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Effects of oxygen and nitrate on growth of *Escherichia coli* and *Pseudomonas aeruginosa* in the presence of organic solvents

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Abstract *Escherichia coli* and *Pseudomonas aeruginosa* grown in the presence of certain harmful organic solvents become susceptible to these solvents during the cultivation. This susceptibility is conspicuous in the stationary phase of growth. The organic solvent tolerance levels of these microorganisms were maintained when the oxygen concentration was kept high. The tolerance levels were maintained also when these organisms were grown with nitrate present under anaerobic respiratory conditions.

Key words *Escherichia coli* · *Pseudomonas aeruginosa* · Organic solvent tolerance · Respiration · Oxygen · Nitrate

Introduction

Hydrophobic organic solvent and aqueous medium form a two-liquid phase culture system for several microorganisms. Various organic solvents used in this system are toxic to microorganisms. The toxicity is correlated inversely with log P_{ow}, which is defined as the common logarithm of the partition coefficient (P_{ow}) of the organic solvent between *n*-octanol and water (Inoue and Horikoshi 1991; Leo 1993). An organic solvent with a lower log P_{ow} value is more harmful to microorganisms. Each bacterium has its own intrinsic tolerance level to organic solvents. It has been proposed that the tolerance level of each microorganism is represented by two terms: the index solvent and the index value (Inoue and Horikoshi 1989; Aono et al. 1994b). The index solvent is the most toxic one among the organic solvents which the organism tolerates. The index value is the log P_{ow} value of the index solvent. This concept provides a useful guide to making microorganisms grow in the presence of organic solvent.

Recently, there have been an increasing number of cases where water-insoluble organic compounds were used as substrates for bioreactors of bacterial cells (de Smet et al. 1983; Harbron et al. 1986; Favre-Bulle et al. 1991; Van Sonsbeck et al. 1993). Some bioreactors use organic solvents to dissolve the substrates (Aono et al. 1994a; Doukyu and Aono 1997). *Pseudomonas* spp. and *Escherichia coli* strains were mainly used in these systems. To achieve high efficiency of bioconversion, it is obviously essential that the bacterial cells are kept constantly viable throughout the cultivation. However, the frequency of viable cells in a microorganism culture is low in the presence of harmful organic solvent. We devised cultivation methods to maintain cell viability under these conditions.

In this report, we describe effects of oxygen concentration and respiration on the cell viability of microorganisms growing in two-liquid phase systems using harmful organic solvents.

Materials and methods

Strains, culture media, and growth conditions

The bacterial strains used in this study are shown in Table 1. *Pseudomonas aeruginosa* PAO3292 was provided by Dr. Y. Tanji (Tokyo Institute of Technology, Yokohama, Japan). *P. aeruginosa* PAO3292 and *Escherichia coli* W3110 (Kohara et al. 1987) were grown in LBGMg medium consisting of 1% Bacto-tryptone (Difco Laboratories, Detroit, MI, USA), 0.5% yeast extract (Difco), 1% NaCl, 0.1% glucose, and 10 mM $MgSO_4$ (Aono et al. 1991). The medium (100 ml) was placed in a 500-ml Erlenmeyer flask and overlaid with an appropriate organic solvent. The flask was sealed with a silicon cap. The cap was pierced with a needle attached to a syringe for sampling from the gas phase or the

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Table 1. Bacterial strains

Strain	Characteristics	Organic solvent tolerance (log P_{ow})				Index solvent	Index value	Tested solvent
		n-Hexane (3.8)	Cyclohexane (3.4)	*p*-Xylene (3.1)	Toluene (2.8)			
P. aeruginosa PAO3292	Derivative from PAO1	+	+	+	−	*p*-Xylene	3.1	Cyclohexane
E. coli W3110	K-12, F^-, IN (*rrnD-rrnE*)1	+	+	−	−	Cyclohexane	3.4	*n*-Hexane

log P_{ow}, common logarithm of partition coefficient of organic solvent between *n*-octanol and water.

medium of the culture. The flasks were rotated at 160 rpm at 30°C.

