Mass Cultivation of *Bacillus thuringiensis* var. *kurstaki* in Fed-Batch Culture for High Spore Count and Improved Insecticidal Activity

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Batch and fed-batch cultivation of *Bacillus thuringiensis* var. *kurstaki* was carried out to achieve high cell and spore density with maximum insecticidal activity. Cell cultivation was done in fully equipped fermentors under controlled conditions at different feed rates. Increased cell mass, high spore density with maximum insecticidal activity were achieved with fed-batch cultivation system. With the appropriate supply of glucose and other nutrients, it was possible to grow the cells to >50 g (dry weight)/l density and achieve a spore concentration of 3.9x10⁶/ml having high insecticidal activity.

**Keywords**: insecticidal activity, fed-batch fermentation, sporulation, crystal potency, glucose feeding

Biological control of various insects, pests and other disease vectors is an extremely important research area related to environment, health and hygiene, and economic aspects of any country. Different varieties and strains of *Bacillus thuringiensis* (Bt) are the most commonly and widely used microbial insecticides. Large quantities of cell biomass with high insecticidal activity are essential for practical applications. Therefore, it is desirable to optimize medium composition and culture conditions to maximise cell biomass production. Insecticidal potency of these organisms has been found to be determined by high productivity of the endosporules in association with the toxic protein crystal. One reasonable way to increase spore production and insecticidal activity is to achieve high cell density and subsequently allow sporulation to occur (Kang et al., 1992). A number of nutritional, biochemical, physical and genetic factors have been identified for the induction and maintenance of the toxigenic titre of the cell biomass (Arcas et al., 1984, 1987). The response of the cell population towards the available nutrients and the changes in the existing microconiches substantially affects the induction and formation of spores/crystals. Fed-batch culture has been widely used for increasing cell mass or product concentration (Kang et al., 1992; Liu et al., 1994). It has an additional advantage of controlling the extra cellular environment and creation of nutrient deficiency to initiate sporulation. This paper presents the effect of several feeding strategies on vegetative cell growth, spore formation and crystal protein content, during the cultivation of *B. thuringiensis*.

*B. thuringiensis* var. *kurstaki* HD-1 obtained from the Bacillus Genetic Stock Centre, Ohio State University, USA (stock No. 4D6) was maintained on the slants of Schaeffer's sporulation medium at 24°C. For fermenter studies, a modified medium (Arcas et al., 1984) was used. The fermentation batches were run in a computer coupled 14-L New Brunswick fermenter. In all the experiments, the temperature was controlled at 30°C, speed of agitation was set at 800 rpm, and pH was controlled within 6.5 to 7.5 by automatic addition of 2N NaOH solution. Exponentially growing cells were used for inoculating fermenters. For batch fermentations, 6 to 8 litres of medium was prepared in the fermenter. Magnesium sulphate and glucose solutions were autoclaved separately and, after cooling, mixed with the rest of the medium. Fed-batch experiments were conducted by first calculating the total requirements of all the nutrients in order to achieve a specific final cell density in the broth. These were then divided into components to be dissolved in the initial solution and those to be fed along with the glucose solution. Feed media were filter sterilized. The fed-batch experiments were started with a batch fermentation, and feeding was initiated between 4 to 6 hrs when the cells had attained a decreased acceleration state as indicated by the oxygen uptake of the culture. Samples were analyzed at intervals and at the end of fermentation.

The reagents were obtained from Sigma Chemicals, USA. Glucose was measured by using the assay kit. Spores were counted using haemocytometer and Nikon YS2-H microscope. Total crystal protein in the broth was analysed (Lowry et al., 1951). The samples
of crystal protein content were prepared by first centrifuging the samples at 4°C for 10 min at 10,000 rpm. The sedimented pellet containing spore-toxin complex was washed twice with distilled water, dried at 80°C in an oven and centrifuged in 0.1 N NaOH solution. The final solution was centrifuged at 10,000 rpm for 10 min. The supernatant, containing dissolved crystal protein, was used in total protein analysis. After fermentation, potency of the crystal in the broth samples was measured using the bioassay method (Dulmage et al, 1971), conducted on *Trichoplusia ni* (cabbage looper) larvae. Eggs of *T. ni* for the first generation were obtained from the Biological Control of Insects Research Laboratory (USDA, Columbia, MO).

