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Screening and characterization of biosurfactant producing novel marine bacteria *Bacillus tequilensis* KM 15 and its application for plant growth promotion

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Abstract

Biosurfactants are a class of surface-active secondary metabolites produced by a wide range of microorganisms. The current study is aimed at isolating bacteria from different marine water samples, screening for biosurfactant activity, characterizing them, and investigating traits for plant growth promotion. Fifty morphologically distinct bacteria were isolated from various marine water samples using the enrichment culture method. Ten isolates were chosen and further screened for plant probiotic properties such as indole acetic acid production, biofilm formation, and antifungal activities based on biosurfactant activity [penetration assay, microplate assay, oil-displacement method, emulsification assay (EA), and emulsification index (EI%)]. Among the ten isolates tested, Bacillus sp. KM15 demonstrated good EI (68.7) percent and EA (210), as well as high indole acetic acid production, biofilm formation, and antifungal activity (75%). The bacterial isolate KM15 has shown high biosurfactant production, yielding 340 mg L^{-1}. With the aid of 16S rRNA gene sequencing, isolate KM15 was identified as *Bacillus tequilensis.*

Keywords

Biosurfactants, Secondary metabolites, Plant growth-promoting traits, Emulsification index, Emulsification assay, Antifungal activity

Introduction

Increasing agricultural production is a major concern in meeting the growing requirements of the world's population. Given the proven toxicity of synthetic fertilizers and pesticides, the application of green compounds is now required for sustainable agriculture. Biosurfactants are produced by numerous plant-associated and rhizosphere microbes [1]. These biomolecules play a key role in quorum sensing and thereby, govern plant-microbe interactions among rhizosphere bacteria. Biosurfactants can be used in agriculture to kill plant pathogens, increase nutrient bioavailability, and promote bioremediation, all of which improve soil quality [2]. 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 biosurfactant production and plant growth promotion [3].

Biosurfactants are a class of surface-active, structurally diverse secondary metabolites that are produced by many microorganisms. These are amphiphilic molecules that accumulate between fluid phases and reduce surface tension at the surface and interface [4,5,6]. Biosurfactants have higher efficiency and stability, lower toxicity, and improved environmental compatibility at high salinity, pH, and temperatures; can be synthesized from renewable feedstock [7,8] and are highly biodegradable

compared to chemical surfactants. Biosurfactants have the scope for commercial production to meet the demand for applications in food, cosmetics, pharmaceutical industries, and agriculture [9, 10]. Various microbial biosurfactants 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 biosurfactants have the potential to be widely used in agriculture to improve pollutant biodegradation, thereby improving agricultural soil quality which indirectly affects plant growth and plant-microbe interaction [11, 12].

Numerous reports are available on the screening of biosurfactant-producing bacteria using crude oil-contaminated soils and aqueous environments [13]. Rhizosphere microbes produce biosurfactants, implying that they could play a key role in plant-microbe interactions and facilitate plant cultivation in terms of impeding plant diseases [14]. Many biosurfactant-producing strains from *Bacillus* and *Pseudomonas* species, for example, were successful in combating a variety of plant diseases [15,16]. In this sense, the current work is focused on: (a) isolation of bacterial strains from various marine water samples (b) screening bacterial isolates for biosurfactant production using different standard methods, (c) Identifying the best biosurfactant producer and test for plant probiotic properties and biosurfactant production (yield) using different media and Isolate showing good plant growth-promoting properties and high biosurfactant production will be further identified using 16S rRNA sequencing.

Materials and methods :

Enrichment technique for isolating biosurfactant-producing bacteria

Marine water samples were collected from the Nellore mangrove area, Krishnapatnam port, and Kandikuppam mangroves in Andhrapradesh. The enrichment culturing was carried out according to Dubey and Juwarkar [17]. For enrichment, a marine water sample (10 mL) was added to a mineral salts medium (90 mL), which was then amended with kerosene (1 mL, 5 mL, or 10 mL) and incubated for 72 hours at 37°C, 180 rpm. Enriched samples were serially diluted, spread plated on nutrient agar medium, and incubated for 24-48 hrs at 37°C. Morphologically different bacterial isolates were picked and sub-cultured on Zobell marine agar medium (Hi-media). The selected isolates were first gram stained and tested for spore formation. Pure cultures were preserved at -70 °C as glycerol stocks for further studies.

