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Cloning and Expression of a cry III Gene Isolated from the Local Habitat into a Modified Strain of *Bacillus thuringiensis*

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Abstract: *The cryIII gene, isolated from a locally isolated Bacillus thuringiensis strain,CAMB #30382 (isolated from grain dust of Shakargarh,Punjab,Pakistan), highly effective against red flour beetle Tribolium castaneum, was amplified through Polymerase chain reaction (PCR) by using of specific primers, The cryIII gene was cloned in HindIII digested and dephosphorylated expression vector pHB201. For this purpose, the DNA was run through 0.8% agarose gel and was eluted by means of DNA extraction kit.The transformation was done into an acrystalliferous strain of Bacillus thuringiensis, 4D22, by electroporation method . The positive clones were selected onto the petri plates containing LB X-gal/IPTG/Chloramphenicol. The Bacillus thuringiensis containing the gene of interest were also confirmed through PCR, and restriction analysis. Total protein extract from the transformed and non transformed Bt. strains were collected through affinity column chromatography.*

Key words*: Acrystalliferous strain, Bacillus thuringiensis, Biotoxicity, Expression*

I. Introduction

To increase the efficiency and persistence of Bt. toxins for field use, crystal protein genes are cloned in variety of the microorganisms. A number of toxin genes effective against lepidopteran, coleopteran and diapteran larvae from different strains of Bt. have been cloned & expressed in *E. coli*, *Bacillus subtilis,* Pseudomonas, clavibacter, Baculovirus, and nuclear polyhederosis virus (Haider *et. al*., 1988). *Bacillus thuringiensis* (Bt.) being a Gram-positive bacterium is widely used in agriculture as a biological pesticide. It is one of the potential and successful biological alternatives of chemical insecticides. Its biological activity mainly resides in a parasporal protein inclusion body, or crystal (Aronson *et al.,* 1986; Hofte and Whiteley, 1989; Schenpf *et al.*, 1998). Bt. produces several insecticidal crystalline proteins (ICP/Cry proteins) at the time of sporulation. These Cry proteins include alpha (α), beta (β), gamma (γ) and delta (δ) endotoxins. The β-exotoxins and δ-endotoxins are used for the control of pests and vectors of disease. The crystalline delta endotoxins are predominantly synthesized as long, inactive protoxins that are activated by proteolysis in the insect gut. The examples include Cry 1, Cry4A, Cry4B, having molecular weights of 130 to 140 kDa are processed to active 65 to 70 kDa toxins (Gill *et .al*., 1992,; Hofte and whitely; 1989), while Cry2A, Cry3A, Cry10A and Cry11A are naturally truncated toxins with molecular weights ranging from 65-80 kDa. Sequence analyses of many genes have suggested that significant changes in the activity spectrum can be attributed to comparatively small changes in amino acid sequences (Rahat 1998).

The δ-endotoxins are mostly used in agriculture by organic and other growers to control agronomically important pests (Dulmage, 1981; Guillet *et al*., 1990; Mulla, 1990).

Most strains are active against lepidopteral larvae, but some are toxic against dipteral (Federici *et al*., 1990) or Coleopteral species (Kreig *et al*., 1983).

Protection of storage grains and other food products from different pests has become a serious issue (Haque et al., 2005)

Most recently, strains have been identified selectively toxic to different species from several invertebrate phyla arthropods (Mainly insects), nematodes, flatworms and protozoa (Feitelson *et al*., 1992), and hymenoptran (Garcia-Robles *et al*., 2001).

Biological control methods being practiced successfully includes the use of pheromones for trapping or disruption of mating behavior, insect growth regulators that interfere with larval development, parasitoids, fungi, viruses and bacteria, which debilitate or cause death in the infected insect (Way and van Emden, 2000).

Chemical insecticides have been valuable in the control of insect pests hence stabilizing even increasing agricultural yields. Besides being uneconomic now-a-days, their indiscriminate and large scale use has given birth to serious problems of pest resistance in plant protection, industry public health and ecology. The exclusive use of non-selective products has disturbed the ecosystem by destroying non-target organisms and killing friendly insects, especially pollinators, raising the need to develop biological control agents.

