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Exploring Endophytic Bacteria in Weed and Wild Plants as a New Frontier for Biosurfactant Production

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ABSTRACT:
Endophytic bacteria, residing within plant tissues without causing harm, have emerged as a promising source of biosurfactants due to their ability to produce surface-active compounds with diverse applications. This study explores the biosurfactant-producing potential of bacterial endophytes isolated from wild and weed plants. The isolates were screened for biosurfactant production using qualitative assays Oil spreading/Displacement test, Drop collapse test, Cetyl trimethyl ammonium bromide (CTAB) agar method, Lipolytic activity, Chemical characterization of type of bio-surfactant, and Emulsification assay techniques E24%. This research underscores the untapped potential of endophytic bacteria as sustainable sources of biosurfactants, paving the way for eco-friendly solutions in various biotechnological fields.

KEYWORDS: Bacterial endophytes, Wild and weed plant, Biosurfactant production.

INTRODUCTION:

Surfactants are amphiphilic molecules composed of hydrophilic and hydrophobic moieties that accumulate at the surface or interface of the two immiscible fluids. Through the formation of aggregate structures such as micelles, they reduce surface/interfacial tensions. Majority of the surfactants today, are chemically produced from petrochemical feedstock and applied in a wide variety of industrial processes for their abilities like detergency, foaming, wetting, emulsification, dispersing, and solubilization [1, 2].

A biosurfactant is a surface-active compound produced by microorganisms such as bacteria, fungi, or yeast. These are the amphiphilic compounds with the ability to accumulate between fluid phases and are produced on microbial cell surfaces or can be secreted extracellularly [3]. Biosurfactants, also called as green surfactants are surface active compounds produced by microbes. These compounds are biodegradable and non-toxic, thus they do not accumulate in the environment [4]. Biosurfactants enhance the bioavailability of hydrophobic organic compounds, which makes them a good agent for cleaning up the environment. The hydrophilic moiety of the bio surfactants can be a carbohydrate, an amino acid, a phosphate group, or alike compounds whereas the hydrophobic moiety is mostly the fatty acid carbon chain. This property helps reducing the interfacial and surface tensions, making them potential candidates for enhancing oil recovery [5]. A number of microorganisms have been stated to produce a number of classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants.

Due to the interesting properties of biosurfactant such as lower toxicity, higher degree of biodegradability, higher foaming capacity and optimal activity at extreme conditions of temperatures, pH levels and salinity, they have been increasingly attracting the attention of the scientific and industrial community [6]. The application of bio-surfactant is reviewed elsewhere [7, 8].

In the soil ecosystem, plant-associated microbes play a central role, working as natural partners that facilitate local and systemic mechanisms in plants to defend against adverse environmental conditions. Usually, several microbes are found to acquire nutrients from various plants, where some interactions are beneficial and some are harmful to the host. Endophytes are microorganisms that inhabit plants. Endophytes are known to promote the growth of host plants in several ways, including defence against pathogens, nutrient solubilization and production of plant growth-promoting hormones [9].

Wild plant can be exists in various ecosystem as they have characteristics like adaptation, high tolerance to biotic and abiotic stresses as well as more sustainable and resilient to climatic changes [10]. Because these plants grow in extreme condition, we hypothesized that the endophytes inhabiting in this plant makes the enormous plant growth in such conditions. The present investigation was planned to explore the potential of two selected weed plants namely *Wedelia urticifolia* (terrestrial weed) and *Lantana camara* (terrestrial weed) in a way that can have positive attributes outweigh the negative ones.

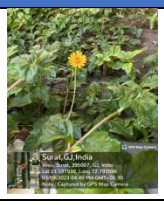
However, many of the former reports pertain to the abilities of endophytic fungi to perform biodegradation, while only a few describe endophytic bacteria and their biosurfactant production ability. We believe that this is the first report on biosurfactant production by endophytes isolated from wild and weed plant. That is why, the purpose of the study was to investigate the potential of culturable endophytic microorganisms isolated from selected wild and weed plant plants for the production of biosurfactants, as indirect plant promoting factors.

