ISOLATION OF NITROSONOMAS IN PURE CULTURE

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Although it has been 68 years since Winogradsky (1890) accomplished the first isolation of nitrifying bacteria, the methods employed have not been improved significantly, and pure cultures remain incredibly difficult to obtain. The literature records numerous attempts to secure Nitrosomonas in pure culture. Nevertheless, the organism has seldom been isolated by dilution techniques. In the relatively few cases where success was claimed, the procedures were tedious and difficult, and the results often uncertain (Frankland and Frankland, 1890; Heubütt, 1929; Engle and Skallau, 1937; Bömeke, 1939). The majority of successful isolations have resulted from the plating of enrichment cultures. However, the colonies formed by Nitrosomonas on washed agar or silica gel are not more than 100 µ in diameter and micromanipulation is required to pick colonies or single cells (Kingma Boltjes, 1935; Meiklejohn, 1950).

The most frequently cited explanations for the difficulties encountered in obtaining Nitrosomonas in pure culture by dilution techniques are: (a) the autotrophic nitrifiers develop very slowly; (b) the heterotrophic contaminants in enrichment cultures develop at a rate equal to or greater than that of the nitrifying bacteria; and (c) the solution media employed by Winogradsky, and subsequently used by others for the cultivation of Nitrosomonas, contain insoluble carbonate which adsorbs the cells and prevents dispersed growth.

A recent report by Goldberg and Gainey (1955) resolved conflicting views regarding the role of surface phenomena in nitrification by demonstrating that appreciable quantities of particulate matter were not essential for rapid nitrification in solution medium. This suggested that the isolation of Nitrosomonas by dilution techniques should be reconsidered using media as free from particulate material as possible. The present report describes such media in which the development of Nitrosomonas in enrichment cultures was greater than that of associated heterotrophic bacteria. The magnitude of the ratio of the number of cells of Nitrosomonas to that of heterotrophic bacteria was adequate to permit isolation of Nitrosomonas in pure culture by dilution techniques.

EXPERIMENTAL METHODS AND RESULTS

Although the medium employed by Goldberg and Gainey (1955) was free of particulate material and permitted rapid nitrification it contained soil extract and was not defined chemically. Therefore, preliminary studies were made of the development of a nitrifying enrichment culture in various chemically defined solution media formulated to be relatively free of particulate matter. A simple salt solution of the following composition was found to be satisfactory: Na2HPO4, 3.1 g; KH2PO4, 1.1 g; MgSO4·7H2O, 0.1 g; (NH4)2SO4, 0.5 g; FeCl3·6H2O, 14.4 mg; CaCl2·2H2O, 18.4 mg; distilled water, 1000 ml. The (NH4)2SO4 was sterilized separately and added aseptically. The reaction of the medium was pH 7.2. Attempts to eliminate the trace of insoluble precipitate in this medium by use of chelates (ethylenediaminetetraacetic acid and N-hydroxyethyl ethylenediaminetriacetic acid) or by substitution of tris(hydroxymethyl)aminomethane buffer for the phosphate buffer were not successful in that nitrification was reduced. The influence of concentration of (NH4)2SO4 and pH on the numbers of Nitrosomonas and heterotrophic bacteria that developed in the medium was determined.

Studies were made using 100 ml quantities of medium in 250 ml Erlenmeyer flasks. All flasks were incubated at 28 C on a rotary shaker. The inoculum was a nitrifying enrichment culture obtained from soil and transferred serially in a conventional CaCO3-containing solution me-
at intervals of 1 month for a period of 2 years. Three treatments were employed. In the
first, flasks received only an initial increment of (NH₄)₂SO₄ (10.6 mg nitrogen); in the
second treatment, flasks received a second increment of (NH₄)₂SO₄ after 7 days; and in the third treat-
ment, the flasks received 3 increments of (NH₄)₂SO₄, the initial amount and additional quantities
after 7 and 14 days of incubation. The reaction of the solutions of this last treatment was re-
adjusted to the original level (pH 7.2) with sterile NaOH at weekly intervals. At the time
of inoculation and at 7 day intervals for 3 weeks thereafter each flask was tested for numbers of
heterotrophic bacteria, numbers of Nitrosomonas, concentration of nitrite, and pH. The numbers
of heterotrophic bacteria were determined by plate counts using nutrient agar. The numbers
of Nitrosomonas were determined by dilution in solution medium. Five tubes were used at each
dilution and the most probable numbers of Nitrosomonas were obtained by reference to the
tables of Halvorson and Ziegler (1933). The tubes were incubated for 30 days and then tested for
the presence of Nitrite. Nitrite was determined using the Griess-Ilosvay reagent and a Klett-
Summerson colorimeter. pH was determined electrometrically. All treatments were tested in
triPLICATE. Representative results are presented in table 1.