In some experiments, the organisms were grown under nitrate respiration conditions. PAO3292 and W3110 were grown in LBGMg medium supplemented with 50 and 60 mM of $NaNO_3$, respectively. The flasks containing the medium were degassed and flushed with N_2 gas three times to reduce the O_2 ratio in the gas phase below 2% (v/v) (Davis et al. 1989; Thomas et al. 1994).

Measurement of solvent tolerance levels of microorganisms

Cells freshly grown in LBGMg medium overnight were suspended in cold 0.8% saline to give a suspension of approximately 10^6–10^7 cells/ml. A drop of the suspension (5 μl) was placed on LBGMg agar to form a circle of 7–8 mm in diameter. After the saline penetrated into the agar, one of the organic solvents was overlaid to a depth of about 2 mm. The plates were incubated at 30°C for 24 h in a closed vessel to avoid evaporation of solvent. Confluent growth of cells was considered an indicator of tolerance to the solvent tested.

Measurement of colony-forming units (CFU)

The number of colony-forming units (CFU) per milliliter of culture was determined as the number of cells forming colonies on LBGMg plates.

Measurement of oxygen ratio

The oxygen ratio in the gas phase in the culture vessel was measured by a gas chromatography apparatus (GC-9AM; Shimadzu, Kyoto, Japan). Sample gas was injected onto a molecular sieve 5A column (3 mm by 150 cm) at 80°C and eluted with argon. The oxygen molecule was detected with a thermal conductivity detector. Normal air was used as the standard gas containing 21% (v/v) O_2.

Materials

Organic solvents used were the highest qualities that were commercially available. The log P_{ow} values of compounds were calculated by the addition rule (Leo 1993).

Results and discussion

Effect of O_2 ratio on the cell viability of *P. aeruginosa* and *E. coli* in the presence of organic solvents

Every microorganism can grow in a medium overlaid with an organic solvent with a log P_{ow} that is greater than or equal to the index value. However, the number of viable cells of the microorganism is extremely low compared with the number of total cells estimated on the basis of the culture turbidity or cell mass when the organism was grown in the presence of organic solvent with log P_{ow} value near the index value. The numbers of viable and total cells are significantly discrepant, especially in the stationary phase of growth. This curious phenomenon has been described by several investigators (Favre-Bulle et al. 1991; Cruden et al. 1992; Weber et al. 1993; Aono et al. 1994b). Such a discrepancy is not found when the organism is grown in the presence of a nonharmful organic solvent with a log P_{ow} that is much greater than the index value.

We assumed that the low frequency of viable cells was due to a shortage of oxygen in the culture containing a large number of the cells. *P. aeruginosa* PAO3292 and *E. coli* W3110 tolerate *p*-xylene (log P_{ow} 3.1) and cyclohexane (log P_{ow} 3.4), respectively (Table 1). These organisms were grown in LBGMg medium overlaid with organic solvent with a log P_{ow} value near the relevant index value under high or usual aerobic conditions. When the O_2 ratio in the gas phase of the culture was kept at 20%–50% (v/v) by blowing-in of O_2, *P. aeruginosa* PAO3292 grew normally in the presence of cyclohexane (Fig. 1b). The number of viable cells was maintained at high levels (CFU 4×10^9/ml) even after 24 h. The O_2 ratio decreased to approximately 1% at 12 h due to O_2 consumption by the aerobic respiration when O_2 was not blown into the flask (Fig. 1a). The number of viable cells increased at first and then fell to around 10^6/ml after 12 h. The culture turbidity was not so discrepant between these two cultures (results not shown). These results showed that the proportion of viable PAO3292 cells growing in the presence of cyclohexane was improved by an excess supply of oxygen, indicating that the organism required a sufficiently high level of O_2 supply to tolerate cyclohexane fully.