Bacterial screening for biosurfactant production :

Fifty isolates were selected and evaluated for biosurfactant activity using the following methods: penetration assay, microplate assay, oil-spread method, emulsification assays (EA), and emulsification index (EI) [18].

Microplate assay:

The microplate method for the determination of biosurfactant activity detects the change caused by surfactant in optical distortion in an aqueous medium [19]. In the microplate, 100 µL culture supernatant of each bacterial isolate was added in separate wells and kept undisturbed over a graph paper, with wells oriented over the grid of 1 mm^2. Biosurfactant activity is confirmed by grid image distortion, which is then compared with positive and negative controls.

Penetration assay :

The penetration test is based on the interaction of two insoluble phases that results in color change [16]. To all 96 wells of a microplate, hydrophobic paste (150 µL) made of silica gel and engine oil was added. Then it was covered with oil (10 µL), cell-free supernatant (90 µL), and safranin (10 µL), observed for color change and thereby biosurfactant activity.

Oil displacement method :

The oil spreading experiment was carried out by Plaza et al. [20]. In this test, a Petri plate with distilled water (30 mL) was taken and engine oil (1 mL) was added in the center. 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 biosurfactant activity, the oil-free clearing zone displaces oil, also known as oil displacement activity. Distilled water (without surfactant) and Triton X-100 were used as negative and positive controls.

Emulsification index (EI%) :

Based on microplate, penetration assay, and oil displacement method results, ten isolates were chosen for further investigation. An equal volume of culture supernatant (2 mL) and kerosene (2 mL), was vigorously mixed for 10–15 min and left undisturbed for 24 hrs. The emulsification index was calculated as per the following formula (1).

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

Emulsification assay :

Emulsification assay was carried out according to the method described by Satpute et al. [21], with minor modifications. To 3 mL of culture supernatant, 0.5 mL of engine oil was added, vortexed for 2 minutes, and left undisturbed 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.

Screening for plant growth-promoting traits :

Plant probiotic traits such as the production of indole acetic acid, biofilm formation, and antifungal activity were tested for the top ten best isolates showing biosurfactant activity.

Indole acetic acid production :

Individual bacterial isolates were inoculated in a grid pattern on LB agar plates (Luria Bertani's media) with 5 mM L-tryptophan to screen for IAA production using sterile toothpicks. A 45 mm diameter nitrocellulose membrane disc was overlaid on top of the agar plates and incubated until the colonies grew to a diameter of 2 mm. The nitrocellulose membrane was withdrawn from the plates and placed on Whatman No.1 filter paper soaked in salkowski reagent (perchloric acid 50 mL, 0.5 M ferric chloride 1.0 mL). A distinct red halo on the membrane in the vicinity of the colony is marked as IAA positive [22].

Biofilm Quantification :

LB media was inoculated with 24 h bacterial culture (1 %), vortexed well, and incubated for 15 days at 28 °C. After incubation, the broth was removed from the tubes and the tubes were washed with saline (0.85 % NaCl) for quantitative analysis of biofilm production. After washing, the tubes were air-dried, and the cells attached to the tubes were stained with one percent crystal violet solution and incubated for 45 minutes. Following the incubation period, the crystal violet solution was removed and the tubes were rinsed with water. Biofilm was visible at this point as purple rings formed on the sides of each tube. Dissolving dye in 95% ethanol and measuring absorbance at 600nm with a spectrophotometer (Shimadzu, Japan) yielded the biomass of attached cells (biofilm) [23].

