CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 PROCESS OPTIMIZATION BY HEAT MEASUREMENTS IN PILOT SCALE BIOREACTOR

Biological process consists of various dependent and independent biochemical reactions. Heat exchanged due to these biochemical reactions will provide insight into the metabolic activity of the organism during cultivation process. The importance of the heat measurements and its application in the fermentation process has paved a way for its exploitation in the commercial fermentation processes. In addition, extensive work has been carried out in bench scale bioreactors towards heat measurements during the cultivation and its implications. Hence, it was decided to study the feasibility of measuring the metabolic heat production rate in the pilot scale bioreactor. These studies were carried out in pilot scale bioreactor to understand the significance of online heat monitoring and its application in commercial large-scale industrial fermentations. This heat measurement study in pilot scale bioreactors necessitated the modifications in the existing bioreactor and development of program in LabVIEW for data acquisition, calculation and control. Also, attempts will be made for improving the existing process on the basis of metabolic heat production rate during growth for the synthesis of ICP in the complex medium using the strain, Bacillus thuringiensis subsp. galleriae. The focus during this feasibility study will be on the identification of the critical parameters and its online measurement. This identified critical parameter has to
reflect the changes in proportion with the heat production rate during the cultivation and creating a source for commercial exploitation.

3.1.1 Modifications in pilot scale bioreactor

The original setup of the pilot scale bioreactor is shown in the figure 3.1. The bioreactor's external walls were insulated with mineral wool to avoid any heat loss to the environment from the cooling jacket. Also, exhaust portion from the reactor lid to the outlet humidity probe through the exhaust air filter was insulated with mineral wool to avoid any possible enthalpy change in the air. Major modification was carried out in the cooling water jacket portion by installing two temperature probes at the inlet and outlet of the jacket. Inlet and outlet airflow paths were also modified to accommodate the humidity probes. The other modifications and added components were:

a. High precision temperature transmitters were installed for accurate measurements. These transmitters were highly sensitive with 5-20mA output for the temperature range of 20 - 40°C.

b. Flow indicator for online measurement of cooling water circulation rate in the jacket was installed in the jacket circulation circuit. Due to the operational difficulties in maintaining the bioreactor temperature with bypassing of chilled water on the opening of the solenoid valve during control, it was removed during cultivation. With the operating speed of the jacket circulation pump, flowrate of the water under circulation was observed to be 45 l/min. This value was
Figure 3.1  The original setup of the pilot scale bioreactor with accessories indicated for its operation.  
[source: Bioengineering AG operating manual]
used in the calculation of the metabolic heat production rate during the cultivation.

c. Humidity probes were provided at the inlet and outlet for air. These probes could measure the temperature of the air also.

d. Wattmeter for the online measurement of total power consumed by the stirrer motor was connected to the power source for the motor.

e. Calibration heater with 500 W rating was installed in the bioreactor. Its controller was interfaced with the computer for control purpose.

f. Power source of 24V for temperature probes and galvanic isolators for the protection of data acquisition board from spikes and surges were used.

g. Relay box for the conversion of the digital signal from the computer to 220 V AC were used for the following operations during the cultivation: (i) solenoid valve in controlling the cooling water flow for the bioreactor temperature control (ii) Actuating the feed pump in accordance with the heat signal for feeding external sterile solution as feed (iii) Actuating the alkali or acid pump for the control of pH at the specified value.

The pilot scale bioreactor setup after the modifications is shown in the figure 3.2. In addition, modifications and installed components were indicated in the above figure.
Figure 3.2 The modified setup of the pilot scale bioreactor used for the heat measurement study. The variables monitored online through data acquisition are represented by the symbol shown separately in the figure. TI - Temperature probe, QI - Wattmeter and calibration heater, HI - Humidity probes, FI - Flowmeter, WI - Load cell. [source: Bioengineering AG operating manual with modifications indicated]
3.1.2 Program for data acquisition and control

The program for the data acquisition and control was developed in LabVIEW, a visual programming environment. The program was developed in separate hierarchical modules with each module performing data acquisition, calibration, calculations, process control, display of data and data storage functions. All the above mentioned modules will be under the control of main supervisory program with provision for changing the parameters in the program thereby facilitating user-friendly application. The basic programming structure was adopted from the program BioOPT developed in EPFL, Lausanne. The developed program was used in all the subsequent cultivations pertaining to heat measurement studies.

3.1.3 Control of bioreactor temperature

During the cultivation, temperature of the pilot scale bioreactor has to be maintained at the optimum for the favorable environmental conditions. The performance of the original (Bioengineering AG) temperature controller in the pilot scale bioreactor was found to be unsatisfactory. Also, in the heat measurement study the flow of heat has to be quantified online with the maintenance of the bioreactor temperature at the constant value. Hence, fluctuations in the bioreactor temperature during the control action could be comprised on the heat transfer rates. This has made the development of the temperature controller with satisfactory performance as a necessary one for the heat measurement study. Hence it was decided to develop a computer based cascade control system for maintaining the temperature of the pilot scale bioreactor due to its distinct advantages.
3.1.4 Development of temperature controller

The temperature of the pilot scale bioreactor has to be accurately controlled at the specified value with the chilled water alone. Heat source was not provided for increasing the temperature as this heat addition could not be accounted in heat balance calculations. The proportional-integral (PI) mode cascade controller was developed for the temperature control in the bioreactor. Since the derivative mode could lead to fluctuations in the process signal, it was not included in the control algorithm. The control algorithm was written in the LabVIEW program used for the heat measurement study. The solenoid value in the jacket circuit was used as the final control element. On the execution of the control action, solenoid valve was opened thereby providing an outflow of water from the top of the cooling jacket and inflow of chilled water into the jacket at the bottom. The measured bioreactor temperature was given as input variable to the reactor temperature controller and the output from this controller was given as setpoint to the jacket temperature controller. The logarithmic average water temperature in the jacket estimated using the measured jacket inlet and outlet water temperature was given as input variable to the jacket temperature controller. The output from this controller was given to the solenoid valve through the relay for converting the digital signal from the computer into analog 220 V signal. The control signal will be the time for which the solenoid valve should be opened and the control action was executed in cycles of 10 secs.

Provision was made in the program so that the values of proportional constant, P and integral constant, I used in the control algorithm could be changed. When the measured process variable was outside the temperature range specified in the program, control action was made stringent by modifying
the P and I values involved. Also, due to the possible differences in response time in the reactor and the cooling jacket, separate P and I values was used in reactor and jacket temperature control programs. The values for control parameters specified in the program for the control of temperature was decided by the following trial and error method. During this study, reactor was maintained at 30°C. Constant heat flow of 500 watts was provided as step input of disturbance for 1.0 hour through the calibration heater. The controller will respond to this external disturbance with the specified control parameters in order to maintain the bioreactor temperature at 30°C. Same procedure was repeated by changing the control parameters to different values in an arbitrary fashion. The temperature profiles of bioreactor, jacket inlet and outlet water during this control experiment are shown in figure 3.3. The different values of the control parameters used were indicated inside the graph. These profiles indicated the performance of the control system at different values of the control parameters with the step heat input. It was observed that the response of the control system with the control parameters, P/I for reactor temperature controller: 1/0.0025 and for jacket temperature controller: 2/0.02 as satisfactory. Hence, these values were employed as default values in the temperature control system in all subsequent experiments carried out in the pilot scale bioreactor.

3.1.5 Development of heat transfer model

Schematic representation of various heat fluxes during the aerobic microbial cultivation in a bioreactor are shown in the figure 3.4. The overall heat balance over the pilot scale bioreactor was given as:

$$Q_p = Q_{acc} + Q_j + Q_a + Q_c + Q_g - Q_h - Q_{sc}$$  \hspace{1cm} (3.1)
Figure 3.3 The performance of the developed cascade control for pilot scale bioreactor temperature with step input in heat ($Q_h$) as disturbance. The profiles of water temperature at the jacket inlet ($T_{ji}$) and outlet ($T_{jo}$) during the process of deciding the control parameters P and I by trial and error method are shown. The different values of P/I used for the study are shown inside the graph.
Figure 3.4 The schematic representation of various heat fluxes encountered in the pilot scale bioreactor during cultivation.

$Q_p$: Heat generated by the metabolic activity of the culture
$Q_j$: Heat transferred to the cooling water in the jacket on execution of control action
$Q_w$: Heat carried away by the cooling water in the jacket on heat transfer
$Q_s$: Power consumed by the stirrer for agitation
$Q_{sc}$: Heat dissipated into the medium on agitation
$Q_g$: Heat lost through the flow of exhaust gas
$Q_e$: Heat flux through the headspace of the bioreactor
$Q_a$: Heat generated on dilution and neutralization by the addition of alkali for pH control
$Q_{sa}$: Heat lost or generated during sampling
The individual heat transfer rates were calculated according to the mathematical equations mentioned below. The overall heat balance over the reactor during the cultivation in pilot scale bioreactor was used to calculate the metabolic or process heat flux from the individual heat transfer rates. Among the different heat fluxes encountered, heat transfer due to the dilution and neutralization ($Q_a$) of the added acid or alkali for the pH control was found to be insignificant as explained in subsequent discussions. Since the reactor was insulated, heat transfer through the headspace or cold spot of the bioreactor ($Q_e$) could be insignificant and contribute to the baseline heat signal. In addition, heat lost or generated through sampling port ($Q_{sa}$) on sampling was considered negligible due to less frequent sampling and the sampling method adopted. Other individual heat transfer rates including rate of heat accumulation inside the bioreactor ($Q_{acc}$) were calculated online using the variables measured online during the cultivation. Then the metabolic heat flux could be calculated using the developed program by adopting the overall heat flux model as mentioned previously.

Individual heat transfer rates were given by,

\[ Q_{cc} = mC_p\frac{dT_r}{dt} \]  \hspace{1cm} (3.2)
\[ Q_j = UA(T_r - T_{jav}) \]  \hspace{1cm} (3.3)
\[ Q_c = F_wC_pW(T_{jo} - T_{j0}) \]  \hspace{1cm} (3.4)
\[ Q_g = f(RH_i, T_{gi}, RH_o, T_{go}, F_a) \]  \hspace{1cm} (3.5)
\[ Q_{sc} = f(Q_s) \]  \hspace{1cm} (3.6)
The proportionality constants involved in the above individual heat flux models have to be determined for its application in the heat measurement studies. Hence, calibration experiments were performed in the pilot scale bioreactor with distilled water to estimate the constants involved in the above models. Once the constants were estimated, individual heat transfer fluxes could be established during the cultivation with online monitoring of the variables involved in the model.

3.1.6 Calibration experiments

3.1.6.1 Specific heat capacity calibration

Calibration was carried out in the pilot scale bioreactor with distilled water of 200 l working volume. Online data acquisition was carried out for all the possible variables from the bioreactor. In particular, bioreactor temperature was followed during the transient period of time for this study. During this calibration experiment, heat dissipation to the environment has to be negligible. Hence, circulating pump in the jacket was stopped to prevent the circulation of cooling water in the jacket. This ensured that the heat transferred to the cooling water in the jacket to be insignificant. Also, heat loss to the environment was made insignificant by following the bioreactor temperature only for the initial period of heating thereby avoiding the attainment of steady state. The calibration heater was used as the heat source of 500 watts. Since the possibility of heat loss was negligible, 500 watts of heat has to be utilized for the rise in temperature of the reactor and its contents depending upon its specific heat capacity. This resulted in linear rise in the temperature of the bioreactor. Also, this linear rise in temperature was observed only during the initial period of the experiment. After this initial period, non-linear rise in temperature could be due to the influence of heat loss to the environment. This rate of increase in
temperature will be proportional to the specific heat capacity of the reactor and its contents. The value of $mC_{pr}$ of the reactor and its contents was estimated to be 783.75 kJ/K from the slope of the plot of bioreactor temperature with time. This estimated value for $mC_{pr}$ will be used in metabolic heat flux calculations during the cultivation of \textit{Btg} in pilot scale bioreactor.

3.1.6.2 Lumped heat transfer coefficient (UA) calibration

The heat transfer rate into the jacket cooling water could be expressed as, $Q_i = UA(T_r - T_{jav})$. The dependency of the heat transfer rate on the temperature difference could be established using the proportionality constant, $UA$ designated as lumped heat transfer coefficient. In this, $U$ is the heat transfer coefficient and $A$ is the area for heat transfer. The area for heat transfer could vary depending on the gas holdup inside the bioreactor. This gas holdup could further depend on aeration and agitation. Since the heat transfer rate has to be estimated from the online measurement of temperatures during cultivation, the value of $UA$ has to be determined for the actual operating conditions.

The bioreactor containing 200 l of distilled water was stabilized at 30°C using the developed cascade control system. On stabilization, the cooling jacket inlet and outlet temperature will attain a constant value for maintaining the bioreactor at 30°C. After maintaining the bioreactor at this steady state for 0.75 hours, constant heat input of 500 watts was provided through the calibration heater immersed in the bioreactor. This constant heat input facilitated to attain a new steady state. The cooling jacket inlet and outlet water temperature will be constant during this steady state. The additional heat generation and heat losses due to agitation, aeration and heat transfer to the
environment could be accounted during this experiment. Since the calibration was carried out at a fixed operating condition, the above factors could be assumed constant. Also, the average jacket water temperature attained was less than that at the beginning without heat input. As before after maintaining this steady state for 0.75 hours, heat input was stopped by switching off the heater. Hence, original steady state was obtained in terms of temperature. The difference in the average jacket water steady state temperatures with and without heat input was calculated. This difference in temperature was due to the heat provided through the calibration heater on the basis of the steady state conditions achieved.

Under steady state conditions, this temperature difference should be proportional to the heat dissipated from the calibration heater with the proportionality constant of UA. By integral method, UA was obtained by dividing the constant heat input in watts by the quantity of decrease in the average jacket water temperature due to the additional heat input. This experiment was carried out at different aeration with fixed agitation. Also, the effect of agitation on UA was studied at different agitation.

Experiments were carried out at 100, 200 and 300 RPM with varying aeration. The estimated values were tabulated as in the table 3.1. The temperature profiles in the pilot scale bioreactor during varying aeration at fixed agitation of 200 RPM are shown in figure 3.5. Also, the temperature profiles during varying agitation without aeration are shown in figure 3.6. It was observed that increase in aeration at fixed agitation has resulted in insignificant differences in the value of UA during the fixed agitation of 100, 200 and 300 RPM. But, slight increase in the value of UA was observed when the agitation was increased at fixed aeration. This indicated that variation in
Table 3.1 The lumped heat transfer coefficient, $UA$ at different levels of agitation and aeration estimated from the calibration experiments in pilot scale bioreactor. Power consumed by the stirrer ($Q_s$) and power dissipated as heat ($Q_{sc}$) into the medium with agitation and aeration are also given in the table.

