CHAPTER 3

RESULTS AND DISCUSSION

The first part of the work was aimed at enhancing the expression of binary toxin genes of \textit{B. sphaericus} by recombinant DNA technology. Despite the fact, that the use of organisms other than \textit{E. coli} as recipients of foreign DNA continues to increase, most of the commercial proteins produced by recombinant technology so far are mostly from \textit{E. coli}. The availability of various mutant strains, efficient promoters, different expression and purification vectors and requirement of inexpensive media for large scale production are the desirable features that favour \textit{E. coli} as a suitable candidate for the expression of foreign proteins (Korz \textit{et al.}, 1995).

3.1 EXPRESSION OF BINARY TOXIN GENES USING EXPRESSION SYSTEM

\textit{B. sphaericus} is a non-sugar utilizing organism and requires complex media for its growth (Russell, 1992). The high cost of fermentation is a major limiting factor for large scale production of \textit{B. sphaericus}. This is very crucial in terms of cost effectiveness of biological pesticides when compared to chemical pesticides. Therefore, economic production of MLPs could be achieved if the genes coding for the MLPs are transferred to a sugar utilizing organism such as \textit{E. coli}. Moreover, in \textit{E. coli}, the MLPs can be expressed throughout the growth phase unlike \textit{B. sphaericus}, where the expression of the binary toxins occurs only during sporulation. The expression of MLPs genes of \textit{B. sphaericus} in \textit{E. coli} under the control of inducible promoters
have been previously reported (Baumann et al., 1991; Oei et al., 1992; Thanabalu et al., 1993).

The genes coding for the binary toxins have been expressed in *E. coli* mainly to study the structure-function relationship of individual proteins (Davidson et al., 1990; Oei et al., 1992). Consequently, attempts to enhance the expression of MLP genes in *E. coli* would greatly facilitate using *E. coli* as an alternative host to *B. sphaericus*.

The toxicity level of the recombinants expressing MLPs against mosquito larvae have been low when compared to the parental *B. sphaericus* strains (Broadwell et al., 1990). A high level of toxicity has been achieved when these genes were expressed under the control of the strong and inducible 'tac' promoter (Rajamohan et al., 1992) using lactose as inducer. However, this system required a careful monitoring of growth conditions and glucose concentration prior to induction by lactose. In the present work, the binary toxin genes were expressed under a specific and more efficient T7 expression system which has the added advantage of resistance to catabolite repression by glucose.

### 3.1.1 Recloning of the binary toxins in pRSET B vector

The parental clone pAR5, carrying a 3.6 kb *Hind*III *B. sphaericus* DNA fragment, coding for the binary toxin was restricted with the enzymes *KpnI* and *Hind*III. This 3.1 kb fragment was directionally cloned into the multiple cloning sites of the vector pRSET B (2.9 kb) restricted with *KpnI* and *Hind*III. *E. coli* PR722 was used as the host for the transformation of ligation mixture. One of the transformants containing the recombinant plasmid was selected and designated as clone MS15 (Fig. 3. 1). In order to get a deleted version
Figure 3.1  Restriction analysis of plasmid pMS15.
Lanes, M. λ/HindIII Molecular weight marker
1. pAR5 restricted with Pst I
2. pAR5 restricted with KpnI and HindIII
3. pRSET B linearised after KpnI and HindIII
4. pMS15 restricted with Pst I
5. pMS15 restricted with KpnI and HindIII
of this plasmid for use as an internal control in competitive PCR experiments, the plasmid from the clone MS15 was isolated and restricted with Clal which has two internal sites and two DNA fragments (5.1 kb and 0.9 kb) were obtained. The 5.1 kb fragment was self ligated, transformed and the recombinant clone obtained was designated as SV18 (Fig 3.2). The construction of the plasmid pMS15 and its derivative pSV18, pMS51 and pMS42 is shown in figure 3.3.

3.1.2 Sub-cloning of 51 and 42 kDa toxin genes

In order to express the 51 and 42 kDa proteins individually, the parental clone (pMS15) was restricted with EcoRI enzyme and two DNA fragments (4.2 and 1.8 kb) were obtained. The larger fragment was religated and transformed into E. coli MV1190. A recombinant clone was identified, and designated as pMS42, which expressed the 42 kDa protein alone. Similarly, the parental clone (pMS15) was restricted with EcoRV enzyme and three DNA fragments (0.5, 0.8 and 4.7 kb) were obtained. The larger fragment was religated and transformed into E. coli MV1190 and a recombinant clone (pMS51) was identified. This clone showed the expression of the 51 kDa protein. These two clones were confirmed by restriction analysis (Fig 3.4) and used for expression and purification of 51 and 42 kDa proteins. For expression studies these clones were transformed into E. coli (BL 21) which carried the prophage containing the gene for T7 RNA polymerase.

3.1.3 Purification of the binary toxins from E. coli

The clone (pMS15) was transformed into E. coli (BL21) for overexpression and inclusion bodies were isolated. Briefly, the inclusion
Figure 3.2. Restriction analysis of plasmid pSV18.

