3.0 MATERIALS AND METHODS

3.1 Enrichment and isolation of microorganisms

a. Sample collection

Soil samples were collected from different areas like agriculture land and
surrounding areas of pesticide, plastic and polymer industries, Himayat Baugh Nursery,
Aurangabad. B. A. M. University campus, Aurangabad. Chikhalthana, Daulatabad,
Waluj. Similarly water sample was collected from hypersaline Lonar Lake. A total 78
samples were collected and 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.

b. Enrichment

Enrichment was performed by successive sub-culturing of soil samples. One gram
of soil sample was used as the inoculum suspended in mineral salt glucose (MSG)
medium (kulkarni, et. al., 2005) containing glucose, 4; K₂HPO₄, 0.65; KH₂PO₄, 0.2;
MgSO₄. 7H₂O, 0.09 and FeSO₄, 0.01gL⁻¹ (pH 7. 3). Medium was sterilized at 121°C for
20 min. in 250 ml Erlenmeyer flask and 100 ppm of P-nitrophenol (PNP) was added
aseptically to the sterilized and cooled medium. The flask was incubated at 30°C on
rotary shaker. After 7 days 2ml of this culture was transferred to 100 ml of fresh medium.
The process was repeated for a total four transfers step by step.

c. Isolation

After one month of acclimatization, the last enrichment culture flask was used to
isolate microorganisms on mineral salt agar containing 100 ppm P-nitrophenol. Standard
protocols were used to isolate bacterial, fungal and actinomycetal cultures. The
morphology and grams nature for bacteria was identified by Gram’s staining,
Lactophenol 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.

3.2 Screening of isolates

a. Primary screening

The primary screening was carried on the basis of ability of the culture to grow in presence of PNP (100 ppm). The PNP degrading microorganisms were selected on the basis of change in the colour of the medium from yellow to colourless.

b. Secondary screening

Secondary screening was carried out on the basis of rate of biotransformation of PNP. For this purpose all the selected isolates were inoculated in to MSG broth containing PNP (100 ppm) and after 48 hours of incubation % degradation of PNP and production of nitrite was measured. The PNP concentration (µg/ml) and nitrite production (µg/ml) was estimated spectrophotometrically at 400 nm (λmax of PNP) and 520 nm (Trivedi and Goel, 1986) at 0 hr and 48 hrs respectively.

c. Intact cell preparation

MSG broth containing 100 ppm of PNP was inoculated with selected bacterial, actinomycetal and fungal strains at 1% inoculum level. These flasks were incubated on rotary shaker at 100 rpm for 48 hrs. After 48 hours cells were harvested by centrifugation at 1000 rpm for 10 min. The cell pellet was washed with physiological saline to remove the traces of growth medium. These intact cells were suspended in saline and were used further as inoculum to study growth parameters.

From the selected 15 bacterial strains, most efficient strain B15 was screened based on maximum degradation (% degradation in terms of PNP). From amongst the selected 4 actinomycetal strains, the most efficient strain A5 was screened. While in case of 4 fungal strains, the most efficient strain R8 was screened based on maximum degradation of PNP. These strains were used further to study growth parameters like
optimization of pH, temperature, growth profile activity, substrate concentration, and effect of carbon and nitrogen sources.

3.3 Detection of intermediates of biotransformation

The metabolic pathway for the screened strains B15, A5 and R8 were studied to find out the intermediate of metabolism. The products of biotransformation were detected and confirmed by TLC and HPLC analysis.

3.3.1 Detection of enzymes by tracing the metabolic pathway

Identification and quantification of intermediates was performed based on UV-visible spectra, Thin layer Chromatography and HPLC analysis by comparison with the standards. To examine intermediates of PNP biotransformation by three culture strains B15, A5 and R8. The culture supernatant was collected, acidified with 1ml of 6 M HCl, and extracted twice with 100 ml of Chloroform;diethyl ether (1:1, v/v).

The organic extract was evaporated to dryness at 20ºC and the residue dissolved in 2 ml of methanol for TLC and HPLC analysis.

