International Journal of Advanced Biotechnology and Research(IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol6, Issue1, 2015, pp21-32
http://www.bipublication.com

Production and Characterization of Biopolymer by Plant Growth Promoting Bacterial Strain *Cronobacter malonaticus* BR-1

Parth V. Bhatt and BRM Vyas*

Department of Biosciences, Saurashtra University, Rajkot, India

*Corresponding author: Email: brmvyas@hotmail.com,

Tel: +91-281-2444295; Fax: +91-281-2586419

[Received 09/12/2014, Accepted-22/12/2014]

ABSTRACT:

Rhizobacterial isolate *Cronobacter malonaticus* BR-1 having multiple plant growth promoting activity produced 2.5 mg/lit exopolysaccharide (EPS), and solubilized inorganic phosphate (220-371 µg/ml) under varying physiological conditions like temperature, pH and salt. EPS was purified and analyzed biochemically by HPTLC and GC-MS for the major amino acid and fatty acid moieties. It suggested glutamic acid as a major amino acid moiety whereas palmitic acid, linoleic acid, elaidic acid and stearic acid as major fatty acid moieties. Quantitative analysis of biopolymer suggested presence of 27% sugar and 2.5% protein. Biopolymer production at different pH, temperature, incubation time and effect of sugar as a sole carbon source was evaluated. Pot trial experiments using barley plants inoculated with *Cronobacter malonaticus* BR-1 showed statistically significant increase in the root and shoot length and plant.

Keywords: Exopolysaccharide, Rhizobacteria, Phosphate solubilization, GC-MS, Phosphate solubilization, *Cronobacter malonaticus* BR-1

[I] NTRODUCTION

Microbial flora represents one of the vital components of soils as they are involved in various biotic activities of the soil ecosystem making it dynamic for nutrient turn over and sustainable crop production [1-2]. Certain bacteria stimulate plant growth by mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds like pesticides [3-7]. Indeed, the bacteria lodging around/in the plant roots (rhizobacteria) are more versatile in transforming, mobilizing, solubilizing the nutrients compared to those from bulk soils. Therefore, rhizobacteria are the dominant driving force in recycling the soil nutrients and

consequently, crucial for soil fertility [8]. Currently, the biological approaches for improving crop production are becoming popular among agronomists and environmentalists following integrated plant nutrient management systems. In this context, the ongoing rigorous research worldwide receive great impetus to explore a wide range of rhizobacteria possessing novel traits like heavy metal detoxifying potentials [9], pesticide degradation/tolerance [10-11], salinity tolerance [12-13], biological control of phytopathogens and insects [14-17] along with the normal plant growth promoting properties such as, phosphate solubilization, production of phytohormones, siderophores [18-19], 1-aminocyclopropane-1-carboxylate deaminase, hydrogen cyanide (HCN), ammonia, nitrogenase [20] etc. Hence, diverse symbiotic (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) and non-symbiotic (*Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azospirillum, Azomonas*) nitrogen fixing rhizobacteria are now being used worldwide as bio-inoculants to promote plant growth and development under various stresses like heavy metals herbicides [21] insecticides, fungicides, salinity [22], etc.

Exopolysaccharides (EPS) are the most active constituents of soil organic matter [23]. EPS comprise the most important part of extracellular matrix that often represents most of the bacterial weight [24]. Bacteria produce EPS in two forms: (1) slime EPS and (2) capsular EPS. EPS are found in a wide variety of complex structures [25]. The important roles exhibited by EPS are (1) protective, (2) surface attachment, (3) biofilm formation, (4) microbial aggregation, (5) plant–microbe interaction, and (6) bioremediation [26]. Some physical and chemical properties of EPS are useful in industries for stabilizing, thickening, coagulating, gelling, suspending, film forming, and water-retention capability in different industries like detergents, textile, paper, paints, adhesive, beverages, and food [27]. Some EPS-producing bacteria like *Pseudomonas* have the ability to survive even under drought due to the production of their EPS [28]. Bacterial EPS are hydrated compounds with 97% of water in polymer matrix which impart protection against desiccation [29]. The EPS protect these bacteria from desiccation under drought stress by enhancing water retention and by regulating organic nutrient carbon [30]. Due to enzymatic activities of EPS, they help in heavy metal transformation and degradation of organic recalcitrant compounds [31]. Water availability in the soil also affects the soil structure. Plants treated with EPS producing bacteria *Azospirillum* showed resistance to water stress [32] through improvement in the soil structure and soil aggregation.