M, λ/HindIII Molecular weight marker;

Lanes,
1. pMS15 restricted with Pst I enzyme
2. pMS15 restricted with Cla I enzyme
3. pSV18 restricted with Pst I
4. pSV18 restricted with Kpn I and HindIII
5. pMS15 restricted with Kpn I and HindIII
Figure 3.3. Construction of the plasmid pMS15 and its deletion derivatives pSV18, pMS51 and pMS42.
Figure 3.4. Restriction analysis of plasmids pMS51 and pMS42

M. λ/HindIII Molecular weight marker

Lanes, 1. pMS15 restricted with *Pst* I

2. pMS15 restricted with *Eco RI* and HindIII

3. pMS42 restricted with *EcoRI*

4. pMS15 restricted with *EcoRV*

5. pMS51 restricted with *EcoRV.*
bodies were finally solubilized in 50mM Na$_2$CO$_3$ buffer (pH 9.3) and used for further analysis. It was observed that in the Na$_2$CO$_3$ buffer (pH 9.3) the solubility of the 42 kDa protein was low when compared to that of 51 kDa protein. The binary toxins were further solubilized by increasing pH (pH 7.0 to 11.0) and it was observed that the 51 kDa protein showed higher solubility at pH 7.0 to 9.0 whereas the 42 kDa protein showed higher solubility at pH 10.0 to 11.0. These results show that there is a differential solubility of the binary toxins in Na$_2$CO$_3$ buffer (Fig. 3.5).

3.1.4 Expression studies using recombinant clones

The recombinant, clone MS15 (E. coli PR722, pMS15), expressed larvicidal activity against mosquito larvae at low levels (LC$_{50} = 3$ μg ml$^{-1}$). This indicated that the putative Bacillus promoter present upstream of the 51 kDa gene was functional but poorly recognized in E. coli. In contrast, the expression of the MLP genes in clone MS16 (E. coli BL 21, pMS15) was high in induced as well as uninduced cultures (LC$_{50} = 20$-30 ng ml$^{-1}$). The MLP expression levels of clone MS16 were comparable to that of the parental B. sphaericus strains (LC$_{50} = 16$ ng ml$^{-1}$). The expression of MLP under uninduced conditions could be due to the "leaky" expression of the T7 RNA polymerase, being regulated under lacLIV promoter. The leaky expression of T7 RNA polymerase gene in the uninduced condition is an inherent property of T7 promoter and expression system as reported earlier (Studier, 1991; Mertens et al., 1995). Further, the presence of a few molecules of T7 RNA polymerase is sufficient to drive higher level transcription of genes cloned under T7 promoter (Dubendorff and Studier, 1991; Studier, 1991).

Unexpectedly, the induction of clone MS16 with different concentrations of IPTG (0.25 mM to 1mM ml$^{-1}$) did not further enhance the MLP expression.
Figure 3.5. Solubility of binary toxins at different pH. The inclusion bodies purified from MS16 cells were solubilised in 50mM Na₂CO₃ buffer at varying pH (pH 7.0 to 11.0) and analysed in SDS-PAGE.

M. Molecular weight marker

Lanes: 1. Solubilised at pH 7.0
2. Solubilised at pH 8.0
3. Solubilised at pH 9.0
4. Solubilised at pH 10.0
5. Solubilised at pH 11.0
This may be due to the fact that the mRNA of the MLP genes is translated using the insert translation initiation signal, present upstreams of the 51 and 42 kDa coding sequences. The strong translation initiation sequence (T7 gene 10 RBS) of the vector could not be utilized due to the presence of translational stop codons in upstream of 51 kDa coding sequence. The expression thus relied on the transcription from the T7 promoter and translation from the insert RBS. These putative RBS might not be amenable for very high level expression of MLPs as it has also been observed by Broadwell et al., (1990).

Rifampicin specifically inhibits the activity of _E. coli_ RNA polymerase, and not the T7 RNA polymerase (Studier et al., 1990). The addition of rifampicin to MS16 cells induced with IPTG showed no inhibition of the MLP synthesis, but a slight increase in the MLP synthesis (LC$_{50}$ = 10 to 20 ng ml$^{-1}$) was observed (Fig. 3. 6).

### 3.1.5 Production of MLP in batch cultivation

The recombinant clone MS16 grew well in the minimal medium (M9) with selection pressure of ampicillin. The utilization of the substrate glucose, paralleled with growth. The specific MLP synthesis remained constant throughout the batch but the total MLP production increased with increase in biomass. As mentioned earlier the MLP synthesis was observed under uninduced condition. On account of the nature of the lacUV5 promoter, there was no catabolite repression of gene expression, thus resulting in a constitutive expression of the MLP genes (Fig.3. 7). Consequently, the production of MLP lends itself for considerable improvement by altering the growth conditions suitably.
Figure 3.6. SDS-PAGE analysis of binary toxin genes expression under induced and uninduced conditions expression.

M. Molecular weight protein marker;

Lanes, 1. proteins purified from uninduced MS16 cells
2. proteins purified from MS16 cells induced with IPTG
3. protein purified from IPTG and rifampicin treated MS16 cells.
Figure 3.7. MLP (binary toxins) synthesis in batch cultivation condition using recombinant clone MS16.

+ , Biomass measured as OD 500 nm; ▲, Glucose;
★, Productivity (Total protein concentration/LC 50); ●, LC 50.
3.1.6 Studies on plasmid stability in continuous cultivation

In order to assess the ability of the strain, the clone MS16 was subjected to continuous cultivation in a chemostat at a low dilution rate of 0.15 h\(^{-1}\). The cultivation was carried out for more than 40 generations. The MS16 cells collected at various intervals up to 40 generations were plated on LB agar plates with and without ampicillin. The growth of MS16 cells on selective and non-selective plates revealed that there was no apparent loss of plasmids up to 40 generations. This was confirmed by larvicidal assay of these cells. Further, the physical intactness of the MLP genes from the clone MS16 after several generations of growth was established by competitive PCR experiments. In these reactions, the same primers could amplify the normal and deleted MLP genes from plasmid isolated from MS16 and SV18 cells respectively. Two PCR products with the sizes of 1.8 (for the internal control, pSV18) and 2.6 kb were observed from plasmids isolated from cells (MS16). It was observed that there was no variation in the sizes of the amplified PCR products showing the intactness of the MLP genes even after forty generations (Fig.3.8).

