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Screening, Isolation And Molecular Identification Of Biosurfactant Producing Bacteria From Rhizosphere Soil Samples

Saritha Pendyala and Madhuri Kulkarni*

Dept of Biotechnology, Mahatma Gandhi University, Nalgonda 508001, Telangana, India

Abstract

Biosurfactants (BS) are a class of secondary metabolite that has surface active properties and are produced by large number of microorganisms. The current study is aimed at isolation and screening of BS producing bacteria from different rhizosphere samples, characterize them and study for BS production in different media. Using the enrichment culture method, 80 morphologically different bacterial isolates were isolated from various rhizosphere samples. Twenty isolates were chosen and further screened for BS activity [penetration assay, microplate assay, oil-displacement method, emulsification assay (EA) and emulsification index (EI%)]. Among the twenty isolates tested, Bacillus sp. RMS25 demonstrated good EI percent and EA, as well as high BS production. The bacterial isolate RMS25 has shown high BS production, yielding 562 mg L^{-1}. The isolate RMS25 was identified as Bacillus vallismortis using biochemical techniques and the 16S rRNA gene sequence. Molecular identification of the isolate RMS25 was performed by16S rRNA sequencing, and the sequences were analysed using NCBI BLAST. The BLAST findings for isolate RMS25 revealed a 99 percent match with the Bacillus vallismortis database sequence. The sequences were submitted to GenBank and assigned the accession number MW468676.

Keywords Biosurfactant; Secondary metabolites; Emulsification index; Emulsification assay; Molecular identification

Introduction

Increasing agricultural production is a major concern to meet the growing requirements of world's population. Given the proven toxicity of synthetic fertilizers and pesticides, the application of green compounds is now required for sustainable agriculture. BSs are produced by rhizosphere microbes; these biomolecules govern plant–microbe interactions by playing critical roles in quorum sensing among rhizosphere bacteria. BSs can be used in agriculture to kill plant pathogens, increase nutrient bioavailability, and promote bioremediation, all of which improve soil quality. These biomolecules have the potential to replace the harsh surfactants that are currently used in the multibillion-dollar pesticide industry. Thus, extensive research on environmental isolates is needed to investigate their potential for BS production.

BSs are a class of structurally diverse secondary metabolites that are surface active and produced by a large number of microorganisms. These are amphiphilic molecules that accumulate between fluid phases and reduce surface tension at the surface and interface. (Mukherjee et al., 2006; Ongena and Jacques, 2008). BSs have higher efficiency and stability, lower toxicity, and improved environmental compatibility at high salinity, pH and temperatures, can be synthesised from renewable feedstock (Romero et al., 2007, Mulligan CN, 2005) and highly biodegradable compared to chemical surfactants. Based on the foregoing benefits, BSs have the potential to be commercially manufactured for a number of applications in agriculture, food, cosmetics, and pharmaceutical industries (I. M. Banat et al., 2010, L. Rodrigues et al., 2006, M. Nitschke et al., 2007, M.Kanlayavattanakul 2009 and N. Lourith). Various microbial BSs have been shown to have antimicrobial activity against phytopathogens and have been used as a promising biocontrol agent in the pursuit of sustainable agriculture. These BSs can be widely employed in agriculture to increase pollutant biodegradation, consequently enhancing agricultural soil quality and, in turn, indirectly influencing plant growth and plant microbe interaction.

Numerous varieties of BS producing bacteria isolated from crude oil contaminated soils and aqueous environments (Sachdev and Cameotra, 2013) have been reported. BSs are produced by a vast number of plant-associated and rhizosphere bacteria, suggesting that they may have vital role in plant-microbe interactions and aid plant cultivation by preventing plant diseases (Moore et al., 1989). Many BS-producing strains from Bacillus and Pseudomonas species, for example, were successful in combating a variety of plant diseases. (Sachdev and Cameotra, 2013). In this sense, the present aims at (i) isolation of bacterial strains from various marine water samples (ii) screening bacterial isolates for BS production using different standard methods, (iii) Identify the best BS producer and test for plant probiotic properties and BS production (yield) using different media and (iv) isolate showing good plant growth promoting properties and high BS production will be further identified using 16S rRNA sequencing.

Materials and methods

Soil sample collection

Rhizosphere soil samples of different crop fields were collected from different areas (Choutuppal, valigonda, aregudem, bhuvanagiri and katrev) of Nalgonda, Telangana, India. Before collecting soil samples, the debris was cleared. The site was dug up to a depth of 10–20 cm below the surface, and 10 g of soil was collected in a sterile tube and brought to the laboratory, where it was stored at 4°C until analysis.

