Effects of Bioactive Substances on the Growth of Lithotrophic Ammonia-Oxidizing Bacteria

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Abstract

The effect of synthetic local anesthetics and related agents, lidocaine, procainamide, tetracaine, and dibucaine on the growth rate of *Nitrosomonas europaea* was studied under lithotrophic culture conditions. The results were compared with those with saponin and benzyl thiocyanate, which have been known to be bioactivators for some kinds of microorganisms. Except for saponin, the growth rates of the five kinds of the chemicals supplemented cultures were found to be statistically higher at 25℃. Lidocaine was the most effective growth stimulant, followed by procainamide, tetracaine, dibucaine, and benzyl thiocyanate. Addition of saponin showed no positive and negative effects on the bacterial growth rate under the experimental conditions studied. These agents could be utilized as stimulators for the slow-growing *N. europaea*.

Key words: benzyl thiocyanate, cell growth, dibucaine, lidocaine, local anaesthetics, *Nitrosomonas europaea*, procaine, procainamide, saponin, tetracaine

1. Introduction

Removal of nitrogen compounds is a key problem in modern wastewater treatment systems, and their biochemical depletion is achieved in a two-stage process. The first stage is nitrification, or conversion of ammonia to nitrate; the second stage is denitrification, or reduction of nitrate to gaseous nitrogen end products (Casey, 1997). Denitrification is accomplished by a wide assortment of respiratory bacteria representing most genera and physiological types (Zumft, 1992).

Microbial nitrification is an aerobic process and is generally believed to proceed by two steps: (*i*) conversion of ammonia to nitrite by *Nitrosomonas* spp., and (*ii*) conversion of nitrite to nitrate by *Nitrobacter* spp. (Abeliovich, 1992). The overall chemical oxidation is described by the equation: $NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$.

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The nitrifying bacteria are lithoautotrophs for whom energy for growth derives from oxidation of inorganic nitrogen. The growth rate of nitrifiers is estimated to be some 10–20 times slower than the growth rate of heterotrophs which are responsible for carbonaceous BOD (biochemical oxygen demand) removal. Of the two species responsible for nitrification, *Nitrosomonas* has a lower growth rate than *Nitrobacter*. Therefore, the growth rate of the former is normally rate-limiting for the nitrification process. To increase the efficiency of microbial nitrification two approaches have been considered: addition of bioactivators to improve endogenous microorganisms' nitrification capabilities, and use of microbial starter cultures to accelerating the epuration process (Thonart et al., 1996).

A variety of compounds are known to enhance growth and/or differentiation in plants, algae, and bacteria (Cachita-Cosma & Ardelean, 1996). These include synthetic local anaesthetics such as procaine and lidocaine (Suzuki et al., 2000). Additionally, benzyl thiocyanate, $C_6H_5CH_2SCN$, has been used since the late 1950s to enhance chlortetracycline biosynthesis by *Streptomyces aureofaciens* in laboratory and industrial fermentations (Novotna et al., 1995). Benzyl thiocyanate and about 40 analogues were examined by Priestap (1987) *vis-à-vis* their influence on tetracycline biosynthesis by *S. aureofaciens* var *mediolanum*, and the structural elements most stimulatory to tetracyclinogenesis determined. Furthermore, addition of saponin reportedly improves the anaerobic digestion of sewage sludge (Nagasaka et al., 1999).

Though the modes of action of these compounds and their breadth of structural diversities remain to be fully explored, use of growth stimulators to accelerate bacterial nitrification is of considerable theoretical and practical interest. This study aimed at establishing whether it is possible to stimulate of the growth rate of *Nitrosomonas europaea* using lidocaine or other synthetic local anaesthetics, bezyl thiocyanate, and/or saponin.

2. Materials and Methods

2.1. Organisms

Nitrosomonas europaea IFO 14298 (Institute for Fermentation, Osaka, Japan) was grown at 25℃ on a mineral salts medium which had the following basal composition (per L): HEPES, 12.0 g; KH_2PO_4, 0.5 g; $(NH_4)_2SO_4$, 5.0 g; $MgSO_4 \cdot 7H_2O$, 5.0 g; Fe-EDTA, 10.0 mg; $CaCl_2 \cdot 2H_2O$, 20.0 mg; $MnCl_2 \cdot 4H_2O$, 20 μg; $NaMoO_4 \cdot 2H_2O$, 5 μg; $ZnSO_4 \cdot 5H_2O$, 5 μg; $CoCl_2 \cdot 6H_2O$, 1 μg; $CuSO_4 \cdot 5H_2O$, 1 g. The pH of the basal medium was adjusted to 7.7 prior to autoclaving.

