CHAPTER III
MATERIALS AND METHODS

3.1 Sample Collection

*Bacillus subtilis* was isolated from soil sample that was collected from the University campus. *Pseudomonas fluorescens* was isolated from rhizosphere soil (soil in the vicinity of the root) of the plant, *Catharanthus roseus*, found growing as a weed, in and around the University campus. The soil samples were taken to laboratory in sterile containers and stored in clean, dry place at room temperature for further studies.

3.2 Isolation of cultures

The cultures were isolated from their respective samples through the serial dilution technique. A quantity of 1g of soil was taken in a conical flask containing 100ml of sterile distilled water. An aliquot of 1 ml of the soil suspension was transferred into a test tube containing 9 ml sterile distilled water, making the dilution factor as $10^{-2}$. From that tube, 1ml was again transferred to test tube with 10 ml distilled water for a final dilution of $10^{-3}$. Likewise, a series of test tubes with 9 ml distilled water consecutively to obtain different dilutions till $10^{-7}$.

From each tube, 0.1 ml of culture was spreaded on sterile petriplates containing Nutrient Agar (Hi Media Laboratories Ltd., Mumbai, India) and incubated at 37 °C for 24h. After incubation, the plates were checked for white, swarmed mucoid colonies which could tentatively be noted as *Bacillus* sp. and yellow mucoid colonies for *P. fluorescens*. The cultures presumed to be *P. fluorescens* were also inoculated on selective media, Pseudomonas F agar (HiMedia Laboratories Ltd., Mumbai, India) and incubated for 24 h at 37 °C.

The cultures were then subjected to morphological identification, biochemical characterisation and subsequently gene sequencing for the confirming the two isolated cultures.
3.3 Morphological Identification

3.3.1 Gram staining

Principle

Crystal violet permeates cells. Iodine is subsequently used as mordant to form CV-I (crystal violet-iodine) complex, so that dye cannot be easily removed. Treatment with ethanol decolorizes by dissolving liquid layer from G-ve cells and remaining excessive unbound dye. The removal of liquid layer enhances the leaching of primary stain (CV) from the cells into surrounding solvent. The solvent dehydrates the G+ve cells, closing the pores as the wall shrink during dehydration. As a result, diffusion of CV-I complex out of the cell is obstructed and the cells remain stained purple. A counterstain of safranin is applied to the smear, to dye the decolorized G- cells, with a pink color.

Procedure

On a clean, dry slide, a drop of distilled water placed to which a loopful of culture was added. The mixture was spread to a very thin smear. The slide with the culture was heat fixed. 2-3 drops of crystal violet dye was added onto the smear and left for 1 min. After which the smear was washed with running tap water. Gram’s iodine of 3 drops was added and left for 1 min. After washing with water, few drops of ethanol was added for 30 sec. To the washed smear, 2 drops of saffranin was added and left for 2 min. The water- washed slide was air dried and viewed under light microscope with 100X magnification.

3.3.2 Endospore staining (Schaffer – Fulton method)

Materials required

Culture broth, malachite green, saffranin, distilled water.
Principle

A primary stain, malachite green, is used to stain the endospore. Because endospore has a keratin covering and resist staining, malachite green will be forced to enter the endospore by heating, which acts as mordant. Water is used to remove excessive dye, acts as the decolorizing agent, as endospores are stain resistant. Endospores are equally resistant to destaining, thus retains primary stain while vegetative cells lose the stain. Cells get stained pink on counterstaining with saffranin.

Procedure

A very thin smear was prepared with a loopful of culture mixed in a drop of distilled water was placed on a clean, dry slide. The slide was placed carefully on stand mounted on boiling water bath. The smear was fully covered with malachite green stain, and was allowed to fix the smear for 10 min. Then the slide was washed with water, completely to remove excess unbound stain. Few drops of saffranin was added to counterstain and left for 2 min. The slide was later washed and viewed under microscope with 100X magnification.

3.3.3 Negative staining

Materials Required

Culture broth, India Ink or Nigrosin, Distilled water

Principle

Capsules are mainly carbohydrate metabolites tightly bound to cell wall of microorganisms which are also found to constitute nucleic acids and proteins. Capsules are characterized by poor staining of standard dyes. Capsule staining can be accomplished by using India ink. The negatively charged dye interacts with the negative ions of the bacterial capsules and cell wall, due to which repulsion of dye occurs. Thus under microscope, the cells with capsule appear bright with no stain while the background appears dark.
**Procedure**

A drop of Indian ink is placed at one end of a clean slide. One drop of culture broth was added to the dye and mixed well. With the help of another slide, the mixture was swiped throughout the slide to form a thin, transparent smear. The air dried slide is then observed under microscope with 100X magnification.

