CHAPTER - 3
FERMENTATIVE PRODUCTION OF *Bacillus thuringiensis* AND STRAIN IMPROVEMENT FOR ENHANCING THE LARVICIDAL ACTIVITY OF *BT*
Dulmage (1981) has suggested that most efficient ways of increasing the potency of 
*Bt* and reducing the cost of fermentation products are through (1) changes in 
nutritional balance and/or (2) through discovery or selection of improved strains of *Bt*. 
These two factors have to be considered independent of each other. For example, 
Dulmage (1989) had shown that change in media composition affected toxicity as 
well as specificity among the two insect pests tested *viz.* *Trichoplusia ni* and *Heliothis virescens*. Thus, it becomes imperative to target selected insect pests by 
choosing suitable media and *Bt* isolates.

Several investigations have been carried out to determine the nutritional requirement 
for growth, sporulation and crystal formation (Dulmage, 1970; Arcas *et al.* 1984; 
Kang *et al.* 1993; Nickerson and Bulla, 1974). In general, media consisted of a carbon 
and energy source like glucose, nitrogen source such as peptone or yeast extract, 
ammonium Sulphate and mineral salts. In addition, several micronutrients like Mn**, 
Ca**, Zn** and Fe** have been also recommended. The presence of Mn** is 
considered essentially for growth and endospore formation in *B. subtilis*. Potassium 
is also important for cell growth, endotoxin formation and sporulation (Wakisaka 
et al. 1982). Recently, the use of gruel hydro for the formulation of a new medium for 
δ-endotoxin production of *Bacillus thuringiensis* subsp. *kurstaki* was reported. A new 
medium was formulated based on the hydrolysate of gruel, a cheap and abundant by 
product of semalino factories, supporting growth and δ-endotoxin synthesis. It was 
reported 1 g/l δ-endotoxin in 4.5 g/l total dry biomass produced (Zouari *et al.* 1997). 
Manasherob *et al.* (1998) reported germination, growth and sporulation of *Bacillus 
thuringiensis* subsp. *israelensis* in excreted food vacuole of the protozoan *Tetrahymena pyriformis*. 
3.1 : Fermentative Production of *Bt* : Nutritional Requirement for the Production of *Bt* : Factors Affecting Growth, Sporulation and ICPs Production:

Production of bioinsecticidal preparation is basically a biomass production process, provided the microorganisms requirement for sporulation and ICPs formation are fulfilled. In this respect the use of carbon source such as glucose, a nitrogen source like yeast extract and the presence of potassium, magnesium, manganese and some micronutrients appears to be essential. *Bt* var. *kurstaki* HD-1 was found effective against *Helicoverpa armigera* and hence was selected for studies with respect to standardisation of fermentation conditions for maximum growth, sporulation and ICPs production. These studies were carried out at shake flask level and using 2.0 L capacity bioreactor.

3.1.1: Inoculum Size:

Inoculum size of 1% (v/v) (10^8 spores/ml) was found to promote uniform growth with more than 95% sporulation. In all subsequent experiments inoculum size of 1% (v/v) was maintained. Stage of inoculum is also an important factor of sporulating *Bt*. The final product, ICPs obtained at the end of fermentation, largely depends on sporulation efficiency of the cells. Twelve hour old vegetative cells and spore suspension were used as inoculum. With vegetative cells as inoculum variation in growth was observed, but when spores were used as inoculum, growth was found uniform with short lag phase. This may be due to germination of the spores at the same time.

