

## MATERIALS AND METHODS

### 3.1. TEST ORGANISM

*Sclerotium rolfsii* Sacc. has been used for the production of extracellular polysaccharides during the course of present study. It is an omnivorous fungus that commonly occurs in the tropics, subtropics, and other warm temperate regions, especially the Southern United States, Central and South America, West Indies, Southern European countries bordering the Mediterranean, Africa, India, Japan, Philippines, and Hawaii (Aycock, 1966).

The fungus was classified and placed in the form genus *Sclerotium* by Saccardo (1913) as it formed differentiated sclerotia and sterile mycelium. The fungi included in this genus are characterized by production of small tan to dark brown or black spherical sclerotia with internally differentiated rind, cortex, and medulla. *S. rolfsii* was reported as the best known member of the genus by Punja and Damiani (1996).

*Sclerotium rolfsii* is a soil borne plant pathogen, causing diseases on a wide range of agricultural and horticultural crops. The first report of the fungus pathogenicity dates back to 1892 in association with tomato blight in Florida (Weber, 1931). The wide host range, prolific growth, and ability to produce persistent sclerotia contribute to the large economic losses associated with this pathogen. In spite of being a pathogenic organism, *S. rolfsii* has found many uses beneficial to humanity. It produces extracellular polysaccharides which can be used

as viscosifying agents in food, petroleum and pharmaceuticals, industries (Vinarta *et al.*, 2006; Iyer *et al.*, 2006; Coviello *et al.*, 2005). Typical photograph of *Sclerotium rolfsii* and its taxonomical position is shown in Plate 3.1.

### **3.1.1. Source of *Sclerotium* Strains**

Fungal strains were obtained from National Agriculture Research Council (NARC), Islamabad and Fungus Culture Collection, University of the Punjab, Lahore. These cultures were originally isolated from chickpea infected plants in different localities of Pakistan (Table 3.1), were identified as *S. rolfsii* and stored on potato dextrose agar (PDA) slants.

### **3.1.2. Purification, Sub-culturing and Maintenance**

The fungal strains obtained on PDA slants were transferred/sub-cultured by growing on freshly prepared PDA plates. The fresh PDA was prepared as described below:

Two hundred (200 g) of thoroughly washed and peeled fresh potatoes were cut into pieces, boiled to softness, and blended in a Waring blender for two minutes. The resulting paste was mixed with 20 g dextrose, and 15 g agar in 1 L distilled water (Sarma *et al.*, 2002). The mixture was autoclaved for 15 minutes at 121°C. After cooling to approximately 45°C, PDA was poured into the pre-autoclaved Petri plates. Three sclerotia of each strain were inoculated on PDA plates in triplicate and incubated at 28°C and their growth behavior was visually

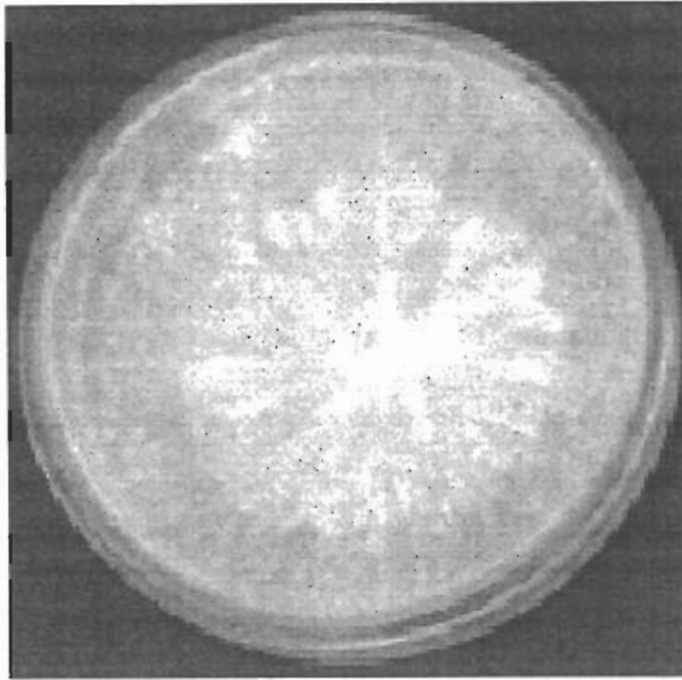


Plate 3.1: Typical photograph of *Sclerotium rolfsii* Sacc.

Kingdom: Fungi  
Division: Basidiomycota  
Class: Basidiomycetes  
Order: Agaricales  
Family: Typhulaceae

Table 3.1: Source/location of fungal strains.

<b>Isolate</b>	<b>Source/location</b>
D1	Fungus culture collection, University of the Punjab, Lahore.
D2	Dera Ismail Khan
D3	Fungus culture collection, University of the Punjab, Lahore.
D4	Dera Ismail Khan
D5	Chakwal
D6	NARC (Farmer Field)
D7	Chakwal
D8	NARC (Research Field)
D9	Dera Ismail Khan

observed for 7 days. All strains were purified by repeated transfers and adaptation on PDA plates.

### **3.1.3. Method of Inoculation**

For obtaining standard amount of inoculum, 10 mm diameter circular disc taken from the margin of an actively growing colony (3-4 days old) was punched out with the help of a self-designed cutter and placed onto the center of the plate with mycelial side facing downwards (Plate 3.2). The inoculations were done in laminar flow hood. After each inoculation the cutter was sterilized on flame, cooled by dipping in methylated spirit and again sterilized on flame; the same procedure was repeated three times to ensure complete sterilization of the cutter. For each experiment the same method was adopted for inoculation on solid medium using petri plates.

## **3.2. CHARACTERIZATION OF FUNGAL STRAINS**

The fungal strains were characterized by studying their growth behavior on PDA plate, mycelial compatibility (morphology and inter-relationship among different strains) and molecular characterization.

