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Nagarathinam Arunkumar
ICAR- Central Institute for Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India

Jainullabudeen Gulsar Banu
ICAR- Central Institute for Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India

Nagarajan Gopalakrishnan
ICAR- Central Institute for Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India

Arkalgud Hiriyannaiah Prakash
ICAR- Central Institute for Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India

Correspondence
Nagarathinam Arunkumar
ICAR- Central Institute for Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India

Isolation, screening and characterization of microbial surfactants producing wax degrading bacteria from cotton mealybugs, *Phenacoccus solenopsis* Tinsley and *Ferrisia virgata* Cockerell (Homoptera: Pseudococcidae)

Nagarathinam Arunkumar, Jainullabudeen Gulsar Banu, Nagarajan Gopalakrishnan and Arkalgud Hiriyannaiah Prakash

Abstract
The physiological role of microbial surfactants production in microorganisms includes antimicrobial activity and break down of complex hydrocarbon substances to make the substrates readily available for uptake in adverse environmental conditions. Wax degrading bacteria are a significant group of microorganisms facilitated mainly by the production of microbial surfactants to emulsify waxy substances. Bacterial strains isolated from cotton mealybugs were screened for biosurfactant production. Eight strains (PSAD 1, PSAD 2, PSAD 3, PSAD 5, PSAD 6, PSAD 7, PSAD 8 and PSAD 9) isolated from the carcass of mealybug species, *Phenacoccus solenopsis* (Tinsley) and *Ferrisia virgata* (Cockerell) showed positive results. Among all the isolates, PSAD 2 and PSAD 7 showed maximum biosurfactant potential of 142.14 mm^2 and 115.93 mm^2, respectively. Based on morphological and biochemical profiles, the isolates were tentatively identified belonging to the genera *Bacillus*, *Enterobacter*, *Pseudoxanthomonas, Pseudomonas* and *Serratia*. The results clearly confirmed the ability of the WDB isolates to utilize wax substrates through microbial surfactants production.

Keywords: Wax degrading bacteria, cotton, mealybugs, biosurfactant, characterization

1. Introduction
Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When an insoluble substrate like wax is given as carbon source, bacteria facilitate their diffusion into the cell by producing a variety of enzymes and microbial surfactants (biosurfactants) [1]. These microorganisms are ubiquitous in nature and recognized widely as wax degrading bacteria (WDB). Microbial surfactants are heterogeneous surface active amphiphilic molecules that accumulate at the boundary between two immiscible fluids or between a fluid and a solid. By reducing surface (liquid-air) and interfacial (liquid-liquid) tension, they reduce the repulsive forces between two different phases and allow them to mix and thus enhance the solubility properties like chemical surfactant [2]. Based on the chemical composition and microbial origin, microbial surfactants can be classified as mycolic acid, glycolipids, polysaccharide-lipid complex, lipoprotein or lipopeptide. Among them, phospholipid and particularly lipopeptide-based biosurfactants have potent pharmacological activities [3].
The application aspect of WDB started with the scientific investigation of Roper (2004) [4] who reported actinomycetes belonging to *Rhodococcus* spp. and *Mycobacterium* sp. successful in degradation of wax in soil by surfactants produced and reducing soil water repellency. Recently, application of WDB in sustainable agriculture production through decomposition of waxy rich agricultural residues, amelioration of water repellent soils and biocontrol of wax coated insect pests is gaining importance [5]. Microorganisms belonging to the genera *Pseudomonas*, *Rhodococcus*, *Flavobacterium*, *Micrococcus*, *Alcaligenes*, *Aspergillus* and *Penicillium* capable of degrading paraffin wax were isolated from hydrocarbon rich environments [6]. Nitschke *et al*, (2004) isolated five biosurfactant producing isolates belonging to *Bacillus* sp. (LB5a, LB2a, LB262, LBB and LB1) from agro industrial wastes [7]. Previously, biosurfactant producing WDB were isolated mainly from oil and industrial waste

contaminated sites, but this study focuses on isolation of WDB associated with the various species of mealybugs. Bacteria such as *Erwinia persicinus*, *Pseudomonas plecoglossicida*, *Pseudomonas putida*, *Brevibacterium casei*, *Staphylococcus gallinarum*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Exiquobacterium acetylicum*, *Exiguobacterium undae*, and *Micrococcus caseolyticus* isolated from the sweet potato whitefly, *Bemesia tabaci* Genn. confirm the diversity of microorganisms associated with insect pests [8] and there is a possibility of utilizing these native bacteria for the control of the same. Salunkhe *et al*. (2013) with the aim to develop a biocontrol tool for mealybug management, isolated three novel wax degrading isolates of *Serratia marcescens*, *Pseudomonas aeruginosa* and *Bacillus subtilis* from the carcass of pink mealybug, *Maconellicoccus hirsutus* [9]. Hence, the research work was framed with the objective to isolate and characterize microbial surfactant producing WDB associated with various species of mealybugs infesting cotton for their possible applications in the future.