Enrichment technique for isolating BS-producing bacteria

The enrichment culturing was performed as reported by Dubey and Juwarkar (2001). For enrichment, 10 gm of soil sample was added to 90 mL of mineral salts medium in 250 mL flasks, which was then amended with 5 % (v/v) sterile crude oil and incubated for 5 days at 37°C, 200 rpm. The enriched samples were further serially diluted, plated on nutrient agar medium and incubated at 37°C for 24-48 hrs. Morphologically distinct bacterial isolates were selected and sub-cultured on Zobell marine agar medium (Hi-media). Gram's staining and spore staining was performed for the selected isolates to

characterize colonies of pure cultures. For future studies, pure cultures were preserved at -70 °C as glycerol stocks.

Bacterial screening for BS production

Preliminary screening of bacterial isolates for BS activity (qualitative) was evaluated using following methods: penetration assay, microplate assay, oil-spread method, emulsification assays (EA) and emulsification index (EI). For all the screening assays, supernatant of 24 h old cultures grown in nutrient broth was used.

Microplate assay:

The microplate method is a qualitative assay for determining BS activity that relies on a change in optical distortion in an aqueous media generated by surfactant (Vaux and Cottingham, 2001). Each bacterial isolate's culture supernatant (100 µL) was added separately into microplate wells and left undisturbed on graph paper, with wells aligned across a $1mm^2$ grid. Grid image distortion confirmed the presence of BS activity, which was then compared to positive and negative controls.

Penetration assay

The penetration test is based on the interaction of two insoluble phases that results in color change (Maczek et al, 2007). To all 96 wells of microplate, 150 µL hydrophobic paste made of silica gel and engine oil was added. Then it was covered with 10 µL of oil, 90 µL of cell free supernatant and 10 µL of safranin and observed for color change and thereby BS activity.

Oil displacement method

The oil spreading experiment was carried out in accordance with Plaza et al, 2006. In this test, distilled water (30 mL) was taken in a pertiplate, and 1 mL of crude oil was added in the center of the distilled water-containing plates. Finally, a 15 µL sample of culture supernatant was dropped into the water, and the displacement of oil above the water surface was observed. If culture supernatant has BS activity, oil-free clearing zone displaces oil, also known as oil displacement activity. Distilled water (without surfactant) and with Triton X-100 were used as negative and positive controls.

Lipase assay

As stated by Kokare et al. (2007), 10 µL of 24h old cultures were spot-inoculated on Tributyrin Agar medium, incubated at 37 °C for 48 h, and inspected for a distinct zone of lysis surrounding the colonies.

Blood haemolysis test

It was decided to test for Haemolysis activity by adding 5 percent of sheep blood to Nutrient Agar Medium (NAM). For the 48-hour incubation period, each isolate was spot-inoculated with 20 µL of 24 h old culture. A distinct zone of haemolysis around the bacterial colonies on the plates after incubation indicated that BS activity was present (Anandaraj and Thivakaran, 2010).

Emulsification index (EI%)

Based on microplate, penetration assay, and oil displacement method results, ten isolates were chosen for further investigation. A 1:1 ratio (2mL each) of culture supernatant and kerosene vigorously mixed for 10–15 minutes, and left unruffled for 24 hours. Emulsification index was calculated using following formula (1).

$$\text{Emulsification index (EI\%)} = \frac{\text{Height of the emulsion}}{\text{Total height}} \times 100$$

Emulsification assay

The emulsification assay was carried out in accordance with method described by Satpute et al. (2008), with slight modifications. A 0.5 mL of hydrocarbon oil (engine oil) was added to 3 mL of culture supernatant, vortexed for 2 minutes, and then left at room temperature for 1 hour to observe for the separation of aqueous and oil phases. At 400 nm, the absorbance of the aqueous phase was measured.

Production of BS

Twenty BS-producing isolates were tested in three different media: NB broth, mineral salts medium (MSM) ($FeSO_4$ (0.01), $MgSO_4.7H_2O$ (0.5), KH_2PO_4 (0.5), $NaNO_3$ (0.5), K_2HPO_4 (0.5) and KCl (0.1) g/L], pH 7 and Zobell medium (Hi-media). In a separate set of experiments, bacterial isolates were inoculated in 100 mL media, prepared in a 250 mL flask, and incubated at 37 °C, 180 RPM for 48 hours. The experiment was done with two replications. After 48 hours, the cell free supernatant was collected by centrifugation at 10,000 rpm, the pH was adjusted to 2.0 with 6 N HCl (Dubey and Juwarkar, 2001), and the sample was stored overnight at 4 ºC. After centrifugation at 12,000 rpm for 20 minutes, the precipitates were extracted twice with methanol.

