CHAPTER 3

RESULTS AND DISCUSSION

There are several shortcomings for the study of growth kinetics and regulation of sporulation and delta-endotoxin formation in batch cultures in contrast to an advanced cultivation system like the continuous culture. However, there are only a few investigations eliciting the biological feedback for step disturbances in a steady state continuous culture of *Bacillus thuringiensis* (B. t.). As this approach would offer further insight into the growth kinetics, physiology and regulation of toxin formation, attempts have been made in the present investigation, to understand the behaviour of *Bacillus thuringiensis* subsp. *galleriae* (B. t. g.) under continuous culture conditions. It was also attempted to express and characterise a lepidopteran specific toxin gene in *E. coli*.

3.1. ASSESSMENT OF CARBON SOURCES

Initially attempts were made to grow the organism in a semi-defined medium. Shake flask experiments were conducted to select appropriate substrate for the growth. Several carbon sources in terms of C-equivalent concentrations were investigated as supplements to glucose yeast extract medium (GY) by suitably replacing glucose. Among the six carbon sources tested, namely, glucose, maltose, fructose, galactose, sucrose and lactose, the economic yield coefficient for glucose was found to be the highest (Figure 3.1). Therefore, glucose was used as a substrate for further batch and continuous culture studies.
Yield coefficient ($Y_{eco}$)

Figure 3.1 Economic yield coefficient ($Y_{eco}$) of *Bacillus thuringiensis* subsp. *galleriae* in different carbon sources. Substrates were supplemented in carbon equivalent concentrations. Glu=Glucose; Mal=Maltose; Fru=Fructose; Gal=Galactose; Sue=Sucrose; Lae=Lactose.

Figure 3.1 Economic yield coefficient ($Y_{eco}$) of *Bacillus thuringiensis* subsp. *galleriae* in different carbon sources. Substrates were supplemented in carbon equivalent concentrations. Glu=Glucose; Mal=Maltose; Fru=Fructose; Gal=Galactose; Sue=Sucrose; Lac=Lactose.
3.2 STEADY STATE BEHAVIOUR OF *Bacillus thuringiensis* subsp. *galleriae*

3.2.1 Preliminary x-D diagram

The x-D diagram of *B. t. g.* in GY medium was constructed (Figure 3.2) in order to monitor the steady state biomass and substrate profile. There was a gradual increase in biomass concentration with increase in dilution rate. The maximum specific growth rate of the organism was found to be 0.38 h\(^{-1}\). When the dilution rate was further increased beyond the maximum specific growth rate, the system experienced a washout and the biomass productivity dropped with a sudden rise in the glucose concentration to 3.4 g l\(^{-1}\). Maximum biomass productivity was found to be 0.3 g l\(^{-1}\) h\(^{-1}\) at a dilution rate of 0.38 h\(^{-1}\). It was also found that with increase in dilution rate the residual glucose concentration increased indicating suboptimal medium components.

Steady state specific substrate uptake rate, Yield coefficient, Yx/s and biomass productivity were determined at every steady state. There was a small increase in the yield coefficient with increase in apparent specific growth rate (Figure 3.3) indicating glucose utilised at lower dilution rates was not fully converted to biomass. Part of the glucose might have been diverted to meet higher energy needs at low dilution rates to overcome the stress offered by severe substrate limitation. This is described as maintenance requirement of the organism. The yield profile observed from the preliminary x-D diagram was not optimal from the production point of view. In addition to this, as discussed latter, the organism segregated into spontaneous asporogeneous variants. Therefore, it is essential to further understand the key factors which could enhance the stability, biomass productivity and delta-endotoxin formation in *B. t. g.*

**N.B.**
The maintenance requirement is expressed as maintenance coefficient (m), which is defined as the rate of substrate used for maintenance function.

Dilution rate is equal to specific growth rate at every steady state.
Figure 3.2 Steady state and substrate profile of \textit{Bacillus thuringiensis} subsp. \textit{galleriae} grown in GY medium. Steady state was determined based on biomass and substrate profiles.
Figure 3.3 Steady state biomass productivity ($P$), specific substrate uptake rate ($qs$) and yield coefficient ($Y_{x/s}$) of *Bacillus thuringiensis* subsp. *galleriae*. Yield coefficient was determined based on the consumed substrate.
3.2.2 Strain instability and formation of spontaneous asporogenous variants.

To examine the growth of \textit{B. t. g.} under carbon limited conditions, an x-D diagram was constructed using GY medium as described earlier. Experiments were also carried out to understand the transient behaviour of the system by pulsing different medium components. Under these conditions it was observed that there was a drift in the gas exchange rates after the return of the cultures to steady state. Whenever a steady state continuous culture is disturbed by a pulse, the system experiences a transience. After the washout of the residual components of the pulse, the system is expected to regain the original steady state. However, in the present study it was observed that there was a drift from the steady state gas exchange rates before and after the pulse. The following could be the reasons:

1. A drift in the baseline of the gas analyser output,
2. Entry of new strain during pulse,
3. Strain instability

The drift in the baseline of the gas analyser was ruled out by frequent calibration and careful measurements. In order to examine the presence of contaminants, if any, samples were drawn frequently and plated on nutrient agar and incubated at 30°C overnight and the culture examined for colony morphology. No morphological variation could be detected. However, prolonged incubation (48 hours) at 30°C revealed the existence of translucent colonies in contrast to the pigmented wild type (Figure 3.4). These translucent colonies were later confirmed to be asporogenic variants of the wild type. It was also understood that the drift in the gas exchange rates could be attributed to the gradual replacement of the wild type strain by the variants. However, a question still remains unanswered: that how can one make sure that the variants are from the chemostat and not from the preinoculum? Hence, preinoculum was developed in such a way that the pre-batch for the continuous culture was always started with spores by inactivating the vegetative forms in the reactor.
Figure 3.5 Variation of wild type and spontaneous asporogenic variants of *Bacillus thuringiensis* subsp. *galleriae* under chemostat culture conditions at a constant dilution rate of 0.04 h\(^{-1}\). At this dilution rate glucose was completely consumed.
The changes in the populations of wild type and the variants as a function of cultivation time in the chemostat were determined at a constant dilution rate of 0.04 h⁻¹. At that dilution rate, it was earlier observed (Preliminary x-D-diagram) that there was no residual glucose present in the medium, which represented a carbon stress condition. It can be seen from the data represented in Figure 3.5 that there was an increase in the number of Spo⁻ variants with an increase in the duration of the cultivation time in continuous culture. At the end of 168 hours, the Spo⁻ variants had completely replaced the wild type. When these variants were examined under a phase contrast microscope, they revealed the presence of more than one inclusion body. These inclusion bodies were less when compared to the bipyramidal crystals seen in the wild type strain. Even after prolonged cultivation, the cells did not revert back to Spo+ Cry+ wild type strain indicating that this was a permanent genetic loss. It has been observed earlier that in strains blocked at or before stage n, multiple parasporal inclusion bodies in each cell were often produced that were asymmetrical in nature (Johnson et al., 1980). However, their results pertain only to conditions where mutagenic agents have been used; no information is available regarding the stability of either the mutant or the wild type strain under batch or continuous cultivation studies.