2.2. Growth experiments

The growth rates of *N. europaea* were measured using a Bioscreen C Microbiol-

(b)

(a)

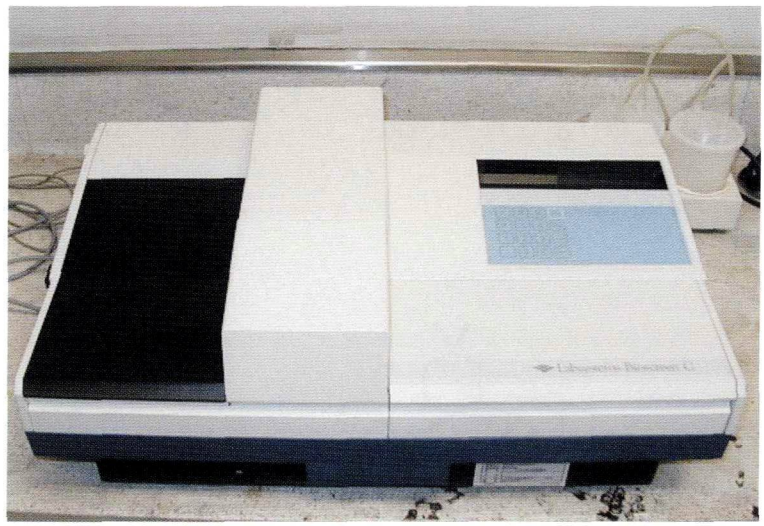
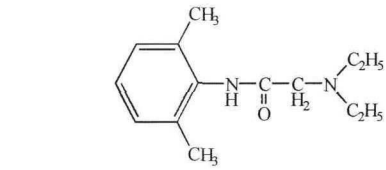


Fig. 1 Bioscreen C Microbiology Reader for obtaining growth curves of microorganisms (a), and 100-well microtitre plate used with this Reader (b).

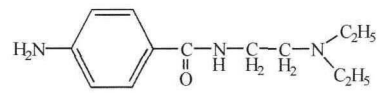
ogy Reader (Thermo Labsystems Oy, Vantaa, Finland); increases in optical density at 405 nm (OD_{405}) were monitored automatically every 30 min at 25℃ (A photograph of the apparatus is shown in Fig. 1). Data analysis was performed using the SigmaPlot 4.0 (SPSS, Inc., Chicago, Illinois, U. S. A.).

2.3. Test Chemicals

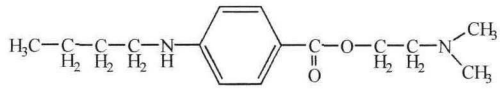
Lidocaine hydrochloride (>99%), procainamide hydrochloride (>99%) and benzyl thiocyanate (97%) were obtained from Sigma-Aldrich Japan (Tokyo, Japan), and tetracaine hydrochloride (>98%) and dibucaine hydrochloride (>98%) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Saponin ("Cica" Reagent) was a product of Kanto Chemical Co. Inc. (Tokyo, Japan). The structures of these compounds are presented in Fig. 2. Stock solutions of prospective growth effectors were prepared in basal medium, stored at −20℃ in tubes protected from light, and added to cultures as needed to give the desired concen-



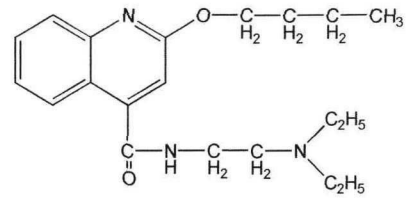
lidocaine



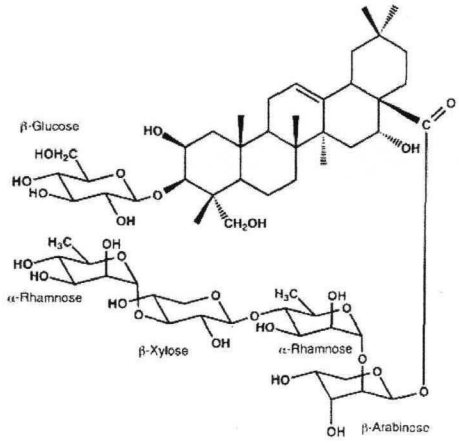
procainamide



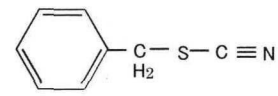
tetracaine



dibucaine



saponin



benzyl thiocyanate

Fig. 2 Structures for chemicals tested.

trations.

