (10 mM Tris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 1.5 Units of *Taq* DNA polymerase. The PCR was performed for 30 cycles at 60 °C annealing temperature and 2 minutes extension time. The PCR products were purified using ultraclean DNA purification kit (SIGMA USA, https://www.sigmaaldrich.com). The quality and quantity of PCR cleaned up product and plasmid DNA were checked through agarose gel.

Preparation of plasmid DNA (pET 28a) for ligation

E. coli (DH 5 α) strains harbouring pET 28a vector was cultured in LB kanamycin broth at 37 °C for overnight and isolated the pET 28a by using sigma plasmid isolation kit (Sigma, USA) as per the instruction given by the manufacturer. Quantity and quality of DNA were analyzed through agarose gel electrophoresis and nanodrop reading (Thermo USA https://www.thermofisher.com)

Preparation of insert and vector for ligation

The Restriction digestion of insert and vector was carried out with specific buffers as per the enzyme manufacturer for both the enzymes sets to produce recombinant protein with and without tag as described earlier. Digestion was inactivated and purified by PCR clean up kit.

Ligation and transformation

The purified Cry 2Ax (1.9 Kb) gene was ligated with pET 28a (5.4 Kb) vector for 1 hour under 22 °C for both experiment. The ligation mixtures were transformed into DH 5α competent cells by heat shock method separately (Alexandrine Froger and James E. Hall 2007). Transformed colonies were picked from the LB Kan plate for further confirmation through colony PCR (Michael E. Woodman 2008), plasmid PCR (Sigma USA) and restriction digestion (Loenen, 2014). Plasmids DNA isolated and purified from the positive clone of DH 5á were transferred into BL 21 strain for expression. Preparation of *E. coli* competent cells and

transformation were done followed by standard protocol described in the Sambrook *et al*. (1989).

Confirmation of clones by colony PCR, Plasmid PCR and Restriction digestion

E. coli strain (DH 5α) having recombinant plasmid was screened by plating the transformed colonies in LB kan (50 ppm) plate. It was further confirmed by colony and plasmid PCR and restriction digestion. Gene specific primers as mentioned above were used for colony and plasmid PCR. The confirmed colonies were used for restriction digestion. Digestion of recombinant plasmid was done with two set of restriction enzyme as per two types of clones. Eco *R1* and Hind III for Cry 2Ax gene without tag, Whereas, NCO 1 and Hind III for Cry 2Ax gene with tag. Double digestion and single digestion were carried out to confirm the clone. In case of double digestion Eco R I and Hind III were used for gene without tag. NCO I and Hind III was used for gene with tag. In case of single digestion only one restriction enzyme was used *viz*., Eco R I alone and Hind III alone for recombinant plasmid without tag. Nco I alone and Hind III alone for recombinant plasmid with tag. The results were confirmed by agarose gel electrophoresis.

Expression of cry 2Ax gene

E. coli (BL 21) harbouring recombinant plasmid of pET 28a was cultured in LB broth containing 50 µg of kanamycin / ml at 37 °C overnight. BL 21 plain strain without plasmid was taken as a negative control. One percent mother culture of recombinant strains of two clones *viz*., Cry 2Aax with tag and without tag were inoculated into 25 ml LB broth under 37 °C separately until to reach 0.6 OD at 600 nm. 1mM of IPTG (Isopropyl β-D-1-thiogalactopyranoside) was inoculated into the log phase culture for induction and kept under 30 °C for 6 hrs. The broths were used for protein extraction by following the procedure described by Shantanu *et al.* (2003). The expression of the gene was analyzed by SDS -PAGE on separating gel of 9 per cent w/v acrylamide (Laemelli, 1970).

Quantification of cry 2Ax protein

Proteins were quantified using known concentration of BSA as a standard. BSA Stock (10µg/µl) was diluted into 0.5, 1.0, 2.0 µg for SDS in such a way to calculate the concentration of recombinant protein after dilution. Dilutions were made based on protein expression in SDS PAGE through several experiments. Since the Cry 2Ax protein with tags showed more expression, maximum dilutions *viz*., 1/7 and 1/10 and in case of Cry 2Ax protein without tags showed less expression 1/3, 1/4 and 1/5 dilutions were taken for comparison and quantification. Protein quantification was done by following the procedure described by Bradford (1976).

