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Bioemulsifier-producing *Bacillus subtilis* from Railway Soil

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Abstract: A novel enrichment combined with a rapid screening method was employed to isolate bioemulsifying strains of *Bacillus subtilis*. Among a total of twenty isolates from railway soil at six geographically distant sites, ten produced bioemulsifiers for soybean oil and crude oil. Qualitative drop-collapse assays indicated the bioemulsifiers were surfactants.

Keywords: *Bacillus subtilis*; bioemulsifier; enrichment; railway soil

Introduction

Hydrocarbonoclastic and emulsifier-producing bacteria occur at sites of chronic hydrocarbon exposure, including railway soils (1, 2, 3, 4). Among the bacteria found at these sites is *Bacillus subtilis* (*B. subtilis*) (5). It can produce bioemulsifiers (6) and products that are commercially valuable (7). Because of its importance, interest in discovering new *B. subtilis* strains with desirable properties is high. Soil is the principal environment where that search begins.

The usual first step to isolating *B. subtilis* from soil is selecting for heat-resistant spores by pasteurization. This is followed by plating on a complex growth medium, a non-selective step that permits viable spores of virtually any *Bacillus spp*. in the sample to form colonies. Many colonies of multiple species must to be sorted through, often with great time and effort, to isolate *B. subtilis*. One strategy for increasing efficiency is enriching for biofilm formation when isolating epiphytic *B. subtilis* strains from roots (8). Another is pre-screening on differential media to identify surfactin-producing strains where natto is the source material (9).

Many members of the genus *Bacillus* can assimilate ammonia as the sole source of nitrogen for growth, but most of those also require growth factors. Knight and Proom observed, while working with over two hundred soil samples, that the colonies appearing on defined medium containing ammonia as the sole nitrogen source without growth factors were almost exclusively *B. subtilis* (10). This suggests that plating initially on an ammonia-containing medium, instead of a complex medium, might be useful as an enrichment for *B. subtilis* after pasteurizing a soil sample.

Experiments using local soil samples supported this (LaPolla and Benoit, unpublished). When soil samples were pasteurized and subsequently plated on defined medium containing ammonia as the sole nitrogen source without growth factors, only *B. subtilis* formed colonies. However, when the same samples were plated on nutrient agar, other *Bacillus spp.* also formed colonies,

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most notably members of the *Bacillus cereus* group corresponding to Group 1A (11).

This report describes experiments employing an ammonia-based defined medium, combined with a rapid screening method, to efficiently isolate bioemulsifier-producing *Bacillus subtilis* from railway soil.

Materials and Methods

Organisms. Control strains used in assays were the surfactant producer *B. subtilis* 3A22, (ATCC 21332, Bacillus Genetic Stock Center, Columbus, OH) and *B. subtilis* W168 which does not produce surfactant (gift of W.C. McDonald).

Medium and culture conditions. M81 contained (g/L): K_2HPO_4, 14; KH_2PO_4, 6; $(NH_4)_2SO_4$, 2; Mg_2SO_4, 0.2; glucose, 8; Na_3citrate, 1; and trace amounts of $MnCl_2$,

$CaCl_2$, and $FeCl_3$. M81 essentially is the medium of Spizizen (12) with additional glucose and trace elements. It was formulated to resemble the optimized medium of Willenbacher, *et al.* for maximizing surfactin production by their *B. subtilis* strains (13). M81 was supplemented with ultrapure agar (15g/L; Affymetrix/USB) to make M81 agar. Liquid cultures were grown to stationary phase. All cultures were incubated at 37C.

Soil samples. Soil samples were collected into sterile 2 ml plastic cryotubes (Fisher Scientific) from railways in Abilene, TX; Merced, CA; Alliance, NE; Durand, KS; Gravette, AR; and Noel, MO. Samples were transported to the lab at amibient temperature and stored at 4C until used.

Soil pasteurization. Soil samples were suspended by vortex mixing in four times their volume of sterile distilled H_2O, gently agitated for 1h, mixed again, and allowed to settle. One ml then was withdrawn and pasteurized at 70C for 30 mins.

Plating and identifications.100 µl of the pasteurized soil suspensions were spread onto M81 agar plates and incubated until colonies appeared (usually 48-72h). Up to four of the predominant colony types were selected from each plate for identification by physological and morphological criteria.

Screen for stable foam formation. Capped tubes containing M81 cultures were vigorously shaken vertically by hand for 5 seconds and allowed to stand at ambient temperature for 10 minutes. Cultures that formed a stable foam were scored as positive and subsequently were used in assays of emulsifier activity and in the drop-collapse test. Cultures that did not form stable foam were discarded.

Emulsifying activity assay. Emulsifying activity (EA_{1h}) was measured by the method of Patil and Chopade (14). M81 cultures were centrifgued at 2990 rcf for 20 minutes. Three ml of the resulting cell-free supernantant was combined with 0.5 ml of either soybean oil (Crisco) or crude oil (West Texas Intermediate, Pride Refineries) by vigorous vortex mixing for 2 min. The resulting emulsion was incubated at 37C for 1h. The absorbance at 400 nm of the aqueous phase then was recorded immediately against a blank of M81 that had been similarly prepared. Absorbance was converted to emulsifier units/ml (EU/ml) where 0.01 absorbance units = 1 EU/ml. EA_{1h} is expressed as the number of EU/ml after the 1h incubation.

