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Optimization and Chemical Characterization of Exopolysaccharide produced by *Pseudomonas* sp. Isolated from Wheat Rhizosphere

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Enrichment technique was implemented to isolate and detect osmotolerant EPS-producing bacteria from the rhizosphere of wheat growing under semi arid tropical soil. The highly osmotolerant isolate, identified as a *Pseudomonas* species by 16S rDNA analyses, produced EPS, as evidenced by colorimetric and gas chromatographic analyses. Quantitatively the EPS production was influenced by the osmotic stress. Exopolysaccharides (EPS) produced from *Pseudomonas* were studied as affected by different carbon, nitrogen sources, C:N, pH of culture media. The FT -I.R. and GC-mass spectroscopy data of purified polysaccharides from the organism revealed presence of many functional groups in the EPS.

Key words: Exopolysaccharides**,** *Pseudomonas*, C: N, carbon, FT -I.R., GC-mass spectroscopy.

Soil moisture stress is a major constraint for crop production in semi arid tropical regions. An efficient, low cost strategy for the soil moisture stress management is the use of osmotolerant microorganisms. It is well documented that beneficial microorganisms colonize the rhizosohere/ endorhizosphere of plants and promote growth of the plants grown under hydric stress through various mechanisms(Arshad *et al*.2008; Sandhya *et al*.,2009 ; Naseem and Bano, 2014). Microorganisms secrete high molecular weight compounds in the environment known as the extracellular polymeric substances or EPS. The significance of EPS in the bacterial attachment and colonization on the root surface ((Bashan and Holguin, 1997), and in soil aggregation (Bashan *et al*. 2004) is reported. Exopolysaccharides possess unique water holding and cementing properties, thus play a vital role in the formation and stabilization of soil aggregates and regulation of nutrients and water flow across plant roots through biofilm formation (Roberson and Firestone 1992; Tisdall and Oades 1982). Alami *et al*. (2000) observed a significant increase in root adhering soil per root tissue (RAS/RT) ratio in sunflower rhizosphere inoculated with the EPS-producing rhizobial strain YAS34 under drought conditions.EPS biosynthesis and composition are a function of a variety of environmental factors. Critical factors for exopolysaccharides production include, are bacterial growth phase, carbon source, nitrogen source, oxygenation rate, temperature, and pH. Exopolysaccharides can be formed from a variety of carbon substrates (Sutherland, 2001).

The composition of EPS material produced varies widely from one bacterial species to another. Additionally, EPS biosynthesis and composition are a function of a variety of environmental factors. Critical factors for exopolysaccharide production include, bacterial growth phase, carbon source, nitrogen source,

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oxygenation rate, temperature, and pH (Prathima *et al*., 2014). Various studies have found that EPS production is favoured under conditions of nitrogen limitation. Under such conditions, any excess sugars remaining can be used specifically for polysaccharide synthesis. The pH optimum for exopolysaccharide production depends on the individual bacterial species may differ from optimal pH for bacterial growth, for most species it is near neutrality (Vermani, *et al*. 1996). For aerobic bacteria, oxygen concentration determines exopolysaccharide production. Bacterial growth (biomass) tends to increase Exopolysaccharide production is often favoured by sub-optimal growth temperatures. The present investigation was aimed to identify the parameters for the enhanced EPS production and characterize the EPS-produced by moisture stress tolerant Pseudomonas spp. isolated from rhizosphere of wheat crop cultivated under semiarid areas of northern India.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from two regions; Bharatpur (pH 6.8) in Rajasthan, IARI (pH 7.18) in New Delhi. At each location ten sub samples of soil from top 15 cm were collected and bulked. All the samples were brought to the laboratory and stored at 4^0C till use.

Enrichment and isolation

Nutrient broth (NB) with different concentrations (1%, 5%,10%, 15%, 20% 25%, 30%, 35%, and 45%) of polyethylene glycol (PEG6000) were used for enrichment of soil sample (1g). Enrichment was brought from 1% PEG in NB and after incubation at 28°C under shaking conditions (120 rpm) for 24 h, growth was estimated by measuring the optical density at 600 nm using a spectrophotometer. The growth of the isolates at various stress levels was recorded. Culture able to grow at maximum stress (45% of PEG) was purified by streaking on nutrient agar (NA) plate. Isolated cultures were preserved on slant-form at -20°C temperature.

