CHAPTER III

MATERIALS AND METHODS

CHAPTER 3

3.1 Sampled sites and plants: Medicinal plant sample was collected from the botanical garden of the institute of agriculture science, Banaras Hindu University (BHU), Varanasi (25.5-N 82.9-E, elevation 279 ft. /85 m (Aly and Debbab, 2010), India, in March 2014. The plant was identified on the basis of its external shape and feature. For this research, the root of a fully grown and healthy plant which was inside the soil was taken. Root samples used in the research were collected in sterile polyethylene and brought to the lab by placing them in an icebox;

The specimen of this plant was kept in a freeze at a temperature of 4 ^{o}C to protect it from external microbial infection. And immediately the process was started for isolation of endophytic bacteria.

3.2 Isolation of Entophytes Bacteria:

3.2.1 Surface sterilization: Prior to root samples, the soil was thoroughly washed with clean tap water to properly remove the soil. To do this whole process, three methods can be adopted so that complete sterilization of the root sample can be done.

The method I samples were deep in 70% ethanol for 2 min (Zin et al., 2007).

Method II root samples were surface sterilized by 70 % ethanol for 2 min and sterile double distilled water for 2 times, 0.5 % sodium hypochlorite for 2 min and sterile double distilled water for 3 min for 2 times (Sun et al., 2008).

Method III then

The flask was completely stirred by adding 4-5 drops of detergent in 200 ml sterile distilled water in a 500 ml flask, after that tween 80 or 01%, mercuric chloride was also used (SRL Chemical Co., St. Louis, Mo, USA) for 2 min. (Anjum and Chandra, 2015)**.** Every method was performed in triplicate.

complete surface sterilization has done through the second method. From which got a kind of pure root piece which have forwarded in further research. After this, Here, information was also given about the sterilization of plant root pieces.

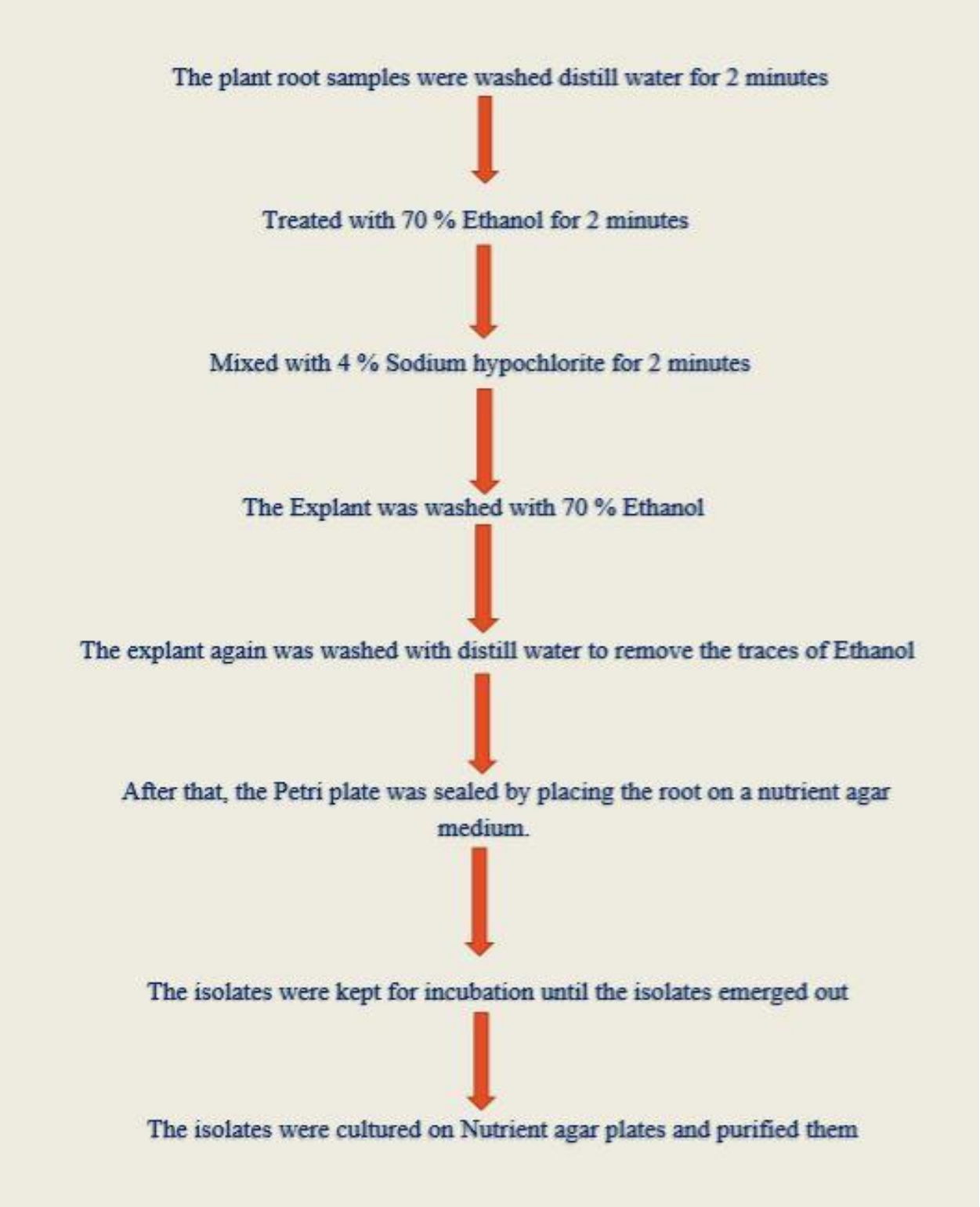


Figure 3.1: Schematic diagram of surface sterilization process of root sample.

3.2.2 Sterility check: At each stage of the surface sterilization process, root samples were washed in double distilled water. Complete sterilization was confirmed by Last washing water of root sample placing on the nutrient agar, and incubated at 32ºC for 48 hrs. and checked for promising microbial growth. This root sample can be used for research if growth of any microbes is not observed on nutrient agar.

3.2.3 Endophytic bacteria isolation, purification and preservation: For this purpose, the surface-sterilized root segments of *Adhatoda beddomei* were cut into a small piece of about 1-1.5 cm and transferred to sterile Petri dishes containing sterile nutrient media (Malt extract 3g/L, beef extract 3g/L, peptone 5g/L, dextrose 10g/L, and agar 2.5 gm/L). pH of medium was adjusted to 7.0 to 7.5. The inoculation medium was supplemented with Ketoconazole (150 µg/ml) and Fluconazole (100 µg/ml) to check fungal growth. The Petri dishes were incubated at 32°C and regularly observed till the outcome of endophytic bacterial cells. After 3 to 4 days, some bacteria have grown around the root segment when viewed carefully. Which were transferred to Petri dishes containing NAM (nutrient agar media) medium devoid of antibiotics? The identification of the endophytic bacteria was based on their characteristic morphology of colony, color and pattern of cells formation.

