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Screening and optimization of exopolysaccharide producing bacteria from rhizospheric region of direct seeded rice

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Abstract
Exopolysaccharide (EPS) are high molecular weight substances mainly comprise of polysaccharides but also contains non-sugar components like protein, nucleic acid and lipids which mainly originated from microbial origin. With the rising awareness of microbial EPS in various fields, rice rhizospheric microorganisms received broad attention and play a crucial role in protecting crops during the stress condition. The present study was to obtain exopolysaccharide producing bacteria from direct seeded rice. Soil samples were collected from rice rhizospheric region (0-15cm depth). Thirty-seven organisms were isolated and identified by morphological, biochemical and microscopic analysis. Out of thirty-seven isolates eighteen isolates produced mucoid substances and having Gram -ve in nature. After screening with Polyethylene glycol (PEG-6000), six isolates tolerate maximum level of stress (-0.73MPa). The production of six isolates were ranged from 3.4 to 5.5g/l. The six potential isolates were optimized with temperature, pH, incubation period, salt concentration and carbon source. We found that, 37 ^{0}C and 7.5 pH were optimum for EPS production. The production rate was decreased with increasing pH and salt concentration. Sucrose was the suitable carbon source for production of EPS. The finding of the study implies the isolates have potential to produce EPS from rice rhizospheric region. Further study should aim to molecular identification EPS producing bacteria linking with drought tolerance and water use efficiency under direct seeded rice.

Keywords: Exopolysaccharide, rice, microorganisms, rhizosphere

Introduction
Rice (*Oryza sativa*) is one of the important staple food crops that feed 60% of the world's population and influences the livelihoods and economy of many people (George, 2018) [1]. Rice is grown over an area of about 149 million ha with an annual production of 600 million tones (Di Pippo *et al*., 2013) [2]. Rice production and food security mainly depend on the irrigated lowland rice system. But, fresh water scarcity, competition for water use, growing population, rising demand for food, climate change and global warming, higher cost of irrigation, and poor availability of labour have threatened to cultivate the rice. To overcome this problem EPS could one of the options to enhance the water holding capacity (Padhy *et al*. 2013) [3], because the EPS released either in the form of capsule or slime materials which is absorbed by soil clay. In details the Exopolysaccharides (EPS) are high molecular weight substances mainly comprise of polysaccharides but also contains non-sugar components like protein, nucleic acid and lipids which mainly originated from microbial origin (Sheng *et al*., 2010) [4]. The bacterial EPS are synthesizing into two forms that is capsular EPS and slime EPS. Microbial mediated EPS have many advantages like help in soil aggregation (Sandhya *et al*., 2009) [5], help in movement of water in earth crust (Rossi *et al*., 2012) [6], formation of biofilm, where they act like a reservoir of water and nutrients (Castro *et al*., 2014) [7], thickening gelling, emulsifying, pharmaceutical and industries (Kour *et al*., 2013) [8]. The important features of EPS which are biodegradable in nature. EPS can release under extreme environmental condition like pH and temperature (Kodali *et al*., 2009) [9]. Some bacteria also have a peculiar character of producing enough EPS to coat more than 500 particles per day (Underwood *et al*., 1995) [10]. Probably, it may possible to alleviate the drought stress in rice by increasing the population of EPS producing bacteria in the rhizospheric region and also the moisture stress in soil is a major limiting factor for crop production. Therefore, it is important to characterize and optimize the EPS producing bacteria from rice rhizospheric region.

Material and methods

Sample collection

Soil sample were collected from rice rhizospheric region at experimental farm of Orissa University of Agriculture and Technology, Bhubaneswar (20º 15' N latitude, 85º 15' E longitude; 39m above sea level), in the eastern part of tropical India. The soil sample (0-15cm depth) were immediately transported to the laboratory and store at 4^0C for microbiological analysis. The soil type laterite with clayey sand texture (42.8% clay, 26% silt, 31.2% sand), bulk density 1.16 g cm^{-3}, total C and N were 0.79% and 0.077%, respectively.

