Bull. Environm. Contam. Toxicol. 26, 38-41 (1981)

Influence of Azo Dye on Synchronized Cells of *Bacillus subtilis*

Toshihiko Ogawa, Eiichi Idaka and Chizuko Yatome

Department of Synthetic Chemistry, Gifu University, Kakamigahara, Gifu 504, Japan

Biological treatment has been utilized generally as a method for the purification of waste waters from dye factories. Most synthetic dyes, however, show not only low degradability but also high toxicity to microbes. These properties have prevented practical biological treatment. We have therefore been investigating the toxicity and biodegradability of dyes. It is unknown whether dyes inhibit the growth of cells or the division of cells and also whether dyes can be assimilated autotrophically, although it has been confirmed that azo dyes may be degraded in a particular condition by specific strains (HORITSU *et al* 1977, IDAKA *et al* 1978, KAPPELER *et al* 1978, MEYER *et al* 1979). In the present investigation, steps of synchronized cells of *B. subtilis* to inhibition by azo dyes were studied, and specific susceptibility to dyes and the related compounds as a sole nitrogen source was tested.

EXPERIMENTAL

Microorganism

B. subtilis (IFO 3022) used in this work was supplied from the Institute for Fermentation, Osaka.

Preparation of synchronized cells

B. subtilis was cultured in a medium containing glucose 0.07%, sodium citrate 0.1, $(NH_4)_2SO_4$ 0.2, K_2HPO_4 1.4, KH_2PO_4 0.6, $FeCl_3 \cdot 6H_2O$ 0.0002, $MnCl_2 \cdot 4H_2O$ 0.0002 and $MgSO_4 \cdot 7H_2O$ 0.02 (DAWSON 1963). When the transmittance of cell suspension became 60%, glucose had been almost consumed and cells were in the state of starvation. On incubation for 1 hr, cells divided to form a population composed only of juvenile cells. The cell suspension was centrifuged and the cells were collected. Incubation was repeated again and the resultant highly synchronized cells were harvested. Reciprocal shaking culture was carried out with T-type test tube. The cell weight (MLSS) was determined after it had been washed with phosphate buffer, centrifuged and collected, and dried for 2 hr at 70°C *in vacuo*. The generation time and the extent of cell division were roughly estimated from the synchronized culture curve.

DNA content

Cells were suspended in phosphate buffer, disrupted with an ultrasonication, and treated with 5% perchloric acid solution at 90°C for 15 min. DNA content was estimated from the uv absorption of the solution at 260 nm (SCHNEIDER 1946, OGUR 1950).

RESULTS AND DISCUSSION

An example of the synchronous growth curves of *B. subtilis* is

0007-4861/81/0026-0038 $01.00

shown in Fig. 1. Virtually no difference could be recognized between the curves of the case involving *p*-aminoazobenzene (PAAB) and that of the case without it, during the period from the 1st to 2nd

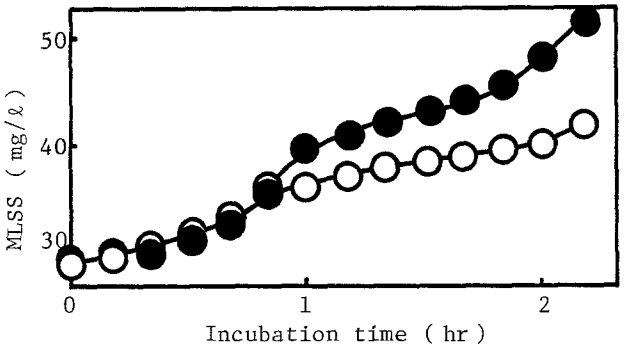
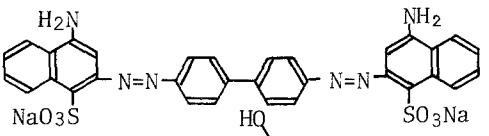
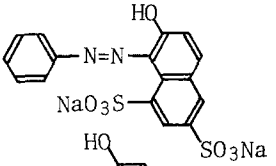
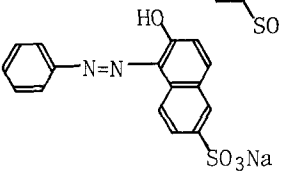
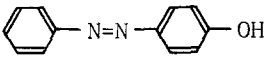
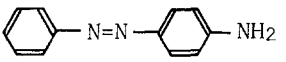


Fig. 1 Synchronous growth of *B. subtilis*
temperature 37°C, pH 7.0, conc. of dye 1×10^{-4} mol/ℓ
○ PAAB, ● control

cell division, namely, generation time. This behavior was also observed when other azo dyes were used. However, the MLSS-increase due to cell division was less in the case where dyes were added as compared with the case without dyes. The rates of the MLSS-increase in the cases with dyes to that without dyes are listed in Table. Direct Red 28 showed almost no inhibition of the cell division. It

TABLE

Rate of MLSS-increase during the cell division

Dye (1×10^{-4} mol/ℓ)	Formula	Rate of Δ MLSS (%)
Direct Red 28		102
Acid Orange 10		88
Acid Orange 12		80
p-Hydroxyazobenzene (PHAB)		67
p-Aminoazobenzene (PAAB)		64
Control		100

has also been found that direct dyes hardly affected the respiratory inhibition in activated sludge (OGAWA *et al* 1978a). The reason may be that the molecular volume of the dyes is too large to permeate a cell. Disulfonic dye (Acid Orange 10) was less inhibiting than monosulfonic dye (Acid Orange 12). PHAB and PAAB, which have a smaller molecular volume, showed more inhibition than any other dyes tested. These results are consistent with those obtained for respiratory inhibition (OGAWA *et al* 1978b).