Each isolate was developed when it was ensured that each isolate originated from a single same colony. All isolated bacteria colonies were identified separately and preserved on the NAM medium for further identification. (Shah et al., 2010). Uncontaminated culture was preserved on NAM slant in test tubes at 5 to 10 ^{0}C. Every tube was labeled with full batch number, the code

number of the host plant and the full name of the bacteria and the date of storage were Properly Mentioned. Several replicates were prepared for each isolates and appropriate media was used for every isolate. In this way selected 03 main isolate (endophytes bacteria) which produced the most metabolites. Of these three, that endophyte bacterium was also forwarded to research which produced the most metabolites. This is endophytes bacteria Number Three (EFB-03). EFB-03 which produced maximum bioactive metabolites'

3.2.4 Steps to avoid contamination: Some steps were kept in mind during

Experimental procedure-

- The plant material was selected carefully and during the selection process it was kept in mind that plant not to affect by any disease.
- Long term storage of plant material was avoided because long term storage sometimes results in contamination.
- Fresh plant material was chosen for isolation of bacterial species.
- Plates were regularly observed for outcome of bacterial cells and as the growth appears growth pattern was observed.
- The cultured plates were examined regularly and if any plates suspected for contamination, complete batch was discarded.

Contamination can avoid by following all the steps. And it is important to take care of all these steps.

3.2.5 Morphological Identification of Endophyte Bacteria: Different species of endophytes bacteria were initially identified based on different morphological characteristics. Different Bacterial cultures were grown on different suitable culture medium at specific culture conditions to make their identification easy and appropriate. During incubation period all the plates were regularly examined for generation of bacterial cells. Some bacterial species are looked like grape flakes. A total of 06 isolates have been identified and all these cultures of bacteria are stored in seed culture media and kept for further Bioactive Metabolites production.

Scanning electron microscope is used in microbiology so that can get to know about the size, arrangement, cell-cell communication and division of bacteria and their different aspects. SEM is a type of power full tool that gives us information about the morphological characteristics of microbial cells, interactions with each other and their natural habitats. And at the same time, there is also information about how their movement is from one place to another and how they depend on the plant. Taking advantage of the SEM (Hitachi S-570 SEM, Hitachi High Technologies, Tokyo, Japan) endophytes bacteria was investigated in the present study. For scanning electron microscope inspection, a single colony of endophytes bacteria was set in a 2–3% Glutaraldehyde solution for 3–4 hrs. at room temperature, and wash for 15 minutes using 0.1 M phosphate buffer, this process is repeated 3 times. In the next step, the bacteria cell was dehydrated in various grades of ethanol (20%, 40%, 60%, 70%, and 85%) for three times, this process was done 3 times for 15 minutes. And finally, the bacterial cell is washed with acetone for 3 minutes. After this, the cells were dried with CO_2 help and mounted on aluminum stubs and using a scanning electron microscope attached to a computer, different images of bacteria have been drawn to study its morphology (Hayat, 1989).

3.3 Cultivation and Crude Metabolite Extraction at Small Scale: The pure bacteria culture was inoculated into 500 ml Erlenmeyer flask containing seed medium: 0.5 gm of Starch, 0.5 gm of Glucose, 0.1 gm of Peptone, 0.5 gm of NaCl, 0.2 gm of $(NH_4)_2SO_4$, 0.1 gm of $MgSO_4.7H_2O$, 0.1gm of K_2HPO_4 and 0.2 gm of $CaCO_3$ in 100 ml of distilled water. The flask was incubated on a shaker with 120 rpm at 32°C for 1 day and used as seed stock for antibacterial compound production. The seed culture was then transferred to starch casein broth (production medium- Starch 10.0g, Glucose 10.0g, Beef Extract 2.0g, Soyabean meal 10.0g, NaCl 4.0g, K2HPO4 0.5g, MgSO4.7H2O 0.5g, CaCO3 2.0g, Casein 3.0g) at 10% inoculum concentration at Incubated at 32^0C and 150 rpm for 05 days. Total of 10 flasks were used in order to collect about 1 Littre volume of broth for bacterial isolate.

After proper centrifuging 1-liter bioactive metabolites, the cells were first cleaned in distilled water 3 times, and then break the cells through sonication to find out if there are any bioactive metabolites intracellular products. After complete confirmation, these products are extracellular rather than intracellular. Bacterial broth evaporates through rotator vacuum dryer so that can get solid residue. The residues were stored at 30 ^{o}C temperatures to preserve their activity and during research a small portion of the residues were dissolved into dimethyl sulfoxide (DMSO) for characterization of the bioactive metabolites' residue.

