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Pattern Recognition Analysis of In Vivo Enzyme-Substrate Fluorescence Velocities in Microorganism Detection and Identification

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A spectrometric technique is presented that combines most of the important criteria necessary for efficient detection and identification of microorganisms. These criteria include simplicity of experimental design, various degrees of sensitivity and selectivity, convenience, and total reaction times of less than 15 min. The study takes advantage of the inherent extracellular enzymes present in living as opposed to dead, non-enzyme-producing organisms. Sequentially, these are harnessed in in vivo reactions with a substrate containing a select organic functional group that is known to be cleaved or hydrolyzed by a certain enzyme. The substrate is tailored so that one of the products can be induced to fluoresce, and by using a conventional spectrofluorimeter the rate at which the fluorescence appears can be recorded. By subjecting the same bacterial sample to a number of different enzyme substrates, a pattern of fluorescence response rates emerges from a 7 by 7 microorganism-substrate matrix. Detection limits ranged from 3.6×10^2 to 3.5×10^8 cells per ml for the *Bacillus globigii*-indoxyl acetate and *Escherichia coli*-diacetylfluorescein pairs, respectively. The specificity and versatility of the method for bacterial determination is demonstrated in probing different bacterial enzymes through their spectrally active metabolic products.

Microbial detection and identification is important in a diverse array of scientific concerns. Recently, new analytical methods have been developed to probe the physical, chemical, and biological characteristics of microorganisms. A common goal in these endeavors is the search for either an exogenous probe or indigenous organism characteristic that would be fundamental in microorganism detection and identification in a fairly straightforward, convenient manner. These methods include pyrolysis mass spectrometry (7, 11, 12, 21), microbial phospholipid fatty acid extracts (15), countercurrent chromatographic bacterial separation (10), excitation-emission matrices with a video fluorometer of whole-cell supernatants (19) and differential dye-cell wall binding (20), time-resolved fluorescence with wavelength-dependent lifetimes (3), resonance Raman spectra of UV-excited organisms (9), staining organisms in blood cultures (18), and the enzyme-linked lectinosorbent assay (5, 8) for detecting and differentiating *Bacillus anthracis* from closely related bacilli. Recent methods developed to probe in vivo enzymes include the chromogenic α-glucosidase substrate probe in distinguishing *B. anthracis* from other bacilli (17), the fluorogenic 4-methylumbelliferone-β-D-glucuronide assay of β-glucuronidase in *Escherichia coli* (16), and the fluorometric assay of the β-lactamases (4) with penicillin and cephalosporin as substrates for a variety of gram-positive and gram-negative organisms. These methods span various degrees of complexity of design, sensitivity, specificity, convenience, and time required to generate sufficient data.

We offer a spectrometric technique that combines the attractive features of most of the important criteria necessary for efficient microbiological detection and identification. The study takes advantage of the inherent extracellular enzymes present in living as opposed to dead, non-enzyme-producing organisms. Sequentially, these are harnessed in in vivo reactions with a substrate containing a select organic functional group that is known to be cleaved or hydrolyzed by a certain enzyme. The substrate is tailored so that one of the products can be induced to fluoresce, and by using a conventional spectrofluorimeter the rate at which the fluorescence appears can be tracked and recorded. By subjecting the same bacterial sample to a number of different enzyme substrates, a pattern of fluorescence response rates emerges. The experimenter can then use a pattern recognition set of standard microorganism-substrate fluorescence response curves, and together with the experimentally derived rates of fluorescence generation, a microbial analysis can be produced. Family taxonomic level and order of magnitude concentration analyses appear to be feasible with the pattern recognition method.

MATERIALS AND METHODS

Materials. Organisms used in this study were *B. subtilis* subsp. *niger* (*Bacillus globigii* ATCC 9372), *B. pumilis* ATCC 7061, *Saccharomyces cerevisiae* ATCC 4110, *Pseudomonas stutzeri* ATCC 11607, *Proteus vulgaris* ATCC 13315, *Serratia marcescens* ATCC 8101, and *E. coli* ATCC 4352.

Bacto-tryptose agar was purchased from Difco Laboratories, Detroit, Mich. Monobasic and dibasic potassium phosphate salts, Tris, dioxane, methanol, and acetone were obtained from Fisher Scientific Co. Methylcellosolve was purchased from Union Carbide Corp. All substrates (see Table 1) were purchased from Sigma Chemical Co., St. Louis, Mo., except for the following: *N*-methylindoxyl myristate, Isolab Inc., Elkhart, Ind.; indoxyl acetate, Nutritional Biochemicals Corp., Cleveland, Ohio; resazurin, Allied Chemicals and Dye Corp., New York, N.Y.; and luminol, Bios Laboratories, Inc., New York, N.Y.

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Methods. The microorganisms were grown on tryptose plate cultures for 48 h. Heavy organism growth was transferred to sterile test tubes with 2 ml of 0.1 M KH_2PO_4 sterile buffer (phosphate buffer); pH 7.5. Centrifugation at 3,500 rpm for 0.5 h and suspension of the pellet in phosphate buffer were performed to remove residual tryptose growth medium, and the suspensions were stored at 4°C. The microorganism suspensions were maintained in a vegetative state in phosphate buffer without organic nutrients. All analyses were conducted and repeated daily within 1 to 72 h after each suspension was prepared.