<table>
<thead>
<tr>
<th>AGITATION 100 RPM</th>
<th>Aeration, l/min</th>
<th>Lumped heat transfer coefficient ($UA$), watts/K</th>
<th>Power consumed by the stirrer ($Q_s$), watts</th>
<th>Power dissipated into the medium ($Q_{sc}$), watts</th>
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<tr>
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<td>50</td>
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<td>66.98</td>
<td>2.68</td>
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<th>AGITATION 200 RPM</th>
<th>Aeration, l/min</th>
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<th>$Q_s$</th>
<th>$Q_{sc}$</th>
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</table>

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<th>AGITATION 300 RPM</th>
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<th>UA</th>
<th>$Q_s$</th>
<th>$Q_{sc}$</th>
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<table>
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<th>NO AERATION</th>
<th>Agitation, RPM</th>
<th>UA</th>
<th>$Q_s$</th>
<th>$Q_{sc}$</th>
</tr>
</thead>
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Figure 3.5 Profiles of water temperature at the jacket inlet ($T_{jj}$) and outlet ($T_{j0}$) during the calibration experiment in pilot scale bioreactor for the estimation of lumped heat transfer coefficient, $UA$ at varying aeration. The experiment was carried out by varying the aeration and maintaining the agitation at 200 RPM.

Figure 3.6 Profiles of water temperature at the jacket inlet ($T_{jj}$) and outlet ($T_{j0}$) during the calibration experiment in pilot scale bioreactor for the estimation of lumped heat transfer coefficient, $UA$ at varying agitation. The experiment was carried out without aeration by varying the agitation.
heat transfer coefficient was influenced more by the agitation in comparison with that of the aeration. Thereby, the influence of agitation on gas holdup and heat transfer area was pronounced more in comparison with aeration. This could be due to the complex interaction of the aeration on the gas holdup and heat transfer area. Also, increase of approximately 0.15 bar per 50 liters/min increase in aeration was observed in the backpressure at the headspace of the bioreactor. This increase in the backpressure was not considerably higher to influence the power consumption at fixed agitation with varying aeration. But, increase in aeration has resulted in decrease in the power consumed at fixed agitation. Also, this increase was insignificant at the agitation of 100 RPM in comparison to 200 and 300 RPM. In particular, increase in agitation at constant aeration has resulted in the increased power consumption for stirring. This correlated well with the difference in the power consumption under gassed and ungassed condition of fluid hydrodynamics in the two-phase system.

3.1.6.3 $Q_{\text{sc}}$ Calibration

Friction in the stirrer motor gearbox and seals reduces the energy transmitted to the fluid. Therefore, the electrical power consumed by stirrer motor is always greater than the power dissipated into the culture medium by an amount depending on the efficiency of the drive. In the metabolic heat flux calculations, the quantity of heat dissipated into the medium by the stirrer ($Q_{\text{sc}}$) should be accounted. The wattmeter used for power measurements was calibrated at the factory itself. Hence, it was decided to understand the relationship between the quantity of heat dissipated into the medium and the agitation employed during cultivation.
Reactor was stabilized at 30°C with 100 RPM and 100 l/min. The jacket water inlet and outlet temperature reached a steady state value. After maintaining at this steady state for 0.75 hours, agitation was increased to 150 RPM with other operating conditions remaining same as before. This increase in agitation would have resulted in the increased heat dissipation into the medium. This has resulted in the attainment of new steady state temperature for jacket inlet and outlet. This new steady state was obtained so that the bioreactor temperature could be controlled at 30°C. The average jacket water temperature during this steady state was calculated. The difference in the average jacket temperature between the condition at 100 RPM and 150 RPM was calculated. This resulted difference was due to the heat transfer into the jacket because of heat dissipation on increasing the agitation from 100 RPM to 150 RPM corresponding to the increase of 50 RPM. The increased agitation has resulted in the constant heat input, which could be calculated using heat transfer model for heat flow into the cooling jacket. UA corresponding to the increased operating conditions has to be employed to calculate the amount of heat dissipated into the medium from the reactor and average jacket water temperature. Further, agitation was increased in steps of 50 RPM and the rate of heat dissipation into the medium was estimated in each step of increase. The temperature and power consumption profiles during this calibration experiment are shown in figure 3.7. This experiment has facilitated to obtain the relationship between the heat dissipated into the medium and agitation. This estimated relationship could be used in the overall heat balance for the metabolic heat flux calculations.

Since the total power consumed by the stirrer could be measured online with the installed wattmeter, alternate method could be used. The water in the bioreactor was drained with little water remaining in order to prevent the
Figure 3.7 Profiles of water temperature at the jacket inlet ($T_{ji}$) and outlet ($T_{jo}$) during the $Q_{sc}$ calibration experiment in pilot scale bioreactor. $Q_{sc}$ indicates the power dissipated on agitation as heat into the medium. The experiment was carried out with aeration of 100 l/min and by increasing the agitation in steps of 50 RPM.
mechanical seal from damage. With this condition, total power consumed by the stirrer motor was measured at different agitation. This facilitated to establish a relationship between the power consumed by the stirrer and agitation under unloaded conditions. The power consumed under these conditions could have dissipated to overcome the friction in the mechanical system. Hence, this quantified power consumption could be detected from the total power consumed during the cultivation in order to obtain the heat dissipation rate into the medium alone. In comparison with the previous method, total power consumption was monitored online with the wattmeter. Further, above calculations were incorporated in the program developed for online metabolic heat production rate monitoring during the cultivation.

3.1.6.4 $Q_g$ Calibration

The heat lost through the air should be accounted in the overall heat balance model to determine the metabolic heat production rate. This rate of heat loss through aeration could be quantified from the air flowrate and its properties at the inlet and the outlet of the bioreactor. The properties of the air include its temperature and relative humidity. The calculations were carried out according to Owens (1985). The equations employed in the calculations are given in Appendix. These established equations provided the relation of rate of heat transfer into the air from the properties of air. These equations were incorporated in the program developed for the heat measurement study. Hence, the online monitoring of the variables mentioned above has facilitated to estimate the rate of heat loss through the air ($Q_g$) during the cultivation. Further, this individual heat transfer rate could be used to estimate the metabolic heat production rate during the cultivation.
3.1.7 Effect of alkali addition on the heat production rate

During the cultivation in the pilot scale bioreactor, pH was controlled in the initial period by the external addition of 5N NaOH. There was a possibility of heat generation by this added alkali on dilution and neutralization inside the bioreactor upon addition. In order to understand the influence of alkali addition on the metabolic heat production rate, studies were carried out in the pilot scale bioreactor by stimulating the NaOH addition.

The bioreactor temperature was controlled at 30°C with 200 RPM as agitation and 100 liters/min as aeration. The pH control was simulated by the addition of 5N NaOH at a constant flowrate of 30 ml/min for a period of 1.0 hour. Pulse feeding was also done twice at the flowrate of 240 ml/min for 1.0 minutes. Process heat signal and temperature profiles during this study are shown in the figure 3.8 with feeding time indicated by the arrows. In the overall heat flux model, 670 watts/K was used for UA corresponding to the operating conditions employed during the experimental simulation. There was no appreciable disturbance in the heat signal as observed in their profiles. Also, during this study maximum pH of 12.11 was reached. These data indicated that the heat of dilution and neutralization on addition of alkali into the bioreactor could be considered negligible. This observation could be due to the larger volume of the water in the bioreactor in comparison with the amount of alkali added. Hence during the microbial cultivation heat generated on addition of alkali (Q_a) could be considered insignificant in the calculations for the metabolic heat generation.

3.1.8 Batch cultivation of Btg in glucose – yeast extract (GY) medium

The reactor temperature was stabilized at 30°C using the cascade control system. 10 litres of mid-exponential phase grown Btg was transferred
Figure 3.8 Profiles of bioreactor temperature ($T_r$), water temperature at the jacket inlet ($T_{ji}$) and outlet ($T_{jo}$) and process heat production rate during the study on the effect of alkali addition in pilot scale bioreactor. The pH control was simulated by the addition of 5N NaOH at the flow rate mentioned in the figure.
into pilot scale bioreactor containing 200 l of sterilized glucose – yeast extract (GY) minimal medium with composition as mentioned in materials and methods. The heat production rate during the cultivation of the \textit{Bt} was estimated using the heat flux models as mentioned previously. The metabolic heat production rate and the CO$_2$ composition in the exhaust gas from the fermentor are shown in the figure 3.9. On inoculation, lag phase was observed due to the change in the fermentation medium from NB to GY medium. The cell density, residual glucose concentration and dissolved oxygen profiles during the cultivation are shown in figure 3.10. It was observed that dissolved oxygen and the residual glucose in the medium were not limiting the biological process. Although, there was a significant reduction in the residual concentration of glucose during the first 2 hrs of cultivation, this observed decrease in terms of consumption by the culture was not reflected either in the heat production rate or dissolved oxygen concentration. This could be attributed to the change in the metabolism of the culture from a highly complex proteineous medium to a minimal medium leading to the accumulation of intermediates. This metabolic shift would have resulted in the expression of additional enzymes and synthesis of metabolites/intermediates.

It was reported that depending upon the growth rate of the culture yeast extract could also be utilized as carbon source through the fermentative pathway (Mignone and Rossa 1996). Hence, the change in the consumption of nitrogen source from the NB medium to GY medium could have resulted in the small drop observed at approximately 2.5 hours of cultivation. This adapted culture have grown in a exponential fashion with corresponding heat production rate reaching maximum of 341.64 watts at 4.2 hours of cultivation. The amount of glucose consumed during this period was significantly less leading to the possibility of utilization of organic acids especially acetate during this period. This was further substantiated by the pH and alkali feeding profile as shown in
Figure 3.9  Profiles of metabolic heat production rate ($Q_p$) and CO$_2$ composition in the exhaust gas during the batch cultivation of *Btg* with GY medium in pilot scale bioreactor. The maximum metabolic heat flux was observed to be 341.64 watts at 4.2 hrs of the cultivation.

Figure 3.10 Profiles of residual glucose, cell density and dissolved oxygen concentration during the batch cultivation of *Btg* with GY medium in pilot scale bioreactor during heat measurement study.
the figure 3.11. During this period, there was a decrease in the alkali solution due to its feeding into the pilot scale reactor for the maintenance of pH at 7.0. After the maximum heat production, significant drop was observed in the heat signal. This could be due to the limitation imposed on the culture to change the metabolism from vegetative to the sporulation phase as an effect of a stringent response.

The sporulation phase has resulted in the gradual decrease in the heat production rate due to the changed metabolic activity in comparison with the growing phase. The metabolic heat produced by the culture was estimated using the models taking into account all the possible sources of heat transfer. During the entire cultivation, the jacket inlet and outlet water temperature profiles has definite correlation with the heat production rate as shown in figure 3.12. This indicated the heat transfer across the reactor into the cooling water present in the jacket was significant and represented the metabolic heat in direct proportions as shown in the figure 3.13. It was observed that the heat transfer across the jacket ($Q_j$) and heat carried away by the cooling water ($Q_w$) during cultivation were similar with constant difference as indicated in figure 3.14. This difference in the estimated heat transfer rates has resulted in the shift in the metabolic heat production rate profile from the baseline. In addition, $Q_w$ could not be used in the heat flux calculations due to its noisy characteristics as observed in figure 3.14. Due to the significant heat transfer into the cooling water, the resulting increase in the difference in the jacket water inlet and outlet temperature as shown in figure 3.14 could be used as the indicator for online metabolic status of the culture. Also, power consumed for stirring and properties of the air used for aeration during the cultivation are shown in the figure 3.15 and 3.16. The change in the rheological properties of the culture broth during cultivation was insignificant due to the less cell density achieved.
Figure 3.11 Alkali feeding and pH profiles during the batch cultivation of \( \text{Btg} \) with \( \text{GY} \) medium in pilot scale bioreactor. pH was controlled at 7.0 with the addition of alkali at 5N during the cultivation.

Figure 3.12 Temperature profiles of the cooling water at the jacket inlet (\( T_{ji} \)) and outlet (\( T_{jo} \)) during the batch cultivation of \( \text{Btg} \) with \( \text{GY} \) medium in pilot scale bioreactor. \( T_r \) indicates the temperature in the bioreactor.
Figure 3.13 Profiles of heat transfer flux across the jacket into the cooling water ($Q_j$) and heat carried away by the cooling water ($Q_w$) for the maintenance of bioreactor temperature at 30°C during the batch cultivation of $Btg$ in GY medium.

Figure 3.14 The profile of difference in the estimated heat transfer rate ($\Delta Q$) across cooling jacket and heat carried away by the cooling water during the batch cultivation of $Btg$ in GY medium. This difference was due to the variation in the estimated $UA$ from the actual value. Also, difference in the jacket inlet and outlet temperature ($\Delta T$) on heat transfer during cultivation is shown in the figure.
Figure 3.15 Profiles of power consumed by the stirrer ($Q_s$) and the power dissipated into the medium ($Q_{sc}$) during the batch cultivation of $Btg$ with GY medium in pilot scale bioreactor.

Figure 3.16 Profiles of relative humidity (RH) and temperature of the air ($T_g$) at inlet and outlet during the batch cultivation of $Btg$ with GY medium in pilot scale bioreactor. Subscripts $i$ and $o$ indicates the inlet and outlet position respectively.
This has resulted in constant profiles for the power consumed for agitation and power dissipated into the medium. Also, variations in the temperature and relative humidities of air were observed to be insignificant even with little fluctuations in the inlet air humidity. This could be due to variation of the environment and change in the efficiency of water removal from the compressed air. Also, during the sporulation phase the microscopic observation indicated the uniformity in the culture population with spores and ICP intact.

The observation of heat and CO\textsubscript{2} profile has lead to the inference of having constant ratio during the exponential growth indicating the culture under balanced condition of carbon flux through the metabolic network. This cultivation of \textit{Btg} on GY medium has resulted in sporulation with 2.5x10\textsuperscript{8} spores/ml with ICP concentration of 1.0068 mg/ml. On the basis of these heat production data, it was clear to adopt heat measurements as online tool for the monitoring of the metabolic activity of the culture during cultivation in pilot scale bioreactor. The baseline heat signal before inoculation was observed to be 221.55 watts due to differences in the proportionality constants involved in the heat flux model estimated with water and the nutrient medium. Hence as discussed earlier, the net maximum heat flux in reference to the baseline signal observed on metabolic heat generation was 341.64 watts.

The understanding of the growth of the \textit{Btg} in bench scale calorimeter and pilot scale bioreactor with reference to the metabolic heat generation could yield appreciable data for analysing the possibility of scaling-up on the basis of heat yield. Also in the bench scale calorimeter, cultivation of \textit{Btg} was carried out with GY(5X) medium with the working volume of 1.7 litres indicating the availability of nutrients at high concentrations. The plot of total heat produced in joules with the cell density in g/l has indicated the possibility
of having two different heat yields (kJ heat evolved per g cell dry weight formed). This could also be determined by the plot of the rate of heat evolution by the culture (watts/l) as a function of the biomass production rate (g/l/hr). The first phase has relatively high heat yield \( \frac{Y_{QX}}{X} = 3.416 \text{ kJ/g} \) due to the influence of the unconsumed complex media sources and metabolites from the inoculum and biomass yield, \( Y_{X,S} = 0.0383 \text{ g/g} \). In the second phase, it was observed to be 1.5199 kJ/g and 2.375 g/g respectively. This indicated the pronounced effect of \( Y_{X,S} \) on heat yield. Also, it was reported that the nature of the carbon and energy substrate has a profound effect on the heat released by the microbial culture. In addition, amount of heat generated per unit biomass formed depends on the enthalpy content of the substrate (Stockar and Marison 1989). The two-phases obtained during the cultivation were similar to the \( E. \ coli \) grown on the glucose - yeast extract medium (Birou et al 1987).