MATERIALS AND METHODS:

Collection of plant sample:

Plants of *Wedelia urticifolia* and *Lantana camara* were collected at the flowering stage during the summer season (April- May) from a farm in Surat, Gujarat (Figure 1). Its identity was authenticated by expert. The plant materials were brought to the laboratory and washed carefully under running tap water to remove any adhering dirt and debris [11].

TABLE 1: List of selected plants with their geographic location and images

SR NO.	PLANT NAME	LOCATION	IMAGE
1.	*Wedelia urticifolia*	Lat: 21.137939 Long: 72.793596	
2.	*Lantana camara*	Lat 21.161553 Long 72.777154	

Isolation of endophytes:

The roots were separated, cut into small pieces of about 2-3 cm in length, disinfected in 70% ethanol for about 30 seconds and then surface sterilized with 0.1% $HgCl_2$ for 3 minutes. The tissue was then repeatedly rinsed about 10-12 times with sterile distilled water. To check the efficacy of the process of surface sterilization method, sterility test was performed. After the final wash, aliquots of water (0.1 ml) diluted with nutrient broth were transferred to a nutrient agar plate (NA) incubated at 28°C ± 2°C for 48 hours. No microbial growth after 48 hours of incubation ensure the effectiveness of the surface sterilization process and subsequent isolation procedures were carried out. The root tissues were aseptically macerated with homogenizers, serially diluted and plated in triplicate on Nutrient agar (NA) plates [11]. Isolates were selected on the basis of variation in morphology, purified and maintained on NA slants and preserved at 4°C for further investigations [12].

Morphological and biochemical characterization:

The isolates were characterized morphologically and biochemically. Morphological characterization includes Gram staining, appearance of colour, elevation, size, shape, surface, margin, pigmentation and opacity. Biochemical characterization was conducted by the biochemical tests like Utilizations of carbohydrates and organic acids using Methyl-Red (M-R) test, Voges-Proskauer (V-P) test, Citrate utilization test, Urea hydrolysis test, Nitrate reduction test, Catalase test, Triple sugar iron agar test was carried out [13].

Screening methods for biosurfactant production:

Oil spreading/Displacement test: In the oil spreading technique developed by [14], a volume of 30 ml of distilled water was introduced into a Petri dish, followed by the addition of 1 ml of motor oil at the centre. A volume of 20 microliters of the culture supernatant obtained from the bacterial broth was placed on the surface of the oil layer. The Petri dishes were carefully examined for any area where the oil had moved, and the size of the moved area was measured.

Drop collapse test: To evaluate the capacity of the biosurfactant to reduce the surface tension between water and hydrophobic surfaces, a droplet of water was subjected to a collapsing test. A droplet of 25 µl of extracted biosurfactant was placed onto parafilm. The process of the droplet flattening and spreading on the surface of the parafilm was observed for a duration of seconds or minutes. Afterwards, methylene blue (which did not affect the shape of the droplets) was introduced to the water stain and let to dry. The diameter of the dried droplet was then measured and recorded [14].

Cetyl trimethyl ammonium bromide (CTAB) agar plate method: This method can detect the production of anionic biosurfactant, rhamnolipids specifically. The medium used for this purpose is an agar based on mineral salts, a low solubility carbon source (glycerol, vegetable oil, and hydrocarbon), blue methylene and CTAB. The CTAB is a cationic salt that reacts with the rhamnolipids, this complex (CTAB-rhamnolipids-blue methylene) can be visualized by presence of traslucid halo around the colony growth on the agar plates [14].

Lipolytic activity: The lipase assay medium was prepared and the cell free filtrate of the biosurfactant producer was used as a source of lipase enzyme. Lipolytic activity was detected by clearing zones around the hole in comparison to the turbid background of the assay plates [15].

Chemical characterization of type of bio-surfactant: As glycolipids, lipopeptides, phospholipids are the main three types of biosurfactants, three methods are used to identify the type of biosurfactant [16]

1. Phenol-Sulfuric test to identify for glycolipids,
2. Biuret test to identify for lipopeptides,

3. Phosphate test to identify for phospholipids

Phenol-Sulfuric acid test: 1 ml of cell-free supernatant added in a test tube and 1 ml of 5% phenol added. To this mixture, 2-5 ml of concentrated sulfuric acid was added drop by drop, until orange colour was developed. Development of orange colour indicated the presence of glycolipids.