### **3.2.1. Growth of Fungal Strains on PDA Plates**

In order to study morphological characteristics, each strain was further grown on PDA plates. Each strain was inoculated on five Petri plates and incubated at 28°C for seven days. Morphological characteristics documented were i) growth,

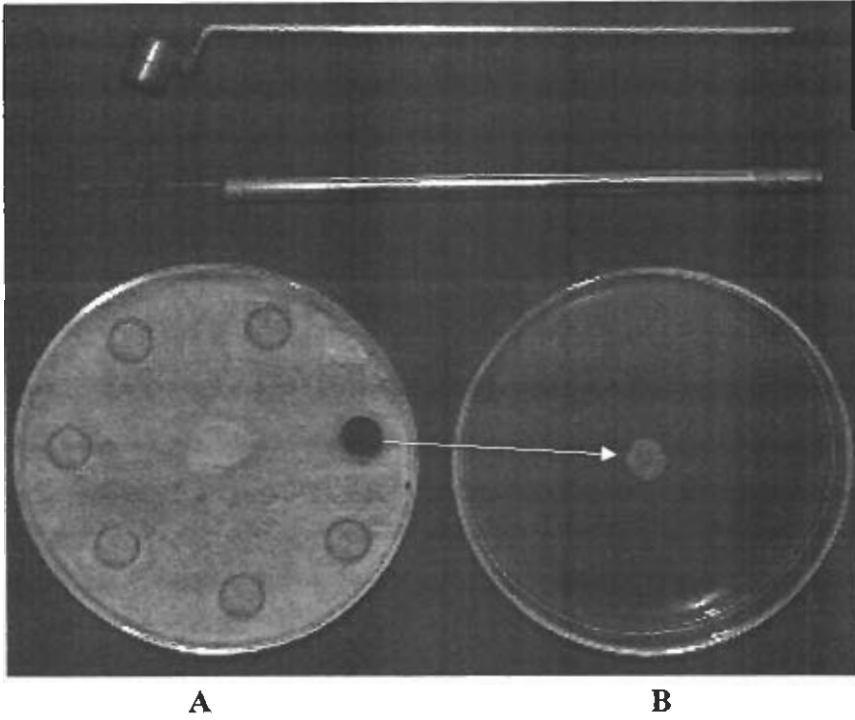


Plate 3.2: Inoculation of fungal culture (disc) from plate A to B.

ii) mycelium condition, iii) radial colony diameter, iv) development of sclerotia, and v) color, type and location of sclerotia. The observations were recorded at 12 hours interval up to 3 days and then at 24 hours intervals for 7 days.

### 3.2.2. Mycelial Compatibility/Incompatibility

All strains of *S. rolfsii* were subjected to mycelial compatibility reaction on PDA plates in order to identify the morphological similarities/differences among various strains as described by Punja and Sun, 2001. Eight fungal strains in combination of three at a time were tested. A total of 56 combinations were prepared using statistical formula as shown below:

$${}^N C_n = \frac{N!}{(N-n)! n!}$$

Where N = Total number of strains i.e. 8

n = Number of sample strains i.e. 3

The PDA plates were marked into three portions. Ten mm diameter mycelial disc of the respective strain was inoculated on each portion of PDA plate as mentioned in Section 3.1.3. Five Petri plates for each combination were inoculated and plates were incubated at 28°C for 15-20 days and examined daily for the presence of clearing zone at the region of mycelial contact.

### 3.2.3. Molecular Characterization

#### 3.2.3.1. RAPD analysis

Genetic similarity and diversity among eight strains of *S. rolfsii* was assessed using randomly amplified polymorphic DNA (RAPD) analysis as described by Punja and Sun (2001).

#### 3.2.3.2. DNA extraction

DNA was extracted from the mycelial mat of each strain grown on PDA plates for seven days at 28°C. Mycelium (200 mg) was transferred to 1.5 mL microcentrifuge tubes containing liquid nitrogen. Subsequently, DNA extraction was carried out by the method of Punja and Sun (2001).

The mycelial mass was thoroughly ground into a fine homogenate using pallet pestle mixer. Eight hundred microlitre ( $\mu\text{L}$ ) of lysis buffer (200 mM Tris pH 8.0; 500 mM NaCl; 100 mM ethylene diaminetetra acetic acid (EDTA) pH 8.0; 2.0 percent sodium dodecyle sulphate (SDS); 1 percent 2-mercaptoethanol) was added to the tubes, mixed well and incubated at 24°C for 30 minutes. Afterwards 400  $\mu\text{L}$  extraction buffer (phenol/chloroform/isoamyl alcohol, 25/24/1, v/v/v) was added followed by gentle stirring on a vortex mixer till an emulsion was formed. The emulsion was centrifuged at 6000 x g for 5 minutes in a microcentrifuge (Eppendorf, Germany) at room temperature. An aliquot of the upper aqueous layer (600  $\mu\text{L}$ ) was collected, mixed with an equal volume of extraction buffer, and re-centrifuged. The upper layer was discarded. and to the lower aqueous layer 10  $\mu\text{L}$  of RNase A (10 mg mL<sup>-1</sup>) was added and the mixture was incubated at 37°C for 30

minutes. The DNA was precipitated by addition of 2.5 volumes of ice cold ethanol (70 percent) and the tubes were placed at  $-20^{\circ}\text{C}$  for overnight. The mixture was centrifuged at  $14000 \times g$  for 30 minutes at  $4^{\circ}\text{C}$ . The pellet was collected and suspended in 70 percent ethanol and re-centrifuged. Finally, the pellet was air-dried for 10-15 minutes, re-suspended in  $100 \mu\text{L}$  of Tris-EDTA (TE) buffer having 10 mM Tris-HCl (pH 8.0) 1 mM EDTA. The DNA concentration was estimated by electrophoresis in 1 percent Agarose gel containing 0.05 percent ethidium bromide. The DNA concentration was estimated with reference to Lambda DNA marker under UV illumination. The DNA concentration was further confirmed spectrophotometer. The stock solution of DNA was prepared at a final concentration of  $100 \mu\text{g mL}^{-1}$  and used at final working concentration of  $100 \text{ ng } \mu\text{L}^{-1}$  for subsequent RAPD analysis.

#### **3.2.3.3. Primer selection for DNA amplification**

The gene link (GL) decamer primers (Gene Link, USA) set A and B were initially screened to detect polymorphism among eight strains of *S. rolfsii*. The GL Decamer set A primers indicated higher degree of polymorphism, were selected for the diversity analysis studies. The DNA sequences ( $5'$ - $3'$ ) of ten primers in GL decamer set A, used for DNA amplification are given in Table 3.2.

#### **3.2.3.4. DNA amplification and agarose gel electrophoresis**

The RAPD-PCR reaction was carried out in  $50 \mu\text{L}$  volume. The PCR reaction contained  $1 \mu\text{L}$  template DNA ( $100 \text{ ng } \mu\text{L}^{-1}$ ),  $1 \mu\text{L}$  ( $4.5 \text{ pMol}$ ) of respective primers,  $1 \mu\text{L}$  dNTPs ( $10 \text{ mM}$  dNTP stock),  $5 \mu\text{L}$  PCR buffer (Tris HCl

Table 3.2: RAPD primers and their sequences.

