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Growth Inhibition of *Bacillus subtilis* by Basic Dyes

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Dyeing at factories is usually carried out in conjunction with desizing, scouring and finishing. The total drainage thus contains many water-soluble organic substances (Toyota 1976, Tashiro 1979, Nakao et al. 1975). Since these are not adequately eliminated by physical treatment, the water is purified in many cases by biological treatment such as the activated sludge method. However, dyes, bichromates, etc. present in the water often cause growth inhibition of microbes and make purification more difficult (Hashimoto and Fujita 1972). Dye toxicity has been frequently studied with respect to sterilization (Horiguchi 1969) and mutation (Brown et al. 1978, Haveland-Smith et al. 1979) for medical application, but not much in regard to water-treatment (Inoue and Honda 1970, Inoue and Honda 1971). The influence of basic dyes on the growth rate and nucleic acid content of cells was investigated in the present study to elucidate inhibitive reactions in the biological treatment of waste dye-liquor.

MATERIALS AND METHODS

The dyes listed in Table 1 were used following recrystallization from ethylalcohol. *Bacillus subtilis* (IFO 3022), a bacterial population in activated sludge, was obtained from Institute for Fermentation, Osaka. This strain has been found to be an azo-assimilating bacterium by the authors (Horitsu et al. 1977, Idaka et al. 1982). Spizien medium was used for the asynchronous culture and consisted of 0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate-$2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.0002% $FeCl_3 \cdot 6H_2O$, 0.0002% $MnCl_2 \cdot 4H_2O$ and 0.5% glucose. The medium composition of the synchronous culture was the same as that described above but contained 0.0072% glucose as well. The cell population was synchronized by the stationary phase method. These precultivate broths, 1 ml each, were inoculated in 1000 ml mediums, and the cultures were shaken at 37°C. The cell concentrations of the asynchronous cultures were determined by measurement of transmittance at 660nm, and those of the synchronous cultures were read under a microscope using Thoma's hemacytometer. After

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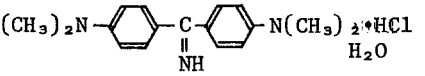
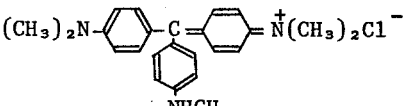
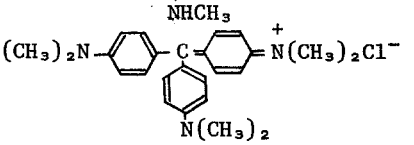
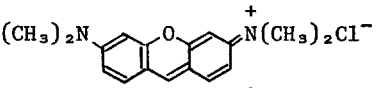
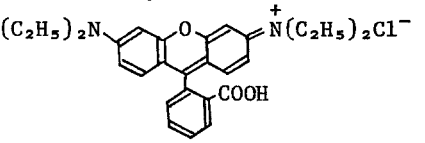
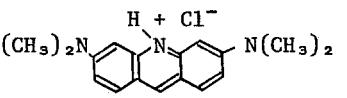
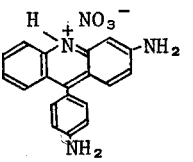
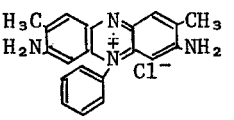
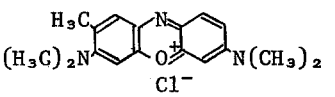
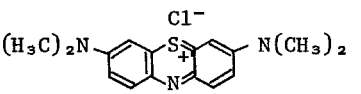
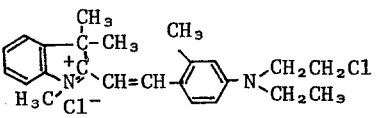
Table 1. Structures, n- and ϕ-Values of Dyes