From the 26 substrates, 6 were chosen for comprehensive analysis in the present study. Assay conditions for each substrate are as follows. (i) Indoxyl acetate: to 1.8 ml of sterile phosphate buffer, 0.1 ml of a 1:100 acetone dilution of a solution of 10 mg of indoxyl acetate per ml of acetone and 0.1 ml of the bacterial sample are added together. (ii) Indoxyl-β-D-glucoside: to 1.8 ml of sterile 0.1 M Tris, pH 7.5 (Tris buffer), 0.1 ml of a 1:100 aqueous solution of a solution of 6.31 mg of indoxyl glucoside per ml of Tris buffer and 0.1 ml of bacterial suspension are added together. (iii) 4-Methylumbelliferone-β-D-glucoside (4MU-glucoside): to 1.8 ml of sterile Tris buffer, 0.1 ml of a solution of 3.38 mg of 4MU-glucoside per ml of Tris buffer and 0.1 ml of bacterial sample are added together. (iv) 4MU-phosphate: to 1.8 ml of sterile Tris buffer, 0.1 ml of a 1:10 aqueous solution of 12.67 mg of substrate per ml of Tris buffer and 0.1 ml of bacterial suspension are added together. (v) 4MU-β-D-galactoside: 2.0 mg of 4MU-galactoside is dissolved in 40 ml of phosphate buffer, and 0.1 ml of a bacterial sample is added to 1.9 ml of the substrate solution. (vi) 3-Indoxyl phosphate: to 1.8 ml of sterile Tris buffer, 0.1 ml of a 1:10 aqueous solution of 2.57 mg of indoxyl phosphate per ml of Tris buffer and 0.1 ml of a bacterial suspension are added together.

Each substrate-organism suspension was briefly mixed with a piece of parafilm over the cuvette. The initial fluorescence velocity-versus-microorganism concentration log profiles were obtained by serially diluting a portion of the organism stock suspension and, using the same assay conditions, noting the initial fluorescence rate of response.

A Farrand MK-2 fluorimeter was used in the spectral kinetic studies. Excitation and emission slits were 1 and 5 nm, respectively. The assay temperature was controlled to 26 ± 1°C. Bacterial concentration was determined by viable plate count. Pour plates of tryptose agar with samples of bacteria were prepared in triplicate for each assay sample (most-probable-number procedure). Fluorescence quantitation was performed by determining the slope of a tangent to the initial portion of the fluorescence generation curve. Each experimental data point represents the average of one to three assays. The spectrofluorimeter settings were calibrated with a borosilicate glass standard (λ_{ex} = 310 nm, λ_{em} = 350 nm) purchased from Farrand Optical Co. Two methods of analyzing the fluorescence rate data and the respective microorganism concentrations were used. Each method embodies a different statistical concept that relates to the type of question and application that are sought from a set of experimental data.

Linear regression analysis becomes the method of choice when an experimentally measured dependent variable is used to predict the value of an independent variable (microorganism concentration in this study) with the aid of standard response curves. In the present case, standard response curves are portrayed by linear regression lines with hyperbolic limits of 95% confidence. Microorganism concentration can be extrapolated from the experimentally derived initial rate of fluorophore fluorescence and the linear regression line, and the range of concentration values in which the extrapolated microorganism concentration will be found 95% of the time can be determined from the hyperbolic error limits of the particular regression line (2, 13).

The bivariate normally distributed population statistical analysis is a method that delineates regions of response (here in two-dimensional space). That is, the method generates a boundary that portrays how the independent and dependent variables are statistically correlated with no implied cause and effect relationship. The boundaries are ellipses with their major axes equivalent to that of the linear regression line of the same data set; however, that is the extent of their similarity. The elliptical boundary directly implies a region, as opposed to an interval, in which one would expect to find a certain percentage of the total population of independent-dependent variable response pairs of a given subject (microorganism-extracellular enzyme). This concept is critical in the determination of microorganism identity in a sample, because different substrate-organism standard regions can be observed that contain an experimentally observed initial fluorescence velocity for a given substrate. Each set of regions for a given substrate is cross-referenced in the determination of the identity of the microorganism(s).

Equation 1 embodies the mathematical construct used to generate the elliptical regions of microorganism responses (1, 6, 23):

$$\frac{(x-\bar{x})^2}{\sigma_x^2}+\frac{(y-\bar{y})^2}{\sigma_y^2}-\frac{2r(x-\bar{x})(y-\bar{y})}{\sigma_x\sigma_y}=5.991\,(1-r^2) \quad (1)$$

where σ_x^2 and σ_y^2 are the variance of the x and y variables, respectively; $\bar{x}$ and $\bar{y}$ are the means of the x and y variables,

TABLE 1. Enzyme substrates

Substrate	Enzyme probed	Response[a]
Indoxyl acetate	Lipase/esterase	+
N-Methylindoxyl acetate	Lipase/esterase	+
N-Methylindoxyl myristate	Lipase/esterase	+
β-Naphthyl acetate	Lipase/esterase	+
α-Naphthyl acetate	Lipase/esterase	+
4MU-heptanoate	Lipase/esterase	+
4MU-acetate	Lipase/esterase	+
5-Bromoindoxyl acetate	Lipase/esterase	+
5-Bromo-4-cloro-3-indolylacetate	Lipase/esterase	+
4MU-galactoside	β-D-Galactosidase	+
5-Bromo-3-indolyl-β-D-galactoside	β-D-Galactosidase	−
Naphthol-AS-BI-phosphate	Phosphatase	−
4MU-phosphate	Phosphatase	+
β-Naphthyl phosphate	Phosphatase	−
5-Bromo-4-cloro-3-indolyl phosphate	Phosphatase	−
3-Indoxyl phosphate	Phosphatase	+
Indoxyl-β-D-glucoside	β-D-Glucosidase	+
4MU-glucoside	β-D-Glucosidase	+
6-Bromo-2-naphthyl-β-D-glucoside	β-D-Glucosidase	+
4MU-glucuronide	β-D-Glucuronidase	−
7-Ethoxycoumarin	Monooxygenase	−
Glycyl-L-phenyl-β-naphthylamide	Aminopeptidase	−
β-Naphthyl sulfate	Sulfatase	−
3-Indoxyl sulfate	Sulfatase	−
Luminol	Oxidase/dehydrogenase	−
Resazurin	Dehydrogenase	+