3.3.1.1 Thin layer chromatography (TLC)

20 µl of the concentrated extract was applied to silica gel TLC plate (0.25 mm layer of silica gel, Merck, India),which was developed in benzene:dioxane:acetic acid (60:36:4, v/v). After drying, the plate was sprayed with freshly prepared ferric chloride: potassium ferricyanide reagent. Rf values of PNP and its metabolites were compared with those of the authentic PNP, hydroquinone and 4-nitrocatechol standards (Lueng, et. al., 1997).

3.3.1.2 HPLC analysis

The HPLC analysis was carried out using a model equipped with a photodiode array detector system. The compounds were separated on a BDSC18 (250×4. 6 MMID×5µM) silica column using 1% glacial acetic acid in methanol and 1% glacial acetic acid in HPLC grade water at a ratio of 80:20 as the mobile phase. Flow rate was 1.
0 ml/min; injection volume was 15 ul and the compounds were detected in the range 220-400 nm. Under these conditions, samples were compared with authentic PNP, hydroquinone and 4-nitrocatechol (Arora, 2012).

The metabolic pathway was traced on the basis of intermediates.

### 3.3.2 Location of enzyme

Based on the results of metabolic pathway amongst bacteria, actinomycete and fungi B15, A5 and R8 strains were selected for further studies, the enzyme activity was estimated in cell supernatant as well as cell lysate.

#### 3.3.2.1 Cell lysate preparation

The actinomycetal cell mass thus obtained was initially washed with sterilize physiological saline. Repeated washing was done to obtain cell mass free from medium components.

After obtaining the cell mass, the cells were suspended in sterile saline and were sonicated at 7 Hertz for 10 min. in cold condition. After sonication, the cells were then again centrifuged to obtain the debris free cell lysate which was further used for as a source of intracellular enzyme.

#### 3.3.2.2 Enzyme unit

One unit of enzyme activity was defined as the amount of enzyme required for the disappearance of 1µmol of substrate (PNP) per minute.

#### 3.3.2.3 Specific activity

The decrease in the A400 (corresponding to the \(\lambda_{\text{max}}\) of PNP) was monitored on spectrophotometer and from the protein content of the assay solution, the specific activity of the enzyme was calculated and expressed in micromoles of PNP degraded per minute per mili gram of protein.
3.3.2.4 Enzyme assay

Cell supernatant and cell lysate were used as a source for extra and intra cellular enzyme for monooxygenase assay. The monooxygenase assay were carried out at room temperature in a quartz cuvette in a total volume of 1 ml. The standard assay contained 0.1 mM PNP, 0.4 mM NADPH, and 4 mM MgSO$_4$ plus enzyme in 20 mM phosphate buffer (pH 7.5), (Zeyer, et al., 1988).

3.3.2.5 Protein estimation

Protein content was determined by using Biuret method. Bovine serum albumin was used as a standard for estimating proteins.

3.4 Identification of the efficient PNP degrading isolates

The selected isolates (B15, A5 and R8) were identified by means of various morphological, physiological and biochemical characterization tests using the standard procedures (Cappuccino and Sherman, 2004; Bergey’s Manual of Systematic Bacteriology edition IV). Various biochemical tests for the identification of the potent isolate are as follows: Sugar utilization, temperature tolerance, NaCl resistance, pH tolerance, starch hydrolysis, urea hydrolysis, catalase production, H$_2$S production, nitrate reduction test, oxidase test, IMViC test, resistance to antibiotics (Ningthoujam et al., 2012).

3.4.1 Biochemical test for B15 and A5

a. Catalase production

Test cultures were grown freshly on Trypticase soy agar slants on to which were added a few drops of 3% (v/v) hydrogen peroxide. Culture tubes were observed for the formation of nascent oxygen in the form of bubbles which was indicative of a positive reaction for catalase production.
b. Citrate utilization

This test was carried out in Simmon’s citrate medium. Individual test cultures were streaked on to the prepared slants. Inoculated slants were incubated for 24-48 hr at 37°C. Positive reaction for citrate utilization by the culture was indicated by change of colour of medium to deep blue (alkaline reaction).

