STUDIES ON HETEROTROPHIC NITRIFICATION VIA
_Pseudomonas fluorescens_
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ABSTRACT

Bacteria (_Pseudomonas fluorescens_) was identified as heterotrophic nitrifiers. The present investigation was carried out to study heterotrophic nitrification activity in nutrient liquid medium with pH 6, 6.5, and 7. The growth at pH 6.5 was higher than that of pH 6 and 7. During the exponential growth phase the intermediate products of heterotrophic nitrification activity which excreted into the culture media (nitrite, nitrate and hydroxylamine) were increased significantly at pH 6,5 than pH 6 and 7. At a constant C/N ratio 3:1 glutamine was identified as a better nitrogen source for heterotrophic nitrification than the other organic N-containing components: arginine and asparagine.

INTRODUCTION

Heterotrophic nitrification was first described in 1894 for a fungus (Stutzer and Hartleb, 1894). For a long time heterotrophic nitrification was thought to be restricted to old or stationary-phase fungal or bacterial cultures and to play only a minor role in the biogeochemical cycle of nitrogen (Brown, 1988). In recent years, great number of heterotrophic (chemoorganotrophic) soil bacteria belonging to different genera are capable of nitrification, i.e. oxidation of ammonia to nitrite and/or of nitrite to nitrate, in addition of being able to use inorganic nitrogen (e.g. ammonium-salts) as a substrate for nitrification (Papen et al., 1989). Heterotrophic nitrifiers can also use organically bound nitrogen (e.g. in proteins, peptides and amino acids) as precursors of nitrification (Verstraete, 1973 and Kilham, 1987). It has to be stressed that the information available about heterotrophic nitrification, especially about the products produced in different media and under different conditions is extremely scarce among bacterial genera.

Leifi (1985) described that many heterotrophic organisms isolated from acid forest soils were able to oxidize NH$_4^+$ and NO$_3^-$ to NO$_2^-$. Acidophilic cultures (pH 4.0) were more effective in nitrification than neutro/alkaliphilic ones (pH 6.0 and 8.0).

Stroo et al., (1986) described that nitrate was formed from ammonium at pH 3.2 to 6.1 in suspensions of a naturally acid forest soil. The maximum rate of nitrate formation occurred at pH 5.0. A strain of Absidia cylindrospora was isolated from this soil and was found to produce nitrate and nitrite in a medium with alanine at pH values ranging from 4.0 to 4.8.

Papen and Rennenberg (1999) demonstrated that a Alcaligenes faecalis strain produced nitrite, nitrate, nitric oxide (NO) and nitrous oxide (N$_2$O) in both peptone-meat extract medium and a defined medium with ammonium and citrate as the sole nitrogen and carbon sources. Furthermore, they demonstrated that production of NO$_2^-$, NO$_3^-$ and NO and N$_2$O under aerobic conditions occurred shortly after cultures started growth and that production
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proceeded during the whole logarithmic growth phase. NO$_2^-$ and NO$_3^-$ production rates were higher for those in media at alkaline pH.

The aim of this work was to study the production of, nitrite, nitrate and hydroxylamine by *Pseudomonas fluorescens* bacteria during growth in defined culture media supplied with different amino acids and determination of the effect of the medium pH on the growth rate and production of, nitrite, nitrate and hydroxylamine.

**MATERIALS AND METHODS**

**Bacterial strain:**

*Pseudomonas Fluorescens* strain was isolated from the soil of the experimental farm, Fac. Agric., Minia University and identified according to Bergey's Manual (1964).

**Media:**

1- **Nutrient liquid medium**

Bacteria was grown in nutrient liquid medium (Dowson, 1957) the pH of the medium was adjusted to pH 6, 6.5 and 7 with 5% NO$_3$CO$_3$ or 1N NaOH after autoclaving.