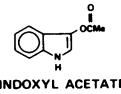
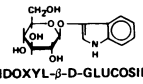
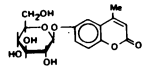
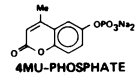
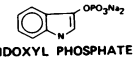
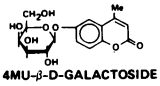
[a] Fluorescence observed with some or all of the organisms; −, no generation of fluorescence with the seven organisms at the highest concentration of each organism listed in Table 5.

respectively; $(\bar{x},\bar{y})$ represents the centroid of the bivariate population; r is Pearson's correlation coefficient; and the constant 5.991 represents a chi-square variate with two degrees of freedom that is exceeded only 5% of the time ($\chi^2_{0.05}$).

RESULTS

The seven organisms used in this study were probed for their extracellular enzymatic action with substrates that are known to react with in vitro enzymes and yield inducible fluorescent products. Table 1 presents an analysis of the fluorescence response of various enzyme substrates with the seven test organisms, and from these candidates a total of seven compounds were chosen for the microorganism characterization analysis. A number of enzyme-substrate reactions that were not chosen for comprehensive reaction rate analyses are nevertheless worth noting. All seven organisms displayed activity with the compound 4MU-acetate, but spontaneous hydrolysis of the substrate was prohibitively high, 0.36 ΔF/min in 0.1 M phosphate buffer, pH 7.5. For the compound 6-bromo-2-naphthyl-β-D-glucoside, a response of 0.004 ΔF/min was obtained with 2.0×10^7 cells of *B. pumilis* per ml by monitoring the fluorescence of the product 6-bromo-β-naphthol, which is similar to the response obtained with the substrate indoxyl glucoside (see below). A response of 0.05 ΔF/min was noted with 4.0×10^7 cells of *Proteus vulgaris* per ml and 0.04 ΔF/min with 5.0×10^8 cells of *Serratia marcescens* per ml, with a background fluorescence rate of 0.015 ΔF/min monitoring resorufin fluorescence from resazurin. The analysis of the substrate diacetylfluorescein (DAF) is presented elsewhere (22), and a summary of the other substrate hydrolysis reactions and their spectral parameters is given in Table 2. Figure 1 shows the emission spectra of both fluorescent products. Control analyses are reported elsewhere (22) concerning the determination of enzyme activity in dead (nonviable) intact bacteria and in the supernatant and suspended cell pellet after centrifugation.

TABLE 2. Extracellular enzyme reaction parameters

Substrate	Fluorescent product	Enzyme
 INDOXYL ACETATE	 INDOXYL[a]	LIPASE
 INDOXYL-β-D-GLUCOSIDE	INDOXYL	β-D-GLUCOSIDASE
 4MU-β-D-GLUCOSIDE	 4-METHYLUMBELLIFERONE[b]	β-D-GLUCOSIDASE
 4MU-PHOSPHATE	4-METHYLUMBELLIFERONE	PHOSPHATASE
 INDOXYL PHOSPHATE	INDOXYL	PHOSPHATASE
 4MU-β-D-GALACTOSIDE	4-METHYLUMBELLIFERONE	β-D-GALACTOSIDASE

[a] λ_{ex} = 395 nm, λ_{em} = 470 nm.
[b] λ_{ex} = 365 nm, λ_{em} = 445 nm.

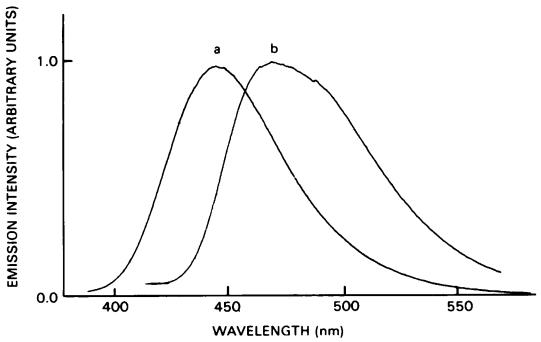


FIG. 1. Emission spectra of the fluorescent products in the bacterial enzyme-substrate assays: a, 4MU, λ_{ex} = 365 nm; b, indoxyl, λ_{ex} = 395 nm.

Optimum assay solution conditions were determined for the enzyme-catalyzed reactions by varying the buffer salt, pH, substrate, substrate solvent, and concentration. An analysis is presented for indoxyl acetate. Figure 2 portrays the effect of indoxyl acetate concentration on the initial velocity of fluorescence with the esterase enzyme supplied by *B. globigii*. A bell-shaped response curve is observed. At the chosen substrate assay concentration of 3.1×10^{-5} M, the effect of substrate solvent was noticed in that acetone afforded a higher initial fluorescence rate than dioxane, methanol, and methylcellosolve. Figure 3 presents the effect of pH on the initial fluorescence rate of the *B. globigii* esterase reaction. Phosphate buffer produces a maximum fluorescence velocity closer to neutral pH conditions than Tris and was therefore used for indoxyl acetate reaction studies at pH 7.5. For the phosphate and glucoside substrates, Tris was the buffer of choice.