Isolation and Screening of EPS producing bacteria

To isolate the EPS producing bacteria, 1g of soil sample was suspended with 0.85% of saline solution (Subair *et al*., 2015) [11]. Isolation of EPS by serial dilution methods and followed by the spread plate techniques by using a selective media like Tryptic Soya Agar (TSA) (casein peptone;15.0 gm l^{-1}, soya peptone;5.0 gm l^{-1}, sodium chloride; 5.0 gm l^{-1} and agar; 15.0 gml^{-1}). Plates were incubated at 28 ± 2 ^{0}C for 48h. The mucoid colonies were screened and maintain in a pure culture on TSA medium. Isolated colonies were identified on the basis of morphological, biochemical and microscopic observation. The identification was done by using Bergey's Manual of determinative bacteriology 9th edition.

Bacterial growth under water stress

Bacteria growth was estimated by using tryptic soya broth (TSB) with different water potential (-0.05, -0.15, -0.30, -0.49 and -0.73MPa) and was prepared by adding accurate concentration of polyethylene glycol (PEG 6000) (Michel and Kaufmann, 1973) [12]. After preparing the broth, 1% overnight raised fresh culture of bacterial isolates was inoculated. Three replications were prepared for each isolate and for each concentration. Incubates the bacterial culture in rotary shaker at 120rpm for 24h at 28 ^{0}C. Growth was measured by using spectrophotometer (Remi-180, India) at 600nm at various stress label (Sandhya *et al*., 2009) [5].

Gram reaction test and biochemical characterization of EPS producing bacteria

The EPS producing bacteria was characterized by gram staining, where colonies of bacteria taken from a pure culture and loopful of bacteria was mixed with a 3% KOH solution on a glass slide, after complete mixing, it shows string like up to 0.5 to 1cm long. This indicates that bacteria show gram negative (-) and vice versa is gram positive (+) (Subair and Darwisah, 2105) [13]. The biochemical like mannitol and motility test, oxidase and catalase activity, citrate utilization, fermentation of glucose, maltose, dextrose, galactose, raffinose, trehalose, manose accordingly. Antibiotic susceptibility test was secerned by measuring the zone of inhibition on solid medium using antibiotic discs (Himedia, India) of different concentration (Power *et al*., 2013, Krithinga *et al*., 2014) [14, 15]. The antibiotic profile like chramphenicol (30µg/ disc), streptomycin (10µg/ disc), ofloxacin (5µg/disc), gentamycin (10µg/disc), norfloxacin (300µg/disc), ciprofloxacin (5µg/disc).

Production, extraction and purification of EPS

Isolated organisms were used for production of EPS. The bacterial isolates were maintained in TSA slants. Production was carried out in 250ml conical flasks having 50ml TSA broth. Media were sterilized at 121 ^{0}C for 15mins. After cooling of the media, the loopful of organism was inoculated. The flasks were incubated at rotary shaker at room temperature for 72h. After incubation, cells were harvested by centrifugation for 20min at 10000rpm. After centrifugation, ice-cold isopropanol (10ml) was added and stored at 4 ^{0}C. The precipitated material was collected and centrifuged for 20min at 10,000rpm. Pellets were dried at 100 ^{0}C and weight the dried EPS (Power *et al*., 2013) [14].

Optimization of exopolysaccharide

To study the effect of different parameters, 1% inoculum containing 5*10^6cells/ml were inoculated in 100ml of production medium. EPS production was optimized under different environmental and nutritional condition i.e. incubation period (1-5days), pH (6.5, 7, 7.5, 8), Temperature (30, 37, 45 ^{0}C), Carbon sources (glucose, lactose, fructose, sucrose) (Power *et al*., 2013) [14].

Results

Isolation and Screening

A total of 37 bacteria were isolated from rice rhizospheric soil (0-15cm depth) by using Tryptic Soya Agar (TSA) media (Table 1). The bacterial growth under media shows the diversity of morphological character, out of 37 isolates 18 isolates were suspected to produced exopolysaccharide and bacterial colony forming slime. The isolates were named as MD1 to MD37. Out of 18 isolates 6 isolates were forming very slimy in nature (Fig.1)

