CHAPTER - III

MATERIALS AND METHODS

1] Enrichment and Isolation of microorganism

1.1 Sample Collection

Soil samples were collected from different areas like agriculture land, like Chikhalthana, Daulatabad, Shegaon, University, Nursery, pesticide, plastic and polymer industries. Similarly water sample from Lonar lake containing high salt concentration was collected. Total 60 samples were collected (Fig 1a &1b). Samples were brought to the laboratory for examination on the same day to avoid any changes in physical, chemical or biological characteristics. The samples were stored at 4°C in a refrigerator.

1.2 Enrichment

The enrichment culture technique as modified by (Bandopadhyay 1976) was used. Elective enrichment has been carried out by adaptation and acclimatization using basal salt broth containing (KH₂PO₄ 1.5 gm; K₂HPO₄ 3.5 gm; MgSO₄ .7H₂O 0.19 gm; Yeast extract 50 mg; Trace element; pH 7.5; Distilled water 1000 ml.) (Appendix I), Medium was sterilized at 121°C for 20 min. Benzonitrile 0.05% was added aseptically to sterile and cooled medium. Initially one gm of soil sample and one ml of water sample was used for pre enrichment. After initial incubation of 7 days at 30°C aerobically on rotary incubator shaker the culture broth was transferred at 5% (v/v) into fresh medium containing rise concentration of benzonitrile. The
process was repeated for a total four transfers by step by step raising the concentration of benzonitrile (0.05 to 0.2%).

1.3 Isolation:

After one month of acclimatization, the last enrichment culture flask was used to isolate microorganisms on basal salt agar containing 0.2% benzonitrile. Standard protocols were used to isolate bacterial, fungal and actinomycetal cultures. The morphology and grams nature for bacteria was identified by Gram’s staining, cotton blue staining for fungi and high power microscopic observation for actinomycetes. The pure cultures were maintained on selective medium like nutrient agar for bacteria, casein starch agar for actinomycetes and potato dextrose agar for fungi for healthy cell mass. The percent distribution of all types of isolates was diagrammatically represented using pie chart.

2. Screening of Isolates

2.1 PRIMARY SCREENING

Primary screening of all 111 isolates was carried out by growing all isolates into basal salt broth containing benzonitrile (0.2%) at 30\(^\circ\) c for 48 hours on rotary shaker. The screening was based on amount of ammonia produced which was estimated by Nesslers method. After 48 hrs samples were centrifuged at 10,000 rpm for 10 min. Cell supernatant was used for estimation of ammonia using Nesslers method. To one ml of cell supernatant, one ml Nesslers reagent was added. After addition brown colour was formed and ammonia was estimated at 500 nm. Ammonia concentration was determined using standard graph of ammonia (Appendix 2 Expt No.
2). Results were expressed in the form of number of microorganisms producing ammonia in specific range.

2.2 SECONDARY SCREENING

Seventeen bacterial, 11 fungal and 6 actinomycetes were screened based on maximum production of ammonia. Further screening was carried out by selecting strains giving maximum activity at either of three distinct pH viz. 4, 7 and 9. The methodology described below was used for selected bacteria, fungi as well as actinomycetes.

2.2.1 Preparation of intact cells

(100 ml) Basal salt broth was prepared and autoclaved at 121°C for 20 min. After sterilization 0.2% benzonitrile was added and was inoculated with selected bacterial, actinomycetes and fungal strains at 1% inoculums level. These flasks were incubated on rotary shaker at 100 rpm for 72 hrs. After 72 hrs cells were harvested by centrifugation at 10,000 rpm for 10 min. Washing of cell pellet was carried out using 5 ml sterile physiological saline. After centrifugation the cell pellet was suspended in 10 ml of saline and store in freeze and this intact cell suspension was used further to study various factors.

