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Characterization of *Nocardia amarae* as a Potent Biological Coalescing Agent of Water-Oil Emulsions

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Nocardia amarae grown in a liquid medium induced coalescence of emulsions which differed in type, composition of the organic phase, and structure of stabilizing emulsifiers. De-emulsifying activity varied with the type of growth medium, culture age, and postharvest treatment. Based on extraction and degradation studies, it was concluded that de-emulsifying properties are due to the bacterial cell surface. Thus, bacteria may provide a new source of de-emulsifying agents.

The present studies were aimed at demonstrating that whole bacterial cultures have de-emulsifying properties toward a variety of emulsions, that culture age and growth medium are variables in controlling the de-emulsifying activity of the whole culture, that the cells are the active agents in the whole culture (although their activity could be modulated, in principle, by other components within the medium), and that the cell surface properties responsible for the de-emulsifying activity are stable toward a variety of conditions likely to be encountered in industrial applications.

MATERIALS AND METHODS

Nocardia amarae (strain B-8176 from Northern Regional Research Laboratory, Peoria, Ill.) was grown on a rotary shaker (250 rpm) in 500-ml flasks containing 200 ml of medium (1% yeast extract, 1% dextrose, and 4% hexadecane in tap water). Cells suspended in the growth medium were tested for their ability to break the emulsions described in Table 1.

Emulsions were prepared by adding aqueous and organic components to a 10-ml test tube and mixing them with a Vortex-Genie (Fisher Scientific Co., Fair Lawn, N.J.) at maximum speed until no further emulsification occurred. Emulsion type (water-in-oil [W/O] or oil-in-water [O/W]) was determined on the basis of whether a saturated kerosene solution of oil red O or a saturated aqueous solution of crystal violet diffused into the emulsion and whether the emulsion could be dispersed into kerosene or water. De-emulsification was initiated by adding a specified volume of cell suspension or control (the suspending medium) to the emulsion and shaking it for an additional 30 s. The height of the emulsion within the test tube was followed with time, and a half-life ($t_{1/2}$) was calculated by assuming that emulsion decay could be approximated as a first-order reaction. This assumption provided the best empirical fit to the data over the majority of the decay process.

RESULTS AND DISCUSSION

Seven-day-old whole cultures (cells plus medium) were tested for their ability to break a variety of emulsions (Table 1). The cells were grown at 22°C (±2°C) on 1% yeast extract (Difco Laboratories, Detroit, Mich.), 1% dextrose (laboratory grade, Fisher), and 4% hexadecane (99% pure; Humphrey Chemical Co., North Haven, Conn.). The data in Table 1 indicate that suspensions of *N. amarae* cells in growth medium significantly reduced emulsion stability of (i) both W/O and O/W emulsions, (ii) emulsions with the same emulsifier but different organic phases, and (iii) emulsions with the same organic phase but different stabilizing emulsifiers. Variations in the $t_{1/2}$ of the L92 (K) emulsion in the presence of suspensions from flasks A and B may reflect different biomasses within the two flasks. Because biomass was not measured in these studies, comparisons between flasks are not possible; however, $t_{1/2}$ for emulsions exposed to suspensions from any one flask are comparable.

Growth of *N. amarae* in a 10- and 20-liter batch was completed in a New Brunswick CMF-128 S 28-liter fermentor at 22°C and with agitation by two propellers rotating at 250 rpm. Aeration was at 15 liters per min through a sintered metal sparger. The medium pH was not controlled during growth.

Table 2 presents part of the data from two growth studies: one with a soluble substrate (1% dextrose and 1% yeast extract) in tap water and

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TABLE 1. Composition of emulsions used to test bacteria-induced de-emulsification and $t_{1/2}$ of these emulsions when mixed with 1 ml of a suspension of *N. amarae* cells in their growth medium

