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Microbial diversity as a source of useful biopolymers

Eugene Rosenberg

Department of Molecular Microbiology & Biotechnology, Tel Aviv University, Ramat Aviv, Israel

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INTRODUCTION

Bacteria are specialists. In any particular ecological niche, one bacterium or a limited number of strains dominate. These bacteria have adapted through evolution to be able to survive in this niche during the long periods when growth is impossible, and then when nutrients become available, they can outgrow their competitors. The fast growth of microorganisms depends largely on their high surface-to-volume ratio, which allows for efficient uptake of nutrients and release of waste products. The price that the microorganism pays for the high surface-to-volume ratio is that it is totally exposed. All of the components on the outside of the cell, flagella, fimbria, capsules, outer membrane proteins and extracellular enzymes and other polymers, must be able to function under the specific conditions of the ecological niche. For example, if the conditions are high salt and low pH values, then the exocellular polymers must be active at high salt and low pH. On the other hand, the conditions inside the cell are well buffered. It is probably for this reason that the 'Unity of biochemistry' was discovered inside the cell, whereas the 'Diversity of the microbial world' is best expressed on the outside of the cell.

During the last 20 years, our laboratory has attempted to take advantage of the diversity of exocellular polymers to isolate and characterize surfactants and enzymes of potential commercial interest. A list of some of the microbial polymers that we have studied is presented in Table 1.

RAG-1 EMULSAN

Acinetobacter calcoaceticus RAG-1 was isolated by enrichment culture on a medium containing crude oil as the sole carbon and energy source, ammonium sulfate, phosphates and sea water [8]. One of the characteristics of this strain is that during growth on crude oil or model substrates it produces an extracellular polymeric emulsifier, referred to as emulsan [9,10]. The chemical and physical properties of emulsan are summarized in Tables 2 and 3. The polymer consists of a repeating trisaccharide of three different *N*-acetyl aminosugars with fatty acids linked via *N*-acyl and *O*-ester linkages [1]. The average molecular weight is approximately one million.

The unusual property of RAG-1 emulsan is that it binds avidly to oil/water interfaces, thereby stabilizing oil-in-water emulsions. Several lines of evidence indicate that the hydrophobic fatty acid side-chains are on the inside of the oil droplet and the hydroxyl groups and uronic acid residues of the anionic polymer are oriented towards the water. Thus, emulsan forms a strong film on the oil surface. The only way to remove this film and break the emulsion is with a specific emulsanase [16,17]. Fig. 1 shows a composite photomicrograph of an oil-in-water emulsion, stabilized by 0.1% emulsan (lower frame). If the emulsion is allowed to stand for several days or is centrifuged, the oil droplets, 3–5 μm in diameter, rise and form a cream, containing about 70% oil and 30% water (Fig. 1, upper frame).

Emulsan has several interesting applications. First, it is effective in cleaning petroleum and other hydrophobic materials from surfaces, such as cargo compartments of oil tankers and barges, and oil-contaminated barrels and pipes. It is particularly applicable when the use of chlorinated solvents is not advisable for environmental or safety reasons. Second, emulsan can be used to lower the viscosity of heavy oils, thereby reducing the energy necessary to transport heavy oil in pipelines. Third, emulsan can be used to stabilize emulsions in the food, cosmetic and paint industries, because it is biodegradable and not toxic. Fourth, emulsan has been used to efficiently remove uranium from water [22].

What is the natural role of emulsan? One of the natural roles of emulsan may be in regulating desorption of the

Correspondence to: Eugene Rosenberg, Department of Molecular Microbiology & Biotechnology, Tel Aviv University, Ramat Aviv, Israel.

TABLE 1

Bacterial polymers discussed in this article

Bacterial polymer	Type of polymer	Properties	Potential applications
A. calcoaceticus RAG-1 emulsan	Polysaccharide: lipid	Stabilizes oil-in-water emulsion, binds cations	Oil cleaning, viscosity reduction, general emulsifier, concentrates UO_2^{++}
A. calcoaceticus BD4 emulsan	Polysaccharide protein complex	As above	As above
A. calcoaceticus A2 biodispersan	Polysaccharide	Dispersant, surfactant	Dispersing agent, grinding aid
B. stearothermophilus xylanase	Protein	Hydrolyses xylan at pH 9, 65°C	Biobleaching pulp

producing strain from hydrophobic surfaces. Consider *A. calcoaceticus* RAG-1 growing on the surface of crude oil in seawater. During the growth phase, the bacteria accumulate emulsan in the form of a minicapsule on the cell surface [2]. This bacterium uses only relatively long-chain *n*-alkanes for growth. After these components are used, RAG-1 becomes starved, although it is still attached via fibriae [15] to the oil droplet, enriched in aromatics and cyclic paraffins. Starvation of RAG-1 causes release of the minicapsule of emulsan [2]. It is postulated that this released emulsan forms a polymeric film on the *n*-alkane-depleted oil droplet, thereby desorbing the starved cell. In effect, the 'emulsifier' frees the cell to find fresh substrate. At the same time, the depleted oil droplet has been marked as used by now having a hydrophilic outer surface to which the bacterium cannot attach.