In majority of the mutation studies with strains of B. t. by other workers, it was observed that the resulting variants were translucent or colourless in nature (Yousten, 1978, Wakisaka et al., 1982). When strains B. t. were grown at 42°C, the elevated temperature above 30°C induced the formation of colourless translucent variants. The translucent colonies thus obtained failed to sporulate and the sporulation frequency was found to be between 30 to 300 unlike in the present study that it was observed that the variants did not revert back to wild type. It was also pointed out that the variants exhibited enhanced growth over the wild type. It is to be mentioned here that the studies carried out by Yousten (1978) were confined to surface cultures in mere petri plates where local variations in growth environment do exist. It has been observed in the present study that the variants exhibited faster growth rate over the wild type under continuous culture conditions and has been proved for the first time that under severe limitations, the variants are potential competitors. Wakisaka
etal. (1982) observed colourless colonies when *B. t.* cells were treated with mutagenic agents. Major portion of the variants isolated by this method failed to produce active delta-endotoxin except one of the isolates that produced active form of delta-endotoxin at a cell density of $10^{10}$ to $10^{11}$ which would help to obtain spore free formulations. However, no growth kinetics data for the mutants has been reported in the above study which will be necessary for extrapolating the data to large scale studies. Another study involving *Bacillus thuringiensis* subsp. *yunnanensis* grown at $27^\circ$C and pH 7.4 led to the formation of small spherical bodies even in the Spo+ cells and the frequency of active crystal bearing cells were 0.8 - 0.9 % of approximately 1200 cells. This investigation indicates inadequacy in the design of growth medium / physical parameters as normally *B. t.* strains are grown at $30^\circ$C and pH 7-7.2. The fast segregational instability encountered even under batch cultivation revealed the impact of physical parameters on the strain instability and delta-endotoxin formation among strains of *B. t.*. The variants isolated in the study were grown in a complex medium. Even after repeated subculturing no reversal was encountered. However, the lack of crystal toxin production is not necessarily accompanied by absence of plasmids as it was observed with many *B. t.*, isolates namely *kurstaki* HD73, HD41, *alesii* and *galleriae* (Carlton and Gonzalez, 1980).

Strain instability was also encountered with other entamopathogenic bacteria namely *Bacillus sphaericus* ISPC-6 which exhibited larvicidal activity against mosquito larvae. In this strain the biocidal principle was coded by the chromosomal DNA. In spite of this, a rapid loss of biocidal potency was encountered when the organism was repeatedly subcultured in a complex medium, namely, NYSM. The variants isolated after fourth subculturing exhibited a thousand fold lower potency than that of the parental strain (Rao and Mahajan 1990). However, this is much slower rate when compared to *B. t. g.* where complete replacement of the wild type in continuous culture took place after approximately 67.5 generation times. These variants are smaller and light brownish in nature in contrast to the colourless variants in *B. t. g.* while the wild type *Bacillus sphaericus* strain was medium sized and dark brownish in colour. However, the kinetics and biochemical basis for the segregation and loss of biocidal potency were not demonstrated by them.
Figure 3.6 Oxygen Transfer Rate (OTR) and Carbon dioxide Production Rate (CPR) as a function of cultivation time at a constant dilution rate of 0.04 h$^{-1}$.
Gas exchange rates were followed (both OTR and CPR) throughout the cultivation time at a lower dilution rate of 0.04 h\(^{-1}\). Interestingly, there was an increase in the gas exchange rates accompanying the increase of the population of Spo\(^-\) variants (Figure 3.6) at a constant dilution rate of 0.04 h\(^{-1}\). This reflects a changed physiological response of these variants to nutrients. Indeed this observation substantiates the upward shift observed in the gas exchange rates in pulse experiments. The maintenance requirements of these variants might be less than that of the wild type. Thus with the replacement of the wild type by the variants, the energy not required for the maintenance function by the wild type might have been spilled which in turn is accepted by the terminal electron acceptor, namely, oxygen. Thus an increase in gas exchange rates (both OTR and CPR) were observed. Obviously the Spo\(^-\) variants have some selective advantages over the wild type, an important prerequisite of this kind of segregation (Kunkel et al., 1992). Loss of certain biosynthetic functions had led to the selective advantage over the parental strains (Harder and Kuenen, 1977).

In majority of the mutation experiments, both by mutagenic agents as well as by spontaneous process, the resulting variants were less pigmented. The loss of the biosynthetic pathway for the production of the cell wall pigment might have reduced the metabolic burden, which might increase the growth rate of the variants over the wild type strain. This colourless nature could also be due to the loss of S-layer protein on the surface of the cell wall (Luckevich and Beveridge, 1989). However, the variants isolated in the present study were not subjected to cell wall characterisation.