S. No.	Primer name	Primer sequence (5'-3')
1	A-01	CAGGCCCTTC
2	A-02	TGCCGAGCTG
3	A-03	AGTCAGCCAC
4	A-04	AATCGGGCTG
5	A-05	AGGGGTCTTG
6	A-06	GGTCCCTGAC
7	A-07	GAAACGGGTG
8	A-08	GTGACGTAGG
9	A-09	GGGTAACGCC
10	A-10	GTGATCGCAG

buffer), 5  $\mu\text{L}$   $\text{Mg Cl}_2$  (25 mM stock), 2.5  $\mu\text{L}$  gelatin(1 percent) and taq polymerase 0.5  $\mu\text{L}$  (5 units  $\mu\text{L}^{-1}$ ). Deionized water was used to make the total reaction volume up to 50  $\mu\text{L}$ . In order to ensure the reproducibility of the RAPD reaction, appropriate negative controls (without DNA template) were also run.

PCR tubes were placed in thermocycler (Eppendorf, Germany) for DNA amplification. The cycling parameters were: pre-incubation for 10 min at 94°C followed by denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute and extension at 72°C for 2 minutes while final extension was carried out at 72°C for 10 minutes. The total cycles were 40 for amplification and then the samples were kept at 4°C till further use.

The amplified samples (15  $\mu\text{L}$ ) by adding 5  $\mu\text{L}$  of loading buffer (0.1 percent bromophenol blue, 0.05 percent xylene cyanol FF and 30 percent glycerol) were loaded on 1 percent agarose gel. The gel was then electrophoresed using TE buffer for 4 hours at 100 volts in an electrophoresis apparatus (BioRad). The gel was stained with ethidium bromide (0.1 percent) and photographed under UV illumination using gel documentation system.

### **3.2.3.5. Data analysis**

The fungal strains were compared with each other using their RAPD-PCR profiles and bands of DNA fragments were scored as present (1) and absent (0) for each of the decamer primer used. For the data collection very very faint bands were not included. Genetic similarity matrix was generated on the basis of similarity

coefficients. A dendrogram based on the similarity coefficients was constructed by using un-weighted pair group method of arithmetic means (UPGMA) as described by Nei and Li's (1979).

### **3.3. EFFECT OF DIFFERENT FACTORS ON GROWTH OF FUNGAL STRAINS**

#### **3.3.1. Effect of Temperature on Growth of Fungal Strains**

PDA plates inoculated with test strains were incubated at 24, 28, 32 and 36°C, respectively. Five petri plates were inoculated for each strain as mentioned in Section 3.1.3. and incubated at respective temperatures in an incubator. Growth of fungal strains and production of sclerotia was recorded after 10 days of incubation.

#### **3.3.2. Effect of pH on Growth of Fungal Strains**

Fresh PDA was prepared and its pH was adjusted to 4, 6, 8, and 10 with 0.5 N hydrochloric acid and/or sodium hydroxide, respectively. After autoclaving, it was poured into petri plates. Fungal strains were inoculated as mentioned in Section 3.1.3. Five petri plates/strain/pH levels were inoculated and incubated at 28°C. Growth of fungal strains and production of sclerotia were recorded after 10 days of incubation.

#### **3.3.3. Effect of Sodium Chloride on Growth of Fungal Strains**

Disc (10 mm diameter) from each test strain grown on PDA plate was inoculated on to PDA plates containing 0, 2, 4, and 8 percent NaCl. Five petri

plates/strain/salt concentrations were inoculated as mentioned in Section 3.1.3. and incubated at 28°C. Visual observations regarding growth behavior and production of sclerotia were recorded after 10 days of incubation.

### **3.4. INOCULATION OF FUNGAL STRAINS IN LIQUID MEDIUM**

For inoculation of liquid medium, 10 mm diameter disc from culture plates kept in refrigerator were cut out with the help of sterilized cutter and inoculated into fresh plates containing PDA. The disc was placed upside down in the centre of the plate and incubated at 28°C. Five petri plates were inoculated for each strain. The disc of 10 mm diameter taken from edge of 3-4 day old culture from one plate having best growth was used to inoculate the liquid medium.

#### **3.4.1 Composition of Growth Medium**

All strains were grown in basal mineral medium with slight modification in the medium described by Farina *et al.* (1998). To prepare one litre of mineral medium in gL<sup>-1</sup> was: NaNO<sub>3</sub>, 3.0; K<sub>2</sub>HPO<sub>4</sub>.3H<sub>2</sub>O, 1.3; KCl, 0.5; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5; FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.05; Yeast Extract, 1.0; Citric acid, 0.7 and 25 gL<sup>-1</sup> sucrose as carbon source instead of 20 gL<sup>-1</sup> was added and pH was adjusted to 4.5.

#### **3.4.2. Growth of Fungal Strains in Liquid Medium**

An experiment for studying growth behavior and biopolymer (extracellular polysaccharides, EPS) production by fungal strains in liquid medium was carried out in shake flasks. The discs of 10 mm diameter from the edge of 3-4 day old culture on petri plate were punched out with sterilized cutter and inoculated to 50

mL medium in 300 mL flask, as inoculum. The flasks were incubated on rotary shaker at 100 rpm for ten days. It was observed that as the disc was inoculated, generally it got immersed in the medium and sometimes it was inverted during the course of shaking. This hindered active mycelial growth of the fungal strains. Therefore, the disc was held on the side of flask just above the surface of the medium in such a way that it stuck to the wall of the flask. As growth of fungal culture on the disc progressed, it got dragged inside the medium and continued proliferation. Visual growth observations that is patch type, patch color, development of sclerotia and their color, type, location, broth color and broth jelly were recorded. After 10 days of incubation, pH of the culture medium was also noted.

The amount of dry biomass was determined followed by washing with water and centrifugation of culture broth at 5000 revolution per minute (rpm) for 20 minutes and subsequent drying at 105°C till constant weight. The net weight of biomass was determined gravimetrically. The EPS produced was determined after its precipitation from cell free culture filtrate and drying at 105°C to constant weight. It was observed that as the growth of fungal culture progressed, mycelial mat was formed on the surface of the medium which limited the supply of oxygen to the cells underneath the mycelial mat. Bunches of sclerotia were also developed on the surface of the mycelial mat.

Viscosity of 5 mL culture filtrate in terms of time of flow (seconds) in 20 mL syringe was determined. The syringe method was standardized by using high viscosity synthetic polymer i.e. carboxy methyl cellulose (CMC). A stock solution

of CMC (0.1 percent) was prepared. Different dilutions from the stock solution that is 20, 10, 5, 2.5, 1.66 and 1.25 times in distilled water were prepared. Twenty mL syringe was held in stand and filled with one dilution at a time. Then the solution was allowed to flow under gravity at room temperature. Time taken to flow the solution from 10 mL to 5 mL mark was noted and standard curve was drawn. This method was developed for initial screening of viscous culture broth produced by the fungal strains, used in the present study.