The present study has highlighted the need for a continuous search for the optimal expression conditions of the MLP genes in heterologous hosts that are economically viable for the development of alternatives to chemical pesticides. While newer hosts are being discovered for the expression of these toxins (Liu et al., 1996), until their cost effectiveness is proven, use will be limited to known host organisms with proven economic productivity. It still remains to be improved in terms of environmentally safe downstream recovery and application of the MLPs derived from the recombinant strain under field conditions.
Figure 3.8. Competitive PCR for assessment of MLP genes stability.

PCR amplification of plasmid DNA isolated from clone MS16 grown for different generations with pSV18 as internal control in all the reaction mixtures.

M: Molecular marker λ DNA-HindIII digest
Lanes, 1. 1st generation; 2. 5 generations;
3. 10 generations; 4. 20 generations;
5. 30 generations; 6. 40 generations;
7. PCR amplification of pSV18;
8. PCR amplification of pMS15.
3.2 PROBING THE MECHANISM OF ACTION OF BINARY TOXINS BY SITE-DIRECTED MUTAGENESIS

In order to understand and prevent the emergence of resistance by insects against biological insecticides it is important to study the mechanism of action of insecticidal toxins. The mechanism of action of *B. thuringiensis* insecticidal toxins have been explored considerably (Dean et al., 1996) whereas the understanding the mechanism of action of *B. sphaericus* binary toxins is yet to be studied in detail. The mode of action of *B. sphaericus* binary toxins is different from *B. thuringiensis* insecticidal toxin. In the case of *B. thuringiensis* only one peptide is involved in receptor binding and insertion into membrane whereas in *B. sphaericus* two peptides (binary toxin) are required for biological activity.

Gut binding studies using fluorescent labeled deletion derivatives of 51 and 42 kDa proteins have helped to localize the functional regions of binary toxins. This study showed that the N-terminal region of the 51 kDa is involved in gut binding and its C-terminal region is essential for localized binding of 42 kDa. N-terminal deletion of 42 kDa failed to show localized binding which indicates that this region is required for interaction with the C-terminal region of 51 kDa. On the basis of these results, a model for the mechanism of action of binary toxin has been suggested. A hypothetical model is shown in figure (3.9). This model suggests that the N-terminal region of 51 kDa is involved in receptor binding and the C-terminal region of the 51 kDa and N-terminal region of 42 kDa are essential for their interaction. This is followed by internalization of the toxin complex (Oei et al., 1992). However, the nature of the receptor and the intracellular mechanism which lead to larval death is yet to be investigated.
Figure 3.9. Hypothetical model for mechanism of action of *B. sphaericus* binary toxin. BBM, Brush border membrane vesicles.
Deletion of a considerable number of amino acids in binary toxins sometimes led to a change in the structure of the toxin as evidenced from mobility differences of some of the deletion derivatives (Clark and Baumann, 1990; Oei et al., 1993). Hence, studies using mutant binary toxins, without deleting the amino acids, will be good candidates. In this study an attempt has been made to study the mode of action of binary toxin using alanine substituted mutant binary toxins generated by site-directed mutagenesis.

3.2.1 Alanine scanning mutagenesis to identify amino acids in functional domains of the binary toxins

For site-directed mutagenesis Uracil containing single stranded DNA of binary toxin genes were obtained by transforming *E. coli* CJ236 with plasmid pMS15. The procedure for site-directed mutagenesis (Muta-Gene M13 *in-vitro* mutagenesis kit, Bio-Rad) was followed as described in manufacturer's manual. Mutant clones were identified by screening the transformants by DNA sequencing, by Sanger’s dideoxy method following the manufacture’s (United States Biochemicals) instructions.

Since the three dimensional structure of the binary toxins was not known the mutant primers were synthesized on the basis of predicted secondary structure. Based on computer prediction and published results (Oei et al., 1992) twelve sites at the N- and C-terminal region of binary toxins were targeted for mutagenesis. The diagrammatic representation of amino acid sequences of binary toxins and the amino acids selected for site-directed mutagenesis is shown in figure (3.10). The secondary structure of the binary toxins were predicted using Maxhom multiple alignment program (EMBL). The amino acids selected for mutagenesis and their secondary structure prediction is given in figure (3.11). In the absence any of previous
Figure 3.10. Amino acid sequences of 51 and 42 kDa toxins.

* - Indicates the amino acids subjected for alanine substitutions.
Figure 3.11a. Secondary structure predictions for 51 kDa toxin. The regions selected for site-directed mutagenesis is represented.

