CHAPTER 3
MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1. Isolation of urease producing microbial isolates from soil samples

3.1.1. Collection of soil sample

Soil samples were aseptically collected from farm fields of Shoolini University (Solan district of Himachal Pradesh, India), which was sprayed with urea fertilizer for more than five years (as per the reports by the farmers). Different parameters such as pH, temperature and moisture content of soil were measured at the time of soil collection.

3.1.2. Isolation and purification of soil bacterial and fungal isolates

The soil samples were collected in aseptic bags and brought to the laboratory. One gram of each collected soil sample was subjected to serial dilution. 1 g of soil was added to 9 ml of sterile distilled water and shaken vigorously. The suspension was centrifuged at 800 rcf for 5 min to remove the insoluble particles. The supernatant was serially diluted up to $10^{-6}$ dilution. 100 µl of undiluted sample and $10^{-6}$ dilution was plated on nutrient broth (NB) (Appendix-I) containing 2 % agar and 0.2 % urea (spread plate method) for bacterial isolation, whereas it was potato dextrose agar (PDA) medium (Appendix-I) for fungal isolation. Plates were incubated for 3 days at 37 °C for bacterial growth and at 30 °C for fungal growth. After 3 days, bacterial and fungal colonies appeared on the NB agar plates and PDA plates respectively. Isolated bacterial colonies that appeared on the agar plates were purified by three successive streaking on NB agar medium and were preserved as 50% glycerol stock at -80 °C. Isolated fungal colonies which appeared on PDA were purified by hyphal tipping technique and preserved by suspending their conidia in normal saline containing 0.1% Tween-80 at -80 °C.

3.2. Screening of bacterial and fungal isolates for urease enzyme

Isolates were screened for extracellular urease enzyme based on qualitative and quantitative assays.

3.2.1. Qualitative assay

Potential isolates were selected by performing urease test that determined the ability of an organism to cleave urea and releasing ammonia by the action of urease enzyme. The
production of ammonia raises the pH of the medium, which changes the color of the medium from orange-yellow to deep pink. For this, isolated bacterial colonies were streaked on Christensen’s urea agar medium (Appendix-I) and fungal isolates were inoculated into urea broth medium. Cultures were incubated for 4 days at 37 °C for bacterial isolates and at 30 °C for fungal isolates. The isolate that showed faster urease response and changed the color of the medium into deep pink was selected (Christensen, 1946). E. coli strain (DH5α) served as a control.

3.2.2. Quantitative assay

For this, urease activity in supernatant was determined by measuring the production of ammonia from urea by using Nessler’s reagent (Kayastha and Das, 1999). Reaction mixture contained 0.1 ml of supernatant, 1 ml of urea (0.2 M) and 0.9 ml of 0.05 M Tris-acetate buffer (pH 7.5) and was incubated at 30 °C for 10 min. After 10 min, reaction was stopped by inactivating the enzyme with 1 ml of 10% trichloroacetic acid (TCA). The mixture was then centrifuged at 12,800 rcf for 10 min and 1 ml of Nessler’s reagent was added to the supernatant to estimate the formation of ammonia. The reaction mixture was made up to 50 ml with distilled water, while swirling. The yellow color produced was measured at 405 nm and urease activity was calculated from the standard graph of ammonium chloride. Ammonium chloride standard curve was prepared by making different dilutions of NH₄Cl solution (1 mg/ml). One unit of urease activity was defined as the amount of urease that produces 1 µmole of ammonia in one min at 37 °C (0.05 M Tris-acetate buffer containing 0.2 M urea). The isolates that showed high level of urease activity were selected for further use.

3.3. Identification of bacterial isolate by morphological and biochemical characterization

Urease producing bacterial isolate was subjected to Gram’s staining (Bergey et al., 1994) for microscopic identification and morphological and biochemical characterization according to Bergey’s Manual of Systematic Bacteriology (Claus and Berkeley, 1986). Morphological features (morphology of cell and colony) and biochemical tests (indole test, methyl red (M.R.) reaction test, voges-proskauer (V.P.) reaction test, citrate
utilization test, oxidase test, catalase test, casein hydrolysis, starch hydrolysis and motility tests) were performed for urease producing bacterial isolate.