In treatment 1 the concentration of (NH₄)₂SO₄ limited the development of Nitrosomonas. In
treatment 2, where a second addition of (NH₄)₂SO₄ was made after incubation for 7 days, pH
came the factor limiting development of Nitrosomonas. The greatest numbers of Nitroso-
monas were obtained in those flasks that received 3 increments of (NH₄)₂SO₄ and had the
reaction of the medium adjusted to pH 7.2 at weekly intervals (treatment 3). In each case
the numbers of heterotrophic bacteria increased greatly during the first week of incubation,
decreased the second week, and remained relatively constant thereafter. In treatments 2 and 3
the ratio of the number of cells of Nitrosomonas to those of heterotrophic bacteria exceeded 100,
indicating that the isolation of Nitrosomonas in pure culture by dilution techniques was possible.

| Time | Treatment | pH | NO₂⁻-N | No. of Nitro-
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<tr>
<td>weeks</td>
<td></td>
<td></td>
<td>µg/100 ml</td>
<td>No. of Nitrifying</td>
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<tr>
<td>0</td>
<td></td>
<td>7.2</td>
<td>0.2</td>
<td>1/1</td>
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<tr>
<td>1</td>
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<td>7.3</td>
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<td>2</td>
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<td>6.4</td>
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<tr>
<td>3</td>
<td>3</td>
<td>6.3</td>
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For 1, 10.6 mg NH₄⁺-N was added initially; for 2, 10.6 mg NH₄⁺-N was added both initially and
after 1 week incubation; for 3, flasks received additions of 10.6 mg NH₄⁺-N initially and after
incubation for 1 and 2 weeks, and at the latter 2 periods the reaction was readjusted to pH 7.2
with sterile NaOH.

To test this possibility, a nitrifying enrichment culture was transferred to the phosphate buffered
medium, additional increments of (NH₄)₂SO₄ were provided to each flask, and the pH of the
solutions was readjusted at 7 day intervals as in treatment 3 of the previous experiment. After
3 weeks, 1 ml aliquots were taken from each flask and diluted serially (10⁻¹ to 10⁻¹⁰) in
medium in test tubes. The tubes were incubated for 30 days and then tested for nitrite and hetero-
trrophic bacteria. The following 4 procedures were used to detect heterotrophic bacteria: (a) 1 ml
aliquots were incorporated into pour plates using nutrient agar; (b) 0.1 ml aliquots were used to
inoculate the surface of nutrient agar plates; (c) 1 ml and 10 ml aliquots were added to flasks of
nutrient broth; and, (d) double strength nutrient broth was added directly to the tubes. Of 69
tubes inoculated with 10⁻⁶ to 10⁻¹⁰ dilutions of the nitrifying culture, 24 (35 per cent) were found
to be free of heterotrophic contaminants and to contain Nitrosomonas in pure culture.

Although the procedures described above yielded pure cultures of Nitrosomonas without
great difficulty, the necessity of adding (NH₄)₂SO₄ in increments and of adjusting the pH at weekly intervals was laborious and greatly increased the chances of contamination. A study of the influence of medium constituents including buffer concentration and pH on the development of Nitrosomonas resulted in the preparation of a more satisfactory medium. The composition of this medium was Na₂HPO₄, 13.5 g; KH₂PO₄, 0.7 g; MgSO₄·7H₂O, 0.1 g; NaHCO₃, 0.5 g; (NH₄)₂SO₄, 2.5 g; FeCl₃·6H₂O, 14.4 mg; CaCl₂·2H₂O, 18.4 mg; distilled water, 1000 ml; final pH, 8.0. This differed from the medium used in previous experiments in that the buffer concentration was increased from 0.03 to 0.1 M and sufficient (NH₄)₂SO₄ (53 mg nitrogen) was supplied initially to permit relatively profuse development of Nitrosomonas. In addition, the reaction of the solution was increased from pH 7.2 to 8.0. This medium proved satisfactory for the preparation of nitrifying enrichment cultures as well as for the isolation and maintenance of pure cultures of Nitrosomonas.

