CHAPTER 2

MATERIALS AND METHODS

2.1 MICROORGANISM

*Bacillus thuringiensis* var. *galleriae* was obtained from the laboratory strain collection and maintained on Nutrient Broth (NB) agar plates at 4°C. The culture was also maintained as spore stock at −20°C. Culture maintained in NB agar plates was used for the inoculum.

2.2 CHEMICALS

Yeast extract, peptone and soyabean meal were obtained from HIMEDIA laboratories. Dextrose, ammonium sulphate, salts and trace elements were obtained from Merck. Corn steep liquor was obtained from a local company.

2.3 MEDIA AND FEED SOLUTIONS

Nutrient Broth (NB) medium: (g/l)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
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</tbody>
</table>

1.5% agar was added for Nutrient Broth agar.
Glucose Yeast extract (GY) medium: (g/l)

Glucose 3.4
Yeast extract 1.0
Ammonium sulphate 1.7
KH₂PO₄ 0.2
MgCl₂.6H₂O 0.04
CaCl₂.2H₂O 0.02
FeSO₄.7H₂O 0.0001
ZnSO₄.7H₂O 0.0001
NaCl 0.02
Trace elements 100 µl
pH 7.0

Trace elements stock solution (g/l): (Kuhn et al 1979)

MnCl₂.4H₂O 15.13
ZnSO₄.7H₂O 0.25
H₃BO₃ 2.5
CuSO₄.5H₂O 0.125
Na₂MoO₄.2H₂O 0.125
CoNO₃.6H₂O 0.23
H₂SO₄ 2.5 ml

GYA Medium: (Sachidanandham et al 1996)

GY medium containing following amino acids – 40 mg/l each of aspartic acid and glutamic acid and 20 mg/l each of threonine, tryptophan, glycine, valine, phenylalanine, cysteine, isoleucine.
Complex medium: (g/l)
Soyabean meal 16.0
Glucose 26.0
Corn Steep liquor 7.0
Sodium chloride 5.0

Feed solutions for fedbatch cultivation in bench scale bioreactor:
Glucose solution (0.75 l) 80.0 g/l
Yeast extract solution (0.25 l) 80.0 g/l
Ammonium sulphate solution (0.2 l) 13.6 g/l

Feed solutions for fedbatch cultivation in pilot scale bioreactor:
10 l of glucose solution at two different concentrations of 104 g/l (4X) and 650 g/l (25X).

2.4 BENCH SCALE BIOREACTOR

2.4.1 Cultivation conditions

Cultivations were carried out in a 1.5 l bioreactor (Bioengineering AG, Wald, Switzerland) with a working volume of 1.0 l. During cultivation, pH was controlled at 7.0 by the addition of 2N H₂SO₄ and 2N NaOH. Cultivation was started with 500 RPM as agitation and 1vvm as aeration. Dissolved oxygen concentration in the bioreactor was maintained above 30% of saturation by adjusting aeration and agitation manually. Temperature was maintained at 30°C by circulating chilled water at 15°C and heating by an in situ heater. Balance containing load cell was used for measuring the weight of the reactor and its accessories. Foam was controlled by the manual addition of 10% polypropylene glycol (PPG) as antifoam agent.
2.4.2 Inoculum development

A single colony of *Bacillus thuringiensis* var. *galleriae* from Nutrient agar plate was inoculated into 10ml of NB medium and incubated overnight at 30°C in static condition. This was used as seed inoculum for the next step. This culture was then inoculated into 100 ml of NB medium and incubated at 30°C for 5-6 hours at 125 RPM in a water bath shaker (ORBITEK). This was used as inoculum for the cultivation in bench scale bioreactor. 10% of preculture growing in the exponential phase was used in each stage of inoculation. Microscopic observation was carried out periodically to check the sterility of the culture.