Bacillus thuringiensis based biopesticides are effective in variety of situations. However in terms of reliability, spectrum of activity, speed of action & cost effectiveness, their performance is considered to be poorer than

chemicals. *Bacillus thuringiensis* is rod-shaped, aerobic, gram positive spore forming, ubiquitous, soil dwelling bacteria which is an insect pathogen and produces highly specific crystal protein called delta endotoxins. These toxins have a great potential for various agricultural & forestry pests as well as human disease vectors. The use of Bt. as biological control agent has several advantages over chemical pesticides. It has a narrow and highly specific host range & it is harmless to non-target insects. Bt. proteins are not harmful to vertebrates and beneficial insects.

There are no receptors in the gut of mammals, including human beings, for delta endotoxins and proteins from Bt. tested so far, are degraded within 20 seconds due to action of digestive enzymes in mammals (Krattiger, 1997).

Although this organism has large variety of field applications, Bt. products are not as potent as chemical products. Because they are rapidly inactivated by exposure to sunlight or other environmental factors, Bt. products act slowly, have narrow spectrum & are not stable in other environment factors (Pustazai, 1991, Tamez Guerra *et. al*., 2000). It is the potency and spectrum of activity of high yields toxins that is required for economic viability and acceptability rather than their low ecotoxicity & other ecological advantages. The production of antibiotics is usually improved by strain selection and by varying cultural conditions. It is likely that the production of the toxin could similarly be improved (Smith, 1982). A recombinant *Bacillus thuringienesis* strain expressing additional cryI gene under the control of cry3A gene expression system yields more crystal protein than wild type strain (Sanchis, 1999). It may be possible to increase the total amount of toxin produced in a *Bacillus thuringiensis* strain (Komono, 2000). Moreover the workers, Sanchis *et al*., (1999) have reported the development of broad spectrum non viable, asprogenic recombinant strain of *Bacillus thuringiensis* with greater potency.

To increase the efficiency and persistence of Bt. toxins for field use, crystal protein genes are cloned in variety of the microorganisms. A number of toxin genes effective against lepidopteran, coleopteran and diapteran larvae from different strains of Bt. have been cloned & expressed in *E. coli*, *Bacillus subtilis,* Pseudomonas, clavibacter, Baculovirus, and nuclear polyhederosis virus (Haider *et. al*., 1988).

Amplification techniques can be employed to express foreign genes in a choice host there by offering a technically feasible and commercially viable strain (Jorgensen *et. al.,* 2000). Bhattacharya (2000) isolated the hyper toxic mutant strains of *Bacillus thuringiensis* var. *israeliensis* by mutagensis of the parent strain. Coyle *et al*., 2000, conducted the field experiments to determine the efficacy of two *Bacillus thuringiensis* Berliner formulations, Novoder and Raven, for controlling cotton wood leaf beetle chrysomella seripta. They reported that both formulations reduced cotton wood leaf beetle defoliation damage after a single application giving high efficacy for control of cotton wood leaf beetle under the conditions and concentrations evaluated.

Nariman (2007), in his study detected and isolated different *cry* genes toxic towards the larvae of members of the orders lepidopteran, dipteran and coleopteran in some local *B. thuringiensis* isolates based on PCR method with specific primers, as well as predicted their insecticidal activity.

Managing the insect pests that affect economically important plants and vectors of human diseases is a major concern world wide in food production, storage of various grains and human health.

II. Material and Methods

Bacterial Strains and Plasmids

An acrystilliferous strain (4D22) of *Bacillus thuringiensis* used in the present study, was very kindly supplied by the Culture Collection Laboratory, Centre of Excellence in Molecular Biology, Punjab University, Lahore. These samples were collected from different areas of Pakistan. Most of the strains selected for the study were isolated from wheat grain, wheat dust, pulse dust, soil and dead insects.

Bt. plasmid vector p-HB201 was obtained from Promega.