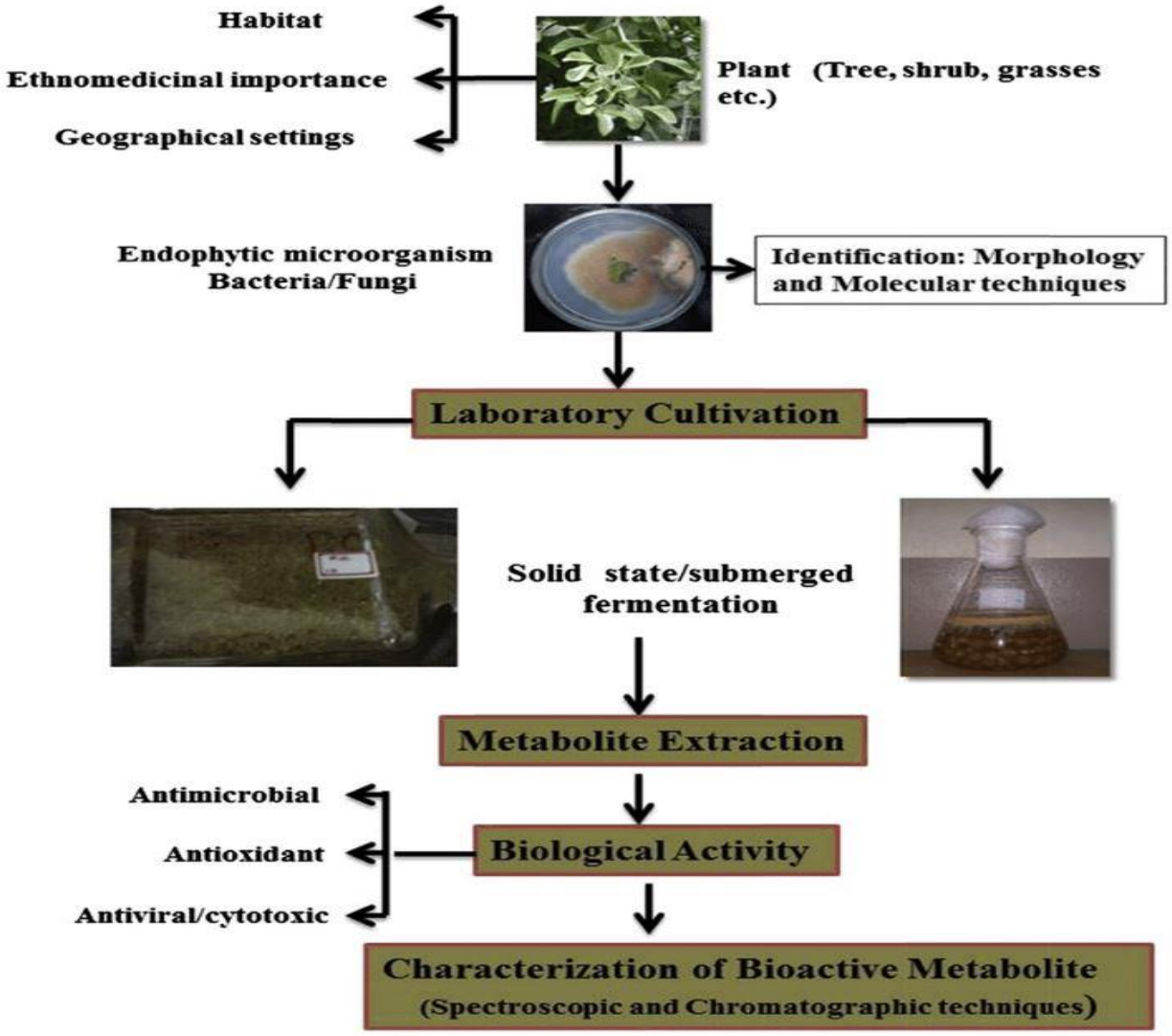


Figure 3.2: schematic diagram of isolation of endophytes bacteria form plant root and production of bioactive metabolites from endophytic bacteria.

3.3.1 Antimicrobial Activity of Bioactive metabolites against different Microorganisms: According to prior screening. Endophyte bacteria produce highly antibacterial and antifungal bioactive metabolites and exhibit their activity unlike various bacteria and fungi. And the bacterium and fungus, unlike which antiproperty shows, is the name of the bacteria and fungus *Escherichia coli, Streptococcus pneumonia, Bacillus subtilis, Staphylococcus hyicus, B. sphaericus, Staphylococcus aureus, pseudomonas aeruginosa.* And the same bioactive metabolites show antiproperty against some human pathogenic and plant pathogenic bacteria and fungi, the name of the human pathogenic and plant pathogenic bacteria and fungus is *Candida albican, Sclerotium rolfsii, Sclerotinia scleratiourum, Fusarium species and Penicillin sp.*

The inhibitory effect of endophytic bacterial extracts was checked using the improved Boer – Kirby method (Bauer et al. 1966). To test the antimicrobial activity of bioactive metabolites against various disease-causing microbes in plants, various plant pathogenic bacteria and fungi were collected from the Institute of Agriculture Science Banaras Hindu University (IAS-BHU) Varanasi. Each microorganism was inoculated with an overnight in the same way each bacteria culture was inoculated as it was for fungal pathogens; Each NAM plate was inoculated with each bacterial suspension. The microbial suspension was uniformly spread using a sterile glass spreader. The sterile paper disk, which is 6 mm in size, were placed on the center of each Petri plates and was sealed with parafilm by adding crude of 15µl bioactive metabolites. And these bacterial plates were kept at 35 ^{o}C temperature for 24 hours. and fungal plates at 28 ^{0} C for 48 hours. respectively. After 24 to 48 hrs. the zone of inhibition was measured and expressed in diameter in millimeter. Microbiology Institute of Medical Science BHU Varanasi India has also investigated the activities of our bioactive metabolites in contrast to various pathogenic non-pathogenic bacteria. The

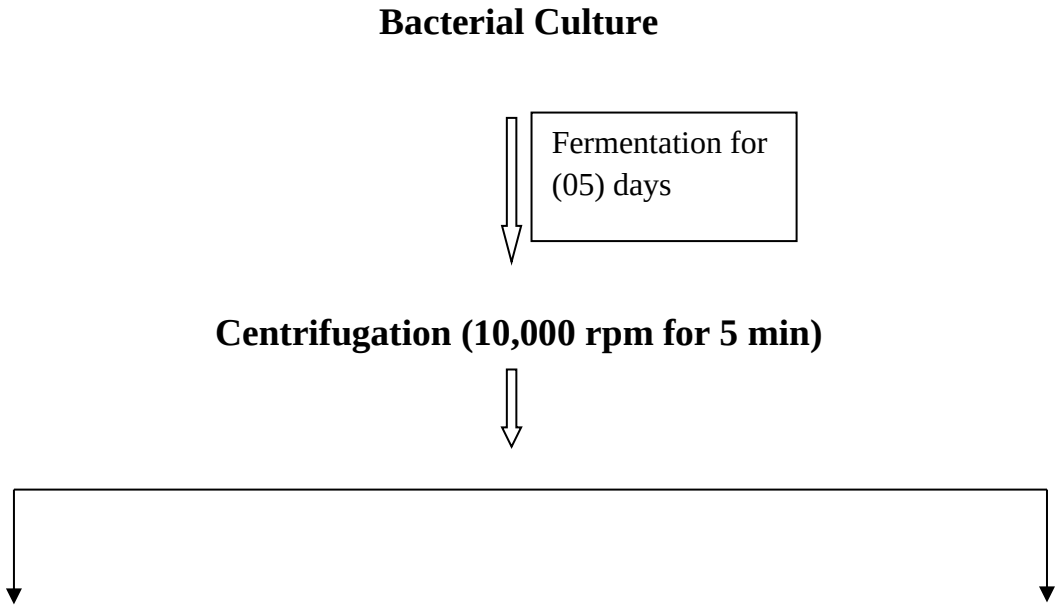
pathogenic non-pathogenic bacteria list is as follows (*E. coli, B cereus, B subtilis, Staphylococcus aureus, B.megaterium, Helicobacter pylori, Streptococcus pyogenes, Multiple drug resistant S. aureus, Chlamydia pneumonia, Corynebacterium amycolatum, Mycobacterium tuberculosis, S. epidermis, Pseudomonas aeruginosa, S. epidermis, Salmonella typhimurium, Salmonella typhi, Streptococcus pneumonia, Staphylococcus epidermidis, Corynebacterium diphtheria, Vibrio sp., Clostridium difficile, Klebsiella pneumonia*). The best bacterial isolates were selected as potential strains for further research based on the results of preliminary investigations. Three replicates of each experiment were maintained to avoid errors in measurements.