2.4.3 Biochemistry analyzer

Biochemistry analyzer YSI SELECT 2730 (Yellow Springs Instruments Inc.) configured for online measurement was used for the online monitoring of residual glucose concentration in the bioreactor during cultivation. This biosensor based glucose analysis was carried out using the glucose oxidase enzyme immobilized in the membrane. This glucose oxidase enzyme oxidizes glucose into hydrogen peroxide and concentration of $\text{H}_2\text{O}_2$ was measured in an electrochemical cell. The current measurement from the electrochemical cell was found to be proportional to the glucose concentration. The membrane module consists of cellulose acetate and polycarbonate membranes in addition with the enzyme immobilized membrane for efficient operation. In addition, biochemistry analyzer was operated with a microprocessor installed in it. This facilitated the entry and storage of measuring parameters for subsequent calibration and analysis operations.
2.4.4 Filtration system

Online analysis of residual glucose concentration was carried out in the medium from which cells were removed by the filtration module. This further helps in preventing contamination from the sampling end and fouling of the immobilized enzyme membrane. The filtration module used was Spirosep 90TM, tangential flow filtration system. The exposed membrane area of the filter is 45 cm² and is made from polyethersulphone packed with a non-woven polypropylene matrix. The pore size of the filter membrane is 0.22 microns. The membranes have good thermal stability and low fouling characteristics. Being a tangential flow filtration, there is less concentration polarization with better performance.

2.4.5 Computer and accessories

Feed pump (Matson Warlow 503U) and biochemistry analyzer were interfaced with the computer (Digital Celebris GL 5133) through the DAQ channel board (AT-MIO-16DE-10, National Instruments) and interfacing hardware (NB-MID-32X, National Instruments). Data acquisition from bench scale bioreactor was also carried out with the above hardware. Graphical programming for Instrumentation, LabVIEW (National Instruments) was used for the development of data acquisition and control application programs.

2.5 PILOT SCALE BIOREACTOR

2.5.1 Cultivation conditions

Cultivations were carried out in the 300 l pilot scale bioreactor (Bioengineering AG, Wald, Switzerland) of 200 l working volume with all
available accessories and control systems. This bioreactor has a water jacket with heat exchanger and circulation pump to maintain the bioreactor temperature. This pilot scale bioreactor was modified for heat measurement studies. Sterilization was carried out with the steam generated from treated water in the steam generator installed by SPIC - SMO. LPG (Liquified Petroleum Gas) was used as fuel in the steam generator. Bioreactor temperature was maintained at 30°C using the chilled water available from the chilling plant installed by SPIC - SMO. Centrifugal pumps were used for the circulation of water through the bioreactor and chilling plant. Aeration was carried out at controlled conditions by measuring the airflow rate through magnetic flowmeter. The inlet air pressure was maintained at 2.0 bar by the pressure regulating valve at the air inlet. Compressed air was obtained from the single stage double cylinder reciprocating air-cooled compressor (ELGI). Foam was controlled by the addition of crude polypropylene glycol through a peristaltic pump on control signal from the level controller. Controllers for pH (Bioengineering AG) and agitation (SIEMENS SIMOREG) were used with pH controlled at 7.0 by the addition of 5N NaOH during cultivation.

Hygrometer (ROTRONIC AG) was installed in the air inlet and outlet as modification for measuring the temperature and humidity of air. Wattmeter for measuring the power consumed by stirrer, power source for temperature measurements and control unit for calibration heater were fabricated at EPFL, Lausanne, Switzerland. Further, modifications in cooling water jacket for installing temperature probes and flowmeter (Brooks Instrument, FISHER-ROSEMOOUNT) were done at EPFL, Lausanne, Switzerland. Temperature measuring element (RTD, Pt100) and its transmitter (RUEGER 82000) were used in the heat measurement studies. Exhaust gas analyzer (Bioengineering AG) containing CO₂ analyzer (BINOS 100) was used
for analyzing the CO₂ composition in the exhaust gas from fermentor. It was calibrated using the calibration gases of different CO₂ compositions. Peristaltic pump (Matson Warlow 503U) interfaced with the computer was used for feeding the external sterile solutions during fedbatch operation.

2.5.2 Computer and accessories

Data acquisition of all the possible parameters (25 Nos.) were carried out through the DAQ channel board (AT-MIO-64E-3, National Instruments) and interfacing hardware (NB-MID-32X, National Instruments) by the computer (Dell OptiPlex GX1). The entire program for data acquisition, calibration, calculation, storage and display were developed in the software, LabVIEW (National Instruments) as separate modules with the supervisory control of main (front) module.