Biuret test: 2 ml of cell-free supernatant was heated at 70◦C for 10min.10 drops of 1M NaOH solution added to the solution. To this mixture added 1% copper sulphate drop by drop, to observe a violet or pink ring, which indicates the presence of lipopeptides.

Phosphate test: To 2 ml of cell-free supernatant, 10 drops of 6M Nitric acid was added and heated at 70◦ C for 10 5% ammonium molybdate was added drop by drop to mixture until yellow color is formed, and then the formation of yellow precipitate, which indicates the presence of phospholipids.

Emulsification assay techniques E24%: A total of 2 ml of cell-free supernatant was introduced into screw-capped tubes holding an equal volume of distilled water. The solution was combined with 1 ml of either soybean or diesel oils as a substrate. After vigorous vortexing for 2 minutes, the tubes were left undisturbed for 1 hour to allow the separation of the aqueous and oil phases. The absorbance at 540 nm was then measured. The aqueous phase was meticulously extracted and the optical density at 540 nm was measured and compared to the negative control, which consisted of un-inoculated soup. Emulsification activity was quantified by determining the optical density at a wavelength of 540 nm. The experiments were conducted in duplicate. A total of 2 ml of cell-free supernatant and 2 ml of diesel oil were combined in screw cap tubes and vigorously vortexed for 2 minutes to create a combination. The mixes were left to incubate at ambient temperature for a duration of 24 hours. The emulsion index (E24%) was subsequently determined by dividing the height of the emulsion layer by the combined height of the oil, emulsion, and aqueous layers [3]

RESULTS:

Isolation of endophytes: A total of eighteen endophytes were isolated from roots of *Wedelia urticifolia* and *Lantana camara* plants. The isolates obtained were from sample plants from ecologically very diverse habitats. A total of twenty two bacterial isolates were picked up on the basis of their colony characteristics.

TABLE 2: List of all isolated endophytic strains from selected plants

PLANT NAME	ISOLATED STRAIN NAME	TOTAL ISOLATES
Wedelia urticifolia	WUR1, WUR2, WUR3, WUR4, WUR5, WUR6	06
Lantana camara	LCR1, LCR2, LCR3, LCR4, LCR5, LCR6, LCR7, LCR8, LCR9, LCR10, LCR11, LCR12	12
Total		18

Screening methods for biosurfactant production:

Eighteen endophytic strains were screened for biosurfactant production using several methods. The results of these screening tests are shown in Table 3.

TABLE 3: Results of screening methods for biosurfactant producing bacterial endophytes

Strain code	Oil displacement	Drop collapse	CTAB agar	Lypolytic activity	Type of biosurfactant	E24%
WUR1	-	-	-	-	Glycolipid	-
WUR2	-	-	-	-	Glycolipid	36.66
WUR3	-	-	+	+	Phospholipid	25.50
WUR4	1.2 cm	-	-	-	Glycolipid	23.33
WUR5	2.3 cm	+	+	-	Phospholipid	52.45
WUR6	-	-	+	-	Glycolipid	17.50
LCR1	-	-	-	-	Phospholipid	-
LCR2	-	-	-	+	Glycolipid	-
LCR3	2.6 cm	-	-	-	Glycolipid	45.00
LCR4	-	-	-	-	Lipopeptide	-
LCR5	2.8 cm	+	-	+	Glycolipid	52.50
LCR6	-	-	-	-	Glycolipid	21.66
LCR7	3.9 cm	+	+	+	Glycolipid	62.45
LCR8	-	-	-	-	Lipopeptide	-
LCR9	-	-	-	-	Glycolipid	32.50
LCR10	-	-	-	-	Glycolipid	42.85
LCR11	-	-	-	-	Glycolipid	38.50
LCR12	-	-	-	-	Glycolipid	48.25

Oil spreading/Displacement test: The thin oil film obtained by adding used engine oil facilitates visualization of the clear zone displaced upon the addition of cell-free culture supernatant. To evaluate the ability of the tested strains to produce biosurfactants, oil displacement diameter (ODD) was measured. Oil displacement diameters recorded after the addition of cell-free supernatants of strains WUR4, WUR5, LCR3, LCR5 and LCR7 were larger than 1cm. Strain LCR7 had the largest ODD, while strain WUR4 had the lowest, measuring 3.9 cm and 1.2 cm, respectively.