A comparison of the rates of hydrolysis of ester compounds with the in vivo *B. globigii* esterase is given in Table 3. Except for the substrate DAF (22), reaction mixtures contained 1.8 ml of phosphate buffer, 0.1 ml of an acetone solution of the substrate, and 0.1 ml of the microbial sample.

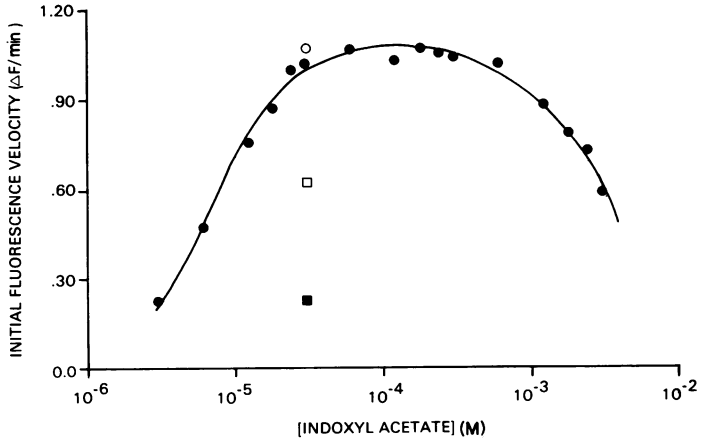


FIG. 2. Effect of indoxyl acetate concentration and substrate solvent on the initial velocity of fluorescence with a constant *B. globigii* concentration of approximately 10^7 cells per ml. Substrate solvents: acetone (○), dioxane (●), methanol (□), and methylcellosolve (■).

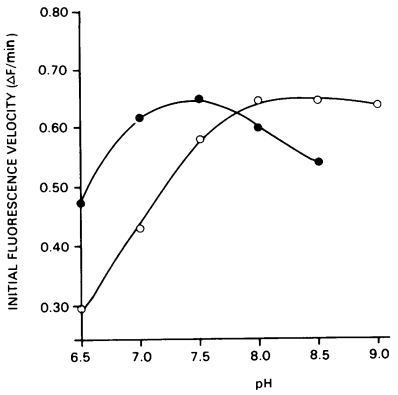


FIG. 3. Plot of the initial rate of fluorescence versus pH and buffer salt with a constant *B. globigii* concentration of approximately 10^7 cells per ml. Symbols: ●, phosphate buffer; ○, Tris buffer.

DAF and indoxyl acetate were the ester substrates of choice because of their high rates of enzyme reactivity. These two substrates display 10- to 1,000-fold-higher activities than the other seven ester substrates even though 10 times less of them was used in the assays.

Standard substrate curves for substrate-organism standard regions are shown in Fig. 4 through 9. Together with Fig. 1 and 2 in reference 22, they constitute a pattern recognition set obtained with the seven different organisms and organic compounds. An analysis of the organism-substrate reactivities can be conducted as outlined in the Methods section. For clarity, selected microorganism-substrate 95% hyperbolic confidence limits are presented. The following sections detail the in vivo extracellular enzyme response patterns with the different substrates.

Indoxyl acetate. Figure 4 presents the relative rate of fluorescence response with the organisms and indoxyl acetate. The organism responses vary to the extent that a number of generalizations can be made. At concentrations of less than 10^6 cells per ml, essentially no esterase reaction interference was observed with *Pseudomonas stutzeri*, *Proteus vulgaris*, *Serratia marcescens*, and *E. coli* in the detection of *B. globigii* and *B. pumilis*. At similar concentrations of less than 10^4 cells per ml, *B. globigii* would be the only detectable organism in the presence of the other six organisms. *S. cerevisiae* and *B. pumilis* display similar rates of reaction, whereas the two organism groups of *Serratia marcescens* and *Proteus vulgaris* and of *E. coli* and *Pseudomonas stutzeri* display virtually identical lipase activity within each group, the two groups themselves being closely related in terms of lipase activity.

Indoxyl-β-D-glucoside. Despite a fairly high detection limit of approximately 10^6 cells per ml, the fluorescence response was quite marked in that only the bacilli displayed activity (Fig. 5).

4MU-glucoside. The pattern of response with another glucoside substrate was quite different, with at least an order of magnitude higher sensitivity. The bacilli along with *S. cerevisiae* and *Pseudomonas stutzeri* were able to generate fluorescence response sets (Fig. 6). However, all four organisms displayed similar reaction rates.

4MU-phosphate. By probing the phosphatase enzyme, a different group of organisms displayed a fluorescence response (Fig. 7). *E. coli* appeared to be the most sensitive and reactive to the phosphate substrate, while similar concentrations of *Serratia marcescens* and *Proteus vulgaris* caused approximately a 5% fluorescence interference, as estimated from the major axis of each 95% confidence limit ellipse. Although *B. globigii*, *Serratia marcescens*, and *Proteus vulgaris* displayed similar kinetics, each contributed no more than approximately a 5% fluorescence interference in the presence of similar concentrations of *E. coli*.

3-Indoxyl phosphate. As opposed to the 4MU derivative, *S. cerevisiae* was detected with only a 10% interference effect in the presence of *Serratia marcescens* at similar concentrations (Fig. 8). The presence of *E. coli*, *Pseudomonas stutzeri*, and *B. pumilis*, however, obscured the fluorescence response of *S. cerevisiae*, whereas *B. globigii* and *Proteus vulgaris* had no fluorescent response at the indicated bacterial concentrations.

4MU-galactoside. The response with this substrate was apparent in that only *E. coli* and *Serratia marcescens* produced fluorescence while the other five organisms displayed no activity (Fig. 9). The responses were, however, similar in β-galactosidase activity.