E. coli viable cells are maintained stably in the presence of organic solvents whose log P_{ow} values are much greater than the index value (Aono et al. 1994). However, the number of viable cells of *E. coli* W3110 grown in the presence of

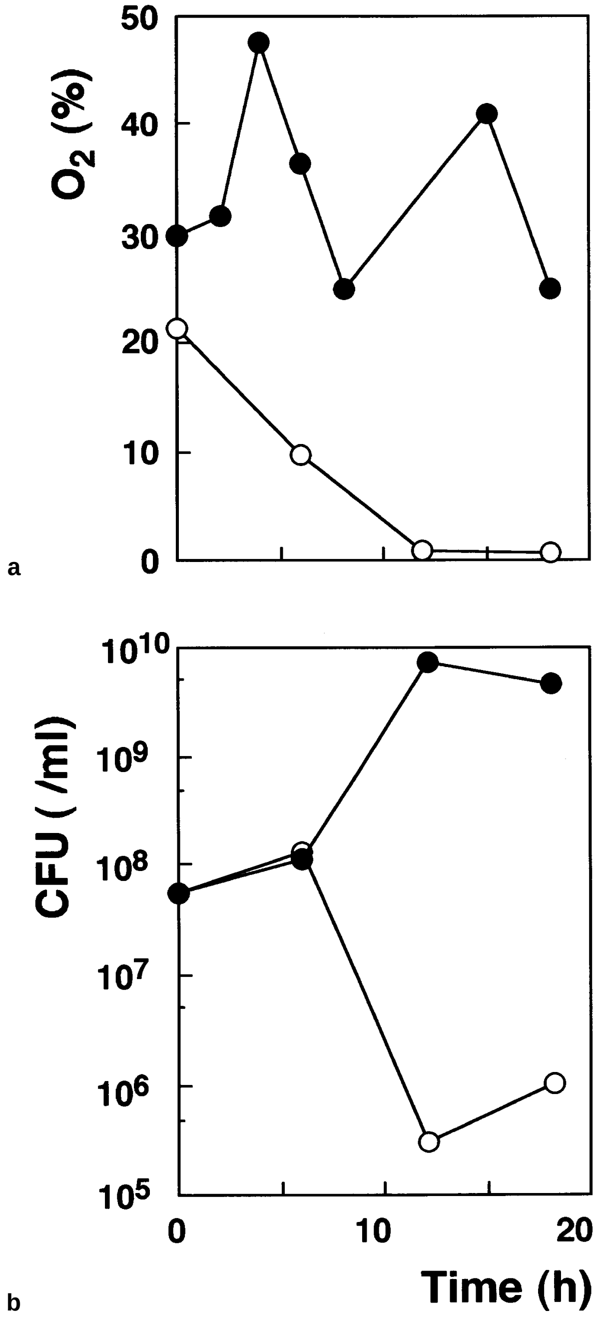

Fig. 1a,b. Effect of O_2 ratio on cell viability of *P. aeruginosa* PAO3292 in the presence of cyclohexane. *P. aeruginosa* PAO3292 was incubated in 100 ml of LBGMg medium in the presence of cyclohexane (20 ml) in two sealed 500-ml flasks at 37°C. The O_2 ratio in the gas phase was maintained at 20%–50% (v/v) by blowing O_2 into the flask (*closed circles*). The O_2 ratio in the other flask (*open circles*) was not controlled. During the incubation, the O_2 ratio (**a**) and the number of viable cells (**b**) were measured periodically. CFU, colony-forming units

