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Effects of surface-active chemicals on microbial adhesion

W.K. Whitekettle

Microbiology/Biotechnology Department, Betz Laboratories, Inc., The Woodlands, TX, U.S.A.

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SUMMARY

A simple, continuously circulating fed-batch culture system of microorganisms was designed and used to study the adhesion of mixed microbial cultures to surfaces of 316 stainless steel, Admiralty brass, and wood. The adhesion of the microbes to the surfaces was monitored by scanning electron microscope analysis. Eighteen non-toxic, non-ionic, or anionic surface-active compounds were tested for efficacy as inhibitors of microbial adhesion to stainless steel and wood surfaces. A rating system was devised to correlate efficacy with the degree of biomass adhered to 316 stainless steel, although correlation could not be made with wood. A correlation was also found between the ability of a compound to lower surface tension and its ability to prevent microbial adhesion.

INTRODUCTION

The adhesion of microorganisms to surfaces of various compositions has been shown to have serious consequences in ecological, medical, and industrial situations [3,6–8,12,21]. Microorganisms can readily attach to a wide variety of surfaces in a defined series of steps and in a layering progression that is fairly consistent within a given group of organisms [5,8]. The initial colonizers, frequently specific bacteria, are able to adhere due to their ability to produce exocellular polymers composed primarily of non-ionic and anionic polysaccharides [6,7,10]. Further colonization of the bacterial layer by succeeding microbial types then appears to take place. In addition to the serious ecological and medical implications, these adherent communities, which form layers on surfaces of pipes, heat exchangers, etc., are responsible for tremendous financial losses to industry in the forms of increased fluid flow resistance, loss of heat exchange, and increased biomass deposition. These sessile microbial types are also strongly implicated in microbially induced corrosion [1,9,11,14].

Until the advent of the scanning electron microscope, this adhesion phenomenon was very difficult to study. The scanning electron microscope has become a primary means of researching the types of microorganisms and order of progression of surface colonization by microbial communities [6,15,16,20]. A variety of devices has also been proposed to monitor the progression of surface colonizations in ecological and industrial situations. Some have proven valuable, while others have been overly complex for routine industrial use [4,17,18].

Attempts have been made to eliminate surface-colonizing microorganisms in industrial situations through the use of toxic compounds, such as chlorine and industrial microbiocides. Unlike the situation with planktonic microorganisms, this approach has met with only limited success, as it appears that considerably higher concentrations of toxic materials are needed to inhibit the growth of sessile microbial forms than are required to inhibit these same organisms when in the planktonic state [1,2,9,19–20]. Increasing the concentrations of toxic materials to get at the sessile groups is costly, results in increased environmental burdens, and threatens non-target organisms.

This paper describes a simple and inexpensive fed-batch culture system used to study the adhesion of microorganisms to industrially significant surfaces, and to determine the efficacy of a variety of non-toxic surface-active compounds as inhibitors of microbial adhesion.

MATERIALS AND METHODS

Fed-batch continuous circulation culture systems

Continuous circulation microbial adhesion tanks (MAT) were developed to establish and maintain mixed microbial cultures in a continuous state. MAT consisted of 9.0 l working volume HDPE tanks, drilled and fitted with PVC piping and joints, which incorporated bypass loops having PVC coupon holders (Fig. 1). Coupons of

Correspondence: W.K. Whitekettle, Microbiology/Biotechnology Department, Betz Laboratories, Inc., The Woodlands, TX 77380, U.S.A.

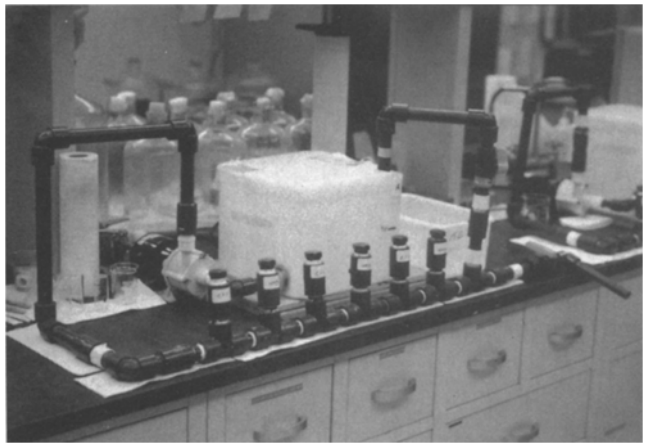


Fig. 1. MAT unit as described in Materials and Methods. High-density polyethylene tank (9.0 l working volume) can be seen with attendant pumping system. PVC bypass loop with coupon holders in foreground.

either wood, Admiralty brass, or 316 stainless steel were fixed to the coupon holders (polyethylene fasteners) and were then lowered into the mainstream of the flowing culture medium (Fig. 2). Pumps used for the continuous circulation of the culture medium were set to a constant flow rate of 37.7 l/min. During the course of each experimental series, 2 l of spent medium was removed from each MAT unit daily and the volume was replaced with an equal volume of fresh culture medium, in order to maintain the fed-batch nature of the cultures. MAT units were sterilized between experiments by circulating hypochlorite solutions at pH 5 until a free chlorine residual of at least 1.0 μg/ml was maintained for 1 h. Sterile conditions were confirmed by observing standard plate counts showing no growth 48 h later.