Morphological and biochemical characterization of selected bacterial isolate

One potential bacterial isolate was chosen for further research based on the findings of the BS activity screening. It was identified morphologically by Gram's staining and biochemical tests like Catalase test, Methyl red test, Indole test, Citrate utilization, Voges-Proskauer test, starch hydrolysis, and Glucose fermentation test. The Bergey's Manual of Systematic Bacteriology was used to conduct all of the assays (Sneath et al., 1986).

Molecular identification of RMS25 using the 16S rRNA gene sequence

Molecular identification of bacterial isolate RMS25 was performed by sequencing with universal 16S rRNA primers (MACROGEN, Seoul, Korea). Mega-4 bioinformatics software was used for phylogenetic analysis.

Results and Discussion

Enrichment culturing was used to select BS producers from rhizosphere soil samples collected from various crops, yielding 80 morphologically distinct bacterial isolates. Using Gram's staining and spore staining, all isolates were identified as Bacillus spp. and were chosen for further research. Qualitative analysis of BS activity of all isolates was assessed using microplate assay and penetration assay.

The optical irregularity of the surface changes when a surfactant-containing fluid is applied to a microplate; pure water has a flat surface in a hydrophobic well, whereas surfactant-containing fluids have concave and uneven surfaces. The BS activity was indicated by a change in colour from translucent red to hazy white within 15 minutes of starting the penetration assay (see figure). All 20 isolates had a distinct zone on crude oil ranging in diameter from 25 to 40 mm, with RMS25 having a diameter of 35 mm. All twenty isolates showed a zone of haemolysis ranging in size from 3 to 16 mm in diameter in a blood haemolysis test, with RMS 25 having a diameter of 16 mm. Each of the 20 isolates produced a distinct halo around the colonies on tributyrin agar plates, indicating lipase activity. The zone size ranged from 5 to 15 mm in diameter (data not shown), with RMS25 having the largest zone size of 15 mm in diameter. A total of 20 isolates were chosen for further investigation based on the results of the penetration and microplate experiments (Table-1).

S.No.	Isolate number
1	R1
2	R5
3	R8
4	R10
5	R14
6	R16
7	R19
8	R20
9	R25
10	R30
11	R33
12	R40
13	R44
14	R48
15	R52
16	R61
17	R66
18	R72
19	R75
20	R78

Table-1 List of best isolates showing positive results for BS activity

The oil displacement assay detects BSs in a more sensitive and time-efficient manner. The size of oil clearance zone in presence of BS is defined as oil displacement activity (Morikawa et al., 1993). A linear relationship exists between the amount of surfactant and the diameter of zone of clearance. This method is quick and simple to implement, require only a small volume of sample and does not require any special equipment. All 20 isolates exhibited a clear zone with crude oil.

In the current study emulsification assay and emulsification index were performed with kerosene. Among 20 isolates, isolate Bacillus sp RMS25 showed high EI percentage (EI%) 68.7 on engine oil, followed by isolates R14, R30 and R72, which showed 62.5% (Table 2). Bacillus sp. RMS25 demonstrated high emulsification activity [EA units (Eu)/mL] 213.9 on engine oil, followed by isolates R8, R20 and R75, which showed 211.9, 208.4 and 210.0 (Table 3).

S.No	Isolate No.	Engine oil	
		EH	EI%
1	R1	1.6	50.0
2	R5	1.4	43.7
3	R8	1.9	59.3
4	R10	1.6	50.0
5	R14	2.0	62.5
6	R16	1.2	37.5
7	R19	1.4	43.7
8	R20	1.4	43.7
9	R25	2.2	68.7
10	R30	2.0	62.5
11	R33	1.6	50.0
12	R40	1.5	46.8
13	R44	1.4	43.7
14	R48	1.3	40.6
15	R52	1.8	56.2
16	R61	1.3	40.6
17	R66	1.7	53.1
18	R72	2.0	62.5
19	R75	1.7	53.1
20	R78	1.9	59.3

Table-2 Emulsification index (EI%) of the selected isolates on kerosene

S.No	Source of sample	Engine oil	
		O.D	EA
1	R1	1.519	151.9
2	R5	2.034	203.4
3	R8	2.119	211.9
4	R10	2.063	206.3