Sequencing analysis

Positive clones were sent for sequencing analysis with T_7 promotor primer for confirmation. Sequence results were subjected to vector screening using 'Vecscreen programme' of the NCBI to identify vector region. Homology analysis was carried out using Basic Local Alignment Search Tool (BLAST) programme of NCBI (Altschul *et al*., 1990). The remaining gene region was blast with Cry 2Ax gene for homology assessment and confirmation of the clones.

Bioassay for insecticidal activity

Recombinant proteins *viz.,* Cry 2Ax with and without tags were tested for toxicity assay along with BL 21 plain strain protein as absolute negative control. Semi-synthetic diet (Patel *et al*. 1968) dispensed inside the 1.8 ml cryovial (Tarson®; 1 cm dia.) were inoculated with 7 days old larva of *Helicoverpa armigera* and *Spodoptera litura*. 10 µl recombinant proteins were spread on the surface of the vial using a sterile glass rod and allowed to dry for 30 min. Ten vials were maintained for each treatment with three replications. Larval mortality was recorded periodically for seven days.

RESULTS

PCR followed by the sequencing results confirmed the 1.9 Kb size of cry 2Ax gene size. The result of the colony (Fig. 1) and plasmid PCR of the recombinant clone showed positive amplification (1.9 Kb size). Restriction digestion result confirmed the clones 100% (Fig. 2). Double digestion of recombinant plasmid produced two bands *viz*., 5.4 Kb and 1.9 Kb. In which 5.4 Kb indicates vector size and 1.9 Kb indicates insert region. In case of single digestion produced single band 7.3 Kb size which includes vector and insert region. The results clearly indicated the positive ligation of cry2Ax gene within vector region. Protein expression of recombinant strain showed 65 kDa which is equal to cry 2Ax protein of *Bt* (Fig. 3). Protein quantification result

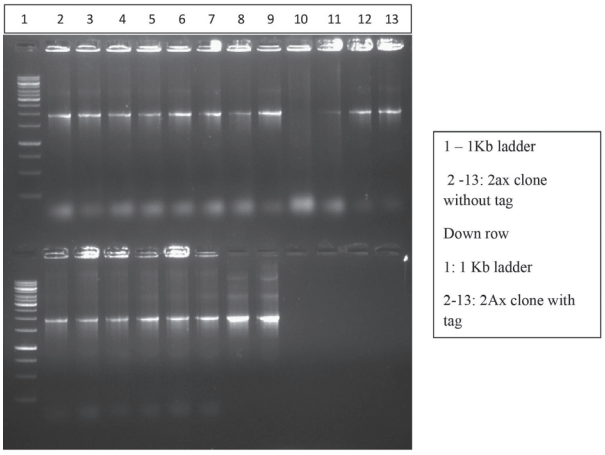
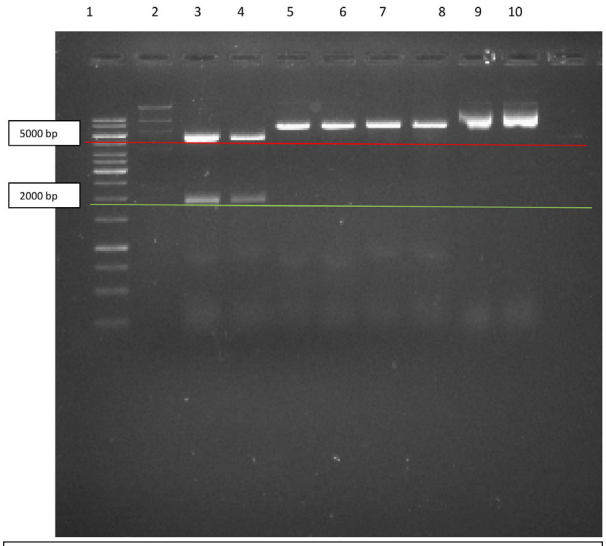


Fig. 1. Confirmation of transformed colonies of 2Ax in pET 28a of BL 21 Alpha strain through colony PCR