3.1.2: Effect of pH:

During vegetative growth of *Bt*, pH of the medium falls to acidic side (6.0 - 6.5), which rises to alkaline side towards the end of exponential growth as shown in Figs. 3.1.1 and 3.1.8. The fall in pH is due to accumulation of acids like lactic, pyruvic and acetic acid which are consumed at the end of vegetative phase and pH rises to alkaline side under normal fermentation conditions (Benoit *et al*. 1990; Dulmage, 1971;
Pearson and Ward, 1988; Yousteen and Rogoff, 1969). However, low pH suppresses sporulation, affecting final yield of ICPs production. This occurred when high concentration of glucose is used. pH adjustment is not required at low concentration of glucose. Incremental feeding of glucose along with pH adjustment would allow use of higher substrate concentration. In fact, it is difficult to define "too high" glucose concentrations, since it depends on the concentrations of the nitrogenous nutrients in the medium. Bt tends to produce alkaline materials from nitrogenous nutrients that can neutralize the acidic compound produced from the sugars. Hence, it is essential to have a relative balance of sugar and nitrogen materials.

3.1.3: Effect of Media Composition:

There is no one medium that is best for all the cultures of Bacillus thuringiensis, hence attention was paid to nutritional studies, since a medium may be sufficient to support vegetative growth and not be sufficient for sporulation.

In basal glucose yeast extract salt medium (GYS) containing glucose, 0.1 g%; yeast extract, 0.2 g% and ammonium Sulphate, 0.2 g%, the biomass of 2.30 g/l and insecticidal crystal protein about 0.25 mg/ml were obtained with 95% sporulation within 24 h. In this medium yield and productivity were 108 mg crystal protein/g biomass and 0.0104 g/l/h respectively (Fig. 3.1.1). Various carbon sources were used to see their effect on crystal protein production. Among the various carbon sources studied, Glucose was found an adequate carbon source (Fig. 3.1.2). Sharp crystals were produced in the glucose yeast extract salt medium having 0.5 g% glucose concentration. Amorphous crystals were formed due to decrease in pH of the medium when glucose concentration above 1 g% used. Besides glucose, glycerol was found to be a better carbon source but was not feasible economically to use for the production of Bt. Production of Bacillus thuringiensis with respect to biomass and crystal protein was studied at various glucose concentrations. Glucose concentration at 0.8 g% resulted in more biomass and protein but the crystals were amorphous when observed microscopically. At 0.5 g% glucose, sharp crystals were formed. The biomass and
FIG. 3.1.1: Profile of fermentative production of *B. thuringiensis* var. *kurstaki* HD-1 in basal Glucose yeast extract salt medium (GYS).

The error bars represent the standard deviation (SD).
FIG. 3.1.2: Effect of different carbon sources on the production of *B. thuringiensis* var. *kurstaki* HD-1 (Btk HD-1).

The error bars represent the SD.
crystal protein obtained were 3.8 g/l and 0.4 mg/ml, having yield 118 mg crystal protein/g biomass and productivity 0.093 g/l/h respectively (Fig. 3.1.3). When higher concentration of glucose is to be used, the pH of the medium should be controlled which decreases below 6.0. Glycerol also gives higher biomass and spores but glycerol is not a cheap carbon source. It is possible that the use of higher concentration of glucose (10-20 g/l) could be convenient for increasing yields but this can only be performed with controlled pH. It was necessary to use an adequate organic nitrogen source. Effect of different nitrogen sources on growth, biomass, crystal protein production and sporulation were studied (Fig. 3.1.4). Yeast extract, beef extract, casein and peptone were found to be the best nitrogen sources. Effect of various concentrations of yeast extract was also studied. Yeast extract at the concentration (1 g%) gave optimum crystal protein yield (Fig. 3.1.5).

Effect of different inorganic nitrogen sources on the growth, biomass, crystal protein production and sporulation were studied (Fig. 3.1.6). The use of inorganic nitrogen as a sole source of nitrogen was completely inadequate. Inorganic nitrogen sources were supplemented with 1 g% yeast extract to support the growth, sporulation and ICPs production.

The adequate inorganic nitrogen source was ammonium Sulphate supplemented with yeast extract. The effect of various concentrations of ammonium sulphate on the production of Bt were studied. Optimum concentration of ammonium sulphate was 0.2 g% (Fig. 3.1.7).