2.2.2 Effect of pH

Basal salt medium100 ml was prepread containing 0.1 % glucose and distributed as 10 ml in each tube. All tubes were sterilized at 121°C for 20 min. After sterilization to each tube benzonitrile was added at a concentration of 0.2 %. The broth inside
tube was adjusted to various pH viz. 4, 7 and 9 using the appropriate buffers and inoculum was added at 0.5% level. All tubes were incubated on rotary shaker at 100 rpm at 30°C for the 72 hrs. After 72 hrs. 10 ml sample was removed, subjected to centrifugation at 10,000rpm for 10 min. Ammonia was estimated from each supernaent by Nessleres method.

Based on above experimental data bacterial, fungal and actinomycetal cultures were selected giving maximum ammonia production at three distinct pH and used further for optimization of growth parameters.

2.2.3 Growth Optimization

To optimized temperature, nitrogen and carbon sources, to determine incubation period, substrate concentration etc. the intact cell of specific strains selected was used further. The protocol for intact cell preparation was same as mentioned above.

2.2.4 Effect of Temperature

Basal salt medium(100 ml) was prepread containing 0.1 % glucose and distributed as 10 ml in each tube. All tubes were sterilized at 121°C for 20 min. After sterilization to each tube benzonitrile was added at a concentration of 0.2 %. Intact cells were added at 0.5% (v/v) level. All these tubes were incubated at various temperature viz. 10°C to 60°C. for 72 hours. After 72 hrs.culture broths were centrifuged at 10,000 rpm for 10 min. Ammonia was estimated from each supernatent by Nessleres method.
2.2.5 Effect of Nitrogen sources

Basal salt medium (100 ml) was prepared and distributed 10ml quantity in each tube. All tubes were sterilized at 121\(^0\)c for 20 min. After sterilization benzonitrile at 0.2% level was added and then all tubes were inoculated using 0.5% (v/v) intact cell of all selected strains. Then tubes were supplemented with various nitrogen sources like tryptone, ammonium chloride, ammonium sulphate, malt extract, casein at 0.1% concentration. All tubes were incubated on rotary shaker at 100 rpm at 30\(^0\)c for the 72 hrs. After 72 hrs. 10 ml sample was removed, subjected to centrifugation at 10,000 rpm for 10 min. Ammonia was estimated from each supernatant by Nessleres method.

2.2.6 Effect of carbon sources

Basal salt medium (100 ml) was prepared and distributed 10ml quantity in each tube. All tubes were sterilized at 121\(^0\)c for 20 min. After sterilization benzonitrile at 0.2% level was added and then all tubes were inoculated using 0.5% (v/v) intact cell of all selected strains. The all tubes were supplemented various carbon sources (0.1%) like Maltose, Glucose. Lactose Fructose, Sucrose. All tubes were incubated on rotary shaker at 100 rpm at 30\(^0\)c for the 72 hrs. After 72 hrs. 10 ml sample was removed, subjected to centrifugation at 10,000 rpms for 10 min. Ammonia was estimated from each supernatant by nessleres method.

2.2.7 Effect of substract concentration

Basal salt medium 100 ml was prepared, to it glucose was added at 0.1% concentration and distributed 10 ml quantity in each tube. All tubes were sterilized at 121\(^0\)c for 20 min. After sterilization
benzonitrile ranging from at 0.1% to 1% level was added and then all tubes were inoculated using 0.5% (v/v) intact cell of all selected strains. All tubes were incubated on rotary shaker at 100 rpm at 30°C for the 72 hrs. After 72 hrs. 10 ml sample was removed, subjected to centrifugation at 10,000 rpm for 10 min. Ammonia was estimated from each supernatant by nessler's method.

2.2.8 Effect of Incubation Period

Basal salt medium 100 ml was prepared and distributed 10 ml quantity in each tube. All tubes were sterilized at 121°C for 20 min. After sterilization benzonitrile at 0.2% level was added and then all tubes were inoculated using 0.5% (v/v) intact cell of all selected strains. All tubes were incubated on rotary shaker at 100 rpm at 30°C for the 72 hrs. After 72 hrs. 10 ml sample was removed, subjected to centrifugation at 10,000 rpm for 10 min. Ammonia was estimated from each supernatant by nessler's method. After 48, 72, 96, 112 hrs samples were removed and same procedure applied.