The culture medium was modelled after a paper mill white water; the composition of the medium is listed in Table 1. The pH of the culture medium was maintained

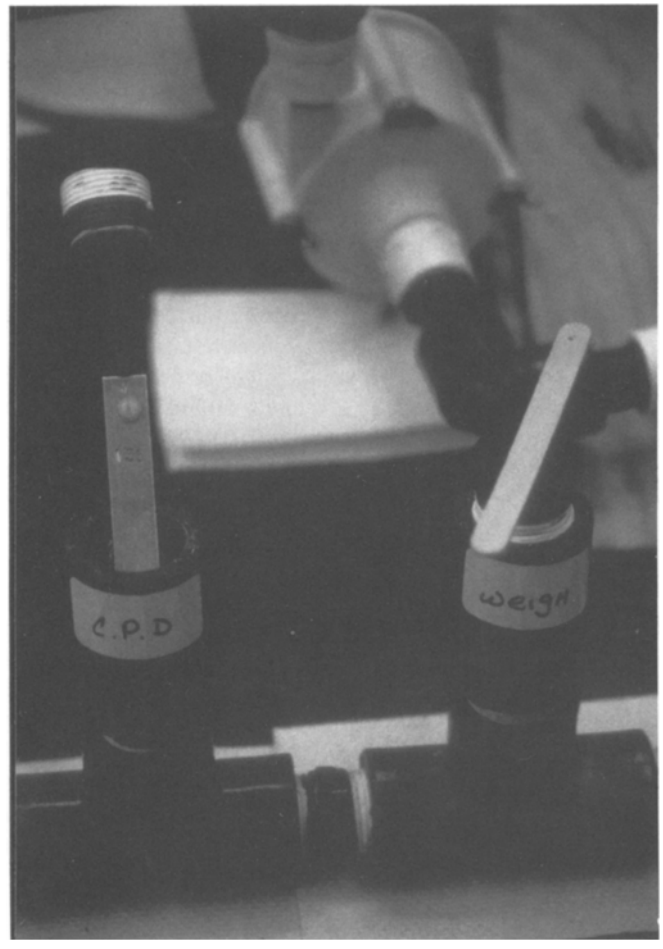


Fig. 2. Close-up photograph of two coupon holders from MAT unit bypass loop. Coupons of 316 stainless steel, brass, or wood were bolted to coupon holders with polyethylene fasteners and lowered into recirculating culture medium.

TABLE 1

Adhesion medium composition[a]

		Per liter
Stock solution A:	$MgSO_4 \cdot 7H_2O$	5.0 g
	K_2HPO_4	200.0 g
	NH_4NO_3	60.0 g
	Citric acid	50.0 g
Stock solution T:	$CaCl_2$	4.4 g
	$ZnSO_4 \cdot 7H_2O$	0.8 g
	$MnCl_2 \cdot 4H_2O$	2.0 g
	$FeCl_3 \cdot 6H_2O$	5.4 g
	$CuSO_4 \cdot 5H_2O$	0.4 g
	H_3BO_3	0.06 g
	Na_2MoO_4	0.04 g
	Conc. HCl	10.0 ml
Final use composition:	Stock solution A	20.0 ml
	Stock solution T	1.0 ml
	Yeast nitrogen base	2.0 g
	Glucose	1.0 g

[a] Water used is sterile, > 16.5 megOHM-CM water. Stock solutions A and T are steam-sterilized at 121 °C for 20 min. Final use solutions are filter-sterilized by passing medium through sterile 0.45-μm pore filters.

between pH 4.5 and 4.8, while the temperature was controlled at 32 °C.

Microbial composition of the fed-batch mixed culture

The inocula used in establishing the fed-batch mixed culture were each generated by producing individual shake flask cultures of the three organisms used in the studies. The medium specified in Table 1 was used to generate the individual batch cultures. *Klebsiella pneumoniae* (Institute of Paper Chemistry (IPC) – 500) was grown in shake flasks at 32 °C to a turbidity of $A_{450} = 0.8$. *Pseudomonas aeruginosa* (ATCC 10145) was grown in shake flasks at 32 °C to a turbidity of $A_{450} = 0.6$. *Aspergillus niger* (I.P.C. 144) was grown on solid culture medium at 28 °C for 5 days. The spores were harvested by gentle washing of the culture surface with sterile distilled water and adjusted to an absorbance of $A_{450} = 1.3$. 20 ml of each of these standardized cultures was then used to inoculate each 9 l MAT unit.

Preparation of coupon samples for scanning electron microscope analysis

Coupons exposed to the culture medium in the bypass loop were removed at designated times and sectioned into approximately 1.5 × 1.5 cm squares. A randomly chosen square was fixed in 0.5% (v/v) glutaraldehyde in potassium phosphate buffer, pH 7.7, for 30 min at 4 °C [13]. The sample was then dehydrated in progressively more concentrated aqueous ethanol solutions with final steps in 50/50 ethanol/amyl acetate and 100% amyl acetate. The samples were critical point dried (Denton critical point dryer apparatus, Cherry Hill, NJ) and sputter-coated using gold leaf (Denton, Cherry Hill, NJ). The scanning electron microscopy analyses were done using an Amray Model 1200 B (Amray, Bedford, MA).