1 – 1Kb ladder, 2 - λ Hind III (36 ng)
3 – Double Digestion of plasmid DNA (2Ax with tags Eco R1 and Hind III)
4 – Double digestion of plasmid DNA (2ax without tags Nco I and Hind III)
5 – Single digestion of plasmid DNA (EcoR1)
6– Single digestion of plasmid DNA (NCo 1)
7 – Single digestion of plasmid DNA (Hind III)
8 – Single digestion of plasmid DNA (Hind III)
9 – Plasmid DNA 1 (2Ax with tags)
10 – Plasmid DNA 2 (2Ax without tags)

Fig. 2. Restriction digestion of Plasmid DNA from *E.coli* clone harbouring pET 28a + 2Ax with and without tags

revealed 2.0µg / µl of protein from the clone with tag whereas 0.8 µg /µl for clone without tag. It is clearly noticed that the protein quantity from recombinant *E. coli* strain was more than *Bt* (0.4 µg/ µl). Figure 3 statistical analysis showed the expression modulation among the experiments were

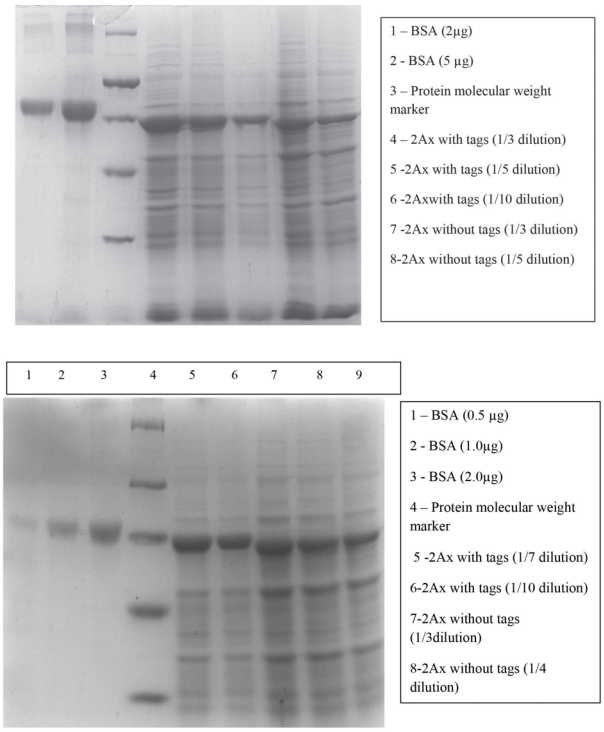


Fig. 3. Quantification of protein using BSA standard

significantly different. Sequence analysis result indicates 100% homology of recombinant gene with *Bt* gene as like database. Bioassay result with *H. armigera* and *S. Litura* exhibited 100% mortality for cry 2Ax protein without tag whereas 70% for cry 2Ax protein with tags (Table 1). Statistical analysis of the bioassay result revealed that significant variations were observed between the activity of recombinant protein with and without tags.

DISCUSSION

Shuffling of gene is predominantly followed practices in the current life science research to favour the welfare of human being. Many genes have been studied by heterologus expression system to characterize it's phenotype, genotype and its expression pattern. In general, every organism has chaperones mechanism to protect the protein for its function by repair the misfolding (Paul J. Muchowski, 2002). But in case of recombinant system such mechanism may not perform as it does not involve native proteins of the same organism. This may cause variation in expression and activity of the recombinant gene. Since this study aimed to understand the protection efficiency of recombinant protein structure and its function by tagging and

Table 1. Bioassay of recombinant Cry 2Ax protein with and with tag from *E. coli* against *Helicoverpa armigera* and *Spodoptera litura*

Treatment	1 Day	2 Day	3 Day	4 Day	5 Day	6 Day	7 Day
Control	0	0	0	0	0	20 %	20 %
HD 1(Cry 1, 2)	10%	80%	100%	100%	100%	100%	100%
2Ax without tag	0 %	30%	90%	100%	100%	100%	100%
2Ax with tag	0 %	10%	30%	40%	50%	60%	70%
SEd							
CD							