3 Identification

The isolates selected after secondary screening were further identified by means of morphological, and 16/23s r RNA sequence analysis.

3.1 Identification of bacteria

The morphology of bacterial cultures was determined by performing Gram's staining. Further the Bacillus cultures were identified by 16 s r RNA analysis performed at NCCS, Pune.
3.2 Identification of Fungi

Identification of fungal strains F12, F18 & F19 was carried out at Department of Botany, Vasantrao Naik College, and Aurangabad.

3.3 Identification of actinomycetes

The Actinomycetes were identified by cover slip slide method in laboratory and then identified microscopically. Similarly the Actinomycetes were identified from Microbiology Department, Dr.B.A.M.U. Sub-center, Osmanabad.

4. TERTIARY SCREENING

The metabolic pathway for the screened and identified stains was studied to find out the intermediate of metabolism. The products of biotransformation were detected and confirmed by GLC and IR analysis. From analytical studies the strains which are showing one step biotransformation was selected.

The screened cultures used for further studies are:

Bacterial: 1. *Bacillus pumilus* (S15), *Bacillus mojenevisis* (S14), (C8)

Fungi: *Aspergillus fumigatus* (F12, F18, F19)

Actinomycetes: *Nocardia sp.* (A9), *Streptomyces sp.* (A11, A15)

4.1 Analysis by GC and IR

The isolated strains were cultured aerobically at 28°C for the 3 days. The cells were centrifuged, washed with physiological saline and suspended in 0.1M potassium phosphate buffer, pH 7.0. The reaction mixture for the screening of benzonitrile producing strains contained 100 μmole of potassium phosphate buffer, 300 μmole of benzonitrile as substrate, washed cells from 3 ml of culture broth in a
total volume of 1.0 ml. The reaction was carried out at 30°c for 1 hr. with moderate shaking and terminated by addition of 0.2 ml of 1 N HCL. The supernatant was separated by centrifugation and analyzed by GC and IR spectroscopy.

GC Conditions: The supernatant was analyzed with a Chemito Gas chromatography, Model GC -7610 equipped with flame ionized detector. The column used was stainless steel silicon 30, packed with porapackQ (80 to 100 mesh) operational conditions were: column temperature, 200°c; injection and detector temperature 151°c and 201°c. The carrier gas was N₂ at 40 cm³/min.

4.2 TLC studies for detection of benzamide

The above supernatants were separated by thin layer chromatography for detection of benzamide and benzoic acid. Silica gel (G) containing plates were spot inoculated and then they were placed on a chromatography chamber containing the solvent systems Chloroform: Butanol: Water (10: 25:65). The separation was carried out for 20 minutes. Then the plates were removed from the chamber and dried in an oven. Locating reagent Ninhydrin (0.2% in ethanol) was sprayed over the plate. The results were for development of brown color for presence of benzamide and violet color for benzoic acid.
5 LOCATION OF ENZYME

5.1 Cell lysate preparation

For location of enzyme the intact cells (0.5% (v/v) of *Bacillus pumilus*, *Aspergillus fumigatus* and *Streptomyces* spp. were inoculated into basal salt medium containing benzonitrile at 0.2% level, All flasks were incubated on rotary shaker at 100 rpm at 30°C . After 72 hrs. The cells were separated from culture flask by centrifugation at 10,000 rpm for 10 min. The pallets were washed twice and suspended in 5 ml of potassium phosphate buffer (0.05 M pH 7.5 and then sonicated using (10 times 10 sec at intervals of 50sec). The cell lysates were prepared by centrifugation. The cell supernatant and lysates were used as a source of enzyme and the enzyme assay has been carried out for benzonitrilase as mentioned below.

5.2 Enzyme Assay - To 50 micromole of potassium phosphate buffer (pH 7), 3 micromole of benzonitrile was added as substrate, And an appropriate amount of enzyme (0.45ml cell supernatant and lysates) in total volume of 0.5 ml and reaction was carried out at 30°C for,20 min. The reaction was terminated by addition of0.2ml of 1N HCl The supernatent was separated and analyzed for ammonia production using Nesslers method. Protein concentration from cell supernatant and lysates was determined by Lowry’s method.

