Israel Journal of Entomology

Vol. XXIH (1989) pp. 239-246

PROCESS OPTIMIZATION AND SCALE UP OF THE *BACILLUS THURINGIENSIS* FERMENTATION

S.E. VECHT-LIFSHITZ[1], M. GANDMAN[2], AND E. ZOMER[3]
[1]Biotechnology Unit, Dept. of Biological Chemistry,
Hebrew University of Jerusalem, Givat Ram, Jerusalem 91904, Israel;
[2]Unikoor, POB 1172, Jerusalem, Israel; [3]Dept. of Tropical Public Health,
Harvard University, Boston, USA.

ABSTRACT

Growth, sporulation and lysis of *Bacillus thuringiensis (B.t.)* cells, grown in a 121 fermentor, were monitored continuously by a microcalorimeter (bioactivity monitor LKB 2299). Process Development was carried out in this benchtop fermentor using analytical grade substrates at low cell concentrations (10^8 cells/ml) to avoid oxygen limitation in the bioactivity monitor cell. The data gathered was used to calculate heat output and corresponding oxygen demand. This information was used to perform an energy balance for *B.t.* production. This was compared with pilot plant data using industrial grade substrates at high cell concentrations (10^9/ml).

Growth and sporulation kinetics, oxygen demand, and energy balances were calculated and compared wilh the microcalorimetric data. The results showed that the bioactivitythermograms provided accurate information about the fermentation process which may be applied to industrial scale fermentations. The bioactivity monitor has potential use as a tool for preliminary medium optimization, and for defining mass and energy balances. Adaptation of this technique for scale-up can save much of the research and development costs of industrial processes.

Further development of this monitoring technique could lead to its use for optimization of real- time control in industrial processes.

INTRODUCTION

The bioactivity monitor is an instrument which continuously monitors very small heat fluctuations associated wilh biological events in living organisms. Its real time monitoring is both direct and nondestructive. It has been used to monitor the growth and metabolism of microorganisms (Forrest, 1969; Nichols et al., 1979), tissue cells (Monti, 1980), and invertebrates (Gnaiger, 1983). It has been used to observe viral infection of cells (Voitsekhovsky, 1972) and to observe ecological phenomena.

It is well known that microbial growth processes generate heat. This heat effect may be measured directly, or as a function of oxygen uptake (Wang et al., 1979), or by monitoring changes in ATP levels (Alkinson and Mavituna, 1983). The removal of this heat is a well known problem associated with large scale fermentations. Calorimetric measurement of that heat evolution is an important parameter for describing the metabolic activity of the specific process. Measuring heat fluctuation in a continuous flow system may supply real time information on the growth process which can be used for optimization studies, or control of the studied process. Thermogram data yield estimates of the specific and total heat outputs (Nichols et al., 1979).

This study was initiated in order to assess te usefulness and reliability of the flow microcalorimeter (bioactivity monitor) as a tool for scale-up of fermentation processes and for defining engineering parameters. It was connected to a fermentor in continuous flow mode as described below.

The bioactivity monitor described has two major limitations to the scale-up of an aerobic process, namely, oxygen limitation in the calorimetric cell, and clogging in the connecting tubes at high cell densities or when using industrial grade substrates. Thus, all optimization studies must be performed using low cell concentrations and fine chemicals. This may limit the reliability of scale-up based on these results. However, the bioactivity monitor has an advantage over the spectrophotometer, namely, its operational accuracy is not limited by the turbidity of the culture.

The microbial process examined was the *Bacillus thuringiensis* fermentations for endotoxin production. The endotoxin is of commercial importance as a species-specific microbial insecticide (Dulmage, 1980; Margalit et al., 1983; Sneh et al., 1981). The temperature control of such a process is particularly important as the bacterial growth and its sporulation have different temperature optima.

MATERIALS AND METHODS

Microorganisms and Media

The bacterial strain employed was *Bacillus thuringiensis* Berliner (isolate no. *B.t.* 24). It was maintained on slants and prepared for inoculation as described previously (Sneh et al., 1980).