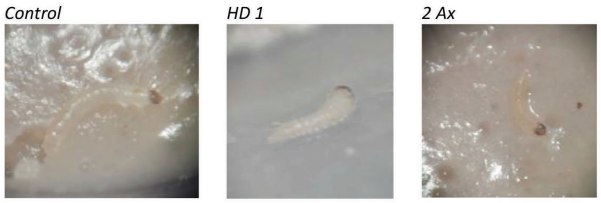


Fig. 4. Bioassay of recombinant Cry 2Ax protein with and with tag from *E.coli* against *Helicoverpa armigera* and *Spodoptera litura*

hence, this study holds its important.

The experimental result revealed protein expression was higher (2.0 µg /µl) from transformed colonies with tag than without tags (0.8 µg /µl). There was low quantity of other extracellular protein along with 2Ax protein with tags but high amount of extracellular protein were observed in case of 2Ax protein without tags. This is due to the positive effect of tags on protein folding, stability and structure. This current findings were supported by Li *et al*. (1997) who has reported the hetrologous gene expression in *E .coli* can leads to production of insoluble and (or) non-functional target proteins. This is often due to the absence of cofactors of post translational modifications required for function, stability or folding.

Coexpression of multiple genes in *E. coli* such as the members of a stable multi protein complex or a protein with a chaperone (Rivas *et al*., 2001 and Bross, 1993 year) can alleviate these problems. Affinity tags are important in protein purification. They can be helpful for stabilizing proteins or enhancing their solubility (Terpe, 2003). A final preparation of enzymes exhibited purity higher than 95% with tag as ascertained by SDS-PAGE. This tag will separate the new born protein from other protein mixture of the cytoplasm.

However the efficacy of recombinant protein against *Helicoverpa armigera* and *Spodoptera litura* has significant differences. Protein without tag showed 100% mortality whereas with tag showed 70% toxicity. This may be due to the impact of tags on recombinant protein. Hakansson *et al*. (2000) reported proteins with a His tag may vary slightly as far as their mosaicity and diffraction to the native protein. Small size tag grafting has no effect with expression, if the size of the tag is more for grafting may alter the expression. Past studies says small size tags have no impact on the activity of the recombinant protein but the size of the tag increases may leads to decrease the activity of the recombinant protein. So the nucleotide sequence of the tag and its coding mechanism, inactivation before tagging with recombinant protein has to be studied further clearly.

The experimental result revealed that the grafting of the recombinant protein cry 2Ax with His and fusion tag leads increases (3 fold) expression but 30% decreases the activity o the protein. Appropriate selection and inactivation of the tag before grafting is important for getting more proper activation of the recombinant protein in *E. coli.*

CONCLUSION

The current study concluded grafting with His and fusion tag improve the stability, quantity and purity of the recombinant protein but 30% decrease the activity of the recombinant protein. With reference to quantity of protein expression in *E. coli* with tags (2.0 µg/µl) showed threefold increase over *Bt* (0.4 µg/µl). So this recombination will a helpful for production of biopesticide formulation require low active ingredient.

REFERENCES

Alexandrine Froger and James, E. Hall, 2007. Transformation of plasmid DNA into *E.coli* using the Heat shock method. *Journal of Visualized Experiments*. (6): 253.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.W. 1990. Basic local alignment Search Toll. *J. Mol. Biol.* 215: 403-410.

Bradford, M.M. 1976. A rapid and sensitive method for

the quantification of microgram quantities utilizing the principle of protein dye binding. *Anal. Biochem*. 72(6): 248-254 doi:10.1016/0003-2697(76)90527-3.

Bravo, A.S., Likitvivatanavong, S., Gill and Soberón, M. 2011. *Bacillus thuringiensis*: a story of a successful bioinsecticide. *Insect Biochem Mol Biol*. 41: 423–431.

Bravo, A.S. 1997. Review Phylogenetic relationships of *Bacillus thuringiensis* delta-endotoxin family proteins and their functional domains. *A. J. Bacteriol*. 179(9): 2793-801.