In order to investigate the effect of shaking and non-shaking (stationary) one of the strains D8 was grown on mineral medium containing 1 and 2 percent sucrose. Two sets of flasks were inoculated. One set was kept stationary while the other was incubated on rotatory shaker at 100 rpm for 21 days. From the gravimetric analysis of EPS and biomass produced, it was concluded that culture grown under non-shaking conditions produced comparatively more EPS as compared to cultures grown under shaking conditions. The experiment was repeated and following options were tried to enhance the production of EPS by the fungal strains.

#### **Option I**

After growth of fungal strains in liquid medium, 10 mL of the 6-fold medium was added to the flasks on 6<sup>th</sup> day of the experiment and the experiment was continued up to 21 days under non-shaking condition. EPS was precipitated by ethanol and estimated gravimetrically. It was noted that the recovery of EPS was improved. In order to avoid the possibilities of contamination due to addition of nutrients, it was planned that 6-fold medium will be used instead of 1-fold medium.

It was further concluded that under non-shaking conditions, the quantity of EPS was increased.

## Option II

In order to improve the supply of oxygen and increase in surface area for growth of fungal cultures, 50 mL of medium and 100 g plastic beads (polyethylene) were added to the culture flasks. After inoculation of the test culture, the flasks were incubated at 28°C for 21 days without shaking. The culture flasks were slightly rotated daily in order to ensure supply of oxygen to cells. Based upon the results of above experiments, it was decided that further experiments are to be carried out with 6-fold medium containing beads under non-shaking condition.

### 3.4.3. Selection of Medium and Culture Conditions

In order to verify the effect of medium composition, shaking, non-shaking and hand mixing in liquid medium with and without beads on production of EPS, four sets of experiments with strain D8 were carried out, as detailed in Table 3.3.

In above experiments, basal mineral medium as described by Farina *et al.* (1998) was used for 1-fold medium. However, for 6-fold medium, each ingredient of the medium was multiplied by 6. A total of 48 flasks containing 50 mL of respective medium in 300 mL flasks were autoclaved. Sucrose was autoclaved separately and added into 1 and 6-fold medium at the rate of 2.5 percent. Ten mm diameter disc from 3-4 day old culture of strain D8 was used as inoculum. One set of flasks was incubated at 28°C for 21 days whereas another set was placed on

Table 3.3: Effect of various shaking methods on production of EPS.

<b>Exp. No.</b>	<b>Shaking</b>	<b>Non-Shaking</b>	<b>Hand-Shaking</b>
1.	1-fold medium	1-fold medium	1-fold medium
2.	6-fold medium	6-fold medium	6-fold medium
3.	Beads with 1-fold medium	Beads with 1-fold medium	Beads with 1-fold medium
4.	Beads with 6-fold medium	Beads with 6-fold medium	Beads with 6-fold medium

rotary shaker (30 rpm) at 28°C. Third set of culture flasks were kept in incubator at 28°C for 21 days. However, the contents of the flasks were daily mixed by slightly tilting the flask in such a way that cells growing on the surface were shifted inside the medium while the cells growing inside are brought to the surface. This process facilitated an even supply of oxygen to the cells. It was observed that sclerotia were also developed in flasks containing beads; however, sclerotia were not developed in the liquid medium but only the mycelial growth as shown in Plate 3.3. The production of sclerotia was accompanied by the synthesis and release of EPS.

Based on the results of above experiments, it was decided that 6-fold mineral medium along with beads under hand mixing conditions are to be selected for subsequent experiments.

### **3.5. OPTIMIZATION STUDIES**

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#### **3.5.1. Optimization of Levels of Carbon and Nitrogen**

During preliminary studies, three strains i.e. D4, D7, and D8 were found to be fast growing; produced comparatively more EPS and their culture filtrate took comparatively more time while flowing through syringe. Therefore, these strains were selected for optimization studies. Optimization of levels of two major nutrients that is carbon and nitrogen were carried out under the above selected conditions for EPS production by three fungal strains D4, D7, and D8.

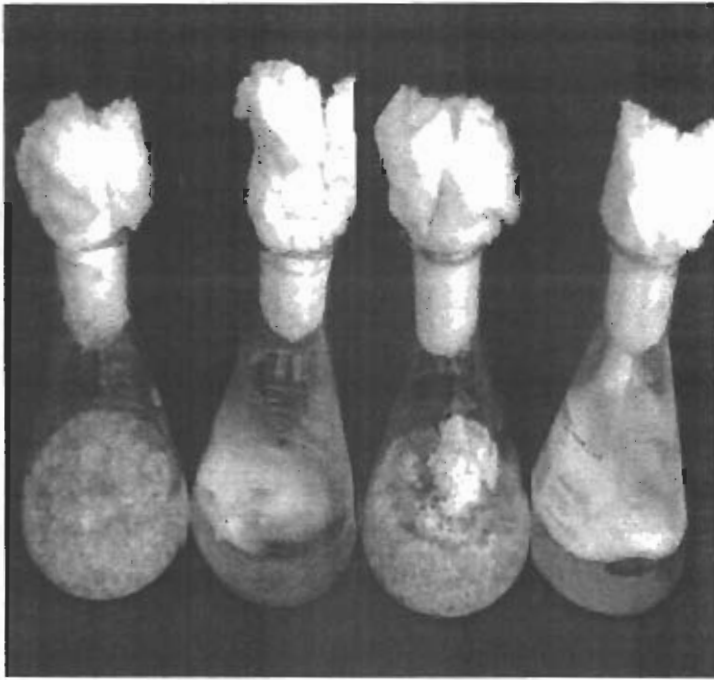


Plate 3.3: Comparison of growth and EPS production in liquid versus beads containing medium.

### **3.5.2. Optimization of Carbon Source**

To examine the effect of two carbon (C) sources; glucose and sucrose on mycelial biomass and EPS production, two sets of experiments were carried out in 144 Erlenmeyer flasks (300 mL), one for glucose and another for sucrose. In each flask 50 mL of the 6-fold medium along with 100 g beads were autoclaved. Both carbon sources were provided at the concentration of 2, 4, 6, 8, 10, 12, and 14 percent. After inoculation of the test strains (discs) from the edge of 3-4 days old cultures of D4, D7, and D8, the flasks were incubated at 28°C for 21 days. All determinations were carried out in triplicate. The un-inoculated flasks (in triplicate) were reserved as control. The contents of the flasks containing beads were daily mixed by hand by slight rotation of the medium upside down.