2.5.3 Data acquisition and control

The transmitters in the pilot plant were connected to the computer by shielded cables. Data acquisition was carried out with the programs written in LabVIEW. 200 scans were performed to represent a data on acquisition with the scan rate of 2500 scans/sec. Calibrations were carried out with known indicated values to identify the slope and intercept for the linear correlation of acquired voltage with the actual value of variables. This was used to convert the acquired voltage in their respective channels into a real value of the variable. These online acquired values were continuously averaged (moving average) to avoid the pronounced influence of noise on data acquisition. These smoothened data were used in calculations involved in the program. The online acquired and calculated data were stored in a data file for its retrieval and analysis. The
above data were also displayed in the front panel of one of the modules for visual observation during cultivation.

2.5.4 Sterilization and startup

The pilot scale bioreactor was washed and rinsed with distilled water. The medium components were added into the bioreactor according to the specific requirements for the cultivation. The bioreactor and its contents were sterilized using steam at 4.0 bar pressure. Heat was transferred to the bioreactor through plate type heat exchanger available at the water jacket circulation unit. The bioreactor was maintained at 121°C for 20 mins. During sterilization, original temperature controller (Bioengineering AG) was used. After sterilization, bioreactor was cooled to the operating temperature using the cooling water at 15±5°C. When the bioreactor temperature reached 95°C, exhaust valve was opened to avoid the formation of vacuum during cooling down operation. When the bioreactor temperature reached 80°C, aeration was initiated with low flow rate to avoid any substantial loss of water from the bioreactor. When the bioreactor temperature reached the operating temperature of 30°C, temperature controller was switched to the cascade one executing from the digital computer.

2.5.5 Inoculum

Inoculum was prepared as mentioned previously in the initial steps. 100 ml of the exponentially grown culture was transferred into 1.0 l NB medium and incubated at 30°C for 5-6 hours at 125 RPM in a shaker (ORBITEK). This was used as an inoculum for 10.0 l NB medium in 20 l bioreactor (Bioengineering AG). Temperature, agitation and aeration were
maintained at 30°C, 250 RPM and 1 vvm respectively after inoculation in the 20 l bioreactor. The cultivation was carried out for 6-8 hours and the exponentially grown culture was used as inoculum for the 300 l pilot scale bioreactor. It was transferred into the bioreactor through sterile silicon tubing by means of a peristaltic pump.

2.6 OFF-LINE ANALYTICAL METHODS

2.6.1 Biomass estimation

Biomass concentration in terms of optical density was measured using spectrophotometer (Hitachi U2000). Cell dry weight was measured by filtering 10 ml of culture broth through a preweighed membrane (Zetapor, 0.22 μm). The membrane was washed with 5 ml of distilled water. It was then dried in the microwave oven at 500 watts for 2 minutes. The heated membrane was cooled down to the room temperature in a desiccator and its weight was measured. The weights of the membrane were measured in the micro-balance (Sartorius). The cell dry weight was obtained from the difference in weights measured. This cell dry weight measurement was correlated with optical density measurements of the culture broth. This correlation was found to be:

\[ \text{Cell density, g/l} = 0.1933 \times \text{O.D} + 0.0444 \]  

[R – squared: 0.9963]. In subsequent experiments, optical density was measured during the cultivation and cell dry weight in g/l was estimated from the above equation.

2.6.2 Glucose concentration analysis

The offline analysis of residual glucose concentration was carried out in the biochemistry analyzer (Yellow Springs Instruments Inc.). The principle of operation of biochemistry analyzer was described previously. The samples
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from the bioreactor were centrifuged (SORVALL RC – 5B plus) at 12000 RPM
for 10 minutes at 4°C. After centrifugation, supernatant was collected and
stored at -20°C. 25 μl of this sample was used in the analysis. The analysis was
carried out in duplicates and the average was taken.

2.6.3 Sporecount estimation

The sterile culture was heated to 70°C for 10 minutes in a water bath.
This heat-treated culture was diluted to different dilutions of the order of 10^{-13}
in saline solution (0.9% NaCl). 50 μl of this sample at various dilutions was
added and spreaded across NB agar in the petriplate. The plates were incubated
overnight at room temperature. The heat resistant spores will grow into a
colony on germination. Number of colonies obtained on incubation indicates
the actual number of heat resistant spores in the culture broth. This experiment
was done in duplicates with different dilutions of the initial sample. The
concentration of spores was estimated from the number of colonies observed,
amount of diluted sample and its dilution.