From the results obtained, it was indicated that the best nitrogen source was a combination of yeast extract and ammonium Sulphate. When concentration of ammonium sulphate was increased upto 1 g%, higher values of biomass was obtained but the pH shifted to acidic side with the appearance of amorphous crystals. The use of ammonium sulphate as a sole source of nitrogen was completely inadequate which proved that Bt requires some amino acids or peptides, which are essential for growth.
FIG. 3.1.3 : Effect of different Glucose concentrations on the production of Btk HD-1

The error bars represent the SD.
FIG. 3.1.4: Effect of different nitrogen sources on the production of Btk HD-1

The error bars represent the SD.
When H$_2$O$_2$ treated yeast extract was used in the medium, which destroyed some amino acids like cysteine and methionine resulted in poor growth. Ammonium Sulphate was found to have synergistic effect in combination with yeast extract in enhancing growth and sporulation of Bt. On the basis of the results obtained in the present investigation, GYS medium was modified.

The final composition of the modified GYS medium was as follows: glucose, 0.5 g%; yeast extract, 1 g%; ammonium Sulphate, 0.2 g%; K$_2$HPO$_4$, 0.06 g%; MgSO$_4$, 0.04 g%; MnSO$_4$, 0.006 g% and CaCl$_2$, 0.008 g%. When Bt was grown in the modified GYS medium at shake flask, biomass, 4.5 g/l; crystal protein, 0.72 mg/ml and $2.5 \times 10^{12}$ spores/ml were obtained at the end of 60 h (Fig. 3.1.8). In this medium yield (160 mg crystal protein/g biomass) and productivity (0.012 g/l/h) was obtained. In the medium containing glucose (0.5 g%) and yeast extract (1 g%) C:N ratio was found to be 3:1 and for glucose (0.5 g%) and ammonium sulphate (0.2 g%) C:N ratio was 12:1.

The production of ICPs by Bt could be limited by growth factors, which could influence the sporulation rate. In Bt massive sporulation may be initiated by the depletion of key nutrients, such as carbon, nitrogen and phosphate sources. Inhibition of sporulation under protein rich medium was also observed (Pearson and Ward, 1988). Good balance of carbon and nitrogen concentration along with mineral salts and sufficient aeration was very much essential to ensure high degree of substrate utilization, sporulation and ICPs production. Scherrer et al. (1973) have reported that increased glucose concentration affected protein yield as well as toxicity with formation of amorphous inclusions.

Potassium ions has long been known as an essential mineral for bacterial growth. But its metabolic role in cell division is not clear. The effect of potassium on ICP production and sporulation may provide a key to elucidating the metabolic role of potassium in bacterial morphogenesis and cell division. Certain concentration of
FIG. 3.1.5 : Effect of different concentrations of Yeast extract on the production of Btk HD-1.

The error bars represent the SD.
Effect of different Inorganic nitrogen sources on the production of Btk HD-1.

The error bars represent the SD.

Inorganic N sources

FIG. 3.1.6 : Effect of different Inorganic Nitrogen sources on the production of Btk HD-1.

The error bars represent the SD.
potassium is essential for the fermentative production of ICPs by the isolates of *Bt*. Wakisaka *et al.* (1982) reported that the presence of potassium ion in the growth medium was essential for formation of active ICPs. Similar studies were carried out by Foda *et al.* (1985), using *Bt* var. *entomocidus*. They have shown marked increase in both yield and activity of the ICPs with increase in dipotassium hydrogen phosphate concentration up to 0.2 M.

Manganese could not be substituted for potassium. Phosphate ions stimulated poly-β-hydroxybutyrate formation. The sporulation of *Bt* and several other *Bacilli* were suppressed on the potassium deficient medium. Potassium plays an essential role in *Bacillus* cell growth, ICP production and in sporulation. Magnesium Sulphate is one of the important components of media ingredients for *Bt* production (Goldberg *et al.* 1981). They used a complex medium including soypeptone and increased the spore yield 120-fold and insecticidal activity, 83-fold by the addition of 2 g/l magnesium sulphate.

