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Bacteria with capacities of production of biosurfactants isolated from native plants of Baja California, México

Bacterias con capacidad de producción de biosurfantantes aisladas de plantas nativas de Baja California, México

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Abstract. The aim of the present study was to isolate autochthonous microorganisms with biosurfactants capacities from the rhizospere of the cotton (*Gossypium* spp.), cachanilla (*Pluchea sericea*) and salicornia (*Salicornia bigelovii*) in the Mexicali valley. The biosurfactant activity and biosurfactant productions by the strains isolated from the rhizosphere of the cotton (Bs-Alg), cachanilla (Bs-Cach) and salicornia (Bs-Cach01) were determined using oil spreading technique and emulsification activity with corn, olive, soybean and diesel oils, respectively. The analysis of the 16S rRNA showed that strains Bs-Alg, Bs-Cach and Bs-Cach01 are closely related among them and with respect to all *Bacillus subtilis* with more than 99% similarity values. Results showed that all strains had biosurfactant activity. However, Bs-Cach was the only strain that showed a significant biosurfactant activity with all the vegetable oils and diesel. Finally, the isolation of a biosurfactant producing *Bacillus subtilis* strain from native plants of Mexicali valley displayed a substantial potential for production of biosurfactants that can be applied to food industry.

Keywords: Mexicali valley; Biosurfactants; *Bacillus subtilis*; Native plants.

Resumen. El objetivo en el presente estudio fue aislar microrganismos nativos con capacidad biotensioactiva de la rizosfera de algodón (*Gossypium* spp.), cachanilla (*Pluchea sericea*) y salicornia (*Salicornia bigelovii*) en el valle de Mexicali. La actividad del biotensioactivo y producción del biosurfactante por las cepas aisladas de la rizosfera de algodón (Bs-Alg), cachanilla (Bs-Cach) y salicornia (Bs-Cach01) fueron determinados con la técnica de dispersión en aceite y actividad de emulsificante con aceites de maíz, olivo, soya y diésel, respectivamente. El análisis de 16SrRNA mostró que las cepas Bs-Alg, Bs-Cach y Bs-Cach01 están estrechamente relacionados entre sí y con una similitud del 90% con respecto a *Bacillus subtilis*. Los resultados mostraron que todas las cepas tenían actividad biotensioactiva. Sin embargo, B-Cach fue la única cepa que mostró una actividad biosurfactante significativamente con todos los aceites vegetales y diesel. Finalmente, el aislamiento de una cepa de *Bacillus subtilis* procedente de plantas nativas del valle de Mexicali con la capacidad de producir biotensoactivo mostró un potencial para la producción de biotensoactivos que pueden ser aplicados a la industria alimentaria.

Palabras clave: Valle de Mexicali; Biotensoactivo; *Bacillus subtilis*; Plantas nativas.

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INTRODUCTION

Biosurfactants are molecules with a polar and a non-polar region, and hence considered amphipathic. They can be produced by extracellular or intracellular microorganisms, and can reduce surface tension at the air-water interface between two immiscible liquids or between the solid-water interface (Yu et al., 2011). These compounds can be grouped as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds (Biermann et al., 1987). Biosurfactans have been used for (1) serving various environmental problems such as bioremediation of hydrocarbon contaminated soils, (2) enhanced oil recovery and (3) transportation of crude oils (Mulligan, 2005). On the other hand, biosurfactants also have potential applications in agriculture, cosmetics, pharmacy, detergents, personal care products, food processing, textile manufacturing, laundry supplies, metal treatment and processing, pulp and paper processing and paint industries (Banat et al., 2000). Biosurfactants are not yet used in food processing on a large scale due to the numerous protocols set by governmental agencies for alternative ingredients. However, the increase in consumer awareness of adverse allergic effects caused by artificial products has stimulated the development of alternative ingredients such as biosurfactants (Salihu et al., 2009; Banat et al., 2010). Additionally, the biosurfactants show several characteristics such as emulsifying, antiadhesive and antimicrobial activities which suggests their potential application as multipurpose ingredients or additives in diverse areas of the food industry (Nitschke et al., 2010; Malavenda et al., 2010).

