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Production of Biodispersan by *Acinetobacter calcoaceticus* A2

E. ROSENBERG,* C. RUBINOVITZ, A. GOTTLIEB, S. ROSENHAK, AND E. Z. RON

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69 978, Israel

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Bacterial strains A2 and HE5, isolated by enrichment culture techniques, were shown to produce extracellular, nondialyzable materials which disperse limestone powders in water. These materials are referred to as biodispersans. Strains A2 and HE5 were classified as *Acinetobacter calcoaceticus* by physiological and genetic tests. An assay for limestone-dispersing activity was developed which is based on the settling time of a standard 10% limestone-in-water mixture. The assay was proportional to biodispersan concentration between 40 and 100 μg/ml. Dispersion was optimum between pH 9 and 12. Phosphate (2 mM) and magnesium (8 mM) ions caused a 50% inhibition of activity. An optimized medium for the production of biodispersan was developed with ethanol as the substrate. Biodispersan was produced only after the bacteria completed their exponential growth phase and continued during the stationary phase, reaching over 4 g of crude biodispersan per liter.

In any heterogeneous system, boundaries are of fundamental importance to the behavior of the system as a whole. It is therefore not surprising that microorganisms, having large surface-to-volume ratios, produce a wide variety of surface-active agents. Surface-active compounds from microorganisms have been the subject of a number of reviews (3, 14, 18). Essentially all of the microbial surfactants that have been isolated and characterized either lower the interfacial tension between oil and water or stabilize (or destabilize) hydrocarbon-water emulsions. These amphipathic molecules have potential applications in tertiary oil recovery, transport of heavy oils, and enhancing hydrocarbon degradation.

It occurred to us that bacteria might also produce extracellular surfactants which adhere to the surfaces of inorganic minerals. Such surfactants might prove useful in mining and manufacturing processes. To examine this concept, limestone (calcite) was chosen as a model mineral because it is inexpensive, available in pure form, well characterized structurally, and widely used in common products such as paints, ceramics, and paper (5).

In this paper, we present data on the isolation and characterization of two strains of *Acinetobacter calcoaceticus* which produce extracellular polymers which disperse limestone in water. These polymers are referred to as biodispersans. Subsequent reports will be devoted to a description of the physical and chemical properties of biodispersan (14), its immunological characteristics, and its use in the manufacture of paper.

MATERIALS AND METHODS

Unless stated otherwise, the limestone rock used in this study was a white carbonate of high purity (>99% $CaCO_3$) quarried in the south of Israel, obtained from American-Israeli Paper Mills Ltd., Hedera, Israel. Jerusalem limestone, kindly provided by Y. Kolodny (Hebrew University), was used in some preliminary experiments. The limestone rock was ground in a Norton porcelain jar mill with cylindrical Burundem grinding pellets (50% limestone; roller speed, 290 rpm; 6 h). After grinding, the limestone particles were separated from the water and dried at 90°C. The dried powder was then passed through a 325-mesh screen.

Bacterial strains and growth conditions. *A. calcoaceticus* BD4, isolated by Taylor and Juni (17), was provided by N. Kaplan. Strain RAG-1 (ATCC 31012) has been described previously (15). *A. calcoaceticus* A2 and HE5 were isolated during this study by an enrichment culture procedure (1, 16) with AC medium (0.2% sodium acetate, 0.2% KNO_3, 0.02% $MgSO_4 \cdot 7H_2O$ and 40 mM KH_2PO_4-Na_2HPO_4 buffer, pH 6.0). Flasks (125 ml) containing 10 ml of AC medium were inoculated with soil and incubated at 30°C with vigorous shaking for 48 h. Samples of the turbid culture were then streaked onto AC agar (AC medium solidified with 2% agar). Colonies appearing after 48 h at 30°C that contained nonmotile oxidase-negative coccobacilli were considered possible *Acinetobacter* species and transferred to brain-heart infusion agar (Difco Laboratories, Detroit, Mich.).