The underlined amino acids were subjected for alanine substitutions.
### 42 N - Terminal Prediction

**protein:** predict

- **length:** 371

<table>
<thead>
<tr>
<th>AA</th>
<th>PHD sec</th>
<th>Rel sec</th>
<th>detail</th>
<th>prH sec</th>
<th>prE sec</th>
<th>prL sec</th>
<th>subset: SUB sec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **MRHALDFISFIPTEGYRVQMDYNSYEPCHAPSAPGDMTEIERSRENQKQFFPP**
- **E EEEE EEEE EEEE EEEE EEEE**

- **PHD sec**
- **Rel Bee**
- **detail:**
- **prH sec**
- **prE sec**
- **prL sec**
- **subset: SUB sec**

---

### 42 C - Terminal Prediction

<table>
<thead>
<tr>
<th>AA</th>
<th>PHD sec</th>
<th>Rel sec</th>
<th>detail</th>
<th>prH sec</th>
<th>prE sec</th>
<th>prL sec</th>
<th>subset: SUB sec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **EXPGITDQDQWTGRLNIGAGOFPLRNGKNTFGECFTYDRTQIPRTPLCAGCF**
- **E EEEE EEEE EEEE EEEE EEEE**

- **PHD sec**
- **Rel sec**
- **detail:**
- **prH sec**
- **prE sec**
- **prL sec**
- **subset: SUB sec**

---

**Figure 3.11b.** Secondary structure predictions for 42 kDa toxin. The regions selected for site-directed mutagenesis is represented. The underlined amino acids were subjected for alanine substitutions.
reports on the functional role of amino acids located in these targets the selected amino acids were substituted by alanine. Alanine, being amino acid having only a methyl group as side chain, will not impose extreme electrostatic or steric effects (Cunningham and Wells, 1989) and exists abundantly in both buried and exposed secondary structures (Rose et al., 1985), was selected for substitution in all selected sites. The binary toxin genes were mutated in twelve selected sites and the mutant genes were expressed in *E. coli* after identification by DNA sequencing.

### 3.2.2 Identification of essential amino acids in functional domains of binary toxins

Wild type and mutant toxins were purified as inclusion bodies from *E. coli*, and individually bioassayed against *Culex* mosquito larvae. These toxins were analysed by SDS-PAGE (Fig. 3.12 a, b). The replaced amino acids, their location and biological activity for all the mutants have been presented in Table 3.1. In general the bioassay results were compatible with earlier reports (Clark and Baumann et al., 1990; Oei et al., 1992) suggesting that the amino acids localized in the N-terminal and C-terminal regions play an important role in determining the biological activity.

Alanine substitution in the N-terminal of 51 kDa protein (51N1, 51N2) did not affect the biological activity. This could be due to the fact that these amino acids have been localized upstream to the proteolytic cleavage sites and the mutated amino acids could be removed during proteolytic processing and leaving intact the active fragments. This result is in agreement with previously published results suggesting that these amino acids are not crucial for biological activity (Clark and Baumann, 1990). Replacement of three amino acids by alanine blocks (51N3 and 51N4)
Figure 3.12 a. SDS-PAGE analysis of wild type and mutant 51 kDa toxins.

M. Molecular weight marker

Lanes, 1. Wild type toxin; 2. mutant 51N1;
3. mutant 51N2; 4. mutant 51N3;
5. mutant 51N4; 6. mutant 51C1;
7. mutant 51C2; 8. mutant 51C3
Figure 3.12 b. SDS-PAGE analysis of wild type and 42 kDa mutant toxins.

M. Molecular weight marker
1. mutant 42N1; 2. mutant 42N2;
3. mutant 42C1; 4. mutant 42C2;
5. mutant 42C3;
<table>
<thead>
<tr>
<th>Mutants Name</th>
<th>Region</th>
<th>Amino acids substituted</th>
<th>Decrease in Toxicity level</th>
<th>LC50 24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. pMS15</td>
<td>WT</td>
<td></td>
<td>Toxic</td>
<td>20ng</td>
</tr>
<tr>
<td>2. 51N1</td>
<td>51-N-ter</td>
<td>K14K17</td>
<td>Toxic</td>
<td>16ng</td>
</tr>
<tr>
<td>3. 51N2</td>
<td>51-N-ter</td>
<td>K14 K17</td>
<td>Toxic</td>
<td>16ng</td>
</tr>
<tr>
<td>4. 51N3</td>
<td>51-N-ter</td>
<td>Y2NLm</td>
<td>Non-toxic</td>
<td>15µg</td>
</tr>
<tr>
<td>5. 51N4</td>
<td>51-N-ter</td>
<td>S2KKm</td>
<td>Non-toxic</td>
<td>15µg</td>
</tr>
<tr>
<td>6. 51C1</td>
<td>51-C-ter</td>
<td>Y39RLm</td>
<td>Non-toxic</td>
<td>15µg</td>
</tr>
<tr>
<td>7. 51C2</td>
<td>51-C-ter</td>
<td>I395Qm</td>
<td>Non-toxic</td>
<td>15µg</td>
</tr>
<tr>
<td>8. 51C3</td>
<td>51-C-ter</td>
<td>K411H412</td>
<td>10 fold</td>
<td>200ng</td>
</tr>
<tr>
<td>9. 42N1</td>
<td>42-N-ter</td>
<td>F22 Y23</td>
<td>Non-toxic</td>
<td>15µg</td>
</tr>
<tr>
<td>10. 42N2</td>
<td>42-N-ter</td>
<td>C24</td>
<td>Non-toxic</td>
<td>15µg</td>
</tr>
<tr>
<td>11. 42C1</td>
<td>42-C-ter</td>
<td>L314L136</td>
<td>100 fold</td>
<td>2µg</td>
</tr>
<tr>
<td>12. 42C2</td>
<td>42-C-ter</td>
<td>R313</td>
<td>Non-toxic</td>
<td>15µg</td>
</tr>
<tr>
<td>13. 42C3</td>
<td>42-C-ter</td>
<td>H325 R327</td>
<td>Non-toxic</td>
<td>15µg</td>
</tr>
</tbody>
</table>

Table 3.1. The table shows the mutant binary toxins, amino acids replaced by alanine and their biological activity against *Culex quinquefasciatus* mosquito larvae. LC50 values are average of triplicates.
affected the biological activity. Previously, it has been reported that deletion of amino acids in 51 kDa (amino acids from 39 to 45) resulted in both loss of biological activity and gut binding to *C. quinquefasciatus* larvae (Clark and Baumann 1990; Oei et al., 1992). *In vitro* binding studies using BBMV have shown that the binding of binary toxins is mediated by the presence of a specific receptor in the susceptible mosquito larvae (Nielsen et al., 1992). Hence, the replaced amino acids in these sites may be essential for gut (receptor) binding.