### 3.2.3 Characterisation of the Spo\(^-\) variants

In order to assess the nature of the inclusion bodies in these variants, western blot analysis of the crystal proteins from the cells solubilized by 0.5M Na\(_2\) CO\(_3\) (Cooksey, 1968) was carried out. It was observed that the soluble fractions did not exhibit the presence of any of the crystal peptides (Figure 3.7). As these cells exhibited the presence of a large number of inclusion bodies, a more vigorous approach of using 5 M urea to solubilize these inclusion bodies was undertaken.
Figure 3.7 Western blot analysis of total alkali cell extracts of wild type and spontaneous variants of *Bacillus thuringiensis* subsp. *galleriae*. Lanes a, c and e; Cells harvested after 9 hours, 11.30 hours and 18.30 hours of wild type *B. t. g*. Lanes b, d and f; Cells harvested from the variants; Lane g; Purified crystal protein from *Bacillus thuringiensis* subsp. *galleriae*; Lane h; Molecular weight standard.
Figure 3.8 Western blot analysis of the crystal peptides from the wild type and Spo- variants. Alkali or urea solubilized peptides and their tryptic digests were separated in a 10 % SDS-PAGE and probed with the antisera raised against the wild type crystals as outlined in methods. Panel A: Whole crystals; Panel B: Tryptic digests of the crystal peptides: Lane a, ASM 37 (Spo- Cry+); Lane b, ASM 27 (Spo- Cry+); Lane c, Wild type (Spo+ Cry+).
(Schoner et al., 1985). In two of the representative variants, the western blot profiles of the inclusion bodies and their trypsin digests were identical to the native crystal peptides (Figure 3.8). These results indicated that the formation of the insecticidal crystals in the Spo− variants was different from that of the wild type and were perhaps present as aggregated inclusion bodies rather than easily solubilizable crystalline forms. The purified inclusion bodies from these variants were also tested for toxicity against *Bombyx mori* (Figure 3.9). They were less toxic than the native crystals from the Spo+, Cry+ strains of *B. t. g.* It is evident that the less defined crystalline inclusions were biologically less effective. Perhaps in the absence of spore formation, the crystal proteins may have been over-expressed in a disproportionate manner, thus resulting in the formation of these aggregates. An appropriate modification of this expression will be invaluable in the production of higher toxin components per cell. In addition, perturbations observed in a chemostat can be useful indicators of strain instability.

3.3 TRANSIENT RESPONSE OF CHEMOSTAT CULTURE OF *B. t. g.*

Since chemostat studies are conducted to measure metabolic responses of microbial cells to the added compounds in a pulse form, a variety of compounds were tested. Initially the medium components of GY medium such as C, N and mineral sources were pulsed in the steady state chemostat. No observable effect was encountered in terms of biomass, substrate and gas exchange rates. In addition vitamins and trace elements were also pulsed. This also resulted in neutral response. It is clear that in this suboptimal medium, there is a pressure to select Spo− Cry+ variants and hence growth conditions have to be evolve for maintaining the stability of Spo+ Cry+ strain. Therefore, further studies were undertaken to find optimal growth medium that increase the stability and biomass productivity of the strain. The response of a steady state continuous culture of *B. t. g.* for a pulse of casaminoacids (2.2 g l−1) is presented in Figure 3.10. The biomass concentration increased to 1.7 g l−1 with the residual glucose concentration close to zero. The yield coefficient in GY medium for glucose was found to be 0.38. However, in the positive region of the transient, the
Figure 3.9 Toxicity assay of the inclusion bodies of wild type and Spo- variants of *B. t. g.* Toxicity was tested against the fourth instar larvae of *Bombyx mori*. Assay were conducted in triplicate. Potency is described as $1/Lc_{50}$, ml/mg.
Figure 3.10 Transient response of continuous culture of *B. t. g.* for casaminoacids pulse (concentration = 2.2 g l\(^{-1}\)) at a dilution rate of 0.2 h\(^{-1}\). The input glucose concentration was 4.5 g l\(^{-1}\).
yield coefficient for glucose had increased to 0.7. Part of the substrate that supported growth must have come from casaminoacids. The biomass productivity was higher (approx. 1.5 g l⁻¹) than the steady state observed in GY medium.

In order to examine whether the positive response is due to nitrogen limitation a sodium nitrate pulse was made. This resulted in neutral response. In order to further understand the growth requirements in terms of specific amino acids, single amino acid pulses were carried out. This also resulted in neutral response. Therefore, it is essential to find out the synergistic effects, if any, among amino acids. These amino acids were grouped based on their general assimilation pathways. They were pulsed systematically either as single group or as many groups together in an additive fashion. It was found that the additive response increased with increase in number of groups (Figure 3.11, Figure 3.12). Only this additive effect of several amino acids present together was identified as growth-supporting (Figure 3.13). In addition to increase the biomass productivity, they increased the glucose uptake. Functionally they acted both as growth factors as well as substrate. Such components in the medium are generally classified as partially substitutable carbon sources (Baltzis and Fredrickson, 1988). Amino acids such as aspartic acid, threonine, histidine, glutamic acid and alanine were already reported as essential for the growth of B. t. g. (Kuznetsov et al., 1984). But results of the present investigation very clearly showed that the strain under study does not exhibit auxotrophy to amino acids. These amino acids support growth in combination.

As far as industrial application of chemostat is concerned, it is well proved that the transient state of chemostat gave higher biomass productivity than the steady state continuous culture. The biological response for disturbances of the steady state was rapid and identification of the growth requirements is relatively easy. The results obtained in transient state culture could be extrapolated to batch cultures to enhance the time space yield of biomass and delta-endotoxin yield.
Figure 3.11 Transient response of *Bacillus thuringiensis* subsp. *galleriae* for amino acids pulse. Magnitude increased with increase in number of groups.
Figure 3.12 Additive effects of amino acid groups on the growth of *Bacillus thuringiensis* subsp. *galleriae.*
Figure 3.13 Transient response of continuous culture of *Bacillus thuringiensis* subsp. *galleriae* for combined amino acids pulse. The amino acids were pulsed at a concentration of 150 mg l\(^{-1}\).
3.4. STEADY STATE BEHAVIOUR IN GYA - MEDIUM

A second x-D diagram was developed in GY- medium supplemented with amino acids (Figure 3.14). The biomass concentration remained constant up to a dilution rate of 0.9 h\(^{-1}\) and a sudden drop in the biomass profile was observed when the dilution rate was raised beyond the maximum specific growth rate of 0.9 h\(^{-1}\). The maximum medium flow rate of 1.35 l h\(^{-1}\) could be used in contrast to the preliminary x-D diagram, where the maximum flow rate was only 0.6 h\(^{-1}\). This is 2.25 times higher than the maximum flow rate that could be used in the preliminary x-D diagram. The glucose was completely consumed at lower dilution rates and increased to 0.6 g l\(^{-1}\) at higher dilution rates from 0.7 h\(^{-1}\). The maximum biomass productivity was found to be 1.82 g l\(^{-1}\) h\(^{-1}\) at a dilution rate of 0.9 h\(^{-1}\). This is approximately three times higher (calculated based on carbon equivalent concentrations) than the x-Dmax found in GY-medium.