DISCUSSION

A summary of the detection limits of the organism-substrate pairs that produce a fluorescent response is given in Table 4. In the determination of the detection limit at which no measurable spontaneous hydrolysis was observed at an instrumental sensitivity of 0.0001 ΔF/min, the detection limit for fluorescence response was arbitrarily chosen as 0.00015 ΔF/min. The 7 by 7 organism-substrate matrix had detection limits ranging from 3.6×10^2 cells per ml for *B. globigii*-indoxyl acetate to 3.5×10^8 cells per ml for the *E. coli*-DAF pair. A question to be raised is the inconsistent differential of the detection limits for the organisms with the ester substrates DAF and indoxyl acetate. The bacilli showed a 10-fold increase in sensitivity to DAF over indoxyl acetate. *S. cerevisiae* and *Pseudomonas stutzeri* showed similar sensitivities to both substrates, and the three remaining organisms displayed detection limits that varied by approximately two orders of magnitude in a comparison of their DAF to indoxyl acetate activities. However, with five of the seven organisms tested, it appears that indoxyl acetate is a more sensitive probe of esterase activity than DAF. With the glucoside substrates both bacilli displayed greater sensitivity to the 4MU versus the indoxyl derivative. However, a different phenomenon was obtained with *Serratia marcescens* and *E. coli* with the phosphate substrates; the former displayed four times greater sensitivity to indoxyl phosphate than 4MU-phosphate, whereas the latter was three times less

TABLE 3. Comparison of *B. globigii* lipase activity to ester compounds[a]

Substrate	Substrate Concn (μM)	ΔF/min
DAF	48	45.0
Indoxyl acetate	31	3.0
5-Bromo-indoxyl acetate	500	0.43
N-Methylindoxyl acetate	47.5	0.33
5-Bromo-4-chloro-3-indoxyl acetate	500	0.31
N-Methylindoxyl myristate	475	0.21
β-Naphthyl acetate	700	0.026
4MU-heptanoate	500	0.026
α-naphthyl acetate	700	0.0028

[a] The concentration was approximately 10^7 cells per ml.

