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An acidic solution (pH 2.5-2.6) with a high oxidation-reduction potential (ORP; about +1,170 mV) and an alkaline solution (pH 11.5-11.7) with a low ORP (about -880 mV) that resulted from electrolysis of 20 mM NaCl (dissolved in a pure water) were tested for their effect on the growth of Streptomyces spp. When spores (2x10\(^{10}\)) were exposed to the electrolyzed solutions (2 ml) for 1 minute, colony formation was totally inhibited by the acidic solution, but little by the alkaline solution although extending the exposure (10 minutes) resulted in a marked inhibition. The 1 minute exposure to their mixture (1:1, v/v) showed a strong inhibition (but weaker than that of the acidic solution). When the unexposed spores were streaked and incubated on ISP No. 4 (inorganic salts - starch medium) agar plate containing a cross density gradient of the acidic and alkaline solutions, a biased growth inhibition toward the acidic solution side was observed although the pH range of the acidic solution end of the plate was around 6.2. It seemed thus unlikely that low pH value contributed to the antimicrobial activity of the acidic solution. It was notable that S. griseus SS-1198 formed a unique morphology on the cross gradient plate.

In addition, clear growth inhibition by the acidic solution was observed without direct contact with spores, probably because of chlorine gas release. Acidic solutions (pH 2.6-2.7) resulting from the electrolysis of 20 mM of Na\(_2\)SO\(_4\) show no significant antimicrobial activity when tested by the cross gradient plate method. It thus seemed likely that chlorine played a key role for the antimicrobial activity of the acidic electrolyzed NaCl solution.

INTRODUCTION

Recently, waters with novel properties, which were arbitrarily designated "activated waters" or "function waters", have been introduced into fields of biological technology in Japan\(^1\). The methods to yield such waters include a variety of physical treatments (e.g., electrolysis, and exposure to magnetic and electric fields). These waters have been claimed to show various biological effects such as antimicrobial and antiviral activities\(^2\) and growth stimulation of plants\(^3\). Detoxification of Euglena cells intoxicated with tributyltin chloride was also reported for a high electric field loaded water\(^3\).

The antimicrobial and antiviral activities were reported for acidic electrolyzed water (EW) resulting from the electrolysis of aqueous NaCl solution\(^2\) by the use of instruments in which anode and cathode were separated by a membrane in order to form two compartments. According to the supplier's claim, the antimicrobial and antiviral activities of the acidic EW is so strong that the EW is powerful enough to kill wide varieties of pathogenic microorganisms and viruses including MRSA and HIV. Eventually, numbers of hospitals and clinics started to use the acidic EW in order to prevent nosocomial infection of pathogenic bacteria such as MRSA.

The scientific basis for the development of the acidic EW is that microorganisms were not found in aqueous environments with both low
pH (lower than pH3) and high ORP (higher than +900 mV)\(^6\). Electrolysis of aqueous NaCl solution by the instruments developed can yield the acidic and oxidative EW (pH lower than 2.7 and ORP higher than +1,100 mV) at the anode side compartment, and the alkaline and reductive EW (pH higher than 11 and ORP lower than –800 mV) at the cathode side. Some other chemical species will also be formed; chlorine gas and probably HClO at the anode side and hydrogen gas at the cathode side.

In spite of such a broad and strong antimicrobial activity, data available for the acidic EW presented an additional problem; i.e. the use of tap waters for electrolysis. Since tap waters available at different places should have different electrolyte profiles, electrolysis of different tap water must yield EWs with different levels of activities. It seemed therefore necessary to use pure water supplemented with a certain concentration of a pure electrolyte in order to establish the unequivocal basis for the biological activities of EWs. In this context, we used Milli-Q water containing 20 mM NaCl for electrolysis in order to examine the antimicrobial activity of the acidic and alkaline electrolyzed solutions. This paper describes their effect on the growth of *Streptomyces* spp. under various conditions.

**MATERIALS AND METHODS**

**Instrument and conditions of electrolysis:**
A batch type of electrolysis instrument, "Super Mini-Water" (Janix Inc., Atsugi, Japan) was used. In this instrument, the anode and the cathode (both made from platinum) were partitioned by a filter, resulting in the formation of the anode and cathode compartments where the acidic and alkaline EWs are accumulated, respectively. Unless otherwise described, 1,300 ml of 20 mM NaCl dissolved in Milli-Q water (Millipore) were subjected to electrolysis under the following conditions: 850 mA/ 7.3–8.3 V for 15 minutes at room temperature (25–27°C). Some of their physico-chemical properties were shown in Table 1.

**Organisms:** Two strains (Nos. 624 and 2021) of *Streptomyces* spp., and *S. griseus* SS-1198 were used.

**Media:** ISP media No. 2 (malt extract - yeast extract agar) and No. 4 (inorganic salts - starch agar) available from Difco were used. According to the method reported\(^7\) previously, media with a cross density gradient of the acidic and alkaline electrolyzed-solutions were prepared as follows. ISP No. 4 agar medium, which was dissolved in Milli-Q water, autoclaved and then cooled down to 60°C, were mixed with an equal volume of the acidic electrolyzed solution. The mixture (40 ml) was then poured into a rectangular dish with a pillow under the one end of the plate. After the medium was hardened with a slope, the plate was placed flat and poured with 40 ml of ISP No. 4 agar medium mixed with an equal volume of the alkaline electrolyzed-solution. After hardening, the surface of plate was dried and kept at room temperature for a few hours until use.