Erlenmeyer flasks (nominal volume 250 ml) containing 100 ml of nutrient broth (Difco 0001 8 g/l) were inoculated from the slants and placed on a rotary shaker for 12 hours at 120 rpm at 29°C. All cultures having an optical density greater than 2.0 at 650 nm, were used as seed inocula for the fermentations. The media used in the different experiments are tabulated in Table 1.

Culture Conditions

A 2% inoculum was aseptically transferred to a 12 l (Bioengineering model) fermentor with a stirrer speed of 500 rpm, air sparge rate of 0.5 vvm at 30°C. The large scale fermentations were performed in two identical 600 l fermentors (Palbam k98-2, POB 18960, Ein Harod, Israel) with 0.5 vvm air sparge rate and 150 rpm at 30°C. Oxygen tension was monitored with an Ingold Oxygen electrode. pH was maintained at 6.0 using a control system with NH_4OH feeding.

TABLE 1

Run No.	Liquid Volume (l)	Carbohydrates (mg/l)	Proteins (mg/l)
1	12	396	156
2	12	396	156
3	12	60	26
4	12	24	24
5	12	9.6	9.6
6	12	4.35	10
7	12	4.80	4.80
8	12	2.40	2.40
9	12	0.213	0.117
10	12	0.22	0.223
11	12	0.254	0.446
12	12	0.097	0.354
13	500	19810	12460
14	500	19810	12460
15	500	19810	12460
16	500	19810	12460

Sporulation quantification, cell counts and bioassays were performed as described previously (Sneh et al., 1980).

Energy Balances

The microcalorimeter (LKB 2277) was connected in continuous flow mode to the bench top fermentor. A small volume (30 ml/hr) of the cell culture was continuously pumped from the fermentor to the microcalorimeter cell, and maximal deflection settings for the heat output were varied from 30–750 uW, according to the medium concentration. The heat generated in the cell was recorded, and thermograms accompanying the growth were obtained.

Sterilization

Preceeding the fermentation, all connecting tubing between the microcalorimeter cell and the fermentor was sterilised by pumping 1% alcohol for 40 min, followed by rinsing with sterile water. The volume of the microcalcorimeter cell was 0.6 cm^3. The length of the polypropylene tubing (internal diameter 0.1 cm) leading from the fermentor to the bioactivity monitor was 120 cm. It was surrounded by a water jacket held at 30°C. Special aseptic seals were designed for the joints between the tubing, fermentor and bioactivity monitor.

On the large scale, two identical fermentors containing 500 l medium were used. Cooling water temperature and flowrates were recorded for energy balance calculations.

One fermentor was used as a control and was not inoculated, yet was agitated and aerated under the same conditions as the inoculated fermentor such that heating effects of the system other than metabolic heat output could be quantified.

Energy Balance Calculations

Total heat outputs from large and small scale fermentations were calculated by numerical integration of the areas under the cooling water curves and thermograms, respectively. Assumptions about the calorific contents of the various media were made with reference to the various producer manuals. Estimations of stirrer heat output and evaporation heat output were made with reference to Wang et al. (1979).

RESULTS AND DISCUSSION

Preliminary results from the batch culture of *B.t.* proved that the bioactivity monitor was both a reliable and sensitive device for monitoring microbial activity. The study was limited to microbial grade substrates only. The thermograms obtained showed that at high cell concentrations anoxia prevailed in the monitoring cell. Thus, in order to obtain accurate quantification of metabolic activity, cell concentrations were kept constantly below 10^8 ml.

It was observed that there was a good correlation between exponential growth, glucose uptake and heat output (measured by the bioactivity monitor) and likewise when glucose was depleted and cell growth ceased there was a sharp decline in metabolic activity (Fig. 1). Sporulation commenced four to five hours after glucose depletion. A second peak in heat output was proportional to the sporulation rate. When the sporulation was completed and cell lysis commenced, another sharp fall in metabolic heat output was observed. Similar phenomena were observed in 500 l fermentations, quantified with cooling water measurements (Fig. 2). For the exponential growth, the area under the thermogram was indicative of the quantity of substrate consumed and of the number of cells formed.