c. Gram’s stain

The heat fixed smear of the individual presumed bacterial and actinomycetal isolates prepared on a clean glass slide was stained with crystal violet for 1min, followed by washing off excess stain with water. Then Lugol’s iodine solution was added and allowed to react for 1 minute. After washing off iodine with water, the smear was treated with 95% ethanol for 30 seconds so as to remove the excess crystal violet. Finally the smear was counter stained with safranin, allowed to react for 30 seconds, washed with water, dried and examined under oil immersion of a compound microscope. Gram positive cells appear as violet colored, while gram negative cells as pink colored. Beside, the cell morphology was also recorded.

d. Indole production

Individual test cultures were inoculated into prepared tryptone broth tubes and incubated for 24 hr at 37°C. To each of the inoculated tubes was added 0.2-0.3 ml of Kovac’s reagent. The formation of a dark red color in the surface layer of the culture broth indicates a positive test for indole.

e. Methyl Red and Voges Proskauer (MR-VP) reaction

These two tests were performed using MR–VP broth. Test cultures were inoculated individually into the broth medium and incubated for 24 hr and 37°C. The culture broth was divided into two parts, one part was used for MR reaction and the other for VP reaction.
f. H₂S Production

This test enables the determination of the ability of the organism to produce H₂S from substrates such as the sulfur-containing amino acids or inorganic sulfur compounds. In this test, the SIM medium contains peptone and sodium thiosulphate as the sulfur substrates; ferrous ammonium sulphate, Fe (NH₄)₂SO₄, which behaves as the H₂S indicator; and sufficient agar to make the medium semisolid and thus enhance anaerobic respiration. The organism was inoculated in the SIM medium tube by means of stab inoculation and incubated for 24 hours.

g. Nitrate reduction

Individual test culture was inoculated into the prepared nitrate broth and incubated for 24 hr at 37°C. To 5ml of the 24 hr old culture was added two drops each of Solution 1 and 2 of the nitrite testing reagent development of orange/ brick red color indicated nitrate reduction to nitrite.

h. Oxidase test

Oxidase enzyme plays a vital role in the operation of the electron transport system during aerobic respiration. Cytochrome oxidase catalyzes the oxidation of a reduced Cytochrome by molecular oxygen (O₂), resulting in the formation of H₂O or H₂O₂. A single line streak inoculation of the organism was done on the solid agar surface of trypticase soy agar. After 24 hours incubation at 37°C, two to three drops of tetramethyl-p-phenylenediamine dihydrochloride was added to the surface the growth of the organism. The presence of oxidase enzyme was checked by observing the color change from pink to maroon and finally to black.

i. Starch hydrolysis

A loopfull of 20 h old broth culture of individual organisms was spotted onto portions of pre-poured plates of plate count agar with 1% soluble starch and incubated at 37°C for 24-48 hr. Incubated plates were exposed in a glass chamber saturated with iodine vapors, to read for the positive/negative action on starch.
j. Urease production

The presence of urease is detectable when the organisms are grown in a urea broth medium containing the pH indicator phenol red. As the substrate urea is split into its products, the presence of ammonia creates an alkaline environment that causes the phenol red to turn to a deep pink. This is a positive reaction for the presence of urease. Failure of a deep pink colour to develop is evidence of a negative reaction.

k. Antimicrobial activity

Antimicrobial activity of the selected isolates *Streptomyces coeruleorubidus* (A5) against various microorganism was carried out e. g. *S. aureus*, *Bacillus subtilis*, *E. coli*, *P. aeroginosa*, *Salmonella abony*, *A. niger*, *A. flavus* etc.

l. Antibiogram of the isolates

*Streptomyces coeruleorubidus* (A5) antibiotics resistance or senstivity was checked by Ampicillin, Vancomycin, Gentamycin, Methicillin, Oxacillin, Teicoplanin, Rifampicin, Amoxycillin, Penicillin, Cefpodoxime, Streptomycine.

3.4.2 Genotypic analysis of the isolates B15 and A5

Morphological identification of bacterial culture was determined by performing Gram’s staining. Further it was identified by 16S rRNA analysis performed at NCCS, Pune, India. Sequence data was aligned and analysed for finding the closest homologous microbe.

Fungal strain R8 was identified at the Department of Botany, Vasantrao Naik College, Aurangabad.