Antifungal activity :

To assess antifungal activity, the dual-culture method was used. *Sclerotium rolfsii*, a soil-borne plant pathogenic fungus, was grown on PDA. A 96-hour-old fungal culture agar block of five-millimeter-diameter was picked and placed on the surface of a fresh agar medium. A loopful of each bacterial isolate (24 holds) was inoculated as a straight line on one edge of a 90 mm diameter Petri plate, incubated at 30 ± 2 °C, and the zone of inhibition was measured after 5 days of inoculation. As a negative control, media plates inoculated with the same fungus and without inoculating bacteria were used. The experiment was performed in three replications and the following formula was used to calculate the reduction in radial growth as well as the percent inhibition over control. (2) [2].

$$I\% = \frac{(C-T)}{C} \times 100$$

Where I = Inhibition% of mycelial growth (growth reduction over control).

C = Radial growth of fungus in the control plate (mm).

T = Radial growth of fungus on the plate inoculated with bacteria (mm).

Production of biosurfactant :

Ten biosurfactant-producing isolates were tested in two different media: NB broth and Zobell medium (Hi-media). In a separate set of experiments, isolates were inoculated in 100 mL media and incubated at 37 °C, 180 rpm for 48 hours. The experiment was done with two replications. After 48 hours, the culture supernatant was collected by centrifugation (10,000 rpm), precipitated by

adjusting the pH to 2.0 with 6 N HCl [17], and incubated overnight at 4 ºC. The precipitates were extracted twice with methanol after centrifugation (12,000g for 20 minutes) [18].

Molecular identification of KM15 using 16S rRNA gene sequence:

Molecular identification of Bacillus spp. isolate KM15 was done by sequencing using universal 16S rRNA primers (MACROGEN, Seoul, Korea). Mega-4 bioinformatics software was used for phylogenetic analysis [18].

Statistical analysis :

Unless otherwise specified, all experiments were conducted in triplicate, and all results are the mean of three independent experiments that produced consistent results. The Microsoft Office Excel 2003 software package was used to compute the ANOVA, means, CV percent, ranking, and standard errors (version 7).

Results and Discussion :

Enrichment culturing was used to select biosurfactant producers from marine water samples collected from various mangrove areas, yielding 50 morphologically distinct bacterial isolates. Based on Gram's nature and sporulation, all isolates were identified as Bacillus spp. and chosen for further research. All isolates were assessed for qualitative analysis of biosurfactant activity using microplate assay and penetration assay.

The alteration in optical irregularity is the principle of microplate assay; surfactant-containing fluid surfaces are concave and irregular in shape, whereas pure water has a flat surface in a hydrophobic well. In the penetration assay, biosurfactant activity was stipulated by an alteration in color from transparent red to cloudy white in 15 minutes. Ten isolates were chosen for further studies based on the results of penetration and microplate assays (Table-1).

Source	Isolate number
Krishnapatnam Port	KP3
Krishnapatnam Port	KP4
Nellore port	NP3
Nellore port	NP10
Nellore beach	NB6
Nellore beach	NB12
Kandikuppam mangroves	KM2
Kandikuppam mangroves	KM8
Kandikuppam mangroves	KM 10

Kandikuppam mangroves	KM15

Table-1. List of isolates showing positive results for biosurfactant production

The oil displacement assay detects biosurfactants in a more sensitive and time-efficient manner. A linear relationship exists between the amount of surfactant and the diameter of the zone of clearance. This method is quick and simple to implement, does not require any special equipment, and requiring only a small volume of samples. All ten isolates exhibited a clear zone with crude oil (Figure-1).