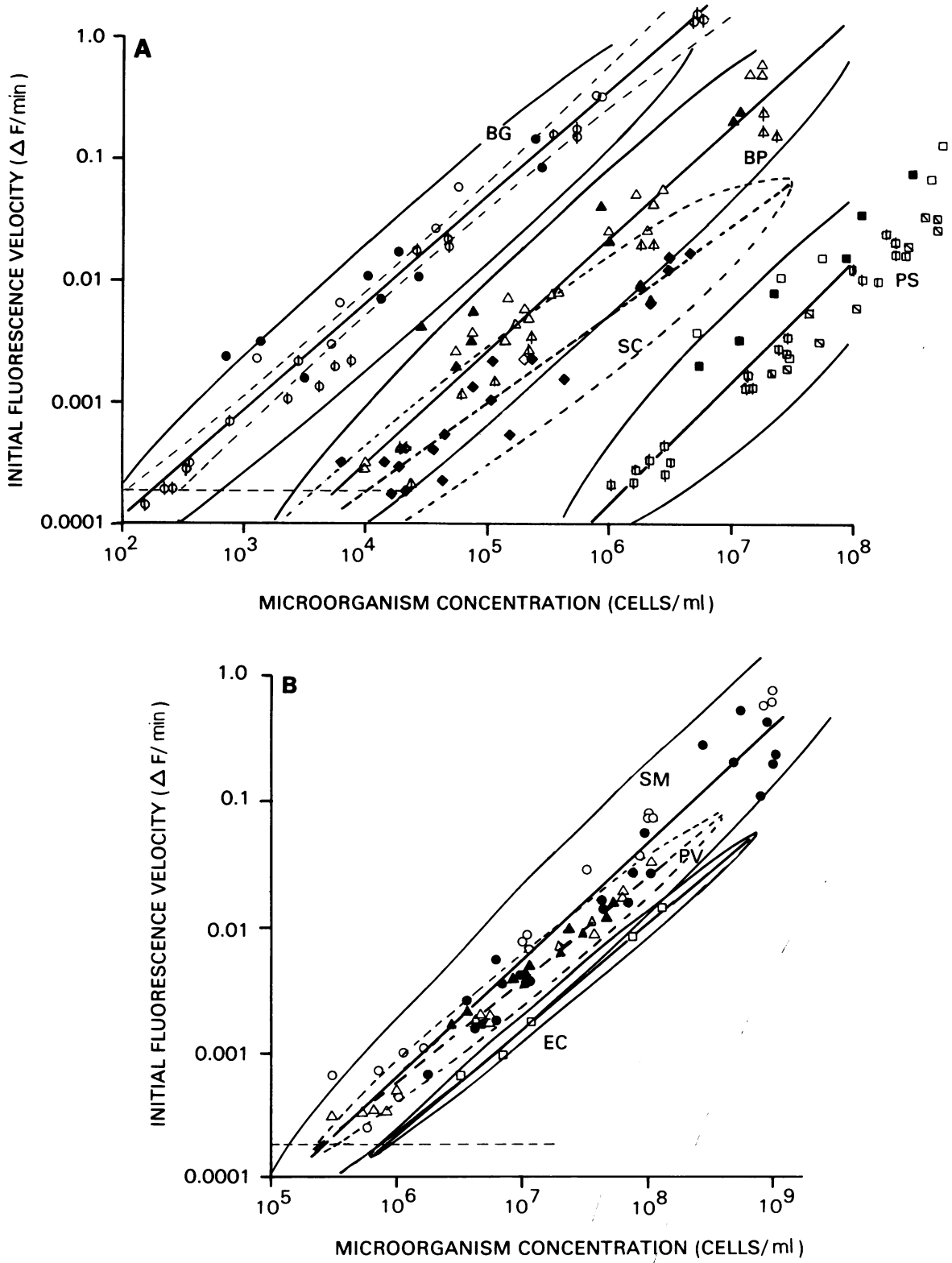


FIG. 4. Initial velocity of indoxyl fluorescence from the substrate indoxyl acetate as a function of microorganism concentration. In this and the remaining figures, each symbol represents a separate microbial suspension. The horizontal dashed line at 0.00018 ΔF/min represents the spontaneous substrate hydrolysis fluorescence rate. In Fig. 4 through 9, elliptical regions comprising 95% of each organism response set are depicted. A 95% microorganism concentration reaction confidence interval is presented for *B. globigii* as a dashed hyperbolic curve. Symbols: (A) *B. globigii* (BG) (○, ●, ϕ); *B. pumilis* (BP) (△, ▲, ⍋); *Pseudomonas stutzeri* (PS) (□, ■, ⧆, ⧅); *S. cerevisiae* (SC) (◆). (B) *Proteus vulgaris* (PV) (△, ▲); *Serratia marcescens* (SM) (○, ●); *E. coli* (EC) (□).

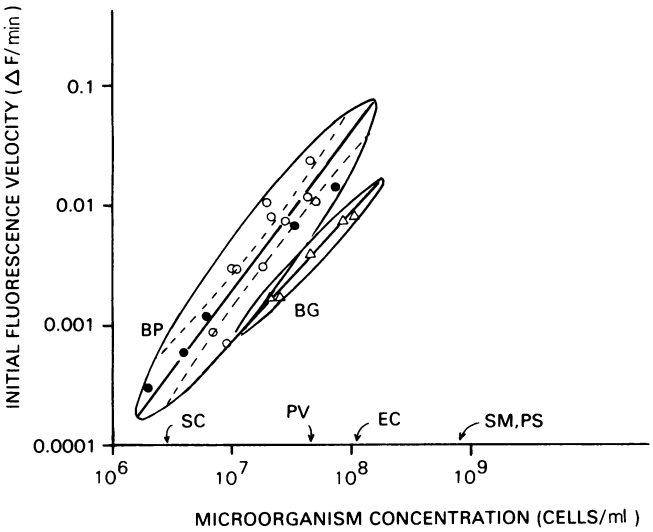


FIG. 5. Initial velocity of indoxyl fluorescence from the substrate indoxyl glucoside as a function of bacterial concentration. A 95% confidence interval is presented for *B. pumilis* as a dashed hyperbolic curve. No measurable substrate spontaneous hydrolysis was observed. Symbols: △, BG; ○, ●, BP. See the legend to Fig. 4 for abbreviations.

sensitive. Possible explanations to describe these observations are the degree of steric factors, substrate polarity and enzyme-cell wall association to enzyme-substrate accessibility in the enzymatic reactions.

Table 5 depicts a qualitative pattern recognition tabulation of the quantitative pattern recognition set presented in Fig. 4 through 9 (and Fig. 1 and 2 of reference 22). A qualitative accounting of microbial responses can be used as a first filter in the determination of microorganism identity and approximate concentration analysis. In the determination of the identity of a sample of an organism(s) of interest, buffered solutions of various enzyme substrates are prepared and placed in separate cuvettes. The initial fluorescent velocity of each substrate-organism pair is derived, and the values are noted on their respective substrate standard curves (e.g., Fig. 4 through 9). The initial fluorescence velocity is used to determine which organism(s) displays that particular reactivity, regardless of organism concentration. By comparing the candidate organism set for each substrate standard curve, various organisms can be eliminated within the standard curve organism concentration limits, because each of the organisms displays orders of magnitude differences in reactivity with different substrates.

A bacterial extracellular enzyme essay that produces a fluorescence signal above the spontaneous substrate hydrolysis background within the established organism concentration response limits, together with substrate solution preparation, takes approximately 15 min to perform. At relatively low organism concentrations, roughly 2 h or less is required to analyze a sample for microbial presence with the substrates presented in Table 5.

An order of magnitude light-scattering microorganism concentration analysis would greatly facilitate the determination of the response region occupied by the sample of interest. The measurement, however, produces a total microbial count without discriminating between viable and nonviable organisms. This piece of information is valuable in that organism-substrate pairs which have detection limits higher than that of the light-scattering concentration determination can be eliminated. However, with the substrate that responds to the lowest concentration of a particular organism, that microbe could still be present in the sample below its lowest detectable concentration limit in a fluorescence velocity measurement. Therefore it is desirable to have as concentrated a sample as possible. A microorganism concentration detection limit less than or equal to 10^6 cells per ml encompasses approximately 50% of the organism-substrate pairs that produced a fluorescent response, spanning at least one pair of each of the seven organisms and six of the seven substrates.