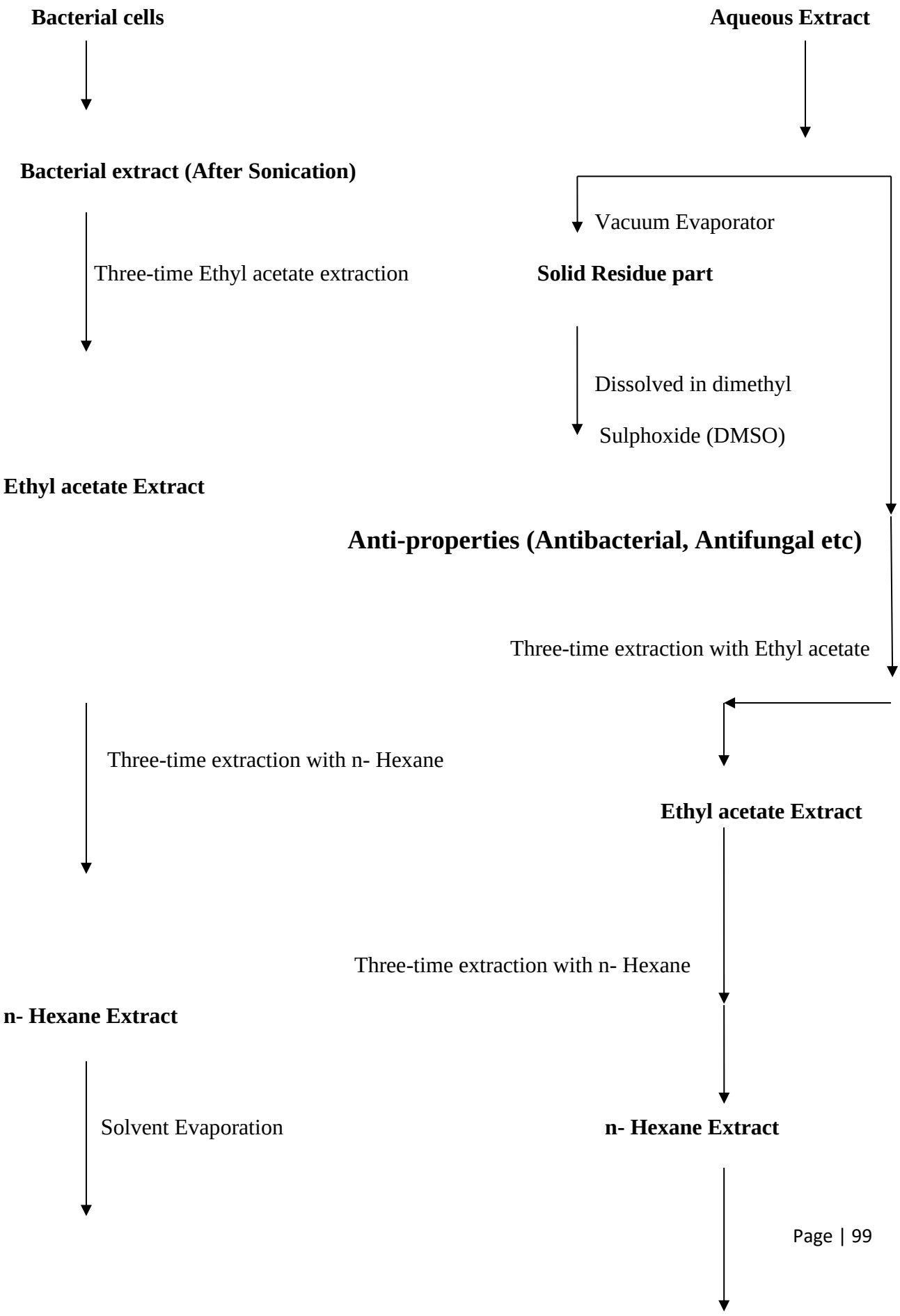
3.3.2 Determination of minimum inhibitory concentration (MIC) Assay: Antimicrobial spectra of microbial bioactive compounds were determined to be the minimum inhibitory concentration against various Gram-positive Gram-negative and fungi (Cappuccino 2002). Three sets of plates were made/maintained for each concentration of the test sample. Muller – Hinton agar and Czapek – Dox agar media were prepared to grow bacteria and fungi. Pure bioactive metabolites were dissolved in dimethyl sulphoxide (DMSO) from 05 to 100 µg/mL and metabolites of this different concentration were used against different bacteria and fungi for the minimum inhibitor test (MIC). The inoculated plates were analyzed after 24–48 hrs. of incubation at 37 ^{0} C for bacteria and 48–72 hrs. at 28 ^{0}C for fungi. The lowest concentration of bioactive metabolites performing anti-property against various bacteria and fungi was taken as MIC of the compound.

3.3.3 Large scale Fermentation and metabolite production: The same culture conditions were provided for large-scale production and small-scale fermentation production. On a large production scale,

About 12 liters of broth were prepared so that the right amount of bioactive metabolite could be found. One of the motives for setting up a large-scale production was to get a specified amount of metabolites to get all our characterization of the compounds. In a large-scale production, the same culture conditions as that of the Small-Scale production. We did a large-scale production with isolated number 03 (EFB-03) was done.

3.3.4 Extraction of Microbial Metabolites: Filtration process was used for removal of bacterial cells and this was done first by using centrifugation (10,000 rpm for 5 min). Thereafter filtered broth was extracted with ethyl acetate, two phases appeared: organic phase and aqueous phase and both phases was separated using separating funnel, dried in the rotatory vacuum drier under 38 ^{0}C till solid residues remain for the both phases.




Bacterial cells
Aqueous Extract
Bacterial extract (After Sonication)
Vacuum Evaporator
Three-time Ethyl acetate extraction
Solid Residue part
Dissolved in dimethyl
Sulphoxide (DMSO)
Ethyl acetate Extract
Anti-properties (Antibacterial, Antifungal etc)
Three-time extraction with Ethyl acetate
Three-time extraction with n- Hexane
Ethyl acetate Extract
Three-time extraction with n- Hexane
n- Hexane Extract
Solvent Evaporation
n- Hexane Extract