The analysis of the data on maximum heat flux could provide additional details regarding the relationship between the cell density and metabolic heat produced. In the pilot scale reactor at the point of maximum heat flux, heat production rate in terms of the cell density was observed to be 5.098 watts/g of biomass. In the bench-scale calorimeter (Padmaja et al 1998), maximum heat flux of 6.27 watts was observed at 3.426 hour of the cultivation with 5.421 watts/g of biomass. Also, specific growth rate of the culture was estimated using the plot of lnX with t and it was found to be 0.3583 in pilot scale and 0.2293 in bench scale calorimeter during the cultivation. This difference in specific growth rate could be due to the dependency of the specific growth rate on the medium constituents and its concentration. In the pilot scale bioreactor, point of maximum heat flux was observed to occur later in comparison with the bench scale calorimeter during the cultivation. Even with relatively higher growth rate in the pilot scale reactor, the observed difference
in the point of maximum heat flux could be attributed to the role of residual medium unutilized in the inoculum. Also at the point of maximum heat flux, no appreciable difference in cell density was observed between the two scales of cultivation. This indicated high carbon source recovery towards biomass as growth in pilot scale bioreactor with respect to high concentration of glucose used in the bench scale calorimeter. The net heat produced from the biological network of reactions reflecting in growth and product synthesis in macroscopic scale could depend on the nature and quantity of substrate utilized under balanced conditions. The analysis of the data obtained indicated the feasibility of scaling up the biopesticide production process in terms of volumetric heat production and heat yield based on the nature of the substrate used for cultivation. Also, the temperature profile in the jacket could help in the estimation of the cooling requirements and online monitoring of the metabolic activity of the culture during cultivation.

As it was reported, the heat production rate depending on the substrate used for cultivation could reflect the efficiency of the metabolic conversion process. The inferences obtained from these studies could be employed to optimize the metabolic process of growth and synthesis of ICP. Further, utilization of agricultural residues and industrial by-products for the production of biopesticides with increased toxicity was reported in the literature (Vora and Shethna 1999; Zouari and Jaoua 1999; Alves et al 1997; Morris et al 1997). This could reduce the cost of production involved. In addition due to the presence of insoluble ingredients in these media, cell dry weight estimations could not be correlated with the growth and metabolic activity of the culture. In these conditions, online heat monitoring could be applied as an efficient tool in comparison with the exhaust gas analysis. Hence, it was decided to optimize the growth and ICP yield by Btg with the cost-effective medium. The medium
developed by Meenakshisundaram and Fernado (1992) based on soyabean meal will be used in the subsequent experiments to enhance the yield of ICP based on the online heat measurements. Batch cultivation was carried out in the pilot scale bioreactor initially to understand the growth kinetics/ characteristics of *Btg* in complex medium with reference to the heat released. This cultivation will provide appreciable data for developing feeding strategies in fedbatch cultivation of *Btg* in the above complex medium.

### 3.1.9 Batch cultivation of *Btg* in complex medium

The constituents of the complex medium were added to the pilot scale reactor according to the composition as mentioned earlier. Temperature of the bioreactor was stabilized at 30°C with the operating agitation and aeration to achieve the baseline in the heat signal. Inoculum of *Btg* was transferred into pilot scale reactor as in the above cultivations. The metabolic heat production rate and the CO₂ composition in the exhaust gas from the fermentor are shown in the figure 3.17. During the period of inoculation, disturbance was observed in the heat signal due to the release of the pressure in the headspace of the fermentor. The exponential growth of the culture was observed from the heat signal with the reduction in lag phase as compared to the one in previous cultivation with GY medium. During this cultivation, cell density of the culture could not be followed by cell dry weight measurements due to the presence of insoluble materials and its subsequent decrease during cultivation on consumption by the culture. The dissolved oxygen, residual glucose and ICP concentration profiles during the cultivation are shown in figure 3.18. Excessive foaming was observed during the initial period of growth due to the presence of highly proteinaceous substances in the medium. The low level of dissolved oxygen as observed in the profile was due to the manipulation carried out in agitation and aeration in order to control the foam.
Profiles of metabolic heat production rate ($Q_p$) and $CO_2$ composition in the exhaust gas during the batch cultivation of $Btg$ with complex medium in pilot scale bioreactor. The maximum metabolic heat flux was observed to be 1327.47 watts at 7.27 hours of cultivation.

Figure 3.18 Profiles of residual glucose, dissolved oxygen (DO) and ICP concentrations during the batch cultivation of $Btg$ with complex medium in pilot scale bioreactor during the heat measurement study.
The glucose in the medium was not consumed totally as indicated by the residual glucose concentration profile thereby indicating that the limitation of the glucose is not a contributing factor. Even with the presence of residual glucose in the medium, consumption of the secreted acidic intermediates/metabolites was started as indicated by the rise in pH profile (figure 3.19). Also, pH control could not be extended during the entire cultivation due to the dilute concentration of alkali solution used. In addition, as observed in the process optimization studies with minimal medium containing glucose and yeast extract in bench scale bioreactor, there seemed be an optimum ratio of carbon and nitrogen sources for the effective and rapid utilization of the carbon source. With the results obtained during the cultivation and the visual observation of the presence of the insoluble soyabean meal residues even at the later stage of the cultivation, the residual glucose unutilized in the medium could be due to the non-availability of the nitrogen source in the utilizable form. This indicated the further scope of identification and improvement of media components and its level by medium optimization studies.

In order to have the foaming under control, manipulations were carried out in agitation and aeration as indicated in their corresponding profiles. This could result in variations in power consumed for agitation and lumped heat transfer coefficient, UA. Since power consumed for agitation was measured online using the wattmeter, changes in the power consumption will be taken care off in the total heat flux model used in the metabolic heat calculations. In contrast, the change in the heat transfer coefficient was insignificant to variations in agitation and aeration as observed in the initial UA calibration experiments. It was observed that the concentration of the ICP remains invariably constant from 13 hours of cultivation as shown in the figure 3.18.
Figure 3.19 Profiles of volumetric air flowrate ($F_a$) and pH during the batch cultivation of $Btg$ with complex medium in pilot scale bioreactor during the heat measurement study.

Figure 3.20 Profiles of relative humidity (RH) and temperature of air ($T_g$) at the inlet and outlet during the batch cultivation of $Btg$ with complex medium in pilot scale reactor during the heat measurement study. Subscripts i and o indicates the inlet and outlet positions respectively.
But, event of sporulation was not completed to release the spores into the medium on microscopic observation. This indicated the completion of the ICP synthesis by 13 hours in the complex medium containing soyabean meal as nitrogen source. The maximum heat production rate during the cultivation was observed to be 1327.47 watts at 7.27 hours of cultivation with volumetric heat yield of 6.6379 watts/liter of culture broth. The sporecount and the ICP concentration were found to be 40.5x10^9 spores/ml and 6.1539 g/l. This enhanced yield of ICP in comparison with the yield in minimal medium as in the previous cultivation indicated the prominent influence of complex sources on the ICP synthesis.

The profiles of the properties of the inlet and outlet air, power consumed by the stirrer (Q_s) and power dissipated into the medium (Q_sc) are shown in the figure 3.20 and figure 3.21. The sudden changes observed in the relative humidity of inlet and exhaust air, Q_s and Q_sc profiles could be due to the change in the backpressure in the fermentor headspace by opening the additional ceramic filter for exhaust. On observation of the heat signal and the CO_2 profile with time, it could be inferred that the disproportionality among them was due to the possibility of metabolic shift in utilizing the nutrients as carbon source. In addition, this observation could also be due to the dissolved oxygen limitation as shown by the dissolved oxygen concentration profile.

The water temperature profile in the inlet, \( T_{ji} \) and outlet, \( T_{jo} \) of the jacket cooling water circulation system was shown in the figure 3.22. The similarity of the temperature profile in the jacket cooling water with the net metabolic heat production rate in direct proportions indicated the future prospects of its application for the online monitoring the culture status in industrial scale fermentations. In this cultivation, an important calculation could
Figure 3.21 Profiles of power consumed by the stirrer ($Q_s$), power dissipated into the medium ($Q_{sc}$) and agitation during the batch cultivation of $Btg$ with complex medium in pilot scale bioreactor during the heat measurement study.

Figure 3.22 Temperature profiles of the cooling water ($T_j$) at the jacket inlet and outlet during the batch cultivation of $Btg$ with complex medium in pilot scale bioreactor. Subscripts $i$ and $o$ indicates the inlet and outlet position respectively. $T_r$ indicates the temperature of the bioreactor
be made on the total heat generated with respect to time from 13 hours to 24 hours during which the sporulation process was observed to be occurring. This indicated the heat production rate of 1648.8 kJ/hr during this period of cultivation from 13 hours to 24 hours of cultivation. This heat could be accounted for the metabolic activity during the process of sporulation till the spore release. The results indicated the profound influence of the complex medium components on the growth and ICP synthesis. The yield of ICP was high enough in the complex medium in comparison with the minimal GY medium. In addition, the presence of insoluble materials in the complex medium interferes with offline cell density measurements. Since follow up of the cell density during cultivation could yield the metabolic condition of the culture, online heat measurements could provide a solution for the online monitoring of culture status under different environmental conditions. This could further be employed for enhancing the yield of products in bioprocesses by adopting appropriate advanced feeding strategies in cultivation.

Prior to inoculation, heat signal got stabilized at the baseline value of 223.85 watts. This baseline heat signal could be due to the variations in the estimated constants of the total heat flux model used as in the previous cultivations. The accurate estimation of the proportionality constants involved in the heat flux model could provide an invariable and stable baseline for net heat production rate for the processes taking place inside the reactor. Since, initial heat transfer coefficient was determined using the water as the model system, it was decided to understand the influence of the insoluble materials on the lumped heat transfer coefficient, UA value. Hence in the complex medium before the inoculation, UA calibration was carried out as described previously. UA was found to be 732.01 watts/K in the complex medium in comparison with the estimated value of 750 watts/K with water under the similar operating
conditions used during the cultivation. As it was observed, the little variations in the constants used in the model for determining the metabolic heat generation will be taken into account in the baseline of the heat signal. To follow our objective of process optimization using heat measurements, it was decided to perform fedbatch cultivation by feeding the external concentrated glucose solution with the reference to heat production rate. As the design of feeding strategy in fedbatch cultivation was critical, development of feeding profile on the basis of heat signal by understanding the metabolic status of the culture could lead to an efficient one.

3.1.10 Fedbatch cultivation of \textit{Btg} in complex medium by feeding glucose solution

The initial glucose concentration in the medium was made half as that in the previous batch experiment i.e. 13 g/l and other components were maintained at the same concentration as before. During this fedbatch cultivation, it was decided to feed the concentrated glucose solution of 25X at 26.0 g/l. The feeding was started after the metabolic heat production rate exceeded 250 watts to ensure that the growth was established in the pilot scale bioreactor after inoculation. Feeding was carried out in proportional to the rate of increase in the metabolic heat production rate. The program for feeding profile was developed in LabVIEW with heat production rate and weight of the feed solution from the load cell as input variables. The output signal will be the digital on-off signal to the feed pump operated at the preset speed. Feed solution was fed at the flow rate of 72.48 ml/min with glucose flow of 47.12 g glucose/min for 1.2 hours during the growth phase. It was done from 3.75 hours to 4.95 hours of cultivation. The feeding was stopped as the rate of increase in the heat production rate was observed to be insignificant. Also during the later
growth phase, increase in both heat production rate and dissolved oxygen concentration has resulted in the feeding of the external glucose solution. Feeding was carried out at the flow rate of 17.73 ml/min with glucose flow of 11.53 g glucose/min for 0.7 hours during this phase. It was done from 9.45 hours to 10.16 hours of cultivation.

The metabolic heat production rate and the CO$_2$ composition in the exhaust gas from the fermentor during the cultivation are shown in the figure 3.23. During this fedbatch cultivation, maximum heat flux of 445.49 watts was observed at 17.03 hours of cultivation with ICP concentration of 1.768 g/l and spore count of 19.9 X $10^{08}$ spores/ml. This maximum heat production rate was less with delay in time of occurrence on comparison with the previous batch cultivations.

The dissolved oxygen concentration and pH during the fedbatch cultivation are shown in figure 3.24. It was observed that the dissolved oxygen concentration was not limiting during the process. Further, pH profile indicated the possibility of accumulation of acetate as observed with decrease in pH and its subsequent consumption in the later stages resulting in the increase of pH. The residual glucose concentration and ICP concentration during the cultivation are shown in figure 3.25. The maximum residual glucose concentration of 27 g/l was observed at 12 hours of cultivation with 1.0 g/l above that present in the initial stage of the previous batch culture. This indicated that this excess glucose concentration could not have caused the inhibition in the culture growth. But this presence of excess glucose at the later stages of the cultivation could have played a significant role in the initiation of sporulation. Also, since the utilization of the glucose was relatively slower in comparison with the glucose feed rate, accumulation of glucose could have resulted in the medium.
Figure 3.23 Profiles of metabolic heat production rate ($Q_p$) and CO$_2$ composition in the exhaust gas during the fedbatch cultivation of $B_{tg}$ with complex medium in pilot scale bioreactor by feeding glucose solution at 25X of 26 g/l. The maximum metabolic heat flux was observed to be 445.49 watts at 17.03 hours of cultivation. The arrows indicate the point at which feeding of glucose solution was started.

Figure 3.24 Profiles of dissolved oxygen concentration (DO) and pH during the fedbatch cultivation of $B_{tg}$ with complex medium in pilot scale bioreactor during the heat measurement study. Feeding was carried out with feed solution at 25X of 26 g/l glucose.
This indicated the possibility of change in the internal metabolic network in response to changes in the environmental conditions provided. Hence, this could have resulted in the enhanced accumulation of intermediates/metabolites which was consumed in the later phase resulting in the diauxic growth pattern with maximum heat flux observed during this phase.