3.3.1. **Gram’s staining** (Bergey *et al.*, 1994)

Gram’s staining was done for the identification of bacteria. Single colony of the bacterial isolate was picked from overnight grown culture plate and was smeared with normal saline on to a glass slide. The culture was uniformly spread to prepare smear and was heat fixed. The smear was covered with 0.5% crystal violet for 1 min and then washed with tap water. After this, the smear was covered with Gram’s iodine solution for 1 min and washed with tap water. Smear was flooded with decolorizing agent, 95% ethanol for 30 sec. After that, smear was covered with safranin for 1 min and washed off. Slide was allowed to air dry and then observed under microscope. Gram positive cells appeared purple, while Gram negative cells showed pink-red coloration.

3.3.2. **Indole test** (MacFaddin, 1980)

This test determines the ability of an organism to split indole from amino acid tryptophan. The bacterial isolate was inoculated into tryptophan broth (Appendix-I) and incubated at 37 °C for 24 h. After this, 4-5 drops of Kovac’s reagent was added. The tube was gently shaken and appearance of red ring indicates indole test was positive. *E. coli* strain DH5α was used as a positive control and *Pseudomonas fluorescens* was used as negative control.

3.3.3. **MR-VP test** (Clark and Lubs, 1918)

MR test is done to know the ability of the organism to maintain stable acid conditions during the fermentation of glucose by overcoming action of the buffering solution. VP test is done to determine the ability of an organism to produce acetyl methyl carbinol (acetoin) from glucose. The bacterial isolate was inoculated into MR-VP broth (Appendix-I). The culture was incubated at 37 °C for 24 h, followed by addition of 5 drops of methyl red for MR test. One ml of 40% KOH and 3 ml of 5% α-naphthol were added for VP test. The culture was gently shaken for 30 sec with caps off to expose the media to oxygen. Change in color of the broth from yellow to dark red indicates that MR
test is positive. Change in color of the broth from yellow to crimson red indicates a positive result for VP test. No change in color indicates negative result. For MR test, *E. coli* strain DH5α was used as positive control and *Enterobacter aerogenes* was used as negative control. For VP test, *Enterobacter aerogenes* was used as positive control and *E. coli* strain DH5α was used as negative control.

### 3.3.4. Citrate utilization test (MacFaddin, 2000)

This test is done to determine the ability of the organism to utilize the citrate as the sole source of carbon for metabolism resulting in alkanity. Bacterial isolate was streaked on Simmon’s citrate agar medium (Appendix-I) and incubated at 37 °C for 4 days. *Enterobacter aerogenes* was used as positive control and *E. coli* strain DH5α was used as negative control. Change in color of the medium from green to blue indicates positive test for citrate utilization.

### 3.3.5. Motility test (Tittsler and Sandholzer, 1936)

Motility test was performed to determine the motility of the bacterium. Bacterial isolate was inoculated into tubes of motility agar (Appendix-I) by stabbing through center of the medium with inoculating needle. The culture was incubated at 37 °C for 24 h. The original stab line diffused out into the medium as the bacteria spread throughout, indicates that the bacterium is motile. *E. coli* strain DH5α was used as positive control and *Staphylococcus aureus* was used as negative control.

### 3.3.6. Starch hydrolysis (MacFaddin, 2000)

Amylase assay was done to demonstrate starch degradation. Bacterial isolate was patched on starch agar medium (Appendix-I). The culture was incubated at 37 °C for 24 h. After that, the medium was flooded with 1% iodine solution for 30 sec. Appearance of clear zone around the colony indicates starch hydrolysis. *Bacillus subtilis* was used as positive control and *E. coli* strain DH5α was used as negative control.

### 3.3.7. Protease test (Brown, 2007)

Protease test was performed to determine the protease activity of bacteria. The bacterial isolate was patched on NB agar medium containing 1% skimmed milk. The culture was
incubated at 37 °C for 24 h. The appearance of zone formation around the colony indicates positive reaction for protease activity. *Bacillus subtilis* was used as positive control and *E. coli* strain DH5α was used as negative control.