Classical name	Structures	ϕ (mol/l)	n
Auramine O		1.8×10^{-4}	0.6
Methyl Violet		6.0×10^{-6}	1.3
Crystal Violet		1.3×10^{-6}	3.1
Pyronine G		6.9×10^{-5}	2.5
Rhodamine B		2.7×10^{-3}	1.1
Acridine Orange NS		2.5×10^{-5}	2.1
Phosphine E		8.0×10^{-5}	2.5
Safranine T		4.2×10^{-5}	2.3
Capri Blue		5.4×10^{-5}	1.2
Methylene Blue		3.0×10^{-5}	2.5
Astrazon Red 6B		$5.5\ 10^{-5}$	0.6

harvesting by centrifugation at 8,000 rpm for 15 min from the cultivate broth, RNA and DNA were each fractionated by the method of Schmidt, Thannhauser and Schneider. Their concentrations were determined respectively by measurement of absorbance at 260 and 270 nm.

RESULTS AND DISCUSSION

The asynchronous growth curves are illustrated in Figure 1. Both the mean growth rate of the cell population at the logarithmic phase and the cell concentration at the stationary phase decreased with the addition of the dyes. Purification at many factories is carried out continuously under conditions that microbes growing at the logarithmic phase exist abundantly in the treatment tank. Thus, the degree of cell growth inhibition at this phase is an important factor for evaluating the influence of a dye on the degree of purification.

The equation for the mean growth rate of a cell-population at the logarithmic phase can be expressed as

$$dC/dt = kC \qquad (1)$$

where C is the cell concentration, t, the culture time, and k, the mean growth rate constant. Setting $C = C_0$ and $t = t_0$ as the initial conditions following integrating, equation (2) is obtained.

$$\ln(C/C_0) = k(t - t_0) \qquad (2)$$

The degree of growth inhibition, H, is defined as

$$H = 1 - (k_d/k_0) \qquad (3)$$

where k_d and k_0 are, respectively, the mean growth rate constants in a system with and without a dye. It has been shown experimentally for many kinds of inhibitive substances that the degree of inhibition may be represented as

$$H = G^n/(\phi^n + G^n) \qquad (4)$$

where n is the exponent indicating inhibitive ability, G, the concentration of the inhibitive substance, and ϕ, the concentration of the inhibitive substance at H = 0.5 (Yanagida 1981). Equation (4) can be rewritten as

$$\log[H/(1 - H)] = n\log G - n\log\phi \qquad (5)$$

H-values were calculated applying the data of the growth curves to equation (2) and (3), and were then plotted using the relation of $\log[H/(1 - H)]$ vs. logG as shown in Figure 2. The values of n and ϕ were determined respectively from the slope of the straight line and G-values at $\log [H/(1 - H)] = 0$ and are listed in Table 1.

A number of dyes used, particularly triphenylmethane dyes, strongly inhibited cell growth, as evident from the fact that the ϕ-values were less than 10^{-4} mol/l. It is well known that triphenylmethane dyes also have strong sterilizing properties (Horiguchi 1969). In the case of xanthene dyes, Rhodamine B was a weaker inhibitor of cell growth than Pyronine G. Hydrophilic anionic groups such as sulfonic and carboxylic acid result generally in lower permeability and accumulation of the compounds in the cells (Kitao 1982). The above observation on Rhodamine B may thus possibly be due to the presence of a carboxylic group.