It was reported that diauxic growth could be observed by heat measurements (Schneiders et al 1995). With crabtree effect in yeast cultivation, diauxic growth was observed with heat signal as reported in Alexander and Jeffries 1990. Under aerobic conditions and at high glucose concentrations higher than 100 mg/l, ethanol was released into the medium until glucose is consumed. Subsequently, ethanol was oxidized. During this transition phase as intermittent heat peak was observed. Also, similar behavior was observed when CO$_2$ evolution was monitored, which could be interpreted as the consumption of pyruvate and acetate formed transiently (Sonnleitner and Fiechter 1992). In addition, insoluble soyabean meal could not be available in the utilizable form for the culture as the enzymes especially proteases needed for its degradation were not expressed due to the disturbance happened in the internal metabolic network. Further, it was observed that the amount of glucose consumed during the cultivation was less and its metabolic flux was diverted away from biomass formation. Hence, it could be concluded that the adopted feeding profile using this concentrated glucose solution could not result in enhanced biomass and ICP formation. The microscopic observation of the culture indicated the presence of heterogenous population of vegetative cells, sporulating cells and released spores from 22 hours of cultivation. This observation also indicated the stress the culture to which the culture was subjected due to the disturbance in the flux of glucose for metabolic activities. Also, the need of constant ratio of carbon/nitrogen for balanced flux as observed in the other part of our study.
using glucose–yeast extract (GY) minimal medium could be correlated. Hence, the disturbance produced on the addition of concentrated glucose solution could have altered the optimum ratio of carbon/nitrogen from that needed for balanced flux. Also, it was observed that there was a little increase in ICP concentration from 14 hours to 26 hours and remained relatively constant till 30 hours of cultivation as shown in the figure 3.25.

Before inoculation, stable baseline in the heat signal was obtained at 197.4 watts. Also, values for proportionality constants involved in the model were used the same as in the previous batch cultivation. The profiles of the properties of the inlet and outlet air, power consumed by the stirrer (Q_s) and power dissipated into the medium (Q_sc) during the cultivation are shown in the figure 3.26 and figure 3.27. Slight fluctuations were observed in the dissolved oxygen concentration, Q_o and Q_sc profiles after approximately 16 hours of cultivation. This was due to the insignificant changes in the flow rates due to the control action of the air flowrate controller. The water temperature profile in the inlet, T_{ji} and outlet, T_{jo} of the jacket cooling water circulation system was shown in the figure 3.28. As in previous cultivations, this temperature profile in the cooling jacket reflected the heat production rate providing a possibility for online metabolic status determination. The above data indicated that the feeding strategy has to be modified to obtain enhanced biomass and ICP yield. Hence, further fedbatch experiments were decided to perform with modified feeding of glucose solution with low concentration of glucose on the basis of the heat production rate.

The external sterile glucose solution to be added into the bioreactor with the specified flowrate was very less in comparison with 200 l present in the pilot scale bioreactor. Hence, high concentration of glucose (25X) was
Figure 3.25 Profiles of residual glucose and ICP concentration during the fedbatch cultivation of \textit{Btg} with complex medium in pilot scale bioreactor during the heat measurement study. Feeding was carried out with feed solution at 25X of 26 g/l glucose. The arrows indicate the point of feeding glucose solution.

Figure 3.26 Profiles of relative humidity (RH) and temperature of the air ($T_{p}$) at the inlet and outlet during the fedbatch cultivation of \textit{Btg} with complex medium in pilot scale reactor by feeding glucose solution at 25X of 26 g/l. Subscripts i and o indicates the inlet and outlet position respectively.
Figure 3.27 Profiles of power consumed by the stirrer ($Q_s$), power dissipated into the medium ($Q_{sc}$), agitation and volumetric air flowrate ($F_a$) during the fedbatch cultivation of $Btg$ with complex medium in pilot scale bioreactor by feeding glucose solution at 25X of 26 g/l.

Figure 3.28 Temperature profiles of the cooling water ($T_j$) at the jacket inlet and outlet during the fedbatch cultivation of $Btg$ with complex medium in pilot scale bioreactor by feeding glucose solution at 25X of 26 g/l. Subscripts i and o indicate the inlet and outlet position respectively.
taken in the initial fedbatch experiment. From the insight provided on the impact of feeding glucose at high concentration over the metabolism of Btg, feeding glucose at low concentration could provide a balance flux of carbon and nitrogen thereby leading to the enhanced yield of ICP. The glucose concentration at 4X was selected so that the total amount of glucose provided would be 0.75 times that of the batch cultivation thereby creating a condition of glucose limitation during cultivation. In addition, experience gained over the fedbatch cultivation of Btg with feeding glucose at high concentration has helped to decide on feeding the glucose at the concentration of 4X at 26 g/l in response to the heat signal. As in the previous cultivation, initial glucose concentration was taken at 13 g/l. The feeding was started as before after the metabolic heat production rate exceeded 250 watts. Feeding of glucose at low concentration was carried out with the program used in the previous cultivation in proportional to the rate of increase in the online estimated metabolic heat production rate. Feeding was carried out at the flow rate of 64.3 ml/min with glucose flow of 6.6873 g glucose/min for 2.47 hours during the growth phase. The feeding was done from 2.589 hours to 5.059 hours of cultivation.

The metabolic heat production rate and the CO₂ composition in the exhaust gas from the fermentor during the cultivation are shown in the figure 3.29. During this fedbatch cultivation, maximum heat flux of 1189.61 watts was observed at 5.893 hours of cultivation with ICP concentration of 6.969 g/l and spore count of 37.67 X 10⁰⁸ spores/ml. There were discontinuities in the heat flux and CO₂ profiles due to the power failures occurred during the cultivation. The profile of CO₂ concentration in the exhaust gas was observed to be in correlation with the metabolic heat production rate. But during the exponential growth phase, increase in CO₂ concentration in the exhaust gas could not be matched with rise in the heat production rate. This could be due to
Figure 3.29 Profiles of metabolic heat production rate ($Q_p$) and CO$_2$ composition in the exhaust gas during the fedbatch cultivation of $Btg$ with complex medium in pilot scale bioreactor by feeding glucose solution at 4X of 26 g/l. The maximum metabolic heat flux was observed to be 1189.61 watts at 5.89 hours of the cultivation. The arrow indicates the point at which feeding of glucose solution was started.

Figure 3.30 Profiles of dissolved oxygen concentration and pH during the fedbatch cultivation of $Btg$ with complex medium in pilot scale bioreactor during the heat measurement study. Feeding was carried out with feed solution at 4X of 26 g/l glucose.
the redirection of carbon flux away from the respiratory pathway of producing CO₂ towards the formation of biomass. This indicated that the present strategy of feeding glucose at low concentration could result in the balanced flux of nutrients towards the formation of biomass. It was reported that the major portion of amino acids for the ICP synthesis has come from the protein turnover of the vegetative proteins (Monro 1961).

The pH and the dissolved oxygen concentration profiles during this fedbatch cultivation are shown in figure 3.30. It was observed that the dissolved oxygen concentration could not be maintained above the limiting concentration due to excessive foaming. Hence, aeration and agitation were manipulated during the cultivation to have the foam under controlled conditions. Increase in pH was observed after the second drop in the heat signal indicating the possible consumption of acidic intermediates produced. Also, residual glucose and ICP concentration profiles are shown in figure 3.31. The residual glucose profile indicated the enhanced uptake of glucose during the growth thereby leading to its depletion by 12.5 hours. This condition would have simulated the culture to undergo sporulation from vegetative phase. It was also observed that the ICP concentration increased from 16 hours to 26 hours of cultivation with the spores released at 26 hours as shown in the profile. In addition, microscopic observation indicated the presence of uniform population of sporulating cells during the sporulation phase of the cultivation. In this cultivation, it was observed that the fedbatch phase with the glucose feeding at low concentration followed by the glucose limited batch cultivation has resulted in the enhanced yield of ICPs in comparison with the batch cultivation. In addition, the total quantity glucose consumed during this fedbatch cultivation was less in reference to the batch cultivation. The resulted enhanced synthesis of ICP with the present feeding strategy could be due to the balanced utilization of glucose
Figure 3.31 Profiles of residual glucose and ICP concentration during the fedbatch cultivation of Btg with complex medium in pilot scale bioreactor during the heat measurement study. Feeding was carried out with feed solution at 4X of 26 g/l glucose. The arrow indicates the point of feeding glucose solution.

Figure 3.32 Profiles of power consumed by the stirrer ($Q_s$), power dissipated into the medium ($Q_{sc}$), agitation and volumetric air flow rate ($F_a$) during the fedbatch cultivation of Btg with complex medium in pilot scale bioreactor by feeding glucose solution at 4X of 26 g/l
preventing its accumulation in the medium. Also, the effective initiation of sporulation process on the depletion of glucose could have to lead to high ICP synthesis rate during this process.

Stable baseline in the heat production rate was observed at 123.88 watts before inoculation in the pilot scale bioreactor. The agitation, aeration and power consumption profiles are shown in the figure 3.32. Also, the temperature and humidity profiles of the inlet and outlet air are shown in the figure 3.33. The heat lost in the form of the enthalpy transfer to air was observed to be constant due to the invariable properties of the air as indicated in their respective profiles. The slight increase in the power consumption profiles after the power failure could be due to the cooling down of the moving parts in agitator and mechanical seal assembly. The temperature profiles at the bioreactor, jacket inlet and outlet during this fedbatch cultivation are shown in the figure 3.34. The observation indicated that the decrease in the average jacket temperature was proportional to the metabolic heat production rate. Hence, the heat flux towards the cooling water on the jacket due to the control of bioreactor temperature at 30°C could form a major and variable portion in the various heat fluxes encountered during cultivation. This observation could be exploited in industrial scale fermentations for the online monitoring of the heat production rate and subsequent inference of metabolic status by continuously measuring the cooling water temperature at the jacket inlet and outlet. This could be carried out with other operating conditions maintained at the constant level during the cultivation.

3.1.11 Impact of medium and process conditions on metabolic heat flux

The significance in heat measurements was substantiated by the detailed understanding of complex relationship between the heat production
Figure 3.33 Profiles of relative humidity (RH), temperature of the air ($T_g$) at inlet and outlet during the fedbatch cultivation of $B_{tg}$ with complex medium in pilot scale reactor by feeding glucose solution at 4X of 26 g/l. Subscripts $i$ and $o$ indicate the inlet and outlet position respectively.

Figure 3.34 Temperature profiles of the cooling water at the jacket inlet ($T_{ji}$) and outlet ($T_{jo}$) during the fedbatch cultivation of $B_{tg}$ with complex medium in pilot scale bioreactor by feeding glucose solution at 4X of 26 g/l. $T_r$ indicates the bioreactor temperature.
rate with the influence of substrate(s) and process conditions on the metabolic process towards biomass and ICP synthesis. The observed maximum heat flux and the estimated cumulative heat produced at different cultivation periods for different media were summarized in table 3.2. The total heat produced at the time of harvest in the batch cultivation of \textit{Btg} with complex medium was observed to be 24.61 kJ/l. From previous studies, cumulative heat output of 17.24 kJ/l and 39.25 kJ/l was reported during the cultivation of \textit{Zymomonas mobilis} with initial glucose concentrations at 40 g/l and 80 g/l respectively (Silman 1986). Further, 24 kJ/g biomass with 60 MW of heat dissipation was observed during the cultivation of \textit{Methylophilus methylotrophus} on methanol as sole carbon and energy source in large scale fermentor (Stockar and Marison 1991). Randolph \textit{et al} (1990) reported the heat production of 11 watts from \textit{Saccharomyces cerevisiae} cultivated in 1.8 l with feed control by employing the ratio of carbon dioxide evolution rate to heat production rate. In a significant observation, 9.8 kJ/l was noticed during the cultivation of \textit{E. coli} with glucose as carbon and nitrogen source and 22 kJ/l with glucose and yeast extract in the medium (Marison and Stockar 1986). In addition, 11.8 kJ/l was observed with its cultivation under nitrogen limitation. These data clearly indicate the influence of carbon and nitrogen sources in the medium on heat production rate, which was also valid for the complex media sources.

The data from table 3.2 indicated that the total metabolic heat produced was observed to be 26.37 kJ/l during the fedbatch cultivation of \textit{Btg} at the time of harvest (26 hours) in complex medium with feeding glucose at 4X concentration of 26 g/l. In particular, heat produced during the initial stages were less with relatively high total heat produced at the time of harvest resulting in high concentration of ICP at 6.969 g/l. This could be interpreted in terms of the correlation of heat production rate with the balanced flux of
Table 3.2  The observed maximum metabolic heat flux and the estimated cumulative heat produced at different cultivation periods for different media under varying process conditions. Baseline heat signal values obtained in each cultivations were also shown.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Mode of operation</th>
<th>Net Maximum Heat flux (Watts)</th>
<th>Baseline Heat signal (Watts)</th>
<th>Cultivation time for Maximum heat flux (hours)</th>
<th>Cumulative heat production, kJ/l</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>Maximum heat flux</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12 hours</td>
</tr>
<tr>
<td>Glucose – Yeast Extract</td>
<td>Batch</td>
<td>341.64</td>
<td>221.55</td>
<td>4.2</td>
<td>2.07</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex Medium</td>
<td>Batch</td>
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<td>223.85</td>
<td>7.27</td>
<td>8.79</td>
</tr>
<tr>
<td>Complex Medium with feeding glucose at 25X</td>
<td>Fedbatch</td>
<td>445.49</td>
<td>197.4</td>
<td>17.03</td>
<td>11.95</td>
</tr>
<tr>
<td>Complex Medium with feeding glucose at 4X</td>
<td>Fedbatch</td>
<td>1189.6</td>
<td>123.88</td>
<td>5.89</td>
<td>5.35</td>
</tr>
</tbody>
</table>
nutrients favoring enhanced biomass and ICP synthesis. On comparison, total heat produced in GY medium was considerably less with respect to complex medium indicating the predominant role of complex media sources on heat production. This characteristic of complex medium could be exploited in large-scale fermentation as an online metabolic status indicator. Also, process of ICP synthesis could be monitored with the total heat produced during the cultivation providing a possibility of indirect measurements of secondary metabolites during cultivation.

3.2 PROCESS OPTIMIZATION WITH CONSTANT GLUCOSE CONTROL

Optimization of bioprocess towards enhancing the yield of biomass and ICP synthesis by $Btg$ was carried out. It was reported that *Bacillus thuringiensis* could be grown in various complex sources for the production of biopesticides (Vora and Shethna 1999; Zouari and Jaoua 1999; Alves *et al* 1997; Morris *et al* 1997). In these media sources, it will be difficult to understand the effects of various contributing components towards biomass and product synthesis. Also, these complex sources could be insoluble and seasonal variations in its composition were also expected. Hence, optimized process could be carried out with the complex media sources by comparison of the components involved on a rational basis. Comparison of components could be carried out after identifying the basal compounds in the media involved. For this purpose, $Btg$ could be grown in minimal medium containing glucose and yeast extract with supplementation of salts. In addition, free aminoacids, short peptides and unidentified growth supporting factors from yeast extract could be available during the cultivation. Hence, attempts will be made to understand the impact of glucose as carbon source and yeast extract as organic nitrogen source.
on the growth and ICP synthesis by *Btg*. This detailed understanding will be further utilized in the process optimization strategies to enhance the yield of biomass and ICP. On identification, macroscopic environment of the microbial culture will be manipulated to provide a constant optimum glucose concentration even with the continuous utilization of glucose. This necessitated a control system for maintaining a constant glucose concentration in the bioreactor. Therefore, it was decided to develop a fuzzy logic based feedback control system for maintaining the residual glucose concentration at the desired level by online monitoring of its concentration inside the bioreactor through biochemistry analyzer during cultivation.