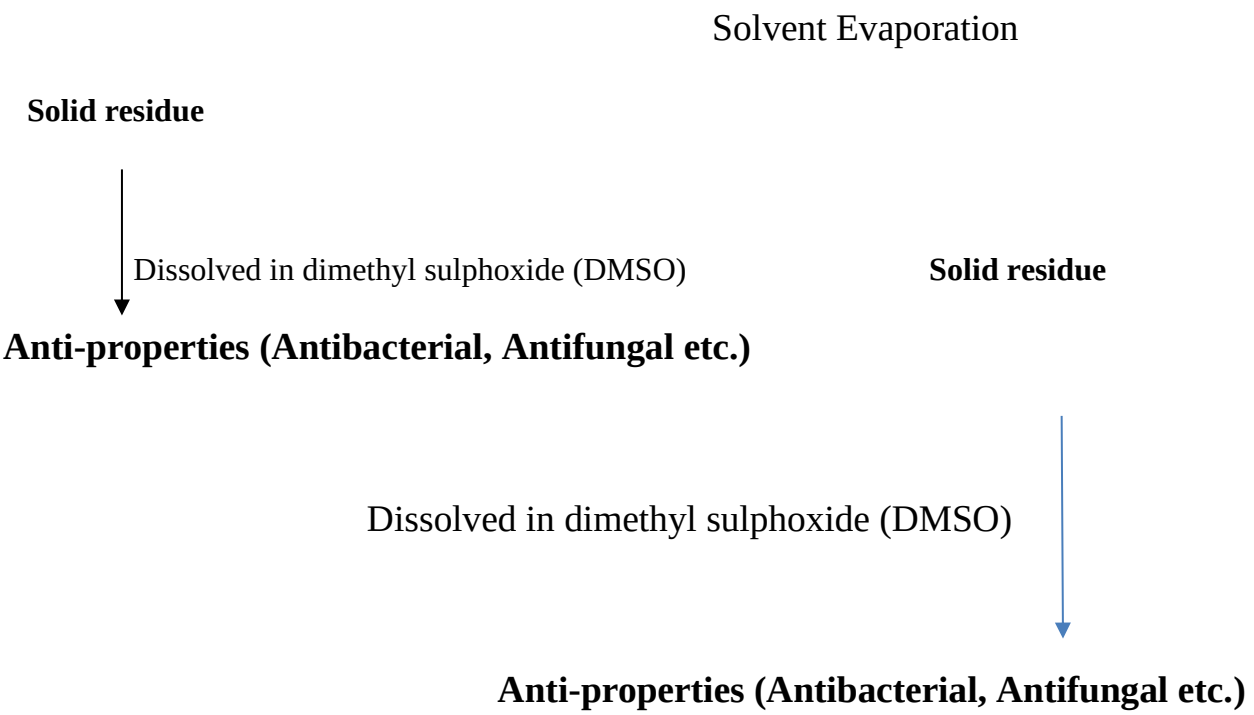


Figure 3.3: Flow chart of Extraction of Bioactive Metabolites.

3.4 Optimization conditions of Bioactive Microbial metabolites production: This experiment was performed to maximize the production of antimicrobial agents and to study its associated parameters. There are various methods of media optimization such as, Streptomyces genome mining, Genetic manipulation regulator, Ribosome engineering, use of heterologous host, signaling molecules, Optimization of culture condition. What has followed here is the state of an optimization culture condition. Through this method, what is the effect on the production by analyzing the parameters of different cultures through this method? Is the production of metabolites decreasing or increasing? And Attempts were made to enhance the antimicrobial activity of Endophytes bacteria bioactive metabolites by optimizing the culture conditions such as incubation period, pH, temperature, carbon, nitrogen sources with different concentration and resonance surface methodology (RSM).

In this, the level of production of metabolites has been checked using various carbons such as glucose, maltose, sucrose, starch, fructose and lactose. And at the same time, the effect of nitrogen at various concentrations has also been investigated through this study to produce bioactive metabolites in this experiment. And through various incubation times, different temperatures and pH, it has also tried to explain what effect they all have on the production of metabolites.

3.4.1 Effects of different carbon sources on antimicrobial metabolites Production: Various carbon sources such as glucose, maltose, sucrose, starch, fructose, and lactose have an effect on the production of bioactive metabolites and cell biomass formation of endophytic bacteria. In this, a separate set was prepared for each carbon. In which each set had a particular content, these contents are as follows (g/L) (Starch 10.0g, Glucose 10.0g, Beef Extract 2.0g, Soyabean meal 10.0g, NaCl 4.0g, K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.5g, $CaCO_3$ 2.0g, Casein 3.0g). Only one carbon source was used in each set. Here carbon compounds were added to starch casein medium in a 1% (w/v) concentration, with $NaNO_3$ (3g/L) being used as a supplement.

In this medium, 10% of the bacteria inoculum (seed media, which had been prepared beforehand) was added. After adding the inoculum, each set was kept at 35 ^{0}C temperatures and 180 rpm for 92 hrs. Final pH of the medium was adjusted to 7.2 (Elliah et al., 2000). After the complete incubation period, Centrifuged the cell at 10,000 rpm for 5 minutes and separated the bacterial cells. After centrifuging, the bacterial cell was dried at a temperature of 70 °C until the cell was completely dried. Then measured dry cells in g/L.

3.4.2 Effects of different Glucose concentration on antimicrobial metabolites Production: For these studies, Different glucose concentrations were used in the medium of this experiment. (0.5%, 1%, 1.5%, 2.0%, 2.5 %. w/v). It has been observed here that at different concentrations of glucose, different types of bioactive metabolites are produced and cell biomass is produced. Fermentation was performed in 1L Erlenmeyer flasks (for each concentration) with constant shaking at 180 rpm for 92 hrs. with same media composition (Each set had the same media composition but the concentration of glucose was different). A final temperature and pH of the medium was adjusted to 35 ^{0}C, 7.2. Further, the optimal levels of the suitable glucose concentration (0.5–2.5% w/v) for good yields of bioactive metabolites production and cells biomass formation were also recorded (Kathiresan and Balagurunathan, 2005). After separating the bioactive metabolites, the bioactive metabolites of different sets were concentrated via vacuum evaporation, Dissolved in DMSO to check the anti-properties of bioactive metabolites; Anti-property check was done against various bacteria and fungi (*Pseudomonas sp., B.subtilis, E.coli, C. albicans*) from bioactive metabolites of each set. The anti-microbial activities were confirmed by paper disk diffusion method. The diameters of the zones of inhibition were noted.

3.4.3 Effects of different nitrogen source on antimicrobial metabolites Production: The conditions and compositions for carbon sources on the production of bioactive metabolism were the same as the conditions and compositions for nitrogen sources. In this experiment different types of Carbone sources were used, such as, KNO_3, $NaNO_3$, NH_4Cl, NH_4NO_3, Beef extract, Peptone and Yeast extract were used. In this also have used the same condition and composition of media.