The data of batch and fed-batch cultivation systems are shown in Table 1. During batch cultivation, maximum cell concentration was recorded parallel with the exhaustion of glucose in the medium verifying that the fermentation was glucose limited in nature and supported production of 5.9 g dry cell mass, which corresponds to a cell yield of 0.74 g dry cells/g of glucose. This cell yield is in agreement with other reported values from situations in which glucose acts as an energy source and culture carbon is derived from complex sources such as yeast extract and corn steep liquor. Growth and sporulation of *B. thuringiensis* maximizes by high levels of aeration (Liu et al, 1994). Dissolved oxygen concentration in the broth was ≥70% of saturation value at all the time and pH was 6.5 and a spore concentration of 1.5 × 10⁹/ml was achieved. Arcas et al (1984) have reported 5.40 g dry cell weight/l with 1.75×10⁷ spores/ml and 3.70 g dry cell weight/l with 1×10⁶ spores/ml at 20 and 10 g/l glucose, respectively. They further suggested that the use of 30 g/l glucose could yield further enhanced cell and spore concentrations, if pH was controlled and kept above 5.5. However, with the improved growth medium, they could enhance sporulation up to 2.1×10⁹/ml. In this study, cell and spore concentration achieved and the toxicity values closely resemble other reports.

In *B. thuringiensis*, sporulation initiated right after the depletion of glucose in batch cultivation (Liu et al, 2000). Approximately, 90% of the sugar was consumed in the first 15 hrs and maximum spore density (2×10⁹/ml) was achieved at 33 hrs of cultivation. While studying sporulation and toxicity profiles of a wild type and rDNA strain, the toxicity of the fermentation broth could not be ascribed to the level of spore density alone. Endotoxin protein concentration also plays an important role. In the study, the substantial crystal protein produced at the end of cultivation also corresponded with highest total protein concentration.

For fed-batch studies, batch fermentations were initiated first. Feed solutions containing glucose, yeast extract, corn steep liquor, and calcium chloride in ratio of 8:4:4:0.041 were fed into the fermenters at constant but different flow rates starting at 4 to 6 hrs after inoculation; glucose concentration in the feed solution was 128 g/l. The data (Table 1) indicates considerable increase in the yield of cells and spores with the increase of feed rate.

For achieving further high cell density and spore production, variable feed rate fed-batch cultivation experiments were undertaken. Sugar feed rates were periodically changed according to a preset pattern designed to meet the demand of cells growing at a constant specific growth rate. In run 5, sugar feeding was initiated at 6 hrs from the start of the batch. In the next two experiments, sugar feeding was initiated at the end of the 4th hr, which resulted in the production of further higher cell concentration (more than 50 g dry cells/l). In the run 5 and 6, spore counts increased and virtually no vegetative cells were seen under the microscope at the end. In the run 7, the process of sporulation was very slow and many nonsporulated cells were observed under the microscope even 15 hrs after the feeding was stopped.

To optimize the growth conditions for producing high cell mass coupled with the production of high spore/crystal complex, the studies (Golberg et al, 1980) reported that fast cell growth in batch cultures supported better sporulation and could achieve a yield of 4×10⁹ spore/ml concentration in chemostat. While studying enhanced sporulation of *B. thuringiensis* by fed-batch cultures (Kang et al, 1992), 1.25×10¹⁰ spores/ml was obtained when glucose and yeast extract were fed intermittently, to grow the cells fast, and subsequently the cells were further allowed to grow in batch mode. However, glucose feeding at high concentration did lead to high cell mass production but spore counts were fairly low. The process of sporulation formation in various Bacillus species has been found to be associated with the specific protein/carbohydrate ratio of the medium and appears to be regulated by nitrogen catabolite repression.

While studying the effect of complex nitrogen source (gruel) on sporulation and toxicity of *B. thuringiensis* (Zouari & Jaoua, 1999), it was observed that sporulation and toxicity was associated with the synthesis of several proteins including proteases.
The specific crystal potency decreased by correlation could be seen and the total crystal protein. This is in agreement with other studies (Dulmage, 1970; Scherrer et al., 1973) in the run 7, where the highest cell density was achieved, and several unusual observations were made which did not correlate with each other.