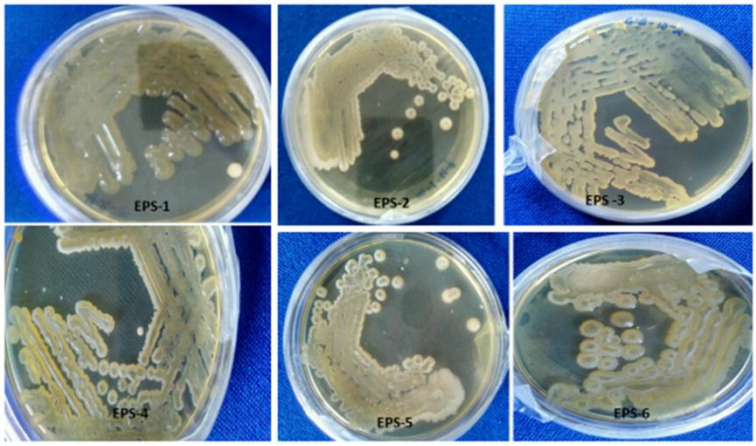


Fig. 1: Six numbers of EPS producing bacteria produced mucoid substances.

Table 1: Isolation of exopolysaccharide-producing bacteria from the rice rhizospheric region under TSA media and its morphological character

Isolate code	Colony diameter (mm)	Shape of colony	Color of the colony in TSA media	Surface of the colony	Margin of the colony	Elevation of the colony	Opacity	Consistency
MD-1	5mm	Circular	Off white	Rough	Entire	Convex	Transparent	Mucoid
MD-2	4mm	Irregular	Off white	Rough	Filiform	Convex	Opaque	Mucoid
MD-3	4mm	Circular	Off white	Rough	Entire	Convex	Transparent	Mucoid
MD-4	5mm	Irregular	white	Smooth	Filiform	Umbonate	Opaque	Mucoid
MD-5	3mm	Circular	Violet	Smooth	Entire	Raised	Opaque	Moist
MD-6	3mm	Irregular	white	Rough	Undulate	Raised	Transparent	Mucoid
MD-7	5mm	Irregular	white	Rough	Undulate	Convex	Transparent	Mucoid
MD-8	4mm	Irregular	white	Rough	Undulate	Raised	Transparent	Mucoid
MD-9	8mm	Irregular	White	Rough	Undulate	Umbonate	Opaque	Moist
MD-10	5mm	Irregular	White	Rough	Undulate	Convex	Opaque	Mucoid
MD-11	3mm	Irregular	Off white	Rough	Undulate	Convex	Opaque	Mucoid
MD-12	6mm	Circular	Off white	Smooth	Entire	Flat	Opaque	Mucoid
MD-13	3mm	Circular	Pale yellow	Smooth	Entire	Raised	Opaque	Moist
MD-14	2mm	Circular	Off white	Smooth	Entire	Raised	Opaque	Moist
MD-15	1mm	Irregular	Yellow	Rough	Entire	Flat	Opaque	Mucoid
MD-16	6mm	Circular	Off white	Smooth	Entire	Raised	Opaque	Moist
MD-17	7mm	Circular	Off white	Smooth	Entire	Raised	Opaque	Mucoid
MD-18	2mm	Circular	Yellow	Smooth	Entire	Flat	Opaque	Mucoid
MD-19	5mm	Circular	Off white	Smooth	Entire	Raised	Opaque	Mucoid
MD-20	2mm	Circular	Off white	Smooth	Entire	Raised	Opaque	Mucoid
MD-21	5mm	Circular	white	Smooth	Entire	Raised	Opaque	Moist
MD-22	2mm	Circular	white	Smooth	Entire	Raised	Opaque	Mucoid
MD-23	8mm	Circular	Off white	Smooth	Entire	Raised	Opaque	Mucoid
MD-24	4mm	Circular	Yellow	Smooth	Undulate	Flat	Opaque	Moist
MD-25	6mm	Circular	Off white	Smooth	Curled	Flat	Opaque	Moist
MD-26	3mm	Circular	white	Smooth	Undulate	Flat	Opaque	Moist
MD-27	3mm	Circular	white	Smooth	Entire	Flat	Opaque	Moist
MD-28	3mm	Circular	white	Smooth	Entire	Flat	Opaque	Moist
MD-29	7mm	Irregular	Whitish	Rough	Undulate	Convex	Transparent	Mucoid
MD-30	3mm	Circular	white	Smooth	Entire	Raised	Opaque	Moist
MD-31	4mm	Circular	white	Smooth	Entire	Raised	Opaque	Moist
MD-32	8mm	Filamentous	white	Rough	Filiform	Flat	Opaque	Moist
MD-33	3mm	Circular	white	Smooth	Entire	Raised	Opaque	Moist
MD-34	3mm	Circular	white	Smooth	Entire	Raised	Opaque	Moist
MD-35	7mm	Irregular	white	Rough	Entire	Convex	Opaque	Mucoid
MD-36	5mm	Circular	Off white	Smooth	Entire	Convex	Opaque	Moist
MD-37	9mm	Circular	Off white	Rough	Undulate	Convex	Opaque	Mucoid