**Examination of antimicrobial activity:**
*Streptomyces* strains were grown at 27°C on ISP No. 2 agar to a sufficient formation of the matured spores. The spores were suspended into 0.85% NaCl and used as the inoculum (about

**Table 1. Physico-chemical properties of electrolyzed solutions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Milli-Q</th>
<th>Anode</th>
<th>Cathode</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.8</td>
<td>2.5</td>
<td>11.6</td>
</tr>
<tr>
<td>ORP (mV)</td>
<td>698</td>
<td>1170</td>
<td>–878</td>
</tr>
<tr>
<td>DO (mg/l)</td>
<td>5.00</td>
<td>13.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Cl (ppm)</td>
<td>0.55</td>
<td>40</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

\* Data was available from the instrument supplier. ORP: oxidation-reduction potential, DO: dissolved oxygen, Cl: chlorine.
4×10^8/ml) unless otherwise mentioned. A portion (50 µl: about 2×10^7) of the spore suspensions were transferred into 2 ml of the electrolyzed solutions, vortexed quickly and stood still at room temperature. At 1 minute after vortexing, a portion (50 µl: 5×10^8) was spread on ISP No. 2 and No. 4 agar plates and incubated at 27°C for 3–5 days.

The antimicrobial activity was also examined on the ISP No.4 agar plate containing the cross density gradient of the acidic and alkaline solutions. Spore suspensions (20 µl) were streaked and incubated at 27°C for 7 days on this plate.

**Observation of Morphology:** A scanning electron-microscope, HITACHI S-5000, was used. For preparation of samples, mycelia grown on the cross gradient plate were subject to fixation with OsO₄ vapor followed by freeze (with liquid nitrogen)-drying and then coating with Pt/Pd.

**RESULTS**

**Antimicrobial activity of electrolyzed solutions:** Fig. 1 shows colony formation from *S. griseus* spores that had been exposed for 1 minute to the acidic and alkaline solutions as well as their mixture. No colonies appeared from the spores exposed to the acidic solution. By contrast, the growth from the spores exposed to the alkaline solution was comparable to that from the unexposed spores. However, antimicrobial activity of the alkaline solution was confirmed in the following experiments. Extending the exposure (10 min.) resulted in a marked reduction (90% or higher) of colony formation. Furthermore, when the unexposed spores (5×10^8) were spread on ISP No. 4 agar plate mixed with an equal volume of the alkaline solution, only 20–30 colonies appeared after incubation at 27°C for 5 days (data not shown). The pH of this plate was about 7.4–7.6 when checked with pH testing papers. On the other hand, the mixture solution showed a clear

<table>
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<th>Ratio of acidic solution to alkaline solution</th>
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<tr>
<td>10:0</td>
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<tr>
<td>1:1</td>
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<tr>
<td>0:10</td>
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Fig. 1. Effect of the exposure to electrolyzed solutions on colony formation. A: ISP No.2, B: ISP No.4.0

<table>
<thead>
<tr>
<th>Alkaline soln.</th>
<th>Acidic soln.</th>
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Fig. 2. Growth on agar plates with the cross density gradient of the electrolyzed solutions. A and B: strains No. 2021 (top) and No. 624 (bottom) were streaked. C: a soil suspension was smeared. Incubation was carried out at 27°C for 7 days. Basal media were ISP No. 4 for A and C, and ISP No. 2 for B.
inhibition of colony formation. Although numbers of colonies appeared from the exposed spores, inhibition level was estimated at higher than 90%. Furthermore, extending the exposure time to 10 min. resulted in the total inhibition of colony formation (data not shown). It was notable that markedly larger numbers of colonies were formed on ISP No. 2 plate than on ISP No. 4 plate on which the spores exposed to the mixture solution were spread.

Fig. 2 shows the growth of *Streptomyces* strains on ISP No. 4 agar (half strength) plate with a cross density gradient of the acidic and alkaline solutions. The growth of all the strains tested was inhibited at the acidic solution side. Similar results were obtained for soil suspensions tested. When the pH of the agar plate was checked with pH testing papers, pH of both ends of the plate was in a neutral range (6.2–6.4 at the acidic solution side and 7.4–7.6 at the alkaline solution side), indicating that the electrolyzed solutions have no significant buffering function. The growth inhibition zone was narrowed when ISP No. 4 medium (neither amino acids nor vitamins present) was replaced with ISP No. 2 medium (nutritionally rich) as the basal medium.