Measurement of attachment to 316 stainless steel, Admiralty brass, and wood surfaces

Coupons of 316 stainless steel, Admiralty brass, and wood (pine) were washed with bleach and acetone solutions. They were then steam-sterilized. These were introduced into the coupon holder loops of duplicate MAT units. Wood, brass, and 316 stainless steel coupons were mixed randomly in both units. Coupons of each type were also placed in uninoculated culture medium for the duration of the experiment to act as controls. Fed-batch cultures were established as described above by inoculating each MAT with cultures of *Klebsiella*, *Pseudomonas*, and *Aspergillus*.

The numbers of microbial cells adhering to the three surface types were determined at t_0 and 6, 12, 24, 48, 72, and 96 h. These were made by direct count in the SEM at either 2000 or 3000 × magnification and the counts reported are the averages of a minimum of 20 randomly chosen fields. The surface of a coupon was scanned at 20 × magnification, marked, and raised to 2000 or 3000 × to obtain a count/field of known area.

Prevention of microbial adhesion to 316 stainless steel and wood by surfactants

MAT units were inoculated and operated as described above. At the time of inoculation, the units were treated with surfactants at an active concentration of 10 μg/ml. Surfactants were primarily anionic or non-ionic in character. One cationic quaternary compound was used. Table 5 lists the surfactants used for this testing. Replacement medium for the treated MAT units contained the same concentration of surfactant. Coupons were removed from the MAT units after 72 h treatment and analysed by SEM as described previously. Inhibition of adhesion was determined by comparison to an untreated control. The surface tension of the recirculating growth medium before and after surfactant dosing was measured with a tensiometer (Keithly Instruments, Inc., Cleveland, OH).

RESULTS AND DISCUSSION

The MAT units as devised proved to be suitable tools for evaluating the adhesion of microorganisms to the test