The suitability of the medium for the isolation of Nitrosomonas in pure culture was determined by inoculation with a nitrifying enrichment culture that had developed in the conventional CaCO₃-containing medium. After 8 days of incubation the culture was examined by the procedures employed in previous experiments. A culture that had been maintained in the conventional CaCO₃-containing medium was tested similarly.

Table 2 shows that the development of Nitrosomonas in the medium containing an insoluble carbonate buffer was limited by pH and that the ratio of the numbers of Nitrosomonas to heterotrophic bacteria was 2. There was more nitrification in the phosphate buffered medium. Although the experiment was terminated before either pH or the concentration of (NH₄)₂SO₄ had become limiting, the ratio of the number of cells of Nitrosomonas to heterotrophic bacteria exceeded 250. Aliquots taken from the phosphate buffered medium and diluted serially in test tubes of medium were incubated for 30 days and tested

<table>
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<td><strong>Development of Nitrosomonas and associated heterotrophic bacteria in two media</strong></td>
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<tr>
<td>Medium</td>
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<tr>
<td>CaCO₃ buffer...</td>
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<td>Phosphate buffer...</td>
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![Figure 1](http://jb.asm.org/) Electron micrographs of Nitrosomonas europaea. The specimen was air dried and shadowed with germanium. A, ×16,000; B, ×20,000.
for nitrite and heterotrophic bacteria. Of 25 tubes inoculated with $10^{-7}$ to $10^{-9}$ dilutions of the nitrifying enrichment culture, 22 (88 per cent) were free of heterotrophic contaminants and were presumably pure cultures of Nitrosomonas.

The isolates obtained by the foregoing procedures appear to be *Nitrosomonas europaea*. The cells are oval, measuring approximately 1.5 μ in length and 1 μ in width (figure 1). They are nonmotile and gram-negative. Pure cultures of Nitrosomonas have been maintained in the phosphate buffered solution medium for more than 12 months without loss of vitality and without recontamination. The culture was deposited with the American Type Culture Collection.

**DISCUSSION**

The recent statement of Meiklejohn (1950) that "it is impossible to obtain pure cultures [of Nitrosomonas] by simple serial transfers in liquid medium" was based on reports such as those of Frankland and Frankland (1890), Gibbs (1919), and Kingma Boltjes (1935) who demonstrated that after numerous transfers contaminants remained in the nitrifying enrichment cultures. Gibbs (1919) found that after 50 transfers heterotrophic bacteria persisted in equal or greater numbers than the autotrophic nitrifying bacteria. The cultures of Nelson (1931) contained 1000 heterotrophic bacteria for each cell of Nitrosomonas, and Hanks and Weintraub (1936) similarly observed greater numbers of heterotrophic contaminants than of Nitrosomonas in nitrifying enrichment cultures. The present investigations support these early studies, in that the ratio of the number of cells of Nitrosomonas to the number of heterotrophic bacteria obtained when nitrifying enrichment cultures developed in a solution medium containing an insoluble alkaline carbonate as buffer, was approximately 2 and of insufficient magnitude for the isolation of Nitrosomonas by dilution techniques.

It is apparent that the inability of previous investigators to isolate Nitrosomonas in pure culture by dilution techniques resulted from the presence of an insoluble carbonate in the media employed. By use of media formulated to be relatively free of particulate matter it has been possible to obtain dispersed growth of nitrifying enrichment cultures and a ratio of cells of Nitrosomonas to those of heterotrophic bacteria that exceeded 250, making isolation of Nitrosomonas in pure culture by dilution techniques a relatively simple matter.

The report of Goldberg and Gainey (1955), which demonstrated that particulate matter was not essential for rapid development of nitrifying bacteria in solution media, and served as the basis for the present investigations, was confirmed in a recent note by Engel and Alexander (1958). The medium employed by these investigators was rendered free of insoluble constituents by the use of a chelate (ethylenediamine di(o-hydroxyphenyl-acetic acid)) and would undoubtedly suffice for the isolation of Nitrosomonas in pure culture by the foregoing dilution procedures.

**ACKNOWLEDGMENT**

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**SUMMARY**

The development of a nitrifying enrichment culture in solution media formulated to be relatively free of particulate matter was investigated. Rapid and dispersed growth of Nitrosomonas was obtained in a medium buffered by phosphate and containing (NH₄)₂SO₄ as a source of energy. Under suitable conditions the ratio of the number of cells of Nitrosomonas to that of heterotrophic bacteria present in the enrichment culture exceeded 250, and isolation of Nitrosomonas in pure culture by dilution techniques was accomplished with comparative ease.

**REFERENCES**


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