Flask	Emulsion symbol	Type	Aqueous Phase (4 ml)[a]	Organic phase (6 ml)[a]		$t_{1/2}$ (h) in presence of cells[b]
			Emulsifier[c]	Solvent	Emulsifier[c]	
A	L92(K)	O/W	0.068% Pluronic L92	Kerosene		8.7
	L92(C16)	O/W	0.068% Pluronic L92	*n*-Hexadecane		6.1
	L92(C10)	O/W	0.068% Pluronic L92	*n*-Decane		2.5
	L92(C6)	O/W	0.068% Pluronic L92	*n*-Hexane		18.9
	L92(C14U)	O/W	0.068% Pluronic L92	1-Tetradecene		33
	L92(C6C)	O/W	0.068% Pluronic L92	Cyclohexane		2.2
	T/S(K)	O/W	0.072% Tween 60, 0.028% Span 60	Kerosene		3.6
	T/S(C16)	O/W	0.072% Tween 60, 0.028% Span 60	*n*-Hexadecane		<1
	T/S(C10)	O/W	0.072% Tween 60, 0.028% Span 60	*n*-Decane		1.3
	T/S(C6)	O/W	0.072% Tween 60, 0.028% Span 60	*n*-Hexane		12.0
	T/S(C14U)	O/W	0.072% Tween 60, 0.028% Span 60	l-Tetradecene		1.0
	T/S(C6C)	O/W	0.072% Tween 60, 0.028% Span 60	Cyclohexane		12.0
B	L92(K)	O/W	0.068% Pluronic L92	Kerosene		<1
	L43(K)	O/W	0.1% Pluronic L43	Kerosene		<10
	S40(K)	O/W	0.1% Span 40	Kerosene		<1
	B/T(K)	O/W	0.09% Brij 72 0.01% Tween 20	Kerosene		<1
	S/P(K)	O/W	0.03% Span 85 0.07% Pluronic P85	Kerosene		<1
	T/S(K)	O/W	0.072% Tween 60 0.028% Span 60	Kerosene		<1
C	S80(K)	W/O		Kerosene	0.078% Span 80	10.5

[a] Five milliliters each of the organic and aqueous phases were mixed to form the S80(K) emulsion.
[b] All emulsions had a control $t_{1/2}$ of >200 h, except the S80(K) emulsion, which had a control $t_{1/2}$ of 70 h.
[c] Emulsifiers were obtained from the following sources: Pluronics (copolymers of polyethylene oxide and polypropylene oxide), BASF Wyandotte Corp., Wyandotte, Mich.; Tween 60 (polyoxyethylene 20 sorbitan monostearate) and Span 60 (sorbitan monostearate), J. T. Baker Chemical Co., Phillipsburg, N.J.; Tween 20 (polyoxyethylene 20 sorbitan monolaurate) and Span 85 (sorbitan trioleate), Atlas Chemical Industries, Brantford, Ontario; Brij 72 (polyoxyethylene 2 stearyl ether), ICI Americas Inc., Wilmington, Del.

one with an insoluble substrate (4% *n*-hexadecane) in a modified mineral salts medium (NH_4NO_3, 0.4%; KH_2PO_4, 0.4%; K_2HPO_4, 0.6%; $MgSO_4 \cdot 7H_2O$, 0.02%; $CaCl_2 \cdot 2H_2O$, 0.0001%; $FeSO_4 \cdot 7H_2O$, 0.0001%; EDTA, 0.00015%). The 200-ml inoculum was grown in shake flasks for 7 days without transfer on the same medium as used in the growth study. Table 2 indicates that comparable biomass concentrations existed at the beginning of log phase growth in both studies.

Table 2 shows that the de-emulsifying activity of 0.5 ml from either crude whole culture (cells plus medium) fluctuated with the culture age. As shown below, the de-emulsifying activity resided with the cells; however, the age-dependent de-emulsifying activity of whole cultures did not always parallel the age-dependent changes in biomass. The fluctuations in de-emulsifying activity of the whole culture were therefore a function not only of the emulsion composition being tested and the biomass concentration at the time the culture was sampled, but also of the concentration of any extracellular compounds in the culture medium such as medium components, cell secretory products, or even cell lysis products which could, in principle, influence the de-emulsifying activity by either interacting at the interface of the emulsion droplet to alter the stability of the emulsion or by adsorption onto the cell surface to alter the de-emulsifying properties of that surface or by both. Detailed studies using cells washed free of medium will be presented in a subsequent communication (W. L. Cairns, D. G. Cooper, and N. Kosaric, *in* J. E. Zajic, ed., *Microbial Enhanced Oil Recovery*, in press). These latter studies indicate that, additionally, the de-emulsifying activity of the cell