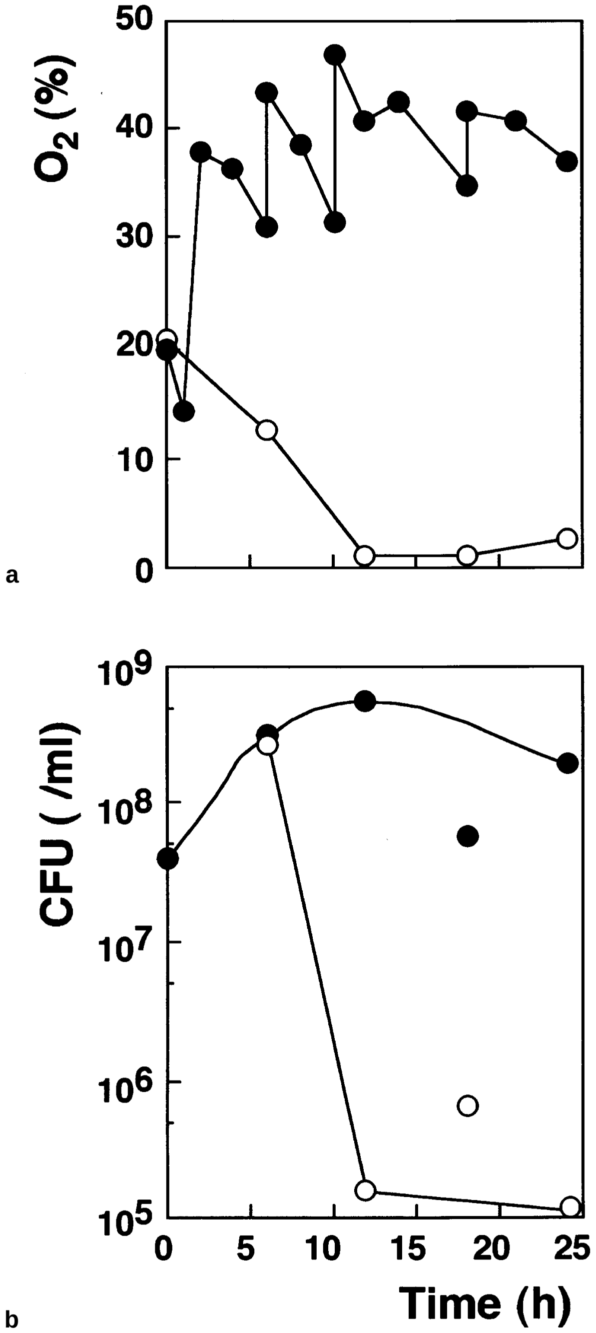

Fig. 2a,b. Effect of O_2 ratio on cell viability of *E. coli* W3110 in the presence of *n*-hexane. *E. coli* W3110 was grown in 100 ml of LBGMg medium in the presence of *n*-hexane (20 ml) in two sealed 500-ml flasks at 37°C. The O_2 ratio was maintained at 20%–50% (*closed circles*) or not maintained (*open circles*). During the incubation, the O_2 ratio (**a**) and growth (**b**) were measured periodically