The biomass productivity, specific substrate uptake rate and yield coefficient determined from the steady state values were shown in Figure 3.15. The yield coefficient remained constant in contrast to the preliminary x-D diagram, where increasing profile in the yield coefficient was observed. This indicated that there was a remarkable reduction in the maintenance requirement by the organism. In addition, there was an increase in specific substrate uptake rate with increase in dilution rate. The maximum specific substrate uptake rate of 2.68 g l\(^{-1}\) h\(^{-1}\) was observed at a dilution rate of 0.9 h\(^{-1}\) in comparison to the value observed with the preliminary x-D diagram, 1.63 g l\(^{-1}\) h\(^{-1}\). The corresponding biomass productivity was far higher than the preliminary x-D - diagram. Periodical examination of culture for sporulation and toxin formation revealed that the strain was stably maintained throughout the cultivation under carbon limiting conditions.
Figure 3.14 Steady state biomass and substrate concentration as a function of dilution rate in a continuous culture of *Bacillus thuringiensis* subsp. *galleriae*.
Table 3.2 Growth constants of Bacillus thuringiensis subsp. galleriae grown in GY and GYA medium.

<table>
<thead>
<tr>
<th>Symbols</th>
<th>GY-medium*</th>
<th>GYA-medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_{\text{max}}$</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>$K_s$</td>
<td>0.13</td>
<td>0.18</td>
</tr>
<tr>
<td>$D_m$</td>
<td>0.32</td>
<td>0.88</td>
</tr>
<tr>
<td>$D_c$</td>
<td>0.38</td>
<td>0.90</td>
</tr>
<tr>
<td>$Y_g$</td>
<td>0.37</td>
<td>0.41</td>
</tr>
<tr>
<td>$m$</td>
<td>0.1</td>
<td>n</td>
</tr>
<tr>
<td>$x$-$D_{\text{max}}$</td>
<td>0.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Indicates apparent values. n = no maintenance requirement was found.
Figure 3.15 Steady state volumetric biomass productivity (P) and Specific substrate uptake rate (qs) and yield coefficient (Yx/s) as a function of dilution rate in a continuous culture of *Bacillus thuringiensis* subsp. *galleriae*. 
Growth constants determined from the steady state data is presented in Table 3.2. Maximum specific growth rate ($\mu_{\text{max}}$) was found to be 0.9 h$^{-1}$. This is far higher than the apparent specific growth rate found in the preliminary x-D diagram. All these data was obtained from a stable population of $B. t. g.$ which could be reliably used for further modelling and predictions. Already there were several attempts made to understand the growth kinetics of *Bacillus thuringiensis* (Blokhina *et al*., 1984; Shakarova *et al*., 1985; Starzak and Bajpai 1991). However, most of these studies were carried out either in shake flask levels or in batch cultures. In contrast data obtained in the present study was from steady state continuous culture. In another study by Arcas *et al* (1987), the generation time was reported to be 57 min. However, there was a variation in the reported specific growth rate from 0.71 to 0.77 h$^{-1}$. Varying specific growth rate with constant generation time is not possible. However, the above investigations might not have considered the statistical variation of 7.79 % in the specific growth rate with increase in substrate concentration. Even though the substrate used in both the studies were the same, higher specific growth rate observed in the present study could be attributed to the variation in the cultivation system and the composition of the medium determined based on the pulse experiments, which led to the stoichiometric supply of the nutrients. Such variation in specific growth rate was reported in many thermophylic bacilli. In shake flask the $\mu_{\text{max}}$ was found to be 0.2 h$^{-1}$; in controlled batch reactor it was found to be 0.5 h$^{-1}$ and in chemostat $D_c$ (Critical Dilution rate) 2.7 h$^{-1}$ (Sonnleitner *et al*., 1982; Cometta *et al*., 1982). The difference in the specific growth rate is significant and cannot be attributed to the analytical error. The cultivation system used in the present study is more reliable compared to that employed by many workers.

Knowledge on these parameters, coupled with mathematical modelling, allows prediction of the productivity of continuous, open, partially-closed and closed bioreactors; thereby it aids the design and optimisation of the production processes. Even though the exact metabolic pathway that increased the substrate transport and utilisation is not known at present, the TCA cycle intermediates supplemented in the medium might have kept the energy metabolism active and no limitation in the internal
Figure 3.16 Steady state oxygen transfer rate and carbon dioxide production rate as a function of dilution rate in a continuous culture of *Bacillus thuringiensis* subsp. *galleriae*.
Figure 3.17 Steady state specific oxygen transfer rate \((q_{O2})\) and specific carbondioxide production rate \((q_{CO2})\) as a function of dilution rate in a continuous culture of *Bacillus thuringiensis* subsp. *galleriae*. 

- \(q_{O2}\)
- \(q_{CO2}\)
aminoacids supply for protein turn over might have led to homogeneous population throughout the cultivation.

3.5. STEADY STATE GAS TRANSFER

Steady states in continuous culture of B. t. g. were mainly determined based on gas transfer rates in addition to biomass and substrate profiles. The air flow rate and impeller speed were kept constant throughout the range of dilution rates studied. In the present study the steady state volume related oxygen transfer rate (OTR) and carbon dioxide production rate (CPR) were determined at every dilution rate using exhaust gas analyser. Unlike in the preliminary x-D diagram, the gas transfer rates remained constant at a given dilution rate under steady state conditions. The gas transfer rates increased with increase in dilution rates (specific growth rate) indicating oxygen unlimiting conditions (Figure 3.16). The maximum OTR and CPR were found to be 0.4 g l\(^{-1}\) h\(^{-1}\) and 0.32 g l\(^{-1}\) h\(^{-1}\) respectively. The respiratory quotient (RQ) remained constant below unity.

Similar profiles as volume related gas transfer rates were observed with specific gas transfer rates (qO\(_2\) and qCO\(_2\)) (Figure 3.17). Increase in specific oxygen uptake rate clearly indicated that full oxidised byproducts were produced at higher dilution rates where vegetative population predominated. From these experimental results it is evident that the culture was always grown under oxygen excess conditions and the growth was limited by glucose. The maximum specific oxygen uptake rate was found to be 0.17 h\(^{-1}\). In the spore formers like the strains of B. t., the coincidence of shift of sporulating population to vegetative form with several metabolic alterations that usually decide oxygen requirement of the organism. In general, these strains exhibited higher oxygen requirements at higher specific growth rates (dilution rate).
The values on the oxygen transfer rate are highly specific for a given organism, cultivation medium and the dimension of the reactor. Therefore, it is not possible to propose a value for oxygen transfer which would suit all cultivation conditions. Also, it is difficult to define oxygen requirements for a sporulated culture. In the present study the specific oxygen uptake rate of 0.08 h\(^{-1}\) supported sporulation and toxin formation at lower dilution rates.