### **3.5.3. Optimization of Nitrogen Source**

Sodium nitrate ( $\text{NaNO}_3$ ) was used as nitrogen (N) source for EPS production by the fungal strains. The level of nitrogen was optimized for three fungal strains (D4, D7, and D8) using glucose and sucrose as carbon source separately. While optimizing nitrogen source, the concentration of carbon source was kept constant whereas level of nitrogen was varied. A total of 108 Erlenmeyer flasks containing 50 mL of 6-fold medium along with 100 g beads, with  $\text{NaNO}_3$  at the concentration of 0.6, 1.2, 1.8, 2.4, and 3.0 percent were prepared and autoclaved. Ten mm diameter disc from culture plates of three strains were inoculated into sucrose and glucose containing media. The un-inoculated flasks (in triplicate) for each set of experiment were reserved as control. All the flasks were

incubated at 28°C for 21 days. The contents of the flasks containing beads were daily mixed by slight rotation of the medium upside down by hand.

Before harvesting the cells, pH of the culture broth was noted with pH meter. Fungal biomass and EPS were separated by the protocols shown in Figure 3.1 and Figure 3.2. Dry weight of EPS and biomass was determined by gravimetric method. The culture filtrate of each strain was analyzed for utilization of total carbon and nitrogen by the fungal strains using the methods described by Azam and Sajjad (2005) and Keeney and Nelson (1982), respectively. Flow time of cell free culture filtrate was determined by flowing 5 mL of culture broth through 20 mL syringe as described earlier.

#### **3.5.4. Assimilation of NaNO<sub>3</sub> with Sucrose as Carbon Source**

Three fungal strains D4, D7, and D8 were grown in 50 mL of 6-fold medium along with 100 g beads. The medium was supplemented with 1.8 percent NaNO<sub>3</sub> (0.3 g N 100 mL<sup>-1</sup>). Sucrose at the concentration of 2, 4, 6, 8, 10, 12, and 14 percent was added to each culture flask. The flasks were incubated at 28°C for 21 days. However, the culture medium was daily mixed by hand as mentioned in Section 3.4.3. After 21 days of incubation, pH of the culture broth was noted. Biomass and extracellular polysaccharide were separated and quantified. Residual carbon and nitrogen in the cell free culture filtrate was analyzed. Percent utilization of carbon and nitrogen was also calculated.

### **3.5.5. Assimilation of NaNO<sub>3</sub> with Glucose as Carbon Source**

In another set of flasks, three fungal strains D4, D7, and D8 were grown on 50 mL 6-fold medium along with 100 g beads. The medium was supplemented with 1.8 percent NaNO<sub>3</sub> (0.3 g 100 mL<sup>-1</sup>). Glucose at a concentration of 2, 4, 6, 8, 10, 12, and 14 percent was separately autoclaved and added to each culture flask. Utilization of carbon and nitrogen was determined by analyzing residual carbon and nitrogen in the cell free broth. Percent utilization of carbon and nitrogen was also calculated.

### **3.5.6. Assimilation of Sucrose with NaNO<sub>3</sub> as Nitrogen Source**

Assimilation of 15 percent sucrose (6.32 g C 100 mL<sup>-1</sup>) by three strains D4, D7, and D8 at various concentrations of NaNO<sub>3</sub> (0.6, 1.2, 2.4, and 3.0 percent) was investigated. The amount of dry biomass and EPS produced at various concentrations of NaNO<sub>3</sub> was quantified. The effect of C/N ratio on production of EPS was also investigated.

## **3.6. PRODUCTION OF BIOPOLYMER (EPS) BY MOST POTENT FUNGAL STRAIN D8 ON OPTIMIZED MEDIUM**

### **3.6.1. Time Course of EPS Production by Strain D8**

The time course of growth, EPS and oxalic acid production, utilization of carbon, nitrogen, and pH profile of strain D8 was studied at various time intervals. Fifty (50) mL of the optimized 6-fold medium with 100 g beads in 300 mL Erlenmeyer flasks was autoclaved. Glucose and sucrose was separately autoclaved

and added to the medium at 8 percent and nitrogen at 0.6 percent. One set of flasks in triplicate were prepared for each carbon source. Ten mm diameter disc from 3-4 day old culture of strain D8 was inoculated and the flasks were kept in an incubator at 28°C for 21 days. However, the contents of the flasks containing beads were daily mixed by slight rotation of the medium upside down.

As the experiment was carried out with beads, taking sample from the flask by the pipette was not possible, therefore, one flask from each set was harvested at 0, 3, 6, 9, 12, 15, 18, and 21 days. Growth curve of strain D8 with respect to production of biomass, EPS, oxalic acid, and utilization of carbon and nitrogen was drawn. The effect of C/N ratio on synthesis of EPS was also investigated.

### **3.7. ANALYTICAL STUDIES**

#### **3.7.1. Determination Dry Biomass**

Biomass determinations were carried out after addition of 100 mL of distilled water and mixing the whole broth (containing beads and mycelial mat) with spatula. The contents of the flask were transferred to 250 mL glass beaker. The mixture was stirred on a magnetic stirrer for 10 minutes. After thorough mixing, the mixture was passed through strainer. The beads along with mycelial biomass were retained on the strainer. After washing with water, the mixture was collected on Whatman No. 1 and placed on to perforated plate. The whole mixture was dried at 105°C to constant weight. After subtracting the bead weight, dry weight of biomass was calculated.

The culture filtrate was centrifuged at 5000 rpm at 4°C for 20 minutes, the mycelial pellet collected on Whatman No.1 filter paper was dried to a constant weight at 105°C. The weight of dry biomass from beads and filter paper was added to determine total dry biomass. The flow diagram for separation of biomass is shown in Figure 3.1. The supernatant was reserved for the extraction of extracellular polysaccharide.

### **3.7.2. Precipitation and Separation of EPS**

To the cell free culture broth, 2 volumes of 98 percent chilled ethanol was added and the solution was mixed gently and kept overnight at 4°C in a refrigerator. After completion of precipitation, the polymer was recovered by winding onto a wire loop. The remaining EPS was collected by passing through sieve. This crude precipitate was further purified by re-dissolving in 50 mL distilled water and re-precipitation by ethanol (two times). Finally the precipitate was pooled and freeze dried under vacuum for more than six hours and weighed according to the method of Farina *et al.*, 2001. The flow diagram for separation of EPS is given in Figure 3.2.