Medium optimization was attempted with the knowledge that under normal conditions (such as inoculum size, pH temperature and oxygen supply), the thermogram of the microorganism would show a characteristic series of changes. These changes can be used to calculate the optimal ratio of protein to carbohydrate. These can also be used to minimize energy wastage and to maximise the yield of biomass per substrate concurrently. In our project maximum sporulation was also required

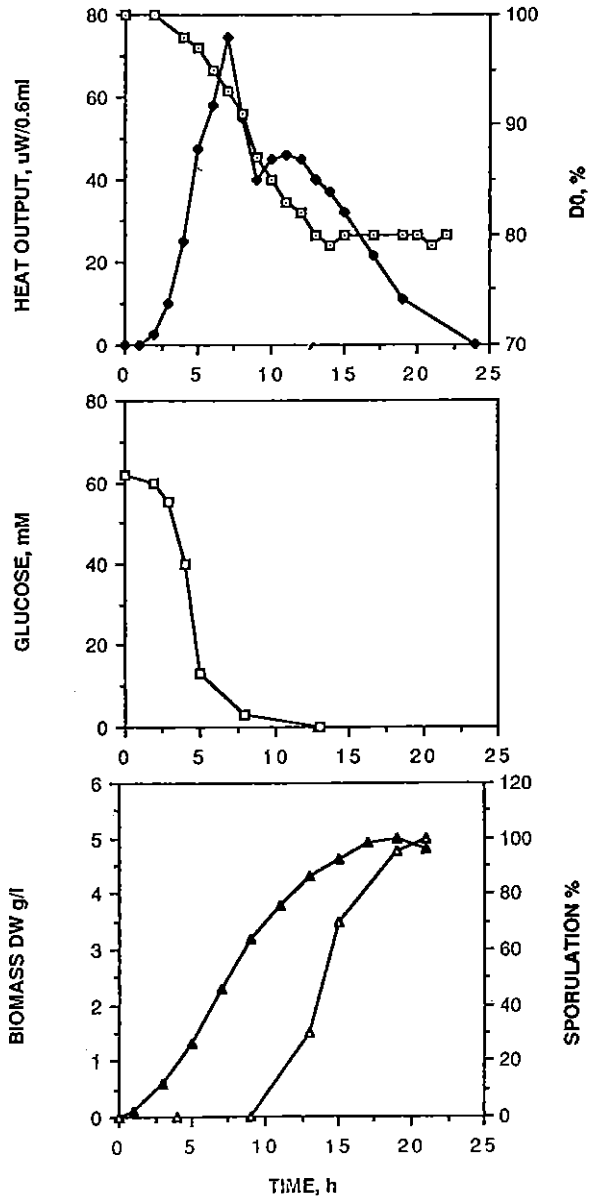

Fig. 1. Fermentation of *B.t.* in 12 l fermentor. (♦) Heat output as measured by bioactivity monitor as in methods, (⊡) dissolved oxygen, (▲) biomass, and (Δ) sporulation.

for maximal yield of bioinsecticidal activity. Results showed that the bioactivity monitor was of use in medium optimization (Fig. 3) and the optimal C:N ratio for growth was 1:2. At this feed ratio, the cellular yield was maximal per unit substrate, yet the energy output was about 30% less than the maximum. It was found that at this feed ratio the sporulation rate was also maximal.