**3.3.4 Motility test (hanging drop technique)**

This technique was carried out to check whether the organism is motile (or) non-motile, which is due to the presence of locomotive organ, flagella. A drop of culture broth was placed in the center of the corner slip. A cavity slide was placed in such a way that the culture hangs into the cavity from the coverslip. The slide was viewed for motility under microscope.

**3.4 Biochemical characterization (IMViC tests)**

**3.4.1 Indole production test**

**Materials Required**

Tryptone water broth, Kovac’s reagent, culture.

**Principle**

This test is based on the hydrolysis of tryptophan into indole and pyruvic acid, using a hydrolytic enzyme (hydrolase) called tryptophanase. Indole produced is detected with Kovac’s reagent. If the organism has the ability to hydrolyse, cherry red ring at the top of the culture broth could be observed, indicating the presence of indole.

**Procedure**

A loopful of culture was inoculated into 5 ml of sterile tryptone water broth containing test tube and incubated at 37 °C for 48 h. After incubation few drops of Kovac’s reagent was added to interpret the result.
3.4.2 Methyl Red test

Materials required

Culture, Methyl Red indicator, MR-VP broth

Principle

Upon fermentation of glucose in MR-VP medium into mixed acids like lactic, acetic, succinic and formic acids and CO₂, H₂ and ethanol, the pH of the medium turns acidic. The pH indicator, methyl red, turns red, if the organism is a mixed acid fermentor.

Procedure

A loopful of culture was inoculated into 2 ml of sterile MRVP broth and incubated at 37 °C for 24 h. After incubation, 3-4 drops of methyl red indicator was added to infer the results.

3.4.3 Voges Proskauer test

Materials Required

Culture, MRVP broth, Barritt’s (A) and Barritt’s (B) reagent.

Principle

This test is carried out to check whether the organism possess the capacity to produce a precursor, 2, 3- butanediol, also called acetoin, during glycolysis, instead of ethanol as metabolite. If glucose could be fermented by organism, medium turns bright red upon addition of reagents.

Procedure

A volume of 5 ml of MRVP broth was inoculated with a loopful of culture and incubated at 37 °C for 24-48 h. To observe the result, 15 drops of Barritt’s (A) reagent was added to the culture broth, following which Barritt’s (B) reagent, about 5 drops, was added.
3.4.4 Citrate utilization test

Materials Required

Culture, Simmon’s Citrate Agar

Principle

The organism is tested for its ability to utilize citrate as the sole carbon source. If citrate is used by the organism, the medium color changes from green to Prussian blue due to alkaline pH. This is detected by pH indicator, bromothymolblue, in the medium.

Procedure

With 5 ml of molten, sterile Simmon’s Citrate Agar, a slant was prepared in a sterile test tube and which a loopful of culture was streaked. The slant was then incubated at 37°C for 24 h.

3.4.5 Carbohydrate fermentation test

Materials Required

Culture, sugars – glucose, fructose, lactose, maltose, mannitol and sucrose, NaCl, water, tryptone

Principle

The ability of carbohydrate fermentation by the organism is being checked. Fermentation begins with the glycolytic pathway. Glucose acts as an electron donor in the fermentation reaction. Pyruvate and metabolic products of glucose act as electron acceptors. The other disaccharides and polysaccharides are hydrolyzed into glucose a converted into glucose then the fermentation happens. Finally the reaction result in acids, ethanol, H₂ and CO₂ and other compounds. When organism ferments, acid organism byproduct, the medium turns yellow due to reduction in pH (acidic). The inverted Durham tubes defect the gas production.
Procedure

To each 5 ml of sterile tryptone water broth containing different sugars (1 % w/v), loopful of culture was inoculated. Each tube contained Durham’s tube. The test tubes were incubated at 37°C for 24 h.