Coverslip and slide culture technique was used to identify the actinomycetal culture in laboratory. The actinomycete culture was grown on starch casein agar plate containing coverslip inserted into the agar medium at an angle about 45°. The culture was allowed to grow over the coverslip. After incubation period of two days, coverslip was removed and placed on a slide for morphological observations. Further it was identified
Studies on Microbial Biotransformation of Para-nitrophenol

by 16S rRNA sequence analysis performed at Xcelris Laboratories, Ahmedabad, Gujarat, India.

Sequence analysis results of actinomycetal isolate was recorded in form of the aligned sequence, distance matrix based on nucleotide sequence homology and phylogenetic tree using neighbor joining method.

3.5 Optimization of physiochemical parameters for maximum growth and efficient biotransformation

3.5.1 Optimization of incubation period

MSG broth containing PNP (100 ppm) was prepared and inoculated with 1% inoculum. The flasks were incubated at 37°C for varying period of incubation time viz. 0, 24, 48 and 72 hrs on a rotary shaker at 100 rpm, and the rate of PNP degradation and nitrite production was measured by standard method as described by Trivedi and Goel (Chemical and Biological Methods for Water Pollution Studies, 1986).

3.5.2 Optimization of pH

MSG broth containing PNP (100 ppm) adjusted to various pH viz. 4 to 9 was prepared and inoculated with 1% inoculum. All flasks were incubated at the optimum incubation time (48 hrs) on rotary shaker at 100 rpm for 48 hours, 10 ml sample was removed, subjected to centrifugation at 1000 rpm for 10 min. and growth observed at 600 nm. The rate of PNP degradation and estimation of nitrite was measured by standard method as described above.

3.5.3 Optimization of temperature

MSG broth containing PNP (100 ppm) adjusted to the optimum pH was inoculated with 1% inoculum of the respective culture and the flasks were incubated at varying temperature viz. 20°C to 60°C at the optimum incubation time (48 hrs) on rotary shaker at 100 rpm and the rate of PNP degradation and nitrite production was measured by standard method as described above.
3.5.4 Optimization of concentration of PNP

MSG broth containing various concentrations of PNP, viz. 100 ppm to 800 ppm was prepared, adjusted to optimum pH and inoculated with 1% intact cells of the respective culture. All flasks were incubated on rotary shaker at 100 rpm for 48 hours at respective optimum temperature and the rate of PNP degradation and estimation of nitrite was measured by standard method as mentioned above.

3.5.5 Optimization of carbon and nitrogen sources

MSG broth containing optimum concentration of PNP was prepared. The medium was supplemented with various carbon and nitrogen sources, viz. dextrose, sucrose, peptone, yeast extract, etc. adjusted to optimum pH and inoculated with 1% inoculum of the respective culture. All flasks were incubated on rotary shaker at 100 rpm for 48 hours at optimum temperature and the rate of PNP degradation and nitrite production was measured by standard method as described above.

3.6 Production of Para nitrophenol degrading enzyme (4-monooxygenase)

For determination of enhance enzyme activity by surface and submerged technique was carried out. MSG broth was prepared with 400 ppm PNP. Then the inoculum of three selected strains Lysinibacillus sphaericus, Streptomyces coerualeorubidas and Aspergillus flavus at 5% level was added to this medium and incubated at 40°C. After 72 hrs cells were harvested by centrifuging at 1000 rpm for 10 min. Protein concentration was estimated by biuret method.
3.7 Downstream processing

3.7.A Step 1-Ammonium sulphate precipitation

Finely powdered ammonium sulphate was added to 80% saturation. The bacterial, actinomycetal and fungul crude enzyme prepared was brought to 60% saturation with ammonium sulphate at pH of 7.5 and kept overnight at 4°C. After equilibration, the supernatant was brought to 80% saturation with ammonium sulphate and centrifuged at 8000 rpm, at 4°C for 10 min. Then the precipitates were collected separately and dissolved in a 0.02 M phosphate buffer at pH 7.5 stored at 4°C for further purification.

3.7.B Step 2-Dialysis

The pre-treated dialysis tubes were used for dialysis of the precipitates collected in step 1. The precipitate were dissolved in 0.02 M phosphate buffer (pH 7.5) and dialysed against 0.002 M phosphate buffer pH 7.5. The dialysed fraction was used to determine enzyme activity and protein concentration and stored at 4°C for the SDS-PAGE and further parameters.