### **3.7.3. Determination of Total Carbon**

The method based on oxidation of organic carbon with hot solution of  $K_2Cr_2O_7$  and  $H_2SO_4$  by the method developed by Azam and Sajjad (2005) was used to determine the organic carbon content of the culture broth. One mL sample was taken into 100 mL glass beaker followed by addition of 5 mL of 2 N  $K_2Cr_2O_7$  and 8.0 mL of concentrated  $H_2SO_4$ . The reactants were kept in microwave for one

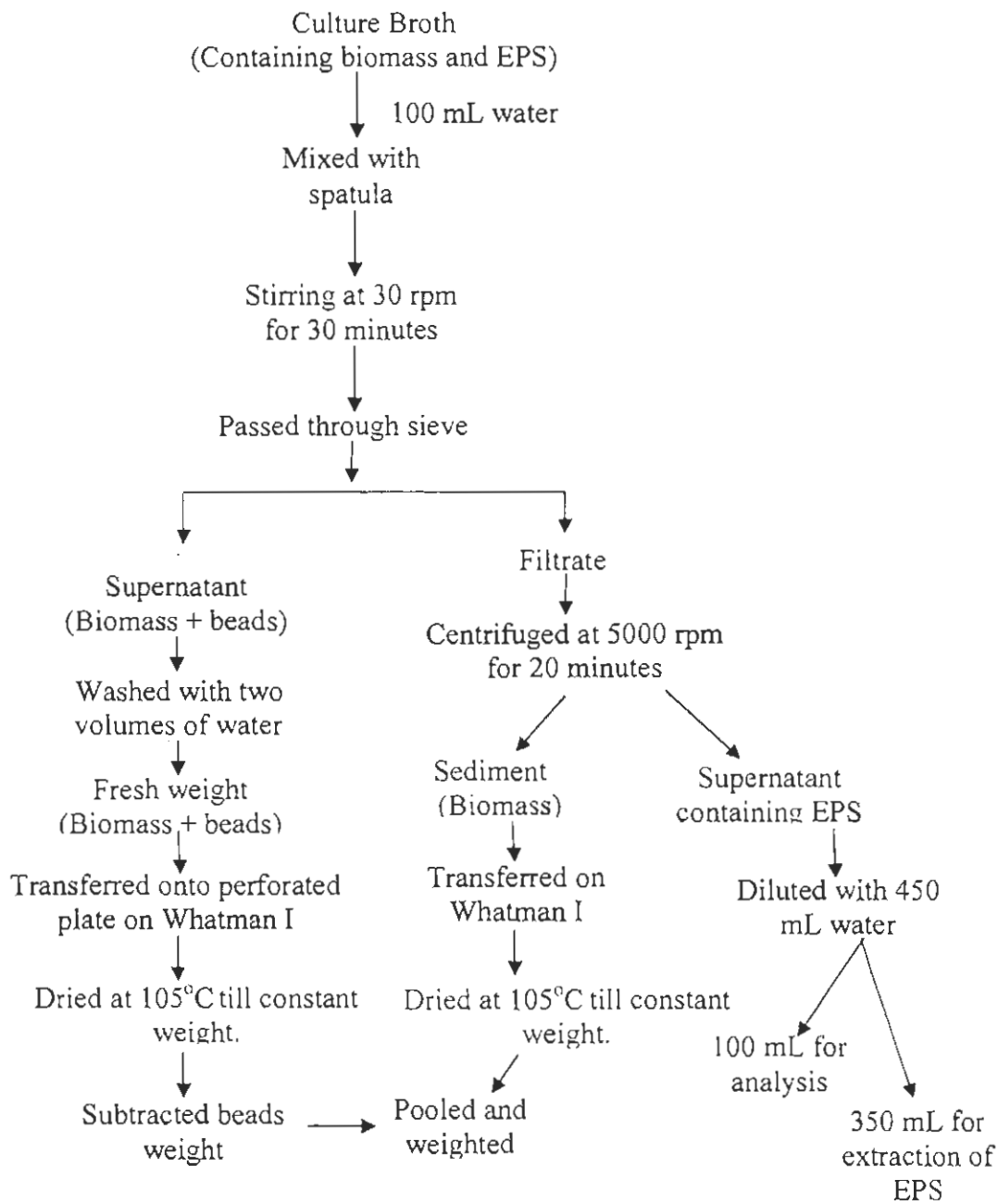


Figure 3.1: Flow diagram for separation of biomass from culture broth.