3.4.6 Urease test

Materials Required

Culture, Christensen-Jensen’s Urea agar, distilled water

Principle

Urease is a constitutively expressed enzyme hydrolysis urea to CO₂ and ammonia. An increase in pH due to the production of ammonia results in a color change from yellow (pH 6.8) to light pink (pH 8.2)

Procedure

An agar slant was prepared by adding 5 ml of molten Christensen-Jensen’s urea agar with test tube and sterilizing it at 121 °C for 15 min at 15 lbs/in². Upon solidification, the culture was inoculated and incubated at 37°C for 24 h.

3.4.7 Triple Sugar Iron test

Materials Required

Culture, Triple Sugar Iron Agar (TSI), distilled water.

Principle

This test is to differentiate bacteria that are capable of fermenting carbohydrates and H₂S production. TSI agar contains 3 fermentative sugars – lactose, sucrose in 1% concentration and glucose in 0.1% concentration. Due to the building of acids during fermentation, pH falls. The acid indicator, phenol red, is incorporated to detect fermentation, by change in color from red to yellow. If decarboxylation of peptone occurs, alkaline pH prevails, thus medium turns deep red. Sodium tri sulfate and ferrous
tri ammonium sulfate detects H₂S production, indicated by black color in the broth of the tube.

Procedure

A TSI Agar slant was prepared in a sterile test tube by adding sterile TSI Agar solution, of about 5 ml, and solidified. A loopful of culture was inoculated on slant and incubated at 37 °C for 24 h.

3.4.8 Starch Hydrolysis

Materials Required

Nutrient starch agar, culture, distilled water.

Principle

The purpose is to check whether the bacterium uses starch, a complex carbohydrate made from glucose, as a source of carbohydrate and energy for growth. Use of starch by microbes is due to the production of hydrolyzing enzyme, α-amylase. Starch hydrolysis can be detected as a halo surrounding the culture, upon adding iodine. If unutilized, the medium remains blue.

Procedure

A loopful of culture was streaked on a sterile petridish containing nutrient starch agar. The inoculated plate was incubated at 37 °C for 24 h. After incubation, iodine solution was added onto the plate, to observe the results.

3.4.9 Casein hydrolysis

Materials Required

Skimmed milk, agar, culture, distilled water
Principle

The enzyme caseinase is secreted out of the cell as an exoenzyme into the surrounding medium catalyzing the breakdown of milk protein, casein, into small peptides and individual amino acids, which are then taken up by organism for energy or used up as a building material. A zone of clearance was observed upon casein hydrolysis.

3.4.10 Gelatin hydrolysis

Materials Required

Nutrient gelatin, distilled water, culture.

Principle

Gelatin gets hydrolyzed in the presence of gelatinases to poly peptides, which are then broken down to amino acids. This results in the liquefaction of the medium.

Procedure

A loopful of culture was inoculated in 5 ml of sterile nutrient gelatin medium. The inoculated tubes were incubated for 24 h at 37°C.

3.4.11 Oxidase test

Materials Required

N,N,N’,N’- Tetramethyl p- phenylenediamine, culture broth, distilled water.

Principle

The oxidase test in a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidised colored product.
**Procedure**

To 5 ml of culture broth, 1 ml of reagent was added and observed for the color change.

### 3.4.12 Catalase test

**Materials Required**

3% hydrogen peroxide, culture broth.

**Principle**

The catalase enzyme serves to neutralise the bactericidal effects of H$_2$O$_2$. Catalase expedites the breakdown of hydrogen peroxide into H$_2$O and O$_2$. This reaction is evident by the rapid fermentation of bubbles.

**Procedure**

A drop of culture broth was placed on a clean slide, to which a drop of 3% H$_2$O$_2$ was added and observed for results.

### 3.4.13 Nitrate Reduction test

**Materials Required**

Culture, sulfanilic acid and N, N dimethyl-1-naphthylamine, nitrate broth

**Principle**

The nitrate reduction test is used to identify the presence or absence of nitrite (after incubation) as an indicator of nitrate reduction. Generally some of the facultative anaerobes are capable of using nitrate as an electron acceptor at the end of the electron transport chain and that reduce nitrate (NO$_3$) usually produce nitrite (NO$_2$). The nitrite will react with indicator and produce red color which denotes nitrate positive.
Procedure

The sterile nitrate broth was inoculated with isolated bacteria and incubated at 37 °C for 24 h. After incubation period, few drops of sulfanilic acid and α- napthylamine were added and a color change was compared with control (nitrate broth with no inoculum).