3.8 Molecular studies of purified enzyme

3.8.1 Spectrophotometric studies

The spectral studies of the dialyzed fractions were subjected to spectrophotometric analysis. Using double beam UV-Vis. Specrophotometer (Chemito) $\lambda_{max}$ was determined and purity was confirmed based on 280:260 ratio.

3.8.2 Determination of molecular weight of 4-monoxygenase by SDS-PAGE

SDS-PAGE was performed according to the method of Sambrook Russel, 1989. The following standard proteins were used for molecular weight determination 97.4 kDa (Phosphorylase B), 68 kDa (Bovine serum albumin), 43 kDa (Ovalbumin), 29 kDa (Carbonic anhydrase) and 14.3 kDa (Lysozyme). Preparation of reagent see in Appendix D.
Studies on Microbial Biotransformation of Para-nitrophenol

Clean grease free plates were assembled. In disposable plastic tubes, appropriate volumes of solutions were prepared and were mixed in order as shown below. Without delay the solution was poured into the gap of the glass plates leaving sufficient space for stacking gel. An overlay of iso-butanol on the top of resolving gel was given so as to minimise the risk of the oxygen diffusing into gel which inhibits the polymerization. After polymerization, the overlay was washed with distilled water several times to remove the unpolymerised acrylamide. In another disposable plastic tube stacking gel was prepared and it was poured over the polymerised resolving gel. Immediately a Teflon comb was inserted into this stacking gel. Any spaces left were filled with the stacking gel with Pasteur pipette.

**Resolving gel preparation:** - 12% polyacrylamide resolving gel was prepared by dissolving 10 ml of 30% acrylamide mix solution, 8.2 ml deionised water, 0.25ml of 10% ammonium per sulphate and 0.01 ml of TEMED. The gel was allowed to polymerize for about 30 minutes by pouring a layer of butanol on the top of resolving gel so as to avoid the oxidation. After complete polymerization, the butanol layer was removed and the layer was washed.

**Stacking Gel preparation:** - On this layer 5% stacking gel was poured. Stacking gel was prepared by mixing 0.83 ml of 30% acrylamide mix with 0.63 ml of 1M Tris (pH 6.8), 0.05 ml of 10% SDS solution, 3.4 ml of deionised water, 0.05 ml of 10% ammonium per sulphate and 0.005 ml of TEMED.

**Sample Preparation:** - Samples were prepared in 1X SDS gel loading solution. 8µl of sample enzyme was mixed with 2 µl of 1X SDS gel loading solution and were kept in water bath for about 2 minutes.

**Electrophoresis of the samples:** - Samples (20 µl) were loaded and the gel was electrophoresed at constant voltage supply of 150 V for 4hours in tris glycine buffer (pH 8.6).

**Staining of gel:** - After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 solution prepared by mixing 50 mg of dye in methanol: acetic acid: water
Studies on Microbial Biotransformation of Para-nitrophenol

(45:10:45). The gel was immersed in 4 to 5 volumes of staining solution for at least 4 hours on a rotary shaker set at minimum 40 rpm.

**Destaining of the gel:** - The gel was destained with mixture of methanol: acetic acid: water (45:10:45) solution without dye. Destaining was carried out for 3-4 hours with constant shaking and changing the destaining solution for 3 to 4 times.

**Visualization of bands:** - Bands obtained thus were analysed on an illuminator of gel documentation unit. Standard molecular weight markers were compared with the samples for determination of the molecular weight of the protein samples. For the record, the picture images were stored.

### 3.9 Characterization of 4-monooxygenase enzyme

The free enzyme was used for the characterization of monooxygenase and to optimize its activity. The enzyme was assayed at wide range of pH (4.0 to 10.0), temperature (30°C to 80°C), substrate concentration and metal ions. Moreover thermal stability and pH stability of the enzyme was also studied.

#### a. Optimization of pH

The enzyme produced by *Bacillus sphaericus* B15, *Streptomyces corelurubidus* A5 and *Aspergillus flavus* R8 was partially purified its pH optimization was carried out over a broad pH ranges (4.0-10.0). Citrate buffer (pH range 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 assay was carried out using 0.2 ml of approximately diluted enzyme. Enzymes unit were calculated by measuring the PNP degradation rate. Buffer preparation see in (Appendix C).