Mutation in the 51C-terminal (51C2) or 42N-terminal (42N2) destroyed biological activity whereas mutant 51C3 retained biological activity. It has been observed earlier by other workers that the deletion of 61 amino acids from the C-terminal of the 51 kDa affected the toxicity whereas deletion of 53 amino acids from C-terminal did not reduce the toxicity (Clark and Baumann, 1990). Deletion of 17 amino acids from the N-terminal of the 42 kDa reduced toxicity whereas deletion of 34 amino acids led to loss of activity (Broadwell et al., 1990; Oei et al., 1992). These reports are in agreement with results obtained in the present study indicating that the amino acids mutated in 51C2 and 42N2 mutants are responsible for interaction between two peptides and mutation in either 51C-terminal or 42N-terminal could affect the interaction of 51 and 42 kDa and eventually lead to loss of biological activity.

Mutant 42C3 showed toxicity equal to that of wild type toxin. In the present study it has been observed that a single amino acid (R_{313} to A_{313}) substitution in mutant 42C2 abolished biological activity, suggesting that this amino acid may be important for internalization of the toxin complex. Earlier it has been reported that deletion of 21 amino acids from the C-terminal of the 42 kDa protein resulted in loss of toxicity which could be due to the absence of internalization of toxin complex (Oei et al., 1992).
3.2.3 Functional complementation of non-toxic mutant binary toxins

From the first part of the mutation studies, it was clearly understood that alanine substitutions in some of the mutants destroyed total biological activity. To understand the causes for loss of biological activity in non-toxic mutants four defective mutants (51N4, 51C2, 42N2 and 42C2) were selected for further investigation. In these mutants the replacement of three amino acids at the N-terminal and two amino acids at the C-terminal regions of 51 kDa (51N4 and 51C2) abolished the biological activity. Wu et al. (1996) have reported that alanine substitutions in CryIIC toxin (loop I region of domain II) has affected biological activity of the toxin against Colorado potato beetle larvae. In the case of 42 kDa protein (42N2 and 42C2) replacement of a single amino acid at the N- and C-terminal regions destroyed biological activity. Chan et al. (1996) have reported that substitution of a single amino acid (Lys-224 to Thr-224) abolished the biological activity of the Mtx toxin (Mtx2) against mosquito larvae. Lu et al., (1994) have reported that alanine substitution in CryIa toxin destroyed the activity of the toxin against Bombyx mori. Similar results have been obtained with CryIb toxin that a single alanine substitution in domain II affected irreversible binding against BBMV of Manduca sexta larvae (Rajamohan et al., 1995).

The intactness of the 51 and 42 kDa genes in non-toxic mutant clones were confirmed by PCR using specific primers. The sizes of the PCR amplified products for 51 and 42 kDa from parental and mutant plasmids were similar. The inclusion bodies isolated from these mutants were analysed by Western blot (Fig. 3. 13). The profiles of mutant binary toxins in SDS-PAGE (Fig. 3. 12a, b) and western blot analysis were similar to the wild type toxins isolated from the parental clone. These results imply that the loss
Figure 3.13. Western blot analysis of wild type and non-toxic mutant binary toxins.
M. Molecular weight marker
Lanes 1. mutant 51N4; 2. mutant 51C2
3. mutant 42N2; 4. mutant 42C2
5. wild type
of activity in these mutants may not be due to loss of expression or structural deletions, but rather due to the mutations at the specific sites on the mutant toxins.

Two reasons may be attributed to explain the cause for loss of activity in these mutants. Firstly, these mutations have been made in the conserved sequences of binary toxins genes. Baumann et al., (1991) have reported that the interchanging or duplications of the conserved blocks present in 51 or 42 kDa coding sequences abolished the biological activity of the binary toxins. Thus any manipulations in the conserved regions is expected to affect the overall function of these toxins. Secondly, it is known that the N- and C-terminal deletions in genes coding for the 51 and 42 kDa proteins reduced or abolished toxicity (Baumann et al., 1991; Oei et al., 1992). The results obtained from the mutations studies are in agreement with reports of other workers suggesting that the replaced amino acids at the N- and C-terminal regions of binary toxins are important for biological activity.

It is not known whether the binary toxins act as monomers, oligomers or multimers. As site-directed mutations could affect the association interphases of toxin moieties and thereby lead to loss of toxicity, a study was undertaken to determine whether individual non-toxic mutants can associate and give rise to functional toxins. The combined effect of the different mutant toxins on the biological activity was determined by mixing two non-toxic mutant products of the same toxin. For example, the mutant 51N4 was mixed with 51C2 and tested for toxicity. Surprisingly, this combination of mutant peptides exhibited toxicity against *C. quinquefasciatus* and *Anopheles stephensi* larvae. The toxicity levels obtained were four fold lower on *Culex* and two fold lower on *Anopheles* larvae compared to the wild type toxin that was purified from *E. coli* using parental clone. In 51N4 and 51C2 the mutations have been performed only in 51 kDa whereas the gene
coding for 42 kDa was intact in both the constructs. This was also observed when the wild type 51 kDa proteins were mixed with 51N4 or 51C2, the toxicity was restored.