Growth and biodispersan production experiments were carried out in ethanol medium (EM) containing (per liter of deionized water) 16 g of ethanol, 14.8 g of $K_2HPO_4 \cdot 3H_2O$, 4.84 g of KH_2PO_4, 4.0 g of $(NH_4)_2SO_4$, 0.4 g of $MgSO_4 \cdot 7H_2O$, and 1 ml of trace elements solution. The trace elements solution contained (per 10 ml) 3.68 mg of $CaCl_2 \cdot 2H_2O$, 6.24 mg of $CuSO_4 \cdot 5H_2O$, 6.04 mg of $FeSO_4 \cdot 7H_2O$, 5.94 mg of $MnSO_4 \cdot 4H_2O$, 4.22 mg of $ZnSO_4 \cdot 7H_2O$, 7.88 mg of $CoCl_2 \cdot 6H_2O$, and 6.96 mg of Na_2MoO_4. After the medium was inoculated, the cultures were incubated at 30°C in a New Brunswick G24 gyratory shaker at 150 rpm.

Preparation of biodispersan. After 3 days of growth of *A. calcoaceticus* A2 in EM medium, the turbid culture broth was centrifuged at 12,000 × g for 20 min. Ammonium sulfate was added slowly to the supernatant fluid with stirring to reach a final concentration of 60% saturation. The resulting precipitate was collected by centrifugation, dissolved in water, dialyzed against distilled water, and lyophilized. The yield of crude biodispersan A2 was 3.5 g/liter.

Analytical procedures. Oxidase activity was determined by replica-plating colonies onto Whatman 3mm filter paper that had been impregnated with 1% *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine hydrochloride. Development of a deep purple color within 10 s indicates a positive test for oxidase.

* Corresponding author.

TABLE 1. Growth characteristics of *A. calcoaceticus* strains

Strain	Growth[a] at 42°C	Utilizable carbon source[b]			
		Glc	Arg	Trp	Tyr
A2	+	±	+	+	+
HE5	+	−	−	−	±
RAG-1	−	−	+	−	+
BD4	−	+	+	+	−

[a] Growth on MS agar supplemented with 2% ethanol.
[b] Tryptophan added to MS agar to a final concentration of 0.01%; all other carbon sources used at 1%. All strains utilized ethanol, acetate, alanine, and proline, but failed to grow on serine; ± indicates weak growth.

Measurement of hydrocarbon-in-water emulsifying activity was determined turbidometrically as described previously (16). Absorbance was read on a Gilford model 240 spectrophotometer. Turbidity was measured in a Klett-Summerson colorimeter fitted with a green filter.

Standard limestone dispersant assay. To 12-ml conical graduated tubes were added 400-mg portions of milled Arad limestone. Various concentrations of biodispersan in distilled water were added to a final volume of 4.0 ml. The tubes were then vortexed on a Thermolyne Maxi Mix (Sybron Corp., Dubuque, Iowa) for 30 s and allowed to equilibrate for 30 min. The tubes were again vortexed for 30 s (time zero) and allowed to stand undisturbed. After 30 min, the upper 2 ml was removed by using a Pasteur pipette. The turbidity of the upper 2 ml was determined. Dilutions were made in distilled water so that the final Klett unit (K.U.) value was less than 150.

DNA transformation assay. The following modification of the method of Juni and Janick (6) was used to examine the ability of suspected *Acinetobacter* strains to serve as DNA donors for the transformation of *A. calcoaceticus* BD413 *trpE27*. An overnight culture of the donor strain in brain-heart infusion medium (0.5 ml) was mixed with 0.5 ml of 0.2% sodium dodecyl sulfate and incubated for 30 min at 60°C. A few drops of chloroform were added, and the mixture was incubated for an additional 30 min at 60°C. The lysed cell suspension was then cooled and diluted with an equal volume of water. Transformation was performed by placing a small amount of bacterial paste of strain BD413, just visible to the naked eye, on a sector of GM agar (7) supplemented with 1 μg of L-tryptophan per ml and then spreading the paste together with a loopful of the lysed cell suspension. After incubation for 24 h at 30°C, a loopful of the developing colonies that appeared were spread onto GM agar and incubated for 24 h at 30°C. Confluent growth or many colonies appearing on the minimal glucose agar indicated positive transformation of BD413 *trpE27* to prototrophy. Controls with no donor DNA or lysates from non-*Acinetobacter* strains showed no colonies.