### 3.3.8. Oxidase test (Kovacs, 1956)

This test is based on the bacterial production of an oxidase enzyme. A strip of Whatman filter paper was soaked in 1% Kovacs oxidase reagent (1% tetra methyl-para-phenylene diamine dihydrochloride) and dried. Single colony of bacterial isolate was picked and smeared on the filter paper. Inoculated area was observed for color change to deep blue or purple within 10-15 sec, which indicates oxidase positive test. MTCC 8115 was used as positive control and MTCC 8515 was used as negative control.

### 3.3.9. Catalase test (Whittenbury, 1964)

This test is performed to detect the presence of the enzyme catalase. A smear of the isolated bacterial colony was prepared on the glass slide. 2-3 drops of H₂O₂ were added to the smear. The occurrence of bubbles within 1 min after the addition of H₂O₂ indicates the presence of catalase. MTCC 8515 was used as positive control and MTCC 8114 was used as negative control.

### 3.4. Identification of fungal isolate by morphological characterization

#### 3.4.1. Lactophenol cotton blue (LCB) staining (Leck, 1999)

Microscopic identification was carried out by Lactophenol cotton blue (LCB) staining. The fungal culture was mixed in a drop of 70% alcohol with L-shaped loop on a microscope slide. One drop of the LCB stain (Appendix-I) was added before the alcohol dried out. The culture was covered with coverslip, avoiding air bubbles. After that, the slide was observed under microscope.

### 3.5. Molecular identification of isolated bacterial and fungal isolates

#### 3.5.1. Amplification of 16S rDNA and ITS region and phylogenetic analysis

For molecular identification, bacterial and fungal isolates were subjected to 16S rDNA and ITS region amplification and sequencing. Genomic DNA of bacterial isolate BS-13 was isolated by the method as described by Sambrook *et al.* (2012). Briefly, the bacterial
isolate was inoculated into 5 ml NB medium and incubated at 37 °C in incubator shaker at 150 rpm. 2.5 ml of overnight culture (OD$_{600}$ ~ 1-1.5) was taken and centrifuged at 10,600 rcf for 2 min. The supernatant was discarded. The pellet was suspended in 500 µl extraction buffer (100 mM Tris-Cl, 50 mM EDTA, 500 mM NaCl and 0.07% β-mercaptoethanol), 4 µl lysozyme (20 mg/ml) and 0.5 µl RNase (10 mg/ml). Sample was incubated at room temperature for 20 min. After this, 50 µl of 10% SDS was added and incubated at 65 °C for 30 min. The sample was centrifuged at 10,600 rcf for 5 min and the supernatant was collected. Equal volumes of phenol: chloroform (1:1) were added and mixed properly. Centrifuged at 10,600 rcf for 10 min and the aqueous phase was transferred to a new tube. The supernatant was treated with 0.1 volume of 5 M NaCl and 2.5 volume of 100% ethanol (i.e. 100 µl supernatant + 10 µl NaCl + 250 µl 100% ethanol). Sample was kept at -20 °C for 2 h. Thereafter, the sample was centrifuged at 15,200 rcf for 20 min and the supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol. Later, the pellet was air dried and finally resuspended in 50 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

Genomic DNA of fungal isolate FS-02 was isolated by the method as described by Choi et al. (1992). The fungal isolate was cultured in 10 ml potato dextrose broth (PDB) medium (Appendix-1) for 5 days at 30 °C. The fungal culture was crushed using pestle and mortar with liquid nitrogen. One ml of extraction buffer (1 M Tris-Cl, 5 M NaCl, 0.5 M EDTA and 0.7% β-mercaptoethanol) was added to it and crushed again. Suspension was transferred to microcentrifuge tubes and 130 µl of 10% SDS were added. The microcentrifuge tubes were then incubated at 65 °C for 15 min. After incubation, microcentrifuge tubes were centrifuged at 6,700 rcf for 10 min and the supernatant was collected. Equal amount of phenol: chloroform (1:1) was added to the supernatant and centrifuged at 6,700 rcf at 4 °C for 10 min. The aqueous phase was collected and phenol: chloroform step was repeated. To the aqueous phase, 2.5 volume of chilled 100% ethanol was added and incubated at -20 °C for overnight. It was then centrifuged at 15,200 rcf at 4 °C for 12 min and the pellet was washed with 1 ml of 70% ethanol. Later, the pellet was air dried and finally resuspended in 50 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Isolated DNA of both bacterial and fungal isolates was run on 1% agarose gel.
DNA was quantified to access the concentration and purity of DNA by using TECAN Nanoquant plate (Infinite 200).