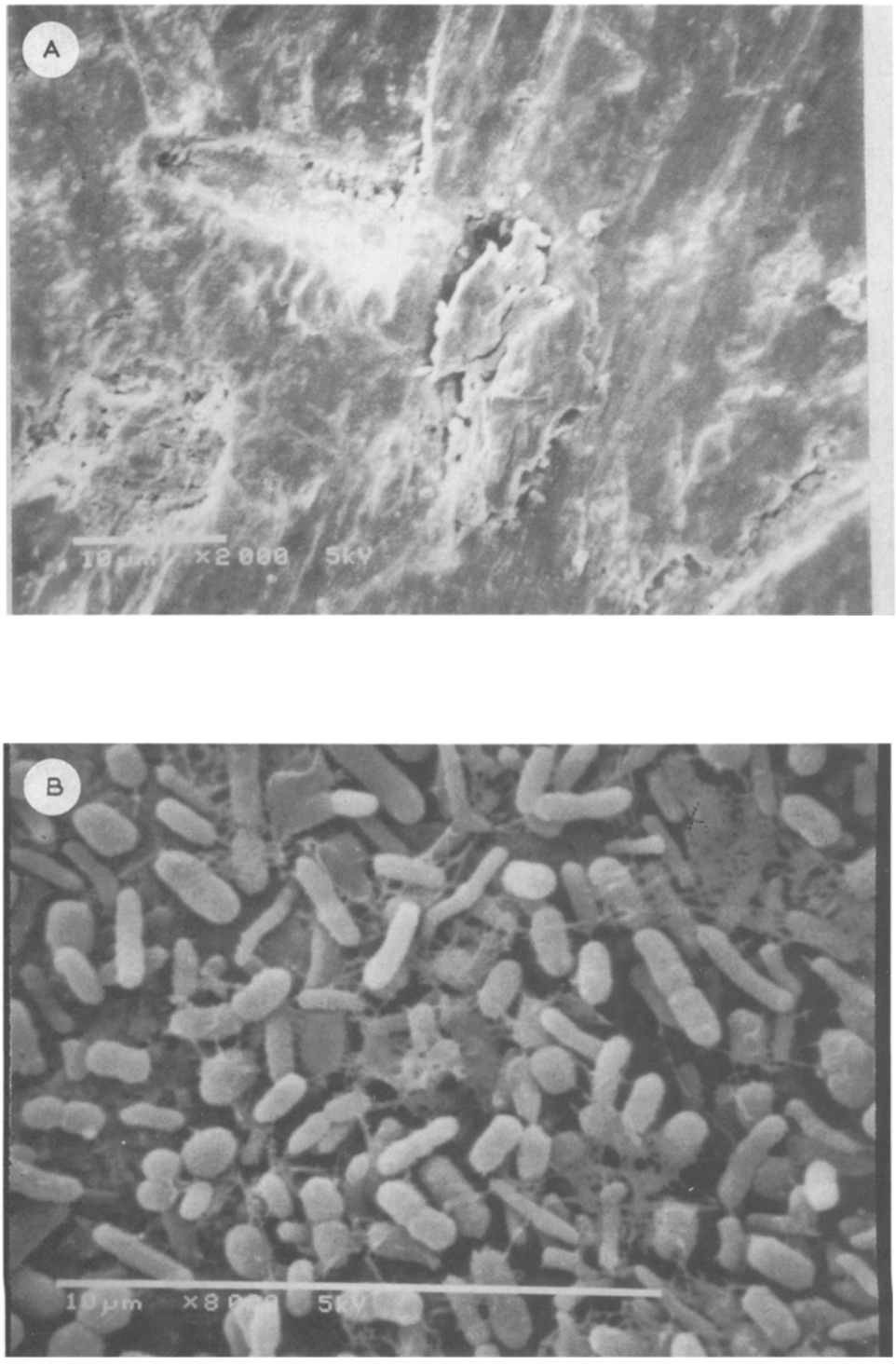


Fig. 3. Scanning electron micrographs of control and MAT-exposed 316 stainless steel coupon surfaces. Exposure was for 24 h. **A.** Control surface presented at 2000× magnification to show overall appearance. **B.** MAT-exposed coupon presented at 8000× magnification to show surface and bacterial/exopolymer detail (Scale bars: 10 μm).

surfaces and for determining the efficacy of surfactants as inhibitors of microbial adhesion. Expensive and complicated devices involving precise machining of pipeline surfaces were not required, nor desired, since much industrial microbial adhesion takes place in areas not influenced by constant or uniform flow characteristics. Reproducible biofilms were obtained with the fed-batch MAT units described.

It is commonly believed that cultures removed from natural environments (in vivo) and maintained by standard laboratory practices (in vitro) readily lose their ability to produce exopolysaccharides and, therefore, their ability to adhere to surfaces [7]. It should be noted that in contrast to this reported information, the in vitro cultures used in this study readily produced glycocalyx (slime) and adhered to the provided surfaces even though these cultures have been laboratory maintained for many years. The *Klebsiella* and *Aspergillus* cultures used in this study have been maintained in our culture collection for over 25 years, having originally been isolated from paper machine systems. The *Pseudomonas* has also been a standard stock culture item available from the American Type Culture Collection for many years. All that seemed to be required for the in vitro bacterial cultures to revert to having sessile properties as a glucose-containing fed-batch environment.

Microbial adhesion to 316 SS, Admiralty brass, and wood surfaces

The bacterial species present in the MAT readily adhered to the 316 stainless steel, Admiralty brass, and wood surfaces present. *Aspergillus* was only able to attach to the wood surface, independently of a bacterial adhesion step. Figs. 3–5 contain representative examples of microbially colonized surfaces of each type after exposure of those surfaces in the MAT for between 24 and 48 h. Controls of each coupon type exposed to the same conditions and sample preparation steps as the culture samples can also be seen in Fig. 3–5. The surface-accumulated cell population determinations are presented in Table 2, along with the average standard planktonic bacterial plate counts performed at the same time the surface samples were removed from the bypass loops for SEM analysis.

Fig. 6 represents the adhesion of bacteria to the three test surfaces over the span of 96 h in the MAT units. Fig. 6 also shows the rate of growth of the planktonic population, which reached a maximum within 24 h and remained at a fixed population density after that time period.

As the data and photographs indicate, microbial attachment to the wood surface was extremely rapid, and heavy surface colonization was accomplished by both bacteria and the mold in under 6 h of exposure. Exopolymer (presumably polysaccharide) production was readily apparent. Subsequent samplings of the wood coupons showed continued layering of bacteria and fungi. It also showed extensive polysaccharide binding to the wood surface.

TABLE 2

Bacterial attachment to coupons

Time (h)	Sessile (cells/cm^2, average)			Planktonic phase cfu/ml
	316 SS	Brass	Wood	
0	0	0	0	1.1×10^6
6	4.5×10^5	0	1.9×10^7	5.2×10^7
12	1.0×10^6	2.5×10^6	4.7×10^7	1.2×10^8
24	7.0×10^6	1.0×10^7	1.2×10^8	1.8×10^8
48	3.4×10^7	1.6×10^7	1.5×10^8	1.7×10^8
72	3.8×10^7	2.0×10^7	–	1.6×10^8
96	8.0×10^7	1.7×10^7	3.2×10^8	1.7×10^8

Admiralty brass showed little surface attachment of bacteria in less than 12 h, but a steady increase occurred in bacterial adhesion in the 12–48-h period of exposure. This rate of adhesion slowed by 72 h and began to show a decline by 96 h exposure in the MAT. This self-limiting surface build-up on Admiralty brass was thought to have resulted from the toxicity of surface-leached Cu^{2+}, Zn^{2+}, or Sn^{2+}, although the specific bacteria used in this study may only produce polymers of limited adhesive strength to Admiralty brass. Preliminary work using SEM energy dispersive X-ray analysis shows compositional changes in the brass surface over time, with loss of Cu^{2+} from the surfaces exposed to bacteria (Table 3). It is speculated that this Cu^{2+} solubilization by the colonizing bacteria may limit the microbial attachment to brass in this experimental setup. This is especially evident in the first 6 h of exposure, when the most dramatic Cu^{2+} loss from the brass takes place. *Aspergillus* appears to be unable to

TABLE 3

Change in surface composition[a] of Admiralty brass over time

Time mat exposed (h)	% Composition		
	Cu	Zn	Sn
6	60.0	31.7	2.3
12	63.2	31.8	1.7
72	61.6	21.6	2.0
6-h control (medium only)	74.3	25.2	0.5
Standard brass composition	71	28	1.0

[a] SEM-EDX analysis of surface metal composition of brass coupons exposed to sterile and contaminated MAT units.