The β-lactamases (4) penicillinase and cephalosporinase are two other extracellular enzymes that could be considered in the pattern recognition set, because an in vivo reaction with the nonfluorescent penicillin and cephalosporin substrates produces fluorescent products. Their investigation is further desirable in that the products are generated within 5 to 15 min at room temperature, producing a blue-green fluorescence with 300- to 350-nm excitation. These

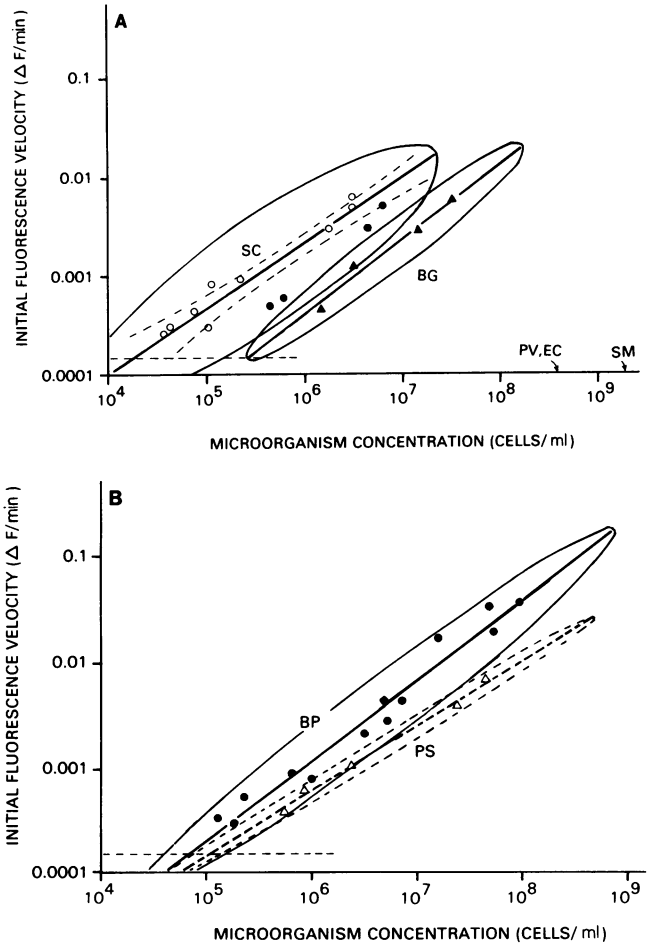


FIG. 6. Initial velocity of 4MU fluorescence from the substrate 4MU-glucoside as a function of microorganism concentration. A 95% confidence interval is presented for *S. cerevisiae* as a dashed hyperbolic curve. The dashed line at 0.00015 ΔF/min represents the substrate spontaneous hydrolysis fluorescence rate. See the legend to Fig. 4 for abbreviations. Symbols: (A) SC (○, ●); BG (△). (B) BP (●); PS (△).

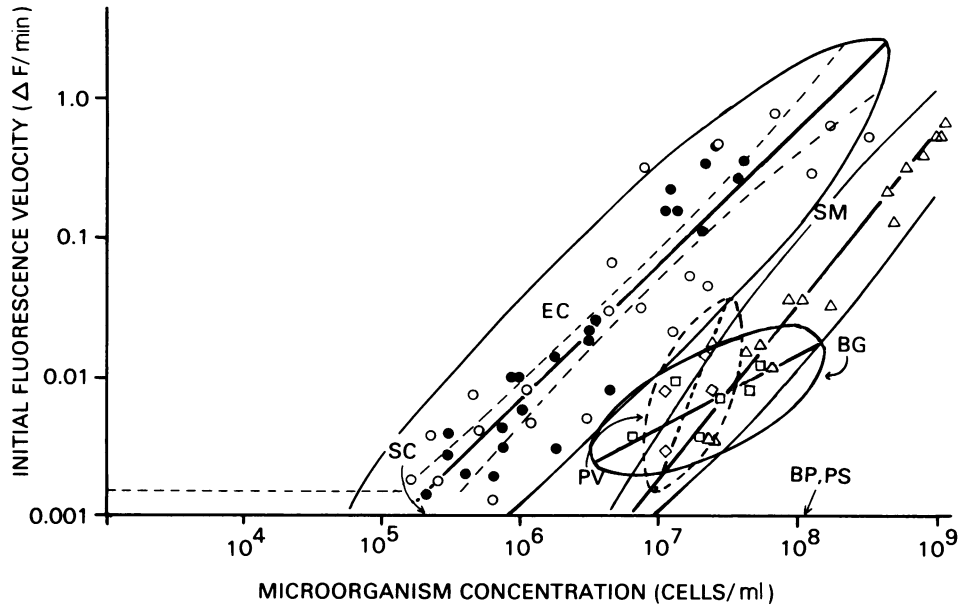


FIG. 7. Initial velocity of 4MU fluorescence from the substrate 4MU-phosphate as a function of microorganism concentration. A 95% confidence interval is presented for *E. coli* as a dashed hyperbolic curve. The dashed line at 0.00015 ΔF/min represents the substrate spontaneous hydrolysis fluorescence rate. See the legend to Fig. 4 for abbreviations. Symbols: EC (○, ●); SM (△); PV (◇); BG (□).

enzymes were found to be readily available in the in vivo reactions in a variety of gram-positive and gram-negative bacteria.

Thiaminase I and II are two other extracellular enzymes found in microbial genera such as *Candida*, *Bacillus*, and *Clostridium* (14). With the aid of an organic base or thiol compound, the enzymes catalyze the cleavage of the substrate thiamin (vitamin B_1) to a fluorescent tricyclic product (24).

The pattern recognition set is of central importance in the technique, because it permits organism discrimination by a process of elimination as well as a rough concentration estimation. An adjunct to the pattern recognition set is a rapid and convenient light-scattering concentration determination. However, for the pattern recognition set to be useful in a practical situation, more microorganisms need to be added to the data base as well as samples from sources other than laboratory cultures, such as clinical isolates, food extracts, and the environment. Currently, the pathogenic *Streptococcus*, *Salmonella*, *Staphylococcus*, *Streptomyces*, and *Candida* genera are being studied.