3.4.4 Effect of different $NaNO_3$ concentration on biomass and bioactive metabolite production: In these studies, the differential concentration was used of NaNO3; These concentrations ranged from 0.1 to 0.7% (v / w) in 1LIt was found that different levels of NaNO3 produce bioactive metabolites from different levels and form cell biomass. Prepared a different set for each concentration and took the same media composition for each set, which was used for a different carbon source. A different concentration of NaNO3 was used for each set. Each Erlenmeyer flasks (set) were kept at 35 ^{0}C and 180 rpm for 92 hrs. After the complete incubation period, Broth was separated from bacterial cells by centrifuge (10000 rpm for 5 min). The broth of different concentrates was kept separate. And all (broth of each set) were concentrated through a vacuum evaporator. Concentrated bioactive metabolites of each set were dissolved in DMSO to check anti-property against various bacteria and fungi (*Pseudomonas sp., B. subtilis, E. coli, C. albicans*) and the activity and quantity of bioactive metabolites were measured by paper disk diffusion method. The diameter of the zone of inhibition that came through bioactive metabolites was measured.

3.4.5 Effect of different p^H on the production of bioactive metabolites: The initial pH levels of the starch casein broth media were adjusted from 3 to 12 and the isolates were grown for 92 hrs. at 180 rpm and 35◦C. In this study also, different sets were prepared for different pH, that too from the same media composition and condition.

The process was adopted as described above to produce biomass and bioactive metabolites. (Effect of different $NaNO_3$ concentration on biomass and bioactive metabolite production) (Srinivasan et al., 1991; Thongwai, N. and Kunopakarn, J. 2007).

3.4.6 Effect of different incubation temperature (^{0}C) on the production of bioactive metabolites and cells biomass formation: Similarly, the optimum temperature for the antimicrobial bioactive metabolite production was determined by incubating the endophytes bacterial strain at temperatures ranging from 20 to 60 °C, while maintaining all other conditions at optimum levels (Saurav and Kannabiran, 2010). In this also, Separate sets were taken for different temperatures whose media composition was same and inoculums were grown for 92 hours at 180 rpm. After 92 hours, the broth of each set was separated from the bacterial cells by centrifugation. And all (broth of each set) were concentrated through a vacuum evaporator. Concentrated bioactive metabolites of each set were dissolved in DMSO to check anti-property against various bacteria and fungi (fungi (*Pseudomonas sp., B. subtilis, E. coli, C. albicans*)) and the activity and quantity of bioactive metabolites were measured by the paper disk diffusion method.

The diameter of the zone of inhibition that came through bioactive metabolites was measured (Griffith and Saker 2003; Lethimaki et al., 1997).

3.4.7 Effect of Incubation Period on Biomass and Bioactive Metabolite Production: The isolates were inoculated into the starch casein broth medium and incubated up to 08 days in a rotary shaker at 180 rpm at 35˚C. In these studies, centrifuged the first set a day later and determined the quantity of cell biomass and bioactive metabolites by the method given above. The sets of the second, third and eighth days were also determined in this way respectively. The cell free supernatant thus obtained was concentrated five-fold in the vacuum concentrator and 50 µl was used to determine antimicrobial activity. The diameter of the zones of inhibition were noted.

3.4.8 Optimization of culture conditions by response surface methodology (RSM): RSM applies the statistical and mathematical approach for modeling, designing, and analyzing the engineering problems. Maximum it is used to optimize the different reaction surface as well as which quantitatively affects the various parameters and relationships between the input parameters and the received response (Parmjit 2008). The Relationship between response and independent variables is unknown. Performing RSM is therefore important because need to know the valid rightful practical relationship between the responses and the set of independent variables. In these studies, the use of RSM to determine the optimum conditions of endophytic bacteria strain for the secondary bioactive metabolite production under a broad range of physical situation was demonstrated. Through these studies, the determined the minimum number of experimental runs, a complete factorial central composite face-centered design (CCFD) with four free changeable and their combinations was used to optimize the response with the region of three-dimensional observation spaces. Design proficient software (Version 7.0 State-Ease, Inc., USA) was used to design the experiments for bioactive secondary metabolites production. Using RSM, the most variables (A, B, C and D) at their optimum levels were recognized for highest response in conditions of Antimicrobial activity of bioactive metabolites considered as the diameter of zone of inhibition. The four independent variable parameters are as follows, Incubation Temperature, pH, Carbon source, Nitrogen source. A total of 32 experiments were obtained using following equation that has 2^4 full factorial CCD for four variables comprising 16 factorial points, 08 axial points, and 06 replicates:

$$N = 2^n + 2n + nc = 2^4 + 2 \text{ X } 5 + 6 = 32,$$

Where N is the total number of experimental runs to be performed, n is number of variables (factors), and nc is number of replicates at center points.

The central coded value of all variables is zero. Low and high range of every variables used in RSM and the absolute experimental plan with values in the definite and coded form are indicated in.

Factors	Symbols	Actual levels of coded factors		
		-1 (low)	0 (middle)	+1 (high)
pH	A	06	07	08
Temperature (^{0}C)	B	29	30	31
Carbon Concentration (%w/v)	C	1.5	2.5	3.5
Nitrogen Concentration (%w/v)	D	0.2	0.3	0.4

Table 3.1: Experimental range of factors studied using RSM in terms of coded and actual factors.

Statistical analysis: The model was statistically analyzed to calculate the analysis of variation (ANOVA). To analyze the fit and the prediction accuracy of the model constructed, correlation coefficients (R^2), adjusted determination coefficient (Adjusted- R^2), root mean square error (RMSE), and absolute average deviation (AAD) were carried out between experimental and predicted data. Experimental errors and reproducibility of data have been attempted through central points. In order to significantly reduce the different effects of uncontrolled factors in this, the experimental and uncooperative sequence was randomized (Cui et al. 2016).

The subsequently degree polynomial equation was used with every changeable to generate an empirical model which correlated the response (bioactive metabolites production) to four variables.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + (\sum_{i=1}^{n} \beta_{ii} X_i)^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j$$

Where *Y* is the predicted response, *β0* is intercept coefficient, *βi* is the linear coefficient, *βij are* the interaction coefficients, *βii* are the quadratic coefficients, *Xi* and *Xj* are coded values of the five additive variables.

3.5 Fractionation of crude Extract: This is a method of purification in a way that can partially purify our solid residual. From which its bioactive metabolites were obtained as a fraction. In the chemistry and pharmaceutical industries, column chromatography is a chromatography method used to separate a different chemical compound from a mixture. Chromatography is capable to divide substances based on differential adsorption of different compounds to the adsorbent; compounds travel through the column at dissimilar rates, allowing them to be separated into different fractions. The technique is broadly applicable, as many diverse adsorbents (normal

phase, reversed phase, or otherwise) can be used with a large range of solvents. The technique can be used on scales from micrograms up to kilograms. A cylinder-shaped glass column maintaining stationary phase (silica gel) is encountered gradually from the top with a liquid solvent (mobile phase) that flows downward the column with the help gravity or external pressure applied. Once the column is prepared, the sample is loaded inside the top of the column. The mobile solvent is then permitted to flow down through the column. The compounds in mixture have diverse interactive capability with the stationary phase (silica gel), and mobile phase, thereby will flow along with the mobile phase at different time intervals or degrees.

