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Rapid Separation and Quantitation of Mixed Microorganisms by Filtration and Bioluminescence (42388)

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Abstract. A membrane filtration/bioluminescence system was developed for the differentiation and quantitation of mixed populations of microorganisms. Samples containing microorganisms were filtered through two membrane filters of descending pore size. The microorganisms retained on the filter contain ATP that can be extracted and measured on the filter via the firefly luciferase–luciferin bioluminescence assay. Results, obtained in less than 20 min, show a good correlation ($r \geq 0.95$) between the light produced and the number of organisms in the sample. Using these techniques, *Escherichia coli* can be separated from yeast or mold and measured in samples containing both microorganisms. When lysostaphin is used to selectively lyse Staphylococci on the filter, the specific quantification of these bacteria among other microorganisms can also be accomplished. The filtration/bioluminescence technique offers the potential of being a rapid and sensitive method to differentiate and detect microorganisms, by selective sizing or lysing, in a variety of samples. © 1986 Society for Experimental Biology and Medicine.

The bioluminescent assay of ATP with the firefly luciferase–luciferin reaction (1) has been used as a rapid and sensitive method for the enumeration of microorganisms and biomass (2, 3). This technique is more efficient than the conventional culturing techniques which require tedious labor and lengthy incubation time. The extracted microbial ATP, measured with the bioluminescence assay, has been shown (4, 5) to be related to the number of viable cells present in the sample. The measurement of microbial ATP, however, is often affected by the presence of nonmicrobial ATP (6). A new technique which combines bioluminescence assay with the filtration procedure is developed in this study. The filtration step is included to concentrate cells and to minimize the effects of nonmicrobial ATP and other soluble substances, thereby lowering the detection limit of the bioluminescence assay (7). During the filtering process, bacteria in samples can be differentiated and separated from yeast or mold by cell sizing, such that each cell type is retained on its respective filter of discriminating pore size. The filtration technique, with the aid of lysostaphin (8), can also be used to separate Staphylococci from other types of bacteria collected on the filter. Therefore, the coupled filtration/bioluminescence (F/B) system provides a sensitive and rapid method for separating and quantitating different types of microorganisms, and potentially can be used to detect microbial cells in a variety of environmental, industrial, and clinical samples.

Material and Methods. *Microorganism cultures.* Microorganism cultures were obtained from the American Type Culture Collection (Rockville, Md.). Microbial cultures harvested at their stationary phase were used for the experiments. Consistent cellular ATP content can be obtained when cells are at a stable physiological state. The colony-forming units (CFU) for *Bacillus cereus, Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) were enumerated on Trypticase soy agar plates (BBL Microbiological Systems, Cockeysville, Md.). The plates were aerobically cultured at 37°C. Yeast (*Saccharomyces cerevisiae* var *ellipsoides*) and mold (*Aspergillus niger*) were inoculated on potato dextrose agar (DIFCO, Detroit, Mich.). Chloramphenicol and chlorotetracyclin–HCl, 100 mg each, were added to 1 liter of potato dextrose agar as described by Koburger (9). The potato dextrose agar plates were counted for CFU after a 48-hr incubation at 25°C. Trypticase soy broth and sabouraud liquid (modified) broth, both from BBL, were used to start the microbial cultures. The stock microorganism culture was used to prepare the filtration samples when it grew to 10^8–10^9 CFU in broth. Serial dilutions from an aliquot of the stock broth were made in 0.0003 *M*

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phosphate buffer (pH 7.3). An array of samples with a 10-fold decrease in microbial concentration was prepared for filtration.

Reagents. Bioluminescent reagent kits designed for the measurement of ATP in microbial cells can be obtained from several commercial sources, such as Analytical Luminescence Laboratory, Inc. (San Diego, Calif.), Boehringer Mannheim GMBH-Biochemica (Mannheim, W. Germany), and LUMAC Inc. Westlake Village, Calif.). Reagents used in this study were obtained from Packard Instrument Co., Inc. (Downers Grove, Ill.). The reagents were prepared and used per manufacturer's instruction. The kit consisted of a firefly luciferase–luciferin preparation (PICOZYME F), a lysing reagent for microbial cells (PICOEX B), and ATP for calibration (PICOCHEC) and internal standardization. A buffer solution (PICOCHEC Buffer) was used to prepare lyophilized reagents.

Lysostaphin (Sigma, St. Louis, Mo.), an enzyme from *Staphylococcus staphylolyticus,* was used as the lysing agent for staphylococcal cells. An aliquot of 150 μl of lysostaphin (10 units/ml) was added to the filter on which bacterial cells were collected (see *Filtration procedure*). The filter was incubated with lysostaphin for 10 min at 37°C to achieve maximal extraction of staphylococcal ATP.