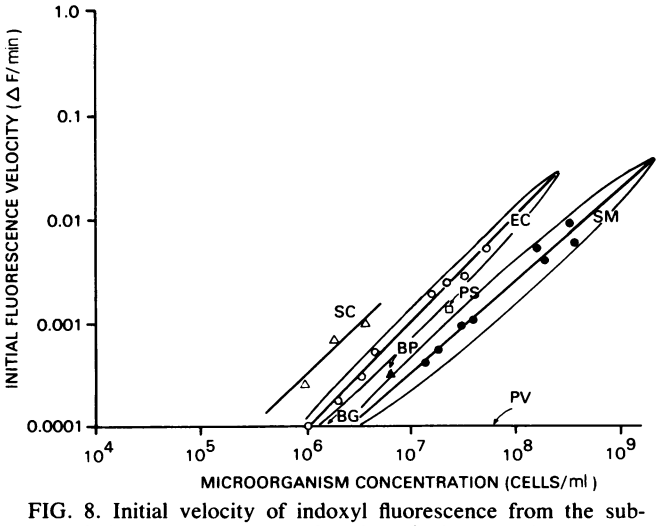


FIG. 8. Initial velocity of indoxyl fluorescence from the substrate 3-indoxyl phosphate as a function of microorganism concentration. No measurable substrate spontaneous hydrolysis was observed. See the legend to Fig. 4 for abbreviations. Symbols: SC (△), EC (○), SM (●), PS (□), BP (▲).

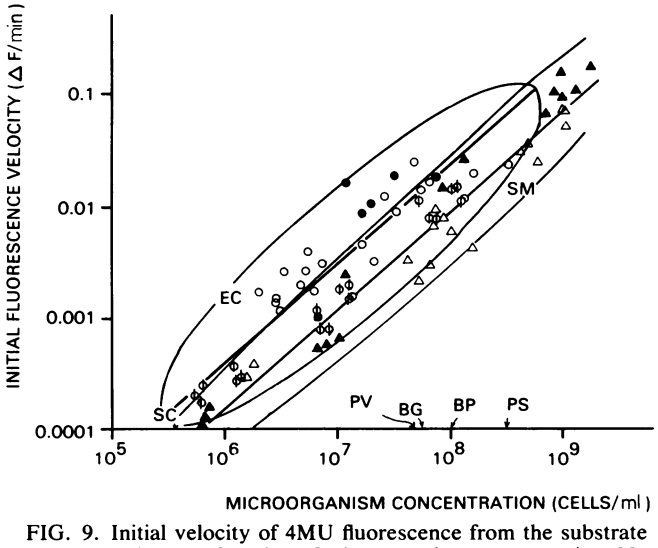


FIG. 9. Initial velocity of 4MU fluorescence from the substrate 4MU-galactoside as a function of microorganism concentration. No measurable substrate spontaneous hydrolysis was observed. See the legend to Fig. 4 for abbreviations. Symbols: EC (○, ●, ϕ); SM (△, ▲).

TABLE 4. Limits of microorganism detection with different substrates in phosphate and Tris buffer systems

Substrate	Response limit[a] (ΔF/min)	No. of viable cells detected/ml						
		B. globigii	*B. pumilis*	*S. cerevisiae*	*Pseudomonas stutzeri*	*Proteus vulgaris*	*Serratia marcescens*	*E. coli*
DAF[b]	0.022	3.8×10^3	1.2×10^5	4.5×10^4	2.3×10^6	8.7×10^7	1.6×10^7	3.5×10^8
Indoxyl acetate	0.00036	3.6×10^2	1.1×10^4	2.5×10^4	2.6×10^6	5.5×10^5	6.0×10^5	1.8×10^6
Indoxyl glucoside	0.00015	2.5×10^6	1.5×10^6	—[c]	—	—	—	—
4MU-glucoside	0.0003	6.5×10^5	1.5×10^5	5.0×10^4	3.0×10^5	—	—	—
4MU-phosphate	0.003	5.0×10^6	—	—	—	1.2×10^7	1.5×10^7	4.1×10^5
Indoxyl phosphate	0.00015	—	2.5×10^6[d]	4.5×10^5	2.5×10^6[d]	—	4.0×10^6	1.5×10^6
4MU-galactoside	0.00015	—	—	—	—	—	1.0×10^6	3.5×10^5

[a] The response limit represents twice the ΔF per minute value of the spontaneous hydrolysis of the substrate.
[b] From reference 22.
[c] —, Not detectable.
[d] Assuming a fluorescence response parallel to that of the other organisms.

TABLE 5. Qualitative microorganism response with different substrates in phosphate and Tris buffer systems

Substrate	Cell concn (cells/ml) giving fluorescent response[a]						
	B. globigii	*B. pumilis*	*S. cerevisiae*	*Pseudomonas stutzeri*	*Proteus vulgaris*	*Serratia marcescens*	*E. coli*
DAF	+	+	+	+	+	+	+
Indoxyl acetate	+	+	+	+	+	+	+
Indoxyl glucoside	+	+	3.0×10^6	1.0×10^9	5.0×10^7	1.0×10^9	1.0×10^8
4MU-glucoside	+	+	+	+	4.0×10^8	2.0×10^9	4.0×10^8
4MU-phosphate	+	1.2×10^8	2.0×10^5	1.2×10^8	+	+	+
Indoxyl phosphate	1.5×10^6	+	+	+	6.0×10^7	+	+
4MU-galactoside	5.5×10^7	1.0×10^8	4.0×10^5	3.0×10^8	5.0×10^7	+	+

[a] Organism concentration below which no fluorescence was observed above the spontaneous fluorescence of substrate. +. Fluorescence observed with greater than or equal to the concentration of organisms presented in Table 4.

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