TABLE 2. Changes in biomass and de-emulsifying ability toward L92(K) and T/S(K) emulsions during growth of *N. amarae*

Growth medium (initial vol)	Time (h) after inoculation of fermentor	$t_{1/2}$ (h) of[a]:		Biomass (g/liter)
		L92(K)	T/S(K)	
1% dextrose + 1% yeast extract in tap water (10 liters)	8.5	54	160	0.08
	23.5	86	94	0.26
	26.5	NA[b]	120	0.50
	31.3	95	>200	1.45
	33.5	93	>200	1.62
	38.3	140	>200	0.74
	55	110	>200	0.64
	71.3	42	>200	0.68
	85.3	26	>200	0.68
	102	20	>200	0.66
	123	22	140	0.49
	143.3	35	130	0.50
	155.5	58	150	0.43
	167.5	46	170	0.46
4% *n*-hexadecane + 0.1% yeast extract in modified mineral salts medium (20 liters)	27	28	94	0.06
	32	39	77	0.19
	45	76	83	1.13
	47	60	84	1.63
	50	56	>200	2.76
	66.8	22	>200	3.28
	75	8.5	>200	3.22
	90.8	10	>200	4.20
	137	9.9	>200	3.6
	155.5	12	>200	3.5

[a] Emulsion $t_{1/2}$ after the addition of 0.5 ml of whole culture (cells plus medium). The addition of 0.5 ml of growth medium as a control did not decrease the $t_{1/2}$ of either emulsion below 200 h.
[b] NA, Not available.

surface is a function of the age-dependent and medium-dependent changes in the structure and biochemistry of the cell surface. Changes in the structure and biochemical composition of *Nocardia* cell walls during growth (1, 2) and with different growth media (3) have previously been reported. The de-emulsifying activity of whole cultures is clearly complex in origin and not a simple function of biomass concentration; however, even with this complexity, the additional expense of cell separation may make whole cultures the choice for industrial application.

N. amarae cells grown in shake flasks for 7 days at 20°C (±2°C) on a mixed substrate (1% dextrose, 1% yeast extract, and 4% hexadecane) in tap water were centrifuged from the whole culture broth and subjected to the procedures shown in Fig. 1. After each procedure, an amount of material equivalent to that found in 0.5, 0.18, or 0.05 ml of the original whole broth was added to the L92(K) emulsion, and an emulsion $t_{1/2}$ was determined. Reagent grade solvents (pentane, chloroform, and methanol) were used in extractions of wet pellets of *N. amarae* cells. Extractions were at 22°C (±2°C) and with slow continuous stirring using a solvent volume/cell pellet volume ratio of 50:1 to 100:1 and a minimum of one solvent change during the 20- to 24-h extraction period. HCl-methanolysis, trichloroacetic acid extraction, phenol extraction, and NaOH-methanolysis were performed on 10 mg of dry chloroform-methanol-extracted cells by standard procedures (5). De-emulsifying activity was found only in association with the bacterial cells or the nonsolubilized cellular residue remaining after solvent extraction.

The major conclusion from the extraction and degradation studies is that de-emulsifying activity is a property of the cell surface. The considerable resistance to both lipid and polysaccharide extracting procedures and the complete loss of activity from the cell fraction only upon vigorous alkaline solvolysis at elevated temperatures suggests that the integrity of the surface is essential for optimum activity and that molecular constituents functional in de-emulsification are firmly bound to that surface.