### 3.6. REGULATION OF SPORULATION AND TOXIN FORMATION IN CHEMOSTAT:

Sporulation and vegetative cells were determined at every steady state. It was found that the culture was stably maintained throughout the dilution rates studied. No segregation instability was encountered as with GY medium. No translucent colony was detected. When continuous culture is to be used for production of *B. t.* it is important that the process potential has to assessed for two major variables. The sporulating capacity of the culture and toxin formation as a function of specific growth rate and the operating dilution rate where biomass productivity and toxin formation are maximum. In the present study the sporulation was examined microscopically and counted by plating method at every steady state. It was observed that there was a decrease in spore count with increase in dilution rate indicating at lower dilution rate (Figure 3.18) the major fraction of the population exists as spores. With increase in specific growth rates, the organism tend to exist as vegetative. These growing cells continuously replace the cells continuously washed out of the reactor. Similar results were obtained by Dawes and Mandelstam (1970) with *Bacillus subtilis* grown in continuous culture at dilution rates of 0.05-0.45 h\(^{-1}\). It will not be possible to achieve complete sporulation in continuous culture as frequency of sporulation is inversely related to specific growth rate of the organism. If such situation occurs, the system will reach a complete washout as there is no vegetative form which can compensate the biomass loss due to washout. Rather there was an increasing probability that a cell would sporulate as the growth rate (dilution rate) decreased (Frankena *et al.*, 1985). Effect of high concentration of substrate on the growth and delta endotoxin production of *Bacillus thuringiensis* subsp. *kurstaki* had been studied...
\( e^{3.18} \) Spore count and larvicidal potency as a function of dilution rate in a steady state continuous culture of *Bacillus thuringiensis* subsp. *galleriae*.
(Arcas et al., 1987). The maximum spore count $1.08 \times 10^{12}$ to $7.36 \times 10^{12}$ with toxin productivity of $1.05 \text{ mg ml}^{-1}$ to $6.85 \text{ mg ml}^{-1}$ when the concentration of glucose was raised from 8 to $56 \text{ g l}^{-1}$ with the corresponding increase in the yeast extract concentration from $4 \text{ g l}^{-1}$ to $28 \text{ g l}^{-1}$.

Samples collected from every dilution rate were subjected to bioassay against *Bombyx mori* for the production of active delta-endotoxin. A decrease in potency was observed with increase in dilution rate. (Figure 3.18). Similar profile was observed with sporulation and delta-endotoxin production. With increase in dilution rate the sporulating population was replaced by the vegetative form with decrease in potency. This indicated that both sporulation and toxin formation occurs simultaneously and they are interdependent. This is also supported by the loss of sporulation led to formation of disproportionate crystals by the variants in GY medium.

As the crystal synthesis is associated with sporulation and the sporulated cells represent only a very small fraction of total cells in the continuous culture, (especially at higher dilution rates) the toxin productivity will be very less. Therefore, for the production of delta-endotoxin from *B. t.* strains lower dilution rates could be employed. It was also understood that limitations with suboptimal concentrations of medium components will not only affect growth, sporulation and crystal synthesis but also the total physiology of the organism. These physiological changes might be adaptations to overcome the strain offered by suboptimal medium components and care must be taken to control the growth conditions employed in production scale, especially in continuous modes of operation.
Figure 3.19 Gas transfer rate profiles in the washout region of continuous culture of *Bacillus thuringiensis* subsp. *galleriae*.
3.7 WASHOUT EXPERIMENT

According to classical Monod model, when the dilution rate exceeded the specific growth rate, the chemostat will reach washout, as the growth rate could not compensate the loss of biomass due to washout. When the dilution rate was shifted from $0.9 \text{ h}^{-1}$ to $1.0 \text{ h}^{-1}$, there was a gradual but sharp increase followed by a sudden decline in gas exchange rate (both OTR and CPR) were observed (Figure 3.19). At the end of the 8th hour from the dilution rate shift, the biomass concentration was close to zero. Four hours after the dilution rate shift, the cells could not compensate the biomass loss due to washout. The system was brought back to lower dilution rate.

3.8 BATCH STUDIES

3.8.1 GROWTH UNDER CONTROLLED CONDITIONS

Growth of B. t. g. was examined under controlled conditions. The pH and the temperature was maintained at 7.2 and 30°C respectively. Impeller speed was controlled at 1500 rpm in order to ensure unlimited oxygen supply. Growth of B. t. g. is shown in Figure 3.20. The initial glucose concentration was 4 g l$^{-1}$ in GY and GY medium supplemented with amino acids. In GYA there was a sharp exponential phase of growth at a specific growth rate of approximately $0.9 \text{ h}^{-1}$ in contrast to the prolonged exponential growth observed with GY medium. In addition higher biomass with complete glucose utilisation was observed with GYA medium. In the late stationary phase of growth decrease in biomass was encountered. This was due to excessive foaming which removed part of the biomass to the foam overlay decreasing the biomass concentration in the bulk liquid. Therefore careful foam control is inevitable.
In order to confirm that small peptides present in the yeast extract is essential for the growth of \textit{B. t. g.} GY medium was supplemented with amino acid groups as shown in Table 3.2. Positive (+) and negative (-) sign indicates presence and absence of growth respectively. From this data it is clear that even in the presence of all amino acids together in GY, no growth was observed. Growth was observed only in the composition containing yeast extract clearly indicated that the strain used in the present study requires yeast extract for its growth.

The results obtained from the chemostat were extrapolated to batch experiments. In addition, substrate inhibition is an associated phenomenon of high cell density cultivations. Substrate inhibition could be realised by assaying biomass and product of interest at higher substrate concentrations. Therefore, in the present study, the effect of glucose concentration (GY medium) on the biomass productivity, sporulation and toxin formation of \textit{B. t. g.} was studied. Cells were grown and harvested during early stationary phase for biomass estimation. It was found that with increase in glucose concentration from 4 g l\(^{-1}\) to 20 g l\(^{-1}\), the biomass concentration increased up to 8 g l\(^{-1}\) with a constant yield coefficient of 0.3. The biomass and economic yield coefficient \((Y_{eco})\) started decreasing beyond the glucose concentration of 28 g l\(^{-1}\) (Figure 3.21, Figure 3.22). In the case of GYA (GY medium supplemented with amino acid mixture whose concentrations are given in the methods), biomass concentration of 12 g l\(^{-1}\) was observed at a substrate concentration of 20 g l\(^{-1}\). The economic yield coefficient was found to be 0.5 - 0.6. However, this started decreasing beyond 20 g l\(^{-1}\) of substrate concentration. This might either be due to oxygen limitation with corresponding increase in osmolarity of the medium. Similar results were observed by many authors with other strains of \textit{B. t.} (Watson, 1970 and Arcas \textit{et al.}, 1985). Similar trends were observed with viable cell counts and spore counts from these cultures (Figure 3.23 & Figure 3.24). High concentration of glucose is also known to reduce sporulation in...
Table 3.2  Growth of *Bacillus thuringiensis* subsp. *galleriae* in the presence and absence of amino acids