2.6.4 Protein estimation

The protein estimation was carried out according to the protocol of
Lowry et al 1951. The samples were solubilized with 0.1 N NaOH at room
temperature. 20 μl of the solubilized protein sample was mixed well with 4.5 ml
of freshly prepared alkali mixture (98 ml of 2% Na₂CO₃ prepared in 0.1N
NaOH; 1 ml of 2% Sodium potassium tartrate; 1 ml of 1% CuSO₄.5H₂O) and
incubated at room temperature for 10 minutes. Then, 0.5 ml of Folin phenol
reagent diluted with water in the ratio of 1:1 was added and incubated at room
temperature for 30 minutes at dark. The absorbance of the protein was
measured at 640 nm with the reagent as blank. A six point calibration curve was always carried out with BSA (Bovine Serum Albumin) as standard protein (1 mg/ml) along with other samples for which the protein concentration has to be measured.

2.6.5 Insecticidal crystal protein (ICP) quantification

The insecticidal crystal protein content was estimated using enzyme linked immunosorbent assay (ELISA) based quantification method (Xavier 1997; Smith and Ulrich 1983). The insecticidal crystal protein was purified from the culture grown in NB medium using NaBr density gradient centrifugation (Baumann et al 1985; Sachidanandham 1993). *Bacillus thuringiensis* var. *galleriae* was grown in 100 ml of NB medium till the spores were released into the medium. The culture broth was centrifuged (Sorvall RC - 5B plus) at 6000 RPM for 15 minutes at 4°C and the pellet was washed with cold 1M NaCl with 10 mM EDTA. The pellet was resuspended in 10 ml of cold 10 mM TE, pH 7.2 and sonicated (Labsonic U, B Braun). Sonication was carried out with the duty cycle of 0.7 for one minute in ice. This obtained free flowing suspension was layered over 20 ml of cold 48% NaBr solution (prepared in 10mM TE, pH 7.2) in corex tubes. Centrifugation (HITACHI HIMAC) was carried out with swing-out rotor at 9500 RPM for 1 hour and 30 minutes at 4°C. On centrifugation, crystals form a ring in the centrifuge tube and it was aspirated carefully. This fraction was washed with 10 mM cold TE to remove NaBr by centrifuging at 13000 RPM for 30 minutes at 4°C. Purified crystal protein was resuspended in 1.5 ml of 10 mM cold TE, pH 7.2 and stored at -20°C. The total protein estimated by the above mentioned method indicates the insecticidal crystal protein concentration. This purified protein was used as the standard in ELISA based quantification method.
Reagents

(i) Coating buffer: It was prepared from 1M NaHCO₃ (4.53% v/v) and 1M Na₂CO₃ (1.82% v/v). The final pH of the solution was adjusted to 9.6. The coating buffer was sterilized and stored at room temperature.

(ii) Substrate buffer: This contains 0.42 g NaHCO₃, 0.62 g Na₂CO₃ and 0.1 g MgCl₂ in 500 ml of the solution. The substrate buffer was sterilized and stored at room temperature.

(iii) PBS (Phosphate buffered saline): It was prepared as a stock solution by dissolving 40 g of NaCl, 1.0 g of KCl, 7.5 g of Na₂HPO₄ and 1.0 g of KH₂PO₄ in 5.0 l of distilled water. pH was adjusted to 7.2 - 7.4 with concentrated HCl and stored at room temperature.