Drop collapse test: Drop collapse assay is a sensitive test, which can give result with a very small amount of surfactant. The biosurfactant droplets do result in a collapsed droplet indicating their effects on reduction of surface tension, in this study the isolate WUR5, LCR5 and LCR7 showed drop collapse than other isolates.

Cetyl trimethyl ammonium bromide (CTAB) agar plate method: CTAB/methylene-blue agar is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactant. If glycolipid biosurfactants are secreted by the microbes growing on the plate, they form dark blue halos. In this study, positive result was obtained by strain WUR3, WUR5, WUR6 and LCR7 when the strain cultivated on a mineral salts plate containing the cationic surfactant cetyl trimethyl ammonium bromide and the basic dye methylene blue.

Lipolytic activity: The results of evaluating lipolytic activity in biosurfactant-producing microorganisms provide valuable insights into their ability to hydrolyse lipid substrates and subsequently utilize them for biosurfactant synthesis. In the present study four strains WUR3, WUR5, WUR6 and LCR7 were found to be positive for lipolytic activity.

Chemical characterization of type of bio-surfactant: Phenol-Sulfuric test, Biuret test and Phosphate test were performed with cell-free supernatant of all isolates to identify the type of biosurfactant produced. The strains WUR1, WUR2, WUR4, WUR6, LCR2, LCR3, LCR5, LCR6, LCR7, LCR9, LCR10, LCR11, and LCR12 strains showed positive results for Phenol-Sulfuric test and thus identify for glycolipids production. The strains WUR3, WUR5, and LCR1 strains showed positive results for Phosphate test and thus identify for phospholipids. The strains LCR4 and LCR8 strains showed positive results for Biuret test and thus identify for lipopeptides production.

Emulsification assay E24%: Emulsification assay is an indirect method used to screen biosurfactant production. The emulsification indexes of the cell-free culture broths demonstrated variations that can be attributed to the different properties of the hydrophobic substrates used. It was presumed that if the cell free culture broth contains biosurfactant then it would emulsify the hydrocarbons present. Here, crude oil was used as the hydrophobic substrate. The E24 of all three organisms are noted in Table 3. Maximum emulsification was observed with strain LCR7 (62.45%) and minimum with WUR6 (17.50%).

DISCUSSION:

The present study describes the isolation of novel biosurfactant producing bacterial endophytic strains from roots of weed plant samples as they provide great resource to discover microorganisms with biotechnological importance [18]. In the last few decades, intense interest has been focused on biosurfactants from microbial origin due to their successful implementations in multi directional areas. It has been known that biosurfactant biosynthesis is coupled with microbial growth when the culture is grow non-water-immiscible substrates. The principle aim of screening new biosurfactant producer endophytic strains from wild and weed plant because wild and weed plants often thrive in harsh, nutrient-limited, or contaminated environments, which may harbour unique microbial communities adapted to such conditions. These bacteria might possess novel biosurfactant-producing capabilities due to selective pressures in these habitats.

Comparison of the screening tests for biosurfactant production indicated that five methods should be considered for evaluation of biosurfactant production [19]. Therefore, five distinct methods including oil displacement assay, drop collapse assay, Cetyl trimethyl ammonium bromide, chemical characterization of type of bio-surfactant and Emulsification assay E24% test were applied in this study.

Among these tests, oil displacement assay is considered the most reliable and sensitive primary screening method [14]. Oil spreading assay results showed corroboration with the drop collapse assay results in the way that the organisms found positive with drop collapse assay were positive for oil spreading assay as well. Previous study [14] explained that the oil displacement area is directly proportional to the surface-active compound in the solution. However, in this study only the qualitative study to check the presence of surfactant was carried out. The results (Table 3) state that Strain LCR7 had the largest ODD, while strain WUR4 had the lowest, measuring 3.9 cm and 1.2 cm, respectively.