A very large number of microorganisms including *Cronobacter* sp. produce variety of EPS with remarkably high moisture holding capacity to maintain minimum moisture in their immediate environment in addition to their plant growth promoting activities [33]. Microbial EPS have been commercialized as possible future industrial commodities for food and in agriculture for the precise encapsulation of somatic embryoid, which offer a greater feasibility for precise delivery of plant growth regulators, fungicides and pesticides [34]**.** Influence of culture conditions on polysaccharide production are reported for various organisms [35]. It has been reported that the use of sugar components e.g. sucrose, dextrose, mannitol, etc. as a sole source of carbon yield more EPS than cell biomass [36]. Minerals and growth factors are also known to regulate EPS yields.

The present study was aimed towards determining the influence of various physicochemical parameters on EPS production by *Cronobacter malonaticus BR-1* and its application for plant growth promotion.

[II] MATERIALS AND METHODS

In this study previously isolated and identified plant growth promoting bacterial strain

Cronobacter malonaticus BR-1 (Gene bank accession number- KC109002) was used [33].

Phosphate solubilizing and biopolymer producing strain was isolated from the rhizosphere of *Ficus religiosa* plant growing in concrete structure. The plant was uprooted gently, roots washed 4–5 times in physiological saline and grown in sterile Pikovskaya's agar, at 30°C for 72 h. The strain was grown on NBRIP medium (National Botanical Research Institute) and maintained on nutrient agar at 4°C.

Inoculum preparation

C. malonaticus BR-1 was grown in 3 ml Nutrient broth (NB) tube at 30°C on shaker (120 rpm). Cultures were centrifuged (10,000 rpm) for 10 min and pellet washed with 1 ml sterile 0.85% NaCl to remove free Pi present in medium, re-suspended in 1 ml 0.85% NaCl and used as inoculum in all experiments.

Screening and Production of EPS

50 ml NBRIP medium in 250 ml flasks were inoculated with *C. malonaticus* BR-1 and incubated at 30°C for 72 h in shaking condition (120 rpm). The medium was centrifuged (10,000 rpm, 15 min) and cell free supernatant amended with double volume of chilled iso-propanol was held overnight with stirring. The precipitated EPS was spooled and dried in oven at 60°C till constant weight [37].

Purification of EPS

The oven dried EPS was dissolved in distilled water and re-precipitated with double volume of chilled iso-propanol and re-dissolved in distilled water, mixed thoroughly, and drops of 5% CTAB were added, until white precipitates appeared. The precipitates were collected by centrifugation, dissolved in 2 M NaCl and re-precipitated with double volume of iso-propanol, and air dried. The purified and dried biopolymer, stored in cool and dry condition was used for biochemical analysis.

Hydrolysis of EPS

The purified biopolymer was incubated with 2.5 N HCl in boiling water bath for 6 h and neutralized with sodium carbonate till effervescence ceased. The hydrolyzed biopolymer was analyzed by TLC for sugars and HPTLC for the amino acids present in EPS.

TLC Analysis of hydrolyzed biopolymer

The TLC analysis of the hydrolysate was performed on silica gel (Merck) plates along with standards, isopropanol-acetone-lactic acid (1:8:1) used as the developing phase, and the monosaccharide(s) in the hydrolysate were detected by spraying with α-napthol reagent followed by heating at 120°C for 5 min in hot air oven.

GC-MS Analysis:

10 mg of extracted biopolymer was suspended in a tube containing 1 ml of choloroform, 0.85 ml of methanol and 0.15 ml of sulfuric acid. All the chemicals used were of AR grade. The tube was sealed and kept in an oil bath at 100°C for 160 min. The contents were allowed to cool and mixed with 5 ml of water. The bottom chloroform phase was taken and used for the analysis. Similarly standards were prepared.