Extraction and quantification of exopolysaccharides

EPS-producing bacteria were cultured in liquid minimal medium with 12.6% K_2HPO_4, 18.2% KH_2PO_4, 10% NH_4NO_3, 1% $MgSO_4.7H_2O$, 0.6%,$MnSO_4$, 1% $CaCl_2$.2H2O, 0.06% $FeSO_4.2H_2O$, 1% sodium molybdate, 1.5% NaCl, supplemented with five sole C sources in 1 l of distilled water for 10 days (Bramchari & Dubey 2006). After incubation for 10 d, the bacterial culture (250 ml) were centrifuged at 15,000 rpm for 20 min at 4°C.The EPS were extracted from the supernatant by the addition of twofold ice cold ethanol (95%), the solution was chilled at 4°C for complete precipitation. EPS were collected from above solution (Kumar *et al*. 2011). EPS extracts were washed with 70–100% ethanol–water mixture; redissolved in distilled water and dialyzed against distilled water at 4°C for 24 h to remove excess salts from EPS. Extracted EPS were lyophilized stored at room temperature (Bramchari & Dubey 2006).

Morphological characterization of the isolate

Colony morphology- Microscopic and macroscopic morphological characteristics of the bacterial colonies was observed after 24 hours of growth period on NA medium. The cell morphology of the isolated bacterial strain was observed under transmission electron microscope (TEM).

Biochemical characterization of the isolate

The highest EPS producing bacterial isolate was characterized for Gram staining, Phenol red test (carbohydrate fermentation test), TSI (Triple sugar iron agar test), Starch hydrolysis, Indol test, MRVP test, Citrate test, Urease test, Gelatin hydrolysis test, Catalase activity, Oxidase test, Nitrate reduction test.

Molecular characterization of the isolate

For molecular characterization, bacterial genomic DNA was isolated and subjected to polymerase chain reaction for amplification of 16S rDNA. The Reaction mixture for PCR was prepared with the following components: 2.5 ìl Buffer (1X), 2.5ìl dNTPS (200ìM each), 2 ìl $MgCl_2$ (1.5 mM), and 0.2 ìl Forward Primer (2pM), 0.2 ìl Reverse Primer (2pM) and 0.4 ìl *Taq* polymerase (2U) with 1 ìl of DNA (30ng). The universal primer sequence of 5' CCAgCAgCCgCggTAATACg 3'as a forward and 5' TACCAgggTATCTAATCC 3' as a reverse used for amplification. DNA amplification was carried out in Thermal Cycler. PCR condition was carried out as follows: predenauration at 94^0C for 5 min, 30 cycles of denaturation at 94^0C for 40 sec, annealing at 55^0C for 1 min, Extension of 72^0C for 1 minute.

Optimization of EPS Production for G7 Bacterial isolates

Following the evaluation of EPS production on each of the five sole carbon sources (Glucose, Fructose, Sucrose, Mannitol, Arabinose), the role of pH, N, C:N was investigated. First, the carbon source which yielded maximum EPS production was held constant while the pH was varied (5, 6, 7, and 8). Then, utilizing the same carbon source, the influence of different nitrogen source was investigated for maximum EPS production. Utilizing the same carbon source, the amount of nitrogen (carbon/nitrogen ratio) was varied (0.025%N, 0.05%N, 0.1%N and 0.2% N) equivalent to C:N values 5:1, 10:1,20:1, 40:1.

Estimation of Carbohydrate and Protein content of crude EPS

The total carbohydrate was quantified by phenol sulphuric acid method proposed by Dubois *et al*., 1956. The protein was estimated by Lowry's method (1951).