This finding was extended with respect to the 42 kDa peptide. The two non-toxic mutants of 42 kDa (42N2 and 42C2) in which the 51 kDa coding sequences were intact, were mixed and tested for toxicity. This combination also resulted in restoration of the biological activity although the toxicity levels were seven fold and three fold lower against Culex and Anopheles larvae respectively, when compared to wild type toxin (Table 3.2). The biological activity was also restored when the 42N2 or 42C2 mutant was mixed with wild type 42 kDa toxin. The toxicity results of individual mutants and combination of mutants against Culex were similar to that against Anopheles larvae except for variation in the levels of toxicity. In general, it is known that B. sphaericus toxins are 4-25 times less effective against Anopheles species. It is probable that the changes in the active domain can enhance the toxic effect against mosquito larvae which is less sensitive to wild type toxin (Berry et al., 1993). Modifications of cell (receptor) binding specificity of MLPs by genetic manipulations could potentially alter the range of susceptible insects (Hazes and Read, 1995).

Since the mixing of the mutant toxins resulted in establishing the biological function of these peptides under in vitro conditions, it was of interest to know whether this phenomenon could also occur under in vivo conditions. To explore this, mosquito larvae were fed with 51N4 mutant and after 4 hours, fed with mutant 51C2 or vice versa. This also restored the toxicity to the same levels seen after in vitro mixing. A similar result was obtained with the mutant derived from the 42kDa gene products. These results clearly established that the complementation may be an in vivo process taking place inside the mosquito gut. The mutant toxic peptide
<table>
<thead>
<tr>
<th>Mutant Name</th>
<th>Aminoacids Substituted With Alanine</th>
<th>Mutated Site in Binary toxin</th>
<th>Bioassay</th>
<th>LC50 Culex1</th>
<th>LC50 Anopheles2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSV15</td>
<td>Wild-type toxin</td>
<td>51 kDa</td>
<td>Toxic</td>
<td>20ng</td>
<td>100ng</td>
</tr>
<tr>
<td>2 51N4</td>
<td>S K K</td>
<td>Non-toxic</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>3 51C2</td>
<td>I N O B</td>
<td>Non-toxic</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>4 42N2</td>
<td>C N</td>
<td>Non-toxic</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>5 42C2</td>
<td>R N</td>
<td>Non-toxic</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>6 51N4</td>
<td>51C2</td>
<td>Toxic</td>
<td>80ng</td>
<td>200ng</td>
<td></td>
</tr>
<tr>
<td>7 42N2 42C2</td>
<td>51C2</td>
<td>Toxic</td>
<td>140ng</td>
<td>300ng</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. The table shows the complementation of mutant binary toxins.

- Protein concentration required to kill 50% of larvae in 24 hrs
- Values are average of triplicates.
- Non-toxic up to 15 μg of toxins

1 *Culex quinquefasciatus* larvae

2 *Anopheles stephensi* larvae
monomers, perhaps, complement each other either by their molecular association in situ or ex situ which results in the availability of functional domain of the toxins.

Based on the above results it became clear that the mutant binary toxins act as dimers or multimers and this may be true with the wild type toxins also. Further it has also been observed that the complementation can be an in vivo process. The amino acids which are essential for toxicity have been identified and further manipulations at these sites to understand the structure function relationship are currently in progress.

3.3 PCR AND NON-RADIOACTIVE GENE-PROBE BASED MONITORING OF B. SPHAERICUS

The release of natural and genetically modified organisms (GMOs) in the environment warrants a close monitoring of the released organisms through simple and realistic methods for their detection. While conventional microbiological methods have been useful, they are time-consuming, error-prone and involve tedious procedures. In addition, these methods lack specificity unless, the microorganisms are endowed with well identified genetic markers such as antibiotic resistance genes. In the case of B. sphaericus, the clearly identifiable genetic markers are the genes coding for the MLPs.

Since B. sphaericus is being released to control mosquitoes in close proximity to human habitats, it becomes imperative that the released organism be monitored carefully, for analysing the stability and also to verify whether there is any transfer of these chromosomally located genes to other organisms that are in close association with the released bacilli. The use of
PCR to identify the presence of deliberately released microorganism, *B. sphaericus*, in the environment is a relatively new approach. PCR has so far been employed mainly for the detection of pathogenic microorganisms like *E. coli* and *Shigella* in water, soil and sludge samples (Bej et al., 1991, 1992; Tsai et al., 1991, 1992). In the present study, the amplification of the MLP genes from *B. sphaericus* was carried out, using specific oligonucleotide primers.

### 3.3.1 Optimization of PCR conditions

Initial experiments were carried out to optimize the PCR conditions to amplify the MLP genes of *B. sphaericus*. The specificity of the selected PCR primers and the product sizes were determined with the cloned MLP genes as well as the total chromosomal DNA of *B. sphaericus*. The sizes of the PCR-amplified products of *B. sphaericus* binary toxin genes were 1.3 (51 kDa) and 1.1 kb (42 kDa), while that for 100 kDa gene was 2.6 kb. Identical product sizes were seen for both the recombinant plasmid DNAs and genomic DNA with all the primers (Fig. 3. 14), indicating the specificity of the primers employed for amplification.