3. Results and Discussion

3.1. Correlation of OD and biomass concentration

It has been suggested that the biomass of *Nitrosomonas* spp. cannot be determined reliably from optical density measurements due to the slow growth rate of this bacterium and the very low biomass concentrations that accumulate; for this

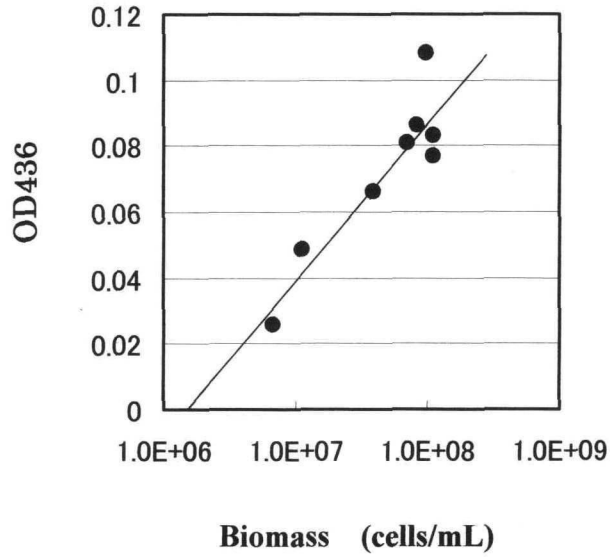


Fig. 3 Relationship between OD_{436} and biomass concentration for *Nitrosomonas europaea*.

reason, growth is usually quantified by measuring the consumption of ammonia or the formation of nitrite and cell nitrogen as a function of time (Bottcher& Koops, 1994). However, Groeneweg et al. (1994) reported a nearly linear correlation (r^2 =0.94) between dry weight of *N. europaea* and optical density at 436 nm.

In the present study, biomass concentrations were determined by *in situ* FISH methodology (Amann et al., 1995), and the relation between biomass concentration and OD_{436} nm was examined (Fig. 3). Figure 3 shows that, in accordance with Groeneweg et al. (1994), there was a good linear correlation (r^2=0.94) between biomass and OD_{436}. Further studies showed that 405 nm was a more sensitive wavelength for monitoring growth of *N. europaea* IFO 14298 in the mineral salts medium, so this wavelength was adopted for all subsequent growth measurements.

3.2. Effects of chemicals on bacterial growth

The influence on growth of *N. europaea* IFO 14298 of lidocaine, procainamide, tetracaine, and dibucaine over the concentration range $10^{-9}-10^{-4}M$ is summarized in Fig. 4. Changes in growth rate are expressed as the ratio of specific growth rate in the presence of test substance to that of cells cultured in unsupplemented medium. Positive effects of these synthetic local anaesthetics on the growth of this bacterium cannot be due to the test compound acting as an auxiliary nutrient, since under our experimental conditions *N. europaea* uses CO_2 as sole source of cell carbon.

Anaesthetics were observed to stimulate replicative growth of *N. europaea* accord-