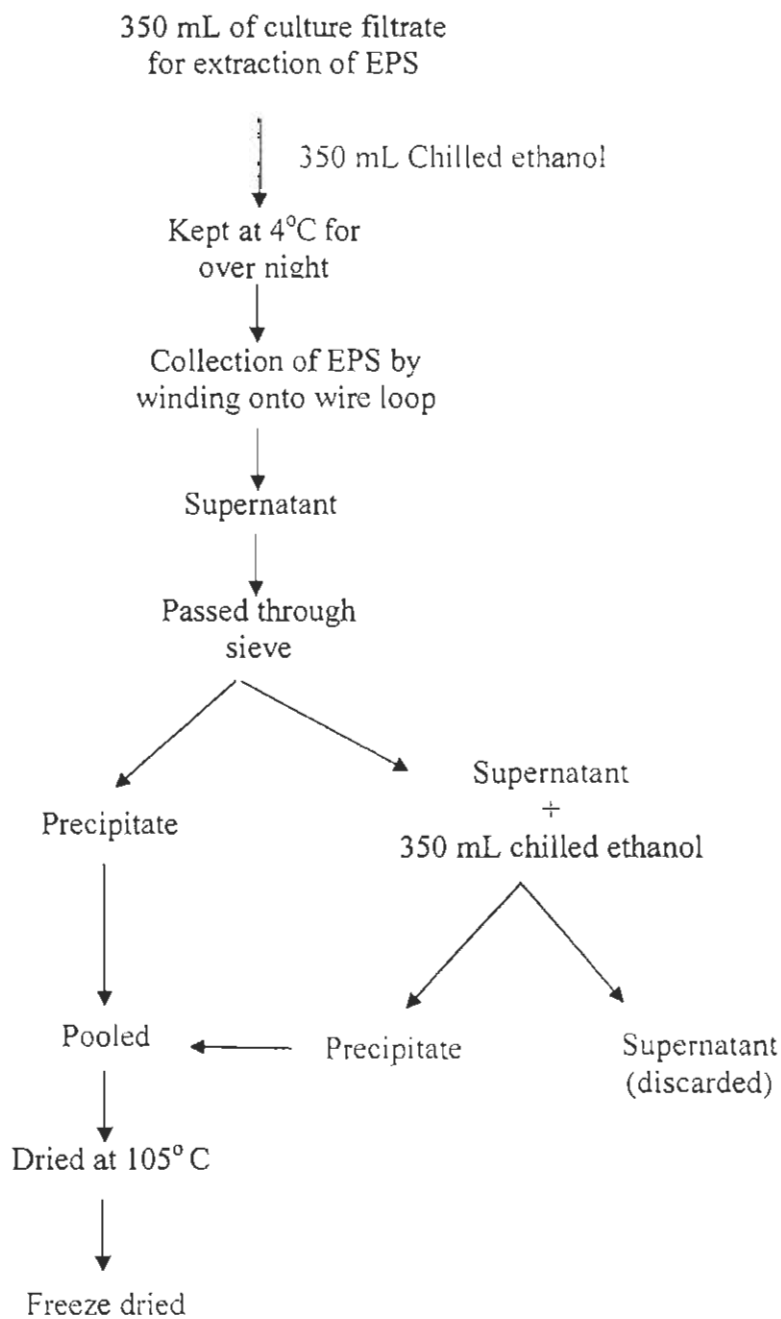


Figure 3.2: Flow diagram for separation of EPS from culture filtrate

minute. After cooling the contents, volume were adjusted to 50 mL with de-ionized water. The absorbance was noted at 590 nanometer (nm) and the carbon content were determined as follows with reference to a standard solution of glucose (1.375 g glucose 100 mL<sup>-1</sup>):

$$\text{Percent C} = \frac{\text{Absorbance (sample)} \times \text{mg C (Standard)} \times 100}{\text{Absorbance (Standard)} \times \text{Volume of Sample (mL)}}$$

#### 3.7.4. Determination of Total Nitrogen

Total N was determined as described by Keeney and Nelson (1982). Five mL of culture filtrate was taken in distillation flask, 0.2 g of heated Mg O (5-6 hrs at 500°C) was added and the flask was attached to the steam distillation unit. The distillate was collected in 5 mL of 2 percent boric acid indicator solution until a volume of about 20 mL was obtained. The flask was detached from the distillation unit and allowed to cool. A portion of the distillate containing NH<sub>4</sub><sup>+</sup>-N was saved for titration. To the remaining distillate 0.1 g Devarda's alloy was added and distillation was carried out for 2-3 hours. This distillate containing NO<sub>3</sub><sup>+</sup>-N+NO<sub>2</sub><sup>+</sup>-N and that obtained with MgO i.e. NH<sub>4</sub><sup>+</sup>-N were titrated against N/50 of 1 N H<sub>2</sub>SO<sub>4</sub>. The nitrogen content in the sample was calculated by using the formula:

$$\text{mg N in sample} = \text{mL of N/50 acid used} \times 0.028$$

#### 3.7.5. Determination of Oxalic Acid by HPLC

Oxalic acid in culture broth was determined with high pressure liquid chromatography (HPLC) by using the method described by Picha (1985). Oxalic acid (0.1 percent) was used as standard. A liquid chromatograph (Perkin Elmer, LC

200) with 300 mm x 7.8 mm internal diameter Aminex HPX-87 H (BioRad) column was used for detection of oxalic acid. Column temperature was maintained at 75°C with an internal column heater. The chromatograph was equipped with injector fitted with a 20 µL sample loop and ultraviolet detector at fixed wavelength of 214 nm. The mobile phase (0.0008 N H<sub>2</sub>SO<sub>4</sub>) was made by diluting reagent grade H<sub>2</sub>SO<sub>4</sub> in HPLC grade water and it was degassed prior to use. Flow rate of the mobile phase was maintained at 0.8 mL/minute. Twenty µL of culture broth filtered through 45 µm millipore filter was used for analysis.

### **3.8. PHYSICAL PROPERTIES OF BIOPOLYMER (EPS)**

#### **3.8.1. Measurement of Viscosity of EPS**

The viscosity of re-hydrated EPS solution was measured by Stabinger Viscometer (RSV 3000) using ASTM D 7042-04 test method. Standard procedures for rinsing and drying of measuring cells were adopted as described in the method and set the internal temperature control of viscometer to the desired measuring temperature. Prior to loading the samples it was assured that the measuring cells were clean and dry.

Five mL of the sample was loaded in the syringe and about 2 mL of the test specimen was poured into the measuring cell. The syringe was left in the inlet opening and started the measurement. After recording the values, further 1 mL of the sample was injected without taking off the syringe and repeated the measurement. Special care was taken that the deviation between two consecutive determinations should not exceed the repeatability precision values as indicated in

the method. After recording the values, the syringe was immediately removed. The measuring cells were rinsed followed by drying. The viscometer recorded three values as the final results, expressed as dynamic viscosity in mPa.s, and kinematic viscosity in mm<sup>2</sup>/s and density in g/cm<sup>3</sup> of the sample.

### **3.8.1.1. Effect of temperature on viscosity of EPS**

The viscosity of 0.25 percent solution of EPS was determined by Stabinger Viscometer at various temperatures like 10, 20, 30, 40, 50, 60 and 70°C. The kinematic viscosity, dynamic viscosity and density of the sample were recorded. The effect of temperature on dynamic viscosity (mPa.s) was evaluated by graph.

### **3.8.1.2 Effect of dilution on viscosity of EPS**

Solution of EPS (0.25 percent) was serially diluted with distilled water as described below and the viscosity of each dilution was determined by Stabinger Viscometer at 30°C. The effect of dilution on dynamic viscosity (mPa.s) was evaluated.

- |  |         |
|--|---------|
| 1. 5 mL sample (0.25percent)           | 5S      |
| 2. 4 mL sample + 1 mL H <sub>2</sub> O | 4S      |
| 3. 3 mL sample + 2 mL H <sub>2</sub> O | 3S      |
| 4. 2 mL sample + 3 ml H <sub>2</sub> O | 2S      |
| 5. 1 mL sample + 4 mL H <sub>2</sub> O | 1S      |
| 6. 5 mL of water                       | Control |

#### **3.8.1.3. Effect of pH on viscosity of EPS**

The initial pH of the 0.25 percent solution of EPS was 4.82. Samples of different pH i.e. 6, 8, 10, and 12 were prepared by addition of 0.1 N NaOH. Viscosity of each solution was measured by Stabinger Viscometer at 30°C. The effect of different pH values on dynamic viscosity (mPa.s) was evaluated.

#### **3.8.1.4. Effect of sodium chloride on viscosity of EPS**

In order to investigate the effect of sodium chloride on viscosity of EPS at different concentrations of sodium chloride (w/v) in 0.25 percent solution of biopolymer was prepared. Total of five different concentrations like 0.5, 1.0, 1.5, 2.0 and 2.5 percent were prepared and all these solutions were subjected to analysis for viscosity at 30°C using Stabinger Viscometer.