RESULTS

Characterization of *A. calcoaceticus* A2 and HE5. The source of the two *A. calcoaceticus* strains used in this study were soil (strain A2) and human hair (strain HE5). Both strains were obtained in pure culture by cloning on AC agar following a standard enrichment culture technique for the isolation of *Acinetobacter*. Strains A2 and HE5 were classified as *A. calcoaceticus* (presently the only species in the genus [2]) by physiological and genetic characterization. The cells were gram-negative, oxidase-negative, aerobic, nonmotile coccoid rods. DNA derived from either strain A2 or HE5 was able to transform competent auxotrophic *A. calcoaceticus* BD413 to prototrophy.

Table 1 compares the growth characteristics of *A. calcoaceticus* A2 and HE5 with two well-studied *A. calcoaceticus* strains, RAG-1 and BD4. Strains A2 and HE5 differed from RAG-1 and BD4 in their ability to grow at 42°C. Strain HE5 differed from the other three strains in that it failed to use arginine as a carbon source; strain A2 differed from strains RAG-1 and BD4 in that it grew on both tyrosine and tryptophan. Strains A2 and HE5 were sensitive to neither the RAG-1-specific bacteriophage ap2 (12) nor the BD4-specific bacteriophage SL-1 (8).

Interaction of *A. calcoaceticus* A2 and HE5 cells and extracellular polymers with limestone. Stationary-phase cultures of *A. calcoaceticus* A2 and HE5 were centrifuged to separate the cells from the extracellular materials. The resuspended cells and dialyzed extracellular materials were then examined for their interactions with ground limestone (Table 2). Limestone flocculated the suspended cells. For example, the absorbance of the upper 1 ml of the A2 cell suspension after standing for 1 h was 13.3 and that of the limestone suspension was 0.9; if there was no interaction between them, then the upper 1 ml of the mixture should have had an absorbance of 14.2. The observed value of 3.4 indicates that most of the cells were flocculated by the limestone. Similarly, the predicted value for the mixture of HE5 cells and limestone was 4.0, whereas the observed value was 2.7.

The extracellular fluids of A2 and HE5 also interacted with the limestone, but in a different manner. The dialyzed materials decreased significantly the settling rate of the powdered limestone. In the presence of the HE5 and A2 supernatant fluids, the turbidities of the upper 1 ml after 1 h were 23.3 and 29.9, respectively, compared with 0.9 for the limestone by itself. As observed by phase microscopy (Fig. 1), the supernatant fluids of A2 and HE5 (data not shown) caused a dispersion of the ground limestone. Small flocs 5 to 40 μm in diameter (Fig. 1A) were dispersed into particles of 1 to 4 μm diameter (Fig. 1B). It follows that the decreased settling rate of the limestone in the presence of the supernatant fluids was due to extracellular dispersing agents produced by *A. calcoaceticus* A2 and HE5. These extracellular dispersing agents did not have any significant hydrocarbon-in-water emulsifying activity. Subsequently, it was discovered (14) that strains A2 and HE5 produced biodispersans with similar properties. Thus, only the data for biodispersan A2 will be presented.

TABLE 2. Interaction of *A. calcoaceticus* A2 and HE5 cells and extracellular fluids with limestone

Strain and fraction[a]	A_{400}[b]			Type of interaction
	Fraction alone	Fraction plus limestone	Predicted value[c]	
A2				
Cell pellet	13.3	3.4	14.2	Flocculation
Supernatant fluid	0	29.9	0.9	Dispersion
HE5				
Cell pellet	3.1	2.7	4.0	Flocculation
Supernatant fluid	0	23.3	0.9	Dispersion

[a] Cells were harvested after 5 days of incubation at 30°C in EM medium. The washed cell pellets were suspended to their original volume; the supernatant fluid was dialyzed against distilled water.
[b] Ground Jerusalem limestone (200 mg) in 2 ml of suspended cells or supernatant fluid was vortexed for 1 min and allowed to stand for 1 h. The upper 1 ml was then removed for determination of turbidity at 400 nm. The limestone control (no bacterial fraction) yielded an A_{400} of 0.9.
[c] Assuming no interactions of the bacterial fraction with limestone.