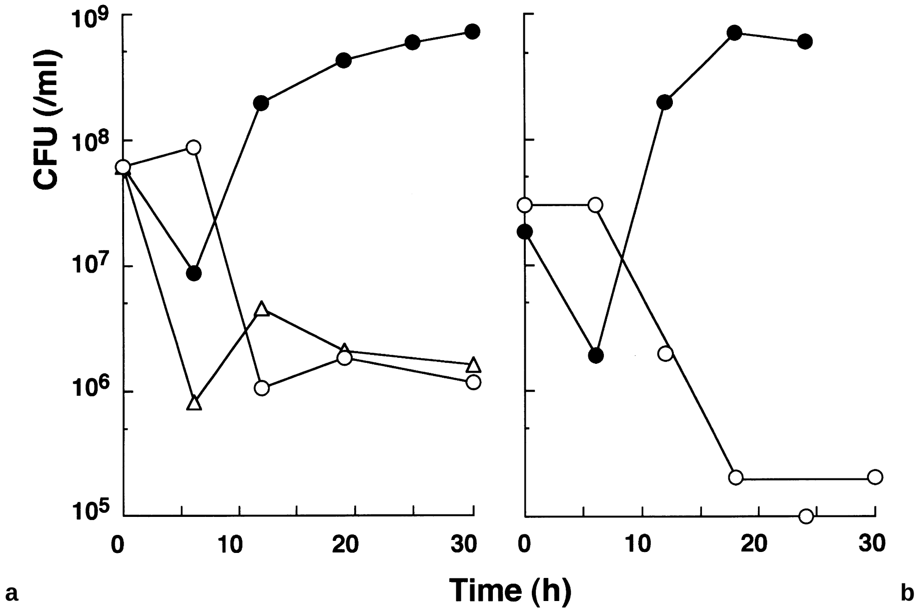

Fig. 3a,b. Effect of nitrate respiration on sustaining organic solvent tolerance in *P. aeruginosa* PAO3292 and *E. coli* W3110. *P. aeruginosa* PAO3292 was incubated in 100 ml of LBGMg medium overlaid with cyclohexane (**a**); *E. coli* W3110 was similarly incubated with *n*-hexane (**b**). The medium contained (*closed circles*) or did not contain (*open symbols*) sodium nitrate. Before the incubation, O_2 in the gas phase was replaced with nitrogen (*open triangles; closed circles*) or not (*open circles*)

n-hexane (log P_{ow} 3.9) fell significantly during the stationary phase of growth (Fig. 2). The O_2 ratio fell to around 1% in the culture when not controlled. At the time, the number of viable cells was only 10^5–10^6/ml. When the O_2 ratio was maintained at 10%–50%, the number of viable cells was kept high. Therefore, it was shown that oxygen contributed to maintenance of organic solvent tolerance levels of *E. coli.*

Improvement of the cell viability by nitrate instead of O_2

As just described, the organic solvent tolerance levels of *P. aeruginosa* and *E. coli* are lowered under the O_2-limited conditions. These organisms can utilize nitrate as an electron acceptor on anaerobic respiratory chains under the micro-aerobic conditions. We examined whether the cell viability of these strains was maintained by anaerobic nitrate respiration instead of aerobic respiration.

When oxygen in the gas phase was replaced with nitrogen and reduced to an O_2 ratio below 2% (v/v), the organism was hyper-susceptible to cyclohexane and did not grow (Fig. 3a). The number of surviving cells was similar to that in the culture in which the O_2 ratio was not controlled. This result supported the conclusion that the low frequency of viable cells was due to O_2 shortage. When sodium nitrate was added to the medium, the organism began to grow in the presence of cyclohexane by nitrate respiration after O_2 was consumed by aerobic respiration. A high number of viable cells was maintained even after 30 h. Also, *E. coli* W3110 grew in the presence of *n*-hexane and maintained cell viability under the nitrate respiratory conditions (Fig. 3b).

Conclusion

Considering the toxicity of organic solvent, it is important to select a suitable bacterium and organic solvent in a two-phase bioconversion system. The maintenance of bacterial respiration with O_2 or nitrate proved effective for sustaining cell viability in the presence of organic solvent for a long period. We expect that the oxygen and nitrate respiration methods described here will be broadly applicable to microbial bioconversion. These methods would allow a wider choice of bacterial strains and organic solvents.

Nitrate respiration relieved *P. aeruginosa* and *E. coli* from a decrease in viability in sealed flasks in the presence of harmful organic solvents. Under conditions in which O_2 was limited, nitrate respiration was effective in maintaining the organic solvent tolerance levels of *P. aeruginosa* and *E. coli.* These results indicated that maintenance of organic solvent tolerance by a sufficient supply of O_2 was due to bioenergy production by aerobic respiration, but not to any requirement for the O_2 molecule itself to oxidize particular metabolites. The organisms can be grown in the presence of harmful organic solvent by anaerobic nitrate respiration. Cultivation utilizing anaerobic nitrate respiration is useful for increasing the medium volume and decreasing the gas phase in a culture vessel. In addition, anaerobic cultivation can reduce the risk of explosion brought about by the concomitant presence of high-concentration oxygen and some flammable organic solvent.

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