Enzyme Unit: One unit of Benzonitrilase was defined as the amount of enzyme which catalyzed the formation of 1 micromole of ammonia per min.

Specific activity: Enzyme unit / mg of protein
6. OPTIMISATION OF FORMULATION MEDIUM FOR PRODUCTION OF EXTRACELLULAR BENZONITRILASE

6.1 Mixture Design Methods

Media components were optimized using mixture design method. The mixture design method was used to create the possible composition in the basal medium. In a mixture experiment, the independent factors were the proportions of different components in a blend. Total proportions of factors in a blend had to be 100%. The measured response of nitrilases production was assumed to be dependent on the relative proportions of components in the mixture. For preparation of various trials see table (Table No.56 Appendix II). Trials of different combinations were prepared as per given in table no. 26 and distributed as 10 ml aliquots into tubes. All tubes were sterilized at 121°C for 20 min. After sterilization benzonitrile at 0.2% level was added and then all tubes were inoculated using 0.5% (v/v) intact cell of all selected strains. All tubes were incubated on rotary shaker at 100 rpm at 30°C for the 72 hrs. After 72 hrs. 10 ml sample was removed, subjected to centrifugation at 10,000 rpm for 10 min. The crude supernatant was used as enzyme source. Ammonia was estimated as end product of benzonitrilase assay by Nesslers method. The results were expressed as enzyme unit (umole/min).

7. Strain Improvement Strain improvement has been carried out by random mutation using uv radiation.

By serial dilution technique a appropriate dilution of each culture was selected and used for uv mutagenesis. The selected dilution was exposed to uv radiations for specific time period as mentioned in the table and then each sample was inoculated into basal
salt broth containing benzonitrile and incubated on rotary shaker for 48 hours. The amount of ammonia was then estimated. Efficiency of strain improvement was determined.

8. Production, Purification of benzonitrilase from Bacillus pumilus S15, Aspergillus fumigatus F19 & Streptomyces sp. A15

Enzyme production at flask level has been carried out using formulation medium optimized by Placket-Burmann and mixture designed. The intact cells of wild and mutant types of all three cultures viz. Bacillus pumilus S15, Aspergillus fumigatus F19 & Streptomyces sp. A15 has been prepared by protocol mentioned earlier. Fermentation has been carried out aerobically on rotary incubator shaker at 30°C for 72 hours using 5% v/v inoculum. The fermentation was terminated and cells were separated from fermentation liquor by centrifugation at 10,000 rpm for 10 min. As the location of enzyme was extracellular in case of Bacillus pumilus and Aspergillus fumigatus the supernatant was subjected for sequential purification of benzonitrilase.

In case of Streptomyces sp. the enzyme was located intracellular therefore first the cell lyzates were prepared as mentioned earlier. Then the lysate was further subjected for purification of benzonitrilase.

8.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation has been carried out by raising the concentration of salt from 40 % to 90%. Every time the
samples were incubated at 4°C for 10 min. Then the precipitate was collected and supernatant was subjected to reprecipitate using elevated level of salt. The precipitate was dissolved in phosphate buffer (pH 7 and 0.05M). The enzyme activity was determined for each fraction. Lastly all fractions were pulled together and enzyme activity was determined. For each fraction the protein has been estimated by Lowery’s method. The pulled fractions were subjected for dialysis.

8.2 Dialysis

The dialysis was carried out against potassium phosphate buffer (0.05M & pH 7) at 4°C overnight and then coentrated on sucrose gradient. The enzyme activity and protein concentration was estimated from dialysed extract.

9. Molecular studies of purified enzyme

9.1 Spectral studies

The spectral analysis has been carried out to confirm and checks the purity of enzyme-protein. The concentration of enzyme was determined colorimetrically (FL) and spectroscopically.