In Mexico, the study of microorganisms capable of producing biosurfactants has focused on isolating mesophilic microorganisms from an oil-polluted coastal in Todos Santos Bay (Ensenada BC, Mexico), and a hydrological region RH-27 Tuxpan Nautla in Veracruz Mexico (Martinez-Toledo & Rodriguez-Vazques, 2013; Morales & Paniagua-Michel, 2013). However, studies on the diversity of *autochthonous* microorganisms in the Mexicali valley, which is characterized by the presence of one of the most extreme climates in Mexico, are scarce. The Mexicali valley is located in northeastern Baja California, Mexico (32.55° N, 115.47° W), has a mean annual rain of less than 75 mm, mean July (high) temperature of 43 °C, and mean January highs of 21.1 °C (García-Cueto et al., 2013). Therefore, the objective of the present study was to isolate indigenous microorganisms with biosurfactant capacities from the rhizosphere of native plants of the Mexicali valley.

MATERIALS AND METHODS

Field collection of rhizosphere soil. Soil samples were collected from the rhizosphere of cotton (*Gossypium* spp.), cachanilla (*Pluchea sericea*) and salicornia (*Salicornia bigelovii*) native plants of Mexicali valley, BC, México. Approximately, 100 g soil adhering to the roots of these native plants was collected in sterilized plastic bags and transported to the laboratory at room temperature for isolation of bacteria.

Isolation of biosurfactant-producing bacteria. One gram of rhizosperic soil of each native's plant species has added to 9.0 mL sterile, distilled water in a 10 mL centrifuge tube, which was shaken for 10 sec. Next, 1 mL soil suspension was transferred to another centrifuge tube containing 9 mL sterilized, distilled water using a sterilized pipette to prepare a 1:100 dilution, and 1 mL suspension was again transferred to another tube containing 9 mL sterilized, distilled water (1:1000 dilution). From the dilutions (10^{-3} to 10^{-5}), 1 mL was transferred to a Petri-dish containing nutritive agar, pH 7.2 ± 0.2 at 28 °C, by the spreading plate technique. The plates were then inverted and incubated at 28 °C for 48 hours. Control and replica plates were maintained for each rhizospheric soil of native plants. After incubation, 81.66 ± 3.05, 33.66 ± 3.21, and 63 ± 1.0 colonies-containing plates were selected from the rhizospheres of cotton, cachanilla and salicornia, respectively. These colonies were stored in nutritive agar slants and kept under refrigeration (4 °C) for further experimentation.

Screening for biosurfactant producers. The preliminary screening assays for biosurfactant production were performed using three methods (blood hemolysis test, oil spreading technique and emulsifying activity test). Twenty four-hour-old cultures of the isolates grown in Tryptic Soy Broth (TSB, Merck) were taken to perform screening tests. All screening tests were performed in triplicate. For the isolation of biosurfactant-producing bacteria, one colony from rhizosphere of cotton, cachanilla and salicornia, respectively, were selected from those colonies that had similar morphologies and cultured on blood agar plates for blood hemolysis test (Carrillo et al., 1996). The Petri dishes were incubated at 30 °C for 2 days, and one strain from cachanilla (Bs-Cach), cotton (Bs-Alg) and salicornia (Bs-Cach01) with a clear halo were selected and marked as positive for the presence of biosurfactant-producing bacteria according to Rodrigues et al. (2006). Finally, these strains were inoculated into liquid 5% Tryptic Soy Broth (TSB, Merck) medium supplemented with glycerol (15% final concentration), and stored at -80 °C until the biosurfactant production assay and molecular identification.

Biosurfactant production assay. The biosurfactant production was carried out using Mineral Salt Media (MSM) with glucose (1%) as the sole carbon source at pH 7 according to the proposal of Yesurethinam et al. (2014). Two hundred and fifty milliliters of MSM were inoculated with 3% inoculum of Bs-Cach, Bs-Alg and Bs-Cach01 separately, and incubated at 30 °C for 24 hours with 150 rpm agitation. After the incubation period, the cells were collected by centrifugation at 9000 rpm for 20 min at 4 °C, and the cell free supernatant was used as crude biosurfactant.

Oil spreading assay to determine surfactant activity. The oil spreading assay was utilized to study the surface activities of crude Bs-Cach, Bs-Alg and Bs-Cach01, respectively. This assay was developed by Morikawa et al. (2000) and can be applied when the activity and quantity of biosurfactant is low. For the oil spreading assay, 30 mL of distilled water were added to a petri dish, and 100 μL of corn, olive and soybean oils and diesel were added to the surface of the water, respectively. Twenty microliters of crude biosurfactant from Bs-Cach, Bs-Alg and Bs-Cach01 was then added to the surface of the oil. After 3 min of contact, the diameters of clear zones of triplicate assays from the same sample were determined. MSM broth without cell free supernatant served as the control.