TABLE 2

Chemical composition of RAG-1 emulsan

Component	%
1 *N*-acetyl hexosamines	70
D-galactosamine	
L-galactosamine uronic acid	
Dideoxy-diaminohexonse	
2 Fatty acids	15
3-hydroxydodecanoic acid	
2-hydroxydodecanoic acid	
3 Water and ash	15

BD4 EMULSAN

A. calcoaceticus BD4, initially isolated by Taylor and Juni [19] produces a large polysaccharide capsule. The chemical structure for the polysaccharide, elucidated by Kaplan et al. [4], is shown in Fig. 2. When grown under nutritionally-limiting conditions, *A. calcoaceticus* BD4 releases the capsule together with protein [3]. The extracellular mixture is a potent emulsifier. Neither the pure polysaccharide nor the protein fraction is active by itself. However, when the pure polysaccharide and protein are mixed together, the emulsifying activity can be reconstituted (Table 4). Apparently, the protein, which is hydrophobic, binds to the hydrocarbon initially in a reversible fashion. The polysaccharide then attaches and stabilizes the oil-in-water emulsion [11]. Many of the potential commercial applications demonstrated for the RAG-1 emulsan can also be performed with the BD4 emulsan.

TABLE 3

Physical properties of RAG-1 emulsan

Measurement	Value
Sedimentation constant	6.06 S
Intrinsic viscosity	570 cc/g
Diffusion constant	5.25×10^{-8} cm^2/s
Partial molar volume	0.712 cm^3/g
Molecular weight	9.8×10^5 (weight avg.)
	9.8×10^5 (viscosity avg.)
Axial ratio	~50
Electron microscopy	Rods (30 Å × 2000 Å)

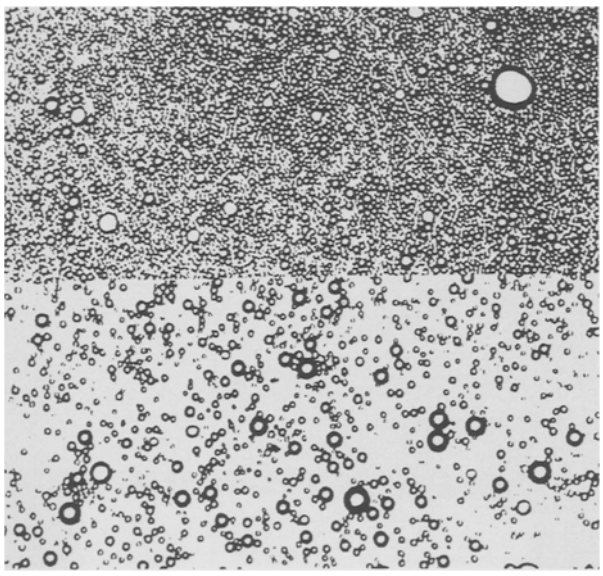

Fig. 1. Oil-in-water emulsion stabilized by RAG-1 emulsan. The lower frame shows the emulsion stabilized by 1 part emulsan per 1000 parts hydrocarbon. The upper frame shows the cream formed by centrifugation of the emulsion.

BIODISPERSAN

The concept that bacteria produce extracellular polymers which can adhere to the surface of liquids and change their surface properties has been extended to solids. *Acinetobacter calcoaceticus* A2 produces an extracellular polymer that binds to ground limestone and thereby prevents floculation in water [12]. When ground limestone is suspended in water, the particles aggregate to form flocs of approx. 25 μm in diameter (Fig. 3, left panel). If the limestone suspension is prepared in the extracellular fluid of *A. calcoaceticus* A2, the particles are dispersed into single units of 3–5 μm (Fig. 3, right panel).

The biopolymer, referred to as biodispersan, responsible for dispersion of finely ground limestone powder, was purified and characterized [13]. An assay for limestone-dispersing activity was developed, based on the settling time for a standard 10% limestone-in-water mixture (Fig. 4). The assay was proportional to biodispersan concentration between 40 and 100 μl/ml. Dispersion was optimum between pH 9 and 12. Biodispersan was produced

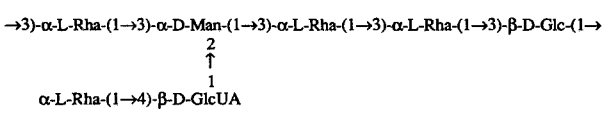

Fig. 2. The chemical structure of *A. calcoaceticus* BD4 capsule polysaccharide.