Effect of media composition on growth and sporulation of *Bt* have been reported (Dulmage, 1981; Faloci *et al.* 1990; Mummagatti and Raghunathan, 1990; Sakharova *et al.* 1988).

Most of the media used in studies on physiology of *Bt* also contain ammonium Sulphate in addition to organic nitrogen source such as peptone or yeast extract (Egorov *et al.* 1990; Nickerson and Bulla, 1974; Avignone Rossa *et al.* 1992; Yousteen and Rogoff 1969; Wakisaka *et al.* 1982). It was also reported that strains of *Bt* fail to utilise ammonium Sulphate as a sole source of nitrogen and requires an additional organic N source such as glutamate to germinate and sporulate (Nickerson and Bulla, 1974). Hence it was necessary to use an adequate organic nitrogen source. Yeast extract was found the most convenient source of organic nitrogen sources because it contains amino acids, vitamins and minerals.
FIG. 3.1.7: Effect of different concentrations of Ammonium Sulphate on the production of Btk HD-1

The error bars represent the SD.
3.1.4 : Effect of Aeration :

*Bacillus thuringiensis* is an aerobic spore forming bacteria and fermentative production of *Bt* is a highly aerobic process. During the present investigation the effect of aeration on the production of *Bt* was studied at shake flask level and using a 2.0 L capacity bioreactor. The effect of aeration on the production of *Bt* at shake flask level was studied at different aeration rates created by taking 10, 20, 30 and 40% working volumes. At these conditions *k*\(_{La}\) values obtained were 19.65, 11.62, 9.02 and 6.89 / h respectively by sulphite oxidation method (Table 3.1.1). The remarkable effect of aeration on the production was observed at 10% and 40% working volumes (wv). At 10% wv i.e. *k*\(_{La}\) 19.65/h, dry weight, 4.8 g/l and crystal protein, 0.81 mg/ml was obtained while at 40% wv i.e. at *k*\(_{La}\) 6.89 / h, dry weight 3.5 g/l and crystal protein, 0.52 mg/ml was obtained (Fig. 3.1.9 & 3.1.10).

Table 3.1.1 : Effect of *k*\(_{La}\) on the rate of crystal protein production in shake flask :

<table>
<thead>
<tr>
<th>OTR</th>
<th><em>k</em>(_{La}) / h</th>
<th>Protein (g/l)</th>
<th>Biomass (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.62</td>
<td>19.65</td>
<td>0.81</td>
<td>4.8</td>
</tr>
<tr>
<td>2.74</td>
<td>11.65</td>
<td>0.72</td>
<td>4.5</td>
</tr>
<tr>
<td>2.12</td>
<td>9.02</td>
<td>0.59</td>
<td>3.8</td>
</tr>
<tr>
<td>1.62</td>
<td>6.89</td>
<td>0.52</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*OTR* - Oxygen transfer rate (mmole O\(_2\)/g biomass/min).

At *k*\(_{La}\) value 19.65/h, yield and productivity were 168 mg crystal protein / g biomass and 0.0135 g/l/h respectively whereas at *k*\(_{La}\) value 6.89/h, yield and productivity were 148 mg crystal protein/g biomass and 0.0086 g/l/h respectively. Production was found to be affected by decrease in the *k*\(_{La}\) value. Higher oxygen transfer rate as well as productivity and yield of *Bt* were obtained at lower working volume (10% wv) than higher working volume (40% wv).
FIG. 3.1.8: Profile of fermentative production of Btk HD-1 in a modified GYS medium.

The error bars represent the SD.
FIG. 3.1.9: Profile of Btk HD-1 production at 10% working volume ($k_{la} 19.65/h$).