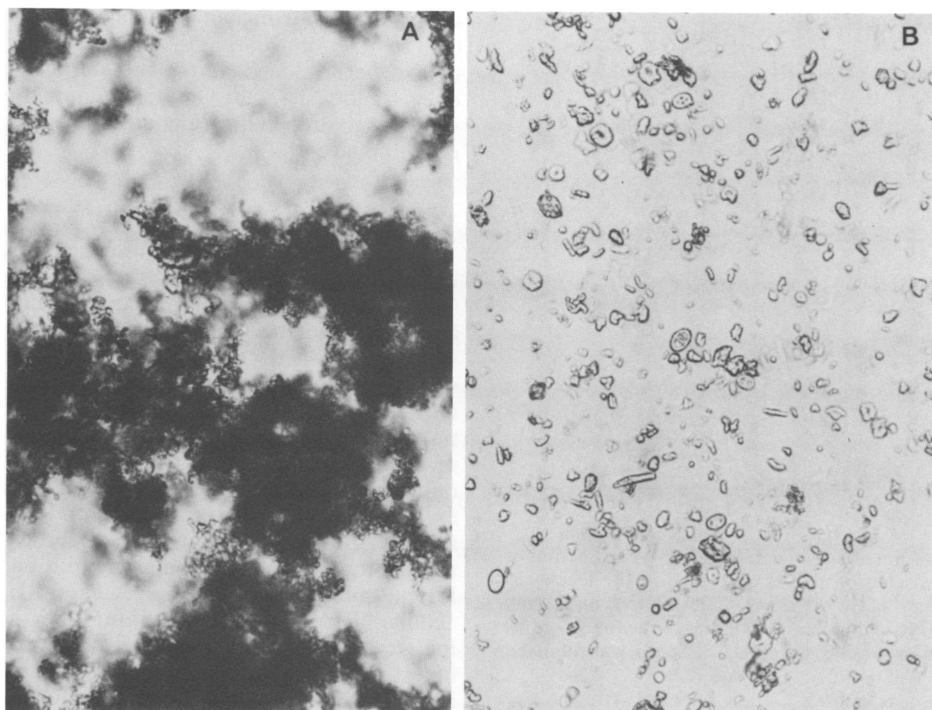

FIG. 1. Phase micrograph of (A) a 10% suspension of limestone powder and (B) the same 10% suspension of limestone dispersed with the extracellular culture fluid of *A. calcoaceticus* A2. Magnification, ×750.

Dispersing activity as a function of concentration. To study the kinetics of biodispersan production and to proceed with the isolation, purification, and characterization of the active component(s), a simple assay for the dispersant was developed (Fig. 2). The assay is based on the fact that aqueous suspensions of ground limestone flocculate and settle rapidly, leaving a clear upper phase in 15 to 30 min. In the presence of a dispersant, however, flocculation is prevented, so that the individual limestone particles settle relatively slowly. Under the standard conditions used here, the turbidity of the upper phase was determined after the suspension was allowed to settle for 30 min. Below 40 μg of crude biodispersan A2 per ml, there was no measurable dispersion of the limestone. Between 40 and 100 μg/ml, the final turbidity of the upper 2 ml increased exponentially from 200 to 10,000 K.U. No further increase in turbidity occurred with higher concentrations. Thus, measurable dispersion began at a limestone-to-crude biodispersan ratio of 2,500:1 and was complete at 1,000:1.