### 3.3.2 Specificity and sensitivity of primers and probes

When any of the primers or their probes were tested with genomic DNA of other *Bacillus* species, absolute specificity was seen only for *B. sphaericus* DNA and no products were amplified from purified genomic DNA of *B. amyloliquefaciens* or *B. thuringiensis* var. *israelensis*. Initially, digoxigenin labeled probe for 42 kDa gene was tested for its sensitivity to detect the presence of *B. sphaericus* cells since this PCR product was detected most intensely from among the three products (Fig. 3. 15 a, b). The
Figure 3.14. PCR amplification of *B. sphaericus* toxins genes.

M. λ DNA -HindIII digest

Lanes: 1, 3, 5 PCR amplification using genomic DNA of *B. sphaericus* as template

Lanes: 2, 4, 6 PCR amplification using plasmid DNA as template

The last lane shows the Molecular weight marker - φX174 HaellII
Figure 3.15a. Specificity of Primers and Probes used for detecting *B. sphaericus*.

Genomic DNA isolated from three strains of Bacilli were used for PCR amplifications:

- PCR amplification using 42 kDa (lanes, 2 to 4) and 51 kDa (lanes, 5 to 7) gene primer

Lanes 1. Molecular weight marker *φ* X174 Haelll digest
2. *B. thuringiensis israelensis*
3. *B. sphaericus*
4. *B. amyloliquefaciens*
5. *B. thuringiensis israelensis*
6. *B. sphaericus*
7. *B. amyloliquefaciens*
Figure 3.15b. Panel A: Southern blot analysis of genomic DNA of three Bacillus strains restricted with HindIII enzyme and probed with digoxigenin labeled probe (51 kDa gene probe).

Slot 1. *B. thuringiensis* israelensis; 2. *B. sphaericus*
3. *B. amyloliquefaciens*

Panel B: Hind III restricted genomic DNA of three Bacillus strains probed with digoxigenin probe (42 kDa gene probe).

Slot 1. *B. thuringiensis* israelensis; 2. *B. sphaericus*
3. *B. amyloliquefaciens*
effectiveness of the lysis method adapted and the sensitivity of the probe were established as follows: Lysates from serially diluted cells of *B. sphaericus*, obtained by the modified lysis method, were PCR amplified using primers A3 & A4 (Primer sequences given in Material and Methods). Only one product of the 1.1kb fragment was seen in these samples in the agarose gel. While ethidium bromide staining could detect the DNA fragment from about 100 cells, the chemiluminescent assay could detect even a single cell (Fig. 3. 16 a, b).

### 3.3.3 Processing of field samples containing *B. sphaericus*

One of the main objectives of this study was to develop a simple sample processing method for the detection of *B. sphaericus* toxins genes in field samples to monitor biopesticide spraying operations and to assess the stability of the toxin gene (hence the sustainability of the *B. sphaericus* toxicity) under field conditions. Sludge and water samples contain several impurities, suspended particles, degraded organic matter and other substances. Several of these (iron and iron complexes, humic acids, etc.) could interfere with the PCR (Tsai *et al.*, 1991). It was demonstrated that an initial clarification of the field samples by centrifugation at 2000 rpm removed all particulates and debris present in the samples and subsequent washing of the pellet with lysis buffer removed other contaminants adhered to the cells. In a mock-up experiment in which a known number of *B. sphaericus* cells were added to the water or sludge samples, well over 95% of the *B. sphaericus* cells added to the field samples remained in the supernatant as confirmed by plating. Further centrifugation of the supernatant at 12,000 rpm sedimented the *B. sphaericus* cells, which were washed, lysed and the lysate was used for PCR amplification.
Figure 3. 16a. Testing the sensitivity of digoxigenin-labelled gene probe.
Lysates from *B. sphaericus* cells (from 10^5 to 1 cell) were PCR amplified. Numbers on top of lanes represent cell numbers.
A: PCR - amplified products analysed in agarose gel electrophoresis
Lane M, Molecular weight marker φX174 HaeIII
Figure 3.16b. Southern blot hybridisation with digoxigenin-labelled probe
Lysates from *B. sphaericus* cells (from $10^5$ to 1 cell) were
PCR amplified. Numbers on top of lanes represent cell
numbers.
3.3.4 Competitive PCR for identifying *B. sphaericus* from field samples

In order to find out if there is any inhibition for PCR amplification from the processed samples, the deleted version of binary toxin gene (pSV18) was added as an internal control to all the PCR reactions. As previously observed, two PCR products in the range of 1.8 kb and 2.6 kb were obtained in all the PCR reactions. These results clearly indicated that there was no inhibition for amplification, nor was there any variation in the PCR product size (Fig. 3.17). When the PCR amplified products from purified genomic DNA or cells were submitted to 'dot-blot' hybridization and chemiluminescence detection the minimum detection limit was either 1 to 5 fg of purified DNA or 1 to 5 cells of *B. sphaericus* (Fig. 3.18). This PCR method was validated with field samples (water and sludge) collected from different locations where *B. sphaericus* had been sprayed. It was evident that only completely processed samples revealed the presence of *B. sphaericus*, thus validating the method of sample preparation (Fig 3.19).

So far, the samples were collected from areas wherein the release of *B. sphaericus* has been made for periods longer than three months. It was observed that no other organism isolated from the environment harbored the MLPs genes (Fig 3.20). This conclusion was arrived at after screening all the other organisms isolated from the samples for the presence of the toxin genes by PCR (Shanmugavelu *et al.*, 1996).