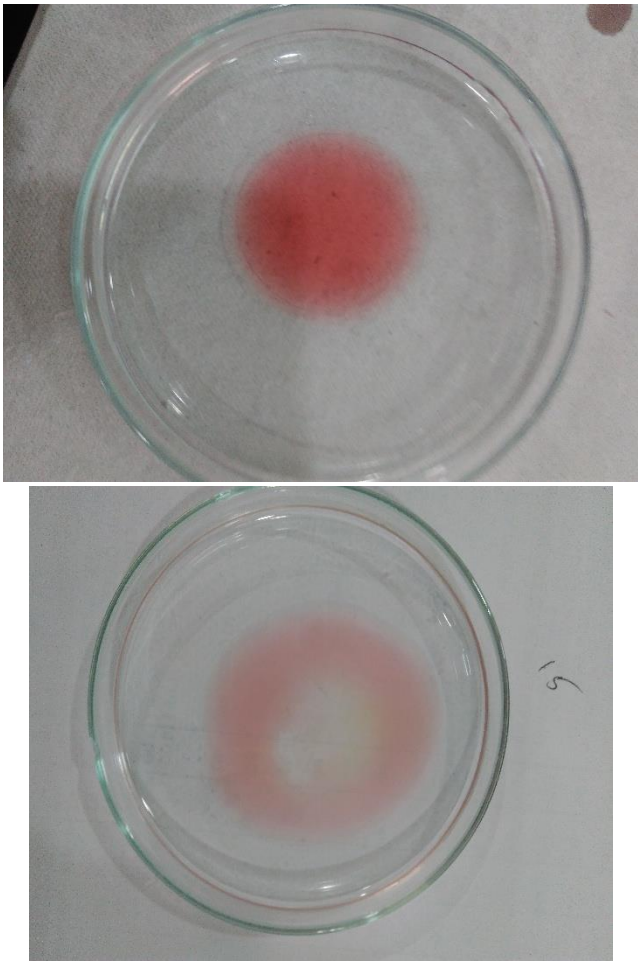

Figure-1. Oil displacement activity showing clear zone

In the current study emulsification assay and emulsification index were performed with kerosene Emulsification assays and emulsification index methods, according to Satpute et al. [21] and Das et al. [24], are critical for screening for potential biosurfactant producers. A study on the marine bacterium *Bacillus subtilis* MTCC 2422 revealed an emulsification index of 68 with kerosene and an emulsification index of 291.4 (EU/mL) with petrol. Among ten isolates, isolate Bacillus sp KM15

showed a high EI percentage (EI%) 68.7 on kerosene, followed by isolates NB6 and KM10, which showed 62.5%. (Table 2). Bacillus sp. KM15 demonstrated high emulsification activity [EA units (Eu)/mL] 210.0 on kerosene, followed by isolates NB6 and KM10, which showed 208.4 and 206.3 (Table 3).

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

Isolate	**Kerosene**	
	EH	EI%
KP3	1.6	50.0(±1.09)^{d}
KP4	1.4	43.7(±1.13)^{e}
NP3	1.9	59.3(±0.09)^{b}
NP10	1.6	50.0(±0.67)^{d}
NB6	**2.0**	**62.5**(±0.61)^{c}
NB12	1.6	50.0(±1.31)^{d}
KM2	1.4	43.7(±1.28)^{e}
KM8	1.4	43.7(±0.57)^{e}
KM 10	**2.0**	**62.5**(±1.34)^{c}
KM15	**2.2**	**68.7**(±0.01)^{a}

Values super scribed by a-e are ranking highest to lowest of significant, the same alphabet is insignificant according to Fischer's least significance difference test ($p<0.05$). Values in the brackets are standard error, values in the column are mean two independent experiments of 4 replications

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

Isolate	**Engine oil**	
	Optical Density	Emulsification Activity
KP3	1.519	151.9(±0.08)^{g}
KP4	1.649	164.9(±0.07)^{f}
NP3	2.012	201.2(±0.07)^{d}
NP10	1.390	139.0(±0.10)^{i}
NB6	2.084	208.4(±0.07)^{b}
NB12	1.442	144.2(±0.33)^{h}
KM2	1.921	192.1(±0.08)^{e}

KM8	1.637	163.7(±0.07)^{f}
KM 10	2.063	206.3(±0.11)^{c}
KM15	2.100	210.0(±0.07)^{a}

Values superscribed by a-i are ranking highest to lowest of significant, the same alphabet is insignificant according to Fischer's least significance difference test ($p<0.05$). Values in the brackets are standard error, values in the column are mean two independent experiments of 4 replications

Plant probiotic trait screening :

IAA is a primary auxin in plants, regulating cell growth and division, and many physiological activities[25]. Furthermore, IAA production by plant-associated microorganisms has been reported [26. 27]. Plant enhancement has been demonstrated using pure cultures that produce IAA. Only a few studies in marine environments have been conducted [28, 29]. All of these studies demonstrate the pervasiveness of bacteria and their importance in promoting plant growth and associated biota. Furthermore, microorganisms, particularly those found in the soil rhizosphere or as free-living soil bacteria, produce IAA. In the present study, among the ten bacterial isolates tested for IAA production, five isolates KP4, NB6, NB12, KM10, and KM15 showed a positive result.