Grams Reaction and Biochemical analysis

Based on the test results, isolates were gram negative bacteria produced slime after reacted with KOH and Gram-positive bacteria are not slimy in nature and also microscopic studied revealed that the 18 isolates of EPS producing bacteria showed gram negative, motile, viable rods having different color morphology, and mucoid colony morphology (Table 2). The isolates showed the presence of oxidase, catalase activity and could utilize citrate as carbon source. EPS producing bacteria showed susceptibility to chramphenicol (30µg/ disc), streptomycin (10µg/disc), ofloxacin (5µg/disc), gentamycin (10µg/disc), norfloxacin (300µg/disc), ciprofloxacin (5µg/disc) which shows the zone of inhibition (Fig 2).

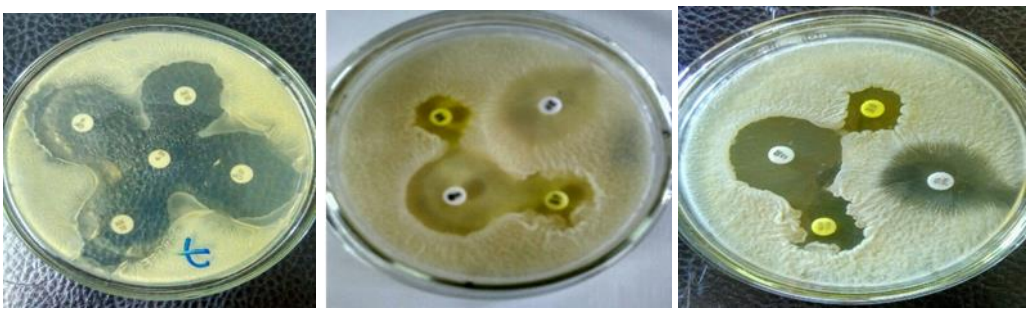


Fig. 2: Antibiotic susceptibly test of EPS producing Bacteria

Table 2: Microscopic and biochemical character of eighteen (18) numbers of EPS producing bacteria

Isolate code	Biochemical tests				
	Mannitol	Motility	Oxidase	Catalase	Gram test
MD (1)	-	+	+	-	-
MD (2)	+	+	+	+	-
MD (3)	-	+	+	-	-
MD (4)	+	+	-	-	-
MD (5)	-	+	-	+	-
MD (6)	-	+	+	+	-
MD (7)	-	+	+	-	-
MD (8)	+	+	-	+	-
MD (9)	+	+	+	-	-
MD (10)	-	+	-	-	-
MD (11)	+	+	-	-	-
MD (14)	+	+	+	+	-
MD (15)	+	+	+	+	-
MD (19)	-	+	+	+	-
MD (24)	-	+	+	-	-
MD (29)	-	+	+	-	-
MD (35)	+	+	+	+	-
MD (37)	+	+	+	+	-

Bacterial growth under different moisture stress conditions

All eighteen isolates were selected according to their morphological and biochemical test and were further evaluated under moisture stress by using different water potential (-0.05 to -0.73MPa) (PEG-6000 Hi-media). The performance is presented in (Fig. 3). Out of eighteen, six numbers of isolates showed higher potential of exopolysaccharide production under different moisture stress conditions.