### 3.2.1 Configuration of biochemistry analyzer for online measurements

The biosensor based online analysis of the residual glucose concentration was carried out using the biochemistry analyzer. In this system, immobilized glucose oxidase enzyme in the membrane module (Yellow Spring Instruments Inc.) oxidizes glucose and releases \( \text{H}_2\text{O}_2 \), which was measured by the electrochemical cell. The released \( \text{H}_2\text{O}_2 \) was found to be proportional to the glucose concentration. Glucose solution at predefined concentration was used as the calibration standard. The voltage signal from the analyzer corresponds to the glucose concentration in the sample. The culture media was drawn in by means of a peristaltic pump in the analyzer. This peristaltic pump was also used for purging the sample tubing with culture media from the bioreactor. After sufficient purging, a preset volume of the sample was aspirated from the sampling chamber and injected into the reaction chamber of the analyzer. At the end of specified reaction time of 30 seconds, glucose concentration was measured using the calibrated analog voltage signal from the analyzer. This analog voltage signal was continuously acquired using a program developed in
LabVIEW through the interface board. The microprocessor in the analyzer facilitates the programming of various parameters involved in the operation of the analyzer. Sampling interval, purging time and flow rate of the sample were specified in the analyzer as 5 min, 1 min and 2500μl/min respectively. Automatic calibration of the glucose analyzer was carried out after analyzing every two samples.

The separation of the cells from the culture medium was done using a filtration module. The sterility in the bioreactor and prevention of fouling in biosensor was maintained using this filtration system. The cell free culture medium obtained as permeate from the filter was used for the estimation of residual glucose. The filtration module used was a tangential flow filtration system with an active membrane area of 45cm² and pore size of 0.22 μm. These membranes were autoclaved at 121°C for 20 min. Insignificant pressure drop across the membrane and better performance was observed with the tangential flow filtration module because of less concentration polarization. Care was taken to ensure low holdup in the tubing and filtration module. The flow through the filtration system was maintained using a peristaltic pump, which circulates the culture medium continuously during the cultivation. The continuous circulation was maintained to prevent any dead volume and unfavourable growth. The retentate from the filtration system was fed back into the bioreactor. During the sampling time, permeate flow was provided by means of a peristaltic pump in the analyzer.

The programs for on-line data acquisition, control system and execution of the control action were written in LabVIEW. The biochemistry analyzer was interfaced with the digital computer through a DAQ Board. The digital trigger of step rise in voltage from 0 to 5 volts from the analyzer was
used for the initiation of the data acquisition for the subsequent sampling periods. Handshaking signal from the computer was helpful to reset the digital trigger before the analysis of the new sample. These handshaking signals also help to ensure the synchronized performance of the analyzer and program with respect to time.

3.2.2 Fuzzy logic control algorithm

Fuzzy logic provides an efficient and feasible way in dealing with unpredictable outcomes in the experimental system. The knowledge acquired over the system can be translated into linguistic rules in the fuzzy language. The needed fuzzy logic feedback controller was developed with error (E) and change in error (CE) as input variables. The measured residual glucose concentration was used both as measured and controlled variable. The difference between the user specified setpoint and measured variable gave the extent of deviation of the system from the setpoint at the sampled time. Change in error is the difference in error in subsequent sampling intervals. The magnitude and sign of the change in error reflects the direction of movement of the system either towards the setpoint or away upon the execution of the control action. The knowledge gained over substrate uptake profile during the cultivation of Bt was applied in deciding the limits for error in the development of input membership function diagram and fuzzy inference rule base in the fuzzy logic control system.

The input variable to the control system namely error was represented in the membership function diagram with five fuzzy subsets as shown in figure 3.35 and labeled with fuzzy labels as Z (zero), S (small), M (medium), L (large) and X (very large). Similarly, representation of the change
Figure 3.35 The fuzzy input membership function of error in residual glucose concentration for the online control of substrate concentration during fedbatch cultivation of \textit{Btg}.

Figure 3.36 The fuzzy input membership function of change in error in residual glucose concentration for the online control of substrate concentration during fedbatch cultivation of \textit{Btg}.
in error was done with three fuzzy subsets labeled as N (negative), Z (zero) and P (positive) as shown in figure 3.36. These two input membership function diagrams were employed for the fuzzification of input variables from its crisp numerical value. The output membership function diagram was also constructed with five fuzzy subsets labeled as Z (zero), S (small), M (medium), L (large) and X (very large) with output voltage range as 0 – 10 volts as shown in figure 3.37. A rule base for fuzzy inference was designed as shown in table 3.3. The membership value of the output fuzzy subset was determined as the minimum value among the membership values of the input fuzzy subset by each selected rule defined in the rule base. This inference procedure was according to min-max algorithm developed by Mamdani (1976). The centre of area method was used for the defuzzification of the output membership value into a crisp real value (Pedryz 1991). In this method, the triangular area formed by the intersection of the fuzzy subset and the membership values in the output membership diagram were approximated as weights \( \alpha_j \) for the corresponding fuzzy subset. The final crisp output control value \( x_f \) was obtained by the average of the value corresponding to the midpoint of the fuzzy subset \( x_j \) with their respective weights \( \alpha_j \), \( x_f = \frac{\sum x_j \alpha_j}{\sum \alpha_j} \). The control signal in the form of varying voltage in the range 0 – 10 volts was supplied to the feed pump through the interface board.

Feed pump was used with varying speed corresponding to the voltage supplied from the control algorithm. The flow rate of the feed pump was calibrated at different speeds by varying voltage input. During the execution of the control action, the feed pump was operated for a constant time period as a pulse feeding. This period of operation of the feed pump could be specified in the program developed. The voltage supplied to the feed pump \( V_f \) can be expressed as \( V_f = 1.5 + V_c \). Since the active culture will have a net glucose
Figure 3.37  The fuzzy output membership function of output voltage to the feed pump for the online control of substrate concentration during fedbatch cultivation of $Btg$
Table 3.3 Fuzzy inference control rules base developed based on the prior experience on cultivation of Btg for the fuzzy logic control system

<table>
<thead>
<tr>
<th>Change in Error</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z</td>
</tr>
<tr>
<td>N</td>
<td>Z</td>
</tr>
<tr>
<td>Z</td>
<td>Z</td>
</tr>
<tr>
<td>P</td>
<td>Z</td>
</tr>
</tbody>
</table>

utilization rate at any point of time, 1.5 volts was supplied to the feed pump immaterial of the magnitude of the control action. The signal voltage from the fuzzy logic control system (Vc) corresponds to the range of 0 – 7.5 volts converted from 0 – 10 volts range in the algorithm. During the control action, sterile medium was fed into the bioreactor at the calibrated flow rate corresponding to the speed based on the voltage supplied to the feed pump for maintaining the glucose concentration at a constant level.

The simulation of the control system was done with the algorithm written in LabVIEW. The output control voltage was simulated for various values of error and change in error which were considered to be in the experimental range. The simulated data for the output control voltage was plotted in MATLAB 4.0 as shown in figure 3.38. The plot demonstrated the relationship between the output and input variables involved in the control system. The output voltage was observed to be above 1.5 volts in all cases substantiating the expression indicated before. This simulation was carried out
Figure 3.38 Simulation results of the control system for the output control voltage with the input of error and change in error of the measured variable—residual glucose concentration.
to predict the performance of the developed control system in practical environment.

3.2.3 Demonstration of fuzzy logic control on residual glucose concentration

The calibration of voltage signal from the glucose analyzer was done by employing standard glucose solutions with known concentrations. The overall experimental setup depicting the control system with all its accessories is schematically represented in figure 3.39. This setup was used for the fedbatch cultivation with need based addition of concentrated sterile feed using the feed pump which was activated during the execution of the control action. During the practical demonstration of the developed control system, it was necessary to operate in higher ranges of cell density with rapid utilization of glucose. With the previous medium development studies carried out, it was observed that GYA medium was supposed to yield relatively high cell density during the time of demonstration of this control system. Hence, concentrated sterile medium (GYA, 3X) was used as feed for the maintenance of residual glucose concentration at a constant level.

In order to demonstrate the performance of the developed fuzzy logic based feedback control system, cultivations were performed with residual glucose concentration control extending over different growth phases of Btg. Initial experiment was conducted with the setpoint at 3.4 g/l of glucose which was in accordance with the glucose concentration in the GYA medium developed (Sachidhanandham et al 1996). The fedbatch cultivation was carried out with the control on residual glucose imposed for a period of 10 hours. The substrate utilization rate depends on the growth phases of the culture during cultivation. Due to different substrate utilization rates, rate of depletion of
Figure 3.39 The schematic diagram of the setup used for the fedbatch cultivation of Btg employing online monitoring and control of substrate concentration.
residual glucose in the medium was observed to vary in a nonlinear fashion. This indicated that the amount of glucose in terms of the flow rate of the external sterile medium has to be varied in order to maintain the glucose concentration at the desired level. This variation in flow rate was dependent on the control voltage supplied to the feed pump by the following relation: 

\[ \text{flowrate in ml/secs} = 0.1779*V - 0.0345 \]

with \( V \) indicating the control voltage supplied to the feed pump. This control voltage was decided by the developed fuzzy logic based feedback control system. Hence during the control period, sterile feed was fed into the bioreactor on the execution of the control action after sampling at every 5 minutes. Increase in culture volume inside the reactor was observed due to this feeding and there was no appreciable loss of culture medium on sampling continuously.

The cell density and residual glucose concentration profiles during the fedbatch cultivation of \( \text{Btg} \) with the setpoint of 3.4 g/l are shown in figure 3.40. The residual glucose concentration profile showed a constant trend without fluctuations in its concentration during the control period and started decreasing when the control was removed due to the utilization of glucose by the organism. The maximum cell density was found to be 3.083 g/l with the specific growth rate of 0.4529. This indicated the relationship between residual glucose concentration and the achieved specific growth rate of the culture. Even with the continuous addition of medium, exponential growth phase could not be extended indicating the significant role of maintaining constant substrate concentration in the bioreactor. This indicated the possibility of maintaining the residual glucose concentration at the desired level during different phases of growth with varying glucose utilization rates.

In order to understand the performance of the control system at the lower range of operating regime, fedbatch cultivation was carried out with
Figure 3.40 Profiles of residual glucose concentration and cell density during the fedbatch cultivation of Btg with the fuzzy logic control system. The control was imposed for a period of 10 hours at the setpoint of 3.4 g/l in residual glucose concentration.

Figure 3.41 Profiles of residual glucose concentration and cell density during the fedbatch cultivation of Btg with the fuzzy logic control system. The control was imposed for a period of 10 hours at the setpoint of 1.0 g/l in residual glucose concentration.
setpoint of 1.0 g/l. The initial glucose concentration in the medium was kept at 3.4 g/l as in the previous case. However, feeding of the medium was started when the residual glucose concentration reached 1.0 g/l. The control period was extended for 10 hours of cultivation at this residual glucose concentration. The cell density and residual glucose concentration profiles of \textit{Btg} at this setpoint are shown in figure 3.41. Maximum cell density of 2.434 g/l was attained during this cultivation. It was observed that the culture has reached the late exponential growth phase with the specific growth rate of 0.5698 by the time residual glucose concentration decreased to 1.0 g/l. Hence, control was established during the stationary/sporulation phase of the cultivation with rate of substrate utilization much less in comparison to other cultivations. Also, medium fed during the control period was found to be significantly less when compared to cultivations at other setpoints. When the control was removed, a decreasing trend in the glucose concentration was observed indicating further utilization of glucose by the organism. In contrast to the previous cultivation, nearly complete utilization of glucose was achieved. The possibility of providing balanced flux based on the relationship between specific growth rate and residual glucose concentration could be appreciated in the previous cultivation. However, this could not be done in this cultivation as the residual glucose concentration was maintained only during the sporulation phase.

On further understanding the performance of the control system in the upper and lower values of the operating regime, it was decided to study the possibility of providing a balanced substrate flux at 2.0 g/l and its role towards the enhancement of cell density. Hence, fedbatch cultivation was carried out with the setpoint of 2.0 g/l with the initial glucose concentration in the medium at 3.4 g/l as before. When the residual glucose concentration dropped to the value of 2.0 g/l, its concentration was maintained at the same level by external
feeding of the concentrated medium. This study was carried out for different control periods during which the glucose concentration was maintained at the constant level for 5 and 10 hours. When the glucose concentration was maintained at 2.0 g/l for 5 hours, it was observed that the control was established during the mid-exponential growth phase of the culture with high substrate utilization rate. The cell density and residual glucose concentration profiles during this fedbatch cultivation are shown in figure 3.42. The maximum cell density attained was found to be 3.44 g/l. Interestingly, final cell density in this case was found to be much higher than the first fedbatch cultivation reflecting the inhibitory effect due to the maintenance of residual glucose concentration at elevated levels on the growth of Btg. In addition, when the control was removed after 5 hours, the glucose profile showed a steep declining trend with time. It could be attributed to the sensitivity of the culture for substrate flux during early phases of growth. Also, on observing the cell density profile during the control period there seemed to be a linear increase in the cell density with constant specific growth rate of 0.1128. This low growth rate could be due to the limiting conditions imposed on the growing culture by low concentrations of the nutrients. Also, this could be due to the accumulation of acidic intermediates during the initial growth phase of decreasing residual glucose concentration and its consumption during the subsequent control period. Before the start of feeding for the control, growth rate of the culture was observed to be 0.5748 indicating the significant role of maintaining the residual glucose concentration at the constant level of 2.0 g/l. It could be inferred that the maintenance of residual glucose concentration at 2.0 g/l has provided a balanced substrate flux resulting in the constant specific growth rate during the control period.

In order to investigate, whether increasing the duration of slow feeding would help in extending the exponential growth phase at the obtained
Figure 3.42 Profiles of residual glucose concentration and cell density during the fedbatch cultivation of $Btg$ with the fuzzy logic control system. The control was imposed for a period of 5 hours at the setpoint of 2.0 g/l in residual glucose concentration.

Figure 3.43 Profiles of residual glucose concentration and cell density during the fedbatch cultivation of $Btg$ with the fuzzy logic control system. The control was imposed for a period of 10 hours at the setpoint of 2.0 g/l in residual glucose concentration.
specific growth rate, experiment was conducted under conditions described previously except the duration of control was extended to 10 hours which gave an encouraging result. During this fedbatch cultivation with the setpoint of 2.0 g/l, higher cell density of 4.08 g/l was obtained as shown in the figure 3.43. As before, linear rise in cell density was observed with constant specific growth rate of 0.0699 during this control period. This difference in specific growth rate in comparison with the previous cultivation could be due to the influence of relatively high specific growth rate during the initial growth phase of the culture. The regulated substrate flux at this level clearly demonstrated the balanced metabolism of $Btg$, which could have prevented the accumulation of metabolic wastes of the organism. These observations indicated the significant role of regulated substrate flux on the growth of $Btg$ and its subsequent synthesis of ICP.