3.5 Effect of UV radiation on EPS yield

The culture suspension was kept under UV lamp at a distance of 50 cm. Each flask was exposed to UV at different time intervals as 10-70 min, with an interval of 10. the irradiated culture suspensions were inoculated on agar plates and incubated at 37°C for 24h. The culture was checked for their morphology and productivity of EPS.

3.6 Isolation of bacterial DNA

Principle

The lysis of the bacteria is initiated by suspending a bacterial pellet in a buffer containing lysozyme and EDTA. In addition to inhibiting DNases, EDTA disrupts the outer membrane of the bacterium by removing the Mg$^{2+}$ from the lipopolysaccharide layer. This allows the lysozyme access to the peptidoglycan. After partial disruption of the peptidoglycan, a detergent such as SDS is added to lyse the cells. Most of the cells will lyse after this treatment and many can even be lysed without lysozyme. Once the cells are lysed, the solution should be treated gently to prevent leakage of DNA. Subsequent steps involve the separation of the DNA from other macromolecules in the lysate. Both phenol and chloroform, with isoamyl alcohol as a defoaming agent are commonly used to dissolve protein from nucleic acids. These reagents also remove lipids and some polysaccharides. Proteolytic enzymes such as pronase or proteinase K are added to further remove protein. Proteinase K is particularly useful enzyme it is not denatured by SDS and works more efficiently in the presence of SDS. The nucleic acids may then be precipitated in ice cold ethanol if the ionic strength of the solution is high. This is followed by RNase treatment to degrade the RNA. The solution may then be
reprecipitated with ethanol, leaving purified DNA in the pellet which can then be dissolved in an appropriate buffer.

**Materials Required**

Culture, Luria Bertani broth, 1 M Tris HCl, pH 8 (adjust with conc. HCl), 0.5 M EDTA, pH 8 (adjust with conc. NaOH), 5% NaCl, 20mg/ml Proteinase K (working conc-20 µg/ml), 10 mg/ml lysozyme (working conc-10 µg/ml), 20% SDS, 1X RNase, Buffer saturated phenol, 70% ethanol, Absolute ethanol, Phenol, Chloroform (1:1), Chloroform: Isoamyl alcohol (24:1), 3 M Sodium acetate, pH 5.2 (adjust with glacial acetic acid), TE buffer (10 mM Tris HCl, pH 8, 5 mM EDTA, pH 8), TES buffer (10 mM Tris HCL, pH 8, 5mM EDTA pH8, 1.5% NaCl)

**Procedure**

A single colony of bacterial culture was inoculated in 10 ml of LB broth and it was allowed to grow at 37 °C for 12 h at 110 rpm. The cells were harvested at 7000 rpm for 10 min at 4 °C. The media was decanted as such as possible and the pellet was tapped gently. The pellet was resuspended in 5 ml of the ice cold TES buffer and 50 µl of 10mg/ml lysozyme was added and incubated at room temperature for 5-10 min. Proteinase K was added to a final concentration of 40µg/ml and was incubated at 55 °C for 10 min. SDS was added to a final concentration of 1% and incubated in a water bath at 55 °C for 45 min. An equal volume of buffer saturated phenol was added, vortexed well and centrifuged at 12000 rpm for 10 min. The aqueous layer was transferred to a fresh tube and 2 volumes of ice cold 70% ethanol was added and was incubated on ice for 5-10 min. The precipitated DNA was spooled to a fresh tube. It was resuspended in 5 ml of TE buffer completely. A concentration of 100 µg/ml of RNase was added and was incubated at 45 °C in a water bath for 15 min followed by the addition of an equal volume of phenol: chloroform (1:1), vortexed well and centrifuged at 12000 rpm for 10 min at room temperature. The tap aqueous layer was collected and extracted once with chloroform: isoamyl alcohol (24:1). The top aqueous layer transferred to a sterile tube and 1/10th volume of 3M sodium acetate was added and 2 volumes of ice cold 70% ethanol was added to precipitate the DNA. It was centrifuged at 10000 rpm to pellet the DNA. The
DNA pellet was air dried and was redissolved in 200µl of TE buffer. A volume of 5 µl of DNA sample was checked using 0.8% agarose gel electrophoresis.