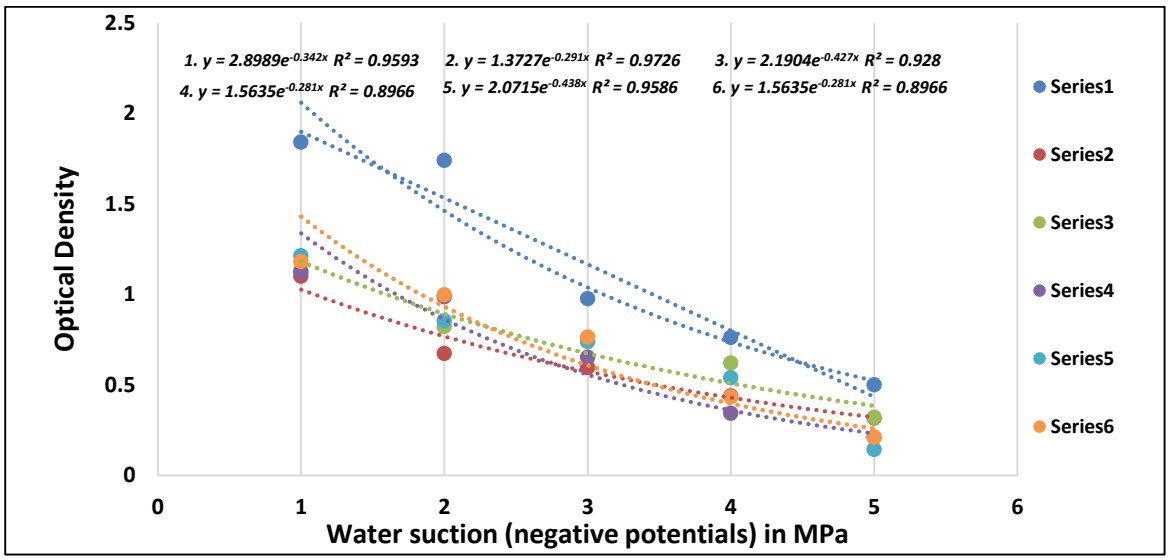


Fig. 3: Bacterial growth (in terms of turbidity in PEG broth) of six isolates under different moisture stress conditions

Production of EPS

Based on the results the EPS measurement was dry weight basis (gram/ liter). The six isolates were used for screening of high amount of exopolysaccharide production was ranged from 3.4 to 5.5g/l (Fig 4).

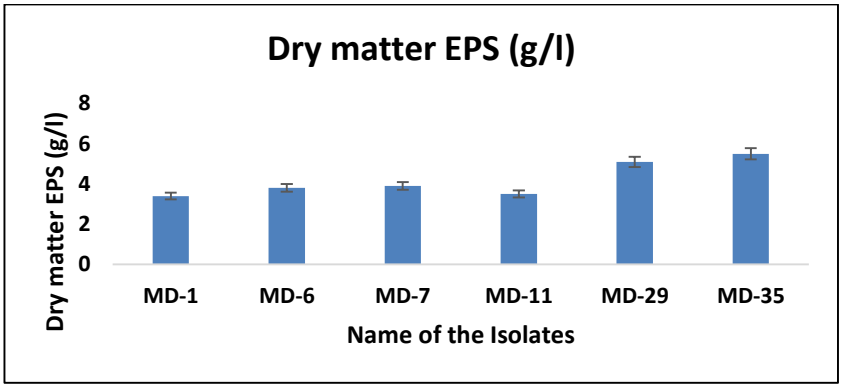
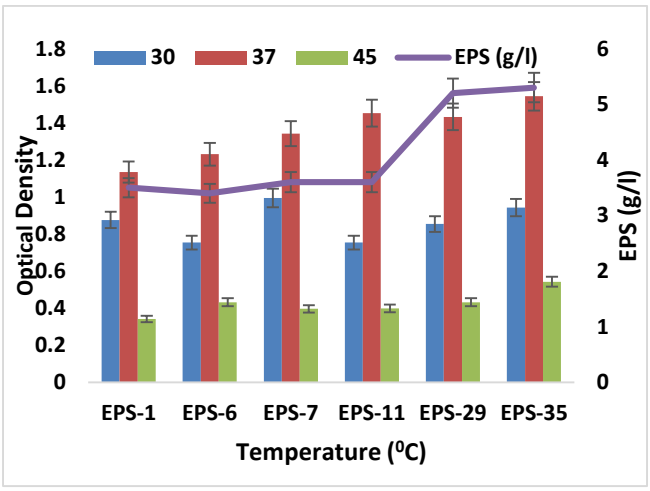