Esterified samples were evaluated by GC-MS. GC Mass spectrophotometer (QP-2010 GCMS, Shimadzu). Column used was DB-5ms (Durabond) capillary column (DB series, Shimadzu, Japan; Mfd by JW Scientific USA) and mass detector used. Helium gas (1 ml/min) was used as carrier gas. Temperature of the injector was 220°C. Temperature program used was 45°C for 7 min; temp ramp of 4°C per min up to 100°C; 10°C/min rise up to 200°C followed by 10 min hold.

Protein assay

Amount of protein assayed according to Lowry et al [38]. The absorbance of the standards and samples were measured at 750 nm and compared to a standard curve obtained by serial dilution of bovine serum albumin.

Carbohydrate assay

The carbohydrate was measured by the phenol-sulfuric acid method according to Dubois et al [39].

Viscosity measurement

Viscosity of fermented broth was measured with Cannon Fenske viscometer at 30°C at 100 rpm with uninoculated medium as reference. Viscosity measured was expressed in terms of centipoises (cP).

Optimization of EPS Production by *C. malonaticus* BR-1

Influence of pH

C. malonaticus BR-1 was grown in NBRIP medium having pH (5 to 8) at 30°C, 120 rpm for 72 h. After incubation the dry weight of EPS measured.

Influence of temperature

Five sets of flasks containing 100 ml NBRIP medium inoculated with *C. malonaticus* BR-1 were incubated at 15, 30, 37 and 45°C at 120 rpm for 72 h.

Influence of carbon substrate

C. malonaticus BR-1 was grown in NBRIP medium with mannitol, sucrose, dextrose, fructose, maltose, or lactose (10 g/l) at 30°C, 120 rpm for 72 h.

Influence of incubation time

C. malonaticus BR-1 inoculated with NBRIP medium was incubated at 30°C, 120 rpm. A set of flasks was harvested after 24, 48, 72, 96 and 120 h of incubation.

Screening for Antagonistic Activity

Potato-dextrose agar was used for assessing the antagonistic activity of *C. malonaticus* BR-1 against major plant pathogens, namely, *Rhizoctonia solani, Fusarium oxysporium, Alternaria solani, Penicillium roqueforti, Aspergillus flavus* and *Aspergillus niger*.

The dual culture method was performed as described by Lim et al [40] for identifying potential isolates possessing antagonistic activity against test pathogens. Fungal culture was cut from the periphery of the growing cultures and placed in the centre of the Petri plate. Control plates were inoculated only with fungus. Petri plates were sealed with parafilm and incubated at 30°C in a BOD incubator for 6 days.

Salt, pH and temperature tolerance

Ability to solubilize phosphate under stress condition was evaluated with NBRIP broth inoculated with the bacterial strain under different physiological conditions. Salt tolerance was evaluated using NBRIP broth containing 2-8% (w/v) NaCl inoculated with *Cronobacter malonaticus* BR-1 (A_{600}=0.1) and incubated for 72 h at 120 rpm at 30°C. After incubation the solubilized phosphate was measured by vanado-molybdate method. The pH tolerance was evaluated using NBRIP broth with pH 4-8 [41]. The temperature tolerance of isolates was tested in NBRIP medium at 28, 30, 37 and 45 °C for 72 h. The tests were repeated at least once and carried out in triplicate.

Pot experiments

A bioassay-based determination of the plant growth promoting ability of the isolate was conducted using barley seedlings in sterile soil under glasshouse conditions. The barley seeds were surface sterilized in 70% ethanol for 2 min and 0.2% $HgCl_2$ for 5 min and washed ten times with sterile tap water. Pure culture in NB at 28°C and diluted to a final conc. of 10^8 cfu/ml in sterile saline (0.85% NaCl) were used to coat surface sterilized seeds by immersing in PGPR suspension (10^8 cfu/ml) for 45 min on a rotary shaker (120 rpm), air-dried, and sown immediately. Control seeds were treated with sterile distilled water. Seeds were sown in plastic pots (15 cm diameter) containing 6 kg of sterile soil (pH 7.2, organic carbon 2.6%, available P 51.2 kg/ha, available K 197.57 kg/ha, iron 34.44 mg/kg) and placed in a glass house. Thinning of seedlings was done 7 days after sowing and ten seedlings per pot were maintained throughout the experimental period. The soil was moistened to 50% of its water-holding capacity. The whole experiment was conducted in three independent trials. For each treatment, the plants of each pot were harvested 3 weeks after the emergence of seedlings and washed; morphological characteristics of each plant were recorded: plant

height, root length, dry shoot and root weights. At harvest, the root system was separated from shoots, and both were oven-dried overnight at 65°C and dry weights were recorded against the control.

