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Bioluminescent Strains of *E. coli* for the Assay of Biocides

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It has been known since the 1940's that the luminous intensity of bioluminescent bacteria is attenuated by exposure to toxic agents (Johnson *et al.* 1954; Johnson *et al.* 1942; Johnson *et al.* 1945; Bulich and Greene 1978). Like the naturally occurring organisms, bioluminescent strains of *E. coli* produced by re–combinant DNA technology also diminish their light output upon challenge with toxic materials (Herson *et al.* 1988).

Biocides are used in numerous industries to prevent microbial growth such as in cooling towers and cutting fluids. Their concentrations must be carefully monitored to assure that ef-ficacious levels are maintained. High concentrations of active biocides may, however, present disposal problems or local hazards to personnel and the environment.

For these reasons, rapid and inexpensive assays for biocidal activity are desirable. The response of bioluminescent strains of *E. coli* to several biocides has been studied to determine their suitability for assaying these compounds in water and in oil-based cutting fluids.

MATERIALS AND METHODS

E. coli ED8654 carrying the plasmid pVFC, which contains the entire *lux* region from *V. Fischeri* (Engebrecht and Silverman 1984; Engebrecht *et al.* 1983) cloned into the Bam H1 site of the tet^r gene of pBR 322, was obtained from Dr. Thomas O. Baldwin. The plasmid was transferred to three other host strains, AB1157, JC9934, and CSH71, which support

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luminescence without the addition of exogenous aldehyde or reduced FMN.

Luminescence attenuation assays are performed by rehydrating a vial of lyophilized bacteria with 1 mL of distilled water and incubating at room temperature (25°C ± 3°C) for 10 min. The reconstituted culture is stored on ice during the assays and maintains relatively constant light output for several hours

The assays are performed by adding 10 µL reconstituted culture, 100 µL of 10% NaCl, and 900 µL of distilled water to a plastic luminometer cuvette. The contents are mixed and allowed to equilibrate for 5 minutes at room temperature. The initial light output (in arbitrary units) is then read with the luminometer. A 100 µL aliquot of distilled water or aqueous toxicant solution is added after 1 min, and in the case of multiple assays, the tubes are set up at 1 min intervals to allow for mixing, reading, etc. Four different concentrations of each toxicant are assayed in each experiment. The concentrations reported in the tables under Results are the final concentrations in the cuvettes. The concentrations of the test solutions are 11 times higher.

The light output is recorded after an additional 10 min incubation at room temperature. Since the light output of the reconstituted culture changes slowly with time in the cuvette, it is necessary to run a water blank simultaneously with the toxin samples and to normalize the data. However, a single reconstituted culture may be used for several hours at the least.

The bioluminescence attenuation data were converted to EC_{50}'s (Effective Concentration for 50% reduction in bioluminescent intensity) by calculating the Gamma (Γ_1) function, which is defined as the percent decrease in light emission divided by the residual percent light emission (Johnson *et al.* 1954; Bulich and Greene 1978). The logarithms of the gamma values were then regressed on the logarithms of the toxicant concentrations. These operations converted the S-shaped response curves to linear relations. The EC_{50}'s were then calculated from the resulting regression equations ($\Gamma_1 = 0$ at the EC_{50}).

The data from each assay tube were normalized by dividing each tube's initial and final light levels by the initial and final emission levels of the control tube to correct for any change of the luminescence of the cells over time.

Stock solutions of biocides and other organic compounds were made up in distilled water and diluted by two or ten fold increments for assay. The biocides, their active ingredients and their manufacturers are listed in Table 1. Based on their manufacturers' data, the percentages of active ingredient of the biocides are as follows: Kathon 886 MW, 14%; Proxel CRL, 33%; Bioban P1487, 70-80%; Grotan BK, 78.5%; and Omadine, Na, 40%. The biocides were diluted on a volume/ volume basis, and the data are presented as concentrations in parts per million (PPM) v/v of these stock concentrates.