Antigen (Insecticidal Crystal Protein) was diluted in the coating buffer in microfuge tubes. 50 μl of this antigen sample was coated per well in the 96 well ELISA plate (CORNING) in duplicates with different antigen concentrations. The plate was incubated overnight at 4°C. Then the plate was washed with PBST (PBS with 0.1% Tween -20) for 6-8 times and with PBS for 2 times in the ELISA plate washer (ELX 50, BIOTEK INSTRUMENTS Inc.). Antigen free sites on the well were blocked by the addition of 200 μl of 2% BSA (Bovine Serum Albumin) prepared in PBS per well and incubated at 37°C for 1 hour. The plate was washed with PBST (PBS with 0.1% Tween -20) for 6-8 times and with PBS for 2 times. 50 μl of diluted specific antibody (primary) for ICP raised in rabbit was added to each well and incubated at 37°C for 2 hours. The antibody was diluted in PBS containing 1% BSA. Then the plate
was washed with PBST (PBS with 0.1% Tween -20) for 6-8 times and with PBS for 2 times. 50 µl of the alkaline phosphatase conjugated goat anti-rabbit antibody (secondary) diluted in PBS containing 1% BSA was added to each well and incubated for 2 hours at 37°C. Then the plate was washed with PBST (PBS with 0.1% Tween -20) for 6-8 times and with PBS for 2 times.

PNPP (p-nitro phenyl phosphate, Sigma Chemicals) was used as the substrate for the enzymatic reaction mediated by alkaline phosphatase conjugated with secondary antibody. It was prepared in substrate buffer at the concentration of 1.0 mg/ml. 200 µl of the substrate solution was added to each well and incubated at dark for 20 minutes at 37°C. The enzymatic reaction was stopped by the addition of 50 µl of 3N NaOH per well. The absorbance was measured at 405 nm in the automated microplate reader (Labsystems Multiskan MS).

Initial standardization was carried out for identifying the linear concentration range of the antigen, dilutions of primary and secondary antibodies. ELISA was performed with varying dilutions of primary and secondary antibodies with the concentration of antigen in the operating range. The sensitivity was found to be relatively high with primary antibody at the dilution of 1:10000 and secondary antibody at the dilution of 1:5000. Hence, these dilutions were employed during the quantification of ICP in subsequent experiments. Further, linear range of the antigen concentration was found to be from 2 µg to 282 µg in 50 µl of the sample added. This antigen concentration range was used for the standard curve in the quantification of ICP from reactor samples. The standard curve gives a linear correlation between the antigen concentration and the absorbance at 405 nm. This was used to estimate the
unknown concentration of ICP in reactor samples from the measured absorbance values.

2.7 SAMPLE PREPARATION

The samples (culture broth) from the bioreactor were centrifuged at 12000 RPM for 10 minutes at 4°C. The pellet was washed with ice cold 1M NaCl with 10 mM EDTA. The pellet was resuspended in 1.0 ml of cold 10 mM TE, pH 7.2 and sonicated (Labsonic 2000, BeBraun). Sonication was carried out with the duty cycle of 0.7 for one minute in ice. Protein concentration was estimated as mentioned before. This sample on dilution to appropriate concentrations was used for the determination of insecticidal crystal protein (ICP) concentration by ELISA method.

2.8 LabVIEW

LabVIEW (National Instruments) is a program development and execution system designed for scientists and engineers, one of its primary applications being online data acquisition and control of complex processes. LabVIEW is the abbreviation for Laboratory Virtual Instrument Engineering Workbench. Further, LabVIEW is an object oriented graphical programming language that simplifies the design and implementation of complex control strategies using a graphical interface. The central concept of LabVIEW is the Virtual Instrument (VI), a functional unit which is symbolized as an icon and may be run on its own or as a subroutine in other higher levels VIs. Virtual instrumentation enables the coding of functions in software, removing the need to build dedicated new instruments. Interactions with the Vis occurs via the front panel with graphical representations of physical controls and indicators.
The front panel overlays the programming sheet with icons corresponding to the front panel controls and indicators. The programs in this graphical programming language defines the data flow and operations by linking icons and mathematical operators.

Extensive and successful applications of LabVIEW in online monitoring and bioprocess control was reported. Online control system for the cultivation of animal cells in a hollow fiber reactor using flow injection analysis was developed with LabVIEW (Stoll et al 1996). Control systems for continuous and fedbatch cultivations were also developed using graphical programming language (Kellerhals et al 1999; Turner et al 1994; Gregory et al 1994). Further, it was used in flux analysis of microbial metabolic pathways with its representations in software from individual reaction elements (icons) which are linked together to show potential flux routes (Regan and Gregory 1995). This was carried out in 3 Escherichia coli mutants with metabolic pathway deletions and insertions. These applications indicates the advantages of LabVIEW over the conventional programming languages for bioprocess monitoring, control and automation.