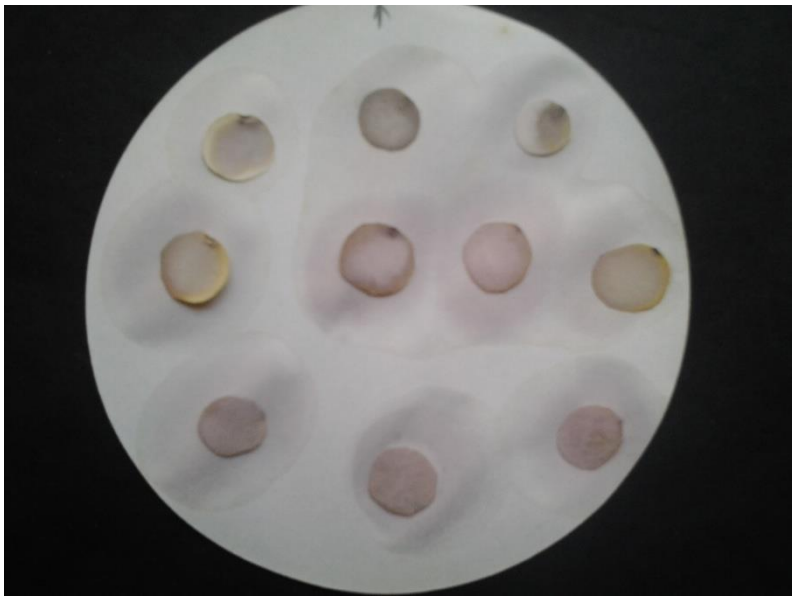

Figure-2. Detection of Indole acetic acid production

Biofilms are multicellular microbial mats with rhizosphere-like structural and functional architecture that influence nutrient response, metabolic processes, and other factors. IAA, a chemical produced by bacteria kindred with biofilms, was identified as an elicitor in microbial quorum sensing [30. 31]. The production of IAA by bacterial isolates suggests that IAA is responsible for sustaining the biofilm

by providing free ammonia, augmenting the availability of micronutrients[32]. In the present study, among the ten isolates tested, isolate KM15 showed high biofilm formation, followed by KM10 and NB6 (Figure-3).