3.7 Molecular Identification of the strain

The molecular identification of the characterized culture was done by analyzing the genomic DNA. PCR analysis was performed with 16SrRNA primers: 27F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1492R (5’- TAC GGT TAC CTT GTT ACG ACT T-3’). A volume of 25µl reaction mixture for PCR was carried out using 10ng of genomic DNA, 1X reaction buffer (10mM Tris HCl pH 8.8, 1.5mM MgCl₂, 50mM KCl and 0.1% Triton X 100), 0.4 mM dNTPs each, 0.5U DNA polymerase and 1mM reverse and forward primers each. The reaction was performed in 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, 72°C for 60 sec and an extension step at 72°C for 10min. The sequencing of 16S amplicon was performed according to manufacturer instructions of Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The 16S rRNA gene sequence obtained from the organism was compared with other Pseudomonas strains for pairwise identification using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and multiple sequence alignments of the sequences were performed using Clustal Omega version of EBI (www.ebi.ac.uk/Tools/msa/clustalo). Phylogenetic tree was constructed by Clustal Omega of EBI (www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny) using neighbor joining method.

3.8 Optimization studies

3.8.1 Pretreatment of agro industrial waste

(a) Cane molasses

Cane molasses, was obtained from a sugar factory. For the study, cane molasses was diluted with distilled water containing sodium dihydrogen orthophosphate, at a concentration of 2 g/L at a ratio of 1:1. The solution was autoclave at 121 °C for 20 min at
15 lbs/in\(^2\). The sterile cane molasses was then filtered and used for fermentation processes.

(b). Rice bran and wheat bran

Bran of rice and wheat were procured from local mills. They were sieved and blended to fine powder. The powdered bran was stored in air tight container to prevent from moisture and pests. Bran, whenever needed was autoclaved at 121°C for 20min at 15lbs/in\(^2\) along with other medium components.

3.8.2 One factor at a time approach

24h culture was used throughout the study. Nutrient broth (pH 7) was inoculated with 2% culture and incubated at 37°C for 24 h. To study the effect of various carbon sources, synthetic sugars like glucose, fructose, lactose, mannose, xylose and sucrose and agro wastes namely cane molasses, rice bran and wheat bran were added at different concentrations of 1, 2, 5, 7 and 10% to each flask with 100ml nutrient broth, then inoculated with 2% culture. Effect of nitrogen sources was experimented by adding different nitrogen components like peptone, yeast extract, beef extract, ammonium chloride and sodium nitrate, at a concentration of 0.1-1.1% with an interval of 0.2, replacing standard nitrogen sources in nutrient broth. Effect of salt concentration on EPS production was checked by adding NaCl at varying concentration of 0.1-1.1% with an interval of 0.2 in nutrient broth. To observe the effect of mineral salts like CaCl\(_2\), FeCl\(_3\), MgSO\(_4\), KH\(_2\)PO\(_4\), CoCl\(_2\), MnCl\(_2\), ZnSO\(_4\), CuSO\(_4\), NaMoO\(_4\) were added at a concentration of 0.01, 0.03, 0.05, 0.07, 0.09, 0.1, 0.5 and 1% to nutrient broth. Effects of vitamins – Vit B\(_1\), ascorbic acid and biotin and amino acids – L-Asn, L-Gln, L-Cys were used at concentrations of 2.5x10\(^{-3}\)% at an interval of 2.5 x 10\(^{-3}\).

3.8.3 Plackett Burman Design

The screening of significant nutrients was carried out using Plackett Burman Design. Based on one factor at a time experiments, the effective nutrient were screened. Based on these nutrients, most essential independent variables (nutrients) were selected, evaluated in varying number of experimental trials according to the selected variables.
Each nutrient was designated as +/- levels, i.e. at two concentrations, low and high. The concentration levels were also selected by one factorial experiment. Plackett Burman design was showed as the first order polynomial model,

\[ Y = \beta_0 + \sum \beta_i X_i \]  

\( (3.1) \)

where, \( Y \) is the response (EPS yield), \( \beta_0 \) is the model intercept and \( \beta_i \) is the linear coefficient and \( X_i \) is the level of independent variable.

This model does not derive the interactive effects but used to screen the nutrients implementing the yield of EPS. The experimental design and statistical analysis of the data were done by Minitab statistical package v 16.0. In the present study, the trials were carried out in duplicates and analyzed EPS was taken as the response. Regression analysis determine the components based on the significant level of 95% (p<0.05).