The error bars represent the SD.
When production of \( Bt \) in 2.0 L capacity bioreactor was carried out, \( k_{La} \) value about 63/h was obtained during the log phase, indicating high oxygen transfer rate during this phase. The effect of aeration was also remarkable when \( Bt \) was produced in a bioreactor. At \( k_{La} \) value 63 / h, dry weight 5.5 g/l and crystal protein 0.92 mg/ml was obtained (Fig. 3.1.11). Yield and productivity were found to be 167 mg crystal protein/g biomass and 0.0153 g/l/h. Specific growth rate obtained was 0.8/h. It was observed that \( k_{La} \) values decreased exponentially during the stationary phase and lowest \( K_{La} \) value was recorded at the end of fermentation. This could be due to less requirement of oxygen and low oxygen transfer rate by increased viscosity of the sporulated \( Bt \) fermented broth. Limited aeration and agitation was provided during the stationary phase to avoid excessive foaming and bubbling out of the fermented broth.

High concentration of oxygen required for growth and sporulation of \( Bt \) was reported earlier (Dingman and Stahly, 1983; Avignone rosa et al. 1992). The requirement for high aeration rates for growth and sporulation of \( Bt \) have also been reported (Pearson and Ward, 1988). Since the rate of crystal protein production depend upon biomass concentration, it is desirable to have a high biomass concentration in the vessel. The medium contains carbon compounds and other nutrients and they allow the organism to grow, initially near the maximum specific growth rate. This rapid growth causes a considerable increase in the initial oxygen transfer rate, so the fermentation medium should be agitated and sufficient supply of air provided to achieve maximum growth during the log phase.

Time course production of the insecticidal crystal protein of Btk during the fermentation was monitored by immunoassay. Production of \( Bt \) ICPs was started 12 h onwards and reached to its maximum at the end of 72 h. Primary powder prepared from the spore crystal complex of \( Bt \) was bioassayed against \( H. armigera \) and \( Spodoptera \) sp. neonate larvae. \( LC_{50} \) value for \( H. armigera \) was 38 ± 1.3 ng/well while that for \( Spodoptera \) sp. was 285 ± 2.6 ng/well, indicated that \( H. armigera \) is highly susceptible to Btk while \( Spodoptera \) sp. is less sensitive to Btk. Spore crystal
FIG. 3.1.10: Profile of Btk HD-1 production at 40% working volume (K_{La} 6.89/h)

The error bars represent the SD.
FIG. 3.1.11: Profile of fermentative production of Btk HD-1 in a bioreactor ($k_{La}$ 63/h)

The error bars represent the SD.
complex harvested from the 10% working volume at shake flask and bioreactor was quantified by dot blot immunoassay. At 10% working volume 78.12 ng crystal protein was detected while that of grown in bioreactor 19.53 ng crystal protein was detected by this method.

3.2 : Strain Improvement for Enhancing the Larvicidal Activity of *Bt* :
Several approaches are being persued to enhance the host range and potency of *Bt* isolates. A combination of conventional microbiological and *in vitro* DNA manipulation methods have been applied. Bacteriophages have been shown mediate exchange of DNA within different *Bt* isolates and between different *Bacillus* species. It is also possible to generate *Bt* strains containing novel combination of protoxin genes by cell mating with the transfer of protoxin-encoding plasmids (Gonzalez *et al.* 1982; Gonzalez and Carlton, 1984) or by the introduction of cloned protoxin genes. Recently conjugation among *Bacillus thuringiensis* under environmental conditions was also reported by Vilas Boas *et al.* (1998). Another strategy for introducing non-resident toxin coding genes into desired *Bt* isolate is by engineering plasmid vectors compatible with the resident plasmids. The spectrum of susceptible insects may be extended by such procedures. The engineered strains may have novel specificities not found for any of the toxin in the donor or recipient strains. An attempt was made to enhance the larvicidal activity of *Bt* by conjugation between two different *Bacillus* species, *Bt* var. *israelensis* and *B. sphaericus*. Since such novel transcipient strains can be develop within different *Bt* strains having enhanced larvicidal activity or broad host range.