2. Materials and methods

2.1 Sample collection

The study was carried out at ICAR-CICR, Regional station, Coimbatore, Tamil Nadu, India during the year 2016. Adult female mealybug samples of *Phenacoccus solenopsis* Tinsley and *Ferrisia virgata* Cockerell, *Paracoccus marginatus* Williams and *Drosicha mangiferae* Green were collected from ICAR-CICR, Regional station, Coimbatore, Tamil Nadu, India and Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The sampling data sheet was filled duly and the mealybug samples were transported to the laboratory in sealed polythene bags. Live insects were anesthetized using CO_2 and subsequently killed by freezing on dry ice for one minute.

2.2 Mealybug wax extraction procedure

Cuticular wax of the mealybugs was extracted by soaking the thawed insects in hexane for 60 seconds. The volume of hexane used for the extraction and elution varied among species, depending on the amount of hexane required to completely submerge the insects. A total of 1500 mealybugs of each species were used in each replicated extraction in order to obtain enough wax for subsequent analysis. The dried extracts were eluted with hexane for 40 min and filtered through cellulose acetate membrane syringe filter (Diameter: 28 mm, Pore Size: 0.2 µm). The materials retained on syringe filter were discarded and filtrates were evaporated to dryness. The white substance consisting of solid wax obtained after drying is taken for the preparation of isolation medium.

2.3 Screening and isolation of wax degrading bacteria

Screening and isolation of wax degrading bacteria was carried out using Davis minimal agar following the standard method with slight modifications [9]. The collected live and dead insect cadavers of the four different mealybug species were immersed in 0.8 % sterile saline solution in 50 ml centrifuge tubes and vortexed for 5 minutes. One milliliter of each suspension was plated onto sterile modified Davis minimal agar containing (g l^{-1}) 2.0 extracted wax of the mealybugs, 1.0 ammonium sulphate, 0.7 dipotassium phosphate, 0.1 magnesium sulphate and 1.8 agar. The microbial colonies producing clear haloes after incubation at 32^{O}C for 48 h were selected for further investigation. The wax degrading ability of the bacterial isolates was further confirmed by streaking on rhodamine B medium containing mealywax as carbon source.

2.4 Screening methods for microbial surfactant production

2.4.1 Drop-collapse test

Wells of microtitre plate were thinly coated with olive oil. Five microlitre bacterial culture grown in nutrient broth at 28ºC under 200 rpm for 24 hrs were added individually to the centre of the microtitre plate well [10]. The biosurfactant producers were detected from the drop collapsing within a minute from the oil coated well. Total of three replications were maintained for each isolate and sterile distilled water was used as a negative control.

2.4.2 Oil-spreading assay

The principle of this method was based on the ability of the microbial surfactant to alter the contact angle at the interface between oil and water. The activity is due to the surface pressure of the biosurfactant to displace the oil. In this technique, olive oil was layered over water in a petri plate and a drop of cell-free extract was added to the surface of oil [11]. The diameter of the clear zone on the oil surface was measured in three replications for each isolate and a sterile water drop was used as negative control.

2.5 Identification and characterization of microbial surfactant positive strains

Positive microbial surfactant WDB strains were grown on nutrient agar plates and the morphological data regarding the form (circular, filamentous, or irregular), elevation (flat, convex, or umbonate), margin, (entire, undulate, erose, or filamentous), and optical features (opaque, translucent, or transparent) of the colonies were recorded. Cells were observed under a microscope (oil immersion, 100×) for gram reaction and spore production [12]. Different biochemical tests *viz*., Indole, Voges Proskauer, citrate utilization tests, catalase test, starch hydrolysis, urease test and nitrate utilization were carried out using KB002 HiAssorted^{TM} rapid biochemical kit (HiMedia, Mumbai, India). Based on the results, the selected isolates were tentatively identified according to Bergey's manual of systematic bacteriology [13].