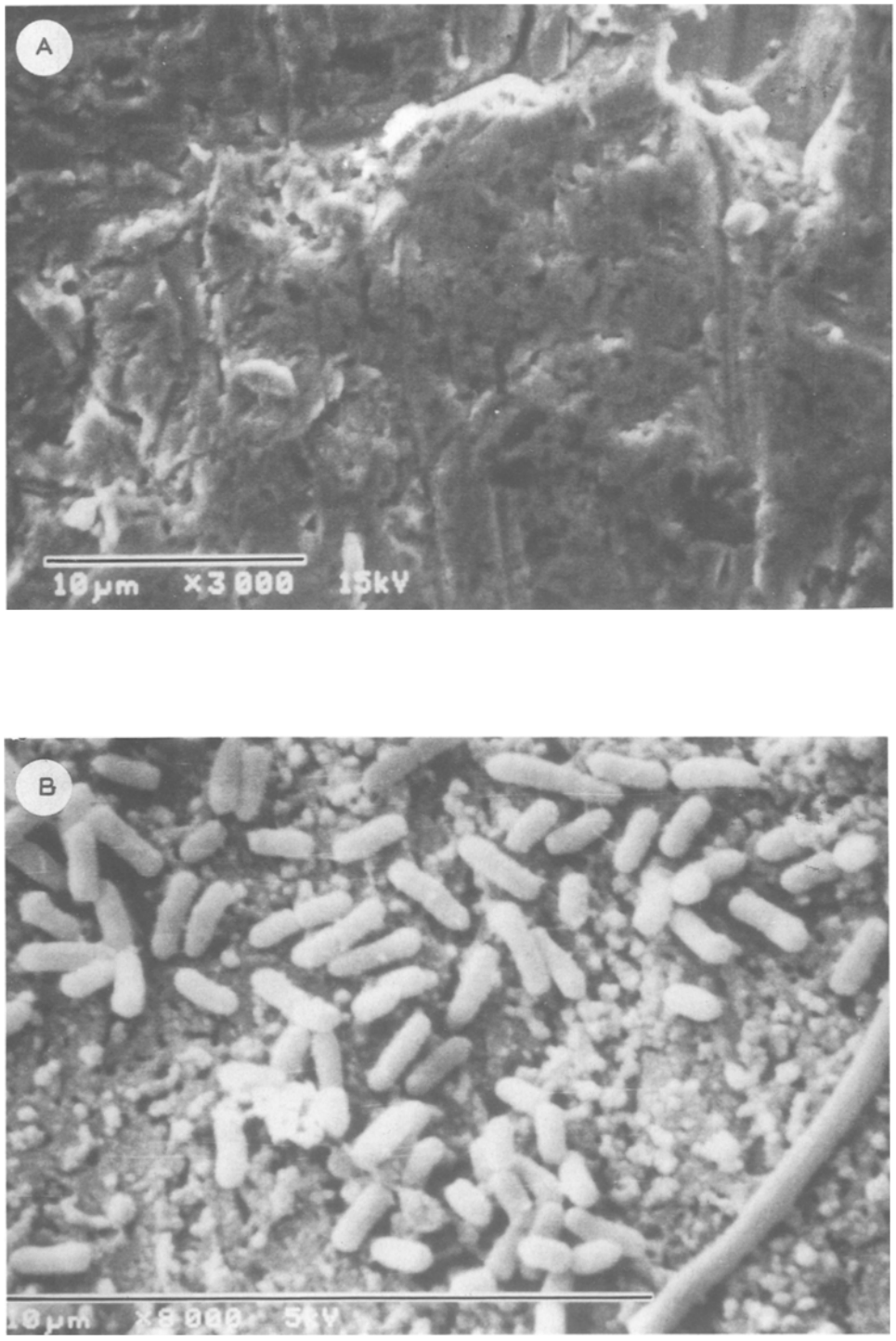


Fig. 4. Scanning electron micrographs of control and MAT-exposed Admiralty brass coupon surfaces. Exposures was for 48 h. **A.** Control surface presented at 3000 × magnification. **B.** MAT-exposed coupon surface presented at 8000 × magnification (Scale bars: 10 μm).

adhere to brass within the first 96 h of exposure of the brass surface, even though bacteria have initially colonized the surface.

The 316 stainless steel surface appears to be readily colonized within 6–12 h of exposure to the fed-batch cultures. The adhesion was predominantly bacterial, and *Aspergillus* spores/mycelium were not present. Unlike the brass, the bacterial colonization of 316 stainless steel continued at a steady rate throughout the 96-h study. As with the wood surface, sufficient surface colonization of the 316 stainless steel had taken place in 3 days to make that time frame a reliable one for evaluating the effects of test compounds on microbial adhesion to surfaces.