9.2 The molecular weight was determined by SDS-PAGE

SDS Page Analysis

Preparation of reagent sees in Appendix II

Procedure -

A) Pouring the SDS- polyacrylamide gels –

1) Assemble glass plates properly by using three spacers.

2) The assembly is sealed by using 15 agarose solution and clamps
3) Prepared resolving gel mixture and fill it into gap between glass plates. Leave sufficient space for stacking gel

4) After polymerization of resolving gel, pour iso butanol on this layer.

5) After 10 min. pour off over lay of iso- butanol and washed top of the gel with deionised water to remove any unpolymerised acryl amide.

6) Prepared stacking gel mixture and pour directly on to the surface of polymerized the resolving gel.

7) Immediately inset a clean comb into a stacking gel solution being careful to avoid trapping air bubbles.

8) Add more stacking gel solution to fill space of comb completely.

9) Place gel in vertical position at room temperature.

B) Preparation of sample and running the gel:

1) Add 10 μ/lit of sample with 40 μ/lit of sample loading buffer.

2) Hit the suspension in boiling water bath for 5 min.

3) After polymerization of stacking gel is over remove the Teflon comb carefully.

4) Mount the gel in Electrophoresis apparatus.

5) Add Tris-glysin electrophoresis buffer to the top and bottom reservoirs.

6) Remove any bubbles that become trapped at the bottom of the gel between the glass plates.
7) Load up to 15 μ/lit of each of the samples in a pre determine order into the bottom of the wells. This is the best done with Hamilton μ/lit syringe or a micro pipettor equipment with gel loading tips with wash with buffer from the bottom reservoir after each sample is loaded.

8) Attach the electrophoresis apparatus to an electric power supply. The positive electrode should be connected to the bottom reservoir.

9) Apply current of 100 V to the gel.

10) After the dye front has moved into the resolving gel, increases the voltage to 150 V and run the gel until the bromophenol blue reaches the bottom of resolving gel.

11) Turn off the power supply.

12) Remove the glass plates from the electrophoresis apparatus and paced them on a paper towel.

13) Carefully remove the plates apart.

14) Mark the orientation of the gel by cutting a corner from the bottom of the gel that is closest to the left most well.

C) Staining and destining of the gel:

1) Stain the gel by using the staining solution which consist of brilliant blue 100mg, methanol 40 ml. glacial acetic acid 10 ml and distilled water for 2 hours.

2) After 2 hours distaining of the gel is carried out by distaining solution contain methanol 40 ml, glacial acetic acid 10 ml and distilled water 50 ml.
3) Band was observed.

10. Immobilization of Enzyme by Entrapment

The whole cells and purified enzyme from wild and improved strains of *Bacillus pumilus S15*, *Aspergillus fumigatus F19*, *Streptomyces* sp.A15 were used for immobilization studies. The immobilization has been carried out by cell entrapment using 3% sodium alginate.

**Procedures**

1. Dissolved 30 gm of sodium alginate in 1 litre to make a 3% solution.

2. Mixed approximately 0.015 gm (w/v) of enzyme/whole cells with 10 ml of 3%(wt.) sodium alginate solution achieved desired hardness.

3. The beads are formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2M CaCl2 solution with a syringe and a needle at room temperature. The bead size can be controlled by pump pressure and the needle guage. A typical hypodermic needle produces beads of 0.5 – 2 mm in diameter. The beads were suspended in the calcium solution to cure for 0.5 – 3 hours.

11 Comparative Kinetic studies

The comparative kinetic studies has been carried out using free enzyme and immobilized cells and enzyme from wild and improved strains of *Bacillus pumilus S15*, *Aspergillus fumigatus F19*, *Streptomyces* sp.A15. The benzonitrilase assay has been performed to
optimized pH, temperature, substrate concentration and vmax and kM. The impact of immobilization on benzonitrilase activity was studied on wild and improved strains.