2- **Sodium citrate medium:**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium citrate</td>
<td>9.50 x 10$^3$</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>9.35 x 10$^3$</td>
</tr>
<tr>
<td>RH$_2$PO$_4$</td>
<td>1.47 x 10$^3$</td>
</tr>
<tr>
<td>MgSO$_4$ 7H$_2$O</td>
<td>1.62 x 10$^4$</td>
</tr>
<tr>
<td>CaCl$_2$ 2H$_2$O</td>
<td>1.63 x 10$^4$</td>
</tr>
<tr>
<td>FeSO$_4$ 7H$_2$O</td>
<td>3.60 x 10$^5$</td>
</tr>
<tr>
<td>Na$_2$EDTA (Titrplex)</td>
<td>3.60 x 10$^5$</td>
</tr>
</tbody>
</table>

The pH of the medium was adjusted to pH 6.5 with 5% Na$_2$CO$_3$ or 1N NaOH prior to autoclaving. Sodium citrate was added as a filter sterilized solution (pH 6.5) after autoclaving and cooling of the rest medium.

**Different amino acids used (μl/ml):**

- Glutamine 9μl/ml
- Arginine 9 μl/ml
- Asparagine 9 μl/ml

The C/N ratio of the medium was adjusted to C/N ratio 3:1 with Glucose. Glucose was added as a filter sterilized solution after autoclaving and cooling.

**Growth conditions of bacteria for studies of the kinetics of heterotrophic nitrification and procedure of sampling.**

The bacteria were grown in liquid cultures, unless otherwise stated, under aerobic conditions on a rotary shaker (120 rpm) at 28°C. Dependent on the experiments, either 100 ml Erlenmeyer flasks containing 40 ml of respective medium or 300 ml Erlenmeyer flasks containing 100 ml medium were used. After inoculation the flasks were closed with cellulose stoppers.
As an inoculum 50-100 μl suspensions of an exponentially growing culture in liquid medium was used. After appropriate time intervals, 2 ml of suspensions were taken under sterile conditions from the growing cultures and were pipetted into plastic cuvettes (4 ml) for determination of optical density (as a parameter of growth, see below) in a perkin spectrophotometer. Thereafter, the suspension was transferred into a 2 ml Eppendorf vessel and was centrifuged at 9500 rpm for 10 minutes at 4°C. Eppendorf tubes were kept separately in a freezer for later analysis of hydroxylamine, nitrite and nitrate produced by the cells grow into the culture medium.

**Determination of growth of bacterial cultures:**

Bacterial growth was followed by measuring the increase in optical density (O.D.) of cell cultures at 660 nm using 4 ml plastic cuvettes. Pure culture medium (not inoculated) served as a blank.

**Determination of hydroxylamine:**

Hydroxylamine was determined by the method of Frear and Burrel (1955).

**Determination of nitrite:**

Nitrite concentrations were determined using the method of Norman and Stucki (1981).

**Determination of nitrate:**

Nitrate concentrations in samples was determined using a modification of the method described by Hanson and Philipps (1981).

**RESULTS AND DISCUSSION**

**Effect of the pH on the growth rate**

The growth rate was determined by measuring optical density (OD), of the appropriate dilutions, at 660-nm (A₀₆₆₀) using spectrophotometer. The effect of the pH on the growth rate of *Pseudomonas fluorescens* strain is given in Table 1. The growth rate was increased dramatically by increasing incubation time. However, the growth rate at pH 6.5 was higher than that at pH 6 and 7. These results revealed the significant role of the pH on the growth rate of *P. fluorescens*. Thus, The pH of the medium can be used as a reliable method to improve growth speed of this strain.

**Effect of the pH on the production of nitrite nitrate and hydroxylamine**

Generally, *P. fluorescens* strain was found to catalyze heterotrophic nitrification under aerobic growth conditions in nutrient liquid medium at pH 6, 6.5 and pH 7. However, the results shown in Table (1) revealed that, the strain tested differed in the amounts of nitrite, nitrate, and hydroxylamine excreted into the medium after 24, 48, 72 and 96 hrs according the pH of the medium.

The presented results demonstrate that the production of hydroxylamine, nitrite and nitrate by *Pseudomonas fluorescens* in medium of pH 6.5 was higher than in the medium at pH 6 and 7.