In particular, the developed control system could be utilized in an effective manner for maintaining the residual glucose concentration at the desired level as demonstrated by the previous experiments. Hence, optimum residual glucose concentration has to be determined so that it could be exploited for enhancing cell density and ICP synthesis. In addition, regulated substrate flux could not be achieved in batch cultures because of continuous decrease in the residual glucose concentration with time during cultivation and the organism is subjected to transient conditions. In this aspect, feedback fuzzy logic based control system could be utilized for operating the bioreactor in fedbatch mode for the cultivation of $Btg$ under regulated substrate flux.

3.2.4 Batch cultivations of $Btg$ in bench scale bioreactor

Glucose being a rapidly metabolizable carbon source represses the TCA cycle enzymes which limits the aminoacid supply for the production of
spores and ICP crystals. Hence, it was reported that the strain of *Btg* segregated to form asporogenic variants in continuous cultures (Sachidanandham and Jayaraman 1993). Also, this could be prevented by the supplementation of aminoacids as reported by the same author (Sachidanandham *et al.* 1996).

Hence, addition of yeast extract at higher initial concentration could act as a source for aminoacids upon hydrolysis during the cultivation. Therefore, it was decided to understand the response of carbon and organic nitrogen source on the growth and ICP synthesis. In addition, these variations in substrates under study were carried out with changes in their initial concentration levels. This will provide an insight into the metabolism of *Btg* with respect to glucose and yeast extract used as carbon and nitrogen source respectively. The above batch studies could be able to provide details regarding the optimum environment with respect to the carbon source employed. Further, this understanding will provide a way for exploiting this flux of nutrients towards the enhancement of biomass and ICP synthesis. Hence, experiments were planned and performed for varying ranges of carbon and nitrogen substrates concentration.

### 3.2.4.1 Analysis of individual batch cultivations

Glucose and yeast extract were selected in the range 3.4 – 34 g/l and 1.0 – 20 g/l respectively with additional salts as mentioned in materials and methods. The higher concentrations were selected to obtain information regarding its role on inhibition for growth and product synthesis. Glucose and yeast extract was mentioned as carbon and nitrogen source respectively in the discussions to follow. Full factorial experimental design was used to decide the concentration levels for each experiment. According to the design suggested by the statistical software STATGRAPHICS 3.1, batch cultivations were carried out in the bioreactor under controlled conditions. Each run was designated as
GY1 to GY9 and initial concentration levels of glucose and yeast extract employed in each cultivations are shown in table 3.4. In addition, combined estimated values on analysis of the samples from cultivations are given in table 3.5.

Table 3.4 The initial concentration levels of glucose and yeast extract as C-source and N-source respectively used in batch cultivations. This levels were obtained from the experimental design employed for the concentration range of 3.4 - 34.0 g/l for glucose and 1.0 - 20 g/l for yeast extract

<table>
<thead>
<tr>
<th>S. No</th>
<th>Medium</th>
<th>Initial Glucose concentration, g/l</th>
<th>Initial Yeast Extract concentration, g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GY1</td>
<td>3.4</td>
<td>1.0</td>
</tr>
<tr>
<td>2.</td>
<td>GY2</td>
<td>3.4</td>
<td>10.5</td>
</tr>
<tr>
<td>3.</td>
<td>GY3</td>
<td>3.4</td>
<td>20.0</td>
</tr>
<tr>
<td>4.</td>
<td>GY4</td>
<td>18.7</td>
<td>20.0</td>
</tr>
<tr>
<td>5.</td>
<td>GY5</td>
<td>18.7</td>
<td>10.5</td>
</tr>
<tr>
<td>6.</td>
<td>GY6</td>
<td>18.7</td>
<td>1.0</td>
</tr>
<tr>
<td>7.</td>
<td>GY7</td>
<td>34.0</td>
<td>1.0</td>
</tr>
<tr>
<td>8.</td>
<td>GY8</td>
<td>34.0</td>
<td>10.5</td>
</tr>
<tr>
<td>9.</td>
<td>GY9</td>
<td>34.0</td>
<td>20</td>
</tr>
</tbody>
</table>

The initial batch cultivation was carried with carbon and nitrogen sources at lower level of concentrations. The profiles of residual glucose...
concentration, cell density and dissolved oxygen concentration during this cultivation of *Btg* in GY1 medium are shown in figure 3.44. The estimated values on analysis of the samples from the cultivation are given in table 3.5. It was observed that glucose in the medium got depleted by 16 hours during the sporulation phase of the culture. Also, pH was observed to be in decreasing trend till the residual glucose concentration reached 1.79 g/l at 4.75 hours. This could be due to the secretion of acidic intermediates into the medium and its subsequent consumption after the depletion of residual glucose. Uniform population of sporulating cells was observed during the sporulation phase with uniform release of spores at 23 hours. It could be inferred that contributing factors for growth from yeast extract was low resulting in less growth of the culture. This indicated the influence of medium components on the metabolism leading to ICP synthesis. In fact, it was reported that the major portion of aminoacids required for ICP synthesis was derived from vegetative protein turnover during sporulation phase (Monro 1961).

In the next batch cultivation, initial yeast extract concentration was increased to 10.5 g/l with glucose concentration at 3.4 g/l. The profiles of residual glucose concentration, cell density and dissolved oxygen concentration during this cultivation of *Btg* in GY2 medium are shown in figure 3.45. The estimated values on analysis of the samples from the cultivation are given in table 3.5. It was observed that glucose got depleted by 5 hours indicating the higher glucose utilization rate. This higher rate of glucose utilization could have resulted due to the increased concentration of yeast extract in the medium. Also, pH was observed to be decreasing till the depletion of glucose in the medium at 4.75 hours. The rate of pH increase due to the consumption of secreted acidic intermediates was found to be relatively rapid. This rapid utilization could be due to the TCA cycle enzymes being expressed after the depletion of glucose concentration, cell density and dissolved oxygen concentration during this cultivation of *Btg* in GY1 medium are shown in figure 3.44. The estimated values on analysis of the samples from the cultivation are given in table 3.5. It was observed that glucose in the medium got depleted by 16 hours during the sporulation phase of the culture. Also, pH was observed to be in decreasing trend till the residual glucose concentration reached 1.79 g/l at 4.75 hours. This could be due to the secretion of acidic intermediates into the medium and its subsequent consumption after the depletion of residual glucose. Uniform population of sporulating cells was observed during the sporulation phase with uniform release of spores at 23 hours. It could be inferred that contributing factors for growth from yeast extract was low resulting in less growth of the culture. This indicated the influence of medium components on the metabolism leading to ICP synthesis. In fact, it was reported that the major portion of aminoacids required for ICP synthesis was derived from vegetative protein turnover during sporulation phase (Monro 1961).

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Table 3.5 The estimated values on analysis of the samples for the batch cultivations with varying initial concentration of glucose and yeast extract as C - source and N - source respectively in bench scale bioreactor

<table>
<thead>
<tr>
<th>S.No</th>
<th>Samples</th>
<th>Cell dry weight, g/l</th>
<th>ICP concentration, g/l</th>
<th>Spore concentration, spores/ml</th>
<th>Biomass productivity, g/l/hr</th>
<th>$Y_{px}$</th>
<th>$Y_{xs}$</th>
<th>$Y_{px}$</th>
<th>Specific growth rate, hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GY1</td>
<td>2.4684</td>
<td>9.1328</td>
<td>9.067E+08</td>
<td>0.1702</td>
<td>2.7262</td>
<td>0.7859</td>
<td>3.6999</td>
<td>0.56±76</td>
</tr>
<tr>
<td>2.</td>
<td>GY2</td>
<td>5.4568</td>
<td>2.5255</td>
<td>6.500E+09</td>
<td>0.7276</td>
<td>0.7406</td>
<td>1.6144</td>
<td>0.4628</td>
<td>0.8179</td>
</tr>
<tr>
<td>3.</td>
<td>GY3</td>
<td>7.3511</td>
<td>6.0175</td>
<td>1.344E+12</td>
<td>0.6392</td>
<td>1.8070</td>
<td>2.2075</td>
<td>0.8186</td>
<td>1.0647</td>
</tr>
<tr>
<td>4.</td>
<td>GY4</td>
<td>13.0922</td>
<td>6.3290</td>
<td>2.513E+12</td>
<td>1.0071</td>
<td>0.3497</td>
<td>0.7233</td>
<td>0.4834</td>
<td>0.8198</td>
</tr>
<tr>
<td>5.</td>
<td>GY5</td>
<td>10.8885</td>
<td>6.7444</td>
<td>1.360E+12</td>
<td>0.7259</td>
<td>0.4133</td>
<td>0.9679</td>
<td>0.6194</td>
<td>0.7775</td>
</tr>
<tr>
<td>6.</td>
<td>GY6</td>
<td>3.0792</td>
<td>3.9313</td>
<td>8.420E+10</td>
<td>0.2799</td>
<td>0.7474</td>
<td>0.8506</td>
<td>1.2767</td>
<td>0.5686</td>
</tr>
<tr>
<td>7.</td>
<td>GY7</td>
<td>3.3576</td>
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<td>7.296E+11</td>
<td>0.2741</td>
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<td>1.3814</td>
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</tr>
<tr>
<td>8.</td>
<td>GY8</td>
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<td>9.</td>
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<td>11.6231</td>
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<td>1.480E+13</td>
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<td>0.3943</td>
<td>0.3696</td>
<td>1.0668</td>
<td>0.9229</td>
</tr>
</tbody>
</table>
Figure 3.44  Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of *Btg* in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 3.4 g/l and 1.0 g/l respectively (GY1). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.

Figure 3.45  Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of *Btg* in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 3.4 g/l and 10.5 g/l respectively (GY2). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.
(Luthy et al. 1982). Uniform population of cells was observed during sporulation phase of the cultivation. The results indicated the significant influence of yeast extract on growth and ICP synthesis. In addition, there could be unidentified contributing factors from yeast extract playing a crucial role in the metabolism of *Btg*.

In order to further understand the effect of yeast extract at higher concentration, batch cultivation was carried out with 20.0 g/l of yeast extract as initial concentration. The profiles of residual glucose concentration, cell density and dissolved oxygen concentration during this cultivation of *Btg* in GY3 medium are shown in figure 3.46. The estimated values on analysis of the samples from the cultivation are given in table 3.5. The increase in the initial concentration of yeast extract has resulted in the enhanced utilization of glucose thereby leading to its depletion by 3.75 hours of cultivation. In contrast to the previous cultivation, exponential growth phase was observed even after the depletion of residual glucose. This could be due to the consumption of yeast extract as carbon source for biomass synthesis as reported by Mignone and Rossa (1996). In addition, microbial population was observed to be uniform during sporulation but there was significant delay in release of spores. This could be attributed to the influence of unconsumed organic nitrogen source on sporulation process.

Since there was a possibility of glucose limiting the growth in previous cultivation, it was decided to increase the initial glucose concentration to next higher level of 18.7 g/l. The profiles of residual glucose concentration, cell density and dissolved oxygen concentration during this cultivation of *Btg* in GY4 medium are shown in figure 3.47. The estimated values on analysis of samples from the cultivation are given in table 3.5. The high concentration of
Figure 3.46 Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of *Btg* in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 3.4 g/l and 20 g/l respectively (GY3). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.

Figure 3.47 Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of *Btg* in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 18.7 g/l and 10.5 g/l respectively (GY4). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.
yeast extract at 20 g/l with 18.7 g/l of glucose has resulted in rapid utilization of glucose thereby leading to its depletion at 13 hours of cultivation. Two phases in growth were observed with decrease in pH till 5.75 hours with 5.25 g/l of glucose as the initial phase. In second phase, glucose and secreted acidic intermediates would have consumed resulting in unbalanced flux of carbon source. Also, release of spores was not observed with heterogenous population even after 41 hours of cultivation. This delay in release of spores could be due to incomplete synthesis of spore components thereby preventing its release (Tyrell et al 1981). The possible presence of excess organic nitrogen source could have interfered with the above process. In particular, amount of ICP synthesised in relation to achieved cell density was relatively less with this initial concentration of glucose and yeast extract.

On the observation of possible interference of excess organic nitrogen source on sporulation, yeast extract concentration was reduced to 10.5 g/l. The profiles of residual glucose concentration, cell density and dissolved oxygen concentration during this cultivation of Btg in GY5 medium are shown in figure 3.48. The estimated values on analysis of the samples from the cultivation are given in table 3.5. The decreased initial concentration of yeast extract with respect to the previous cultivation has resulted in relatively high cell density. But mixed population of vegetative and sporulating cells was observed and release of spores could not be observed even after 40 hours of cultivation. Also, presence of glucose in the medium was noticed even after the initiation of sporulation as pH was observed in the decreasing trend till 6 hours with 11.38 g/l of glucose. Further consumption of glucose during late exponential and sporulation phase could have resulted in the unbalanced flux of carbon and nitrogen sources through the metabolic network thereby influencing the synthesis of ICP in relation to the cell density achieved.
Figure 3.48 Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of Btg in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 18.7 g/l and 10.5 g/l respectively (GY5). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.

Figure 3.49 Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of Btg in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 18.7 g/l and 1.0 g/l respectively (GY6). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.
To further understand the role of organic nitrogen source, its initial concentration was reduced to 1.0 g/l in the next batch cultivation. The profiles of residual glucose concentration, cell density and dissolved oxygen concentration during this cultivation of Btg in GY6 medium are shown in figure 3.49. The estimated values on analysis of the samples from the cultivation are given in table 3.5. In particular, relatively high concentration of glucose with less concentration of yeast extract has caused the heterogenity in microbial population due to nonavailability of growth supporting components from yeast extract as reported by Sachidanandham and Jayaraman (1993). The vegetative cells observed during the sporulation phase could be asporogenic variants as reported by the same author. The inference of high concentration of residual glucose on the expression of TCA cycle enzymes could have influenced the flux of carbon and nitrogen sources. During the cultivation, pH was observed to be decreasing till 7 hours indicating the secretion of acidic intermediates.

With the observations obtained from previous cultivations, batch cultivation was conducted with high initial concentration of glucose at 34.0 g/l in order to understand the effect of excess carbon source. The profiles of residual glucose concentration, cell density and dissolved oxygen concentration during this cultivation of Btg in GY7 medium are shown in figure 3.50. The estimated values on analysis of the samples from the cultivation are given in table 3.5. Excess glucose could have resulted in the repression of TCA cycle enzymes leading to the accumulation of acetic acid as indicated by the decreasing trend of pH till 7.25 hours with 31.05 g/l of residual glucose. In addition, carbon flux through the glycolysis pathway could have decreased as observed by the high concentration of residual glucose after the cultivation. This could be due to the non-availability of organic nitrogen source in the medium. Further, microscopic observation indicated the presence of mixed
Figure 3.50 Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of Btg in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 34 g/l and 1.0 g/l respectively (GY7). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.