Fig. 3 show morphologies of strains 624 and SS-1198 grown on the cross gradient plate. In both strains, normal development of aerial

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Fig. 3. Morphologies formed on the cross gradient plate. Photographs were taken after incubation at 27°C for 9 days (A and B were strain No. 624, and C and D were *S. griseus* SS-1198). Acidic and alkaline solutions electrolyzed for 12 min. were used for making the cross gradient plate.
mycelium and spores was observed at the alkaline solution side (A), while mycelial growth with damage was observed at faded-growth areas (B). The formation of a unique morphology was observed in the growth of *S. griseus* SS-1198 (Fig.3-C), which has never been observed in its growth on ISP media (No. 2, 3, 4 and 5).

**Role of chlorine in antimicrobial activity of the acidic electrolyzed solution:** Fig. 4 shows the effect of the acidic electrolyzed solution on the growth of *S. griseus* SS-1198 without direct contact. In this experiment, after strain SS-1198 was streaked on ISP No. 4 plate, the petri dish was inverted and 1 ml of the acidic solution was put on the inverted lid. Then, incubation was started. Subsequently, no growth occurred even after 7 day incubation, while clear growth was observed on the control plate after 2 day incubation.

On the other hand, Fig. 5 shows the growth of *Streptomyces* spp. on 1/2xISP No. 4 medium with the cross gradient of the electrolyzed solutions made from 20 mM Na₂SO₄ solution. Although an acidic solution with pH 2.6 was obtained, no growth inhibition was observed. The same result was obtained with the acidic electrolyzed solution made from 20 mM NaNO₃ (data not shown).

**DISCUSSION**

The acidic solution resulting from the electrolysis of NaCl solution showed a strong killing activity against *Streptomyces* spp. In addition, the growth inhibition biased toward the acidic solution side was observed when they were incubated on ISP No. 4 plate with a cross density gradient of the acidic solution and alkaline solution. It was reported that the acidic electrolyzed solution showed a broad antimicrobial and antiviral activity². Taken together, it seems likely that actinomycetes are generally sensitive to the acidic solution.

The key factors for the antimicrobial activity of the acidic electrolyzed NaCl solution remain unclear. Although the electrolysis instruments were designed on the basis of that microorganisms cannot survive aqueous environments with pH lower than 3 and ORP higher than +900 mV. Our present study revealed that the clear antimicrobial activity of the acidic solution even if it was neutralized or without direct contact: neutralization of acidic solution resulted in

![Fig. 4. Growth inhibition without direct contact by the acidic solution. *S. griseus* SS-1198 (20 μl of 3x10⁴ (top) and 3 x 10⁶ (bottom)) was streaked and photographs were taken after incubation for 4 days. The acidic and alkaline solutions were put on the lid of an inverted petri dish.](image)

![Fig. 5. Difference in the growth inhibitory effect between the electrolyzed solution from NaCl and the one from Na₂SO₄. 20 mM aqueous solutions of NaCl (A) and Na₂SO₄ (B) were electrolyzed for 12 minutes in order to use for the preparation of the plates. Strains No. 624 (top), SS-1198 (medium) and No. 2021 (bottom) were streaked and incubated at 27°C for 6 days.](image)
ORP level decrease to a range of +100–200 mV (unpublished data). These facts indicate that some chemical species such as chlorine gas and hypochlorous acid (HClO), which are theoretically expected to be formed at the anode side upon electrolysis of NaCl solution, probably play key roles in the antimicrobial activity. The formation of ozone, radicals or active oxygen will also be possible upon the electrolysis and, if present, contribute to the antimicrobial activity.

By contrast, neither chlorine gas nor HClO should be contained in the alkaline solution which is accumulated at the cathode side compartment upon the electrolysis. Therefore, the basis for the weak but clear antimicrobial activity of the alkaline electrolyzed solution remains to be determined, although some unidentified radical may be involved since the antimicrobial activity declined by the addition of a radical scavenger, sodium hyposulfite (unpublished data).

Another point to note is the DO (dissolved oxygen) levels of the acidic and alkaline solutions; DO level is high in the former and low in the latter in comparison with Milli-Q water used for electrolysis (Table 1). These changes in DO level may reflect the change in the structure of water. Although water is often regarded as an inert liquid, it is a reactive substance with unusual properties that distinguish it from most other common liquids. It has been recognized that hydrogen oxide and its ionization products (hydronium and hydroxide ions) are important determinants of the characteristic structure and biological properties of proteins and nucleic acids, as well as membranes, ribosomes, and many other cell components8). In addition, there is a report suggesting that the size of water cluster may play an important role in enhancing biological activity9). It seems therefore likely that the electrolyzed waters form specific structures which may induce biological responses not only in microorganisms but also in higher organisms.

It was also of interest that a unique morphology was formed in *S. griseus* SS-1198 grown on ISP No. 4 plate with a cross density gradient of the alkaline and acidic solutions. This may indicate that the electrolyzed NaCl solutions are capable of inducing some metabolic change in this organism, as we observed an enhanced production of streptomycin by *S. griseus* SS-1198 in a medium supplemented with the alkaline electrolyzed solution (unpublished data).

REFERENCES