11.1 Optimization of pH

The pH activity profile of the partially purified enzyme of three different sources using three different states was studied. The buffers of various pH were used viz. Citrate buffer (pH range 3.0-4.0), Citrate phosphate buffer (pH range 5.0-6.0), Phosphate buffer (pH range 7.0-8.0), and glacial sodium hydroxide (pH range 9.0-10.0). The details of all buffer is given in Appendix II. The standard reaction mixture consisted of 50, umol of respective buffer containing 3 umol of benzonitrile, and an appropriate amount of enzyme (Free enzyme 0.45 ml, 4 beads Immobilized whole cells and enzyme) in a total volume of 0.5 ml. The reaction was carried out at 30°C for 20 min on rotary shaker. The reaction was terminated by addition of HCl (1N of 0.2 ml). The supernatant was separated and analyzed for ammonia production using Nesslers method.

11.2 Optimization of Temperature

As above, the optimum temperature was also determined at which maximum activity of respective enzyme was observed. The activity of enzyme was measured at different temperature ranging from 10°C to 80°C. The standard reaction mixture consisted of 50, umol of selected optimum buffer containing 3, umol of benzonitrile and an appropriate amount of enzyme (0.45ml/4 beads) in a total volume of 0.5 ml. The reaction was carried out at respective temperature for 20 min on rotary shaker. The reaction was terminated
by addition of HCl (1N of 0.2 ml). The supernatant was separated and analyzed for ammonia production using Nesslers method.

11.3 Optimization of Subtract concentration

The buffered substrate of various concentrations was prepared and then the enzyme assay has been carried out as above. The substrate concentration was optimized and then the rate of reaction was determined by calculating \( K_m \). (See in table no.

12 In Ex-situ lab Studies using effluent from pesticide industry

12.1 Pretreatment of effluent.

The effluent (1 Lit.) has been collected from pesticide industry containing residual amount of pesticide. The pretreatment has been given to effluent to remove debris and color. The initial color of effluent was black. It was depigmented by treatment with charcoal followed by filtration using whatmann filter paper.

12.2 Physico-Chemical analysis of effluent

The treated effluent was analyzed to study its various physico-chemical parameters. The physicochemical analysis has been carried out from Government water analysis lab, Aurangabad. The parameters studied were pH, Total solids, Total dissolved solids, and Total suspended solids, Biological oxygen demand, Chemical oxygen demand, Total acidity, and Total hardness, Acidity, Alkalinity and Ammonia.
12.3 HPLC Analysis

The effluent was initially analyzed for detection of benzonitrile using HPLC. The HPLC analysis has been performed in Jeevanrekha Analytical Services, Aurangabad. The HPLC conditions used are given below.

**HPLC Conditions:**

Analysis of references compounds and products were carried out using a Milton Roy 4000 HPLC with a C18 Nuclisil ODS 5μm, 25 cm×4.6 mm Jones chromatography column with a 3 cm× 4.6 mm Nuclisil ODS 5μm guard column. The parameters used for the detection of the compounds were wavelength of 254nm, flow rate of 1.5 ml min⁻¹, a mobile phase between 25-35 % (v/v) phosphoric acid in distilled water. The mobile phase was degassed by helium for 15 min. Injection of 20μl 1:2 dilutions of the supernatant were used and standard curves were constructed for available compounds. Results were recorded in (Fig No.35)

13. In Situ Benzonitrile biotransformation using effluent

13.1 Packing of column using immobilized whole cells

Chromatography Column was fixed to a stand and packed the bottom of column with glass wool. Then the entire column was packed with immobilization beads of benzonitrilase produced from three distinct microbes’ viz. *Bacillus pumilus* S15, *Aspergillus fumigatus* F19 and *Streptomyces* Sp.A15. Results were recorded in (Table no.51) and (Fig No.36)
13.2 Effluent treatment

Then treated effluent was allowed to react with enzyme in immobilized form by passing through column. After an interval of 10 min. 1 ml sample was removed from outlet of column and was used for estimation of ammonia formed by reaction between enzyme and benzonitrile present in the effluent. The samples showing highest production of ammonia were subjected to HPLC analysis for confirmation. The results were recorded in (Fig No.37,38,39)