<table>
<thead>
<tr>
<th>Flask</th>
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<th>6</th>
<th>Growth</th>
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</table>
Figure 3.20 Growth of *Bacillus thuringiensis* subsp. *galleriae* in GY and GYA medium in batch cultures. Cells were harvested during early stationary phase for biomass estimation.
Figure 3.21 Growth of *Bacillus thuringiensis* subsp. *galleriae* in GY and GYA medium.
Figure 3.22 Economic yield coefficient (Yeco) of *Bacillus thuringiensis* subsp. *galleriae* grown in GY and GYA medium.
Figure 3.23 Viable cell count in GY and GYA medium
Figure 3. 24 Spore count in GY and GYA medium
Figure 3.25 Toxin productivity in GYA medium. Delta-endotoxin was assayed by immunodot blot.
strains of *B. t.* (Murrell 1961 and Holmberg et al., 1980). Recently it was reported by Kang *et al* (1992) that at higher concentration of glucose sporulation in *B. t.* was completely inhibited. However, the cultivation was carried out only for 17 hours and at higher glucose concentrations, sporulation is expected to be delayed. Prolonged cultivation would have helped to further increase the sporulation.

The maximum biomass obtained from GYA medium was approximately double than that of GY medium. This clearly indicated that amino acid supplementation helped better substrate uptake and growth led to elevated biomass productivity. The alkali solubilized samples were assayed for toxin productivity through dot blot. It was observed that the minimum protein concentration required to react with the antisera was found to be 0.002 µg in all the samples tested. This indicated that the specific toxin productivity remained nearly constant independent of substrate concentrations (Figure 3.25). This is a remarkable observation from the production point of view.

### 3.9. PRODUCTION OF *B. t. g.* IN INDUSTRIAL MEDIA

*B. t.* is usually grown in low cost industrial media and their composition of presented in Table 1.4. In the present study, *B. t. g.* was grown in defatted soy meal, a proteinaceous medium. It was also observed in our laboratory that, at higher concentrations, soy meal inhibits sporulation and toxin formation in *Bacillus sphaericus* (unpublished data). Therefore, this medium was supplemented with many growth factors identified from the chemostat pulse experiments. As soy meal is a complex medium, accurate estimation of biomass using dry weight methods is a difficult task. Therefore, the biomass profile was assessed in terms of vegetative cell count. The medium supplemented with casaminoacids resulted in high biomass and sporulation profile (Figure 3.26). However, toxin formation was less compared to the other media supplements. It was also found that the casaminoacid and amino acids supplementation resulted in 3 fold and 40 fold increase in toxin productivity respectively compared to the plain soy meal (Figure 3.27). Amino acids
Figure 3.26 Vegetative cell count and spore count of *B. t. g.* grown in complex medium supplemented with different substances.
Figure 3.27 Toxin productivity of *B. t. g.* grown in complex medium namely soy meal supplemented with different growth factors. The medium containing amino acids mixture resulted in highest potency in terms of dot blot unit. SM = Soy meal; Casa = Casaminoacids; AA = Amino acids. Dot blot unit is defined as 1/minimum protein concentration required to react with the polyclonal antisera raised against the purified crystal from *B. t. g.*
supplementation supported biomass growth, sporulation and toxin formation in GY medium as well as in a complex substrate namely soy meal. Amino acids might act as precursors for essential peptide synthesis. There are several reports on the cultivation of B. t. strains in complex substrates. However, the present study clearly indicated that the amino acid addition increased the sporulation and delta-endotoxin synthesis, thus increase in specific delta-endotoxin production was observed (Obeta and Okafor 1984). Even in a highly proteinaceous medium like soy meal, presence of free amino acids helped higher specific delta-endotoxin production. The exact role played by these free amino acids is yet to be resolved.

3.10 PEPTIDE PROFILE OF DELTA-ENDOTOXIN FROM B. T. G.

Larvicidal preparations from B. t. always consists of spore crystal mixture unless the preparation is from an asporogenic mutant / variant. Separation of the crystals from spores and other cell debris is a cumbersome and tedious process involving repeated separations in biphasic systems that generally had to be designed for each subspecies and serotypes (Cooksey 1971). Isopycnic centrifugation of spore crystal mixture in sucrose or caesium chloride gradients succeeded with several strains of B. t. In the subspecies active against lepidopteran larvae, it is known that the crystal protein is a protoxin (Mr. 130,000 to 160,000) which could be processed in vitro or in the insect gut to a toxic peptide of Mr 68,000 (Tyrell et al., 1981). However, electrophoretic analysis of crystals of different subspecies (Calabrese et al., 1980) include that the crystals of some subspecies contain not one but several large peptides and some contain only smaller peptides. Considerable diversity has been reported (Dulmage et al., 1981) with regarding spectrum of insect susceptible to different B. t. strains. In the strain of B. t. g. used in the present study, the delta endotoxin composed of two major peptides of 130 and 68 kDa as shown in Figure 3.30. In contrast, recently, a novel crystal protein isolated from B. t. BTS137J made up of a unique 129 kDa which is toxic to many Lepidopteran and Coleopteran species was reported. Tryptic digests of the protoxin containing only 72 kDa peptide highly toxic to this species was observed (Lambert et al., 1992).
The elucidation of number and nature of the subunits of the crystals of different strains of *B. t.* has been the subject of numerous investigations. However, there is still disagreement between various investigators, mainly due to the following reasons.

1) Method of purification and solubilization of crystal proteins
2) Differences in the *B. t.* strains employed
3) Analytical methods involved in the molecular weight determinations
4) Presence of proteolytic enzymes along with the crystal proteins.

In the present study the spore crystal complex prepared from the sporulated culture of *B. t. g.* was disrupted by sonication and subjected to separation in a 48 % (W/V) NaBr isopycnic density gradient as described in the methods. The white upper layer containing crystals was carefully separated and the constituent peptides of the purified crystals were analysed in a 10% SDS-polyacrylamide gel. Coomassie stained gel revealed the presence of two major peptides of 68 and 130 kDa (Figure 3.28).