Enzymes and Reagents

Restriction enzymes and DNA markers were obtained from New England Biolabs (Beverly, Massachusetts), Bethesda Research laboratories (BRL), Boehringer Mannheim and CAMB Enzyme production lab, HMW and pre-stained protein markers from GIBCO and Bio-Rad. Calf intestine Alkaline Phosphatase was from Boehringer Mannheim and T4 DNA ligase was obtained from New England Biolabs. Qia quick gel extraction Kit was obtained from QIAGEN Inc. Taq DNA polymerase was from CAMB enzyme production laboratory.

Gram Staining

To differentiate between Gram +ve and Gram -ve bacteria, staining method of Christian Gram as described by Bortholomew (1962) was used.Thin heat fixed smear from 24 hrs grown *Bt.* culture was prepared. Slide was flooded with crystal violet or methylene blue for 1min, Stain was poured off and washed with mordant (iodine solution) for 30 seconds. Slides were rinsed with slow running water and then decolorized with

alcohol at a slanting position.The slide was counterstained for 1min with safranin solution and then were washed with tap water and observed under microscope with oil immersion at 100X.Gram positive bacteria gave a blue or purple color, while Gram negative gave a red or pink color.

Malachite Green and Fuschin Staining

Bt cultures grown for 72 hrs. were stained for visualizing spores and crystals with malachite green and fuschin stain by a modified spore- staining procedure of Shaeffer and Fulton (1993) as described by E. Khan (1994, Ph.D Thesis). Smears of sporulated cultures containing spores and crystals were air-dried and heat fixed. Slides were placed above a boiling water bath and flooded with 5% aqueous malachite green. As soon as they started to dry, more were added and after 15 min., they were washed away and blotted off. The slides were seen under microscope at 100X.The spores appeared green while the vegetative cells and crystals deep pink.

SDS Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed in a Bio-Rad Mini protein-ii gel apparatus by the method of Laemli (1970). The acryl amide concentration for the resolving gel was 10% (W/V) and that for stacking gel was 4% (W/V). Purified and crude toxins were run on the gel and stained with commasie stain (0.25% (w/v) commasie brilliant blue R-250, 45.5% (v/v) methanol, 9%(v/v) glacial acetic acid) for 30-60 min. at 65 ^{0}C or overnight at room temperature (sambrook *et al*.1989). The gel was destained in 25% (w/v) ethanol and 7% glacial acetic acid with several changes until the blue background disappeared.

Total cell DNA Purification

For total DNA isolation, the procedure described by Kronstad et.,al. (1983), was adopted. An overnight culture from a single colony of bacterial cells was diluted in SPY medium in 1:100 ratio. Cells were grown at 37 ^{0}C with shaking to optical density 0.8 at 600 nm in 2.8-litre flask with shaking at 200 rpm. Cells were harvested by centrifugation at 4 ^{0}C. at 7K for 10 min. The cell pellet was washed with solution containing 100 mM NaCl, 10 mM Tris-HCl (pH7.9) and 10 mM EDTA. Lysozyme was added to give a concentration of 0.25 mg/ml. Mixture was incubated at 37 ^{0}C for 20 min. 6.25 ml of a solution containing 100 mM Tris-HCl (pH 7.5), 100 mM NaCl and 2% SDS were added to lyse the cells. Preparation was mixed by gently inverting the tubes several times. Incubation was given at 60 ^{0}C, till the preparation was clear. Extraction was done with Phenol-Chloroform mixture. Aqueous layer was removed with the wide bore pippette. 2.5 volume of chilled Ehanol was added and DNA was spooled out by means of a glass rod. DNA was rinsed with 70% Ethanol, air dried and resuspended in 500 ul to 1ml of T.E (10mM Tris-HCL pH 7.9, 1mM E.D.T.A). DNA was dialyzed extensively against cold TE buffer at 4C and concentration measured by taking O.D.260.