Infra Red Spectroscopy Analysis of Crude EPS

Samples of purified EPS were prepared for I.R. analysis according to the method described by Sherborock-cox *et al*. (1984). One mg of the purified EPS was used in I.R. analysis by using salt discs. A mixture made by adding 1mg of EPS samples to 300 mg of pure dried KBr followed by pressing into disc, the whole I.R. spectrum (1000 – 4200 nm) was compared with a known polysaccharide pressed also together with KBr into disc. The spectra were recorded using Perkin-Elmer-1430 spectrophotometer.These were subjected to IR- spectra measurement in the frequency range of 550 and 4000 cm^{-1}.

GC-MS Analysis of Crude Basal EPS

The basal exopolysaccharide was hydrolyzed to monomeric units and transformed in their alditol acetates. 0.1g of crude sample was mixed with 1.25ml of 72% sulphuric acid with a glass stick and incubated for 60min at 30°C. The mixtures were diluted with 13.5ml of distilled water and incubated in boiling water bath for 4 hrs. After incubation, mixtures were cooled and 3.1ml of 32% NaOH (w/v) was added. At the end of hydrolysis, 0.2ml of sample was taken separately and 2ml of 2% sodium borohydride in dimethyl sulfoxide was added. The mixtures were then shaken well at 40°C for 90min. after which 0.2ml of glacial acetic acid was added to decompose excess of sodium borohydride. After cooling, 4 ml of acetic anhydride and 0.4 ml of 1-methylimidazole were added to the solution. The mixtures were then incubated for 10 min at room temperature and then 20 ml of distilled water was added to decompose excess of acetic anhydride. After cooling, 8ml of dichloromethane was added and the mixture was shaken vigorously for total alditol acetate extraction. The upper layer was removed and the lower phase was washed three times with 20ml of distilled water. The dichloromethane was evaporated at 40°C under vacuum and final alditol acetate residues were dissolved in 1ml of dichloromethane and stored at -20°C. Alditol acetates were separated on a 30 m x 0.25mm ID x 0.25ìm film thickness column DB 5ms (Agilent) attached to the GC- 2010 (GCM-QP 2010) SHIMADZU chromatography equipment with a flame-ionization detector and a split injector. High purity hydrogen was used as the carrier gas at a flow rate of 1.40 ml/min. The column temperature was maintained at 200°C and 240°C respectively and 1ìl sample in dichloromethane was injected through a glass-lined splitter, set at 1/90 ratio. The absorption was read between 40m/z and 800m/z.

Data Analysis

Phylogenetic and molecular evolutionary analyses with the sequences of 3 isolates were conducted by using software included in MEGA5. The sequence of 16S rDNA of bacteria was aligned using multiple sequence alignment program CLUSTAL-W.

Statistical analysis

Comparisons between treatments were carried out by one-way analysis of variance (ANOVA) using OPSTAT statistical software developed by HAU, Hisar.

RESULTS

Enrichment and Isolation

Different bacterial isolates from the wheat rhizosphere were observed for a osmotic tolerance by varying concentration of PEG6000 in broth culture, beyond 70% PEG, the survival of bacterial isolates was not observed. Finally 9 isolates from the wheat rhizosphere were found to tolerate moisture stress equivalent to 45% PEG.

EPS Quantification

Among the wheat bacterial isolates, G7 produced significantly and the highest amount of

EPS (330 mg/100 ml or 2.62 g g-1protein biomass) (table 1). There was no significant difference among the isolates G1, G2, G3, G4, G5, G8 and G9 with respect to EPS production per unit of protein biomass. G6 (1.57 g g-1protein) was found significantly higher EPS producer as compared to G1, G3, G4 and G9.

Relationship between the EPS production (by Pseudomonas sp) and osmotic potential of culture media

The cells were subjected to different levels of PEG (0%, 10%, 25%, 35%, 45%) and the corresponding synthesis of EPS was monitored in the growth medium. EPS production was found to be a function of hydric stress with maximum production realised at 35% of PEG. An increase of PEG content in the growth medium from 0 to 35%, a highly significant gain was observed in the amount of EPS. Further increase in PEG from 35% to 45%, EPS production decreased by 14.44% (table 2). Thus further increase in PEG content in the growth medium was ineffective in terms of improving the EPS by the bacteria. This implies that an osmotic stress within in 35- 45% was the maximum which the organism tolerates by enhancing the EPS production. A stress e" 45%PEG was found detrimental on bacterial growth resulting in significant loss of cell count as well as EPS synthesized.