5	R14	1.725	172.5
6	R16	1.442	144.2
7	R19	1.921	192.1
8	R20	2.084	208.4
9	**R25**	2.139	213.9
10	R30	2.002	200.2
11	R33	2.039	203.9
12	R40	1.365	136.5
13	R44	1.390	139.0
14	R48	1.914	191.4
15	R52	1.962	196.2
16	R61	1.637	163.7
17	R66	1.301	130.1
18	R72	1.189	118.9
19	R75	2.10	210.0
20	R78	1.649	164.9

Table-3 Emulsification assay of the selected isolates on engine oil

BS production

BS production by twenty isolates was studied in nutrient broth, mineral salts medium and Zobell medium. Among the 20 bacterial isolates tested for BS production, the bacterial isolate RMS25 exhibited a high BS yield of 562 mg L^{-1} in Zobell medium (Table-4). It was observed that, for all isolates tested, Zobell medium has supported for high BS production compared to MSM and nutrient broth.

Isolate number	BS production (mg L^{-1})		
	Nutrient broth	**Mineral salts medium**	**Zobell medium**
R1	84	110	147
R5	96	104	136
R8	119	189	327
R10	112	142	166
R14	89	115	129
R16	103	130	162
R19	96	112	121

R20	107	119	133
R25	210	346	562
R30	154	181	294
R33	126	143	189
R40	117	128	215
R44	149	168	212
R48	104	115	194
R52	126	142	261
R61	115	127	210
R66	134	114	195
R72	127	245	318
R75	142	219	342
R78	109	210	265

Table-4 BS production by the selected bacterial isolates in different media

Based on BS activity and production, bacterial isolate RMS25 was selected for further studies. It was identified as a rod shaped, motile, Gram-positive. Biochemical tests revealed that the isolate was negative for methyl red test and positive for catalase test, Voges–Proskauer test, indole test, citrate utilization test, glucose fermentation and starch hydrolysis (Table 5). The 16S rRNA gene sequence results were drawn from EzTaxon server BLAST search, and isolate RMS25 was identified as Bacillus vallismortis (Fig. 5).

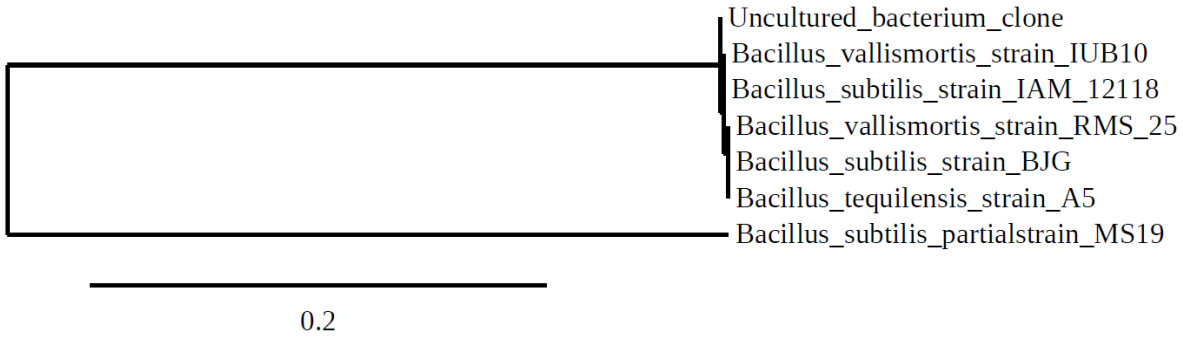

Figure-5. Identification of Bacillus sp. KM15 using 16S rRNA sequencing and construction of phylogenetic tree.

S.No.	Morphological and biochemical tests	Result
01	Gram staining	Gram +ve
02	Motility	Positive
03	Endo spore	Positive
04	Starch hydrolysis	Positive
05	Indole	Positive

06	Voges Proskauer	Positive
07	Citrate utilization	Positive
08	Catalase	Positive
09	Glucose fermentation	Positive
10	Methyl red	Negative

Table 5 Morphological and biochemical identification of the bacterial isolate RMS25.

Conclusion

In the current study, 80 bacterial isolates from rhizosphere soil samples were screened for BS activity using various screening methods. One Bacillus spp. isolate, demonstrated significant BS activity and high BS production, yielding 562 mg L^{-1}. The potential isolate showing high BS activity and production was identified as Bacillus vallismortis RMS25 by 16S rRNA sequencing.

Conflict of interest

The authors declare that they have no conflict of interest for this study.

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