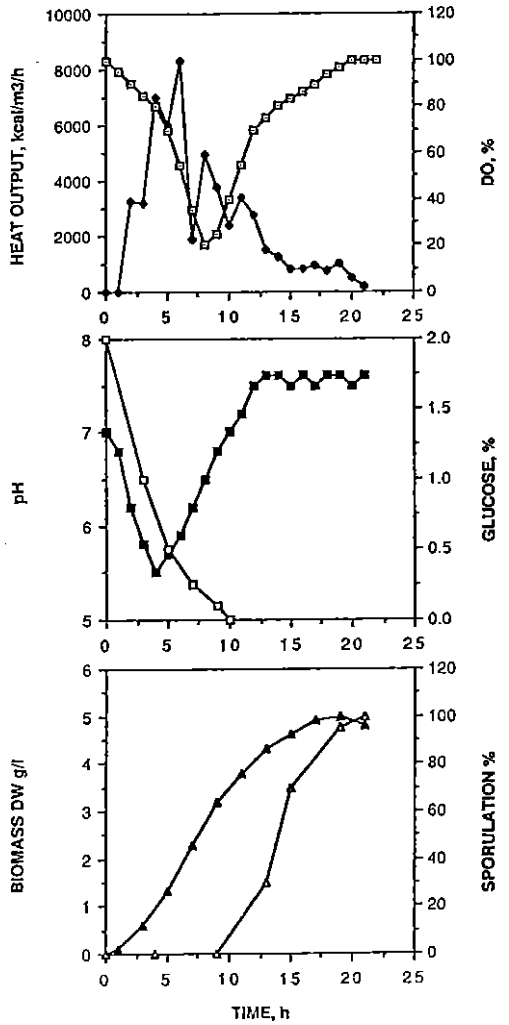

Fig. 2. Fermentation of *B.t.* in 500 l fermentor. (◆) Heat output as measured by bioactivity monitor as in methods, (⊡) dissolved oxygen, (■) pH, (□) glucose concentration, (▲) biomass, and (Δ) sporulation.

Thermogra m data gathered from bench-top fermentor runs were used to define parameters in 500 l (working volume) fermentor. Culture medium was composed of industrial grade ingredients. Calculations were based only on available proteins and carbohydrates in industrial grade substrates as opposed to the total proteins and carbohydrates in the analytical grade substrates. A non-inoculated fermentor was used to estimate heat changes due to agitation, insulation and evaporation. The cooling water flowrate as well as inlet and outlet temperatures were measured and used for the calculation of heat evolution of the *B.t.* fermentation. Heat balances accounted for 97% of the theoretical heat inputs (averages of runs 13–16 (Fig. 4). These measurements were used to construct thermograms for the 500 l runs. Calculation of mass and energy balances for the bench fermentor using the bioactivity measurements accounted for 103% of the theoretical heat inputs (average of

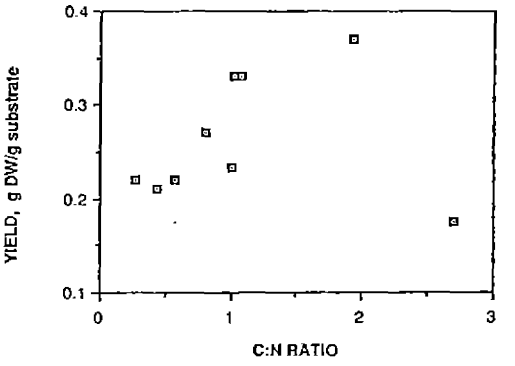

Fig. 3. Maximal cellular yield as a function of the broth C:N ratio.

```
ENERGY BALANCE FOR BACILLUS THURINGIENSIS FERMENTATION (KJ/M3)

ENERGY IN -ENERGY OUT = ENERGY ACCUMULATION
WHERE
     ENERGY IN       =AGITATION ENERGY (E1) + CALORIFIC ENERGY
                      IN FORM OF SUBSTRATES (E2) AND CELLS (E3)
     ENERGY OUT      =ENERGY LOST DUE TO EVAPORATION (E4) + HEAT LOST
                      TO COOLING WATER (E5) + HEAT LOST TO
                                SURROUNDINGS (E6)

ENERGY ACCUMULATION =CALORIFIC VALUE OF UNUSED SUBSTRATES (E7)
                      (IN FERMENTOR) + CELLS PRODUCED (E8)

THUS:-
     (E1)+(E2)+(E3) = (E4) + (E5) + (E6) + (E7) + (E8)
     (E1) + (E2)    = (E4) + (E5) + (E6) + (E7) + ((E8)-(E3))

VALUES FOR LARGE SCALE FERMENTATION (KJ/M3), (average values of runs
13-16)

304819 + 735065      = (E4) + 236298 + 58018 + (338850 + 266434)
                      +112950
         1039885     = (E4) +1012550
ENERGY ACCOUNTED FOR  > 97% OF ENERGY INPUT

VALUES FOR BENCHTOP FERMENTATION (KJ/12L)  (average values from run
nos.9-12)

(Note that the system has temperature control such that only  the
metabolic heat output is measured by the bioactivity  monitor, thus
agitation and evaporation heat outputs, and  surroundings heat gain
cancel each other out):-

Thus (E1) - ((E4)  + (E6)) = 0
With      (E5)      =INTEGRAL OF THERMOGRAM FROM BIO-ACTIVITY MONITOR

          (E2)+(E3) = (E7) + (E8) + (E5)
     118.40  + 1.57 = (E7) + 31.40   + 93.25
     (E7)           = NEGLIGIBLE
ENERGY ACCOUNTED FOR=103.9%
```