Effects of chemicals on microbial adhesion to 316 stainless steel and wood

A variety of compounds known or thought to possess surface-active properties were tested for their ability to prevent the adhesion of mixed microbial populations to wood and stainless steel. The studies centered primarily around non-ionic or anionic compounds of differing chemical natures. Cationic surfactants were not extensively utilized due to their known toxicity and due to the desire to determine whether non-toxic surfactants could prove effective in inhibiting microbial attachment to the stated surfaces. Representative surface areas were observed after 3 days exposure of the surfaces to the surfactant and the results were compared with controls. Attached microbial cells per unit surface area were calculated for each test compound and control. Photographic documentation was made with each surface type and a correlation between the number of attached cells per square centimeter and the photographic representation was made. The devised rating system for 316 stainless steel can be found in Table 4. Fig. 7 shows the photographic appearance of each of the attached count ratings from Table 4. This rating system was utilized for the relatively 'flat' 316 stainless steel surface exhibiting discrete attached units (i.e., individual bacterial cells). A rating system could not be devised for the wood surfaces as there is excessive three-dimensional structure, precluding accurate area measurements, and due to the predominance of *Aspergillus* hyphae (non-discrete units) as the adhering cellular type (Fig. 8). Other types of biomass determinations must be pursued to get accurate data for all surface types, but particularly for the surface of wood.

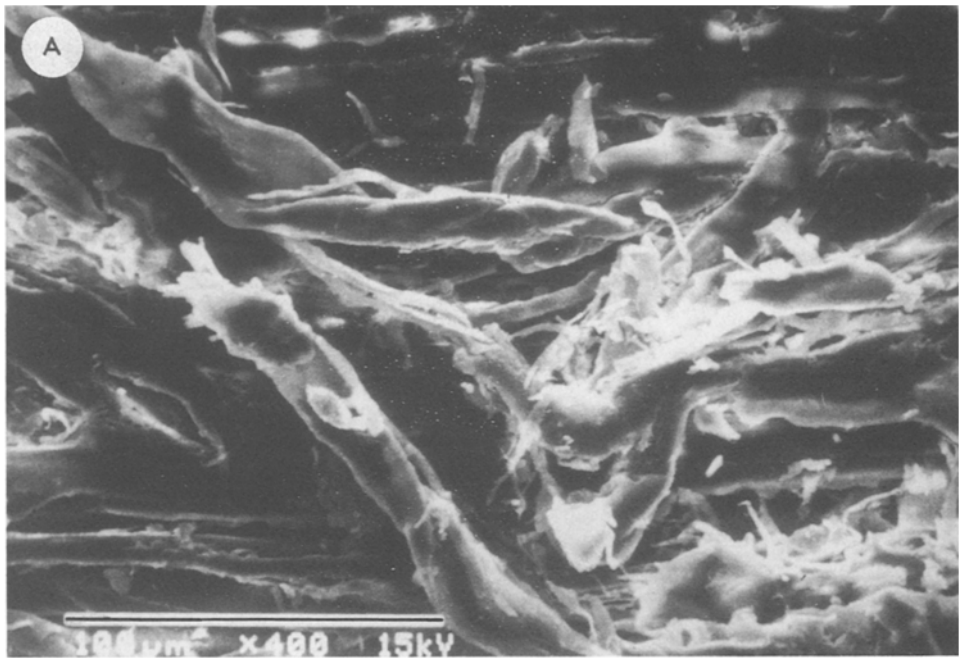
The surfactants tested and their effects on microbial adhesion to 316 stainless steel can be found in Table 5. Excellent efficacy was achieved with many of the non-ionic surfactants, particularly the experimental DP-1200 series dispersants. These materials all gave better than 90% inhibition of microbial adhesion, when compared with untreated controls. The anionic compounds did not show any significant inhibition of adhesion. The only cationic compound tested, a quaternary ammonium salt, exhibited excellent efficacy, but the performance is also due to the toxic nature of the compound. The MAT unit receiving the cationic compound never exhibited growth of the mixed culture, in contrast to all of the tests with the other compounds. The planktonic count in the MAT always exceeded 10^8 cfu/ml within 24 h of inoculation, except in the case of the quaternary ammonium salt, where the planktonic count was only 2×10^2 cfu/ml. The quaternary ammonium compound prevented microbial adhesion primarily by toxicity to the unattached microorganisms, whereas the non-ionic (and in isolated cases, anionic) compounds demonstrated their efficacy via non-toxic surfactant properties. The lack of toxicity of the other actives tested was demonstrated by the rapid growth of planktonic populations in their presence. The low toxicity of these surfactants and the general lack of surface efficacy leads to the contention that a toxic component will be necessary to inhibit the total microbial activity in any industrial system, even if much of the microbial population with surface attachment propensities can be inhibited from becoming sessile. Further evidence for this contention has been shown in that studies directed at removing sessile organisms from surfaces, rather than preventing their attachment, is extremely difficult without the use of either extreme conditions or toxic compounds [2,9,19,20].

The data in Table 5 also show a strong correlation between the efficacy of compounds and their ability to lower the surface tension of the aqueous environment. Those compounds capable of reducing surface tension by ≥ 20 mN/m showed the greatest efficacy as inhibitors of microbial adhesion. Most of these compounds were also non-ionic in nature.

