CHAPTER 2

MATERIALS AND METHODS

ACTERIAL STRAIN

*Agrobacterium radiobacter* NRRL B11291 used in the present study was sourced from Northern Regional Research Centre, Illinois, USA.

Culture maintenance

*A. radiobacter NRRL B 11291* was maintained as lyophilized stock and in glycerol at -20°C. The working stock was maintained on nutrient agar on or as stab.

HEMICALS

Fine Chemicals were procured from Sigma Chemical Company, St. USA. General chemicals were procured locally and were of analytical grade. DL-p-hydroxyphenylhydantoin was obtained from SPIC Pharma Ltd, Madras and Calac Pvt. Ltd. Molasses from Tamil Nadu Sugar Refineries Ltd. CSL was also a gift from SPIC Pharma Ltd.

INSTRUMENTS

Hitachi U2000 UV/Visible Spectrophotometer, Leitz Microscope, Hitachi RP-HPLC, Bioengineering AG Bioreactor, Orbital Water bath shaker
erological waterbath with temperature controller, Hitachi High Speed uge, Hitachi table top centrifuge.

**JLTURE MEDIA**

Various media were made modifying Olivieri's medium for various s.

**m I (Nutrient Medium)**

Consists of yeast extract 3g/L; Peptone 10g/L; NaCl 5g/L; pH 7.3 ed with NaOH.

**m II (Olivieri's Medium)**

Consists of Glucose 10g/L; Yeast extract 3g/L; KH$_2$PO$_4$ 13.6 g/L and 4.7H$_2$O 0.2 g/L and pH 7.0 adjusted with NaOH.

**m III**

Consists of Yeast extract 3g/L; KH$_2$PO$_4$ 13.6 g/L and MgSO$_4$.7H$_2$O 0.2 d pH 7.5 adjusted with NaOH.

**m IV**

Consists of Glucose 10 g/L; KH$_2$PO$_4$ 13.6 g/L; MgSO$_4$.7H$_2$O 0.2 g/L + 7.0 adjusted with NaOH.

**m V**

Consists of Yeast extract 3g/L; KH$_2$PO$_4$ 13.6 g/L and pH 7.5 adjusted aOH.
buffers

- Nitrate-Phosphate buffer 20mM, pH 4.0, 5.0 and 6.0 adjusted with dihydrogen orthophosphate.
- Lysine NaCl - HCl buffer 20mm, pH 4.0, 5.0 and 6.0 adjusted with 2N HCl.
- Tris - HCl buffer 20 mM, pH 6.0, 7.0, 8.0 and 9.0 adjusted with 2N HCl.
- Lysine NaCl - NaOH buffer 20mM, pH 8.0, 9.0, 10.0, and 12.0 adjusted with NaOH.

Methods

oculum Development

A single colony from nutrient agar (NB agar’s medium I) plate was added to 100 mL Erlenmeyer flask containing 10 mL of medium I. This was grown overnight in a water bath shaker at 30°C and 200 rpm.

The next day, the pre-inoculum was transferred to a 500 mL Erlenmeyer flask containing 100 mL of medium I. The actively growing culture at an OD between 0.8 to 1.0 was then used for production studies.

Shake flask studies

The actively growing seed inoculum was used to inoculate in 50 mL or 250 mL Erlenmeyer flask for various studies done in shake flask at 30°C and 200 rpm in water bath shaker.
2.5.3. Design of Experiments

Response surface methodology (RSM) an empirical modelling technique was used to evaluate the relationship between a set of controllable experimental factors and the observed results. RSM is used to determine the optimum response of the cells for the synthesis of hydantoinase and biomass, under a wide range of nutrient conditions. The design of experiments chosen for this study was full factorial central composite design (Box-Wilson 1951) for four independent variables to obtain the combination of values which optimized the response within the region of the three dimensional observation space, that allowed design of a minimal number of experimental runs. The advantage of this design was orthogonality, and this made all computation very simple, all coefficients were estimated independently of one another by using the results of all observations. All regression coefficients were estimated to the same variance, which is minimal and finally these designs had the property of rotatability. The model evaluated the effect of each independent variable to a response. The mathematical relationship of the independent variables and the response was calculated by the quadratic polynomial equation as given below.
EQUATION

\[ y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 \]  \hspace{1cm} (1)

The computation was carried out by multiple regression analysis making use of the least square method. The significant variables of the medium components selected were molasses, ammonium nitrate, sodium dihydrogen orthophosphate and manganese chloride based on the medium substitution studies.