3.8.4 Central Composite Design (CCD)

A central composite design was experimented to optimize the variables, screened by Plackett Burman design that significantly influenced EPS production. Design Expert software (v 8.0.7.1 Stat-ease, Minneapolis, USA) was used to frame the experimental designs and statistical analysis. The independent variables were evaluated at 5 levels (-2, -1, 0, 1, 2) which respective experimental runs and corresponding repetitive central points. The experiments were conducted in 50ml Erlenmeyer flasks with 100 ml medium under non agitating conditions at 37\( ^0 \)C for 48h prepared according to the design. The response obtained to be represented by a second degree polynomial equation as

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{jj} X_j^2 \]  

\( (3.2) \)

where, \( Y \) is the response, \( \beta_0 \) is the constant, \( X_i X_j \) are the input variables, \( \beta_{ij} \) was the second order interactive coefficient, \( \beta_{jj} \) was the quadratic coefficient. The experiments were carried out in triplicates. The response, EPS yield (gL\(-1\)) was the dependent variable.

The 3D graphical plots obtained illustrated the mutual interactions between each significant factor, thus evaluating the optimized medium components. The orthogonal array of the actual and coded values was tabulated.
3.8.5 Optimisation of Environmental parameters

For an enhanced production of EPS, the environmental parameters – temperature, pH, incubation time and inoculums concentration were also optimized by response surface methodology with central composite design. A CCD was framed as mentioned above for optimizing the nutritive components. The actual values of coded levels were tabulated.

3.8.6 Overall process

The sequential steps of the production of exopolysaccharides are given in figures 3.1 and 3.2.

![Overall process of optimized EPS production from Bacillus subtilis](image)

Figure 3.1 Overall process of optimized EPS production from *Bacillus subtilis*
Figure 3.2 Overall process of optimizing EPS production from *Pseudomonas fluorescens*.
3.9 Fed batch Fermentation of EPS

For fed batch fermentation, 500 ml of fermentation basal media was prepared for each culture. 1L of respective carbon source solutions were prepared separately and sterilized. 10 ml of inoculum added to each fermentation broth. For EPS production from *B. subtilis*, 165 ml of 2 % cane molasses was added consecutively with an interval of 24h for 5 days, for an initial concentration of 3.3 %. To obtain biopolymer from *P. fluorescens*, 5% rice bran solution was injected into the culture broth for 5 days at a rate of 250 ml / 24h, enabling a starting concentration of 12.5 %. 5 ml of samples were retrieved for every 24 h to analyze the cell mass, EPS and amount of sugar present in the fermentation medium.

3.10 Immobilisation Technique

To learn the effect of immobilization on EPS production, this study was carried out. Alginate beads were prepared at a concentration of 2% in 0.2 M CaCl$_2$. 1ml of culture sample was encapsulated by adding it in warm sodium alginate solution prior to bead preparation. Various quantities of beads (20, 40, 60 and 80 numbers) were taken in a series of Erlenmeyer flasks containing 100 ml of the optimised medium. All the inoculated flasks were then incubated at 37 $^\circ$C for 48 h (*Bacillus subtilis*) and 30$^\circ$C for 72h (*Pseudomonas fluorescens*).

3.11 Isolation of EPS

The bacterial culture was centrifuged at 11000 rpm for 10 min. The supernatant collected was mixed with equal volume of ice cold ethanol and incubated at 4$^\circ$C for 24 h. The refrigerated solution was then centrifuged at 2500 rpm for 20 min. The obtained pellet was resuspended in distilled water, along with equal volume of ice cold ethanol. The solution was again centrifuged at 2500 rpm for 20 min. The final pellet obtained was lyophilized and weighed.
3.12 Solubility of EPS

A quantity of 100mg was taken in each test tube and added 5 ml of various organic solvents to check the solubility of the isolated exopolymer. Solvents such as methanol, ethanol, butanol, isopropyl alcohol, pentanol/iso amyl alcohol, hexane, chloroform, petroleum ether, ethyl acetate, benzene, toluene were used for this study. Another set of tubes containing 5 ml of concentrated and diluted (10%) sulfuric acid, hydrochloric acid and nitric acid were also tested for EPS solubility. The test tubes were left overnight at room temperature and later checked for solubility.