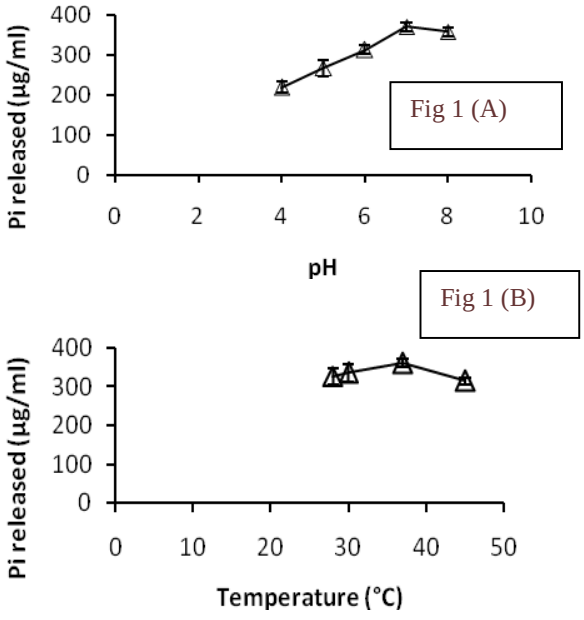
[III] RESULTS

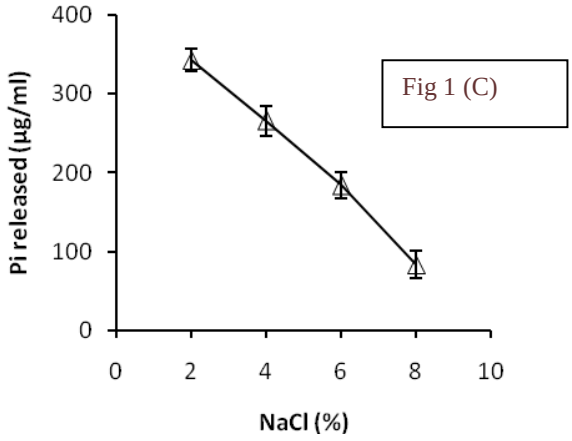
Phosphate solubilization under stress condition

Cronobacter malonaticus BR-1 was screened for its ability to solubilize inorganic phosphate under high salt concentration (2-8% w/v), at varying temperature (28-45°C) and pH (4-8). *C. malonaticus* BR-1 solubilised phosphate at pH 4 and 8 in amount of 220 and 359 µg/ml respectively but optimally at pH 7 (371 µg/ml).

The strain solubilized phosphate in the presence of 2-8% of NaCl. It solubilized 343 and 83 µg/ml phosphate respectively but the amount of phosphate solubilized decreased with increase in salt concentration. *C. malonaticus* BR-1 solubilized phosphate at 28-45°C temperature in the range of 315-328 µg/ml.

Fig. 1 Solubilization of phosphate (A) pH 4-8, (B) 28-45°C and (C) 2-8% NaCl by the 72 h old cultures of *C. malonaticus* BR-1 growing in NBRIP medium.





Biopolymer Production and Purification

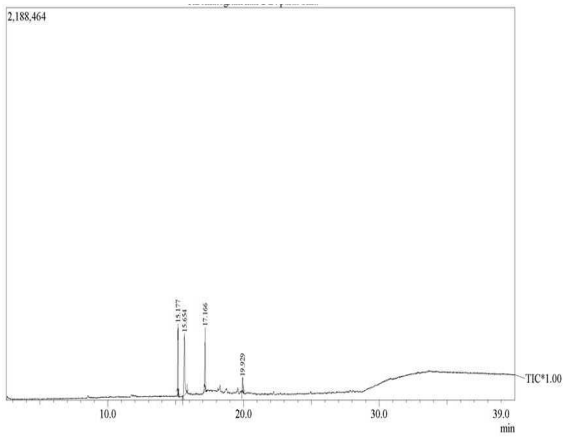
C. malonaticus BR-1 formed mucoid colonies over the surface of NBRIP agar after incubation of 72 h. Culture of *C. malonaticus* BR-1 growing in NBRIP medium became viscous after 72 h of incubation at 30°C on shaker (120 rpm). The cultures were observed to produce 2.5 mg/l biopolymer. The freeze-dried purified polymer of *Cronobacter malonaticus* BR-1 is whitish, has a porous structure and texture like a silicone polymer. It was purified and analyzed for its chemical composition.