#### b. Optimization of temperature

The enzyme produced by *Bacillus sphaericus* B15, *Streptomyces corelurubidus* A5 and *Aspergillus flavus* R8 was partially purified its temperature optimization was
carried out over a range of temperature from 30° C to 80° C, and at the optimum pH using the assay procedure above.

c. Determination of pH and thermal stability on enzyme

The pH stability of the enzyme at optimum pH was studied by varying the time of incubation from 30 min. to 240 min. Similarly the thermal stability at optimum temperature was determined upto 240 min.

d. Optimization of substrate concentration

PNP concentration ranging from 1mg/ml to 10 mg/ml were studied for optimization of enzyme activity.

e. Effect of metal ions on monooxygenase

In order to determine enzyme activity in the presence of different metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}) in the form of metal sulphate were added to enzyme substrate reaction to yield final concentration 4mM. The impact of metal ions on enzyme activity was thus determined. A control of without metal reaction mixture was used to analyse and determine the percent inhibition or induction in enzyme activity (Zeyer, et al., 1986).

3.10 Immobilization studies by using covalent bonding method

Kinetic studies were carried out using immobilized whole cells and immobilized enzyme and compared with free enzyme. The immobilization of enzyme was carried out by using covalent bonding method. This was achieved by using sodium alginate solution. A 3% sodium alginate solution was prepared. For cross linking purpose, the sodium alginate solution was mixed with 1 ml of of enzyme/whole cells. The entrapment was carried out by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2 M CaCl₂ solution with a syringe and a needle at room
Studies on Microbial Biotransformation of Para-nitrophenol

The bead size can be controlled by pump pressure and the needle gauge. A typical hypodermic needle produces beads of 2–4 mm in diameter. The beads were collected, washed with phosphate buffer pH-7.5 and kept in the same buffer at 4-5°C for 24 hrs to remove the unbounded enzyme. Enzyme assay was carried out by using alginate beads equivalent to 1ml of enzyme.

1 ml of enzyme solution corresponded to 15 beads of 2-4 mm diameter i.e. for 0.2 ml enzyme equivalent to 3 beads for enzyme reaction. Using these values further the immobilized enzyme/whole cells was used.

3.10.1 Reuseability of the immobilized enzyme

The immobilized enzyme was put into a sodium phosphate buffer (pH 8.0) at 40°C for 1 hr and then used to study enzyme assay for second and third time. Finally, the residual enzyme activity of the immobilized enzyme was tested and compared after each use.

3.11 Comparative kinetics of free, immobilized enzyme and whole cell

a. Effect of pH on free, immobilized enzyme and whole cell

The effect of pH on monooxygenase activity of free and immobilized preparations was studied by varying the pH value from 4.0 to 10.0.

b. Effect of temperature on free, immobilized enzyme and whole cell

The effect of temperature on free and immobilized forms was investigated from 30°C to 80°C. The heat stability of the immobilized enzyme was compared with that of the free enzyme at their optimum temperature for specified times. Similarly the heat stability of cell was compared at optimum temperature.

c. Comparison of Km and Vmax of free, immobilized enzyme and whole cells

Different concentrations of PNP were prepared ranging from 1 mg/ml to 10 mg/ml. The enzyme assay has been performed as described above. The initial velocity of
enzyme, Km and $V_{\text{max}}$ values were determined for both free and immobilized enzyme from Lineweaver-Burk plot.

### 3.12 Strain improvement programme

Extensive literature survey revealed that not much biotransformation of PNP by actinomycetes has been reported hence the strain improvement and transformation studies were carried out using the potential PNP utilizing actinomycete *Streptomyces coeruleorubidus*.