TABLE 4

Adhesion of bacteria to 316 stainless steel. Correlation of efficacy rating to bacterial count and SEM photographs

Surface bacteria count (cells/cm^2)	Efficacy rating	% Reduction in adhered biomass
10^8	0	0
10^7	1	90
10^6	2	99
10^5	3	99.9


A
x400
15kV


B

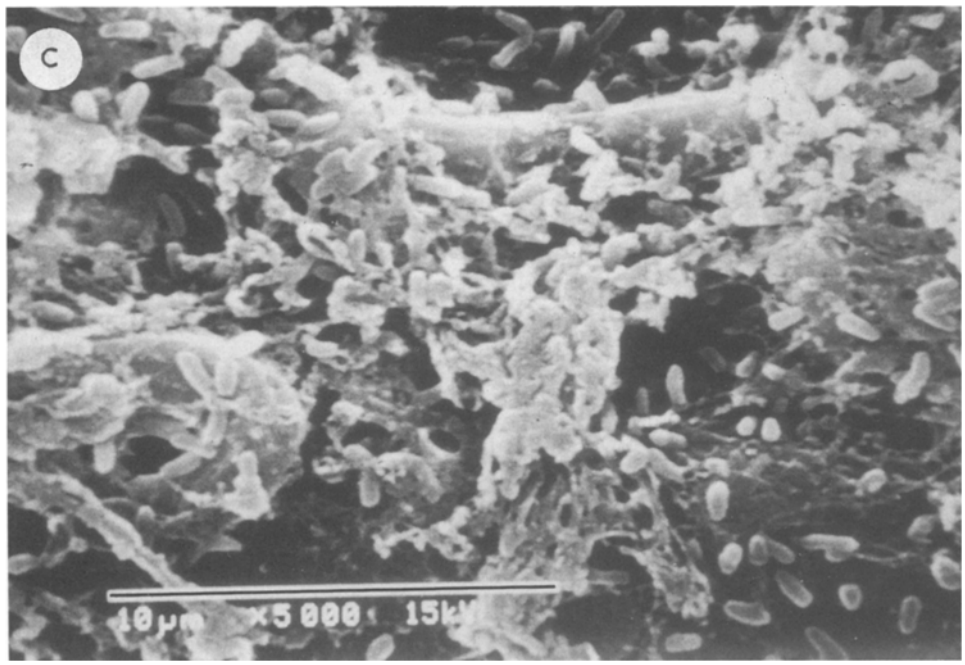


Fig. 5. Scanning electron micrographs of control and MAT-exposed wood coupon surfaces. Exposure was for 24 h. **A.** Control surface presented at 400 × magnification (Scale bar: 100 μm). **B.** MAT-exposed coupon surface presented at 400 × magnification to show mold adhesion (Scale bar: 100 μm), and **C.** 5000 × magnification to show bacterial attachment (Scale bar: 10 μm).

TABLE 5

Efficacy of surface-active compounds against bioadhesion to steel

Compound	Ionic character	Efficacy rating	Surface tension reduction (mN/m)
Emulsan (lipopolysaccharide)	Anionic	0	0.0
Disodium ethylene diaminetetraacetate (EDTA)	Anionic	0	0.2
Polyacrylic acid	Anionic	0	0.8
Sodium lignosulfonate	Anionic	0	1.5
Poly (maleic anhydride) co-diiso-butylene	Anionic	0	5.4
Sodium lauryl sulfate	Anionic	1	7.0
Commercial degreaser	Unknown	1	12.0
Polyvinyl alcohol	Nonionic	1	13.0
Methylcellulose	Nonionic	1	13.0
Polyethylene glycol ether of secondary alcohol	Nonionic	1	20.6
DP-1254A	Nonionic	2	20.8
DP-1254B	Nonionic	2	27.2
Phosphate ester, acid form	Anionic	2	20.4
DP-1253	Nonionic	2	23.7
DP-1252	Nonionic	3	20.0
DP-1255	Nonionic	3	25.0
DP-1223	Nonionic	3	29.0
Alkyl, dimethyl benzyl ammonium chloride	Cationic	3	26.0