Fig. 4. Energy balances for *B.t.* fermentation both on the large scale (5001) and on the small scale (12l).

runs 9–12). These values are not as reliable as they might appear, as the agitation heat input could easily have been 20% greater than the assumed value, due to the impeller configuration in the large fermentors. The assumed calorific values of the cells and substrates could also vary by 10%. It would be more accurate, therefore, to say that these methods accounted for 100 ± 20% of the heat inputs.

The thermograms from the 500 l runs compared well with the microcalorimetric thermograms (Fig. 5). The energy-time curves achieved were almost identical, and there was a good correlation of energy output with exponential growth, sporulation and lysis. Over 90% accuracy was achieved in scaling up practical results with respect to the expected theoretical results. It was also noted that the thermogram curve was more peaked in the large scale fermentations than in the small scale. This could be due to the increased complexity of the medium, or to water flowrate fluctuations. The heat outputs from four runs of 500 l and four runs of 10 l were scaled up to 1000 l, of fixed cell density. Maximal differences were observed initially, due to different lengths of lag phases. The integral area under all eight thermograms considered (not shown), varied by ±15% from the mean. This was a low value of standard deviation, as the cell concentrations, medium compositions, and ambient temperatures varied slightly from run to run.

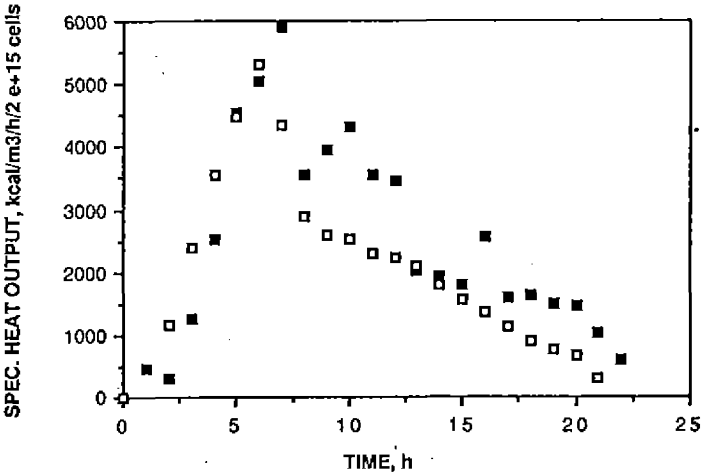

Fig. 5. Comparison of themograms of *B.t.* fermentations (average of 4 runs) on the large scale (■) and on the small scale (□).

CONCLUSIONS

Microcalorimetric monitoring was found to be a useful technique for direct observation of microbial metabolism. Although difficulties were encountered with highly aerobic cultures, use of low cell concentrations enabled reliable monitoring of the bioactivity.

Resultant thermogram data were found to be reliable for scale-up and for predicting optimal conditions in larger fermentors. Since there is a linear relationship between oxygen demand and heat output during the exponential growth phase, one can estimate the required aeration rates and stirrer speeds for industrial fermentations, based on thermogram data from bench-top scale fermentations. Thermogram data also provide useful information about media optimization, which can be used in scaling-up of fermentations.

The phenomenon of sporulation in *B.t.* has been correlated with the synthesis of proteinous toxin active against insects. The thermogram data clearly indicates the time at which sporulation commences. The heat output of the process can be used to improve the regulation of the sporulation

process and also indicate the harvesting time, which may be defined as the time when no further oxygen is required.