Figure 3.51 Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of Btg in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 34 g/l and 10.5 g/l respectively (GY8). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.
population with sporulating and vegetative cells. In particular, release of spores was observed with sporulation initiated cells at 22 hours of cultivation. On this basis, it would be possible to infer that presence of excess organic nitrogen source during sporulation phase has a dominant role in the release of spores. This could be due to its interference on the synthesis of spore components. On contrast, ICP concentration was observed to be relatively more in comparison to the cell density achieved during this cultivation. Hence, excess carbon source could have favored the metabolic activities towards the synthesis of ICP.

On identification of the influence of yeast extract on sporulation process, batch cultivation with increased yeast extract concentration of 10.5 g/l and 34.0 g/l of glucose was carried out. The profiles of residual glucose concentration, cell density and dissolved oxygen concentration during this cultivation of *Btg* in GY8 medium are shown in figure 3.51. The estimated values on analysis of the samples from the cultivation are given in table 3.5. Longer lag phase was noticed during this cultivation due to high initial glucose concentration. Also, presence of glucose in the medium was observed during the time of initiation of sporulation. In addition, pH was observed to be decreasing till 6.75 hours with 21.9 g/l of glucose. Synthesis of ICP was less even in comparison with the relatively high cell density achieved. This indicated the possibility of nutrient flux being directed towards the synthesis of vegetative proteins away from ICP due to the presence of nutrients at high concentrations. In addition, microscopic observation indicated the presence of mixed population without the release of spores after 39 hours of cultivation.

In order to understand the impact of high concentrations of glucose and yeast extract on biomass and ICP synthesis, batch cultivation was carried out with glucose at 34.0 g/l and yeast extract at 20.0 g/l as initial
concentrations. The profiles of residual glucose concentration, cell density and dissolved oxygen concentration during this cultivation of $Btg$ in GY9 medium are shown in figure 3.52. The estimated values on analysis of the samples from the cultivation are given in table 3.5. Due to high initial concentrations of glucose and yeast extract, lag phase was observed during the initial stages of growth as in the previous cultivation. In addition, pH was observed to be in decreasing trend till its depletion. The higher concentration of nitrogen source has resulted in enhanced utilization of carbon source thereby resulting in its depletion by 10.25 hours. Two different exponential growth phases could be observed as in previous cultivation in GY4 medium. These phases could have resulted as response to the metabolic changes occurred due to high concentration of carbon and nitrogen sources in the medium. Depletion of glucose could have initiated the sporulation process after the two phases of growth. Heterogenous cell population was observed during the sporulation phase and also delay in the release of spores as before due to high concentration of nitrogen source interfering with the synthesis of spore components. In particular, flux of glucose as carbon source towards either the biomass or ICP were less as indicated by the yield coefficients $Y_{ph}$ and $Y_{sh}$. This further indicated the possibility of glucose being utilized as energy source through the fermentative pathway. Even with higher concentration of ICP with this initial concentration of glucose and yeast extract has resulted in inefficient utilization of glucose as carbon source for its growth and ICP synthesis. Hence, above environmental conditions could not favor an economically feasible process with effective utilization of glucose as carbon source towards the enhancement of biomass and ICP synthesis.

It was observed during the batch cultivations at varying initial concentrations of carbon and nitrogen source that the ratio of carbon to nitrogen
Figure 3.52 Profiles of dissolved oxygen concentration (DO), residual glucose concentration and cell density during the batch cultivation of Btg in bench scale bioreactor. The initial concentrations of glucose and yeast extract were 34 g/l and 20 g/l respectively (GY9). The cultivation was carried out with controlled conditions of pH at 7.0 and temperature at 30°C.
source in the medium has a predominant role in the metabolic process under study. In addition, understanding the metabolic phenomenon in terms of initial concentrations of carbon and nitrogen sources could provide an insight into the role of nutrient flux on enhanced metabolic activity towards the biomass and ICP synthesis. Hence, data obtained from different batch cultivations were correlated in terms of the initial concentrations of glucose and yeast extract. This analysis could provide a detailed understanding of the role of above nutrients on the process of growth and sporulation during the cultivation.

3.2.4.2 Correlations of data from batch cultivations

The analysis of data obtained during the cultivations of Btg in varying initial concentrations of glucose and yeast extract has facilitated to understand the influence of carbon and nitrogen sources on transient growth conditions. The changing environmental conditions during growth has understood to have an impact on the metabolic process as reported in batch cultures (Avignone and Mignone 1993; Selinger et al 1988; Roy et al 1987). In addition, response of microbial cultures to external macroscopic environment seemed to be complex. This has reflected in inconsistent kinetic parameters for the cultivation of Bt as reported by several authors (Rodriguez and Torre 1996). Hence, understanding in terms of initial concentration levels of glucose and yeast extract could be exploited for enhancing the yield of biomass and ICP.

As initial analysis, maximum cell density achieved in batch cultivations were correlated with initial concentrations of glucose and yeast extract in terms of a second order polynomial equation (3.7). This correlation was established using the statistical software, STATGRAPHICS 3.1 and it was given by,
Cell density, g/l = \(-0.740445 + 0.399777 \times C + 0.820934 \times N - 0.0092374 \times C^2 \\
+ 0.00581828 \times C \times N - 0.0249237 \times N^2\)  
\[\text{R-squared: 94.94\%}\] (3.7)

In the above equation, C and N indicate the concentration of glucose as carbon source and yeast extract as nitrogen source respectively. The response surface plot and contour plot expressing the relationship between the carbon and nitrogen sources for different values of cell density achieved are shown in figure 3.53. It was estimated that there was a maximum cell density of 12.92 g/l at the glucose concentration of 27.85 g/l and yeast extract concentration of 19.7196 g/l. Further, it was observed that increasing the concentration of both carbon and nitrogen sources has resulted in increase in maximum cell density achieved in batch cultivations. In addition, above increase in cell density was observed till 12.92 g/l and then decreased on further increase in carbon and nitrogen sources. Cell density was observed to increase till the point of maximum by maintaining either of the above variables constant and increasing the other variable as indicated in the contour plot. This observation could be due to the consumption of glucose and yeast extract by microbial culture towards the biomass synthesis. The interrelationship between the fluxes of glucose and yeast extract towards biomass synthesis was reported by Mignone and Rossa (1996). Also, rate of increase in maximum cell density achieved was observed to be relatively high with a fixed ratio of carbon and nitrogen sources. This indicated the balanced role of carbon and nitrogen sources in the flux of metabolic resources towards biomass synthesis. The decrease in maximum cell density achieved after the point of maximum could be due to inhibitory level of concentrations of above sources. In particular, heterogenous population of cells was observed during sporulation phase with batch cultivations carried out with high initial concentrations of glucose and yeast extract.
Figure 3.53 The response surface and contour plots for cell density with varying initial concentration of glucose and yeast extract as C - source and N - source respectively in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant cell density.
The non-growth associated synthesis of ICP during sporulation has made its correlation with cell density achieved as a difficult one. It was reported that attainment of high cell density during cultivation need not necessarily result in enhanced synthesis of ICP (Liu et al 1994). Hence, concentration of ICP during harvest was correlated with initial concentrations of glucose and yeast extract during batch cultivations. The obtained second order polynomial was given by,

\[
\text{ICP conc., g/l} = 10.0625 - 0.278235 \times C - 0.876966 \times N + 0.00303985 \times C^2 + 0.0187081 \times C \times N + 0.0309858 \times N^2 \quad \text{[R-squared: 74.19%]} \tag{3.8}
\]

The response surface plot and contour plot showing the relationship between the concentration level of carbon and nitrogen source for constant ICP concentration are shown in figure 3.54. In contrast, point of minimum was identified with ICP concentration of 3.647 g/l with glucose at 31.234 g/l and yeast extract at 4.7221 g/l. The ICP concentration profile was observed to be in contrast to the cell density profile thereby indicating the complex relationship between biomass and ICP synthesis. It was observed that increasing the concentration of both carbon and nitrogen sources beyond the minimum point has resulted in increased concentration of ICP. Also, ICP concentration was found to be increasing towards the lower concentration of carbon and nitrogen sources from the point of minimum. In addition, increase in nitrogen source was more pronounced in resulted ICP concentration in comparison with increase in carbon source. It was reported that 80% of aminoacids needed for ICP synthesis has been obtained from the vegetative protein turnover during sporulation (Monro 1961). Therefore, external influence of nitrogen source in terms aminoacids and short peptides on the synthesis of ICP could be minimum. Hence, process of ICP synthesis could have influenced by the high
Figure 3.54 The response surface and contour plots for insecticidal crystal protein (ICP) concentration with varying initial concentration of glucose and yeast extract as C-source and N-source respectively in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant ICP concentrations.
concentration of nitrogen source rather than its contribution in the form of aminoacids and short peptides. In addition, nitrogen source could influence the synthesis of spore components with delayed spore release on its high concentration.

The complex relationship between ICP concentration (product) obtained to the maximum cell density achieved in batch cultivation could be studied by correlating the product to biomass yield coefficient \( (\text{Y}_{\text{p/x}}) \) with initial concentrations of glucose and yeast extract. The response surface and contour plots employing the obtained second order polynomial are shown in figure 3.55. The shown profile was observed to be similar with that of ICP concentration. Increasing carbon and nitrogen sources has resulted in less \( \text{Y}_{\text{p/x}} \) thereby favoring biomass synthesis till the point of minimum. Hence, minimum point could indicate the redirection in metabolic flux towards biomass and preventing the initiation of sporulation. Further from the point of minimum, \( \text{Y}_{\text{p/x}} \) increased with less cell density at higher concentrations of carbon and nitrogen sources. This could be due to the enhanced uptake of nutrients directed towards ICP synthesis. In particular, with lower concentration of glucose increase in yeast extract has resulted in significant decrease in \( \text{Y}_{\text{p/x}} \) thereby indicating that the culture under the limitation of carbon source has taken the nitrogen source for its growth. This growth, initiation of sporulation, ICP synthesis and spore release during the cultivation of \( Btg \) could be interrelated with external macroscopic environment. The detailed understanding on the influence of the same could be done to exploit for enhanced biomass and ICP synthesis.

The understanding of biomass productivity at different initial concentrations of glucose and yeast extract could provide an insight into the process of biomass synthesis. The relationship between biomass productivity
Figure 3.55 The response surface and contour plots for product (ICP) to biomass yield coefficient (Yp/X) with varying initial concentration of glucose and yeast extract as C - source and N - source respectively in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant product to biomass yield coefficient.
Biomass productivity, g/l/hr = -0.0836716 + 0.0258329 \times C + 0.112221 \times N - 0.000558002 \times C^2 - 0.000298406 \times C \times N - 0.00383196 \times N^2

\[R\text{-squared: 82.32\%}] \quad (3.9)

The response surface and the contour plot employing the above equation are shown in figure 3.56. Maximum biomass productivity of 0.9465 g/l/hr could be identified with initial glucose concentration of 19.435 g/l and yeast extract of 13.8861 g/l. The biomass productivity was observed to be influenced more by nitrogen source in comparison with that of carbon source. In addition, increase in nitrogen source further from the point of maximum has resulted in decreased biomass productivity. This influence of nitrogen source on biomass synthesis could be due to its utilization as carbon source during cultivation. In particular, rate of increase in biomass productivity was observed to be relatively high at a fixed concentration ratio of carbon to nitrogen sources. Hence, this ratio could provide a balanced environment favoring biomass synthesis with needed nutrients, growth supporting factors etc. This fact could be extended further for reasoning the observed decrease in biomass productivity beyond the point of maximum. In addition, concentration level of carbon and nitrogen sources for maximum biomass productivity was observed to be less than that needed for maximum cell density. This clearly demonstrated that presence of carbon and nitrogen sources at lower concentrations could provide a balanced condition favoring the biomass synthesis.

The dependency of biomass productivity on specific growth rate of the culture during logarithmic phase could be understood by correlating the estimated specific growth rate in terms of initial concentration levels of carbon
Figure 3.56 The response surface and contour plots for biomass productivity with varying initial concentration of glucose and yeast extract as C-source and N-source respectively in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant biomass productivity.
and nitrogen sources by a second order polynomial. The response surface and contour plot with the obtained polynomial are shown in figure 3.57. The influence of nitrogen source on specific growth rate of the culture was observed to be significant. With nitrogen source at high concentration, relatively high specific growth rates were noticed with low and high initial concentrations of glucose. This indicated that with yeast extract being utilized as carbon source for biomass synthesis, high specific growth rates could be obtained. In addition, observed high specific growth rate has not resulted in elevated ICP concentrations. Hence, impact of carbon and nitrogen source on specific growth rate was observed to be crucial with redirection in metabolic fluxes with varying concentrations of glucose and yeast extract.

On understanding the flux of carbon source towards biomass, conditions could be manipulated for effective utilization of substrates. Hence, biomass yield coefficient ($Y_{x/S}$) was correlated with initial concentrations of carbon and nitrogen sources. The response surface and contour plot using the obtained correlation are shown in figure 3.58. In addition, biomass yield coefficient was observed to be greater than unity indicating the utilization of components other than glucose from the medium for biomass synthesis. $Y_{x/S}$ was observed to increase with increase in nitrogen source at lower concentrations of carbon source further substantiating the role of yeast extract in the metabolic flux towards biomass formation. This indicated that higher specific growth rate of the culture due to increased concentration of yeast extract has contributed to the enhanced metabolic activity towards biomass formation. In addition, this enhanced flux could not contribute for the ICP synthesis as indicated by ICP concentration profile.

The analysis in terms of ICP productivity and spore productivity could not be carried out in relation with initial concentrations of carbon and
Figure 3.57 The response surface and contour plots for specific growth rate of the culture during the exponential phase with varying initial concentration of glucose and yeast extract as C - source and N - source respectively in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant specific growth rate.
Figure 3.58 The response surface and contour plots for biomass yield coefficient (Yx/S) with varying initial concentration of glucose and yeast extract as C-source and N-source respectively in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant biomass yield coefficient.
nitrogen sources. As ICP was synthesised at the onset of sporulation, time of completion of ICP synthesis should be understood. Hence, ICP productivity in terms of the cultivation period till harvest could yield erroneous values. Also, delay and improper spore release was observed in cultivations with high concentrations of yeast extract. This has led to the estimate of exact time of spore release difficult. Hence, spore productivity also could not be studied in terms of varying initial concentrations of carbon and nitrogen sources.

During sporulation process, vegetative cells were reported to differentiate into spores with synthesis of ICP. Hence, correlation of spores obtained with varying initial concentrations of carbon and nitrogen sources could indicate the effect of above nutrients on the differentiation process. The response surface and contour plot employing the obtained correlation are shown in figure 3.59. Relatively high spore count was observed with high initial concentration of carbon source. In particular, increase in nitrogen source has relatively insignificant influence on the yield of spores. It was also observed in batch cultivations that there was significant glucose left unconsumed after sporulation in the medium with less initial concentrations of yeast extract. In addition, accumulation of acidic intermediates could be high in the above cultivations during growth phase thereby contributing for enhanced spore formation. This indicated the possible flux of carbon source towards the components of spores leading to high spore yield. Also, this fact could substantiate the delay in release of spores observed in batch cultivations. Hence, delay in the release of spores could be due to the incomplete synthesis of spore components by the interference of high concentration levels of nitrogen source. The involvement of differentiation process on spore formation and ICP synthesis during the cultivation has necessitated the detail understanding of the
Figure 3.59 The response surface and contour plots for concentration of spores with varying initial concentration of glucose and yeast extract as C - source and N - source respectively in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant spore concentration.
influence of initial concentration levels of carbon and nitrogen sources on yield of spores.