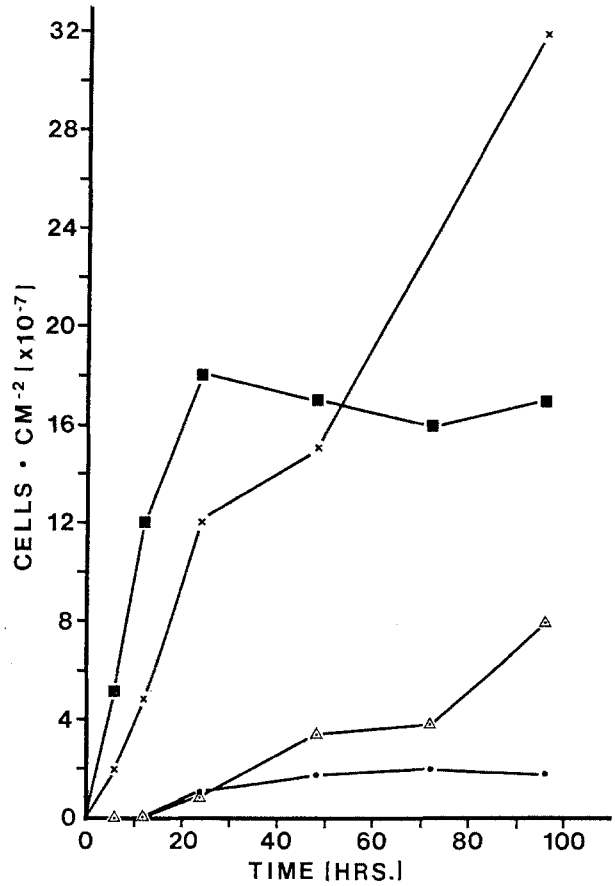


Fig. 6. Adhesion of bacteria to three test surfaces over the course of 96 h. Planktonic bacteria in recirculating medium, shown for comparison purposes: (●) brass; (△) 316SS; (×) wood; (□) planktonic. Planktonic count in cfu/ml. All other units are cells/cm^2.

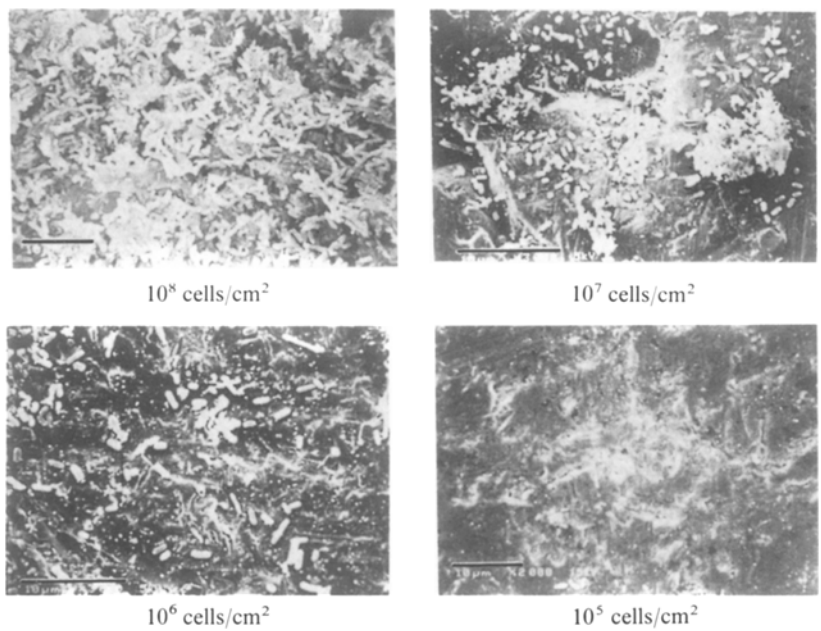


Fig. 7. Scanning electron micrographs of adhesion of bacteria to 316 stainless steel. Photographs represent averaged bacterial counts per square centimeter and are used for rating efficacy of surfactants as microbial adhesion inhibitors (see Table 4). Scale bars: 10 μm.

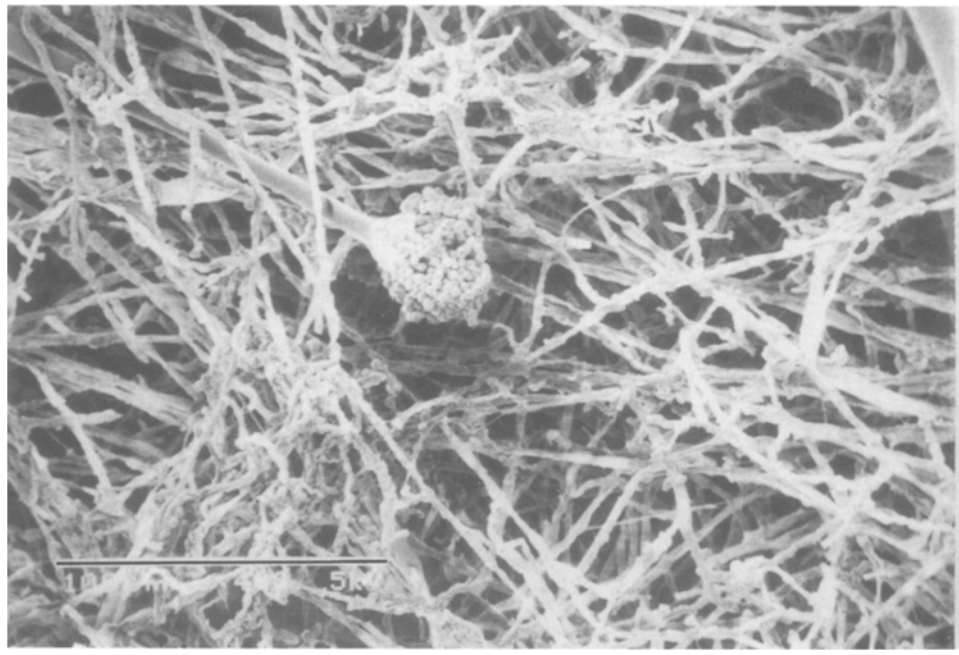


Fig. 8. Scanning electron micrograph of *Aspergillus* hyphae on wood coupon surface at 400 × magnification. Exposure of coupon surfaces to MAT was for 5 days. Scale bar: 100 μm.

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