Morphological and biochemical characterization of higher EPS producing bacterial isolate G7

G7 forms round, smooth, whitish yellow colonies with entire margins and convex elevations. The organism was gram negative, exhibiting rods (plate: 1) with mostly single arrangements. It was motile, non-spore former and various biochemical tests showed that G7 was positive for starch hydrolysis, lipid hydrolysis, casein hydrolysis, dextrose fermentation, NR, gelatin hydrolysis, citrate utilization, catalase and oxidase test. It was negative for lactose fermentation, sucrose fermentation, H_2S production, indole, MR, VP, and urease test.

Table 1. Quantification of EPS produced by different isolates of Wheat crop rhizosphere under hydric stress condition (45% PEG)

Sample	bacterial protein (µg/ml)	EPS (mg/100ml)	EPS (g/g bacterial protein)
G1	1239.33	137.28	1.11
G2	1314.33	162.66	1.25
G3	1309.33	135.74	1.04
G4	1021.00	119.58	1.16
G5	1219.89	158.80	1.30
G6	1191.00	201.94	1.57
G7	1266.56	330.81	2.62
G8	1193.2	168.88	1.32
G9	1258.78	114.49	0.92
CD P=0.05)	N.S.	31.01	0.38

Table 2. Relationship between the EPS production (by *Pseudomonas* sp.) and osmoticpotential of culture media

PEG%	EPS mg/100 ml
0	154.333
10	188
25	305
35	386.667
45	330.81
CD (P=0.05)	32.064

Table 3. Effect of different carbon source on EPS production by (*Pseudomonas* sp.)

Carbon source	EPS(mg/100 ml)
Glucose	138.65
Fructose	160.78
Sucrose	267.93
Mannitol	150.09
Arabinose	151.09
CD (P=0.05)	48.84

Table 4. Effect of Different concentration of sucrose on EPS production by *Pseudomonas* sp.

Sucrose (%)	EPS (mg/100 ml)
0.1	45.05
0.2	65.41
0.5	170.43
1	267.93
2	335.98
3	451.84
4	470
CD(P=0.05)	43.54

Molecular characterization of the isolate G7

The 16S rDNA of G7 isolate was amplified with the 16S rDNA universal primers. A common band of 1.5 kb was present in the PCR product**.** Isolates G7 was identified as *Pseudomonas sp*.

Factor affecting EPS production by *Pseudomonas* sp.

Effect of carbon sources

The concentrations of carbon sources in the media are very important for cell growth and optimal metabolite production (Zou *et al* 2005). Among 5 carbon sources added to the growth medium @ 1% to compare their efficiency to support EPS production, sucrose produced significantly higher amount of EPS (267.93 mg/ 100ml) (table 3). Rest all the carbon sources namely glucose, fructose, mannitol and arabinose were found to support statistically identical levels of EPS production.

Effect of sucrose concentration

Sucrose was identified as best carbon source, therefore different concentrations of sucrose were supplied (0.1%, 0.2%, 0.5%, 1%, 2%, 3% and 4%) in the growth medium at the pH =7, oxygenated condition, mesophilic temperature and NH_4Cl as nitrogen source(0.1%).

At low concentration (0.1%, 0.2%) identical level of EPS was produced and with an increase in sucrose concentration from 0.2% to 0.5%, EPS increased by 160%. With further increase in sucrose concentration with 1%, 2% and 3%, statistically significant increase in amount of EPS was recorded. The magnitude of increase from 0.5% to 1% was 57.2%, from 1% to 2% was 25%, and from 2% to 3% an increased 34.48% in EPS production was recorded. However 4% concentration of sucrose in growth medium did not bring about a significant change in EPS production (table 4).