Filtration procedure. A schematic diagram depicting the complete F/B system is presented in Fig. 1. Filtration samples were prepared by diluting and/or mixing known pure cultures of organisms to different concentrations with phosphate buffer. A 10-ml sample, in a sterile syringe, was pushed (manually or by a syringe pump) upward at a rate of about 2.5 ml/min through two filters connected in tandem. The polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.) were housed in different filter holders. A 25-mm diameter filter was housed in the Swin-Lok holder (Nuclepore Corp.) and a 13-mm filter was housed in the Swinney holder (Gelman Sciences, Ann Arbor, Mich.). During filtration, the larger microorganisms (i.e., yeast or mold) were retained by the larger pore size filter (2- or 3-μm pore, 25-mm diameter), while the bacteria passing through the upstream filter were con-

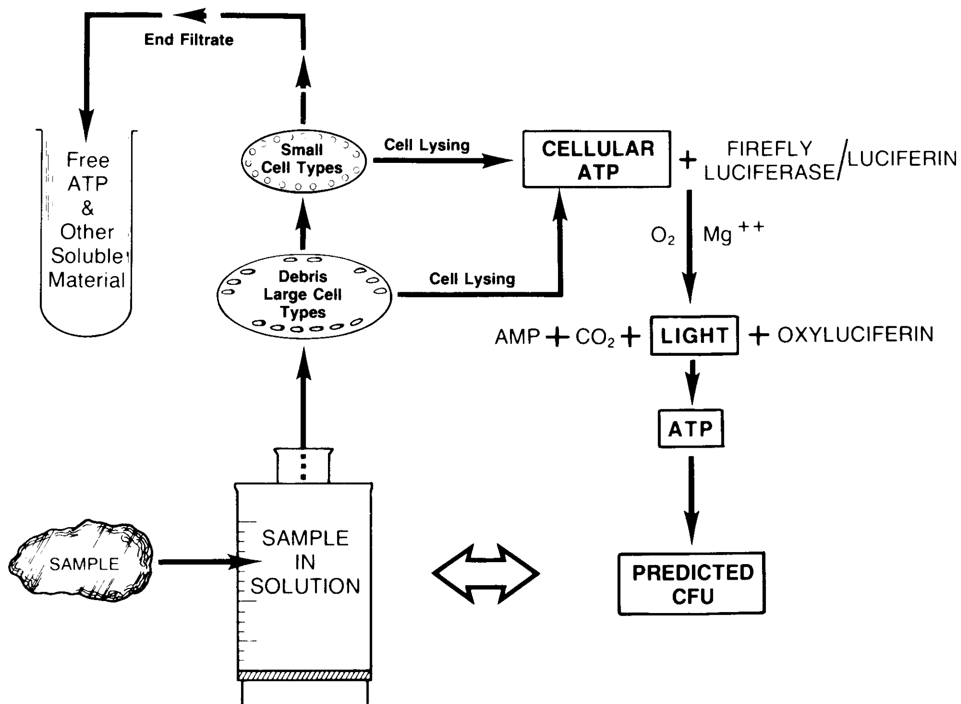

FIG. 1. Schematic drawing of the filtration/bioluminescence (F/B) method.

centrated on the small pore size filter (0.4-μm pore, 13-mm diameter). Soluble ATP and other interfering substances which escaped both filters were voided as the end filtrate. The filters were washed with the phosphate buffer prior to their retrieval for bioluminescence assay. However, the washing was found not to be essential because the number of microorganisms as determined from the measured ATP indicated that there was no residual soluble (nonmicrobial) ATP on the filter.

Bioluminescence assay. The filters were assayed for ATP with the firefly luciferase/luciferin–ATP bioluminescence reaction, and the light response was measured by a luminometer. A luminometer is a photometer specifically designed to measure bioluminescence and/or chemiluminescence. Luminometers vary from a simple manual mode type instrument to a microprocessor-controlled, fully automated system. A PICOLITE Model 6500 luminometer (Packard Instrument Co., Inc.) was used in this study.

The retrieved filter was placed in a 17 by 60-mm glass vial (Kimble–Brockway Tubular Products, Parkersburg, W. Va.), which was then inserted into the sample carrier of the luminometer. As the vial was moved into the photomultiplier detection site, a series of luminescence reagents was automatically injected into the vial. First, 150 μl PICOEX B reagent was added to the filter. The cells were lysed for 2 min at room temperature to release ATP as specific substrate for the firefly luciferase. Second, 100 μl PICOZYME F reagent was added to react with the extracted cellular ATP to elicit a bioluminescence response. Third, the internal standard (100 μl of 10^{-7} *M* ATP) was added for accurate calibration of the light response.