Several of the genes coding these ICPs have been cloned and sequenced (see Review by Hofte and Whitely 1989). In order to further understand the temporal regulation of synthesis of larvicidal principle in *B. t. g.*, the cells were grown in GYA medium and samples were collected at different time intervals. SDS-solubilized samples were load on to SDS-PAGE and electroblotted to nitrocellulose membrane. This was reacted with anticrystal antisera raised against purified and alkali solubilized crystals in rabbits. The immunoreactive peptide profile is shown in Figure 3.28.

It was found that no immunoreactive peptides were detected during the logarithmic phase of growth. The synthesis of epitopes which could be detected by anticrystal antisera started at 8 hours of cultivation. The completion of protoxin (130 kDa) synthesis occurred at 18 hours. Further prolongation of the cultivation led
Figure 3.28 **Panel A**, SDS-PAGE profile of purified toxin from *Bacillus thuringiensis* subsp. *galleriae*. Lane a. Mol. wt standard; Lane b: toxin from *B. t. g.* **Panel B**, Western blot analysis of crude cell extracts from *B. t. g.* grown in GYA medium. Lane a. 2 hours; Lane b. 4 hours; Lane c. 6 hours; Lane d. 8 hours; Lane e. 16 hours; Lane f. 24 hours.
Figure 3.29 Western blot analysis of crude cell extracts of *Bacillus thuringiensis* subsp. *galleriae* grown in soy meal medium. Lane a: 6 hours; Lane b: 18 hours; Lane c: 24 hours; Lane d: 27 hours.
to degradation of protoxin by the proteolytic enzymes even in the carefully processed samples. Therefore, for high potency the culture has to be harvested immediately after the complete synthesis of crystalline inclusion body. However, it was reported that the crystal gene(s) has two types of promoters that can be recognised by different types of RNA polymerase during the logarithmic phase: i.e., each crystal gene has different types of promoters in *B. t*. strains. It was also shown that crystal gene expression during logarithmic phase is regulated at transcriptional level; and the transcriptional rate of the respective peptide might be less which could not be detected by immunological methods (Kanda, 1987).

Similar experiment was conducted by growing the cells in soy meal medium (Figure 3.29) supplemented with amino acids mixture. The samples were collected periodically and subjected to alkali solubilization and analysed through western blot. The immunoreactive peptide profile was similar to that of the cells grown in GYA medium. However, when the cultivation was prolonged beyond 18 hours, the degradation of the protoxin was prominent, which led to the appearance of many smaller molecular peptides. It is known that, soy meal is proteinaceous substrate, which could induce higher amount of protease production. These proteolytic enzymes might have acted upon the protoxin and led to severe proteolytic degradation.

3.11 CLONING OF cryIA(b) GENE IN *E. COLI*

The diversity of *B. t*. strains is due to the protein subunits of their crystals and it was also known that the existence of multiple genes is not very uncommon. The strain *B. t. k.* HD-1 is known to contain three genes coding for larvicidal principle. The three genes could be distinguished based on their southern hybridisation profiles. When the *EcoRI* fragment from the conserved amino terminal of any of these genes is hybridised to the plasmid DNA (from *B. t. k.*) digested with *HindIII*, they can be distinguished as three different bands measure 6.6, 5.3 and 4.5 kb in length. This suggested that the presence of multiple crystal protein genes (Kronstad *et al.*, 1983) that can be distinguished by the distance between the first
Figure 3.30 Partial restriction map of pES 1.

M=MspI; D=DraI; C=ClaI; E=EcoRI; P=PvuII; S=SacI; H=HindIII; A=AccI
Figure 3.31 Preparative agarose gel electrophoresis for the elution of 3.6 kb from pES 1.
internal Hind III site and the 5' flanking HindIII site. The gene cloned from B. t. k. HD-1- Dipel was a "4.5 kb class gene" which is used in the present study. A Sau3A1 partial digested megaplasmid(s) DNA from B. t. subsp. k HD-1 was ligated to the BamHI digested pBR322. The recombinant plasmid containing an insert size, Mr, 11*10^6 was designated as pES1 (Schnepf and Whiteley, 1981). A partial restriction map of pES 1 is shown in Figure 30. The gene consists of two sporulation dependent promoters, the transcriptional and translational start sites followed by a coding region. The coding sequence ends at 200-400 bases distal to the second PvuII site at base 3831 followed by a sequence resembling E. coli transcriptional terminator. (Schnepf et al., 1985). It was found that transcription starts from two temporally regulated, overlapping promoters namely BTI and BTII. In the present investigation whole, 3.6 kb Dra I fragment of the pES 1 containing the structural gene was cloned.

3.11.1 Enhanced expression of crylA(b) in E. coli.

Restriction of pES 1 DNA with Dra I resulted in six fragments of molecular weight ranging from 3.6 to > 0.2 kb blunt ended fragments (Figure 3.31). The 3.6 kb fragment was eluted from agarose gel using DEAE cellulose and subjected to further purification.

The vector used in the present investigation was pMal E, which has many salient features. pMal E vectors were designed as a way of expressing and purifying a cloned peptide by fusing it to maltose binding protein (Guan et al., 1988; Maina et al., 1988). The vector used in the present study was designated as pPR 683 and has a 'tac' promoter and the malE translational initiation signals to facilitate the isolation of the hybrid proteins on cross-linked amylose resin column. Upstream to the promoter is the lac Iq gene, which represses the expression of the operon. A multiple cloning site between malE and lacZ alpha-peptide is available for inserting the foreign DNA and the product of the coding sequences of the insert can form a fusion product with malE in the N-terminal and lacZ in the C-terminal ends. The vector pPR 683 also has a modified polylinker with the factor Xa protease recognition site, inserted in the
Figure 3.32 Map of pPR 683
Figure 3.33 Restriction digestion of the clone pRS 7. Lane 1 pRS 7 Unrestricted; Lane 2 pPR 683 linearised with Hind III; Lane 3 pRS 7 linearised with Pst I; Lane 4 pRS 7 restricted with Pst I and BamHI
Figure 3.34 Western blot analysis of crude cell extracts of recombinant *E. coli* pRS7. Proteins from the cell extracts (100 μg/slot) in the presence of PMSF were separated on to 10% SDS PAGE. Panel A Reacted with anticrystal antisera. Panel B Reacted with anti MBP serum. Lanes a: 2 h (after induction with IPTG); b: 4 h; c: 6 h; d: 8 h; e: 12 h.
BamHI site of the polylinker. This facilitates the separation of the product from the malE protein by specific cleavage (Figure 3.32). This vector was digested with SauI and the sticky ends were fill-in using Klenow fragment of *E. coli* DNA polymerase I and ligated to the 3.6 kb Dral fragment from pES 1 and transformed to an alpha complimenting host of *E. coli* namely PR722. The recombinants were selected on ampicillin plates. About 500 transformants were obtained per µg of DNA. The transformants were replica plated on Amp and Amp IPTG X-Gal plates. The Amp' colourless colonies from X-gal plates were screened for immunoreactivity with anticrystal antisera.