Determination of emulsification activity. The emulsifying activity (EA) of the three strains (BsCach, BsAlg and BsCach01) was assessed against hydrocarbons (diesel), and vegetable oils (corn, olive and soybean). The EA of culture supernatant of the three strains was carried out using the modified emulsification assay described by Cameotra and Bollag (2003). An aliquot of culture supernatant (0.5 mL) was added to 7.5 mL of a 20 mM TM buffer [20 mM Tris-HCl (pH 7) and 10 mM $MgSO_4$] followed by addition of 0.1 mL of each edible vegetable oil and diesel, respectively. After a vigorous vortex for 1 min, the tubes were allowed to stand for 1 h at 30 °C. Finally, the absorbance of triplicate assays from each sample (diesel and vegetable oils) was measured at 540 nm and EA was calculated. One unit of EA was defined as the amount of emulsifier that yielded an absorbance (540 nm) of 0.1 in the assay. MSM broth without cell free supernatant was used as the negative control.

Molecular identification of bacterial isolates. The BsCach (cachanilla), BsAL (cotton) and BsCach01 (salicornia) strains were selected from the colonies based on their ability to form a clear halo on blood agar. Total DNA from Bs-Cach, Bs-Alg and Bs-Cach01 was extracted based on the method of Mendez-Trujillo et al. (2013). The DNA from these strains was amplified by polymerase chain reaction (PCR) with Taq DNA polymerase according to the manufacturer instructions (Invitrogen, Carlsbad, CA, USA). PCR analysis was performed using 25 ng DNA as the template. PCR reactions included the 16S rRNA gene with universal primers 27 forward (AGA GTT TGA TCC TGG CTC AG) and 27 reverse (AAG GAGGTG ATC CAG CCG CA). The PCR reactions were carried out using the following protocol: 94 °C for 5 min (1 cycle), 54 °C for 40 sec, and 72 °C for 1 min (30 cycles). The quality of the PCR reactions analyzed on a 1% Tris acetate EDTA agarose gel, and bands were visualized by staining with ethidium bromide. Images were acquired and stored using the Multidoc-It Digital Imaging system (UVP, Upland, CA, USA). The PCR products were purified using "Purelink® PCR Purification" kit (Invitrogen) and sequencing was carried out using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequence data were analyzed using software provided by the Basic Local Alignment Search Tool (BLAST) of GenBank.

Phylogenetic analysis of bacteria isolates. The three sequences obtained, were compared with other DNA sequences using BLAST program of the National Center for Biotechnology Information (Altschul et al., 1997). The sequences with high similarity to those were removed from the GenBank, and a phylogenetic neighbor-joining tree including the obtained isolates and their closest relatives was constructed using MEGA 4.0.

Statistical analysis. Values were expressed as means and standard deviations of triplicate experiments. Statistical analysis was carried out using the Statgraphics Centurion Software (15.2.06 version).

RESULTS

In the present study, phylogenetic analysis of 16S rRNA genes revealed that the Bs-Cach, Bs-Alg and Bs-Cach01 strains formed a stable clade with the members of the genus *Bacillus* spp., showing a sequence similarity of 99% for the 16S rRNA gene. The neighbor-joining method was employed to construct a phylogenetic tree to illustrate the relationships between the 16S rRNA strain sequences and those of other *Bacillus* species (Fig. 1). Thus, the three strains were designated as *Bacillus subtilis* and their sequences were deposited in the GenBank with accession numbers KF669896 (Bs-Alg), KM212950 (Bs-Cach01), and KC256786 (Bs-Cach). In the present study, the results of the oil spreading assay showed that Bs-Cach strain presented the best results with a zone superior to Bs-Alg and Bs-Cach01, respectively (Table 1). The emulsification activity (EA) of the three biosurfactants produced for bacteria cultures from Bs-Alg, Bs-Cach01 and Bs-Cach was tested by measuring the optical density at 540 nm (Table 2). The results showed that only Bs-Cach showed high EA values when it was exposed to different substrate sources used in the present study (corn, olive, soy and diesel). In contrast Bs-Alg and Bs-Cach01 strains showed an unexpected activity close to zero mostly in diesel and oil olive.