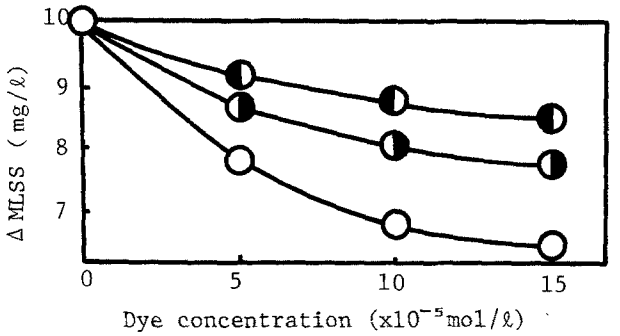


Fig. 2 Relationships between MLSS-increases during cell division and dye concentration
temperature 37°C, pH 7.0, initial MLSS 28mg/ℓ
○ PAAB, ◑ Acid Orange 12, ◐ Acid Orange 10

Synchronous growth curves were determined for the case in which dye concentrations were changed. Hardly any difference in generation time was recognized, and the MLSS-increase during cell division decreased as the concentration of a dye increased. The change of MLSS during the 1st division is shown in Fig. 2. These results suggest that the dyes may inhibit the cell division rather than the cell growth. Hence, the DNA content in the cells incubated in the medium containing PAAB was determined. The result, as shown in Fig. 3, revealed that the synthesis of DNA was inhibited by PAAB.

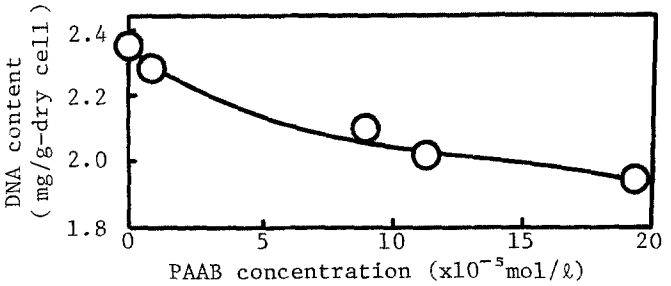


Fig. 3 Relationship between DNA content in cells and PAAB concentration

The assimilative capability of dyes and related compounds was then investigated. When the cells of *B. subtilis* were incubated in the glucose-peptone-meat extract medium (OGAWA *et al* 1974) and also in the medium in which ammonium sulfate or PAAB was used as sole nitrogen source, the growth curves were obtained as shown in Fig. 4. When incubated without a nitrogen source, the cells did not prolif-

erate at all. These findings suggest that PAAB is assimilated as a sole nitrogen source in the initial state of incubation, though its assimilation is inferior to that of peptone-meat extract or ammonium sulfate. *p*-Phenylenediamine and Disperse Violet 1, which have primary amino groups could also be assimilated. However, heterocyclic compounds (imidazole and pyridine) and azo compounds were not assimilated, at least in the initial state of incubation.

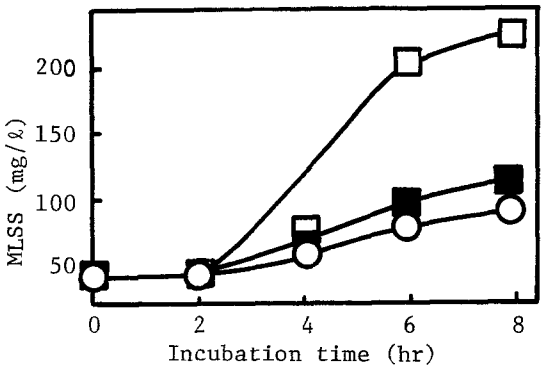


Fig. 4 Synchronous growth curves from changing nitrogen source
temperature 37°C, pH 7.0, medium compositions other than nitrogen source were as follows: glucose 0.6%, $Na_2HPO_4 \cdot 12H_2O$ 0.016, KH_2PO_4 0.1, KCl 0.02, $MgSO_4$ 0.014, NaCl 0.04, $CaCl_2$ 0.02, $FeCl_3$ 0.01. Nitrogen source: □ peptone 0.6% and meat extract 0.4%, ■ $(NH_4)_2SO_4$ 5×10^{-5} mol/ℓ, ○ PAAB 1×10^{-4} mol/ℓ

REFERENCES

HORITSU, H., M. TAKADA, E. IDAKA, M. TOMOYEDA and T. OGAWA: *European J. Appl. Microbiol.* 4, 217 (1977).

IDAKA, E., T. OGAWA, H. HORITSU and M. TOMOYEDA: *J. Soc. Dyers & Colourists* 94, 91 (1978).

KAPPELER, T., U. MEYER, A. von WATTENWYL, K. WUHLMANN and H. ZOLLINGER: *Textile Chemist & Colorists* 10, 10 (1978).

MEYER, U., G. OVERNEY and A. von WATTENWYL: *Textilveredlung* 14, 15 (1979).

DAWSON, P. S. S.: *Can. J. Microbiol.* 9, 671 (1963).

SCHNEIDER, W. C.: *J. Biol. Chem.* 164, 747 (1946).

OGUR, M. and G. ROSEN: *Arch. Biochem.* 25, 262 (1950).

OGAWA, T., E. IDAKA and Y. YAMADA: *J. Soc. Fiber Sci. Tech. Jpn.* 30, 516 (1974).

OGAWA, T., Y. YAMADA and E. IDAKA: *ibid.* 34, 175 (1978a).

OGAWA, T., E. IDAKA and T. KUMAZAKI: *ibid.* 34, 211 (1978b).