3.2.1 : Enhancement of Larvicidal Activity of *B. thuringiensis* var. *israelensis* and *B. sphaericus* by Conjugation :
By mating system transcipients of *Bacillus thuringiensis* var. *israelensis* (Bti) and *Bacillus sphaericus* (Bs) were isolated having tetracycline and streptomyacin resistance. Optimum time for the maximum transcipient formation was found to be 4 h. Cell to cell contact through conjugation tube between donor and recipient cells
was observed under electron microscope (Fig. 3.2.1a). The ICPs produced by Bti and transcipients were observed under electron microscope to study whether there was any morphological difference between the ICPs produced by them. Bti showed the amorphous inclusion bodies while the inclusion of the Bs was associated with spores. Both the above type of ICPs were observed in case of transcipients (Fig. 3.2.1b,c,d).

In order to show that the transcipients produced both the type of ICPs, the sporulated culture of the donor, recipient and transcipients were subjected to SDS-PAGE analysis. In case of transcipients SDS-PAGE profile showed distinct polypeptide bands from that of donor and recipient, indicating production of both the type of ICPs. Lane A and B in fig. 3.2.2A showed polypeptides of Bti and Bs respectively while lane C and D showed polypeptides of transcipients TC1 and TC2. As observed from the gel, lane A showed approximately 130 kDa polypeptide along with 65 and 27 kDa polypeptides. Bs produced three polypeptides 43, 51 and 120 kDa. All these polypeptides were observed in transcipients (Fig. 3.2.2A). To reveal further, whether the transcipients produced both the Bti and Bs ICPs, immunoblotting was carried out. In immunoblot, (Fig. 3.2.2B) lane A showed immunoreactive polypeptide of Bti with anti-Bti antibodies; while lane B having polypeptides of Bs were unable to react with the same antibodies. But the polypeptides of transcipients TC1 and TC2 have reacted with the antibodies of Bti indicating that the transcipients produced polypeptides of Bti. Further, it was clarified by dot blot immunoassay, using both Bti and Bs antibodies and bioassay against *Anopheles* mosquito larvae. Bioassay on *Anopheles* mosquito larvae demonstrated that transcipients were highly toxic. The LC$_{50}$ values for transcipients TC1, TC2 and TC3 were 0.026 ± 0.008, 0.04 ± 0.001 and 0.03 ± 0.012 µg/ml respectively, whereas the LC$_{50}$ value of the donor Bti and recipient Bs were 0.035 ± 0.06 and 0.09 ± 0.03 µg/ml respectively. Increase in larvicidal activity was observed in case of transcipient as compared to Bs (Table 3.2.1). Hence, it is concluded that transcipients produced ICPs of both Bti and Bs.
Fig. 3.2.1: Electron micrograph showing a) conjugation in Bti and Bs; b) ICPs of Bti; c) ICPs of Bs and d) ICPs of transcipient TC1.
Table 3.2.1: LC₅₀ value of Bti, Bs, TC1, TC2 and TC3 against *Anopheles* larvae:

<table>
<thead>
<tr>
<th>Strain</th>
<th>LC₅₀ (µg/ml)</th>
<th>Immunoreactivity with a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-Bti Ab</td>
</tr>
<tr>
<td>Bti</td>
<td>0.035 ± 0.006</td>
<td>+</td>
</tr>
<tr>
<td>Bs</td>
<td>0.09 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>TC1</td>
<td>0.026 ± 0.08</td>
<td>+</td>
</tr>
<tr>
<td>TC2</td>
<td>0.04 ± 0.01</td>
<td>+</td>
</tr>
<tr>
<td>TC3</td>
<td>0.03 ± 0.012</td>
<td>+</td>
</tr>
</tbody>
</table>

a - Immunoreactivity was observed by dot blot analysis.