The activity of the bacterial de-emulsifier toward an L92(K) emulsion was minimally affected by the pH of the emulsion within the range 3 to 10 and by NaCl added to the emulsion in concentrations between 0 and 10^{-3} M. With 20

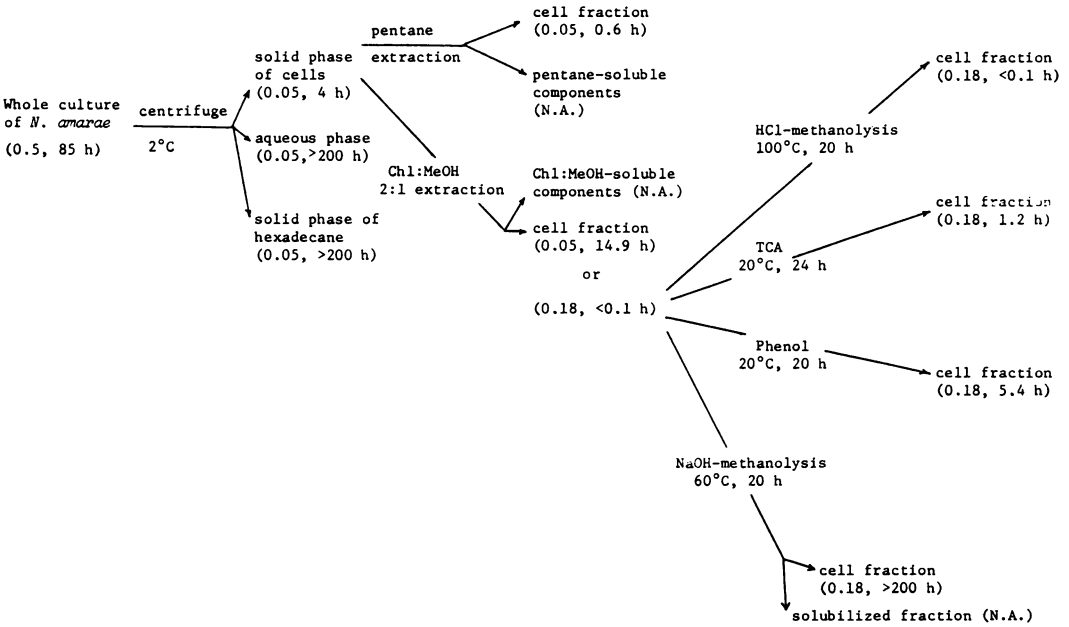

FIG. 1. Procedures used to characterize the *Nocardia* de-emulsifier. The volume (milliliters) of whole broth equivalent (see text) and the $t_{1/2}$ (hours) of an L92(K) emulsion to which this equivalent was added are shown as (volume, $t_{1/2}$) after each procedure. N.A., No activity.

μg of chloroform-methanol (2:1)-extracted cells per 10 ml of emulsion, the emulsion $t_{1/2}$ were reduced to 2 to 3.5% of the control $t_{1/2}$ over the above ranges. This minimal sensitivity of bacteria-induced de-emulsification to pH and NaCl suggests that the de-emulsification mechanism operating in the L92(K) emulsion was insensitive to the degree of ionization of any ionizable groups on the bacterial surface. The significant degree of bacterial de-emulsifier resistance to many chemicals, to pH changes, to NaCl concentration, and also to heat (120°C for 20 min without loss of de-emulsifying activity) is of practical importance for industrial applications.

Fifty times the amount of chloroform-methanol-extracted *N. amarae* cells needed to effect complete de-emulsification of the L92(K) emulsion within 1 min was added to 5 ml of an aqueous solution of L92 emulsifier at the same concentration used to prepare the emulsion. The cells were allowed to sit for 2 h with the emulsifier, and the suspension was then filtered through a 0.2-μm microporous filter (Amicon Corp., Lexington, Mass.) to remove the cells and any adsorbed emulsifier. The interfacial tension against *n*-hexadecane of the L92 solution before the addition of cells and after filtering changed very little (from 5 mN/m to 6.8 mN/m) as measured by a Fisher Autotensiomat, and the concentration of L92 as measured by the number of dilutions required to reach the critical micelle concentration was unchanged. This suggests that the mechanism of bacteria-induced de-emulsification does not involve bulk inactivation of the emulsifier by complexing of it with the bacterial surface.

This paper presents the discovery of a new application of bacteria, namely, de-emulsification. The ability to control the nature of these biological de-emulsifiers through techniques suggested by the present studies raises the possibility that bacteria, alone or in conjunction with other de-emulsifying techniques, may have the versatility to cope with the large variety of existing industrial emulsions (4).

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