Mini Preparation of Plasmid DNA by Alkali lysis method

Plamid DNA was isolated by alkaline lysis method (Brinboin and Dolly, 1979). Single bacterial colony was used to inoculate 5 ml of LB medium containing the appropriate antibiotic and grown at 37 ^{0}C with vigorous shaking for 12-16 hours (overnight). 1.5 ml of overnight culture was shifted to a micro centrifuge tube and centrifuged at 12000 rpm for 3 minutes. The supernatant was decanted and the pellet was re-suspended in 100 ul of ice cold cell suspension buffer (25M Tris Hcl pH 8.0,10 mM EDTA, 50mM glucose,2 mg/ml lysozyme) and incubated for 5 minutes. Then 200 ul of a freshly prepared cell lysis solution (1% SDS, 0.2 % NaOH) was added. After immediately mixing by inverting the tube several times and 5 minutes incubation of lysate at room temperature, 150 ml of ice-cold 3M potassium acetate solution (pH 4.8) was added and again mixed by inversion followed by 15 minutes incubation on ice. Centrifugation was done at 12000 rpm for 15 minutes and clear supernatant was extracted with an equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture. Extracted aqueous phase was carried out at -20 ^{0}C for 15 minutes followed by centrifugation at 12000xg for 15 minutes. The pellet was rinsed with ice-cold 70% Ethanol, air dried and re-suspended in 25 ul of nuclease free water.

Restriction endonucleases digestion of DNA

DNA was digested using restriction endonucleases obtained from New England Biolabs, Gibco BRL, Boehringer Manheim and CAMB Enzyme production Laboratory in an appropriate 1X reaction buffer. Digestion was performed in an eppendorf thermostate at 37 ^{0}C for 60-90 minutes. The digested DNA was eluted by agarose gel electrophoresis as described by Sambrook *et.al*., (1989). One ul of RNAse A (stock: 10 mg/ml) was added in the last 15 minutes of the digestion reaction. The reaction was terminated by adding 1/5 volume loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water).

Polymerase Chain Reaction

Polymerase Chain reaction was done by a modification by Saiki *et.al*., (1988). A total volume of 50 ul contained 2.5 mM MgCl2, 10mM tris-HCl pH 8.0, 100 uM of each deoxyribonucleotide triphosphate (dNTPs), 50 pmol of each forward and reverse primer, one unit Taq DNA polymerase and 5-100 ng of DNA template. Overlaid 50-ul light mineral oil on the reaction mixture. The mixtures were amplified in 35 cycles by programming the PCR machine (M J research) according to the expected length of the PCR product.

To amplify 3.4 kbps, DNA fragment from the genomic DNA of isolate 30385, the PCR programme was as follows:

1 Cycle
95^0C 5 minutes
35 Cycles
95^0C 1 minute
59^0C 1 minute
72^0C 4 minutes
1 Cycle
95^0C 1 minute
59^0C 1 minute
72^0C 4 minutes

Once the PCR reaction was complete, it was kept at 4 ^{0}C till further processing.

Purification of DNA fragments from agarose gel

DNA fragments were eluted from the agarose gel using Qiaquick gel extraction kit (cat #28704). The specific DNA samples were run through 1% agarose gel. The required fragment was cut out of the gel under UV light (by using UV transilluminator), and transferred to an eppendorf tube. The gel slice was weighed and three times volume of buffer QG was added from the kit. In addition it was incubated at 50 ^{0}C in a water bath for approximately 10 minutes. An equal volume of isopropanol was added and mixed gently. This mixture was applied to Qiaquick column and centrifuged for 1 minute. The flow through was discarded and added 0.75 ml buffer PE (wash buffer) added to the column and centrifuged for 1 minute. The flow through and DNA bound eluted from the column, adding 33-50-ul water.

Electro competent Cell Preparation

A single colony from a freshly growing plate was inoculated into 5ml of LB medium and grown with vigorous shaking at 37 ^{0}C until O.D was 0.8 at 550nm. Cells were harvested by centrifugation and pellet washed twice by re-suspension in ice-cold 10% sterile glycerol in sterilized de-ionized water. The cell pellet was then re-suspended in 1ml of 10% glycerol to bring the final cell density to 200-250 O.D 550 units. Cells were aliquoted in 100ul aliquots and stored at -70 ^{0}C.