The 16S rDNA of bacterial isolate was amplified using the universal primers 27F (forward) \(5'\)-AGAGTTTGATCCTGGCTCAG-3') and 1542R (reverse) \(5'\)-AAGGAGGTGATCCAGCCGCAG-3') (Gurtler and Stanisich, 1996). The ITS region encoding 18S rRNA gene of fungal isolate was amplified using the universal primers ITS 5 (forward) \(5'\)-GGAAGTAAAAGTCGTAACAAGG-3') and ITS 4 (reverse) \(5'\)-TCCTCGCTTATTGATATGC-3') (White et al., 1990). PCR reaction mixture was prepared by mixing the following components: genomic DNA (1 ng/µl), MgCl₂ (5mM), each primer (1 µM), dNTPs (0.2 mM), Taq polymerase (0.4 U) and PCR buffer (1X). For both bacterial and fungal isolates, the amplification was performed by initial denaturation of 2 min at 95 °C, followed by 30 cycles of 30 sec at 94 °C (denaturation), 30 sec at 59 °C (annealing), 1.5 min 72°C (extension) and final extension for 10 min at 72 °C in PCR. PCR products were visualized by electrophoresis on 1% agarose gel. Amplified PCR product was purified by gel elution. The desired bands after PCR amplification were extracted from the gel with fine sterile blade and purified with QIAGEN quick Gel Extraction Kit (Qiagen, Hilden, Germany).

The purified PCR product was sequenced at Xcelris labs, Ahmedabad (www.xcelrislabs.com), India. The purified PCR amplicons of bacterial and fungal isolates were sequenced using 27F & 1542R primers and ITS 5 & ITS 4 primers, respectively. The nucleotide sequences obtained for 27F (forward primer) and 1542R (reverse primer) were overlaid to remove the common regions and the complete sequence of 16S rDNA was reconstructed and subjected to nBLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast) against the bacterial 16S rDNA database. Similarly, the nucleotide sequence obtained for ITS subjected to nBLAST analysis against the fungal database. After 16S rDNA and ITS region sequencing, the complete sequences were submitted to GenBank nucleic acid sequence databases (NCBI, USA) for homology searching to obtain gene sequences with high similarity using BLAST tools (Altschul et al., 1990). Molecular Evolutionary Genetics Analysis 6 (MEGA 6.0) (Tamura et al., 2013) was subsequently used to construct a phylogenetic tree using the Neighbour-joining method (Saitou and Nei, 1987) to study the molecular evolution of BS-13 and FS-
02. All positions containing gaps and missing data were eliminated. Tree topology was evaluated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets.

3.6. **Production of urease from microbial isolates BS-13 and FS-02**

3.6.1. **Urease enzyme production from bacterial isolate**

The seed culture was prepared by inoculating a loopful of pure culture in nutrient broth medium. The flask was incubated at 37 °C for 24 h with continuous shaking at 150 rpm. 0.5 ml of seed culture was inoculated in 50 ml of growth medium and incubated at 37 °C for 24 h with shaking at 170 rpm. Growth medium (Smith *et al.*, 1993) used for urease production contained: Glucose 20 g/l; Urea 0.2 g/l; Yeast extract 10 g/l; NiCl₂ 0.032 g/l; Na₂HPO₄ 15 g/l; NaCl 5 g/l. At the end of the incubation period, culture was centrifuged at 12,800 rcf at 4 °C for 15 min. The supernatant was used to determine the extracellular activity of urease enzyme by using Nessler’s reagent as described under Section 3.2.2.