First of all, in this method, bioactive metabolites were added to the silica gel (100-200 mesh). And then loaded on a silica gel column (450 mm x 40 mm)) containing 200 g silica gel (100–200 mesh) using n-hexane solvent. After that had our bioactive metabolites (50g) loaded on silica in column (J. H. Zhao 2012). The column was eluted stepwise at a flow rate of 1 mL min-1. The column was eluted with n-hexane and ethyl acetate gradient (100:0, 90: 10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, v/v). After this, separated each of fractions and kept it. And the weight and anti-property of each fraction was checked against different bacteria and fungus. Thereafter the column was eluted with chloroform and methanol (4:1, 3:2, and 1:1 v/v).

This fraction was also checked weight and anti-property against different bacteria and fungus. Finally, the column was eluted to methanol and acetone (4:1, 3:2 and 1:1 v/v). Anti-property of bioactive metabolism of all these fractions done by the disk diffusion method, and the fraction in which anti-propagation was found was taken into further characterization and the fraction which did not have anti-property was discarded. And the fraction that had anti-property was used in TLC

demonstration (purity was confirmed by TLC) (Augustine et al., 2005). The description of the fraction is as follows,

Figure 3.4: Different stages of column chromatography during fractionation of crude metabolite.

Based on the TLC monitoring, 17 fractions (100 ml each) were collected:

- F-1, 3.5 mg F-8, 1.45 F-15, 0.71
- F-2, 2.20mg F-9, 1.31 F-16, 0.51
- F-3, 1.80mg F-10, 1.31 F-17, 0.36
- F-4, 1.80mg F-11, 1.02
- F-5, 1.50 mg F-12, 0.97
- F-6, 1 .46mg F-13, 0.96
- F-7, 1.50 mg F-14, 0.74

3.5.1 Thin-layer chromatography (TLC): For coating seven 20x20 plates, about 50g TLC silica gel was dissolved in 60ml distilled water. Fine silica gel after coating will not stay on the plate if any type of binder is not included with the mixer.

It will literally fall from the plate during the process in the solvent or fly away. The classical binder used is gypsum (G), which is $CaSO_4$. ½ H_2O, or Plaster of Paris. In most silica gels it is put in at a level of from 10-15% to give good binding to the glass plates. Once such a gel is made, then the time available for pouring into the applicator and pulling the glass plates is limited, as the binder will hydrate and the solution will no longer be a flowing liquid. For a stronger layer, polyvinyl alcohol or polyvinyl pyrollidone can be added to a TLC grade silica gel (without Gypsum) in a level of 1-2% by weight as a polymeric binder. After forming a very thin layer (0.25 mm) all plates were dried in dry air to make cross-linking of the binders until it was completely dried. All these

layers were dried very slowly so that no cracks occur in it. The next day or before use, the plates can be activated if desired, to

remove more of the adsorbed water. If not using instantly, then the water still remaining on an inactivated plate will actually protect the surface more by not allowing impurities to collect on the activated silanol. The plate can be stored in this water and activated for 30 minutes at 110℃ just before use. A mark was made at one end of each plate at a distance of 0.5 cm. At which 10µl differentiated compounds were loaded at a fixed point. Now all the plates were placed in a mixture of methanol ethyl acetate and chloroform (7: 1: 1) to separate the bioactive compound. Each plate was tilted at 60 degrees in a separate beaker.

And 100ml developing solvent was taken into each beaker. The solvent was always below the marked line (The place from which the sample was loaded). And after that all the beakers were covered with aluminium foil so that it could not fall into the air contact. After 15 to 20 minutes, the LTC plate was removed from the developing solvents and all these plates were kept at 100 ° C for 30 minutes to dry.

After the all plates were dried, the orange and brown spots of bioactive metabolites started appearing on it. And when all those plates were sprayed with sulphuric acid, the colors changed to pink-orange. All the plates had spots with different shapes and size.

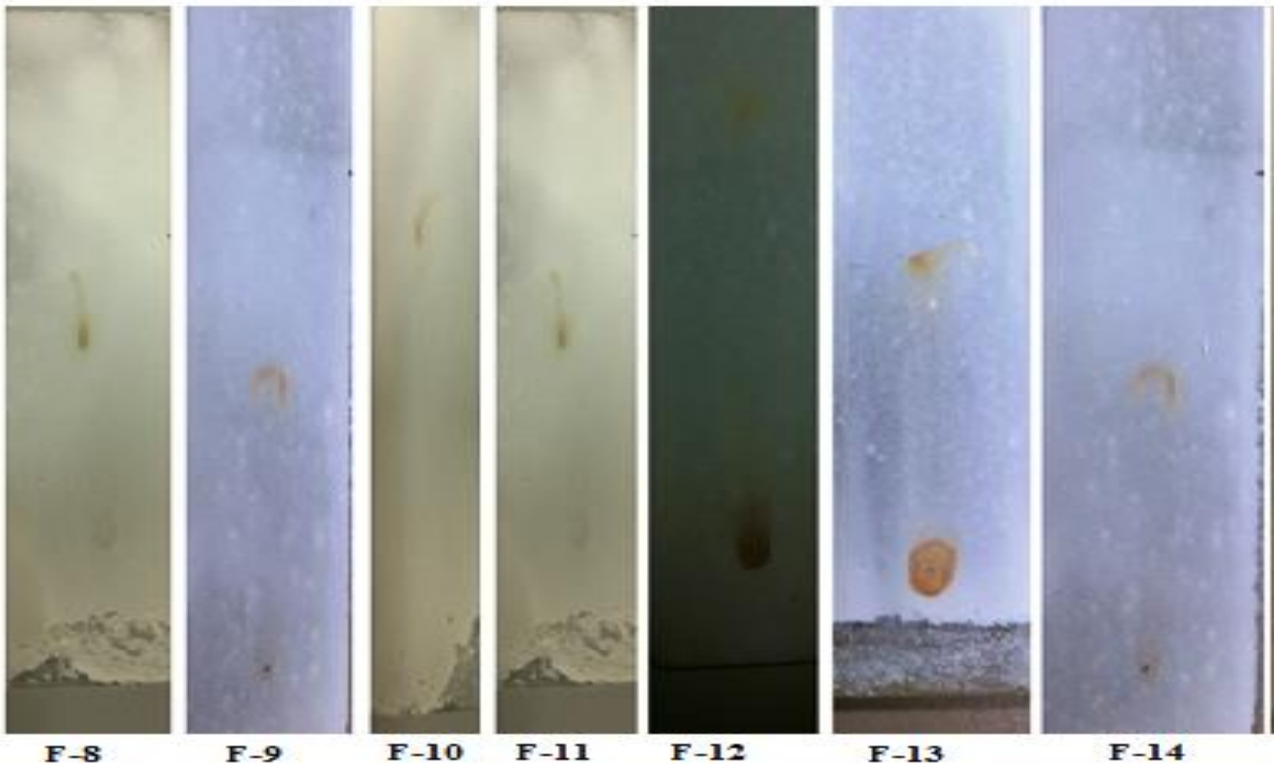


Figure 3.5: TLC of Ten fractionated compounds.