The three cutting fluids used in this investigation and their manufacturers are listed in Table 2. They were diluted to 5% v/v with distilled water for these experiments.

RESULTS AND DISCUSSION

The panel of four strains of bioluminescent *E. coli* was initially tested against the five biocides dissolved in distilled water to approximate conditions in cooling towers and waste water disposal systems. The resulting data are in Table 3.

In the metal working industry, biocides are also added to cutting fluids to prevent microbial growth. Solutions of the five biocides were made up in 5% v/v distilled water dispersions of several oil-based cutting fluid concentrates. Cutting fluids at this concentration correspond to usual shop practice and are not toxic to the cells. Strain ED8654 was selected for these experiments. The data from these studies are presented in Table 4.

These experiments clearly demonstrate that bioluminescent strains of *E. coli* respond rapidly and sensitively to concentrations of biocides in the part per million range. These levels are well below those employed in cooling towers and cutting fluids to inhibit microbial growth. Consequently, assays using these strains should be useful in the the metalworking and air conditioning industries.

Table 1. Biocides

Trade names	Chemical names	Manufacturers
Kathon 886 MW	2-methyl-4-isothiazolin-3-one + 5-chloro-2-methyl-4-isothiazolin -3-one	Rohm & Haas Stamford, CT.
Proxel CRL	1,2-benzoisothiazolin-3-one	Zeneca Biocides Wilmington, DE
Bioban P1487	4-(2-nitrobutyl)morpholine + 4,4-(2-ethyl-nitrotrimethylene) dimorpholine	Angus Chemical Northbrook, IL
Grotan BK	hexahydro-1,3,5,-tris(2-hydroxy-ethyl)-s-triazine	L & F Canada Aurora, Ontario
Omadine, Na	1-hydroxy-2-(1H)-pyridinethione, Na salt	Olin Corp. Stamford, CT

Table 2 Cutting fluids

Trade names	Manufacturers
Add Cool	Ashland Chemical Co., Dublin, OH
Irmco	International Refining and Manufacturing Corporation, Evanston, IL
Ironsides	D. A. Stuart, Willowbrook, IL

Considerable variation is seen in the activity of the biocides with these four strains of *E. coli*. Kathon and Proxel appear to be the most toxic. While the apparent toxicity is lower when the agents are dissolved in cutting fluids, the ED8654 strain is still quite sensitive to the biocides, especially to Kathon. This lowered sensitivity may reflect partitioning of the biocides into the oil of the cutting fluids, thus decreasing the concentration in the aqueous phase. However, the altered sensitivity due to the cutting oils varies the biocide, the largest decrease (approximately 130-fold) occurring for Bioban with only a slight change for Grotan and an intermediate change (approximately 10-fold) for Kathon. These decreases in apparent toxicity may have implications not only for the utility of this assay, but for the efficacy of the biocides in cutting fluids where their purpose is to inhibit bacterial growth.

The strains also vary in sensitivity to the various agents, suggesting that specific strains may be selected for assaying particular toxicants. Strain JC9934 seems to be the most sensitive to all of the biocides under these conditions.

Based on these data, an assay for biocidal activity using these bioluminescent strains of *E. coli* appears to be feasible. This assay may be useful not only in determining the concentration of biocides in wastewater, but also in determining optimal biocide concentrations in the shop on a real-time basis.

Table 3. Sensitivity of strains to biocides in distilled water

Strains	EC50 (ppm)				
	Bioban P1487	Grotan BK	Kathon 886	Omadine, Na	Proxel CRLI XL
AB1157	4.00	12.0	0.47	1.40	0.30
CSH71	0.29	15.0	0.22	0.38	0.06
ED8654	2.00	160.	0.30	1.50	0.23
JC9934	0.08	17.0	0.15	0.20	0.03

Table 4. Sensitivity of strain ED8654 to biocides in cutting fluids

Cutting Fluids	EC50 (ppm)		
	Bioban P1487	Grotan BK	Kathon 886
Add Cool	168	234	4.10
IRMCO	241	101	3.60
Ironsides	68	140	4.80

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