These results agree with findings of (Papen et al., 1991 & Papen and Rennenberg, 1999). Acidovorax-group, heterotrophic nitrification in nature, seems to play an important role, especially in acidic soils, from which autotrophic nitrifiers often can not be isolated, though nitrate production is

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observed in these soils. Furthermore, recent experiments with several fungi and two *Bacillus* spp., isolated from a podzolic brown earth (pH 3.5) showed that these heterotrophic organism exhibited significant rates of heterotrophic nitrification (Lang and Jagnow, 1986). These findings as well as the present data support the view that nitrification can be performed under acidic conditions by more heterotrophs than hitherto was assumed. Similar results were obtained for strain (*Pseudomonas fluorescens*).

Table (1): *Pseudomonas fluorescens* growth rate and production of nitrite nitrate, hydroxylamine during growth in nutrient liquid medium at pH 6, 6.5 and 7.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>O.D. 680 nm</th>
<th>Nitrate (n mol/ml)^{-1}</th>
<th>Nitrate (n mol/ml)^{-1}</th>
<th>Hydroxylamine (n mol/ml)^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6 6.5 7</td>
<td>6 6.5 7 6 6.5 7 6 6.5 7</td>
<td>6 6.5 7 6 6.5 7 6 6.5 7</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.00 0.00 0.00</td>
<td>0.0 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
</tr>
<tr>
<td>24</td>
<td>0.42 0.80 0.56</td>
<td>4.0 9.0 6.0</td>
<td>3.0 6.0 5.0</td>
<td>2.0 4.0 1.0</td>
</tr>
<tr>
<td>48</td>
<td>0.86 1.64 1.20</td>
<td>15 27 14</td>
<td>12 25 10</td>
<td>7.0 10 8.0</td>
</tr>
<tr>
<td>72</td>
<td>1.42 2.28 1.84</td>
<td>18 39 21</td>
<td>16 32 19</td>
<td>10 15 13</td>
</tr>
<tr>
<td>96</td>
<td>1.26 2.70 2.06</td>
<td>10 30 16</td>
<td>10 25 12</td>
<td>9 11 11</td>
</tr>
</tbody>
</table>

O.D. = Optical density

The effect of glutamine, arginine and asparagine on the growth rate

The effect of supplementing with glutamine, arginine and asparagine on the growth rate of *Pseudomonas fluorescens* strain is shown in Table 2 and Fig. 1. In general, the growth rate was increased by increasing incubation time under all experimental conditions. However, supplementing with glutamine, highly affected the growth rate of *P. fluorescens* strains in comparison to the other three amino acids. These data might demonstrate the importance of choosing the most suitable organic nitrogen source for heterotrophic nitrification.

The effect of glutamine, arginine and asparagine on the production of nitrite nitrate and hydroxylamine

Production of nitrite, nitrate and hydroxylamines in medium free of any amino acids were lower than that containing amino acids (Table 2 and Fig. 2). However, a considerable variation was observed between the four amino acids used in this experiment. The results showed that the production rates of nitrite, nitrate and hydroxylamine in medium with glutamine was higher than that of medium with arginine and asparagine.