Among many immunoreactive clones, one clone was selected for further characterisation. Since klenow fill-in altered the Hind III recognition sequence, the insert was cut out with Bam HI and Pst I and resolved in 0.7% agarose gel. The recombinant plasmid pRS 7 containing CryIA(b) gene showed the presence of a 3.6 kb insert (Figure 3.33).

### 3.11.2 Characterisation of recombinant antigen from pRS7

In order to characterise the larvicidal protein from the recombinant *E. coli* carrying the plasmid pRS7, the cells were grown LB amp and induced with IPTG during exponential phase of growth and western blotted with anticrystal antisera. Among many immunoreactive clones, one clone was selected for further characterisation. The total cell extracts were prepared and load on to SDS-PAGE. The protein from SDS-PAGE were electroblotted on to nitrocellulose paper and subjected to immunological analysis. The western blot profile of the recombinant antigen reacted with anticrystal antisera and anti-MBP is shown in Figure 3.34. A 130 kDa peptide reacting with anticrystal antisera was detected. With the protein reacted with anti-MBP, a prominent 130 kDa was detected along with a less reactive truncated peptide of 77 kDa. Even with the anticrystal antisera this peptide could be detected occasionally. Therefore, the appearance of this peptide might be due to proteolytic degradation.
Figure 3.35 Toxicity of pRS 7 tested against fourth instar larvae of *Bombyx mori*. 
3.11.3 Synthesis of *CryIAb* protein in *E. coli*

The recombinant *E. coli* carrying pRS 7 was grown in LB amp and induced with IPTG during exponential phase of growth. In order to study the temporal regulation of ICP gene expression in *E. coli*, the samples were collected at different time intervals were subjected to immunological analysis. The total cell extracts were collected at different time intervals were solubilized with SDS and load on to an SDS-PAGE at a concentration of 100 μg/lane. The western blot profile is shown in Figure 3.34. The synthesis of crystal antigen started during the logarithmic phase of growth and the 77 kDa and the 130 kDa prominently detected at 12 hour of induction with gradual decrease in the intensity of MBP protein. Unlike in *B. t.* the ICP was synthesised at all stages of growth. However, the expression levels were low during exponential phase of growth. Sample collected after 12 th hour of IPTG induction was sonicated and subjected to bioassay against fourth instar larvae of *Bombyx mori*. The mortality was followed up to 78 hours. The toxicity was expressed as potency which is defined as 1/LC 50. Several fold increase in toxicity was observed with recombinant *E. coli* (Figure 3.35) in comparison with the parental clone, pES1. However, this is only 36 % of the expression levels found in the parental strain.
This investigation clearly established that the strain of *B. t. .g.* used in the present study is unable to grow in a chemically defined medium. However, it exhibited growth in a semidefined medium with small amounts of yeast extract indicating that the yeast extract might have supplied peptide(s) essential factor for the growth of *B. t. .g.*

Growth of *B. t. g.* on glucose had clearly indicated that glucose served both as a carbon source and an energy source. The maintenance coefficient (*m*) was found to be 0.09 h\(^{-1}\).

Perturbations observed in a chemostat can be useful indicators of strain instability.

A systematic approach was made to understand the segregational instability of *B. t. g.* in continuous culture.

Further insight into the strain instability indicated that, the tendency of the variants to replace the wild type could be due to the loss of certain biosynthetic functions which may have revealed in terms of loss of sporulation.
The spontaneous asporogenic variants isolated from the chemostat culture still form multiple inclusion bodies in the cell in contrast to the wild type *B. t. g.* which normally forms single bipyramidal crystal at the onset of sporulation.

The purified inclusion bodies from these variants were immunoreactive with anticrystal antisera. The trypsin digested peptide exhibited similar antigenic profile as that of purified crystals from the wild type strain.

These inclusion bodies were nevertheless less toxic to *Bombyx mori*, revealing that these variants after appropriate genetic modifications, could offer a safe biocidal formulation devoid of spores. These variants could also be used as a model system to study the regulation of gene expression during sporulation in *B. t.*.

Stability of the strain increased by appropriately supplementing the medium with amino acid mixtures.

Pulse experiments were clearly established that the amino acid mixture rather than a single amino acid is essential for growth.

The maximum space-time yield of biomass was achieved at a higher specific growth rate of 0.9 h\(^{-1}\). The Pmax was found to be 1.82 g l\(^{-1}\) h\(^{-1}\) which
is 3 times higher (calculated in terms of carbon equivalents) than the productivity observed in GY medium without any precursors.

The sporulation and toxin formation is regulated in a similar fashion and the effect of growth rate on the two process was clearly established.

High Space time yield of biomass was achieved at a higher specific growth rate of 0.9 h⁻¹ and the maintenance requirement was brought down to zero by appropriately designing the growth environment.

The capacity of GYA - medium to support growth, sporulation and toxin formation was further confirmed by batch cultures. The yield coefficient in GYM was 1.66 times higher than GY.

The toxin productivity in soy meal medium supplemented with amino acids was 40 times higher. These results are of great interest and application potential from the industrial production point of view.

Western blot analysis very clearly established that the bipyramidal crystal of *Bacillus thuringiensis* subsp. *galleriae* were composed of two major peptides of 68 and 130 kDa.

The synthesis of delta endotoxin peptides in *B. t. g.* started at 6 hour and led to the completion by 18 hour of cultivation. No immunoreactive
epitopes were detected during vegetative phase of growth. This holds good irrespective of the medium in which the organism was grown. However, intense proteolytic degradation was observed with soy meal.

cryIA(b) gene from B. t. k. HD1 was cloned and expressed in E.coli using MalE vector. A prominent 130 kDa peptide reacting both with anticrystal antisera as well as with anti-MBP was detected. Another peptide of 77 kDa could react only with the anti-MBP was also observed.

Higher toxicity was observed with the recombinant clone pRS7 in comparison with the parental clone.