3.13 Determination of total carbohydrate content (Dubois et al, 1956)

Materials Required

5% Phenol, 96% H$_2$SO$_4$, Glucose (Stock: 1mgml$^{-1}$; working standard: 1mgml$^{-1}$).

Principle

In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This forms colored product with phenol and has a maximum absorption at 490 nm.

Procedure

Aliquots of 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard solution were added into a series of test tubes. Test solution of 0.2 ml volume was pipetted out into another tube. The volume of all tubes was made upto 1 ml with distilled water. The blank was set with 1ml distilled water. A volume of phenol (5 %) was added to each tube and shaken well. The tubes were then left in a water bath (30 °C) for 10 min. The tubes were cooled and optical density was read spectrophotometrically at 490 nm. The concentration of the test sample can be extrapolated from the standard chart (Figure 3.1).
3.14 Estimation of total proteins (Lowry et al, 1951)

**Materials Required**

- Stock - Bovine Serum Albumin (1 mg/ml⁻¹)
- Working standard-100µg/ml⁻¹
- 2 % Sodium Carbonate in 0.1 N NaOH (reagent A)
- 0.5 % CuSO₄. 5H₂O in 1 % Sodium potassium tartarate (reagent B)
- Alkaline Copper Solution- 50:1 (A:B) ; freshly prepared-prior to use
- Folin – Ciocalteau reagent (reagent D)

**Principle**

The principle behind the Lowry’s method lies in the reactivity of peptides with the CU (II) ions under alkaline conditions and the subsequent reduction of Folin – Ciocalteau components - phosphomolybdic and phosphotungstate acid to heteropolymolybdenum blue by the copper catalyzed oxidized of aromatic acids. The Lowry’s method is sensitive to pH changes maintained at 10 – 10.5.

**Procedure**

Aliquots of 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard solutions were added into each test tube. An aliquot of 0.2 ml of test solution was added into another tube. The value was made upto 1ml using distilled water. A tube with 1ml of distilled water served as blank. To each test tube, 5ml of reagent C was added. The solutions were allowed to

![Figure 3.3 Calibration plot for total carbohydrate estimation](image-url)
stand for 10 min. Then 0.5 ml of reagent D was added to tubes and incubated at dark in room temperature for 30 min. The absorbance was read at 660 nm using a spectrophotometer. The concentration of the test sample can be extrapolated from the standard chart (Figure 3.2).

3.15 Fourier Transform Infra Red Spectroscopic Analysis

A quantity of 50mg of lyophilized SNP was taken, mixed with 150mg of KBR powder and ground well to fine mixture. The mixture was pressed to a disc using a hydraulic press. The disc was subjected to FTIR spectral measurement in the frequency range of 4000-600cm\(^{-1}\). The exopolysaccharide was characterized using a Fourier Transfer Infrared Spectrophotometer (Bruker Optics, GmBH, Germany).

3.16 Particle Size Distribution

Particle size of EPS was determined using a particle size analyser, Mastersizer 2000 (v. 5.54, Malvern Instruments Ltd., UK). The technique used light diffraction, which measures the size of the particles. It does this by measuring the intensity of the light scattered as a laser beam passes through a dispersed particulate sample. This data is used to analyse the particle size which created the scattering pattern.
The water insoluble exopolysaccharide powder was mixed thoroughly in distilled water and introduced into the dispersion unit, with a high agitation, dispersed the sample well and directed the sample into the optical bench where the laser beam was passed through the sample and the diffraction pattern was obtained that indicated the size of the particles.

3.17 Nanoparticle Synthesis

A volume of 10ml of the extracted EPS sample was added to 50 ml of 1mM silver nitrate solution. The solution was left at room temperature in dark for 7 days. The change of pale yellow color to dark brown was observed, due to reduction of AgNO₃. The solution was centrifuged at 12000 rpm for 20 min. The pellet was suspended in 5 ml of distilled water. The pellet was dissolved thoroughly and centrifuged again. The step was repeated thrice. The final pellet was dried and the silver nanoparticles were weighed.

3.17.1 UV Visible Spectral Analysis

Primary characterization of SNP was performed by UV-Vis spectroscopy (Model- SL 159, ELICO Ltd, India). An aliquot of 2.5 ml of silver nanoparticle solution was taken in a cuvette and measured with wavelengths ranging between 200-600nm against blank.