Cloning of the cry gene

The genomic DNA, extracted from the Bt. strain 30382, was used to amplify a 3.48 kbp full length cry3 type gene by using specific primers. Ligation of the amplified fragments was done in an expression vector pHB201, for expression studies.

Southern Blotting

Standard Capillary transfer method described by Southern (1975), was used to blot DNA onto nylon membranes. Following agarose gel electrophoresis, DNA was depurinated by immersing agarose gel in 0.25M HCL and shaking gently for 10 min. The gel was then rinsed with distilled water and the gels submerged in denaturing solution (0.5M NaOH, 1.5 M NaCl) for 45-60 minutes. Neutralization was done in neutralization solution (1M Tris-HCL pH 8.0, 1.5 M NaCl) for another 45-60 minutes. The DNA was blotted from the gel by capillary transfer to the membrane using 10X SSC (1.5 NaCl, 0.15M sodium citrate). Blotting was continued overnight to ensure efficient transfer of the DNA. After transfer the membrane was rinsed in 5X SSC (0.7 M NaCl, 0.075M sodium citrate, pH 7.0) at room temperature to remove any gel debris. The DNA was cross-linked to the membrane by exposing to UV radiation for 3 minutes. Pre-hybridization, hybridization and detection of the labeling signal was done using Genius I DNA labeling and Detection Kit (Cat. No. 1093 657), as per instructions of the manufacturer .The membrane was soaked in pre-hybridization solution for 2 hours at 65 ^{0}C. The solution was then replaced with hybridization solution containing 20-50µg /ml of DIG-labelled denatured probe followed by overnight incubation at 65 ^{0}C. After hybridization , the membrane was washed twice (5 minutes per wash), in 2X wash solution (2X SSC containing 0.1% SDS) at room temperature, followed by two washings , 15 minutes per wash, at 65 ^{0}C in 0.5X wash solution (0.5X SSC containing 0.1% SDS) to

remove unbound and non specifically bound probe. To the washed membrane, calorimetric detection procedure with NBT/BCIP solution was applied according to Boehringer Mannheim's instructions.

III. Result

Identification and isolation of Pesticidal gene by PCR Amplification.

For isolation and amplification of the gene of interest, Polymerase chain reaction (PCR) nucleic acid amplification was done using total DNA of 30382 strain, at 59 ^{0}C annealing temperature. The cell DNA was extracted and purified. Specific primers were designed from amino acid sequence of that gene. This set of primers was used to amplify full length cry gene through thermal cycler reaction. Amplified products were analyzed on agarose gel. A single band of expected size of about 3.4 kb was amplified, electrophoretically resolved and purified from agarose gel. Thus the presence of *cryIII* gene (Fig .1) has been confirmed within the strain as has been reported by Khan, (1994).

