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Lysozyme-Sensitive Bioemulsifier for Immiscible Organophosphorus Pesticides

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Two *Bacillus* strains capable of emulsifying immiscible organophosphorus pesticides were isolated by enrichment methods. The emulsifying factor produced by *Bacillus* strain FE-2 has a high molecular weight, is lysozyme sensitive and thermostable, and can be precipitated with trichloroacetic acid or ammonium sulfate; it may be a glycolipopeptide. It is specific for immiscible organophosphorus pesticides and is secreted during growth in the presence of such pesticides.

Organophosphorus pesticides have been used as replacement chemicals for the more persistent organochlorine and mercury compounds (7, 15). Complete microbial degradative pathways for these pesticides have not been established, mainly because of their low miscibility with water and their toxic nature (5, 10, 12).

The problem of low miscibility and solubility in the aqueous medium has been partly overcome for chemicals such as 2,4,5-trichloroacetic acid (2,4,5-T) and 2,4-dichloroacetic acid (2,4-D) by the formation of soluble sodium salts (8) or by the use of detergents (6, 11). Many microorganisms produce surface-active agents (bioemulsifiers or surfactants) during their growth on insoluble compounds such as hydrocarbons (3, 9, 16, 17), and emulsification of 2,4,5-T and related chlorinated compounds has recently been reported (1). No such microbial bioemulsifier had previously been described for insoluble or immiscible organophosphorus pesticides; however, we now report an emulsifying activity, specific for immiscible organophosphorus pesticides, in the culture supernatants of *Bacillus* strain FE-2.

Strains FE-1 and FE-2 were isolated from *O,O*-dimethyl-*O*-[3-methyl-4-(methylthio)phenyl]phosphorothioate (fenthion) enrichment culture obtained from a soil sample exposed to high doses of fenthion. Both strains were tentatively identified as belonging to the genus *Bacillus* (2). Cultures were routinely maintained on Luria agar plates. *Bacillus* strain FE-2 was grown in modified Burk mineral medium (BMM) (13), containing (in grams per liter) K_2HPO_4, 0.2; KH_2PO_4, 0.8; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.0033; $FeSO_4 \cdot 7H_2O$, 0.005; and technical fenthion, 0.5. Cultures were grown at 30°C on a rotary shaker at 180 rpm.

The emulsifying activity was assayed by measuring the turbidity due to the formation of pesticide emulsion by cell-free supernatants of *Bacillus* strain FE-2 that had been grown in the presence of fenthion. To 5.0 ml of culture supernatant in a screw-cap tube (15 by 125 mm) was added 12.5 mg of fenthion as an acetone solution. The suspension was mixed by vortexing for 1 min and was left undisturbed at room temperature (25°C) for 2 h. The turbid emulsion was drawn out carefully, and the optical density was read at 660 nm against water. BMM treated in a similar manner served as a control.

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The cell-free supernatant from cultures grown in the presence of fenthion always led to the formation of emulsions which were stable for more than 30 h at room temperature. No such emulsions were observed when fenthion was shaken with distilled water or BMM. Emulsifying activity was observed with the supernatants of cultures grown in the presence of 0.05% fenthion, 0.05% fenitrothion, or 0.05% glucose. No emulsifying activity was observed with the supernatants of cultures grown in the presence of 1% glucose or in Luria broth (with or without 0.05% fenthion), even though the cell mass obtained under these conditions was greater than that obtained during growth in the presence of 0.05% glucose or 0.05% fenthion. This indicates that the production (secretion) of the emulsifying agent is suppressed when the bacteria are grown in the presence of high levels of glucose or in Luria broth, although these are optimal for cell yields. Similarly, *Rhodococcus erythropolis* secretes anionic trehalosetetraesters with surfactant activity only under growth-limiting conditions (14).

For routine studies of the emulsifying activity, *Bacillus* strain FE-2 was grown in BMM for 40 h in the presence of 0.05% fenthion. There was no further increase in the emulsifying activity beyond 40 h of growth, and the activity decreased slowly after 70 h.

The emulsifying activity was proportional to the amount of emulsifier present, although no emulsifying activity could be detected beyond a fourfold dilution of the culture supernatant. The culture supernatant could be lyophilized to recover the emulsifying activity; the activity was linear up to 20 mg of the lyophilized solid material. As with other bioemulsifiers, the emulsifying activity appears to be caused by the formation of micelles, and the emulsification of fenthion is proportional to the amount of the emulsifier present down to the point, presumably, at which the critical micelle concentration is reached (1, 3, 4).

Maximal emulsifying activity was obtained with 10 to 12.5 mg of fenthion in the standard assay. The emulsifying activity was influenced by the pH, with maximal activity between pH 7.0 and 7.6. The emulsifying activity was lost on dialysis against distilled water, although 12 to 15% of the activity could be recovered on addition of 10-fold-concentrated BMM to the retentate. About 62% of the activity was retained when the emulsifier was dialyzed against BMM.