Bross, P. 1993. Co over expression of bacterial GroESL chaperonins partly overcomes non productive folding and tetramer assembly of *E. coli*. Expressed human medium chain acyl –coA dehydrogenase (MCAD) carrying the prevalent disease causing k304E mutation. *Biochim. Biophys. Acta*. 1182: 264-274.

CAB International, 2011. The 2010 Worldwide Biopesticides Market Summary. London: CPL commercialization en México. *Ciencia UANL*, 2011. 4(2): 143-152.

Crickmore, N. 2000. The diversity of *Bacillus thuringiensis* δ-endotoxins. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C., editors. *Enthomopathogenic Bacteria: From Laboratory to Field Application*. Dordrecht: Kluwer Academic Publishers. 65–79.

Guerra, P.T., Wong, L.J.G. AND Roldán, H.M. 2011. Bioinseticidas: su empleo, produción y comercialización en México. *Ciencia UANL.* 4(2): 143-152.

Hakansson, K., Broder, D., Wang, A.H. AND Miller, C.G. 2000. Crystallization of peptidase T from *Salmonella typhimurium*. *Acta Crystallogr D Biol Crystallogr.* 56: 924–926

Höfte, H. and. Whiteley, H.R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Review.* 53(2) : 242-255.

Kumar, P.A. and Bambawale, O.M. 2002. Insecticidal proteins of *Bacillus thuringiensis* and their application in agriculture. In: *Advances in Microbial Toxin Research* (Ed: R K Upadhyay), pp. 259-280, Kluwer Academic, New York.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T_4. *Nature.* 227: 680-685.

Li, C., Schwabe, J.W., Banayo, E. and Evans, R.M. 1997. Co expression of nuclear receptor partners increases their solubility and biological activities. *Proc. Natl. Acad. Sci.* USA. 94: 2278-2283.

Loenen, W.A., Dryden, D.T., Raleigh, E.A., Wilson, G.G., Murray, N.E. 2014. Highlights of the DNA cutters: a short history of the restriction enzymes. *Nucleic Acids Res* 2014; 42, 3-19. PubMedID: 24141096

Mario S. and Alejandra B. 2008. Avoiding insect resistance to Cry toxins from *Bacillus thuringiensis*; *ISB News Report.* 1-8.

Michael E. Woodman, 2008. Direct PCR for intact Bacteria (Colony PCR). Book : Current Protocols in Microbiology. Wiley online Library. Published online 1 May 2008. DOI : 10. 1002/9780471729259.

Miller JH. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor**,** New york.

Patel, R.C., Patel, J.K., Patel, P.B. and Singh, R. 1968. Mass breeding of *Heliothis armigera* (H). *Indian J. Entomol.* 30: 272-280.

Paul, J. Muchowski, 2002. Protein Mis folding, Amyloid formation, and Neuro degeneration A Critical Role for Molecular Chaperones. *Neuron.* 35: 9–12.

Rivas, E., Klein, R.J., Jones, T.A. and Eddy, S.R. 2001. Computational identification of non coding RNAs in *E. coli* by comparative genomics. *Curr Biol.* 11: 1369–1373.

Sambrrok, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. New York : Cold Spring Harbor Laboratory press.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T. and Christou, P. 2011. *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol. J.* 9(3): 283-300.

Saraswathy, N.K., Vikrant Nian, Sushmita and Ananda Kumar, P. 2008. A fusion gene encoding two different insecticidal proteins of *Bacillus thuruingiensis*. *Indian Journal of Biotechnology*. 7: 204 -209

Shantanu Kumar, V., Udayasuriyan, P., Sangeetha and Bharathi, M. 2003. Analysis of cry 2A proteins encoded by genes cloned from indigenous isolates of *Bacillus thuringiensis* for toxicity against *Helicoverpa armigera***.** *Current Science.* 86(4) 25: 566-570.

Terpe, K. 2003. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol*. 60: 523–533.

Tony, M., Johnson, A.S., Rishi, Pritilata Nayak and Soumitra K. Sen, 1996. Cloning o a cry III A endotoxin gene of *Bacillus thuringiensis var. Tenebrirnis* and its transient expression in *indica* rice. *Journal of Biosciece*. 21 : 673–685.