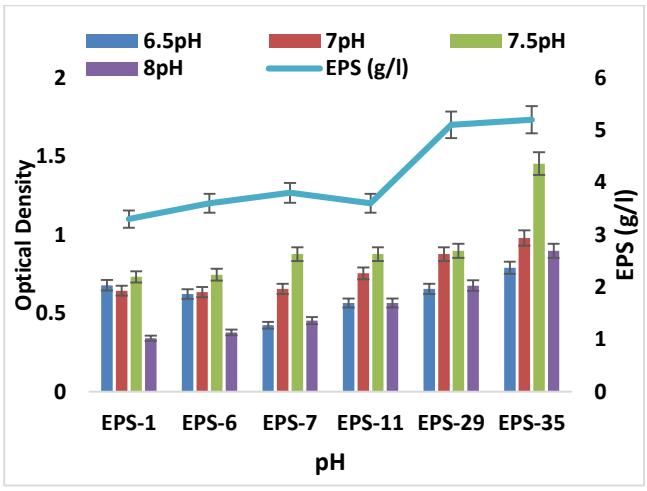
Fig. 4: Production of dry matter exopolysaccharide on EPS production medium for 72 h of incubation

Optimization of EPS producing bacteria

The EPS production was optimized under different environmental conditions (Fig 5). Such as Temperature, pH, incubation period, salt concentration (NaCl mM) and carbon source. The production of EPS was determined during different temperature (30 ^{0}C, 37 ^{0}C and 40 ^{0}C) and pH (6.5, 7, 7.5 and 8). It was found that temperature 37 ^{0}C and pH 7.5 was optimum for production of EPS. Similarly, incubation at 3 Day, salt concentration (NaCl-mM) at 80mM and carbon source of sucrose gives the optimum production of EPS (Sandhya *et al*., 2009) [5].



(a)



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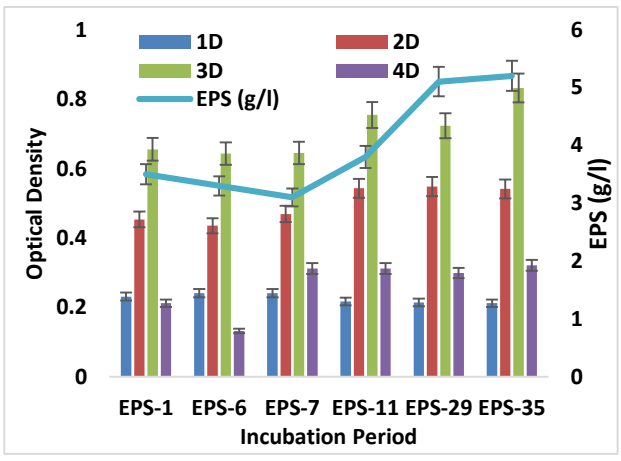

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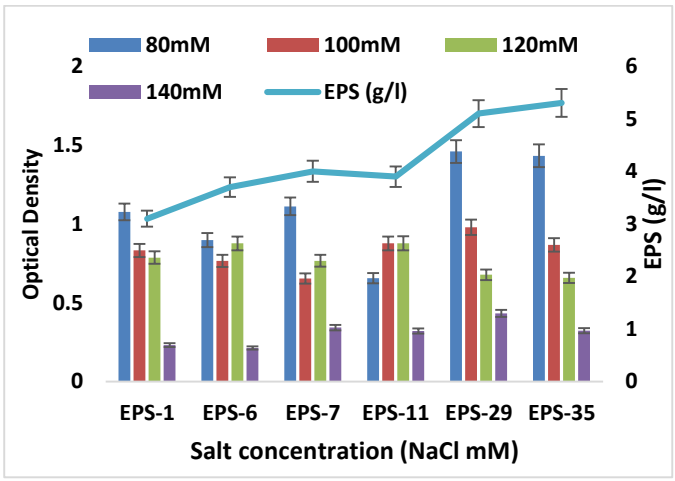
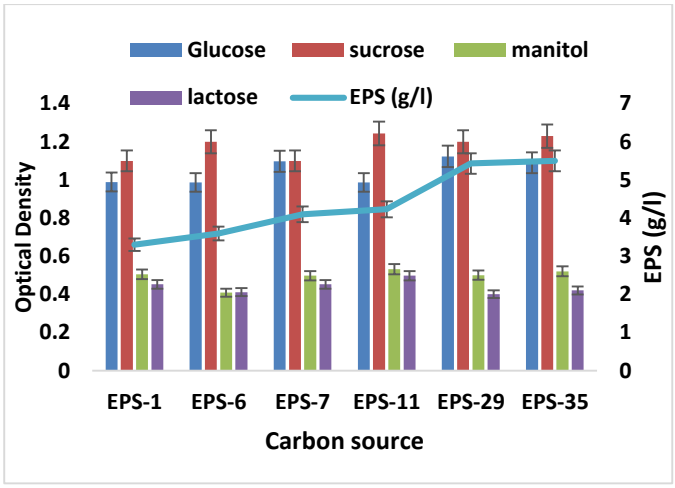