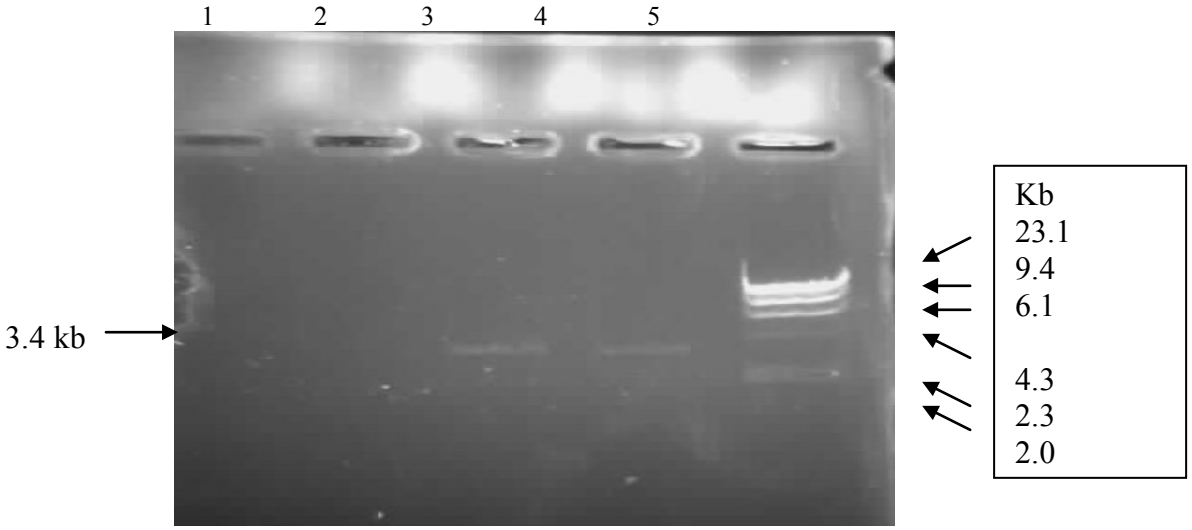


Figure :1: PCR Amplification Of cry3 gene, from DNA, isolated from strain 30382, using specific primers. Lane: 3-4 PCR amplified Cry3 gene, Lane 5: λ/Hind III marker.

Southern Blot anlaysis

Total cell DNA from isolate 30382 was prepared as described in material and method. Restriction digestion was performed at 37 ^{0}C in appropriate buffer for 3 hours. One kb XhoI fragment from PCR amplified *cry3f* gene was used as positive control. The entire DNA was then loaded onto 0.8% agarose gel along with a DNA marker, which was lambda DNA digested with *Hind III* enzyme. The blot after DNA transfer was probed with DIG labeled I kb *Xho I* fragment and hybridization signal was detected with anti-DIG antibody Alkaline phosphate conjugated followed by incubation with the color substrate NBT/BCIP.

The 1 kb positive control and about 4 kb band from digested DNA of isolate 30382 gave positive signal in lane 2 and 4 respectively. No signal was observed in negative control (Figure :2).

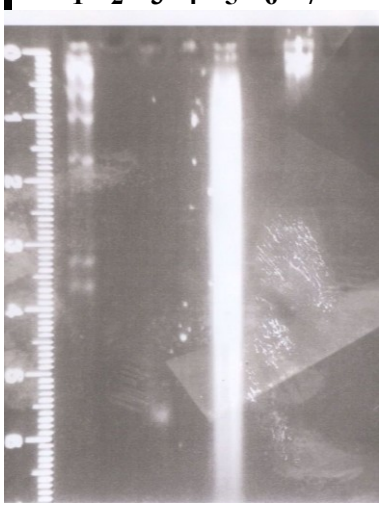


Fig:2a

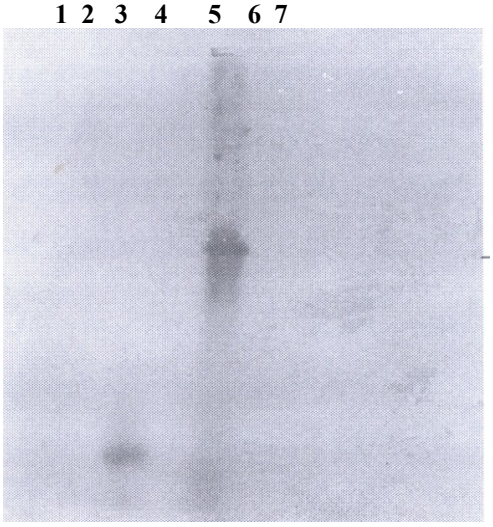


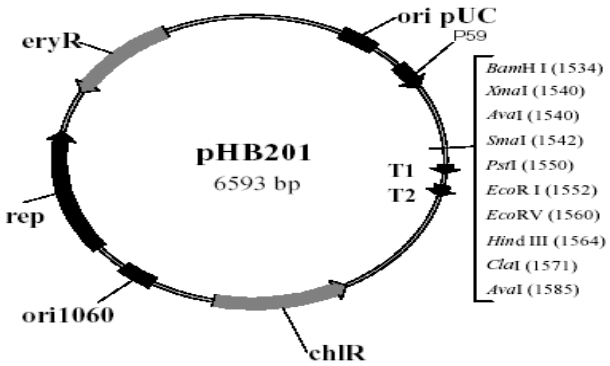
Fig: 2b

Fig .2: Southern blot analysis of genomic DNA from 30382 strain.
Fig .2a:
Lane 1 Lambda DNA digested with HindIII enzyme.
Lane: 3: positive control 1kb fragment
Lane5; DNA from isolate 30382 digested with HindIII
Lane 6: negative control
Fig.2b
Lane 1 ; Lambda DNA digested with HindIII enzyme,
Lane 3; positive control 1kb fragment.
Lane 5: about 4Kb fragment detected with DIG labeled probe
Lane6: negative control