Chemical analysis of biopolymer

The biopolymer contained 27% sugar and 2.5% protein indicating that the polymer was mainly a polysaccharide. Since polysaccharides are a mixture of many saccharides including neutral, uronic acid, and amino sugars, the purified polymer was hydrolyzed with hydrochloric acid to determine the content of sugars. The hydrolyzed and purified polymer showed spots that corresponded to the standard sugar. The Rf value of hydrolysed biopolymer matches with the spot of glucose. Hydrolyzed biopolymer was also ninhydrin positive indicating that it also contained amino acids. HPTLC analysis showed glutamic acid to be the major amino acid moiety in the hydrolysed biopolymer. Polysaccharide from *Cronobacter malonaticus* BR-1 was soluble in organic solvents such as methanol, ethanol, acetone, DMSO, ethyl acetate, and chloroform.

The fatty acid analysis of hydrolysed biopolymer by GC-MS showed presence of palmitic acid, linoleic acid, elaidic acid and stearic acid as major fatty acid moiety (Fig 2).

Fig 2. GC-MS analysis of hydrolysed biopolymer showing presence of four fatty acids palmitic acid, stearic acid, linoleic acid and elaidic acid



Biopolymer production optimization study:

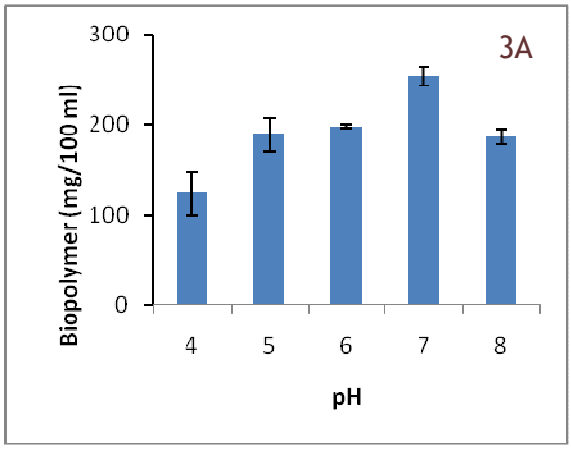
Cronobacter malonaticus BR-1 produced 124-254 µg/100 ml biopolymer during its growth in NBRIP medium having pH 4-8 (Fig 3A). At pH 7 the isolate produced 254 µg/100 ml. Production of biopolymer by the isolate during its growth on sucrose produced 293 µg/100 ml of biopolymer (Fig 3B). At 37°C isolate showed higher production of biopolymer (266 µg/100 ml) (Fig 3C). Incubation period revealed that biopolymer production increased as the incubation time increased up to 72 h. No further increase occurred between 72-120 h. (Fig 3D).

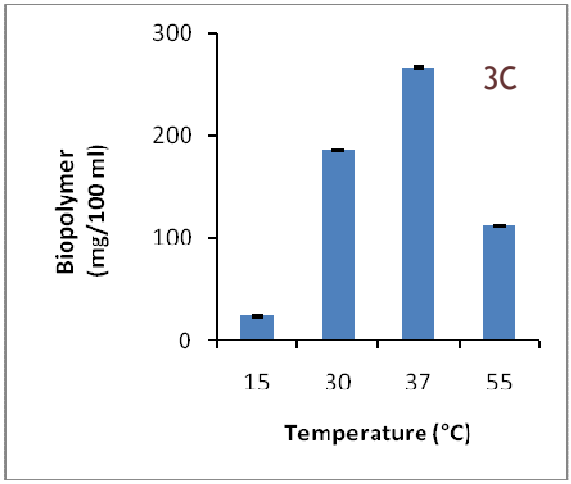
Fig 3 Production of biopolymer by *C. malonaticus* BR-1(µg/100 ml) growing in NBRIP broth with varying pH (3A), (3B) Temperatures, (3C) carbon sources and (3D) incubation times