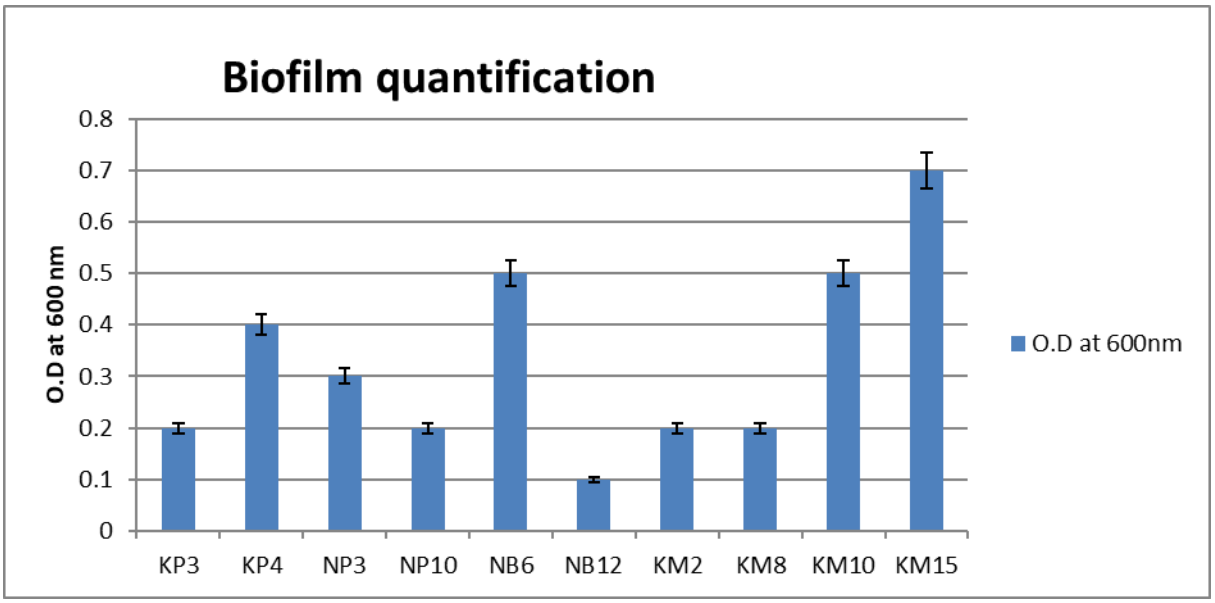

Figure-3 Evaluation of biofilm formation

Antifungal activity against *Sclerotium rolfsii* in vitro :

The results showed that the bacterial isolate KM15 had a noteworthy effect on the mycelial growth of *Sclerotium rolfsii* among isolates tested, followed by KM10 and NB6 could inhibit the mycelial growth of *Sclerotium rolfsii* after 5 days of incubation (Figure-4). According to previous research by Narendrakumar et al.[2], biosurfactant antifungal activity is mediated by its membrane permeabilization properties, and it has been shown to have potent antifungal activity against a wide range of yeasts and fungi. *B. amylolquefaciens* produced iturin A inhibited phytopathogenic fungi [18].

Isolate NB6

Isolate KM10 Isolate KM15

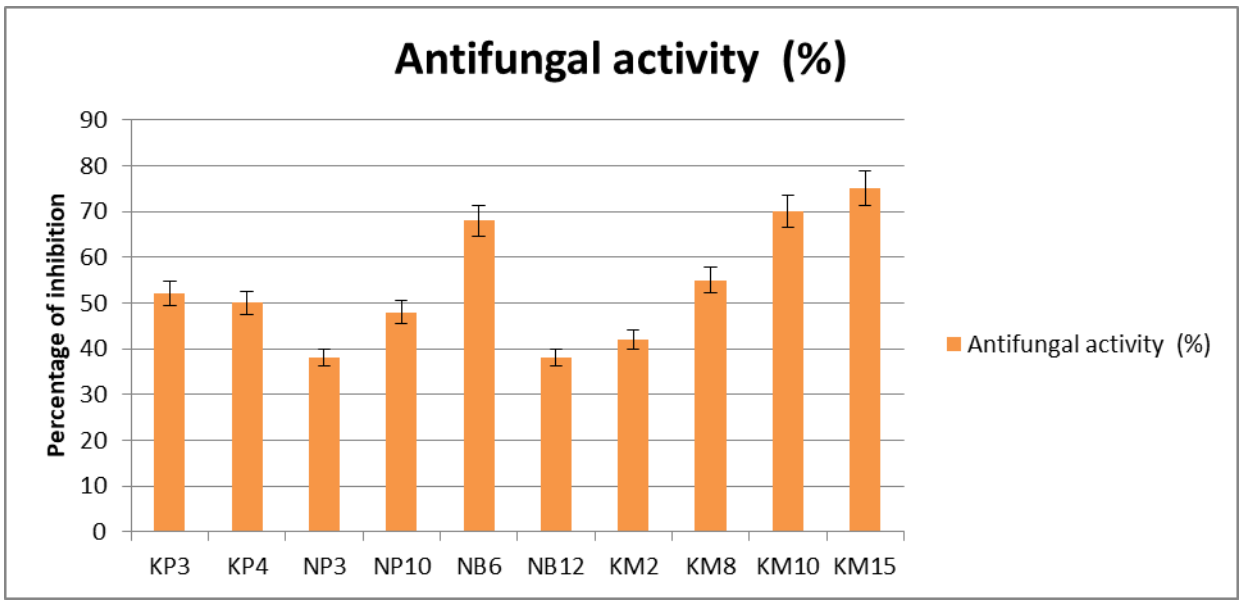

Figure-4. Evaluation of antifungal activity

Biosurfactant production :

Biosurfactant production by ten isolates was studied in nutrient broth and Zobell medium. Among the ten bacterial isolates tested for biosurfactant production, the bacterial isolate KM15 exhibited a high biosurfactant yield of 340 mg L^{-1} in Zobell medium (Table-4). It was observed that, for all isolates tested, the Zobell medium has supported high biosurfactant production compared to the nutrient broth.