The effect of C/N

The effect of C: N was evaluated and C: N ratio 5:1, 10:1, 20:1 and 40:1 was maintained in the

Table 5. Effect of different C:N on EPS production by *Pseudomonas* sp

C/N ratio	EPS (mg/100 ml)
5	141.49
10	284.34
20	397.91
40	205.58
CD (P=0.05)	36.89

Table 6. Effect of of Different pH levels on EPS production by *Pseudomonas* sp

pH	EPS (mg/100 ml)
5	40.38
6	121.79
7	284.34
8	300.13
CD (P=0.05)	28.28

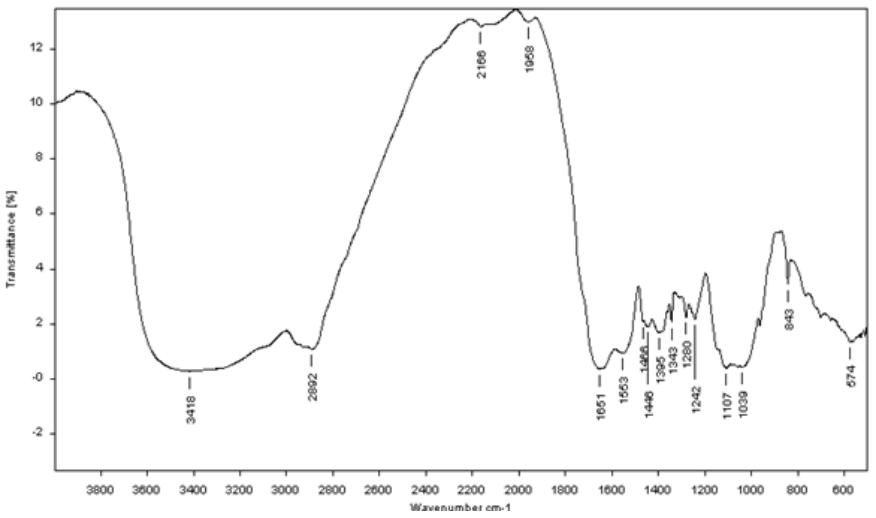

Fig.1. FT-IR absorption spectrum of EPS produced by *Pseudomona*s sp

growth medium. As the C: N ratio increased from 5:1 to 10:1 there was almost 100 % increase in EPS production. Further increase of C: N from 10:1 to 20:1 resulted in an increase in EPS content (39.94%) was recorded. However C: N ratio increased 20:1 to 40:1, a drastic reduction (48.33 %) in EPS amount was observed. Therefore an extremely narrow C: N ratio and extremely wide C: N ratio was found unfavourable for EPS production. A C: N ratio 20:1 was found as the optimum for maximum EPS production under the oxygenized condition and mesophilic temperature (30^0C) (table 5).

Effect of pH on EPS production

Utilizing sucrose as the sole carbon source, EPS production was observed over a range of pH values (5, 6, 7, and 8). It was found that acidic pH (pH =5) was unfavourable and as the pH increased from 5 to 6, a significant increase in EPS (by 200%) was recorded. Maximum EPS production was observed under near-neutral and slightly basic growth conditions (284 mg/ 100ml). When the pH increased from 6 to 7, amount of EPS increased by 133.46%. Further increase in pH from 7 to 8, resulted in no significant effect on EPS production (table 6).

Chemical characterization of EPS

Estimation of carbohydrate and protein content of the EPS

The carbohydrate and protein content of EPS extracted from the *Pseudomonas* sp. was found to be 1564 µg/ml and 284.33 µg/ml respectively.