± standard deviation

The ICPs produced by the donor Bti and transciipients were found cytolytic and not of the recipient Bs, when assessed by the blood agarose plate method (Ingle *et al.* 1993). ICPs of Bti have cytolytic activity (Thomas and Ellar, 1983). The cytolytic activity observed in case of ICPs produced by transciipients may be due to conjugal transfer of the genes encoding ICPs of Bti. Intragenic cell mating is thus a useful mean of transferring both large and small plasmids among the *Bacillus* species. Hence, it is concluded that the cell mating is a conjugation like process and conjugal transfer of large plasmids encoding insecticidal crystal protein genes occur during cell mating. Such transciipients may be used as a potent mosquito larvicidal agent against *Anopheles* and other mosquito species.

3.2.2: Intragenic protoplast fusion between *Bt* var. *israelensis* (Bti) and *B. sphaericus* (Bs)

An approach of protoplast fusion was applied to enhance the host range of Bti and Bs. Protoplast fusion was carried out with Bti (TetR StrS) and Bs (TetS StrR) strains. Protoplast fusion was also carried out with Btk (TetR StrS) and Bs (TetS StrR) strains.

Protoplasts of these strains were isolated using lysozyme to hydrolyse the cell wall. Protoplast formation frequency was not affected, but regeneration frequency was
Fig. 3.2.2: (A) SDS-PAGE and (B) Immunoblot of ICPs of Bti, Bs and transciipients TC1 and TC2.

Lane A - Bti; B - Bs; C - TC1 and D - TC2
affected as the cell age at the time of protoplast formation increased. An eight hour old culture was found to be best regenerated. Lysozyme concentration of 1 mg/ml gave 90-94% protoplast formation. Higher concentration of lysozyme has also been used in an effort to obtain efficient cell wall hydrolysis. However, higher concentration of lysozyme caused less number of protoplast may be due to undesirable effects such as interference with cell wall regeneration due to strong binding of lysozyme to the protoplast membrane or proteolytic activity associated with lysozyme in extract. (Gokhale et al. 1993, Puntambekar et al. 1992). It was observed that, though Bacillus spp. are Gram positive organisms, the mild osmotic shock given by EDTA seems to be favourable for lysozyme activity which is most commonly used in case of gram negative organisms. Addition of 50 mM MgCl₂ was strictly necessary for protoplast viability.

Protoplasts fusion of Bti and Bs was carried out and hybrids were characterized with respect to their phenotypic characters, entomopathogenic property and insecticidal crystal protein profile. All the hybrids were selected on tetracycline and streptomycin containing agar plate. The successful isolation of recombinants using these two strains support the usefulness of protoplast fusion technique in producing intrageneric recombinant Bacillus thuringiensis.

Almost 99% of the Bti and 91% of Bs cells were converted to protoplasts. Regeneration frequencies of protoplasts of parental strains were 43.82% for Bti and 24.9% for Bs respectively (Table 3.2.2).

Most of the hybrids had morphology similar to Bs with respect to spore and crystal inclusion body. The protein profile of hybrid showed 130 kDa polypeptide of Bti and 51 kDa and 42 kDa binary toxin protein of Bs. The hybrids showed the presence of the Lepidoptera and Diptera specific proteins of Bti and also the binary toxin of Bs thereby indicating the expression of crystal genes from both the parent strains.
Table 3.2.2: Protoplast formation and regeneration of *B. thuringiensis* var. *israelensis* and *B. sphaericus*:

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Cell age (h)</th>
<th>Starting No. of cells (Z)</th>
<th>Osmo Resistant colonies (Y)</th>
<th>Regenerated colonies (X)</th>
<th>Protoplast formation frequency*</th>
<th>Regeneration frequency**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bti</td>
<td>8</td>
<td>$1.7 \times 10^8$</td>
<td>$4.9 \times 10^6$</td>
<td>$7.5 \times 10^8$</td>
<td>99.34%</td>
<td>43.82%</td>
</tr>
<tr>
<td>Bs</td>
<td>8</td>
<td>$2 \times 10^{10}$</td>
<td>$4.5 \times 10^8$</td>
<td>$5.43 \times 10^9$</td>
<td>91.11%</td>
<td>24.9%</td>
</tr>
</tbody>
</table>

X) Regenerated colonies include colonies from regenerated cells and osmo resistant cells.