Sub-Cloning, transformation and expression of cry gene into *Bacillus thuringiensis*

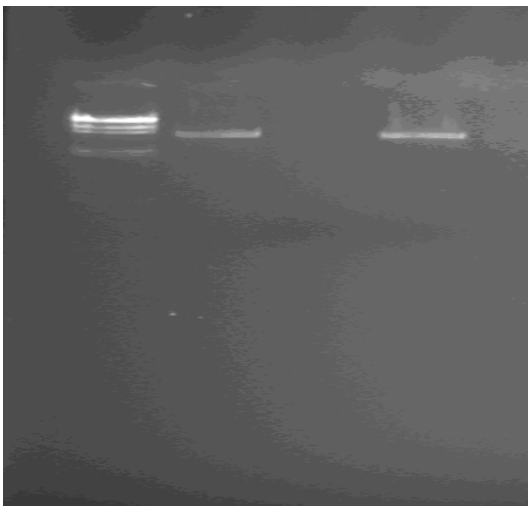
The gene of interest was sub-cloned for expression in a modified strain of *B*t. The *cryIII* gene was cloned in HindIII digested and dephosphorylated expression vector pHB201 (Figure: 3. of vector). For this purpose, the DNA was run through 0.8% agarose gel and was eluted by means of DNA extraction kit (Fig.4) Transformation was done in a modified acrystilliferrous strain of Bt.(4D22) by using electroporation method of the transformation.

The transformants were selected on LB/chloramphenicol X-gal, IPTG. The presence of positive clones was also confirmed through Polymerase chain reaction (Fig:5) and restriction endo-nuclease analysis.



1 2 3 4

Figure :3: Physical map of Shuttle vector pHB201



← 3.4 kb

Fig :4. Elution of Cry3 gene from pHB201/Hind III, for cloning and transformation into an acrystalliferrous strain *of Bacillus thuringiensis*. Lane1: λ/Hind III DNA marker, Lanes: 2-4 3.4kb Cry3 genes isolated from pHB201 plasmid digested with Hind III restriction endonuclease enzyme.

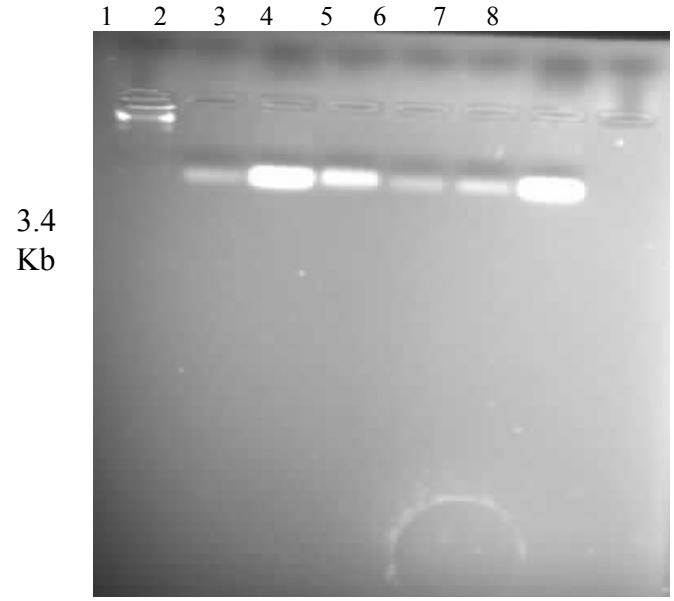


Fig :5: PCR amplification of Cry3 gene cloned within *Bt.* Strain 4D22.
Lane1: λ/Hind III DNA marker.
Lane 2-7; A 3.4 Kb fragment amplified through PCR.