The effect of pH on the dispersing activity of biodispersan A2 is shown in Fig. 3. The natural pH of the 10% slurry of limestone in water was 9.0. Below this pH, the dispersing activity of biodispersan A2 decreased sharply. Between pH 9 and 12 the dispersing activity was relatively constant. Although NaCl, Na_2SO_4, KCl, and LiCl had no effect on the dispersing activity of biodispersan A2, magnesium and phosphate ions (components of the growth medium) strongly inhibited its activity (Fig. 4). Fifty percent inhibition of biodispersan A2 activity occurred at approximately 2 mM K_2HPO_4 and 8 mM $MgCl_2$. It was therefore necessary to dialyze the extracellular fluids of *A. calcoaceticus* before measuring biodispersan activities.

Growth and production of biodispersan. A medium for optimum production (EM medium) of biodispersan was obtained by studying the effect of ethanol, salt concentra-

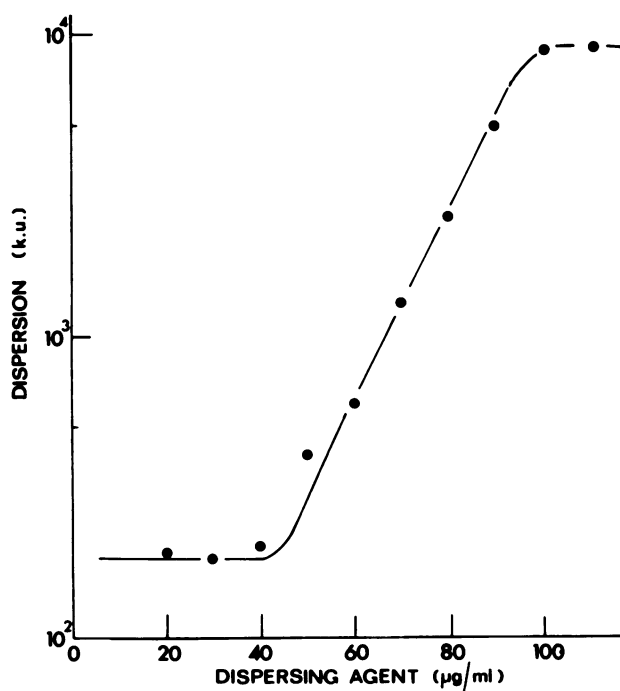

FIG. 2. Standard limestone dispersant assay. Dispersion (corrected K.U.) as a function of the crude biodispersan A2 was determined as described in Materials and Methods, with 400 mg of milled Arad limestone in a total volume of 4 ml. Each point in the figure represents the average of three determinations.

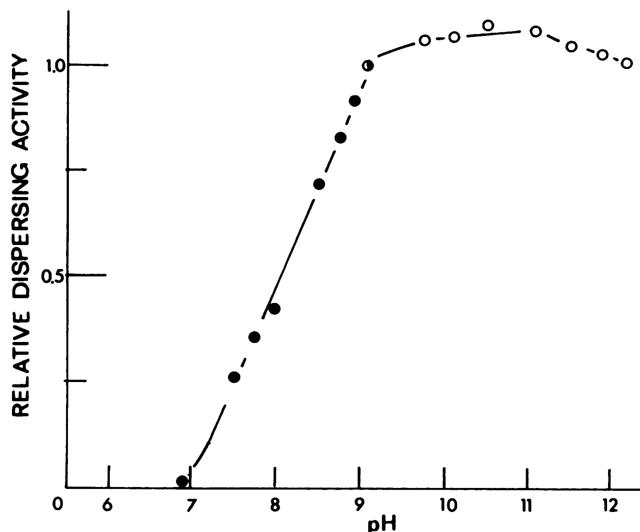

FIG. 3. Dispersion of limestone by biodispersan as a function of pH. The experiment was performed as described in the legend to Fig. 2, with biodispersan A2 at 80 μg/ml. The relative dispersing activities at different pHs are compared with that of the pH 9 control (◐) (no addition of acid or base, 3,400 K.U.). The pH was adjusted with either HCl (●) or NaOH (○).