It is observed that growth and toxicity characteristics of B. thuringiensis var. kurstaki strain are significantly influenced by fermentation conditions. Manipulation of the concentration of medium ingredients and feeding conditions increased very substantially the spore density and insecticidal potency of the culture. However, production of high cell density has not always been proportionately associated with high spore counts and insecticidal activity. High oxygen supply and timely feeding of appropriate concentrations of glucose and other nutrients helped in achieving high density cell mass and raised spore production coupled with high insecticidal potency of the culture.

<table>
<thead>
<tr>
<th>Run</th>
<th>Cultivation conditions</th>
<th>Initial working vol (l)</th>
<th>Total glucose con in feed (g/l)</th>
<th>Feeding hours (after inoculation)</th>
<th>Cell con. dry cell wt (DW g/l)</th>
<th>Spore con. (x10^9/ml)</th>
<th>Crystal protein con. (µg/ml)</th>
<th>Crystal potency (KIU/ml)</th>
<th>Specific crystal potency (KIU/mg crystal protein)</th>
<th>Specific crystal potency (MU/g DW cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>BATCH MODE</td>
<td>8</td>
<td>8</td>
<td>-</td>
<td>5.9</td>
<td>1.5</td>
<td>357</td>
<td>272</td>
<td>262</td>
<td>46</td>
</tr>
<tr>
<td>2.</td>
<td>FED-BATCH MODE II (constant feed rate, ml/min)</td>
<td>6</td>
<td>128</td>
<td>5-23</td>
<td>11.5</td>
<td>3.9</td>
<td>1031</td>
<td>1052</td>
<td>1020</td>
<td>91</td>
</tr>
<tr>
<td>3.</td>
<td>1.6</td>
<td>6</td>
<td>128</td>
<td>6-10</td>
<td>10.4</td>
<td>2.3</td>
<td>684</td>
<td>985</td>
<td>1440</td>
<td>95</td>
</tr>
<tr>
<td>4.</td>
<td>2.6</td>
<td>6</td>
<td>128</td>
<td>5-15</td>
<td>16.0</td>
<td>8.6</td>
<td>1278</td>
<td>658</td>
<td>154</td>
<td>43</td>
</tr>
<tr>
<td>5.</td>
<td>FED-BATCH MODE II variable feed rate; glucose feeding rate g/min</td>
<td>7</td>
<td>165</td>
<td>6-15.9</td>
<td>23.8</td>
<td>30.0</td>
<td>5330</td>
<td>1210</td>
<td>231</td>
<td>51</td>
</tr>
<tr>
<td>6.</td>
<td>0.24-1.24</td>
<td>6</td>
<td>198</td>
<td>4.0-16.7</td>
<td>36.4</td>
<td>39.0</td>
<td>3248</td>
<td>1727</td>
<td>532</td>
<td>47</td>
</tr>
<tr>
<td>7.</td>
<td>0.38-1.08</td>
<td>6</td>
<td>317</td>
<td>4-25.5</td>
<td>53.7</td>
<td>14.5</td>
<td>6089</td>
<td>1680</td>
<td>276</td>
<td>31</td>
</tr>
</tbody>
</table>

Synthesis of proteases fluctuated greatly with the variation of medium composition and complex nitrogen sources. Toxicity was maximum at the end of sporulation accompanied with the release of crystal from spores.

In present communication, with the exception of run 7, increased cell concentration has resulted in increased spore count and insecticidal potency in the broth. In run 7, sporulation was incomplete, insecticidal potency did not increase in spite of increased cell density and high crystal protein content in the system. The specific crystal potency decreased by 30% (48 to 31) in run 7. In runs 5 and 6, the spore count and crystal potency (per ml of broth) increased in proportion to the cell density. The specific crystal potency (per gram of dry cells) remained constant suggesting that the process of achieving high cell density had no impact on production of insecticidal (pro) protein once feeding was stopped. In both these cases, the dissolved oxygen concentration was maintained at a high value all along the fermentation and the concentration of glucose was below detection limits at the time when the feeding was stopped. However, the production of total crystal protein appeared erratic and no correlation could be seen between insecticidal potency and the total crystal protein. This is in the agreement with other studies (Dulmage, 1970; Scherrer et al., 1973) in the run 7, where the highest cell density was achieved, and several unusual observations were made which did not correlate with each other.

References


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