Y) Colonies from non-protoplast were derived from lysozyme treated cells which resisted lysis upon dilution with water.

Z) Starting number of cells.

* Protoplast formation frequency = x-y/x (100)

** Regeneration frequency = x-y/z(100)
3.2.3: Protoplast Formation, Regeneration And Fusion of *B. thuringiensis* subsp. *kurstaki* and *B. sphaericus*:

Protoplast formation, isolation and regeneration was attempted so as to obtain a standardized method for formation and regeneration of Btk and Bs protoplasts, so that this could be used as a tool for genetic manipulation in these strains because protoplast fusion may help in transfer of ICP genes.

In both the cases 8 h old log phase culture of Btk and Bs was used as maximum regeneration frequency was obtained. In Btk 95.65% protoplast formation and a regeneration frequency of 2.038% was obtained when cells were treated with 1mg/ml concentration of lysozyme for 3 h. In Bs 91.71% protoplast formation and 24.9% regeneration frequency was observed when cells were treated with 1 mg/ml of lysozyme for 3 h (Table.3.2.3). For Bs two concentrations of lysozyme were taken (1 mg/ml and 0.5 mg/ml) and treated for 2 and 3 h in both the cases, however 1 mg/ml concentration of lysozyme and treatment for 3 h was better in terms of protoplast formation and regeneration. The formation of protoplasts was monitored microscopically, which in wet mount preparation showed the presence of protoplasts as identified by their altered morphology.

Btk is the only *Bt* strain which is active against many lepidoptera and diptera whereas Bs is effective against *Culex* and *Anopheles* mosquitoes and has the advantage of being capable of surviving in highly polluted water and is resistant to UV inactivation in strong sunlight. Bs is also capable of spread and persistence in aquatic environment making control in inaccessible habitats which are the conditions usually prevalent in environment for field application (Aronson *et al*. 1986, Baumann *et al*. 1991, Burges, 1982).

In order to develop strains which would be able to survive in adverse environmental conditions and to be active against lepidoptera and diptera larvae effectively,
Table 3.2.3 : Protoplast formation and regeneration of *B. thuringiensis* var. *kurstaki* and *B. sphaericus*.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Cell age (h)</th>
<th>Starting No. of cells (Z)</th>
<th>Osmo Resistant colonies (Y)</th>
<th>Regenerated colonies (X)</th>
<th>Protoplast formation frequency*</th>
<th>Regeneration frequency**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Btk</td>
<td>8</td>
<td>5.4 x 10^10</td>
<td>5.0 x 10^7</td>
<td>1.15 x 10^9</td>
<td>95.65%</td>
<td>2.038%</td>
</tr>
<tr>
<td>Bs</td>
<td>8</td>
<td>2 x 10^10</td>
<td>4.5 x 10^8</td>
<td>5.43 x 10^9</td>
<td>91.71%</td>
<td>24.9%</td>
</tr>
</tbody>
</table>

X) Regenerated colonies include colonies from regenerated cells and osmoresistant cells.

Y) Colonies from non-protoplast were derived from lysozyme treated cells which resisted lysis upon dilution with water.

Z) Starting number of cells.

* Protoplast formation frequency = x-y/x (100)

** Regeneration frequency = x-y/z (100)
protoplast fusion mediated by polyethylene glycol (PEG) of these two cultures was attempted.

Protoplasts of Btk (Str<sup>S</sup> tet<sup>'</sup>) and Bs (Str<sup>R</sup> tet<sup>S</sup>) were fused and the hybrids (Str<sup>R</sup> tet<sup>'</sup>) were regenerated on media containing both the antibiotics. Initially 60 hybrids were selected for further study, however after repeated subculture 10 hybrids lost the dual resistance markers property. Fifty hybrids were analyzed for their phenotypes with respect to morphological characteristics and protein profiles. Hybrids sporulated after an incubation period of 5 days whereas the parent culture of Btk and Bs showed complete sporulation within 3 days.