The light response was integrated for 30 sec and translated to a corresponding microorganism level using a standard curve constructed previously. The data obtained from duplicate samples of a minimum of two experiments were reduced and interpreted by an HP-85 computer (Hewlett Packard, Palo Alto, Calif.) which interfaced with the luminometer.

Results. *Bioluminescent standard curve.* A linear correlation ($r \geq 0.95$) between the CFU and ATP concentration is shown in Fig. 2 for each of the microorganisms used in this study. These standard curves were used to interpret ATP data obtained from filters to CFU levels of microorganisms in the sample.

Differentiation and detection of E. coli from yeast. The results of separation and quantitation of *E. coli* and yeast by the F/B method are given in Figs. 3 and 4. In Fig. 3, the CFU levels interpreted from ATP concentrations agree with the CFU as counted on aerobic plates; yeast remained at 10^3 CFU for each sample and *E. coli* reflected the same increasing CFU levels for samples a through e. The microbial spectrum of the samples (Fig. 4) was again accurately determined by the F/B method when each sample was inoculated with increasing numbers of both *E. coli* and yeast.

Differentiation and detection of E. coli from mold. The results of separation and measurement of *E. coli* and mold in various samples are shown in Fig. 5 (A and B). Two different experimental designs are described in the figure legend. The results obtained from both experiments showed that CFU determined by the F/B method correlated well with the CFU enumerated from aerobic plates.

Differentiation and detection of S. aureus with lysostaphin. The F/B method was used to quantitate staphylococcal cells mixed with *E. coli* in a sample (Fig. 6). Lysostaphin was used to selectively lyse *S. aureus,* and the number of *S. aureus* was interpreted from the bioluminescent standard curve (Fig. 2E). Excellent correlation ($r = 0.994$) was found between the originally inoculated level of *S. aureus* and the *S. aureus* concentration predicted from ATP. The presence of *E. coli* in the sample did not contribute additional ATP to be translated into *S. aureus* CFU.

Discussion. A rapid and sensitive method for the separation and enumeration of microorganisms in aqueous samples is presented. The quantitation of microbial cells can be accomplished in a single procedure by passing samples through membrane filters. The microorganisms are concentrated on the filters, and the cellular ATP is extracted for assaying with bioluminescence reaction. The resulting light response is quantified directly on the filter, thereby enhancing the sensitivity of the ATP–bioluminescence assay. Other assay procedures can be easily incorporated into different steps of the F/B method to expand the applicability of this method. For instance, in this study, lysostaphin was added to the 0.4-