The outcome of this part of the present study was the development of a simple, effective and specific detection method for monitoring of the presence of *B. sphaericus* in the environment, without protracted sample treatment steps such as membrane filtration, enzyme treatments etc. as employed in other studies (Bej *et al.*, 1991; 1992; Tsai *et al.*, 1991; 1992).
Figure 3.17. Analysing the suitability of lysates of processed environmental samples for PCR amplifications.

M, $\lambda$/HindIII digest Molecular weight marker

Lanes 1. PCR amplification using pSV18 (internal control)

2. PCR amplification using pMS15 (positive control)

3, 4, and 5, PCR amplification using processed environmental samples.
Figure 3.18. Comparison of sensitivity of lysis method with purified genomic DNA. Genomic DNA (of concentration indicated on top) and crude cell lysates (from cell numbers as indicated below the bottom row) were PCR amplified with primer A3 & A4 and analysed by dot blot hybridization using non-radioactive probe (1.1 kb).
Figure 3.19. Analysis of water and sludge samples for identification of 
*B. sphaericus* using PCR and non-radioactive probe.

M, Molecular weight marker

Lanes, 1. PCR amplification from unprocessed water sample

2, 3. PCR amplification from processed water samples

4. Unprocessed sludge sample subjected directly to PCR

5, 6. Sludge samples processed and PCR amplified

The insert at the bottom is (results described above) the 'dot blot' 
hybridised with non-radioactive probe.
Figure 3.20a. Analysing the MLP genes mobility to other organisms.

A: (Top row). M, \( \lambda /{\text{HindIII}} \) digest Molecular weight marker;
Lanes 1 and 2 are common for both top and bottom row
Lanes 1. PCR amplification using pMS15 (positive control)
2. PCR amplification using pSV18 (internal control)
3 to 11. PCR amplification of processed environmental samples collected from areas where \( B. \ sphaericus \) was applied.

Figure 3.20b B: Bottom row:
Lanes, 3 to 11. PCR amplifications using lysates prepared from cultures of organisms other than \( B. \ sphaericus \). All the reactions (3 to 11) contained pSV 18 as internal control.
Further, the sensitivity of the chemiluminescent method employed in this study eliminated the use of radioactivity.

3.4 CONCLUSIONS AND FUTURE LINES OF WORK

1. Cloning of binary toxin genes of *B. sphaericus* under T7 expression system of *E. coli* resulted in a higher level expression of these proteins. It was observed that the recombinant cells showed equal level of binary toxin expression under both induced and uninduced conditions. The toxicity levels of the recombinant cells against mosquito larvae were comparable to *B. sphaericus* strain.

2. The "leaky" expression of T7RNA polymerase gene being regulated under the lacUV promoter has been found to be responsible for constitutive expression of binary toxins under uninduced conditions.

3. Batch cultivation studies were carried out to analyse the suitability of the recombinant strain for large scale production of mosquito larvicidal proteins. The stability of the cloned binary toxin genes were studied in chemostat. This study clearly established that the binary toxin genes were stable up to forty generations as evidenced from competitive PCR experiment.

4. The binary toxins purified from the recombinant *E. coli* as inclusion bodies were solubilised in Na₂CO₃ buffer at different pH ranging from 7.0 to 12.0. The 51 kDa toxin showed higher solubility at neutral pH whereas the 42 kDa showed higher solubility at pH 11.0 to 12.0.
5. Site-directed mutagenesis technique was used to generate mutant binary toxins and the mutations were made on the basis of the secondary structure prediction. Alanine was substituted at selected sites on binary toxins and the effect of alanine substitution towards biological activity of the mutant toxins were analysed.

6. The bioassay results showed that the alanine substitutions in some sites at N- and C-terminal regions of 51 and 42 kDa toxins resulted in the complete loss of biological activity. These sites were known to be important for gut binding, interaction between 51 and 42 kDa toxins and internalisation of toxin complex. Interestingly, biological activity was restored when two non-toxic mutants of the same proteins (51N4 and 51C2) were mixed and tested for toxicity against mosquito larvae.

7. The restoration of the biological activity by non-toxic mutants could be due to the complementation between them, which results in availability of functional domains, suggesting that the mutant binary toxins act as dimers or multimers.

8. Towards the monitoring of *B. sphaericus* in the environment the conditions for the amplification of MLP genes using PCR were optimised and specificity of the primers and non-radioactive probes were tested. The sensitivity of detection using PCR and non-radioactive probe was from 1 to 10 cells of *B. sphaericus*.

9. An easy sample processing method has been developed to amplify the target sequences in PCR directly from environmental samples. Competitive PCR method has been applied to monitor the mobility of the MLP genes to other organisms.
Based on the results presented above, the following lines of work are suggested for achieving the goals of enhanced expression and production of MLPs of *B. sphaericus* for mosquito control:

- Cloning of 100 kDa gene (Mtx) of *B. sphaericus* upstream to the binary toxin genes in the construct pSV15 for expressing all the MLPs in a recombinant *E. coli*.

- Performing *in vitro* studies such as receptor binding (using ligand blot techniques), batch clamp experiment for demonstrating the role of individual mutant binary toxins. Further mutagenesis in the non-toxic mutants of binary toxins to generate up-mutants.

- Identification of receptor(s) for binary toxins from cDNA library of mosquito cell lines and *in vivo* demonstration of 51 and 42 kDa peptides interaction using yeast two-hybrid system.