3.6.2. **Urease enzyme production from the fungus isolate**

The fungus isolate was cultivated on PDA at 30 °C for 5 days to obtain conidia. The conidia were washed and suspended in normal saline containing 0.1% Tween-80 to give 10⁵ conidia/ml. For submerged fermentation, 1 ml conidial suspension was inoculated in 100 ml of growth medium and incubated on a rotary shaker incubator (200 rpm) at 30 °C for 3 days. The growth medium (Ghasemi *et al.*, 2004) used for the production of mycelia contained: Urea 1.3 g/l; Glucose 20 g/l; MgSO₄.7H₂O 0.5 g/l; KH₂PO₄ 13.3 g/l; K₂HPO₄ 0.34 g/l; CaCl₂ 0.3 g/l; NiSO₄.7H₂O 0.032 g/l. At the end of the incubation period, the culture was filtered through syringe filter (0.25 µm). The filtrate was used to determine the extracellular activity of urease enzyme by using Nessler’s reagent as described under Section 3.2.2.

3.6.3. **Estimation of protein concentration of extracellular fractions of bacterial and fungal isolates**

The protein concentration in the extracellular fractions was determined by Lowry’s methods using bovine serum albumin (BSA) as standard protein (Lowry *et al.*, 1951). BSA stock solution was prepared at 1 mg/ml concentration. The tubes containing different amounts of BSA (0-1 mg) were prepared. To estimate the total proteins, 100 µl
of each supernatant was taken and 0.5 ml of Lowry solution contained alkaline sodium carbonate (2%): CuSO₄ (0.5%): sodium potassium tartrate (1%) (100:1:1) was added. After 10 min of incubation at room temperature, 50 μl of Folin-Ciocalteu reagent was added. After 30 min of room temperature incubation, the absorbance of both the samples was measured at 660 nm using UV-VIS spectrophotometer. A standard curve for OD₆₆₀ verses amount of BSA was plotted, from which concentration of protein in the crude fractions was calculated.

3.7. **Optimization of parameters for urease enzyme from BS-13 and FS-02**

The best urease producing isolates BS-13 and FS-02 were selected for further studies. Microbial metabolic activities are influenced by many physical and chemical parameters. For this, bacterial and fungal isolates were grown in nutrient broth and PDB medium, respectively. Equal number of bacterial cells and fungal spores were inoculated in their respective production medium and incubated at 37 °C for bacterial isolate and 30 °C for fungal isolate.

3.7.1. **Optimization of incubation time for urease enzyme**

To find the optimum incubation time period for urease activity, cultures were incubated for different time periods ranging from 12 h to 60 h with 12 h interval for bacterial isolate and from 24 h to 168 h with 24 h interval for fungal isolate. After incubation, urease activity was estimated by performing enzyme assay to find the optimum incubation time period.

3.7.2. **Optimization of temperature for urease enzyme**

To check the optimum temperature for urease activity, cultures were incubated at different temperatures ranging from 10 °C to 70 °C. Incubation was given for 2 days and 5 days for bacterial and fungal isolates, respectively. After incubation, enzyme assay was performed in order to find the optimum temperature for urease activity.

3.7.3. **Optimization of pH for urease enzyme**

To determine the optimal pH for enzyme activity, different pH values ranging from 4 to 9 of production medium were studied at their optimized temperature. Acidic pH of the
media was set by using 0.1 N HCl and alkali pH was set by using 0.1 N NaOH. After incubation, enzyme assay was performed in order to find the optimum pH of production medium for urease activity.

For bacterial isolate: 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0 pH values of production medium were studied.

For fungal isolate: 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 9.0 pH values of production medium were studied.

### 3.7.4. Optimization of substrate concentration for urease enzyme

To determine the effect substrate concentration, different concentrations of urea were used in the production medium. Enzyme assay was performed in order to find the optimum substrate concentration in production medium for urease activity.

For bacterial isolate: 1, 2, 3, 4, 5, 6, 7, 8, 9 mM of urea concentrations were used.

For fungal isolate: 10, 20, 30, 40, 50, 60 mM of urea concentrations were used.