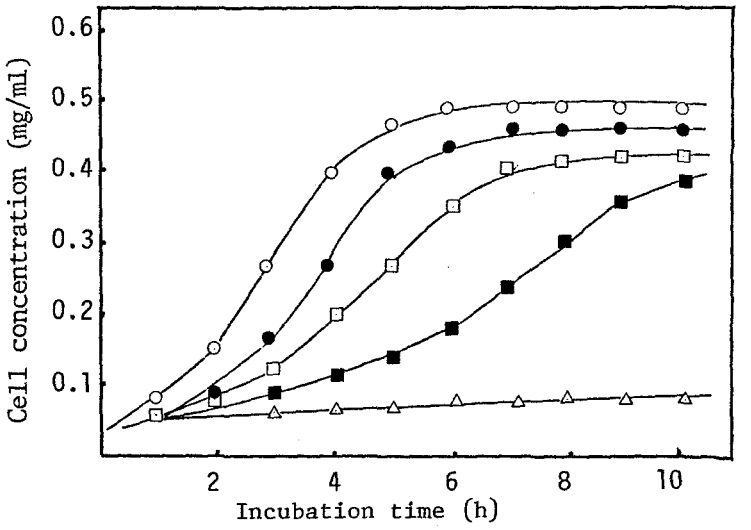


Figure 1. Growth curves of cells containing Methyl Violet in the medium. control (○), concentration of Methyl Violet; 2.7×10^{-6} mol/l (●), 5.1×10^{-6} mol/l (□), 1.0×10^{-5} mol/l (■), 2.0×10^{-5} mol/l (△).

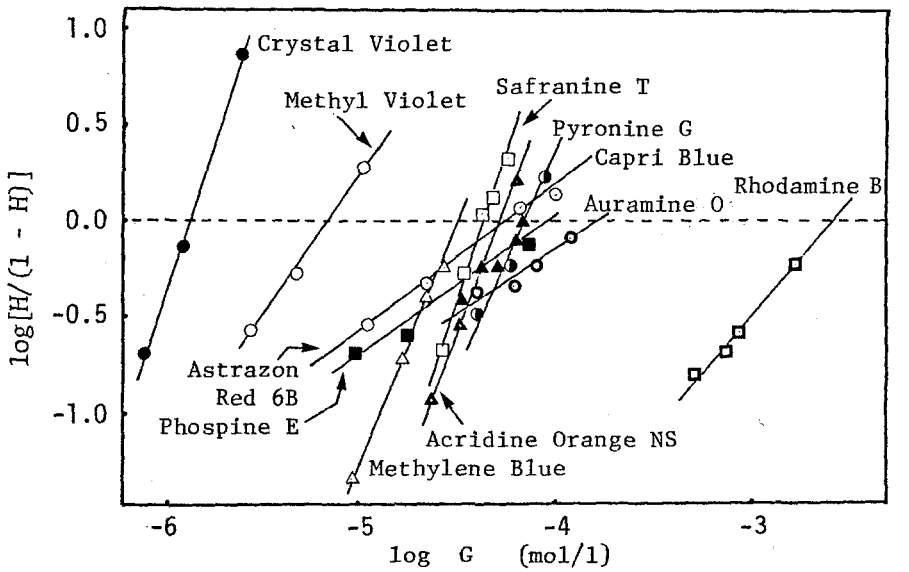


Figure 2. Relation between log [H/(1-H)] and log G in Eq. (5).

The inhibition mechanism should be elucidated so as to determine the influence of the dyes on cell chemical composition. For this purpose, the nucleic acid content was determined for cells harvested, respectively, both at the logarithmic and stationary phases. The content did not appreciably vary throughout the logarithmic

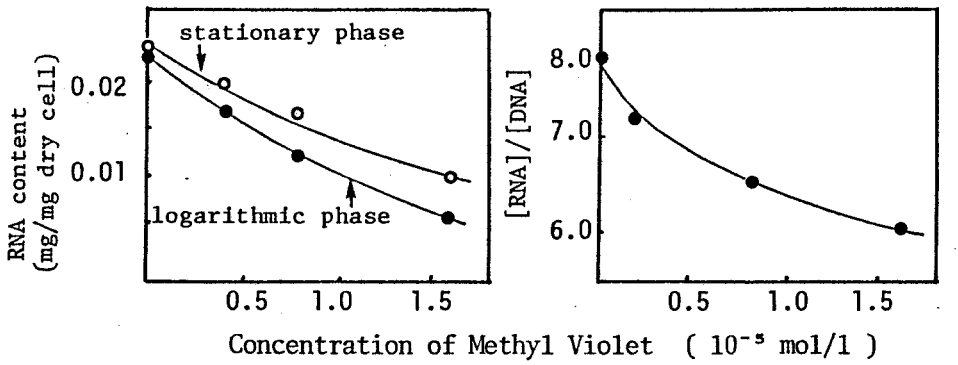


Figure 3. Relation between nucleic acid content of the cells and concentration of Methyl Violet.