3. Results and discussion

The present study was aimed at the exploration and investigation of biosurfactant producing WDB associated with mealybugs infesting cotton. A total of 17 wax degrading bacterial isolates (encoded as PSAD 1 to 17) were obtained from the carcass of adult female mealybug samples and none was retrieved from the live specimens (Table 1). Bacteria with different colony morphology that formed hallow zone on Davis minimal agar (enriched with mealy wax) were purified and taken for further studies. Likewise, Elisa *et al*. (2006) observed wax degrading bacterial isolates from hydrophobic soils of Tiergarten Park in Berlin through enrichment with bees wax mineral salt medium [14]. Occurrence of hydrolases producing WDB from carcass of pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) by using Davis minimal agar media was reported earlier [9].

Table 1: Isolation of wax degrading bacterial isolates from modified Davis minimal agarmedium enriched with mealybug wax

S. No	Insect	Wax degrading bacterial isolates	Population in cfu / gram of mealybug sample
1.	*Phenacoccus solenopsis*	PSAD 1, PSAD 2, PSAD 3, PSAD 4, PSAD 5, PSAD 6,	0.21 X 10^2
2.	*Ferrisia virgata*	PSAD 7, PSAD 8, PSAD 9, PSAD 10	0.40 X 10^1
3.	*Paracoccus marginatus*	PSAD 11, PSAD 12, PSAD 13	0.30 X 10^1
4.	*Drosicha mangiferae*	PSAD 14, PSAD 15, PSAD 16, PSAD 17	0.14 X 10^1

Primary screening of these 17 isolates by drop-collapse test resulted in eight biosurfactant positive bacterial isolates (PSAD 1,2,3,5,6,7,8 and 9) from *P. solenopsis* and *F. virgata* (Table 2). Secondary screening and quantification of the WDB isolates by oil-spreading assay confirmed the results obtained in the primary screening. Drop-collapse and oil spreading techniques were easy and quick to screen for biosurfactant production. It requires a small volume of sample and no specialized equipment [15]. Wax degrading microorganisms synthesize a wide range of surface activity chemicals, such as glycolipids, phospholipids while culturing in hydrocarbon as sole carbon source [16] as noticed in this current experiment wherein olive oil was used as carbon source. Among the biosurfactant positive isolates, PSAD 2 recorded maximum biosurfactant activity of 142.14 mm^2, followed by PSAD 7 (115.93 mm^2) and PSAD 9 (91.08 mm^2); minimum biosurfactants activity was observed in PSAD 8 (45.63 mm^2) and PSAD 6 (40.65 mm^2). Similar results were reported in the assessment of various experimental methods for selecting biosurfactant producing halophilic bacteria [17].

Table 2: Screening and Quantification of microbial surfactants produced by wax degrading bacteria isolated from cotton mealy bugs

S. No	WDB isolate	Rhodamine B assay	Drop-collapse test	Oil-spreading assay Area of dispersal (mm^2) Mean ± SD
1.	PSAD 1	Positive	Positive	78.78±1.92
2.	PSAD 2	Positive	Positive	142.14±2.82
3.	PSAD 3	Positive	Positive	89.12±1.93
4.	PSAD 4	Negative	Negative	No activity
5.	PSAD 5	Positive	Positive	62.32±1.35
6.	PSAD 6	Positive	Positive	40.65±1.39
7.	PSAD 7	Positive	Positive	115.93±3.24
8.	PSAD 8	Positive	Positive	45.63±0.90
9.	PSAD 9	Positive	Positive	91.08±2.38
10.	PSAD 10	Negative	Negative	No activity
11.	PSAD 11	Negative	Negative	No activity
12.	PSAD 12	Negative	Negative	No activity
13.	PSAD 13	Negative	Negative	No activity
14.	PSAD 14	Negative	Negative	No activity
15.	PSAD 15	Negative	Negative	No activity
16.	PSAD 16	Negative	Negative	No activity
17.	PSAD 17	Negative	Negative	No activity