### 3.7.5. Effect of metal ions on urease activity

For this, different salts of metal ions (CuSO₄, ZnSO₄, NiCl₂, CaCl₂, CoCl₂ and FeCl₃) at different concentrations (2, 4, 6, 8 and 10 mM) were prepared. Different concentrations of metal ions were incubated with the enzyme extract and then enzyme assay was performed to determine the percentage of inhibition on activity of urease enzyme.

### 3.8. Enrichment of urease enzyme by (NH₄)₂SO₄ precipitation

Proteins in aqueous solutions are heavily hydrated and with the addition of salt, the water molecules become more attracted to the salt than to the protein due to the higher charge. This leads to interaction between the proteins, results in aggregation and finally precipitation. Crude enzyme was transferred into an ice cold beaker with a magnetic bead. The beaker was placed in an ice tray to keep it chilled and transferred onto a magnetic stirrer. Ammonium sulphate was added slowly until saturation of salt occurred (60%). It was kept on the stirrer for 1 h. Solution was centrifuged at 10,600 rcf for 15 min at 4 °C. The supernatant was discarded and the pellet was collected containing the precipitated protein. The pellet was dissolved in assay buffer (0.05 M Tris- acetate buffer,
pH 7.5) and dialyzed against the same buffer for further analysis and characterization. After that, enzyme assay and protein assay were performed for ammonium sulphate precipitated enzyme extract of both bacterial and fungal isolates (Manual Protocol for Biochemistry protocols, World scientific publishing Co. Pte. Ltd.).

3.9. **Immobilization of ammonium sulphate precipitated urease enzyme**

3.9.1. **Immobilization of ammonium sulphate precipitated urease enzyme by calcium alginate beads**

A 4% solution of sodium alginate was prepared by slowly adding alginate in 0.1 M Tris-acetate buffer, pH 6.5 at 40 °C. The urease enzyme was then added (0.1 mg/ml) to sodium alginate solution. After this, the alginate-enzyme mixture in 20 ml syringe was loaded and the loaded mixture was dropped from constant height into 500 ml of chilled 5% calcium chloride in 0.1 M Tris-acetate buffer, pH 6.5 with constant swirling on a magnetic stirrer. Enzyme gets entrapped in a cage of calcium alginate in the shape of a bead. The beads were washed with buffer to remove remained calcium chloride solution and were stored in buffer at 4 °C for further study (Kayastha and Das, 1999).

3.9.2. **Immobilization of ammonium sulphate precipitated urease enzyme by gelatin powder**

Gelatin powder (6% w/v) used for the immobilization of urease enzyme was swelled in 10 ml phosphate buffer (50 mM, pH 7.5) and heated at 50 °C for 5 min. The mixture was cooled and 0.1 mg/ml enzyme was added. After thorough mixing of the enzyme, organic cross-linker, glutaraldehyde (0.6% v/v) was added. The mixture was constantly stirred at 27 °C and poured on a glass plate to prepare a thin film of the enzyme. The film was stored at 4 °C for 18 h for complete cross-linking and the immobilized enzyme film was washed with phosphate buffer (50 mM, pH 7.5) and cut into small blocks before subsequent experiments (Al-Khafaji et al., 2009).

3.10. **Comparative study of immobilized state of urease enzyme with free state of urease enzyme**

Characterization of free and immobilized urease was studied on the basis of following parameters.
3.10.1. Thermo-stability

The optimal temperature was determined, up to which free and immobilized enzymes can withstand thermal stress. For this, free and immobilized enzymes were incubated at different temperatures (30 °C to 80 °C) for 30 min before the activity was measured. At 10 min intervals, aliquots of 0.1 ml of the incubated enzymes were assayed for activity.

3.10.2. Optimum pH

To determine the optimize pH for urease activity of free and immobilized enzymes, the enzyme reaction was carried out using buffer of different pH ranging from 4.0 to 9.0. Then, enzyme assay was performed at standard conditions as described in Section 3.2.2.

3.10.3. Incubation time

Effect of incubation time of enzyme reaction for both free and immobilized enzyme was determined by varying the time course from 5 to 30 min.

3.10.4 Storage stability

Both the free and immobilized enzymes were stored at 4 °C. The urease activity for both free and immobilized state were determined on intervals of 1, 5, 15, 20, 25 and 30 days under standard conditions.