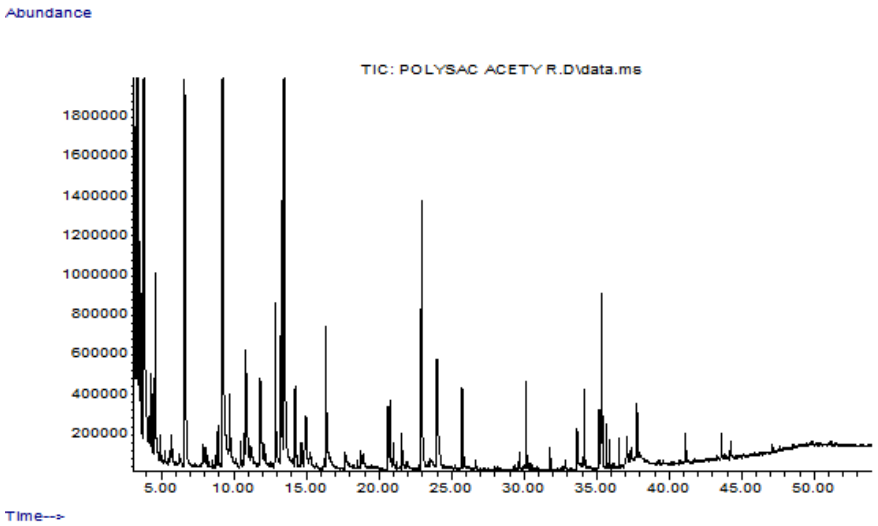

Fig. 2. GC-MS analysis of EPS produced by *Pseudomonas* sp

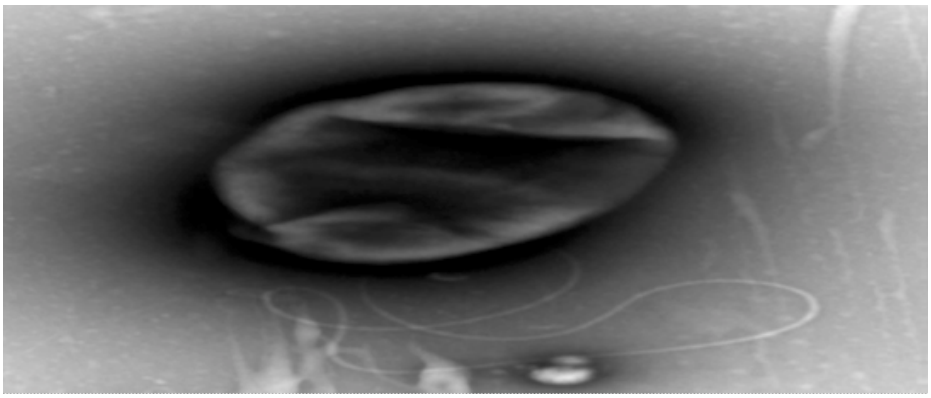

Plate 1. Transmission electron microscopy of G7 bacterial isolates

FT-IR analysis of EPS produced by Pseudomonas

IR spectroscopy of intact basal medium exopolysaccharide (EPS) showed the presence of hydrogen bonded compound, possible acid, amine salt, ether, alkanes alkenes and alkynes, nitro compound, sulphonates and alkyl halides.The IR spectrum of the *Pseudomonas* sp. basal EPS sample showed the band at 1000-1500 cm^{-1} which is characteristic to glycan (Fig. 1).The list of bands at 574 to1651cm^{-1} interval is present. In addition, the spectra showed bands around 1958, 2166, 2892 and 3418. Polysaccharide C-O-C and C-O-P was at 1039 cm-1, absorption range 1242- 1343 was typical for sulphonates (S=O) (table: 10). Absorption 1395, 1446 to 1466, 1553 and 1651 showed the nitro group bended, alkanes, amines and nitro group asymmetrical stretch respectively. In the anomeric region (1000-1600 cm^{-1}) the polysaccharides exhibited the obvious characteristic absorption at 1039 cm^{-1}.

GC-MS analysis of EPS produced by Pseudomonas sp

The analytical techniques like gas chromatography–mass spectrometry (GC–MS) for qualitative and quantitative analyses of the monosaccharides and amino acids of EPS after its hydrolysis is reported (Ortega-Morales *et al*., 2007). The fully methylated products were hydrolyzed with as it , converted in to the alditol acetate and analyzed by GC-MS. Methylation analysis of the exopolysaccharides produced by *Pseudomonas* sp. such as beta-D-galactopyranose at 35.15 RT, alpha-D-glucopyranose at 35.27 RT, alpha-D-mannopyranose at 35.30 RT and beta-D-galactose pentaacetate at 35.35 RT. The electron impact fragmentation patterns were prepared from spectra of derived alditol acetates were prepared from the hydrolyzed EPS (Fig.2).