IV. Expression Studies

The expression study was carried out and it was found that the gene of interest is expressed within the recombinant cells. As the strain was acrystalliferous, toxins remained within the mother cell compartment and thus remained encapsulated within the cell wall. The protein preparation from the recombinant acrystalliferous Bt. Strain (4D22) by column chromatography was resolved on SDS-PAGE (Figure.6).

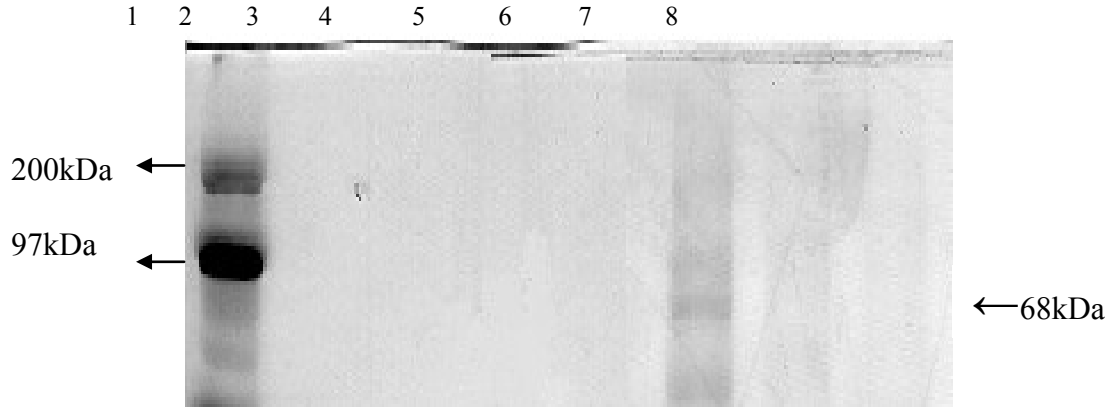


Fig: 6. SDS-PAGE analysis of column purified protein fractions of 30382 protein
Lane 1: Protein standard marker; Lanes 2-8: fractions eluted from column, arrow indicates the protein band at 68kDa.

V. Discussion

E.coli has been the host organism of choice for the expression of cloned cry genes and subsequent characterization of their encoded crystal proteins (Ward *et al*., 1986; Shivkumar *et al*., 1986). However, efficient expression of cry genes has typically required the use of heterologous promoters such as Plac (Oeda et al., 1987), Plac (Haider and Ellar *et al*., 1987) or Lambda PL and PR. Whereas, native ICP promoters are utilized in other *Bacillus* species such as *B.subtilis* (Ward *et al*., 1986) and *B. megaterium* (Donovan *et al*., 1988). Under the Ec promoter of cry1Ac gene, the expression of Cry1Ac protein is about 0.24% of the total protein of the cell (Ge *et al*., 1990; Wong *et al.,* 1983).

The environmental stability of crystal after spraying is important as it determines the duration of pest control and the number of applications needed.

In the present study, the coleopteran–specific *cry III* gene was ligated to an expression vector and transformation was done into an acrystilliferous train of *Bacillus thuringienesis,* by means of electroporation. The toxins remained within the mother cell compartment to form crystal inclusions, which remained encapsulated within the cell wall. Sun light mediated inactivation of *B.thuringiensis* crystals is often cited as the major factor affecting the performance and economic viability of Bt. Based products. Irradiation destroys upto 35% of tryptophan residue in purified *Bacillus thuringienesis* subspecies HD1 and HD73 crystals, resulting in a loss of toxicity (Pustazai *et al.,* 1991).

The results derived from this research signify the importance of cloning and sequencing of the individual gene to study the toxin effect and its mode of action. Exploration of novel gene and novel gene combinations is continued with interesting activities to various pests. Along with cloning and sequencing of the gene of interest, a better understanding of regulation of gene is expected to engineer the protein with potential insecticidal activity.

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