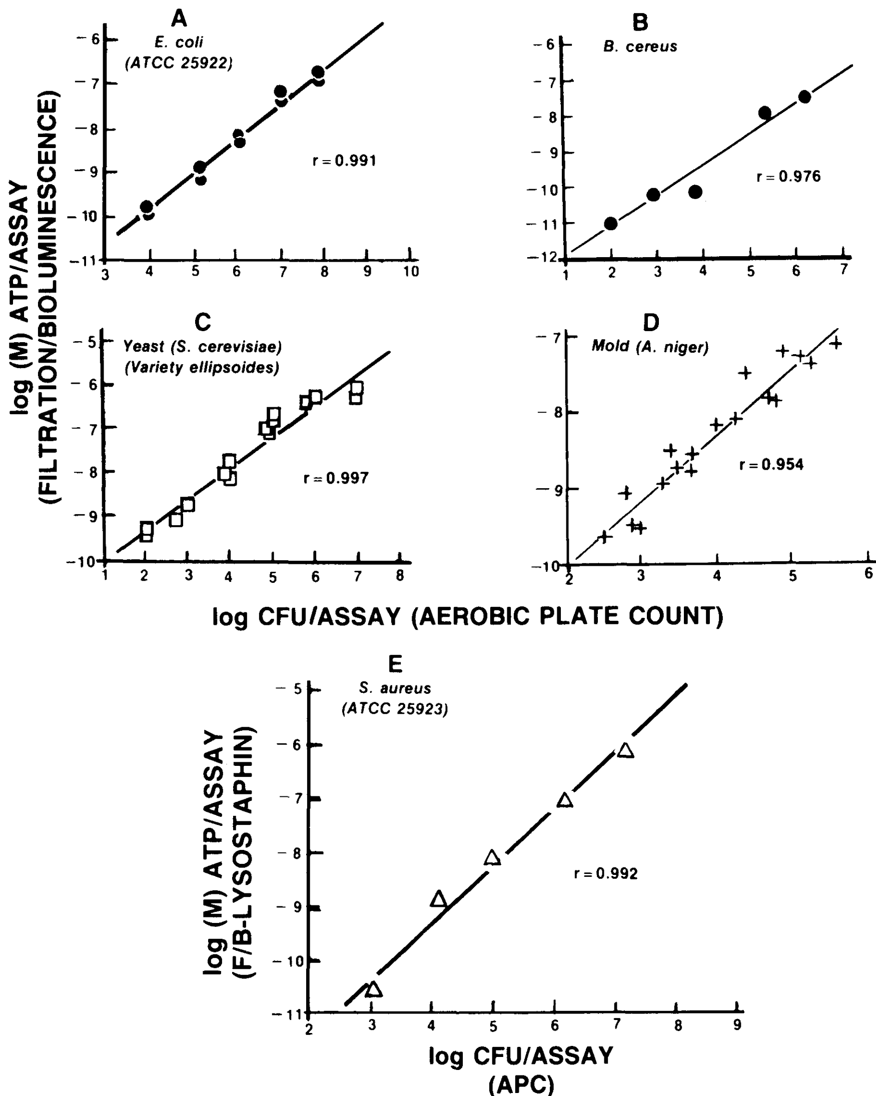

FIG. 2. Bioluminescent standard curves for each microorganism used in this study. Serial dilutions of pure cultures of single organism in 0.0003 *M* phosphate buffer were plated and simultaneously filtered. The CFU results enumerated from aerobic plates (APC) were plotted against the ATP (M) concentrations assayed on the filter by bioluminescence. *S. aureus* (Fig. 2E) was extracted specifically with lysostaphin and other microorganisms were lysed with PICOEX B reagent.

μm filter to selectively extract ATP from staphalococcal cells. Lysostaphin has been characterized (11) as a specific staphylolytic agent, and preliminary results associated with this study also demonstrated that the efficiency of ATP extraction by lysostaphin is higher for *S. aureus* (100%) than for *E. coli* (11%). The lysing step of the F/B method is modified with the lysing of lysostaphin, so that *S. aureus* can be separated from *E. coli* in a sample containing both bacterial cultures (Fig. 6).

It has been found by testing different types of membrane filters, that maximal light response can be recovered from the polycarbonate membrane filters (10). The polycarbonate filter can retain organisms on the surface of the filter, thereby allowing organisms to full exposure to the lysing reagent. The biolumi-

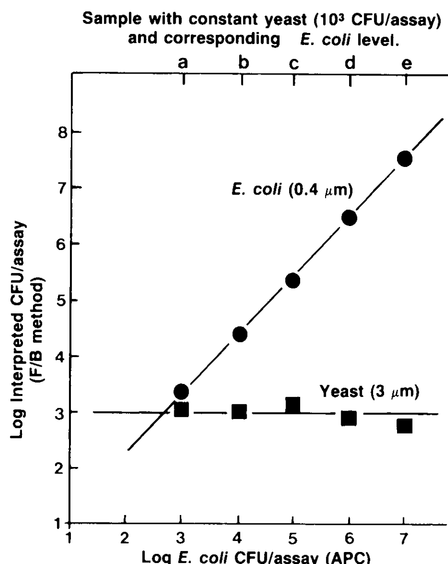

FIG. 3. Separation and quantitation of two types of microorganisms in samples inoculated with *E. coli* (●) and yeast (■). Five samples, each containing a constant level of yeast (10^3 CFU) and an increasing level of *E. coli* (10^3, 10^4, 10^5, 10^6, or 10^7 CFU), were assayed with the F/B procedure. ATP was measured for yeast and *E. coli* collected, respectively, on the 3- and 0.4-μm filters. The aerobic plate count for *E. coli* and yeast was done for each sample.