#### A. Physical mutagenesis by UV irradiation

The starch casein agar slants were used to prepare the spore suspension (880 cfu/ml) of *Streptomyces coeruleorubidus*. A5. This spore suspension was diluted to give $10^{-5}$ dilution (50 cfu/ml) and exposed to UV irradiation (254 nm) at a distance of 20 cm for 30 sec. to 540 sec. 0.5 ml of spore suspension from each exposure was plated on starch casein agar plate by spread plate technique and incubated with a control plate (A. Khattab, *et al.*, 2012). The irradiation was performed in a dark room and incubation was also done in dark in order to minimize the photo reactivation effects. After incubation, number of colonies was counted and percent survival curve for each plate was plotted. The mutant colonies were selected from the plates showing below 50% survival, and checked for biotransformation efficiency (PNP degradation activity). The PNP concentration was estimated spectrophotometrically at 400 nm. The mutant colony was selected for the further experiments as it showed maximum degradation activity as compared to wild type strain A5 and other mutant colonies.

#### B Chemical mutagenesis

##### a. Sodium azide

The selected actinomycete strain, A5, was plated on starch casein agar plates containing varying concentrations (10 to 100 ppm) of sodium azide (Rao, *et al.*, 2010) and incubated at 35°C for 3 days. After incubation the colonies, from the plates showing
below 50% survival, were examined for the post mutation effect on PNP degradation activity.

b. Ethidium Bromide

In this experiment, 500 μl spore suspension of wild strain was added in separate micro tubes. Appropriate quantity of stock solution of ethidium bromide was added to each tube to get the different working concentrations (10μg/ml, 20μg/ml, 50μg/ml and 100μg/ml) of ethidium bromide. Suspension volume was made up to 1000 μl with sterile saline and incubated at 30°C for 1 hour and centrifuged at 12,000 rpm for 5 minutes. The pellet was washed with EDTA and suspended in 0.9% saline solution. 0.1 ml of this was plated on starch casein agar. After incubation the colonies, from the plates showing below 50% survival, were examined for the post mutation effect on degradation activity.

3.13 Molecular level studies of actinomycete

3.13.1 Isolation of plasmid DNA from Streptomyces corelurubidus and transformation into E. coli DH5α to produce efficient new strain

a. Extraction of plasmid DNA

Wild type Streptomyces corelurubidus. A5 and UM1 strain were inoculated in MSG medium in the presence of PNP. Incubated for 48 hr at140 rpm on orbital shaker. Centrifuged for 15 min and mycelial growth was collected. For lysis of cells sonicated in TE buffer. After centrifugation supernatant was collected and incubated for 1 hr at 55°C. Supernatant extracted with equal volume of phenol:chloroform (1:1 v/v) inverted 10 to 12 times then centrifuged for 5 min. After centrifugation aqueous layer was taken and 2.5 volume of chilled ethanol was added, incubated over night at -20°C. Centrifuged for 1000 rpm for 10 min. After centrifugation pellet was dissolved in 1ml TE buffer (Sambrook, et. al., 1989).
b. Confirmation of purity of DNA by spectrophotometric analysis

The extracted plasmid DNA sample was diluted with Tris-EDTA buffer and the absorbance of the sample was taken at the wavelength range of A$_{200}$ to A$_{325}$ nm against T. E. buffer as blank. The purity of DNA was determined by taking the absorbance ratio A$_{260}$/ A$_{280}$.

c. Visualization of plasmid DNA in agarose gel

Agarose gel (0.8% in 1X TAE buffer) was warmed in a flask at 100 °C till its dissolution. Then it was cooled up to 50°C is poured in pre tapped and sealed levelled gel trey. The comb was placed in it such that the wells were near the black (negative) electrode. The gel was allowed to solidify. 1X TAE buffer was poured till the slab was covered. To a 1.5 ml microcentrifuge tube, 5 µl of 10X gel loading duffer and 45 µl of redissolved DNA was added and mixed by tapping. 20 µl of this mixture along with marker and blank dye was loaded in the well made in agarose gel. After the run (50 volts, three forth run of the dye front) the gel was removed from the tray and visualized under UV transilluminator (Vilberlormat, vol. 5339).

d. Preparation of competent cells

A single colony of *E. coli* DH5α was inoculated in 50 ml of Luria Bertini broth. Grown over night and centrifuged at 5000 rpm for 5 min (4°C). After centrifugation pellet was collected. The pellet was dissolved in 50 ml ice cold MgCl$_2$-CaCl$_2$ solution. Then centrifuged at 5000 rpm for 10 min. at 4°C. The pellet was dissolved in 2 ml of 0.1 M CaCl$_2$ solution, then dispensed in pre-chilled eppendorf tubes and stored at 4°C. 0.2 ml volume was stored.