2.5.4. Induction studies

D-hydantoinase was induced by its substrate and substrate analogues in shake flask in molasses medium.

2.5.5. Batch studies

The pre inoculum and seed inoculum was grown as mentioned earlier. The actively growing seed inoculum (OD600 nm 0.8-1.0) was used for batch experiments in bioreactors. The cultivation of \textit{A. radiobacter} was carried out at 30°C, pH 7.5, agitation of 1000 rpm and 1 vvm air flow rate.

2.5.6. Fed Batch studies

The fed batch studies were carried out in 3L bioeractor with a working volume of 2L. The additional nutrients were fed according to pre-designed
strategy based on the respiratory quotients and optimum specific growth rate. The feeding was started during the exponential phase of growth when the cell density reached to 2.0 at 600nm.

2.5.7. Biotransformation studies

Biotransformation of DL-p-hydroxyphenylhydantoin was carried out under nitrogen atmosphere to prevent oxidation of substrate.

Initial experiment were carried out in 10 mL volume in 100 mL stoppered conical flask, with various substrate and A. radiobacter whole cells (DCW) concentrations. The reaction mixture was sparged with nitrogen gas and incubated at 40°C in a water bath under mild shaking condition.

A two liter Bioengineering bioreactor was used for the biotransformation of hydantoin with working volume of 1.5 L. The reaction was carried out for 16 hours and the temperature was maintained at 40°C throughout the run at 700 rpm agitation and under nitrogen gas atmosphere by continuous sparging of nitrogen gas.

2.6. PURIFICATION OF D-HYDANTOINASE

2.6.1. Preparation of crude enzyme

A. radiobacter whole cells were suspended in 20 mM Tris-HCl pH 8.0 and was lysed using French Cell Press at 5000 psi. The cell lysate was centrifuged at 15000 g for 15 minute at 4°C and supernatant was stored at -20°C.
2.6.2. Fractionation of crude lysate with Ammonium Sulphate

The supernatant was fractionated. The protein was precipitated with various saturation of \((\text{NH}_4)_2\text{SO}_4\) fractions. Initially the supernatant was fractionated by slow addition of solid enzyme grade \((\text{NH}_4)_2\text{SO}_4\) to a final concentration of 0-30% (w/v : equilibration time of 1 h at 4°C). The protein was pelleted down by centrifugation at 15000 g for 20 minute. The supernatant was saturated to 70% (w/v). With an equilibration time of 1 h. The protein was pelleted at 20000 g for 20 minutes. The resulting pellet was suspended in 20 mM Tris-HCl pH 8.0.

2.6.3. Dialysis of 30-70% Ammonium Sulphate fraction of protein

The protein fraction after 30-70% ammonium sulphate saturation was dialysed against 500 mL of 20mM Tris - HCl buffer pH 8.0 for 24 hours with three changes of buffer. The dialysed sample was tested for the presence of ammonium sulphate by adding a small aliquot in saturated solution of barium chloride solution.

2.6.4. Heat Treatment

The protein extract from the salting out step at 70% (w/v) \((\text{NH}_4)_2\text{SO}_4\) saturation after dialysis was subjected to heat treatment at various temperature. First for 10 minute at 30°C, then the temperature was increased by step of 5°C with an incubation time of 20 minutes to a final temperature of 50°C. The heat
processed sample was cooled down to room temperature allowed to remain on ice for 2 hours and then centrifuged at 20000 g for 30 minutes at 4°C.

2.6.5. DEAE-Sephadex anion exchange chromatography

The heat treated sample was chromatographed on DEAE Sephadex A-50 anion exchange matrix (20 cm 3 cm bed volume) packed at a flow rate of 300 mL/h and was equilibrated with 20 mM Tris-HCl pH 8.0 buffer. The sample was applied at a flow rate of 180 mL/h and hydantoinase was subsequently eluted at a flow rate of 150 mL/h by 20 mM Tris-HCl pH 8.0 with a step gradient of 0.1 M to 1.0 M of NaCl.