DISCUSSION

In the present study isolation, purification, biochemical and molecular characterization of hydric stress tolerant extracellular polysaccharide (EPS) producing bacterial isolates from the rhizosphere of wheat in the arid soil conditions was undertaken. Using transmission electron microscope (TEM), the morphology of bacterial cells was studied. The maximum EPS was produced by the G7 (*Pseudomonas* sp.) as estimated by chemical method as well as the electron microscopic observations. Under culture conditions quality and quantity of the C source was found to influence the amount of EPS formed by the organism (Mohammed El-Anwar Osman, 2012). Sucrose was the most efficient C source for EPS production, and its concentration increased the polysaccharide production; the maximum EPS production occurred at 3% concentration in the medium. Under culture conditions quality and quantity of the C source was found to influence the amount of EPS formed by the organism. Sucrose was the best C source with the optimum value of C: N being 20:1. Earlier workers have reported that the nutrient content of the growth medium influences the amount of EPS produced (Degeest and De Vuyst 1999, 2000; Godinho and Bhosle, 2009). Furthermore, N starvation is known to shift the metabolism towards the synthesis of polysaccharides as reported by earlier researchers (Sutherland, 2002).

The ability of bacteria to produce extracellular polysaccharides imparts desiccation tolerance has been associated with biofilm-forming capacity (Roberson and Firestone 1992; Costerton,1999; Sandhya *et al*.2009). Biofilm-forming tendency is known as a mechanism by which the bacteria tend to come closer to each other and tide over the stress. For instance, aggregation in biofilm on surfaces provides protection to the bacterial cell against stress.

In the present study, the amount of the EPS produced by the Pseudomonas spp. increased as the moisture stress / osmotic stress level increased .This indicates that the organism tries to retain more of the moisture in the surface polymeric layer (EPS) with an increase in the magnitude of the hydric stress. The high osmotic stress conditions thus appear to stimulate EPS production by this strain. These observations are similar to the earlier reports where microbial cells in activated sludge and biofilms produced more EPS to counteract the adverse effect of heavy metals stress (Priester *et al*., 2006). Similarly, Konnova *et al*., (2001) observed enhanced survival of *A. brasilense* Sp245 under drought stress, when the decapsulated cells were supplemented with their lipo polysaccharide–protein cell coat. Further, the concentration and composition of microbial EPS dramatically changed under stress conditions. The

chemical nature of the EPS produced by the stress tolerant bacteria is a heteropolymer. EPS was readily isolated from culture supernatants, which suggests that the EPS was a slime-like EPS, which may contribute to the formation of a bio film on the root surface.

EPS displayed a strong absorption band of –OH at 3418.0 cm^{-1} and COOH at 1631.48 cm^{-1} showing it to be polysaccharide. An EPS sample produced by this osmotolerant bacterium was found to be heteropolymer-containing different functional groups. The bacterial EPS extracts gave characteristics bands for polysaccharides. Here, carbonyl (C=O) stretching peak and OH stretching peak was at broad and the maximum peak and the band at 1000-1500 cm^{-1}showed the presence of polysaccharides. In case of complex EPS, neutral sugars were identified by their derivatives, alditol acetates by GC-MS. Alditol acetates were often used only to determine the ratios of monosaccharide hydrolysates and our study ,four major peaks present corresponded to D-Galactopyranose, alpha-D-Mannopyranose, alpha-D-Glucopyranose and beta-D-Galactose pentaacetate for *Pseudomonas* sp. EPS.

The production of EPS was increased with influence of nutritional value of the media. The highest amount of EPS was produced with sucrose as the carbon source. C: N ratio 20:1 was found as the optimum for maximum EPS production under the oxygenized condition and mesophilic temperature (30^0C). This is the first report of chemical characterization EPS produced by a osmotolerant Pseudomonas species, which expands our knowledge of the micro-organisms capable of producing these biomolecules. Furthermore, this work shows that semiarid tropical soil environments are potential habitat for bioprospecting EPS-producing bacteria, and that these molecules might be involved in ecological roles of protecting the cells against dessication. The potential of Pseudomonas species as a source of exopolymers (EPS) may be harnessed as a means to impart drought tolerance in crop plants.

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