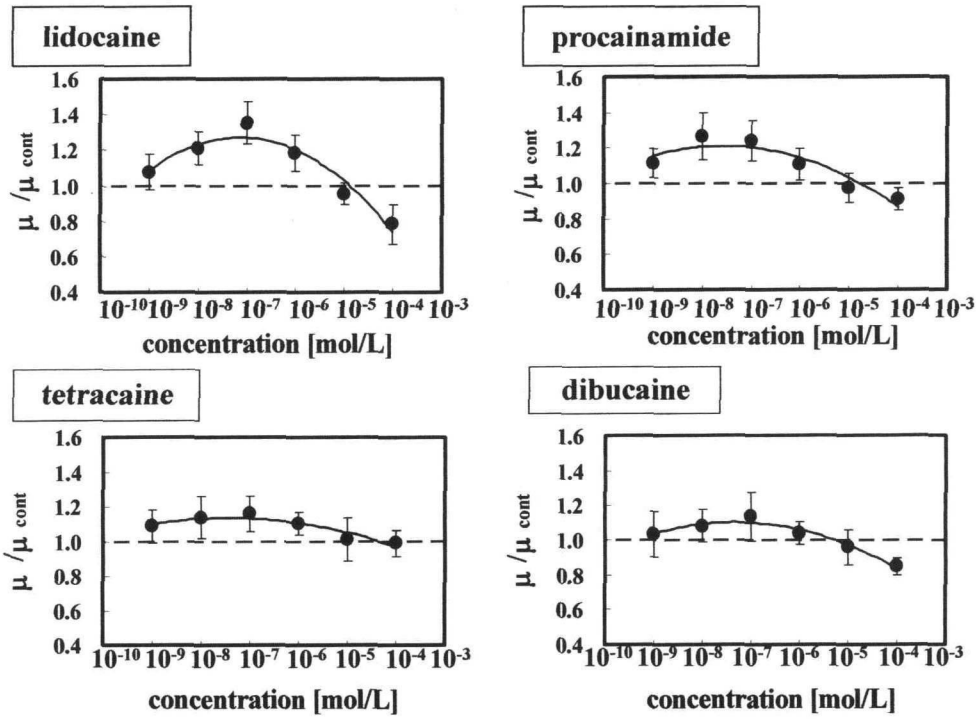


Fig. 4 Effects of local anaesthetic on the lithoautotropic growth of *Nitrosomonas europaea*.

ing to the order lidocaine > procainamide > tetracaine, dibucaine. Maximal stimulation of growth rate was observed with lidocaine. Optimal enhancement of *N. europaea* growth rates by the four anaesthetics varied within a very narrow concentration range: $7.9\times10^{-8}\,M$ for lidocaine, $2.6\times10^{-8}\,M$ for procainamide, $4.0\times10^{-8}\,M$ for tetracaine, and $4.5\times10^{-8}\,M$ dibucaine. Anaesthetic concentrations of $1\times10^{-5}\,M$ did not effect growth rates, whereas concentrations $>10^{-4}\,M$ were inhibitory. The toxicity observed at $\geq 10^{-4}\,M$ anaesthetic may follow from dissipation of the transmembrane potential in *N. europaea* due to the drug's channel-blocking properties (Ohsuka et al., 1994).

Effects of $10^{-3}-10^{2}$ ppm saponin and $10^{-10}-10^{-5}\,M$ benzyl thiocyanate on *N. europaea* growth are shown in Fig. 5. The bacterial response to benzyl thiocyanate resembled the bacterial responses to local anaesthetics over a comparable concentration range, a maximal specific growth rate being observed at $1.7\times10^{-8}\,M$, whereas benzym thiocyanate at the $\geq 10^{-5}\,M$ inhibited growth. In contrast, saponin had no positive and negative effects on bacterial growth rate.

The molecular size, shape, electronic and lipophobic/lipophilic properties of ligands govern their binding affinities to receptors. The structural similarities between

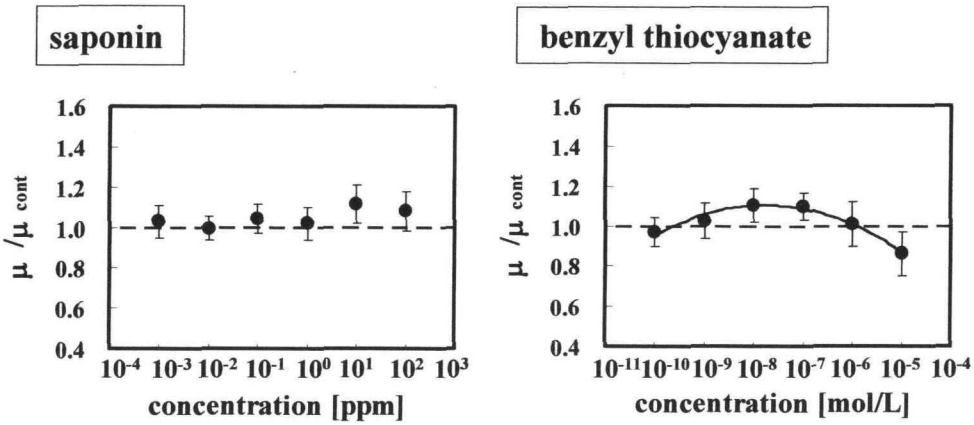


Fig. 5 Effects of saponin and benzyl thiocyanate on the lithoautotropic growth of *Nitrosomonas europaea*.

four local anaesthetics and benzyl thiocyanate could translate into similarities in action and potency towards *N. europaea*.

In conclusion, the effects of six chemicals on the lithoautotrophic growth of *Nitrosomonas europaea* were examined. Bacterial growth rates were increased maximally by 10–26% with appropriate supplementation of lidocaine, procainamide, tetracaine, dibucaine, or benzyl thiocyanate, whereas saponin failed affect bacterial growth rate within the concentration range surveyed.

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