tions, and initial pH on biodispersan production. The growth of *A. calcoaceticus* A2 in EM medium (Fig. 5) consisted of three phases: (i) from 2 to 8 h after inoculation, the cells grew at an exponential rate with a doubling time of approximately 2 h; (ii) after the culture reached a turbidity of 500 K.U. (8 h) growth slowed down, reaching a maximum of 1,500 K.U. at 30 h; and (iii) stationary phase. The pH of the culture medium decreased during the exponential phase from 7.1 to 6.6, during the slow growth phase to 6.4, and finally to 6.0. Measurements of dispersing activity in the dialyzed extracellular fluids indicated that biodispersan increased from 0.08 to 1.3 g/liter during the slow-growth phase and continued to be produced during the stationary phase, reaching over 4 g/liter at 72 h. Only small amounts of biodispersan were found in the extracellular fluids during the exponential growth phase.

DISCUSSION

Extracellular hydrocarbon-in-water emulsifier production is widespread in the genus *Acinetobacter*. In one survey (16), 8 of 16 strains of *A. calcoaceticus* produced high amounts of emulsifier following growth on ethanol-salts medium. Washed cells of *A. calcoaceticus* 2CA2 reduced surface tension, whereas the extracellular fluid was active in deemulsifying kerosene-water emulsions stabilized by a mixture of Tween 60 and Span 60 (9, 10). In the present study, of approximately 200 bacterial isolates obtained from soil, only 2 (strains A2 and HE5) produced significant amounts of extracellular, nondializable, limestone-in-water dispersing activity. Both strains were shown to be acinetobacters by the rigorous transformation assay (6) in addition to physiological criteria. Neither strain A2 nor HE5 produced extracellular hydrocarbon-in-water emulsifiers. Similarly, none of the *Acinetobacter* emulsifiers tested were able to disperse limestone in water. Thus, emulsifying and dispersing activities appear to be due to different materials, and production of dispersants is restricted to a relatively small number of *Acinetobacter* strains.

To understand the effect of pH (Fig. 3) on the dispersing activity of biodispersan, it is useful to consider its chemical structure. As described in the accompanying paper (14), the active component of biodispersan is a polysaccharide that contains at least two different ionizable groups: a uronic acid with a pK_a of 3.1 and an amino sugar with a pK_a of 8.0. At high pH values (above 9), the polymer contains only negative charges due to the carboxyl groups. From pH 9 to pH 7, the amino groups become progressively more protonated,

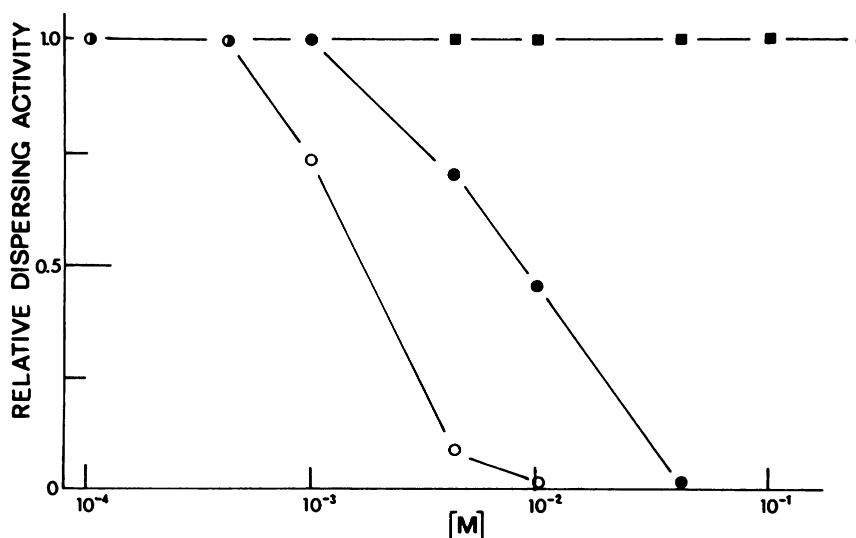

FIG. 4. Effect of salts on dispersion of limestone by biodispersan. The experiment was performed as described in the legend to Fig. 2, with biodispersan A2 at 60 μg/ml. The relative dispersing activity at different salt concentrations is compared with that of the control (no addition of salts, 3,500 K.U.). Symbols: ○, K_2HPO_4; ●, $MgCl_2$; ■, NaCl, KCl, LiCl, and Na_2SO_4.