2.6.6. Octyl-Sepharose CL4B hydrophobic-interaction chromatography

The DEAE-Sephadex column active fraction at a flow rate of 15 mL/h was applied onto an Octyl Sepharose CL-4B column (10 cm 1 cm bed volume) equilibrated with 20 mM Tris-HCl pH 8.0 containing 40% w/v (NH₄)₂SO₄. The hydantoinase was eluted with a decreasing gradient of (NH₄)₂SO₄ in 20 mM Tris-HCl pH 8.0 buffer.

2.7. BIOCHEMICAL ANALYSIS

2.7.1. Sugar Estimation

The residual sugar was estimated using Anthrone method (Gilbert Ashwell, 1957). The anthrone reaction is the basis of a rapid and convenient method for the determination of hexoses, aldopentoses, and hexuronic acid.
The blue-green solution of anthrone with sugar shows an absorption maxima at 620nm.

The anthrone reagent was prepared by dissolving 0.2 g of anthrone in 100mL concentrated sulphuric acid on ice. Glucose in the range of 5-50 μg per 100 μL was used as standard with 1mL of ice cooled anthrone reagent to estimate residual sugar in fermented broth.

2.7.2. Protein Estimation

The protein was estimated using dye-binding assay method of Bradford,(1975). The dye, Coomassie Blue G-250 when dissolved in a acid, turns red-brown colour due to protonation, but when it binds to positively charged protein, it turns blue due to a shift in the pKa of the bound coomassie Blue.

The dye was prepared by dissolving 100 mg of Coomassie Brilliant Blue G - 250 in 50 mL of absolute ethyl alcohol and adding 100 mL of 85% orthophosphoric acid. The dye was made upto 1L with distilled water and filtered to remove undissolved dye particles. Bovine serum albumin was used as a standard protein in the range of 1-20 μg/100 μL with 1mL of dye and incubated for 5 minutes before reading at 595nm.
2.7.3. Hydantoinase Assay

The hydantoinase assay was performed according to the method of Morin et al., (1987) with slight modification. The enzyme was assayed using 5.2mM of DL-p-hydroxyphenylhydantoin as substrate. The reaction mixture contained 0.1mL of toluene treated cells in 1mL of 20mM Tris-HCl (pH 8.0) buffer, 1mL of substrate and incubated for 30 minutes at 50° C under static condition. The reaction was terminated by the addition of 0.5mL of 12% trichloroacetic acid followed by 0.5mL of 10% p-dimethylaminobenzaldehyde in 6N HCl. The precipitated protein was removed by centrifugation and the amount of D-N-Carbamoyl-p-hydroxyphenylglycine formed was determined at 437 nm in a Hitachi U2000 spectrophotometer.

Enzyme activity was expressed as units per mL of culture. One unit of enzyme activity is defined as the amount of enzyme required to produce one micromole of N-carbamoyl-p-hydroxyphenylglycine per minute under assay conditions.

2.7.4. Estimation of D-N-Carbamoyl-p-hydroxyphenylglycine (D-NCHPG) and D-p-hydroxyphenylglycine (D-HPG)

The concentration of D-NCHPG and D-HPG were determined using high performance liquid chromatography according to the method of Deepa et al., (1993) with slight modification. The samples were filtered through 0.2 micron millipore filter and analysed on reverse phase C-18 chromatography column with a dimension of 0.6 mm id and 25 cm long analytical column. The samples
were eluted in isocratic mode and the mobile phase was water, methanol and tetrahydrofuran in the ratio of 96.6:2.4:1 with a flow rate of 1mL/min. The mobile phase and all the samples were degassed by sparging nitrogen gas before using for analysis. The quantitation and purity check was done at 210 nm using UV-VIS photodiode array detector.

2.7.5. Estimation of Dry Cell Weight

A required volume of culture was filtered through a preweighed 0.2 μm Zetapore membrane and dried at 80°C for 24 hours and weighed again to calculate the dry cell weight.

2.7.6. Analysis of exhaust gases from the bioreactor

Online exhaust gas analyses were carried out for oxygen and carbon dioxide with paramagnetic oxygen and infrared analyser respectively. The Oxygen utilization rate and Carbon dioxide production rate was calculated according to the method of Cooney et al.,(1977).