3.6 Spectroscopic Measurements: After column chromatography and confirmation of TLC. Finally, Various Bio-Instruments were used to obtain the final structure of bioactive secondary metabolites. For example, Fourier Transform Infrared spectroscopy (FT-IR), Nuclear magnetic resonance spectroscopy (NMR) and Mass spectrometry (MS). All NMR data were collected on JEOL AL 300 FTNMR. The Mass spectra peak were recorded on Agilent 6520 Q-TOF (ESI-MS) mass spectrophotometer. Chemical shift was expressed in δ (ppm) and coupling constant J in Hz. FT-IR spectra was collected.

3.6.1 Infrared Spectroscopy (IR): IR spectroscopy was further used to conform any functional groups present in any isolated compounds. IR spectrum was recorded in Shidmazu IR spectrophotometer by KBr pellet method. And its data were analyzed by OPUS Software. Solid samples of each compound were made into KBr pellet and after that it was scanned from 400 to 4000 frequencies. IR gives a physically powerful absorption pattern at a particular frequency (400 - 4000) for a particular functional group. Through this scanning different peaks are obtained, and each peak indicts a certain functional group. Resulting peaks were compared to published data of functional groups.

3.6.2 Nuclear Magnetic Resonance (NMR): the structure isolated compounds were elucidated primarily by 1D (homonuclear) and 2D (heteronuclear) NMR spectroscopy. The measurement of 1D and 2D NMR spectra was carried out by Dr. Nagendra at the NMR service, Banaras Hindu University (BHU) Varanasi. 1 D NMR experiments including ^{1}H and ^{13}C were used to locate the atom position and fragments units. Just like that, 2D NMR experiments including HMBC and HMQC were carried out on additional complex molecules for correct assignments of proton and carbon chemical shifts. NMR spectra were recorded on a JEOL AL 300 FTNMR (300 and 75 MHz respectively). NMR spectrometer was coupled with Topspin 2.1 acquisition software. Samples were dissolved in proper solvents (DMSO- d6, $CDCl_3$ and D_2O) for NMR spectrometry. Select solvents based on the solubility of the sample, and simultaneously consider the hydroxyl and an amine group. And analyze the spectra of pure compound using Topspin 2.1 acquisition software. NMR experiments on the samples with very small in mass were carried out using shigemi NMR tubes (100-200 µL samples) (sigma-Aldrich). Signals were recorded in chemical shift (δ) and expressed in parts per million (ppm), with coupling constant (*j*) considered in hertz (Hz).

3.6.2.1 ^{1}H NMR: By these studies, through ^{1}H NMR, we have recorded the data of all isolated compounds. The data records of all the isolated compounds are used to derive the primary structure of bioactive metabolites. The number and type of protons present in each molecule indicate chemical changes and integration. It has often been observed that plurality and coupling constants indicate adjacent protons and their spatial arrangement. And the purity of the compounds can also be determined through ^{1}H NMR.

3.6.2.2 ^{13}C NMR: in these present studies, ^{13}C spectral data was recorded for every isolate compound, to find out the numbers and types of carbon present in all molecule. In these spectra quarter nary and methylene carbon signals appeared in the positive phase, even as methyl and methane signals were apparent in the negative phase of the spectra. CH_3 and CH signals are directed towards the positive phase of the spectrum and CH_2 signal to the negative phase of the spectrum the numbers of quarternary carbon present in the molecules were determined by comparison of ^{13}C.

3.6.3 Mass Spectrometry: By this analysis, MASS was carried out to know the molecular weight of all compounds. (each fraction). In this type of mass spectrometer, the evaporating compound in an empty chamber is bombarded with electrons with an energy of 25.80 eV by electron impact technique (EI). In this type of technology, a high energy is ionized, due to which the organic molecules are fragmented. It is significance to point out that the energy which is required to break down the strongest single bonds in organic molecules is about 4 eV, while to ionize an organic molecule this energy is about 7-10 eV (Elsayed, 2010).

Primarily atoms and molecules can be separated and quantified using a mass spectrometer based on the difference in mass-to-charge ratio (m / z) of ionized atoms or molecules. And in this way, it is used to know the important structure of any compound. There are 4 important parts of mass spectrometry, recorder ionization source, mass-analyzer, and detector. The output of the mass spectrometer is of relative intensity and mass-to-charge ratio (m / z). On the basis of this, the molecular weight of all compounds is derived.

3.6.4 Analytical high-pressure liquid chromatography: In today's date HPLC is a very important technique used for the identification and separation of bioactive natural products, as well as its use in quantifying the bioactive compound.

After columns chromatography of all fractions, HPLC was used for further purification (depending on the amount of these fractions). The purity concentration of the bioactive metabolites compounds was analyzed by analytical HPLC supported with Waters Spherisorb 5 lm ODS2 4.6 X 250 mm analytical cartridge (C-18 column) on a Waters 515 pump; a isocratic Reverse phase system with a 2998 photodiode array detector at 210 nm and the range given was 190–600 nm. Here the flow rate was maintained at 1 ml/minutes; Bioactive metabolites were measured at 254 nm using the Empower 2 software with an additional UV detector. And methanol was also used as a mobile phase. The purified bioactive metabolites were properly mixed with HPLC grade methanol before being injected into the injection port of the HPLC and then filtered using a 0.2 µl Millipore membrane filter. And the sample was run for 15 minutes and the retention time was noted; Based on the percentage of area of the peak the purity of the compound was recognized.

And all peaks are detected at 235, 240, 280 and 340 nm by UV-VIS photodiode array detector.

The part	**Specifications for HPLC**
Pump	Waters 515
Column	Waters Spherisorb (ODS2 4.6 X 250 mm, ID), pre-packe with Eurosphere 100-5 C18, with integrated pre-column
Column thermostat	STH 585
Detector	isocratic Reverse phase system with a 2998 photodiode array detector at 210 nm
HPLC program	Chromeleon (V. 6.3)

Table 3.2: Summary of the specifications of the main parts for the semi-preparative HPLC.

3.6.5 UV-absorption spectrum: Ultraviolet (UV) spectrums were analyzed on the basis of Shimadzu UV-1800 spectrophotometer. Different compounds have been obtained by endophytes bacteria (strain no. EFB-03) were dissolved in methanol at

A concentration of 1 mg/ml and the spectrums were recorded at 200–500 nm range using UV-Probe software.