DISCUSSION

In the present study the hemolytic, oil displacement and emulsification activity measurements were used to isolate *biosurfactant-producing microorganisms* from the rhizosphere of native plants of Mexicali valley. Our results confirmed that cell free supernatant from Bs-Cach01 (salicornia) and Bs-Cach (cachanilla) strains showed the presence of the surface active compound (biosurfactant), and a high surface activity

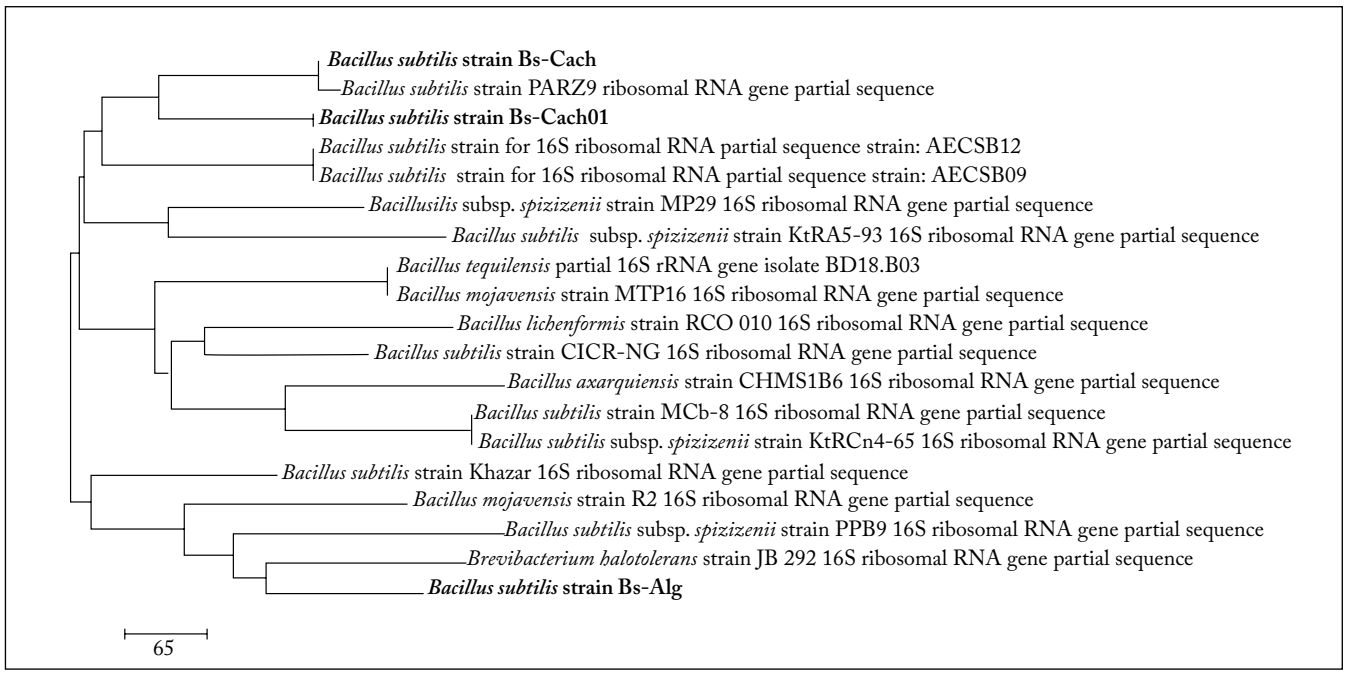

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences constructed using the neighbor-joining method. It shows a close relationship between the 3 strains and the nearest relatives of the genus *Bacillus*. Only values greater than 90% are shown.
Fig. 1. Árbol filogenético basado en las secuencias del gen 16S rRNA construido usando el método de unión al vecino. Muestra una estrecha relación entre las 3 cepas y los parientes más cercanos del género *Bacillus*. Solo se muestran valores mayores a 90%.

Table 1. Comparison of oil spreading efficiency of crude biosurfactant produced by Bs-Cach, Bs-Alg and Bs-Cach01 of natives plants of the Mexicali Valley in different types of oils.
Tabla 1. Comparación de la eficiencia de desparramar el aceite de biosurfactantes crudos producidos por Bs-Cach, Bs-Alg y Bs-Cach01 de plantas nativas del Valle Mexicali en diferentes tipos de aceites.

Source of biosurfactants	Diameter of the clearing zone (mm) in different oils			
	Diesel	Corn	Soybean	Olive
Control (MSM broth)	0	0	0	0
Bs-Cach (Cachanilla)	17.97 ± 0.22	20.6 ± 0.56	9.72 ± 0.56	26.27 ± 1.70
Bs-Cach-Alg (cotton)	0.76 ± 0.67	7.03 ± 0.50	6.25 ± 0.42	7.80 ± 0.54
Bs-Cach01(salicornia)	7.15 ± 0.54	9.24 ± 0.55	6.58 ± 0.38	10.80 ± 0.70

Results are expressed as mean ± standard deviation of values from triplicate experiments.