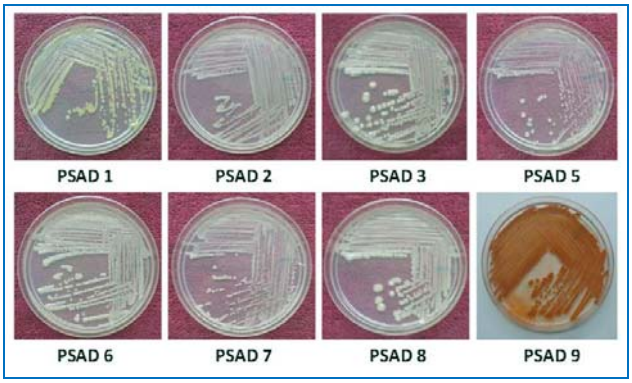

Fig 1: Purified wax degrading bacterial isolates on Nutrient Agar media

Further characterization of eight microbial surfactant positive WDB isolates on nutrient agar media revealed multiple types of colonial and cellular morphologies (Figure 1). Morphological and biochemical characters of the microbial surfactant positive WDB isolates from cotton mealybugs are summarized in Table 3. Wax degrading bacterial isolates, PSAD5 and PSAD7 formed circular, entire, convex papillate and transparent colonies, whereas PSAD1, PSAD2 and PSAD6 formed irregular, effuse, opaque colonies. Gram reaction and spore staining was positive in PSAD5 and PSAD7, whereas negative in the other six isolates. Starch hydrolysis was positive in all the WDB isolates, whereas citrate utilization was recorded positive only in PSAD3, PSAD5, PSAD7 and PSAD8. The biochemical characters of the WDB isolates varied considerably leading to their identification and classification. Based on morphological and biochemical profiles, the eight wax degrading bacterial isolates were tentatively identified as *Pseudoxanthomonas* sp. PSAD1, *Pseudomonas* sp. PSAD2, *Enterobacter* sp. PSAD3, *Bacillus* sp. PSAD5, *Pseudomonas* sp. PSAD6, *Bacillus* sp. PSAD7, *Enterobacter* sp. PSAD8 and *Serratia* sp. PSAD9. Similarly, Etoumi (2007) observed morphological and physiological characters of wax degrading bacteria and classified them as *Pseudomonas* species according to Bergey's manual of systematic bacteriology [18].

Table 3: Morphological and biochemical characterization of wax degrading bacteria isolated from cotton mealybugs

S. No	Characters	Wax degrading bacteria							
		PSAD 1	**PSAD 2**	**PSAD 3**	**PSAD 5**	**PSAD 6**	**PSAD 7**	**PSAD 8**	**PSAD 9**
1.	Colony	Irregular, Crenate, pale yellow	Irregular, Crenate, Dirty white	Circular, Entire, white	Circular, Entire, Creamy white	Irregular, Crenate, Dirty white	Circular, Entire, Creamy white	Circular, Entire, white	Circular, entire, red
2.	Gram staining	-	-	-	+	-	+	-	-
3.	Spore staining	-	-	-	+	-	+	-	-
4.	Shape	Rods	Coccus	Rod	Short rod	Coccus	Rod	Rod	Rod
5.	Starch hydrolysis	+	+	+	+	+	+	+	+
6.	Citrate utilization	-	-	+	+	-	+	+	-
7.	Gelatin hydrolysis	-	-	-	-	-	-	-	+
8.	MR- VP	- (+)	- (+)	- (+)	+ (-)	- (+)	+ (-)	- (+)	- (+)
9.	Catalase test	+	+	+	+	+	+	+	+
10.	Indole test	-	-	-	-	-	-	-	-
11.	Urease test	-	-	+	-	-	-	+	+
12.	Nitrate reduction	-	-	+	+	-	+	+	+
13.	Tentative identification	*Pseudoxanthomonas* sp.	*Pseudomonas* sp.	*Enterobacter sp.*	*Bacillus* sp.	*Pseudomonas* sp.	*Bacillus* sp.	*Enterobacter sp.*	*Serratia* sp.

+ Activity positive, - Activity negative (No activity)

4. Summary

This study clarified and confirmed the biosurfactant efficacy of WDB associated in the habitat of mealybugs infesting cotton. In particular, the WBD *Pseudomonas* sp. PSAD2, *Bacillus* sp. PSAD7 showed promising results during screening. Microbial surfactant producing wax degrading bacteria can provide an environmental friendly solution for major problems of agriculture. In the management of polyphagous pests like mealybugs, these microbial surfactant producing microorganisms could be used as an efficient tool for breaking up the complex wax coating on their cuticle, which otherwise resist insecticidal application. Further investigation in the form of pot culture and field studies are being carried out by the authors for useful application of these WDB in sustainable agriculture production.

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