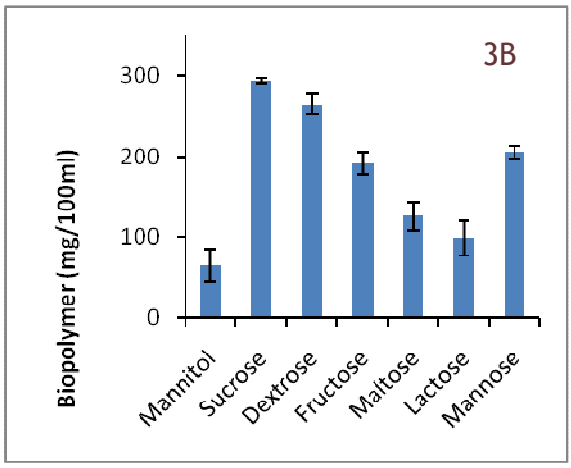
Pot trial experiment

Bacterial coated seedlings of barley were found to be comparably different from the untreated such as morphological parameters like plant height, root length, and fresh and dry biomass.

Isolate significantly improved all the parameters. Significant increase was observed in the root length, shoot length and root fresh weight 162%, 213% and 508%, respectively. On the other hand, shoot fresh weight of the treated seedlings increased by 582%. Increase in the root dry weight was 250% and shoot dry weight increased by 371%. When these morphological parameters were statistically analyzed by ANOVA, results were found to be significant (Table 1)







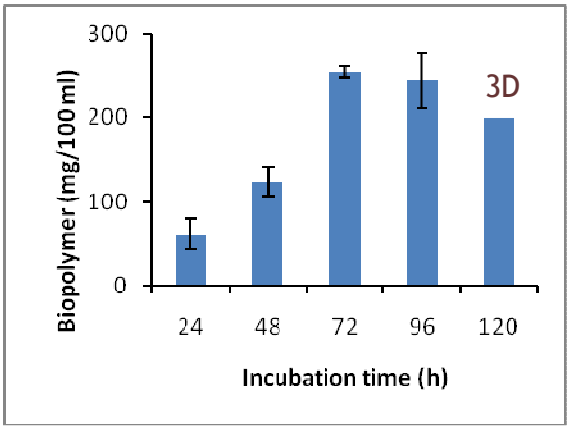


Table 1 Plant growth parameters of Barley plants cultivated from the uncoated and bacterial coated seeds. Results are expressed as means ± SD (n=30). One way ANOVA was performed for each plant section with different letters are significantly different from each other ($P < 0.05$). According to Tukey-Kramer multiple comparison test

	Root length (mm)	**Shoot length (mm)**	**Root fresh weight (mg)**	**Shoot fresh weight (mg)**	**Rot dry weight (mg)**	**Shoot dry weight (mg)**
Control	60.5±2.8	22.9±2.6	25.5±2.5	127.9±20.4	6.8±0.6	15.7±3.6
BR-1	97.7±5.5	48.8±4.6	47.3±3.1	274.9±40.9	14.3±0.5	52.9±2.9
% increase	162	213	508	582	250	371

[IV] DISCUSSION

Many studies illustrated the nature and properties of these unique microbes harboring potential plant growth promoting traits. It is important to search for the region-specific microbial strains which can be used as a potential plant growth promoter to achieve increased crop production.

Different bacterial genera are crucial components of soils. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and crop production [42]. Bacteria growing around/in the plant roots (rhizobacteria) are versatile in transporting, mobilizing and solubilising the nutrients compared to those from bulk soils [43]. Therefore, rhizobacteria are the dominant components in recycling the soil nutrients and consequently, are required for soil fertility [44].

PGPR are commonly used to improve the crop yield, however the need is to explore the indigenous microbial strain for the optimized plant growth promoting activity. This study focuses on the characterization of biopolymer produced by the PGPR isolate having multiple plant growth promoting activities such a bacterial strain was previously isolated and identified .

PGPR strains expressing multiple beneficial functions are known [45]. The isolates exhibited more than two or three PGP traits, promote plant growth directly or indirectly or synergistically [46-47]. Similar to our findings of multiple PGP activities among PGPR have been reported and findings on indigenous isolates of India are less commonly explored [48].