e. Transformation

200µl of competent cells of *E. coli* DH5α with and 100ul plasmid were incubated on ice for 30 min. The tubes were transferred to 42°C for 1 min 30 sec. Thereafter the tubes were placed on ice for 1 to 2 min. 700 µl LB broth was added to it, incubated at
37°C for 45 min. Plating was carried out (MSG medium containing PNP) by spread plate technique. Bacterial colonies which converted the PNP media from yellow to colourless, picked up and transferred to PNP slants, the transformants were inoculated in MSG broth containing PNP (400 ppm). PNP biotransformation was studied after 48 hrs.

3.14 Comparative studies of wild type, mutant and transformant

Optimization of environmental conditions of selected wild type *Streptomyces corelurubidu*, U.V mutant and transformant improved strain for their field application was carried out. Optimization of pH, temperature, substrate concentration and effect of carbon and nitrogen sources.

3.15 Antagonistic assay of the isolates for preapartion of the consortium

In order to determine whether the bacterial, fungal and actinomycetal cultures are antagonist towards each other the antagonistic assay was carried out. For determination of antagonistic assay perpendicular steak method was carried out for B15 and A5. While for fungal strain R8 agar well method was carried out.

3.16 Pot assay

Treatment of PNP amended soil was carried out by using pure culture and consortium. For this purpose soil was sterilized by 3 cycles at 121°C for 20 min. The soil was ammended with PNP and treated with the following combinations.

1. Bacteria
2. Actinomycete
3. Fungi
4. Bacteria+Actinomycete
5. Bacteria+fungi
6. Fungi+Actinomycete
7. Fungi+Actinomycete+Bacteria

Wheat seeds was kept in oven for 1 hr to break the dormancy period. For culture coating purpose on wheat seeds 5 g sterile finely powdered charcoal, jaggery syrup
(dissolved in 2 ml sterile D/W) and 3 ml spore suspension of each culture were mixed in different petri plates, kept half an hour for dryness. These seeds were sowed in pots with 5 ml PNP from 1% stock solution, Total 8 pots were used in this experiment 7 pots for different culture combinations with PNP as mentioned above and one pot contain only PNP as negative control. Seed germination and shoot length was observed for one week.

3.17 Biotransformation using pure culture and consortia

MSG broth (100 ml each flask) was inoculated by 1% pure culture and different set of culture combinations with optimum PNP concentration (400 ppm). Incubated for 48 hr centrifuged for 10 min at 8000 rpm. After centrifugation supernatant was taken for estimation of PNP degradation and nitrite production by spectrophotometrically at 400 nm and 520 nm.

3.18 In Ex-situ lab studies using effluent from pesticide industry

a. Pretreatment of effluent

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

b. 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 laboratory, Aurangabad. The parameters studied were pH, Total Solids (TS), Total Dissolved Solids (TDS), and Total Suspended Solids (TSS), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total acidity, and Total hardness, acidity, alkality and ammonia.

The effluent was initially analyzed for detection of PNP using HPLC. The HPLC analysis has been performed in Jeevan Rekha Analytical Services, Aurangabad. The HPLC conditions used are given as below.
c. HPLC conditions

Analysis of references compounds and products were carried out using a Milton Roy 4000 HPLC with a C18 Nucosil ODS 5µm, 25 cm×4.6 mm. Jones chromatography column with a 3 cm× 4. 6 mm Nucosil 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.

d. Packing of column using immobilized whole cells

A glass column was fixed to a stand the column was sealed with glasswool and silica gel. The column was packed with immobilized beads of isolates Lysinibacillus sphaericus, Streptomyces corelurubidus and Aspergillus flavus individually. The effluent was allowed to pass through the column. Sample was withdrawn at the interval 10 min. for 4 hours. The concentration of PNP was determined and % degradation was calculated.

3.19 Data validation by applying statistical tests using software packages

The data of all the experiments was statistically validated by performing ‘T’ test using statistical Minitab 14 software.