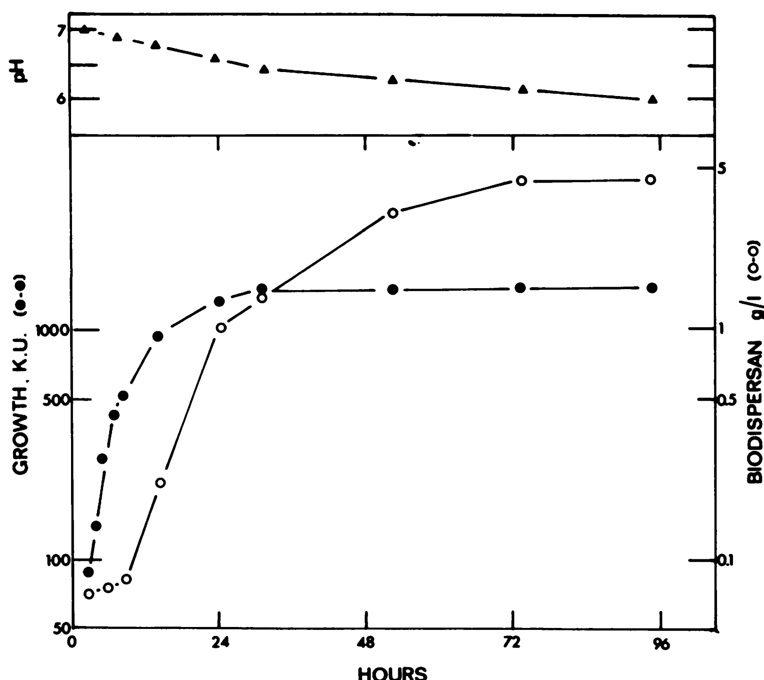

FIG. 5. Production of biodispersan and growth of *A. calcoaceticus* A2 on ethanol medium. EM medium (200 ml in a 2-liter flask) was inoculated with 40 ml of an exponential culture and incubated with shaking for 96 h at 30°C. Samples (10 ml) were withdrawn for determination of turbidity (●), pH (▲), and extracellular dispersing activity (○). The concentration of biodispersan was determined by the standard limestone dispersant assay (Fig. 2) after dialysis of the extracellular supernatant fluid (10,000 × g, 15 min).

yielding a polymer with both negative and positive charges. Above the pK_a of the amino group, the biopolymer would bind to the surface of the limestone particles, imparting a strong negative charge to the surface layer. The resulting electrical double layer causes interparticle repulsion, so that the limestone remains dispersed in the water phase (Fig. 1B). Below the pK_a of the amino group, the bound polymer becomes zwitterionic, weakening interparticle repulsion. In addition, it is possible that flocculation is mediated at pHs below 9 by localized positive charges on one particle interacting with negative charges on a second particle.

During the exponential growth phase, *A. calcoaceticus* A2 had a doubling time of 2 h in EM medium at 30°C. Essentially no biodispersan was detected during the exponential phase. Significant amounts of biodispersan began to appear in the extracellular culture fluid only when growth slowed down and continued to accumulate during the stationary phase, reaching 4 g/liter. This corresponds to a 25% conversion of ethanol into crude biodispersan. The source of the extracellular biodispersan that was produced during stationary phase could be cell-bound material which was synthesized either (i) during exponential phase and released during stationary phase or (ii) de novo during stationary phase. In the case of *A. calcoaceticus* RAG-1, it has been shown that the extracellular emulsan or an emulsanlike precursor accumulates on the cell surface as a capsule during the exponential growth phase (4, 11, 15). When the rate of protein synthesis decreases, emulsan is released into the medium by an energy-dependent process. Immunological techniques are presently being developed for measuring cell-bound biodispersan.

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