phase. The cells were thus harvested at the late logarithmic phase so that a sufficient amount of nucleic acid could be obtained at one time for measurement. One example of the results is shown in Figure 3. RNA decreased with increasing concentration of Methyl Violet; this tendency was more remarkable in the logarithmic than stationary phase, corresponding to difference in the cellular physiological activity at each phase. A similar tendency was also noted in experiments using Acridine Orange NS and Astrazon Red 6B.

It is known in the case of bacteria populations in activated sludge that the growth rate at the logarithmic phase increases in proportion to cellular RNA content (Kaneko and Nanbe 1973). Assuming a similar relation in the cell of the present study, a correlation between cell growth inhibition and that of RNA synthesis should be possible. For confirmation of this, both inhibitions were compared as follows. The ratio of decrement in RNA content to increment in the concentration of Methyl Violet was determined and was shown in Figure 3. The ratios of the dyes, i.e. Methyl Violet, Acridine Orange NS and Astrazon Red 6B, expressed in the units shown in the figure, were respectively 8.5×10^4, 12.7×10^4 and 3.5×10^4; the relative ratios were 2.4:3.6:1.0. The relative ratio of n-values obtained from the data in Table 1 was 2.2:3.5:1.0. The approximate equality of both relative ratios indicated growth inhibition to depend strongly on that of RNA synthesis.

The content ratios of nucleic acid, [RNA]/[DNA], decreased with increasing dye concentration as shown in Figure 3. This has also been observed for the growth inhibition of bacteria by heavy metallic ions (Kaneko and Nanbe 1973). Thus, dyes act more preferencially to lower protein-synthesis than inhibit cell division. Due to the inhibitive action, cell shape varied, as evident from Figure 4. Cells grown under ordinary culture conditions appeared as short rods and those in the presence of dyes, as filaments.

Succesive batch cultures containing a dye was carried out to elucidate the acclimation effects of cells on the dye. The cells were

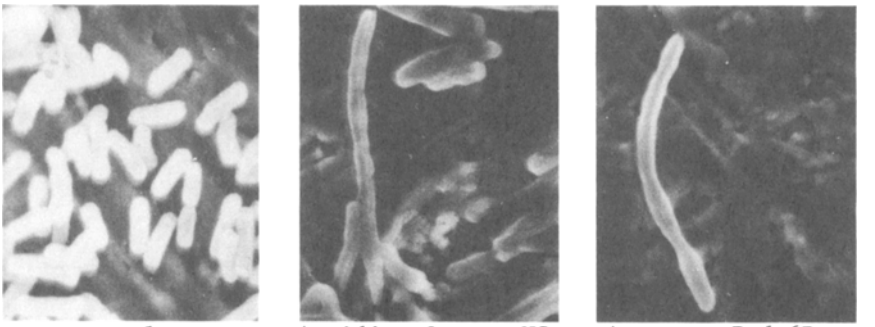


Figure 4. Electron micrograph of cells grown in the culture containing the dye. Manification 10,000X