TABLE 4

Reconstitution of the BD4 emulsan[a]

Fraction	Emulsifying activity (U/mg)
Crude extracellular BD4 fluid	220
Pure BD4 polysaccharide	0
Purified BD4 protein	5
Polysaccharide plus protein	210

[a] Data taken from Kaplan et al. [5].

by strain A2 only after the bacteria completed their exponential phase, and continued during the stationary phase, reaching over 4 g of crude biodispersan per liter.

Purified biodispersan has an average molecular weight of 51400. Titration of the polymer yielded two inflection points: $pK_1 = 3.1$ (1.15 μEq/mg) and $pK_2 = 8.0$ (0.4 μEq/mg). The ^{13}C nuclear magnetic resonance spectrum of biodispersan indicated four methyl groups, four carbonyl C atoms and four signals in the anomeric region (95 to 110 ppm), indicative of the presence of four different monosaccharides. Thin-layer chromatography following acid hydrolysis yielded four sugars: glucosamine, a 6-methyl aminohexose, galactosamine uronic acid and an unidentified aminosugar.

When biodispersan was tested for its ability to be used as a dispersing agent for limestone filler in the paper industry [14], an unexpected result was obtained. The biodispersan not only served as a dispersant, but it was also a surfractant, i.e., it catalyzed the fracturing of limestone into smaller particles. Limestone obtained from the American-Israel Paper Mill was suspended in water (50% w/v) and ground in a laboratory ball mill containing cylindrical Burundem grinding pellets. The average particle diameter following grinding was 3.1 μm in water and 0.1% Dispex N40 (a commercial preparation of polyacrylic acid used as a dispersant in the paper industry) and 1.7 μm in the presence of 0.05% biodispersan (Table 5). Furthermore, the biodispersan-ground limestone was better retained in the paper, 79% compared to 70% and 55%, respectively, for the water control and Dispex experiment. The higher retention and smaller particles with the biodispersan-ground limestone yielded paper with a higher opacity and brightness than the control and Dispex-treated limestones.

Based on the laboratory experiments, several kilograms of biodispersan were prepared in a 15000-liter fermentor, purified and brought to the Paper Mill ball mill for evaluation in grinding limestone (Table 6). Initially, the ball mill was equilibrated at 2 tons per h with water, yielding a particle number average diameter of 2.0 ± 2.4 μm. Then,

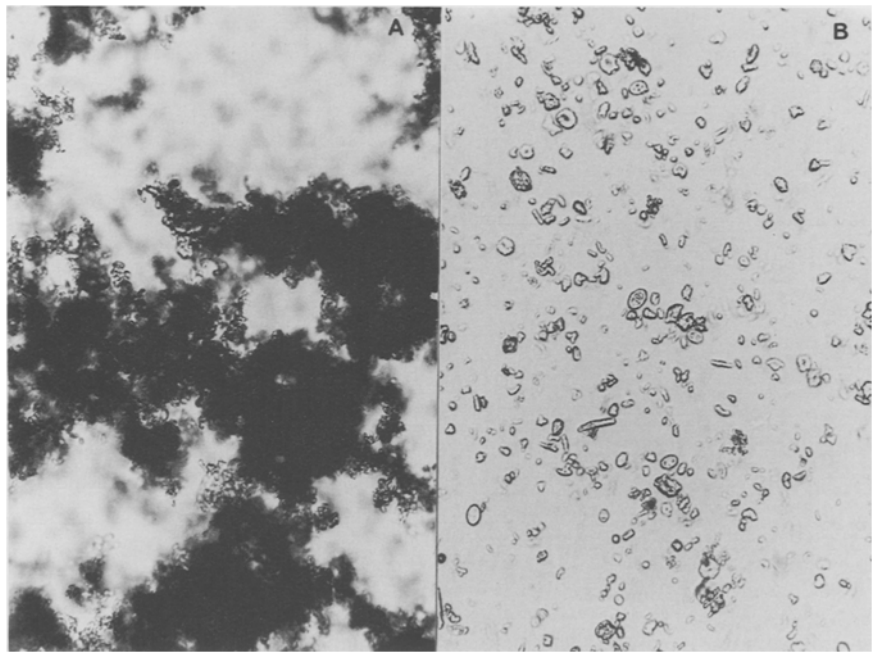

Fig. 3. Deflocculation of ground limestone in water by biodispersan. The right panel shows a photomicrograph of 10% limestone suspension of ground limestone in the extracellular fluid of *A. calcoaceticus* A2. The left panel is 10% limestone in growth medium.

biodispersan was added at a ratio of one part biodispersan to 1000 parts limestone and grinding continued at 2.6 tons per h. When equilibrium was reached, the average particle diameter was reduced to 1.6 ± 1.7 μm. Thus, the grinding was 30% faster, a major saving in energy, with smaller and more uniform particles. When the grinding rate was increased to 2.75 tons per h, the average particle size was slightly smaller than the control.