This fermentation normally takes 48 hours to achieve maximal toxin production as it is produced according to secondary metabolite kinetics. However, with the knowledge obtained by calorimetric methods, a precise harvesting time can be defined, and this can curtail the fermentation time by 10–20 hours, which, in turn, results in a more economic process.

ACKNOWLEDGEMENT

We would like to thank Dr. B. Fridlander (FRM Agricultural Partnership, PO Box 4309, Jerusalem, Israel) for his assistance in the large scale production of *Bacillus thuringiensis*.

REFERENCES

Atkinson, B. and Mavituna, F. 1983. In: The Biochemical Engineering and Biotechnology Handbook. Nature Press, New York. p. 838–853.

Dulmage, H.T. and Collaborators 1980. Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control. In: Microbial Control of Pests and Plant Diseases, 1970–1980. Edit. H.D. Burges. Academic Press.

Eriksson, R. and Holme, T. 1977. Flow Microcalorimetry Applied to Microbial Processes. LKB Application Note 267.

Falcon, L.A. 1971. Use of bacteria for microbial control, In: Microbial Control of Insects and Mites. Edit. H.D. Burges and N.W. Hussey. Academic Press, London.

Forrest, W.W. 1969. In: Biochemical Microcalorimetry. Edit. H.D. Brown. pp. 165–180, Academic Press.

Gandman, M., Vecht, S., Zomer, E. and Erel, Z. 1985. The use of the bioactivity monitor for process optimization and scale-up of the *Bacillus thuringiensis* fermentation. *Biotech. Proc.* p. 665–670.

Goldberg, I., Sneh, B., Battat, E. and Klein, D. 1980. Optimization of a medium for a high yield production of spore-crystal preparation of *Bacillus thuringiensis* effective against the Egyptian cotton leaf worm *Spodoptera littoralis Boisd. Biotechnol. Lett.* 2:419–426.

Gnaiger, E. 1983. Microcalorimetric monitoring of biological activities. Ecological and toxicological studies in aquatic animals. *Science Tools* 30:21–26.

Holmberg, A., Sievanen, R. and Carlberg, G. 1980. Fermentation of *Bacillus thuringiensis* for exotoxin production: process analysis study. *Biotechnol. Bioeng.* 22:1707–1724.

Luong, J.H.T. and Volesky, B. 1983. On microbial activities. In: Advances in Biochemical Engineering. Vol. 28. Edit. A. Fiechter. p. 1–40, Springer Verlag, Berlin.

Margalit, J., Zomer, E., Erel, E., and Barak, Z. 1983. Development and application of *Bacillus thuringiensis* var. *israelensis* serotype H14 as an effective biological control agent against mosquitos in Israel. *Biotechnol.* 1:74–76.

Monti, M. 1980. A new approach to detection of antigen- antibody complexes by microcalorimetric measurements of heat production in blood cells. *J. Immunol. Meth.* 37:29–37.

Navon, A., Wysoki, W. and Kerem, S. 1983. Potency and effect of *Bacillus thuringiensis* preparations against larvae of *Spodoptera littoralis* and *Boarmia (Ascotis) selenaria. Phytoparasitica* 11:3–11.

Nichols, S.C., Prichard, F.E. and James, A.M. 1979. Microcalorimetry studies of energy changes during the growth of *Klebsiella aerogenes* in simple salts/glucose media. *Microbios* 25:187–203.

Poole, R.K. and Haddock, B.A. 1975. Microcalorimetric measurements of heat evolution and their correlation with the oxygen uptake in *Escherichia coli* with genotypically and phenotypically modified electron transport chains. *FEBS Lett.* 58:249–253.

Sneh, B., Schuster, S. and Broza, M. 1981. Insecticidal activity of *Bacillus thuringiensis* strains against the Egyptian cotton leafworm, *Spodoptera littoralis* (Lep:Noctuidae). *Entomophaga* 26:179–190.

Voltsekhovsky, B.L. 1972. Inhibition of cell culture respiration by influenza virus strains of different virulence. *Acta Virol.* 16:313–322.

Wang, D.I.C., Cooney, C.L., Demain, A.L., Dunnill, P., Humphrey, A.E. and Lilly, M.D. 1979. Fermentation and Enzyme Technology. J. Wiley, USA. p. 83–89.