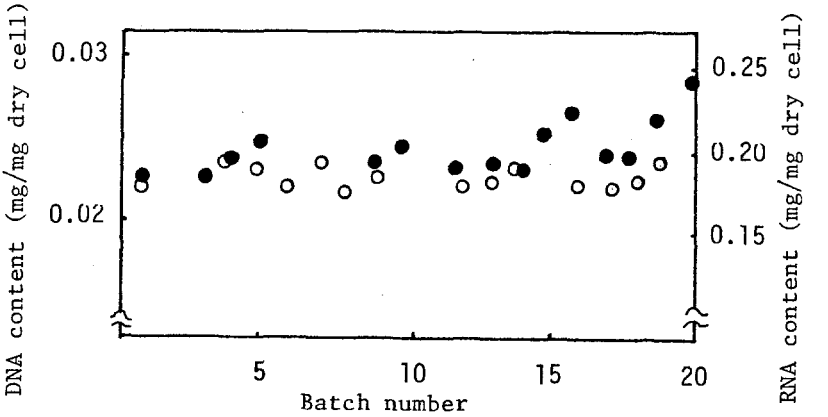


Figure 5. Relation between nucleic acid content and batch number in succesive batch cultures containing Methyl Violet ($3.0X10^{-6}$ mol/l): DNA (○), RNA (●).

inoculated succesively at 12 hr intervals and harvested to determine the nucleic acid content in each batch culture. The results are shown in Figure 5. The DNA content increased little with batch number, and RNA contents somewhat. The physiological activity of the cells was noted to be restored partially by acclimation.

Changes in synchronous growth curves with the addition of dyes were examined to determine the relation between inhibition and cell-age. The results are presented in Figure 6. The first generation time, 55 min, in a synchronous culture without a dye was essentially the same as both the second one and the mean generation time evaluated from the growth curve in the asynchronous culture. It became evident that damage to cells subjected to synchronization was too small to permit the elongation of generation time. Dye addition

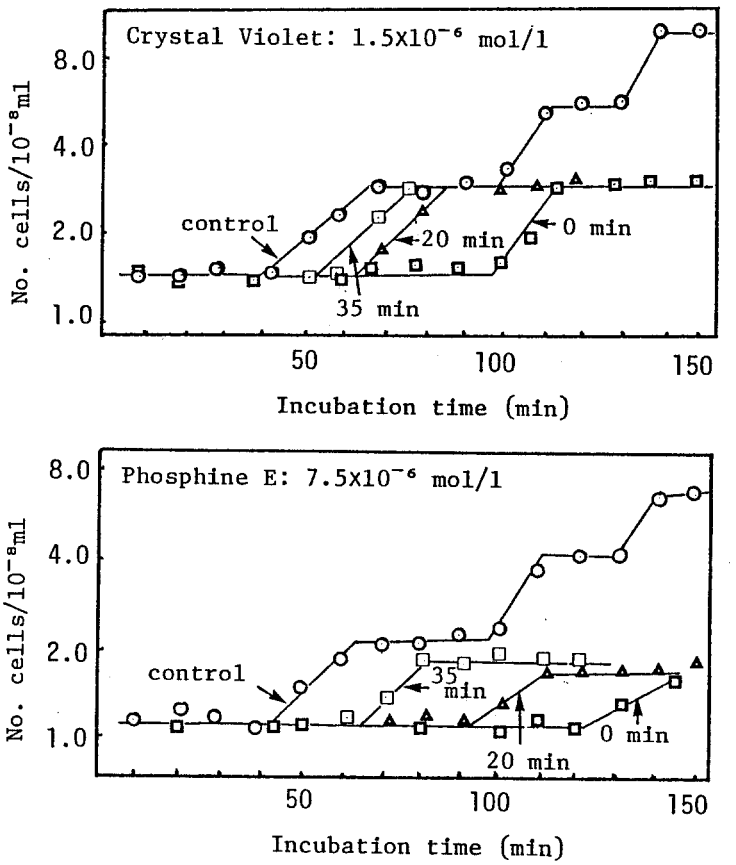


Figure 6. Influence of added dye on synchronous cell growth; Arrows indicate times of the additions.

increased generation time and decreased cell division. The latter means that a part of the cell population took on a static or cidal state. The former was more remarkable when the dye was added to young cells. A similar phenomenon has also been observed on the generation time of tetraphymena, a protozoa, on addition of qunacrine, a triphenylmethane dye (Chou et al. 1968). RNA is synthesized much in the early life of bacteria (Maruyama 1956). Thus, increased generation time results mainly in the inhibition of RNA synthesis. This finding is consistent with the results described previously for nucleic acid content and cell shape.

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