The results obtained indicate, that the C/N ratio alone does not determine heterotrophic nitrification activity, since different organic nitrogen sources (asparagine, arginine and glutamine), which could be used by the bacteria under study as nitrogen sources, at a constant C/N ratio in the medium (3:1) resulted in different production rates of products of heterotrophic nitrification. Glutamine with being the most suitable organic nitrogen source for heterotrophic nitrification.
Table (2) *Pseudomonas Fluorescens*: Production of nitrate, nitrite, and hydroxylamine during growth in ammonium-citrate medium with different amino-acids.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>O.D. 680 nm</th>
<th>Nitrite (n mol/ml) $^3$</th>
<th>Nitrate (n mol/ml) $^3$</th>
<th>Hydroxylamine (n mol/ml) $^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0 0.0 0.0 0.0</td>
<td>0.0 0.0 0.0 0.0</td>
<td>0.0 0.0 0.0 0.0</td>
<td>0.0 0.0 0.0 0.30</td>
</tr>
<tr>
<td>24</td>
<td>0.6 1.2 0.8 0.6</td>
<td>2.0 5.0 4.0 3.0</td>
<td>5.0 7.0 9.0 5.0</td>
<td>1.0 6.0 3.0 2.0</td>
</tr>
<tr>
<td>48</td>
<td>0.8 2.0 1.6 1.2</td>
<td>7.0 15.0 10.0 7.0</td>
<td>9.0 19.0 20.0 10.0</td>
<td>5.0 12.0 8.0 7.0</td>
</tr>
<tr>
<td>72</td>
<td>1.2 3.0 2.0 1.6</td>
<td>11.0 28.0 18.0 18.0</td>
<td>10.0 33.0 29.0 21.0</td>
<td>7.0 15.0 10.0 9.0</td>
</tr>
<tr>
<td>96</td>
<td>1.2 2.6 2.2 2.0</td>
<td>12.0 32.0 25.0 18.0</td>
<td>12.0 38.0 32.0 22.0</td>
<td>8.0 13.0 10.0 9.0</td>
</tr>
</tbody>
</table>

$^3$ Cont. = Control, Glut. = Glutamine, Arg. = Arginine, Asp. = Asparagine
Fig. 1: The effect of glutamine, arginine and asparagine on the growth rate.
Fig. 2: Effect of glutamine, arginine and asparagine on the production of nitrite, nitrate and hydroxylamine.
Therefore, these results agree with findings of Gode (1970) who pointed out that the C/N ratio (usually varied from 3 to 5) is an important factor that has to be considered in employed for cultivation of heterotrophic nitrifiers.

Verstraete and Alexander (1972) found for an Arthrobacter strain that the amount of hydroxylamine produced per cell increased when the C/N ratio in the culture medium decreased from 10:1 to 1:10. However, formation of nitrite per single bacterial cell for this strain turned out to be relatively independent of the C/N ratio.

Von-Gool and Schmidt (1973) obtained relatively high nitrification rates using an Aspergillus flavus strain that was grown aerobically with high concentrations of glucose and peptone. Addition of L-aspartate to the culture medium after depletion of glucose resulted in a only moderate increase in total nitrate produced, but a remarkable response was observed when L-aspartate was added when nitrate production started.

Papen et al., (1991) found that in the constant C/N ratio of 3:1 glutamine was identified as better nitrogen source for heterotrophic nitrification than the other organic N-containing compounds: arginine, asparagine and urea. With glutamine the highest NO-production rates were observed in heterotrophically nitrifying cultures of R. erythropolis HW1-13.

REFERENCES


دراسات على بكتريا التأثر غير ذاتية التغذية عن طريق سيدومناس فلوراسنس

سماء فرحان محمد أحمد

قسم الميكروبولوجيا - كلية الزراعة - جامعة المنيا

تلعب ميكروبيات التأثر غير ذاتية التغذية دوراً هاماً في دورة التروجيج في الردة، ولذلك فإنة تشكل أهمية كبيرة في الفرصة الزراعية. يهدف البحث الحالي إلى دراسة تأثير البكتريا على معدل النمو، وذلك عن طريق الفحص في البيئة على معدل النمو، وبذلك يحتوي نتائج التوقيت والانترات والإنترات لامين على واحد من البكتريا غير ذاتية التغذية وهى سيدومناس فلوراسنس وأظهرت النتائج ما يلي:

- زيادة معدل نمو البكتريا على pH 6.5 و 7 وكان ذلك أيضاً مصحوباً زيادة في إنتاج النواتج الأولية الأساسية وهي القنوات، والانترات، و هييدروكسيل أمين.
- أثناء عملية التأثر عند تهوية البكتريا على pH 6.5 كان الجيل ذا نسبة لذاتي 0.3 أقل مستويات التروجيج لعملية التأثر الهيدروكوفيية بالمقارنة بالأذني وغير الأذنيين من حيث تأثيرها على إنتاج النواتج، والانترات والهييدروكسيل أمين وأيضاً معدل النمو.

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