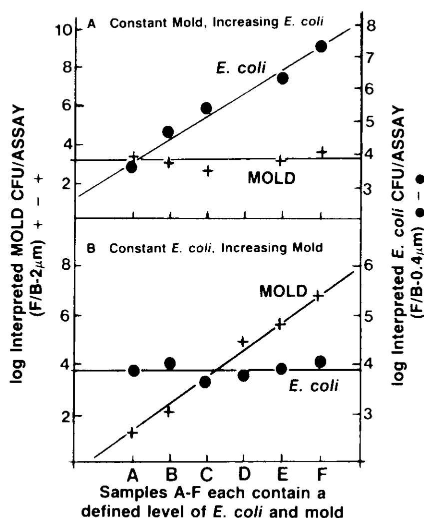

FIG. 5. Differentiation and detection of *E. coli* and mold in samples containing both cultures. (A) Each filtering sample was inoculated with a fixed level (10^3 CFU) of mold and an increasing level of *E. coli* (10^3 through 10^7 CFU in samples A through F). The samples were assayed with the F/B method and the light responses measured on the 2- and 0.4-μm filters were translated into CFU results for mold and *E. coli*, respectively. (B) The experimental design was simply reversed; each sample contained a constant (10^4 CFU) level of *E. coli* and an increasing level of mold (10^1 through 10^6 CFU in samples A through F).

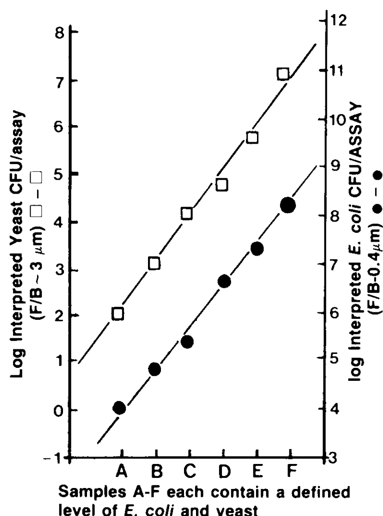

FIG. 4. Yeast and *E. coli* differentiation in samples containing increasing levels of both cultures (10^4 through 10^8 *E. coli* CFU and 10^2 through 10^7 yeast CFU were seeded in samples A through F). Experiments were conducted in the manner described in Fig. 3.

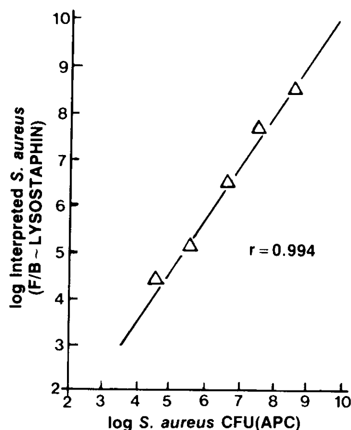

FIG. 6. Detection of *S. aureus* by F/B–lysostaphin assay in samples containing equal concentrations of *E. coli* and *S. aureus*. A 10-fold increase (5×10^4–5×10^8 CFU) in concentration of both *E. coli* and *S. aureus* was inoculated into a series of samples. The samples were assayed with the F/B method. The bacteria collected on the 0.4-μm filter were lysed with lysostaphin to specifically extract staphylococcal ATP.

nescence reaction is carried out on the filter and the light detection is not interfered with by the physical presence of the polycarbonate filter.

The bioluminescence standard curves shown in Fig. 2 indicate that the minimum detectable CFU of *E. coli* (Fig. 2A) is different from that of the yeast (Fig. 2C) and mold (Fig. 2D). This difference is caused by the different cell size and ATP content per cell for each organism (2, 3). The ATP content of a single yeast and mold is approximately 100-fold higher than that of the *E. coli.* Therefore, a slight carryover of the yeast or mold ATP on the 0.4-μm filter could result in over interpretation of *E. coli* CFU in the sample. Results obtained in this study (Figs. 3–5) indicate that very low levels of *E. coli* can be accurately detected by the F/B method in samples containing other microorganisms such as yeast or mold. This proves that a complete separation of *E. coli* (from yeast or mold) is achieved with the discriminating pore size filter. For this reason, the F/B method can probably be used to replace other techniques (12–14) for detecting low levels of bacteria in samples contaminated with other microorganisms.

The F/B method also has limitations in its applications. For example, the method can efficiently separate mold from *E. coli*—small size bacteria, but not from *B. cereus*—large size bacteria often associated with molds in soil and in various food products (15). This limitation is because both *B. cereus* and mold (*A. niger*) are retaind by the same pore size filter (88% of *B. cereus* and 81% of mold are retained on the 2-μm filter). The filtration/bioluminescence technique can be used to differentiate and measure several types of microorganisms (*E. coli,* yeast, and mold). The CFU measured by the F/B method is shown to correlate well with the CFU obtained from aerobic plate count. This technique can reduce the time and labor needed by the conventional incubating and plating techniques, and therefore offers a method for rapid microbiology. The F/B method is also potentially useful for a variety of applications, such as the rapid screening of urine (16, 17) and blood (18, 19) for bacteriological infection, waste and water management (20, 21), disinfection control (22), and quality control of food products (23, 24).

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