3.17.2 Fourier Transform Infra Red Spectroscopic Analysis

A quantity of 50mg of lyophilized SNP was taken, mixed with 150mg of KBR powder and ground well to fine mixture. The mixture was pressed to a disc using a hydraulic press. The disc was subjected to FTIR spectral measurement in the frequency range of 4000-600cm⁻¹. The exopolysaccharide was characterized using a Fourier Transfer Infrared Spectrophotometer (Bruker Optics, GmBH, Germany).

3.17.3 Scanning Electron Microscopy and EDS studies

Morphology of synthesized SNP was examined using Scanning Electron Microscopy (Model-JSM 5610 - Jeol, Japan). Thin films of samples were coated onto a carbon tape and allowed to dry for 5 min. The SEM images were obtained under 30kX magnification. The SEM machine was operated at an accelerating voltage of 20kV. The
low voltage was maintained so that damage to thin sections of sample could be minimized. The presence of elemental Ag in nanoparticles was analyzed using a SEM equipped with an EDS attachment.

3.17.4 Evaluation of antioxidant activity- DPPH Scavenging Activity

Materials required

Vitamin C (Stock-1mg/ml; working standard: 10ml stock in 100ml distilled water), 0.4mM DPPH (Diphenyl Picryl Hydrazyl), test sample

Procedure

Volumes of 0.2, 0.4, 0.6, 0.8 and 1ml of test samples at different concentrations of 200-1000µg were taken in a series of test tubes. The samples were made upto 1ml with distilled water and shaken vigorously. To each test tube, 0.2ml of 0.4mM DPPH was added and incubated for 30 min at dark. The absorbance was read against blank (distilled water) at 517nm spectrophotometrically. Vitamin C (Vc) was used as the positive control. The scavenging activity of the samples was calculated using the formula

\[
\text{Scavenging activity} (\%) = 1 - \frac{A}{B} \times 100
\]

3.17.5 Antibacterial activity

Materials Required

Culture broth, test cultures, Mueller Hinton Agar, Cotton swabs.

Procedure

The antimicrobial activity was determined by well diffusion method. Test organisms namely *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhii* and *Candida albicans* were used for the study. A volume of 100µl culture was spread on sterile Mueller Hinton agar plates and wells were made. A volume of 20 µl EPS reduced SNP were added to each plate and incubated at 37°C for 24h.
3.18 Kinetic studies

The kinetics and modeling of the experiments for cell growth, substrate utilization and product formation were carried out for both the studied cultures. An inoculum of 2% was added to the respective fermentation broths for *B. subtilis* and *P. fluorescens*. The variables changing with respect to time were accounted for every 6h. Once it had attained the stationary phase, the experimentation was ceased. Growth of the cells was determined by dry weight of the cells, substrate consumed was analysed by Dubois et al method and product formed was estimated by weight of lyophilized EPS samples.

The error analysis of each experiment was calculated using the formula

$$\% \text{ error} = \left(\frac{\text{experimental} - \text{predicted}}{\text{experimental}}\right) \times 100 \quad \text{.................. (3.3)}$$

3.18.1 Determination of viscosity and relative viscosity

Viscosity was checked for the culture broth every 6h simultaneously during kinetic experimentation.

![Simple Ostwald's viscometer](image)

**Figure 3.5 Simple Ostwald's viscometer**

A simple Ostwald's viscometer was used for the study (Figure 3.3). The culture filtrate was introduced into the storage bulb (larger), such that the solution reached the measuring bulb (smaller) above the upper mark. Immediately as it started passing down through the capillary, the time taken to flow from the upper mark to the lower mark was noted. Similarly the time taken for the solvent to pass the capillary was also noted. The
time taken and the density of the solution and the solvent were used to calculate the viscosity, which is given as

\[ \eta_1 = \eta_2 \left( \frac{t_1 \rho_1}{t_2 \rho_2} \right) \] ........................ (3.4)

where, \( \eta_1 \) and \( \eta_2 \) are the viscosities of solution and solvent, water, respectively; \( t_1 \) and \( t_2 \) are the time taken to traverse by the solution and water respectively and \( \rho_1 \) and \( \rho_2 \) are the densities of solution and water respectively.

The relative viscosity of a dilute polymer solution, \( \eta_r \), defined as the ratio of the viscosity of the polymer solution to the viscosity of the solvent.

\[ \eta_r = \left( \frac{\eta_1}{\eta_2} \right) \] .......................... (3.5)