Phosphate is abundant in several soils and is one of the major nutrients limiting the plant growth. The overall phosphate use efficiency following phosphate fertilizer application is low because of the formation of insoluble complexes [49]. It is well established fact that improved phosphorous nutrition influences overall plant growth and root development [50]. Hence, frequent application of soluble forms of inorganic phosphate is necessary for crop production it leaches to the ground water and results in eutrophication of aquatic systems. In view of such environmental concerns, current developments in sustainability, research efforts are concentrated on elaboration of techniques that involve the use of less expensive, though less bioavailable sources of plant nutrients such as rock phosphate and by the application of phosphate solubilizing bacteria, the agronomic effectiveness can be enhanced. *Cronobacter malonaticus* BR-1 having multiple plant growth traits solubilized phosphate over a broad range of pH, temperature and salt concentration. Solubilization of phosphate in different physiological conditions may be useful to develop bioformulations and use with the soils

having changing environmental and physiological conditions.

Many bacterial strains producing different kinds of polysaccharides are reported in the literature that exhibit flocculation activity, namely, *Bacillus polymyxa* [51], *Bacillus sp.* A56 [52], *S. paucimobilis* GS1 [53], in the actinomycetes *Rhodococcus erythropolis* [54], *Nocardia amarae* [55], and *Streptomyces griseus* [56]; molds like *Paecilomyces sp.* I-1 [57] and *Pestalotiopsis sp.* KCTC 8637P [58]; and *Myxobacterial* members such as *Nannocystis sp.* NU-2 [59].

Formation of mucoid gummy colonies on NBRIP and increase in the viscosity of NBRIP indicated the ability of *Cronobacter malonaticus* BR-1. to produce EPS. The quantitative yield of EPS produced during submerged fermentation was 2.5 g l^{-1}. Similar yields have been reported for A. faecalis var. *Myxogenes* [60]. Sayyed and Chincholkar [61] have obtained a yield of 1.3 g l^{-1} with *A. faecalis*. When cell free culture supernatant was added to equal volume of iso-propanol (30%) the EPS was found spooling on constantly moving glass rod. The spooled extract when dried at 50°C, a brown colored powder of EPS was obtained. Sayyed and Chincholkar have reported the extraction of EPS produced by *A. faecalis* with 50% iso-propanol.

Chemical analysis of the polysaccharide showed proportion of the total sugar content to the total protein content of was found to be 27 and 2.5% (w/w).

Biochemical analysis of hydrolysed biopolymer showed presence of dextrose as a major sugar moiety in the biopolymer. The HPTLC and GC-MS analysis of hydrolysed biopolymer shows presence of glutamic acid as a major amino acid moiety present in the EPS.

Biopolymer production was optimized and at Exopolymeric compound produced at different temperature, pH, incubation time and in presence of different sugars as a sole source of carbon. Biopolymer production does not vary much in pH range 4 to 8 but it increases with the increase in the incubation temperature up to 37 °C and decreases at higher temperature (55°C). Sucrose supported highest production of biopolymer as compared with other sugars. Production of biopolymer increases as the increase to the incubation time up to 72 h but it does not vary much after further incubation.

Seed inoculated with isolated PGPR strain increased barley plant height and weight. Similar increases in plant height and weight were observed in different crops such as potato, radish plants, sorghum and pearl millet inoculated with *Pseudomonas, Azospirillum* and *Azotobacter* strains.

To our knowledge this is the first report on the production of a biopolymer the multiple plant growth activity possessing *Cronobacter malonaticus* BR-1. It is envisaged that the polymer from this isolate is an attractive candidate for different biotechnological applications. Work addressing its applications are modulatory.

[V] CONCLUSION

The work illustrated the production, purification and characterization of multiple plant growth promoting bacterial strain Cronobacter malonaticus BR-1. Isolate produced 2.5 mg/l biopolymer after 72 h at 30°C in shaking condition. Biopolymer containing glucose and glutamic acid as major carbohydrate and amino acid moieties respectively. Isolate also showed increase in the plant biomass under *in vitro* condition. It was concluded that isolate produced biopolymer at different physiological conditions and which may be used for different biotechnological applications.

ACKNOWLEDGEMENT

DSA Fellowship to Parth V. Bhatt is gratefully acknowledged.

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