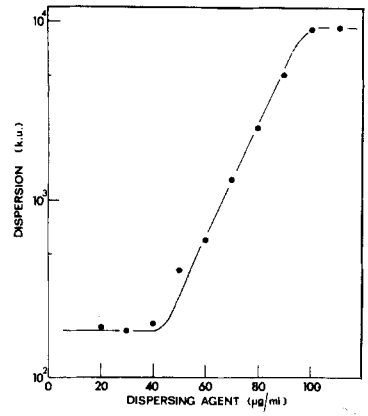

Fig. 4. Dispersion of ground limestone as a function of biodispersan concentration. A 10% suspension of limestone was mixed with different concentrations of purified biodispersan and allowed to stand for 10 min. The upper layer was then diluted in water and the turbidity determined in a Klett photometer.

The mechanism by which biodispersan acts as both a grinding aid and dispersant is not clear. However, based on the chemical data, certain suggestions can be put forth. During the grinding procedure, the pH is between 9 and 9.5. At that pH, biodispersan is an anionic polymer due to the presence of carboxyl groups. We suggest that the polymer enters microdefects in the limestone and lowers the energy necessary to cleave the microfractures. Dispersion itself is not the reason for the surfactant action because Dispex is not a grinding aid, although it is an effective dispersant. When making paper, the pH is adjusted to 7.0. At that pH, the free amino groups of the amino sugars become cationic and assist in the binding to the negatively charged cellulose pulp.

TABLE 5

Paper-making properties of limestone ground in the presence of biodispersan[a]

Parameter	Control	Biodispersan	Dispex
Particle diameter (avg.) (μm)	3.1	1.7	3.1
$CaCO_3$ retained in paper (%)	70	79	55
Opacity (%)	86	88	84
Brightness (%)	86	88	85

[a] A 50% suspension of limestone was ground in laboratory ball mill overnight in water (control), 0.05% biodispersan, and 0.1% Dispex N40.

TABLE 6

Size distribution of limestone ground in the presence of biodispersan[a]

Diameter (μm)	Control (2 T/h)	0.1% Biodispersan	
		(2.6 T/h)	(2.75 T/h)
0.4–0.6	6.3	7.3	6.9
0.6–0.8	5.4	7.3	6.3
0.8–1.0	14.7	19.1	15.5
1.0–1.2	16.3	17.9	15.9
1.2–1.4	14.7	13.2	13.5
1.4–1.6	11.1	9.6	9.5
1.6–1.8	7.2	6.0	7.5
1.8–2.0	6.1	5.8	5.9
Number avg. $\pm$ SD	2.0 ± 2.4	1.6 ± 1.7	1.9 ± 2.3

[a] Grinding was carried out in a continuous flow commercial ball mill, using 40% solids. The particle size distribution was obtained after equilibrium had been reached.

Values are expressed as percentages.

BACILLUS STEAROTHERMOPHILLUS XYLANASE

The production of cellulose pulp from wood chips is a two-step process. Initially, the chips are cooked at high temperature and strong alkaline conditions (Kraft process). During this treatment, most of the lignin is solubilized, yielding a tan pulp. The color that remains is due to residual lignin and lignin derivatives produced during the Kraft process. If the pulp is to be used for manufacturing white paper, diapers or white cardboard, then the pulp must be bleached. The traditional technique for bleaching is the use of chlorine or chlorine dioxide, which oxidizes the aromatic rings of the lignin. However, a significant fraction of the chlorine enters the aromatic rings by substitution reactions, producing the very toxic dioxan and other chlorinated aromatic compounds. Large amounts of these toxic compound are released into the environment causing serious pollution problems. Accordingly, the paper and pulp industry is actively seeking alternative methods for removing the color from unbleached pulp.