Drop-collapse test. The drop-collpase test was carried out by a modification of the method of Jain, *et al*. (15). Ten µl of cell-free M81 culture

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supernatant were spotted onto the surface of Parafilm and observed after 10 mins. A drop that lost its rounded shape and collapsed (flattened) was scored as positive.

Results and Discussion

The six sampled railway soils yielded twenty total colonies of interest, selected from the predominant colony types on individual M81 agar plates. Pure cultures from all twenty colonies were identified as *B. subtilis*. It appears that M81 agar strongly enriched for this organism, as anticipated. Further genetic analyses, however, may show that some isolates are other species among the dozen or so that constitute the virtually phenotypically indistinguishable members of the *Bacillus subtilis* species complex (16,17).

Ten of the twenty *B. subtilis* isolates formed stable foam in M81. See Figure 1 for the appearance of stationary phase cultures of both positive and negative stable foam-formers.

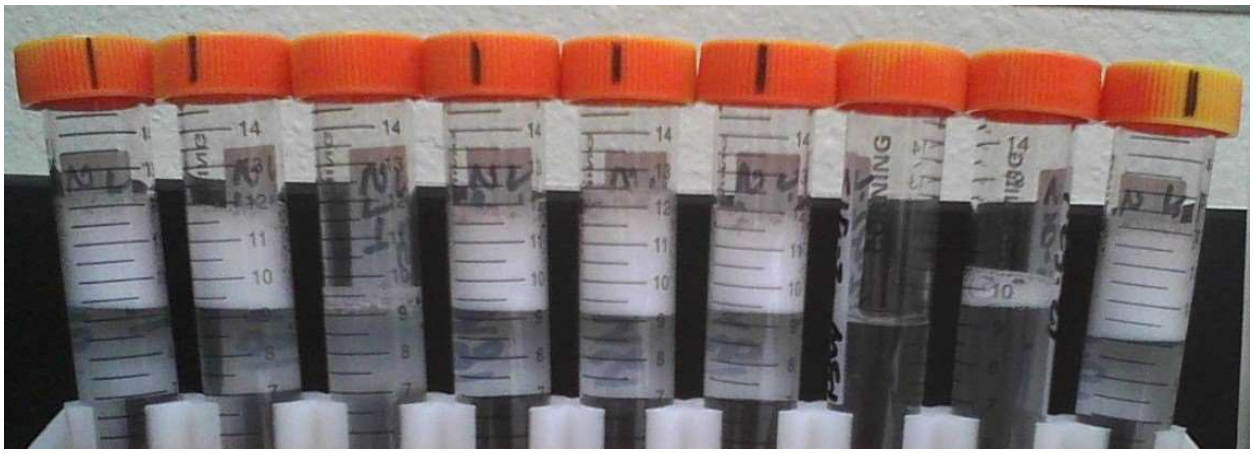

Figure 1. Comparison of stable foam production by *B. subtilis* isolates. .

Shown are M81 cultures of nine B. subtilis isolates. Cultures with marked caps formed stable foam.

Cell-free M81 culture supernatant from the ten stable foam-formers was further assayed for emulsification activity (EA_{1h}). Results are shown in Table 1. The supernatants from all isolates emulsified both soybean and crude oil, suggesting that stable foam formation in M81 broth is a reliable indicator of bioemulsifier production.

Emulsification activity varied by two-fold or more among the *B. subtilis* soil isolates

(Table 1), perhaps because of differences in the amounts or types of bioemulsifiers produced. However, all isolates were positive for the drop-collapse test, meaning that the agent responsible for emulsification likely was a surfactant. Therefore, the differences in EA_{1h} may be due more to differences in the amounts of emulsifier produced than to differences in types since *B. subtilis* is known to produce only a few types of surfactants.

Table 1. Comparison of EA_{1h} and drop-collapse results of cell-free supernatant from M81 cultures of *B. subtilis* isolates.

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Isolate	[a]EA1h		Drop collapse
	soybean oil	crude oil	
S4-2	80	50	+
S6-3	190	100	+
S7-2	105	95	+
S7-3	190	140	+
S9-1	180	110	+
S9-2	120	90	+
S9-3	160	40	+
S10-1	160	90	+
S11-1	150	110	+
S13-3	140	180	+
3A22	100	100	+
W168	15	0	-

Comparison of bioemulsification activity and surfactant production among B. subtilis isolates from railway soil and controls 3A22 and W168. [a]EA1h rounded values of emulsifying activity after 1h incubation expressed as EU/ml.

The occurrence of bioemulsifying *B. subtilis* strains in railway soil is not surprising because the organism is ubiquitous, produces bioemulsifiers, and bioemulsifying bacteria are common at sites of chronic hydrocarbon exposure. However, their occurrence in railway soil previously was unknown. Railway soil appears, then, to be a fertile environment for discovering novel bioemulsifier-producing *B. subtilis* strains, particularly since the methods described provide an efficient way for isolating them.

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