#### **3.8.1.5. Thermal stability of EPS**

Thermal stability of EPS solution was studied at different temperatures using controlled temperature water bath. The solutions (0.25 percent) were subjected to different temperatures for one hour that is 40, 60, and 80°C. The samples were cooled at room temperature for three hours. The viscosity of each solution was measured by Stabinger Viscometer at 30°C.

#### **3.8.2. Measurement of Surface Tension of Culture Broth**

Surface tension of the cell free culture broth was measured by the ring method using KRUSS Tensiometer K10T. After setting up the balance and checking it for zero, about 10 ml of the cell free broth was poured in a standard

cup. The cup was placed on platform below the platinum ring. The platform and the cup were slowly raised until the platinum ring came into contact with the liquid. Then the height of the liquid was gradually lowered so that maximum stretch of the liquid film and the ring was developed. Further lowering the platform crossed the critical limit that sustains the stretch and the ring got detached from the surface of the liquid. The value indicated by the index pointer at the moment the platinum ring got detached from the liquid was taken as the surface tension of the sample (mN/m). Reduction in surface tension in comparison to control was taken as confirmatory test for having surface activity as reported by Cooper and Zajic (1980).

### 3.8.3. Water Holding Capacity of Biopolymer

Water holding capacity of carbohydrate polymers is an important evaluation criterion and was determined for fungal strains D4, D7, and D8 by the difference in weights of fresh and dry EPS. The water holding capacity per unit dry weight of EPS ( $\text{g g}^{-1}$ ) was calculated as under:

$$\begin{aligned}\text{Water holding capacity (g g}^{-1}\text{)} &= \text{fresh weight of EPS} - \text{Dry weight of EPS} \\ &= \text{Water held by EPS} \\ &= \frac{\text{Water held by EPS}}{\text{Dry weight of EPS}}\end{aligned}$$

Water held by the EPS was also determined by centrifugal dehydration using the method described by Mao *et al.* (2001). A known amount of EPS was subjected to centrifugation at 5000 rpm for 10 minutes, three times. After each run

the separated water was removed and quantified. The difference in weight of last run gave the water holding capacity of EPS. The total water contained by the EPS was determined by complete drying of the sample at 105°C till constant weight.

### **3.9. CHEMICAL COMPOSITION OF BIOPOLYMER (EPS)**

#### **3.9.1. Sugar Analysis**

Total sugars present in EPS were estimated by phenol sulphuric acid method described by Dubois *et al.* (1956). Two mL of 5 percent EPS solution and 2 mL of standard glucose solution 50 parts per million (ppm) were taken in separate test tubes in triplicate. To each tube 0.05 mL of 80 percent phenol was added and vortexed for two minutes, 5 mL sulphuric acid was added rapidly to the tube, direct at the surface, without touching the walls of tube, mixed with vortex mixer. The mixture was incubated in water bath at 25°C for 10 minutes. Finally, the test tubes were vortexed again and the absorbance was measured at 490 nm. The concentration of sugars was estimated from the standard curve.

##### **3.9.1.1. Standard curve for glucose estimation**

Glucose stock solution 1000 ppm was prepared by dissolving 1g of glucose in distilled water and volume was made up to 1 L. Ten mL of 1000 ppm stock solution was diluted to 100 mL with distilled water to get the working solution of 100 ppm. Then 2.5, 5.0, 7.5, 10.0 and 12.5 mL of working solution was diluted to 25 mL to achieve the final concentrations of 10, 20, 30, 40 and 50 ppm. Twenty

mL distilled water was used as blank. The absorbance was measured at 490 nm using Spectrophotometer.

### 3.9.2. Protein Analysis

Protein content of EPS was estimated by Bradford micro assay (Bradford, 1976). Two mL of 5 percent EPS solution was taken in 20 mL test tubes in triplicate. Then 1 g of digestion mixture, 0.2-0.3 g of  $\text{Na}_2\text{S}_2\text{O}_3$  and 4 mL of concentrated  $\text{H}_2\text{SO}_4$  was added. Digestion mixture was prepared by mixing 100 g potassium sulphate, 10 g copper sulphate, and 1g selenium metal. It was thoroughly mixed till homogeneous mixture was obtained and the tubes were heated at  $375^\circ\text{C}$  for 2 hours. After completion of digestion (clear solution), the contents of the tubes were cooled and volume was made up to 25 mL with distilled water. This sample was used for analysis of protein.

One hundred  $\mu\text{L}$  of sample was added in a test tube containing 1 mL of Bradford reagent. (Bradford Reagent was prepared by dissolving 100 mg of Coomassie Blue G-20 in 50 mL of 95 percent ethanol). Afterwards 100 mL of 85 percent  $\text{H}_3\text{PO}_4$  was added and the solution was diluted up to 1L. The solution was stirred vigorously for 3-4 hours, kept it overnight and filtered before use. The concentration of protein present in the sample was calculated by using Bovine Serum Albumin (BSA) as standard. The stock solution was prepared by dissolving 1 mg of BSA in 1 mL of distilled water ( $1\text{mg mL}^{-1}$ ). To make working solution of  $100\ \mu\text{g mL}^{-1}$ , to 100  $\mu\text{L}$  of stock solution 900  $\mu\text{L}$  of distilled water was added. In the test tubes 10, 20, 30, 40, 50, 60, 70, 80, 90, 100  $\mu\text{L}$  of the working solution was

taken in triplicate and 1 mL of Bradford reagent was added. The volume of all the test tubes was made up to 2.0 mL by adding distilled water. A test tube containing 1 mL of Bradford reagent and 1 mL of distilled water only (without BSA) was used as blank. The absorbance of the sample and standards was measured at 595 nm within 10-15 minutes.

### **3.10. FERMENTATION STUDIES**

The production of EPS by strain D8 was scaled up in 6 L Erlenmeyer flasks containing 1 L of optimized beads containing 6-fold medium with 15 percent sucrose and 0.6 percent  $\text{NaNO}_3$ . After inoculation of 10 mm disc from 3-4 day old culture of strain D8, the flasks were kept at 28°C for 21 days. The contents of the flasks were daily mixed by hand. After 21 days of incubation, dry biomass and EPS was determined and quantified by gravimetric methods.

### **3.11. STATISTICAL ANALYSIS**

Research data were analyzed by applying appropriate statistical procedure as mentioned by Steel and Torrie (1980). The data recorded for EPS and biomass production by three fungal strains at various levels of sucrose, glucose and nitrogen was subjected to statistical analysis by three factor completely randomized design (CRD) using MSTAT-C program. Least square difference (LSD) test was applied to all the means in order to check their significance.