(d)



(e)

Fig 5: Optimization of exopolysaccharide production at different environmental conditions a) Temperature (^{0}C), b) PH, c) incubation period, d) Salt concentration (NaCl, mM), e) Carbon source (Glucose, sucrose, mannitol, lactose).

Discussion

EPS producing microorganisms play crucial role in protecting crops during the stress condition. Drought stress can make physico-chemical and biological properties of soil unsuitable for soil microbial activity and crop yield. Water availability controls the production and consumption of protein and polysaccharides by the bacteria (Nehad *et al*., 2010) [16]. The purpose of this experiment was to obtain the efficient strain isolated from rice soils which produce high amount of EPS. The Five different isolates from saline/alkaline soil of Baramati region were screened for EPS producing activity (Krithinga *et al*., 2014) [15]. In our case six different isolates from rice rhizospheric soil of OUAT farm was found more EPS producing activity. The pH and temperature of the culture medium is a vital factor that governs cell growth and EPS production (Ali *et al*., 2017) [17]. We found that, at 7 pH and 37^{0}C temperature were optimum for EPS production. The production rate of EPS was decreased rapidly with increasing the pH and temperature (Nehad *et al*., 2010) [16]. Microbes are extreme environment have triggered broad biotechnology interest, which is because of their potential peculiar properties. Some authors describe that the GAP-P45 strain was characterized for gram staining and other biochemical test like oxidase activity, catalase activity (Sandhya *et al*., 2009) [5]. In our study also gram staining and all biochemical tests are using

for characterization of exopolysaccharide producing bacteria. Exopolysaccharides possess unique water holding and cementing properties, it plays a crucial role in the formation and stabilization of soil aggregates and regulation of nutrients and water flow across plant roots through biofilm formation (Ali *et al*., 2017, Tisdall *et al*., 1994) [17, 18]. EPS synthesis is generally favored by presence of carbon source also by another nutrient (e.g., nitrogen, oxygen). A total of seventeen fluorescent Pseudomonas sp. grown under arid and semi-arid conditions were isolated and characterized for drought tolerance. Of these 17 isolates, nine could tolerate maximum level of drought stress (-0.30 MPa). In our study, total of 37 bacteria was isolated and after screening of 37 isolates 18 isolates produced mucoid substances. Further screening water stress potential 6 isolates could tolerate maximum level of drought stress (-0.73 MPa). Out of Eighteen six isolates produced higher amount of EPS. The exopolysaccharide production was optimized under different environmental conditions. EPS production was found to be maximum in 37 ^{0}C temperature at 3 days of incubation. EPS producing bacteria produce a wide variety of EPS which can mainly help in improving soil structure and maintaining water holding capacity of soil.

Conclusion

Thirty-seven bacterial isolates were screened for exopolysaccharide producing bacteria. Out of 37 isolates, six shows higher potential for production of EPS. The growth of these six isolates were optimized with pH, temperature, Salt concentration and different carbon source. These six isolates also show higher production rate among all isolates. Future study enhanced the high EPS production in soil either through culture or microbial intervention would be an area of research in water stress rice cultivation in climate change scenario.

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This is the partial Ph.D. work of Mahasweta Das.

Disclosure statement

No potential conflict of interest was reported by the authors.

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