Table 2. Emulsification activity of crude biosurfactants produced by Bs-Cach, Bs-Alg and Bs-Cach01 of natives plants of the Mexicali Valley in different types of oils.
Tabla 2. Actividad emulsificante de biosurfactantes crudos producidos por Bs-Cach, Bs-Alg y Bs-Cach01 de plantas nativas del Valle de Mexicali en diferentes tipos de aceites.

Source of biosurfactants	Emulsification activity absorbance to 540 nm of different oils			
	Diesel	Corn	Soybean	Olive
Control (MSM broth)	0.39 ± 0.009	0.71 ± 0.001	0.38 ± 0.004	0.50 ± 0.008
Bs-Cach (Cachanilla)	0.007 ± 0.001	0.04 ± 0.003	0.075 ± 0.005	0.0002 ± 0.0004
Bs-Cach-Alg (cotton)	0.044 ± 0.003	0.16 ± 0.002	0.19 ± 0.003	0.037 ± 0.003
Bs-Cach01(salicornia)	7.15 ± 0.54	9.24 ± 0.55	6.58 ± 0.38	10.80 ± 0.70

Results are expressed as mean ± standard deviation of values from triplicate experiments.

in all oils used in this study. However, in this study was not evaluated the surfactant concentration versus the oil spreading activity. Morikawa et al. (2000) reported that the area of oil displacement in oil spreading assays is directly proportional to the concentration of the biosurfactant in the solution.

Similar results were observed by Tomar et al. (2014) and Youssef et al. (2004) in different isolates of microorganisms. Therefore, future studies are necessary to evaluate this possibility using crude biosurfactant from Bs-Cach01 (salicornia) and Bs-Cach (cachanilla) strains. Finally, although the mechanism of oil displacement by biosurfactant has not been fully understood at a molecular level, this assay can be considered as a sensitive and simple method for the measurement of the surface active nature of the biosurfactant (Madhu & Prapulla, 2013). On the other hand, Bs-Cach was only a strain that showed high emulsification activity (EA) on corn, olive, soybean and diesel. These results observed in Bs-Cach were superior to those reported by Olteanu et al. (2011) in new strains of *Bacillus* spp. isolated from different sources. However, the EA from the culture supernatant of Bs-Cach strain was inferior to those of *B. subtilis* B6 and *B. licheniformis* B5 when using corn and sunflower oil, but not olive oil (Sifour et al., 2005). These results indicate that the Bs-Cach strain has both surfactant and emulsifying properties with respect to other strains evaluated in the present study. It is important to consider, however, that even the oil displacement, and use of the emulsification activity are important in the detection of biological surfactants in culture media. These assays are insufficient for the differentiation of bioemulsifiers from biosurfactants. This is due to the fact that bioemulsifiers are best known for emulsification of liquids without significant changes in surface/interfacial tension of their growth medium (Satpute et al., 2008). This could explain the results observed in Bs-Cach01 and Bs-Alg with respect to their emulsifying activity and oil spreading assay.

On the other hand, diverse studies on the evaluation of the emulsifying ability (EA) of biosurfactants are in general related to environmental biotechnology, because many properties of this compound are widely used in remediation technologies of both organic and metal contaminants (Pacwa-Plociniczak et al., 2011). Nevertheless in the present investigation we detected that the biosurfactant and bioemulsifier activity of *Bacillus subtilis* (Bs-Cach) isolated from roots of cachanilla plants form a stable emulsion with oils used in the food industry. This is interesting because the isolation of biosurfactant-producing bacteria from native plants from Mexicali valley displayed a substantial potential for production of biosurfactants and bioemulsifiers that can be applied to the food industry.

CONCLUSION

This study represents the first report about the production of biosurfactant/ bioemulsifiers by *Bacillus subtilis* strains isolated of cachanilla (*Pluchea sericea*), a native plant species of the Mexicali valley. Bs-Cach strain showed the formation of a stable emulsion with oils, suggesting its potential as an emulsifying agent in the food industry. Finally, to our knowledge, use of oil spreading and emulsifying activity represent two simple assays that can be used in the detection of biosurfactant/bioemulsifiers production by indigenous microorganisms.

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