With interdependent activities occurring during cultivation, flux of glucose towards ICP synthesis could be studied by the product yield coefficient, $Y_{ph}$. Hence, $Y_{ph}$ was correlated with varying initial concentrations of carbon and nitrogen sources by a second order polynomial equation. The response surface and contour plot of this polynomial equation are shown in figure 3.60. It was observed that increasing the initial concentration of carbon and nitrogen source has resulted in decreased $Y_{ph}$. In addition, rate of decrease in $Y_{ph}$ was observed to be relatively high at a particular ratio of carbon to nitrogen source. Further, $Y_{ph}$ was observed to be minimum near the concentration level of carbon and nitrogen sources needed for obtaining maximum cell density as discussed earlier. This clearly demonstrated the flux of metabolic resources towards biomass away from the synthesis of ICP. As it was observed, conditions in terms of initial concentrations of carbon and nitrogen sources favoring biomass synthesis could not result in enhanced ICP synthesis.

The analysis of data from batch cultivations in terms of varying initial concentrations of carbon and nitrogen sources indicated the dominant role of balanced nutrient flux towards biomass and subsequently towards spore components and ICP synthesis. Hence, it was decided to perform fedbatch cultivation of $Btg$ by need based addition of substrates with the developed fuzzy logic based feedback control system. This control system could provide a balanced flux of carbon source during cultivation thereby resulting in the enhancement of biomass and ICP synthesis. The optimum glucose concentration level for providing the balanced substrate flux will be decided on
Figure 3.60 The response surface and contour plots for product yield coefficient ($Y_{p/x}$) with varying initial concentration of glucose and yeast extract as C-source and N-source respectively in batch cultivations supplemented with salts and trace elements. The values inside the contour plot indicate constant product yield coefficient.
further analysis of the data obtained from batch cultivations with varying initial concentrations of glucose and yeast extract.

3.2.5 Fedbatch cultivation of *Btg* using fuzzy logic control

Balanced flux of nutrients at any desired level of residual glucose concentration could be provided with the developed control system. In particular, concentration level of glucose in the medium favoring balanced flux of carbon and nitrogen sources towards biomass and ICP synthesis has to be understood. In order to identify the residual glucose concentration needed to enhance the biomass and ICP synthesis, attention was focussed towards understanding the relationship between medium composition in terms of initial concentrations of glucose and yeast extract with specific growth rate and glucose utilization during the batch cultivations. During batch cultivations, microbial cultures were subjected to transient conditions due to continuously varying environment with progress in the metabolic activity. Hence, instantaneous growth rate of the culture and substrate utilization rate was observed to vary during the exponential growth phase. The major change considered in the external macroscopic environment was residual glucose concentration with respect to time due to its utilization by microbial culture.

As initial concentration of glucose and yeast extract at 3.4 g/l and 20 g/l respectively has favored high specific growth rate of 1.0647 during exponential growth phase of the batch cultivation, it was decided to understand the instantaneous growth rate and substrate utilization rate with respect to the residual glucose concentration. Cell density (X) and residual glucose concentration (S) data were taken from the batch cultivation carried out with above mentioned concentrations and correlated with time in terms of a higher
order polynomial using graphical software, GRAPHER 1.09. The polynomial for cell density with time was differentiated once with respect to time in order to obtain an expression relating the instantaneous growth rate of the culture with time \( \frac{dX}{dt} = f(t) \). Similarly, polynomial depicting the relationship of substrate utilization rate with time \( \frac{dS}{dt} = f(t) \) was obtained on differentiation. Simulations were carried out in MATLAB 4.0 employing the polynomials obtained for cell density, residual glucose concentration, instantaneous growth rate and substrate utilization rate by using time interval as 0.01 hours. The data obtained on simulation were stored in a data file thereby facilitating its retrieval and analysis.

On analysis of simulated data, instantaneous substrate utilization rate and growth rate was observed to be maximum at 1.5 g/l and 0.97 g/l of residual glucose concentration respectively. The high substrate utilization rate could have resulted in enhanced accumulation of metabolites and subsequent consumption of the same during the later stages of cultivation for different metabolic activities. Also, biomass formation was dependent on the flux of nutrients through metabolic network thereby reflecting the instantaneous growth rate as a resultant activity due to high substrate flux. Hence, it was decided to maintain the residual glucose concentration at the level of 1.5 g/l for facilitating enhanced glucose utilization in the bioreactor during fedbatch cultivation. The cultivation was started with initial concentration of glucose at 1.5 g/l and yeast extract at 20 g/l. Selected concentration of yeast extract at the level of 20 g/l was in accordance with the batch cultivation studied. In addition, salts and trace elements were added as mentioned in materials and methods. During the fedbatch cultivation, residual glucose concentration was maintained at 1.5 g/l by feeding sterile glucose solution at 80 g/l with the developed fuzzy logic based feedback control system. This external feeding was performed on
execution of the control action after sampling during the cultivation till the initiation of sporulation.

The above feed concentration was selected so that on total feeding (0.75 l) amount of glucose added during fedbatch cultivation could be 68 g for the operating volume of 1.0 l which was twice that of maximum concentration of glucose employed in batch cultivation. Feeding was carried out with the flowrate in ml/sec = 0.1779*V - 0.0345, where V indicates the voltage supplied to feed pump for a time period of 3 secs as specified in LabVIEW program. During feeding, flow rate of glucose was given by, g/sec = 2.22375*V - 0.43125. Also, during cultivation yeast extract was pulsed three times with the flowrate of 3.295 g/min from 4.5 hours at 10 minutes interval with pulsing period of 0.25 minutes. This has resulted in the addition of 0.824 g of yeast extract at the specified period. In addition, ammonium sulphate was also pulsed once for 0.25 minutes with the flowrate of 0.56 g/min at 5.67 hours resulting in the addition of 0.14 g. Concentration of yeast extract and ammonium sulphate in the feed were 80.0 g/l and 13.6 g/l respectively. The pulsing of organic and inorganic nitrogen sources were carried out to avoid any limitation during cultivation when the dissolved oxygen concentration was observed to be increasing. In particular, pulsing of these nitrogen sources could not result in significant change either in cell density or substrate utilization. There was no response even in the dissolved oxygen concentration profile.

The cell density and residual glucose concentration profiles during this fedbatch cultivation are shown in figure 3.61. This cultivation has resulted in 10.753 g/l of cell density with the sporecount of 11.0x10^{12} spores/ml. In particular, specific growth rate of the culture during exponential phase was observed to be 1.1963 with heterogenous population of cells during sporulation.
Figure 3.61 Profiles of cell density and residual glucose concentration during the fedbatch cultivation of *Btg* with residual glucose concentration maintained at 1.5 g/l in the bioreactor by feeding glucose solution with fuzzy logic based feedback control system. The arrows indicate the pulsing of nitrogen sources during cultivation. (YE – yeast extract; AS – ammonium sulphate)

Figure 3.62 Profiles of dissolved oxygen concentration (DO) and reactor weight during the cultivation of *Btg* with residual glucose concentration maintained at 1.5 g/l in the bioreactor by feeding glucose solution with control system.
resulting in the ICP concentration of 12.813 g/l. In addition, pH was observed in the decreasing trend till 3.5 hours of cultivation substantiating the occurrence of metabolic shift with decreased production of acidic intermediates.

Online monitoring of residual glucose concentration was carried out in the biochemistry analyzer using permeate from the filtration unit. Due to this, there was a possibility of biomass being concentrated and thereby reflecting in increased apparent cell density. Hence, weight of the reactor and its accessories were continuously monitored using the online data acquisition program written in LabVIEW. The dissolved oxygen concentration and reactor weight profiles during the fedbatch cultivation are given in figure 3.62. The weight profile of the reactor from the load cell indicated that there was a net increase in its weight. This clearly demonstrated that the cell density achieved was due to growth of the culture. In a significant observation, aeration and agitation were increased to 2 vvm and 1200 RPM respectively during the cultivation to avoid the condition of oxygen limitation for the growing culture. These conditions were relatively high in comparison with the batch cultivations. This could be due to the consumption of glucose as energy source through fermentative pathway thereby resulting in higher production of CO$_2$ by respiration. In addition, yeast extract could have been utilized for biomass formation resulting in relatively higher utilization of oxygen. Therefore, controlling the residual glucose concentration at 1.5 g/l could provide a balanced substrate flux wherein the high initial concentration of yeast extract has resulted in the redirection of glucose through fermentative pathway. Hence, enhanced biomass and ICP synthesis could not be achieved with this strategy in comparison with batch cultivations.

Since high initial concentration of yeast extract has resulted in the consumption of glucose as energy source, it was decided to reduce the initial
concentration of yeast extract to 1.0 g/l. Due to the change in yeast extract concentration, there could be variations in the optimum flux of nutrients during cultivation. Hence, data from batch cultivations with initial yeast extract concentration of 1.0 g/l were used for simulation studies as before to identify the conditions for optimum substrate flux. The residual glucose concentration and cell density data from batch cultivations were correlated with time in terms of a polynomial using GRAPHER 1.09. The obtained polynomials were used for simulation in MATLAB 4.0 with time interval of 0.01 hour. The maxima in instantaneous growth rate and substrate utilization rate with respect to residual glucose concentration were compared with the simulated data from batch cultivations with initial concentrations of glucose/yeast extract at 3.4/1.0, 18.7/1.0 and 34/1.0 respectively. In these cases, respective maxima during the cultivation with 18.7/1.0 and 34/1.0 was observed later in comparison with the cultivation carried out with 3.4/1.0.

On analysis of simulated data from the batch cultivation with initial concentration of glucose at 3.4 g/l and yeast extract at 1.0 g/l indicated that maximum in instantaneous growth rate and substrate utilization rate could be identified with the residual glucose concentration of 2.72 g/l and 2.2994 g/l respectively. During the above batch cultivation, maximum in instantaneous growth rate was observed earlier in time in comparison with that of instantaneous substrate utilization rate. Hence, flux of carbon source at this residual glucose concentration level could favor the biomass synthesis rather than being utilized as energy source. This identified balanced condition could be exploited for enhancing the biomass and ICP synthesis by fedbatch cultivation. Therefore, it was decided to start the cultivation with 2.72 g/l of glucose and 1.0 g/l of yeast extract. During this cultivation, residual glucose concentration was maintained at 2.72 g/l by feeding both glucose and yeast
extract at equal concentrations of 80 g/l. The flowrate of the feed solution containing glucose and yeast extract in ml/sec = 0.1779*V - 0.0345, where V indicates the voltage supplied to feed pump for a time period of 3 secs. The individual mass flow rates of each glucose and yeast extract were given by, g/sec = 1.112*V - 0.2116 so that the feed contains glucose and yeast extract in the concentration ratio of unity.

The cell density and residual glucose concentration profiles during this fedbatch cultivation are shown in figure 3.63. Pulsing of yeast extract for 0.25 minutes at 6 hours and ammonium sulphate for 0.25 minutes at 6.8 hours were carried out during the cultivation with the flowrate of 3.295 g/min for yeast extract and 0.56 g/min for ammonium sulphate. The yeast extract and ammonium sulphate concentrations in the feed were 80.0 g/l and 13.6 g/l respectively. Each pulse has resulted in the addition of 0.824 g of yeast extract and 0.14 g of ammonium sulphate. This pulsing of organic and inorganic nitrogen sources was carried out to understand the limiting conditions during this fedbatch cultivation. The dissolved oxygen concentration and reactor weight profiles during this fedbatch cultivation are given in figure 3.64. During the cultivation, aeration and agitation were increased to the maximum of 1vvm and 1000 RPM respectively to maintain the dissolved oxygen concentration above the limiting condition. This clearly demonstrated the effective utilization of glucose as carbon source with significant increase in its flux towards biomass synthesis. In addition, reactor weight profile from the load cell indicated that there was insignificant increase in its weight. This indicated that the enhanced cell density achieved during this fedbatch cultivation was due to the growth of the culture.

It was observed that the microbial population was uniform during the sporulation stage with uniform release of spores. This was in contrast to the
Figure 3.63 Profiles of cell density and residual glucose concentration during the fedbatch cultivation of \textit{Btg} with residual glucose concentration maintained at 2.72 g/l in the bioreactor by feeding glucose and yeast extract solutions at equal concentration with fuzzy logic based feedback control system. The arrows indicate the pulsing of nitrogen sources during cultivation. (YE – yeast extract; AS – ammonium sulphate)

Figure 3.64 Profiles of dissolved oxygen concentration (DO) and reactor weight during the cultivation of \textit{Btg} with residual glucose concentration maintained at 2.72 g/l in the bioreactor by feeding glucose and yeast extract solutions at equal concentration with control system
observation made in batch cultivations wherein mixed population of vegetative and sporulating cells was observed. Enhanced cell density of 15.992 g/l with spore count of 9.0.77x10^13 spores/ml was obtained during this cultivation. The specific growth rate during exponential growth phase was observed to be 0.688 with the final ICP concentration of 15.705 g/l. The observed specific growth rate was lower in comparison with the previous cultivation. This indicated a possible need of optimum specific growth rate for the enhanced flux of carbon source towards biomass synthesis and subsequently in spore formation and ICP synthesis. This optimum specific growth rate could favor a balanced metabolism during cultivation thereby enabling in the achievement of high cell density and ICP. In addition, external feeding was carried out till the initiation of sporulation and then it was continued as batch cultivation. This further indicated the need for a batch operation during sporulation process for attaining the ICP concentration. This fedbatch cultivation during exponential growth phase has resulted in enhanced cell density with favorable metabolic fluxes for the enhanced synthesis of ICP in subsequent phases of cultivation.

Due to the feeding of glucose and yeast extract on the execution of control action, growing culture would have prevented from being under the condition of inhibition by these components in comparison with the batch cultivations performed at high initial concentrations. This external feeding of glucose and yeast extract at constant ratio has made the culture to grow under balanced conditions substantiating the observations made in batch cultivations. Hence, the adopted fuzzy logic based feedback control system has facilitated in the achievement of high cell density by maintaining the glucose concentration at the optimum level during the fedbatch cultivation. In addition, this balanced flux could have resulted due to the supply of needed aminoacids on hydrolysis of yeast extract added. Further, the constituents of yeast extract could be
standardized and the role of balanced substrate flux in terms of these constituents towards enhanced biomass and ICP synthesis could be understood. This understanding could be employed towards the enhancement of biomass and ICP in complex sources as raw materials used in the production of biopesticides. This could be done by the comparison of fluxes of various basal compounds involved in nutrients used in the fermentation.