Table-4. Biosurfactant production by the selected bacterial isolates

Isolates	Biosurfactant production	
	NB media (mg l^{-1})	Zobell media (mg l^{-1})
KP3	100± (1.70)^{i}	140± (1.06)^{g}
KP4	180± (1.08)^{e}	185± (1.08)^{e}
NP3	135±(1.06) ^{h}	150± (1.02)^{f}
NP10	160±(1.06)^{f}	175± (1.08)^{e}
NB6	250± (1.08)^{c}	275± (1.08)^{c}
NB12	200± (1.70)^{d}	210± (1.08)^{d}
KM2	185± (1.03)^{e}	200 ± (1.08)^{d}
KM8	150± (1.63)^{g}	170± (0.89)^{e}
KM 10	280± (1.03)^{b}	300± (0.64)^{b}
KM15	320± (1.08)^{a}	340± (0.86)^{a}

Values superscribed by a-i are ranking highest to lowest of significant, the same alphabet is insignificant according to Fischer's least significance difference test ($p<0.05$). Values in the brackets are standard error, values in the column are mean two independent experiments of 4 replications

Based on biosurfactant production [33, 34, 35], plant growth-promoting activity, and antifungal activity, bacterial isolate KM15 was chosen for further research. It was identified as rod-shaped, motile, and was Gram-positive. The 16S rRNA gene sequence results were drawn from the EzTaxon server BLAST search, and isolate KM15 was identified as *Bacillus tequilensis* (Fig. 5) and submitted into NCBI accession number MW255676.

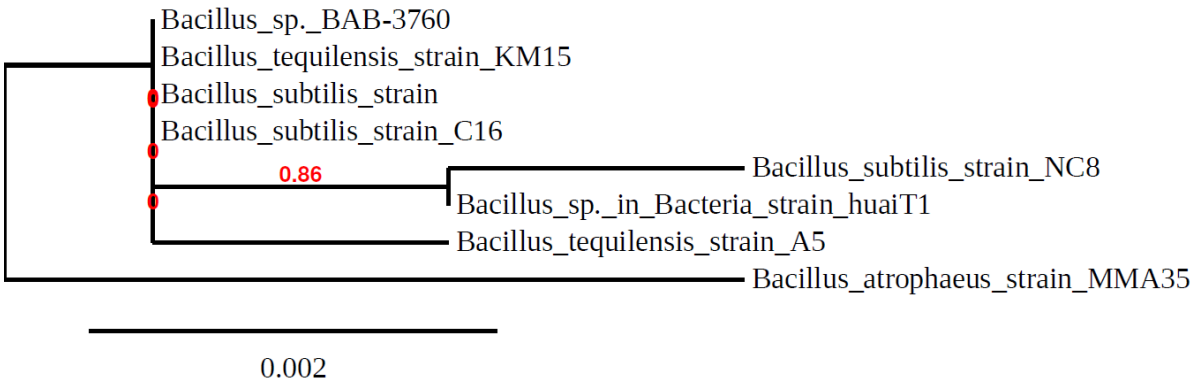

Figure-5. Identification of Bacillus sp. KM15 using 16S rRNA sequencing and construction of the phylogenetic tree.

Conclusion :

The current study used various screening methods to screen 50 bacterial isolates from marine water samples for biosurfactant activity. One Bacillus spp. isolated from water samples of Kandikuppam mangroves, identified as *Bacillus tequilensis* KM15, demonstrated significant biosurfactant activity and plant probiotic traits. Isolate KM15 has shown high biosurfactant production, yielding 340 mg L^{-1}.

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Conflict of interest :

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

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