The drop collapse method depends on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface. There is a direct relationship between the diameter of the sample and concentration of the biosurfactant and in contrast, the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface that cause aggregation of droplets [14]. In drop collapse assay, no activity was detected for distilled water as predicted. The biosurfactant droplets do result in a collapsed droplet (table 3); indicating their effects on reduction of surface tension, in this study the isolate WUR5, LCR5 and LCR7 drop collapse than other isolates.

The CTAB agar plate method exploits the interaction between biosurfactants and cationic surfactants like CTAB. Biosurfactants, being amphiphilic, can form complexes with CTAB and methylene blue, which results in the formation of insoluble bluish green precipitates. In the present investigation, isolates WUR3, WUR5, WUR6 and LCR7 exhibited the biosurfactant productivity which confirmed the presence of anionic biosurfactant as shown in Table 3 above. It has been declared that, CTAB agar plate method is developed for the detection of extracellular rhamnolipids and other anionic glycolipids [14]. Also, previous study [20] used CTAB technique for screening biosurfactant producing isolates and they isolated eleven different bacteria.

Lipolytic activity is closely associated with biosurfactant production, particularly in microorganisms that metabolize hydrophobic substrates such as oils and fats. Lipases, enzymes responsible for hydrolysing lipids into glycerol and fatty acids, play a significant role in microbial biosurfactant production by providing necessary precursors or intermediates for biosurfactant synthesis. Lipolytic activity plays a pivotal role in the production of biosurfactants, particularly when using hydrophobic substrates. By breaking down lipids into biosurfactant precursors, lipase-producing microorganisms provide an efficient and sustainable means of biosurfactant production [15]. In the present study strain WUR3, WUR5, WUR6 and LCR7 were found to be positive for lipolytic activity.

The chemical characterization of biosurfactants is critical for understanding their structure, classification, and potential industrial applications. Biosurfactants are structurally diverse, and their type determines their functional properties, such as surface activity, emulsification, and antimicrobial action. This discussion outlines key methods used to chemically characterize biosurfactants, focusing on determining the type of biosurfactant produced by microorganisms. Characterizing the type of biosurfactant involves identifying the functional groups, molecular structure, and chemical bonds [16]. In the present study 13 isolates were found to be glycolipid type of biosurfactant producers, 3 isolates were found to be phospholipid type of biosurfactant producers and 2 isolates were found to be Lipopeptide type of biosurfactant producers.

Emulsification assay E24% is an indirect method used to screen biosurfactant production. It was presumed that if the cell free culture broth contains biosurfactant then it would emulsify the hydrocarbons present. Emulsifying activity is an indicator of biosurfactant production since surface-active agents can emulsify hydrocarbons by reducing their surface tension [3, 21]. In terms of environmental perspective, emulsification activity is a desirable feature of a surfactant as the anthropogenic production of petroleum, which contains a wide range of hydrocarbons, results in significant contamination that seriously threatens environmental sustainability [22]. Here, crude oil was used as the hydrophobic substrate. The E24 of all three organisms are noted in Table 3. Maximum emulsification was observed with strain LCR7 (62.45%) and minimum with WUR6 (17.50%).

CONCLUSION:

The above mentioned studies emphasize the potential uses of promising endophytic bacteria LCR7 strain for biosurfactant synthesis. The ability of bacterial isolates, specifically isolate LCR7, to produce significant level of biosurfactant as indicated by multiple screening techniques, suggests their potential for exploitation at commercial level. However, fermentation production of these biodegradable and eco-friendly surfactants is limiting due to their increased cost of production at large scale.

The biosurfactants production to an industrial scale remains challenge, to substitute the synthetic surfactants. Despite their environmental advantages and equal performance, commercialization of these molecules remains a challenge. The latter issue can partially be tackled by screening for the research of

better producers and optimizing the fermentation process. In order to reduce cost of production of biosurfactants, there are several methods to extract them from culture medium, like centrifugation or acid precipitation. For environmental applications of biosurfactants, it requires only biosurfactant crude from a free cell culture. For scientific and patent creation purposes it is necessary to have a complete characterization, therefore, spectroscopic, chromatographic and mass spectrometry methods are accurate powerful tools to elucidate novels biosurfactants.

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