Several years ago, Kirk and Yang [7] demonstrated that white rot fungi could significantly reduce the Kappa number (a measure of lignin content) and increase the brightness of unbleached Kraft pulp. Subsequently, it was demonstrated that the biobleaching activity of the fungi was due to lignin peroxidases [6,20]. However, for several technical reasons, the use of fungi for biobleaching is not practical. Also, the use of purified lignin peroxidases, which operate via free radical mechanisms, are not practical because there is only a small net decrease in lignin and the oxidation can weaken the cellulose fibers.

Another enzymatic approach, pioneered by Linko and co-workers in Finland [21], used hemicellulases to detach the lignin from the cellulose fibers. The rationale for this method is that lignohemicellulose is connected to the cellulose by hydrogen bonding of hemicellulose to cellulose. Thus, the hemicellulose acts as a bridge between the lignin and cellulose. By cleaving the hemicellulose with enzymes, the lignin can be solubilized.

By analyzing the problem, together with scientists at the Korsnas paper and pulp mill in Sweden, we came to the conclusion that hemicellulases that are active at about 65 °C and pH 9 would make the process more practical, because lignin is more soluble under these conditions and, more importantly, the large volume of circulating water at the mill is at a high temperature and pH. Accordingly, we isolated by enrichment culture a strain of *B. stearothermophilus* that grew on xylan at pH 9.0 and 65 °C [18]. The extracellular xylanase was purified by adsorption onto carboxymethyl cellulose and desorption in 1 M NaCl.

The purified enzyme, designated xylanase T6, has a calculated molecular weight of 43 811 based on the cloned and sequenced xylanase gene (Shoham, unpublished data). The estimated molecular weight from PAGE was 42 000. The enzyme has a broad pH optimum from 7.5 to 9.0 and a temperature optimum at 65 °C. The enzyme lost no activity at 65 °C for 10 h. Using xylan as the substrate, the enzyme had a V_{max} of 234 units per mg and a K_m of 1.1 mg per ml.

The delignification activity of pure xylanase T6 was tested in the laboratory using partially oxygen-bleached soft wood pulp obtained from the Korsnas paper and pulp company. The net release of lignin was 18% and 10% for 5 U/ml and 2 U/ml, respectively, for 4 h at pH 9 and 65 °C

TABLE 7

Xylanase T6 treatment of softwood Kraft pulp[a]

Treatment	% lignin released	
	Total	Net
Buffer, 2 h	4	–
Buffer, 4 h	5	–
Xylanase, 2 U/ml, 2 h	10	6
Xylanase, 5 U/ml, 2 h	17	13
Xylanase, 2 U/ml, 4 h	15	10
Xylanase, 5 U/ml, 4 h	23	18

[a] Partially oxygen bleached softwood Kraft pulp (Kappa No. = 17.5) was used at a final concentration of 5% dry weight pulp.

[b] pH 9, 65 °C, 20 mM $(NH_4)_2SO_4$.

TABLE 8

Analysis of xylanase T6-treated Kraft pulp

Parameter	Control	Enzyme treated[a]
Kappa No.	17.1	15.4
Viscosity (dm^3/kg)	1061	1072
Pentosan (%)	9.2	7.3
Handsheets		
Brightness (% ISO)	32.6	34.4
Tensile index (Nm/g)	21.2	20.7
Zero-span (Nm/g)	100	103

[a] pH 9.0 and 65 °C for 2 h with 5 U/ml xylanase T6.

(Table 7). Larger quantities (10 g) of the pulp were then treated with 5 U/ml xylanase T6 at pH 9.0 and 65 °C for 2 h and sent to the Korsnas paper mill for analysis (Table 8). Compared to the control (no enzyme), the enzyme reduced the lignin (Kappa No.) and pentosan content without loss in the viscosity of the cellulose. The slight but significant increase in viscosity was probably due to loss of lignohemicellulose, which does not contribute to the viscosity. Handsheets prepared from the pulps demonstrated that the enzyme-treated pulp yielded higher brightness with no significant loss of fiber strength. More significantly, the Cl_2O, Cl_2 required to completely bleach the enzyme-treated pulp was reduced by 35%.

The xylanase T6 treatment is now being optimized and scaled-up. We anticipate that xylanase, as well as possibly other hemicellulases, will soon be used in the pulp industry to reduce or eliminate the use of chlorine-containing compounds for preparation of white pulp.

CONCLUSIONS

The few examples presented here, taken from research performed in my laboratory during the last 15 years, provide additional evidence that bacteria are a rich source of highly specialized polymers, many of which have potential commercial applications. Although modern molecular genetics is a valuable tool for modifying and overproducing proteins, the wonderful diversity of the microbial world remains the major source for discovering new and useful biopolymers. More than ever, the rate-limiting steps in discovering these microbial materials are imagination and techniques for enriching and screening for microorganisms that produce the desired products.

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