The emulsifying factor was resistant to autoclaving at 15 lb/in^2 for 20 min and could partially be extracted with chloroform but not with benzene or heptane (Table 1). It could be recovered after precipitation with trichloroacetic

TABLE 1. Effects of physical and chemical treatments on the emulsifying activity of the *Bacillus* strain FE-2 culture supernatant

Treatment	Emulsifying activity (%)
Autoclaving	67
Solvent extraction[a]	
Chloroform-methanol (2:1, vol/vol)	17
Heptane	0
Benzene	0
Precipitation[b]	
5% Trichloroacetic acid	62
80% Ammonium sulfate	68
25% Polyethylene glycol 6000	0

[a] Culture filtrate was extracted three times, with twice the volume of solvent, and the extraction of emulsifying activity into the solvent layer was checked after the solvent had been evaporated and the residues had been dissolved in the original volume of BMM.

[b] Culture supernatant was treated with the reagent and incubated with gentle stirring at 4°C for 1 h. The precipitate was collected by centrifugation and dissolved in the original volume of BMM. The pH of the samples was brought to 7.0, and the emulsifying activity was checked.

acid or ammonium sulfate but was not precipitated by polyethylene glycol 6000 (5 to 25%). The effect of enzyme treatment on emulsifying activity was also studied (Table 2).

The results showing that the emulsifying factor from *Bacillus* strain FE-2 is nondialyzable, precipitable with trichloroacetic acid and ammonium sulfate, partially extractable with chloroform, and sensitive to lysozyme treatment are compatible with the idea that the emulsifying agent is a macromolecule consisting of carbohydrate, lipid, and peptide. The complete loss of emulsifying activity on treatment with lysozyme indicates that the carbohydrate moiety may contain (β1→4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid. These conclusions were confirmed by (i) inhibition of emulsifying activity in the presence of wheat germ agglutinin, (ii) prevention of such inhibition in the presence of free *N*-acetylglucosamine, and (iii) loss of a specific band in lysozyme-treated supernatant compared with untreated supernatant after electrophoresis on a 7.5% polyacrylamide gel and the presence of emulsifying activity in the corresponding band of untreated supernatant (results not shown).

TABLE 2. Effects of enzyme treatment on the emulsifying activity of the *Bacillus* strain FE-2 culture supernatant

Enzyme[a]	Emulsifying activity (%)[b]
DNase	100
RNase	100
Pronase	88
Protease	75
β-Chymotrypsin	70
Trypsin	100
Lipase	100
Phospholipase C	84
β-Galactosidase	100
β-Glucuronidase	100
Chitinase	100
Lysozyme	2

[a] The culture supernatant was treated with the enzymes (100 μg/ml) for 22 h at 37°C, and the emulsifying activity of the samples was checked.

[b] Emulsifying activity is expressed as percentage of the activity in the untreated culture supernatant.

TABLE 3. Substrate specificity in emulsification[a]

Substrate	State	Emulsifying activity (%)
Organophosphorus		
Fenthion	Liquid	100
Fenitrothion	Liquid	25
Quinalphos	Liquid	48
Ethion	Liquid	122
Diazinon	Liquid	36
Edinfenphos	Liquid	64
Profenophos	Liquid	70
Phosalone	Solid	0
Betasan	Semisolid	25
Organochlorine		
DDT[b]	Solid	0
BHC[b]	Solid	4
2,4-D	Solid	1
2,4,5-T	Solid	2
Hydrocarbon		
Hexane	Liquid	0
Xylene	Liquid	0
Toluene	Liquid	8
Kerosene	Liquid	0
Vacuum oil	Liquid	23

[a] To 5.0 ml of the culture supernatant was added 12.5 mg of organophosphorus or organochlorine pesticide of 0.5 ml of hydrocarbon; the mixture was vortexed for 1 min and kept at room temperature for 2 h.

[b] DDT, Dichlorodiphenyltrichloroethane; BHC; benzene hexachloride.

Only liquid-immiscible organophosphorus pesticides were emulsified (Table 3), indicating that the emulsifying agent shows specificity for both physical and chemical aspects of the substrate. The emulsifying agent from *Pseudomonas cepacia* ATCC 39027 is specific for 2,4,5-T and to some extent for other, related, chlorophenols that are substrates of the dechlorinating enzymes and does not emulsify compounds which are not dechlorinated or utilized as growth substrates (1). Similarly, it seems that *Bacillus* strain FE-2 could metabolize only compounds which are being emulsified. *Bacillus* strain FE-2 has been found to metabolize fenitrothion and methyl parathion (unpublished data).

The emulsifying agent from *Bacillus* strain FE-2 described in this paper could have applications in pesticide formulations and in pesticide disposal. Detailed characterization of the emulsifier awaits its purification, which is in progress.

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