J. Agric. Sci. Mansoura Univ., 28 (5): 3991 - 3999, 2003

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 hydroxlamine) 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 hetrotrophic nitrification, especially about the products produced in different media and under different conditions is extremely scarce among bacterial genera.

Lettl (1985) described that many heterotrophic organisms isolated from acid forest soils were able to oxidize NH_4^+ and NO_2^- to NO_3^-. Acidophilic cultures (pH 4.0) were more effective in nitrification than neutro/alkalophilic 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_2O) 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_2O under aerobic conditions occurred shortly after cultures started growth and that production

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 (1984).

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_2CO_3 or 1N NaOH after autoclaving.

2- Sodium citrate medium:

Sodium citrate	9.50×10^{-3}
NH_4cL	9.35×10^{-3}
$RH_2\ Po_4$	1.47×10^{-3}
$MgSo_4 \times 7\ H_2o$	1.62×10^{-4}
$Cacl_2 \times 2\ H_2o$	1.63×10^{-4}
$FeSo_4 \times 7H_2o$	3.60×10^{-5}
Na2 EDTA (Titriplex)	3.60×10^{-5}

The pH of the medium was adjusted to pH 6.5 with 5% Na_2Co_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 hydroxlamine:.

Hydroxlamine 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 (A_{660}) 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 of pH 6 and 7. These results might revel the significant role of the pH on the groweth 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) reveled 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). Acidovorox-group, heterotrophic nitrification in nature, seems to play an important role, especially in acidic soils, from which outotrophic nitrifiers often can not be isolated, though nitrate production is

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.

Time (hr)	O.D. 680 nm			Nitrite (n mol/ml)$^{-1}$			Nitrate (n mol/ml)$^{-1}$			Hydroxylamine (n mol/ml)$^{-1}$		
	pH											
	6	6.5	7	6	6.5	7	6	6.5	7	6	6.5	7
0	0.00	0.00	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.42	0.80	0.56	4.0	9.0	6.0	3.0	6.0	5.0	2.0	4.0	1.0
48	0.86	1.64	1.20	15	27	14	12	26	10	7.0	10	8.0
72	1.42	2.28	1.84	18	39	21	16	32	19	10	15	13
96	1.26	2.70	2.06	10	30	16	10	25	12	9	11	11

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* strain in comparison to the other three amino acids. These data might demonstrate the importance of chosing 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 aspragine.

The results obtained indicate, that the C/N ratio alone does not determine heterotrophic nitrification activity, since different organic nitrogen sources (asparagine, arginine and glutimne), 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 nitrite nitrate , hydroxylamine during growth in ammonium-citrate medium with different amino-acids.

Time (hr)	O.D. 680 nm				Nitrite (n mol/ml)$^{-1}$				Nitrate (n mol/ml)$^{-1}$				Hydroxylamine (n mol/ml)$^{-1}$			
aa	Cont.	Glut.	Arg.	Asp.	Cont.	Glut.	Arg.	Asp.	Cont.	Glut.	Arg.	Asp.	Cont.	Glut.	Arg.	Asp.
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.30
24	0.6	1.2	0.8	0.6	2.0	5.0	4.0	3.0	5.0	7.0	9.0	5.0	1.0	6.0	3.0	2.0
48	0.8	2.0	1.6	1.2	7.0	15.0	10.0	7.0	9.0	19.0	20.0	10.0	5.0	12.0	8.0	7.0
72	1.2	3.0	2.0	1.6	11.0	28.0	18.0	18.0	10.0	33.0	29.0	21.0	7.0	15.0	10.0	9.0
96	1.2	2.6	2.2	2.0	12.0	32.0	25.0	18.0	12.0	38.0	32.0	22.0	8.0	13.0	10.0	9.0

aa = amino acids, Cont.= Control, Glut.= Glutamine, Arg.= Arginine Asp.= Asparagine

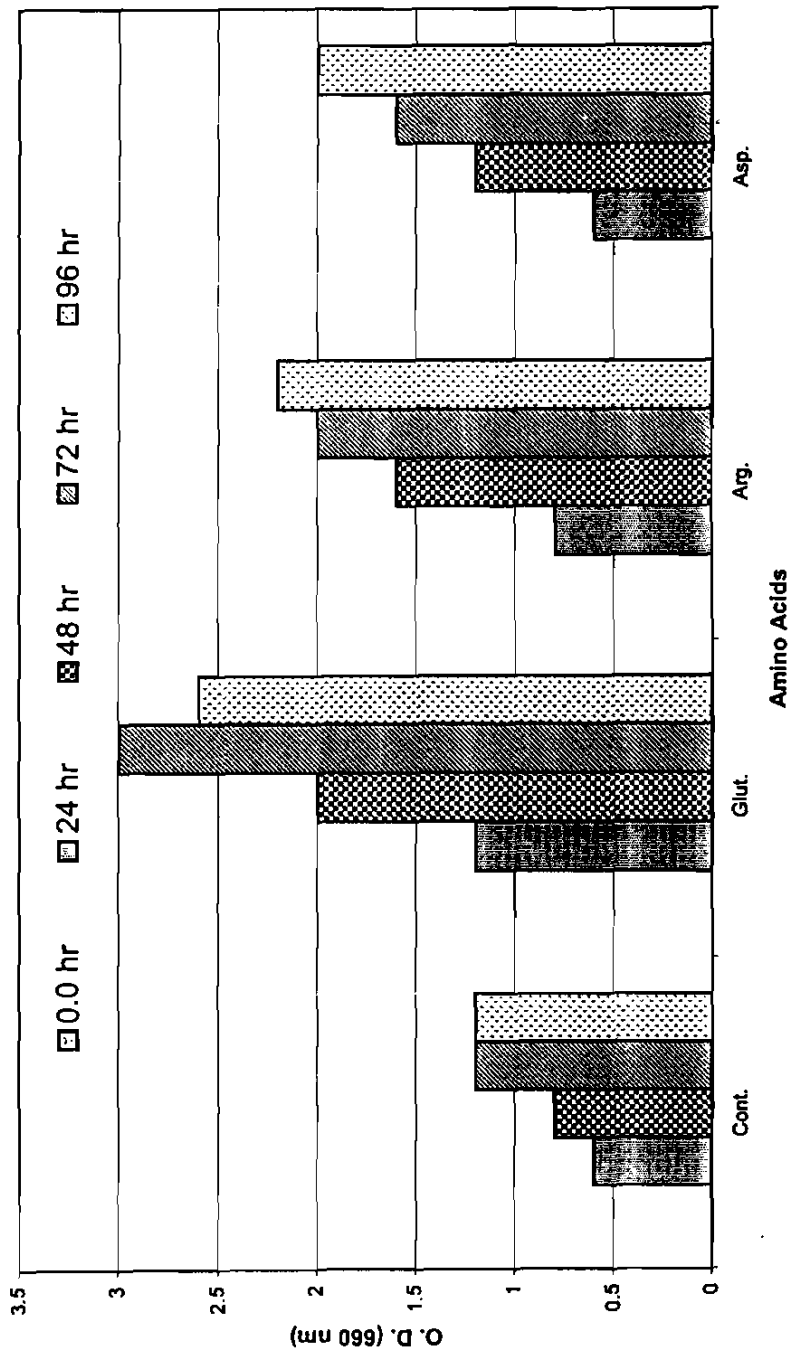

Fig. 1: The effect of glutamine, arginine and asparagine on the growth rate

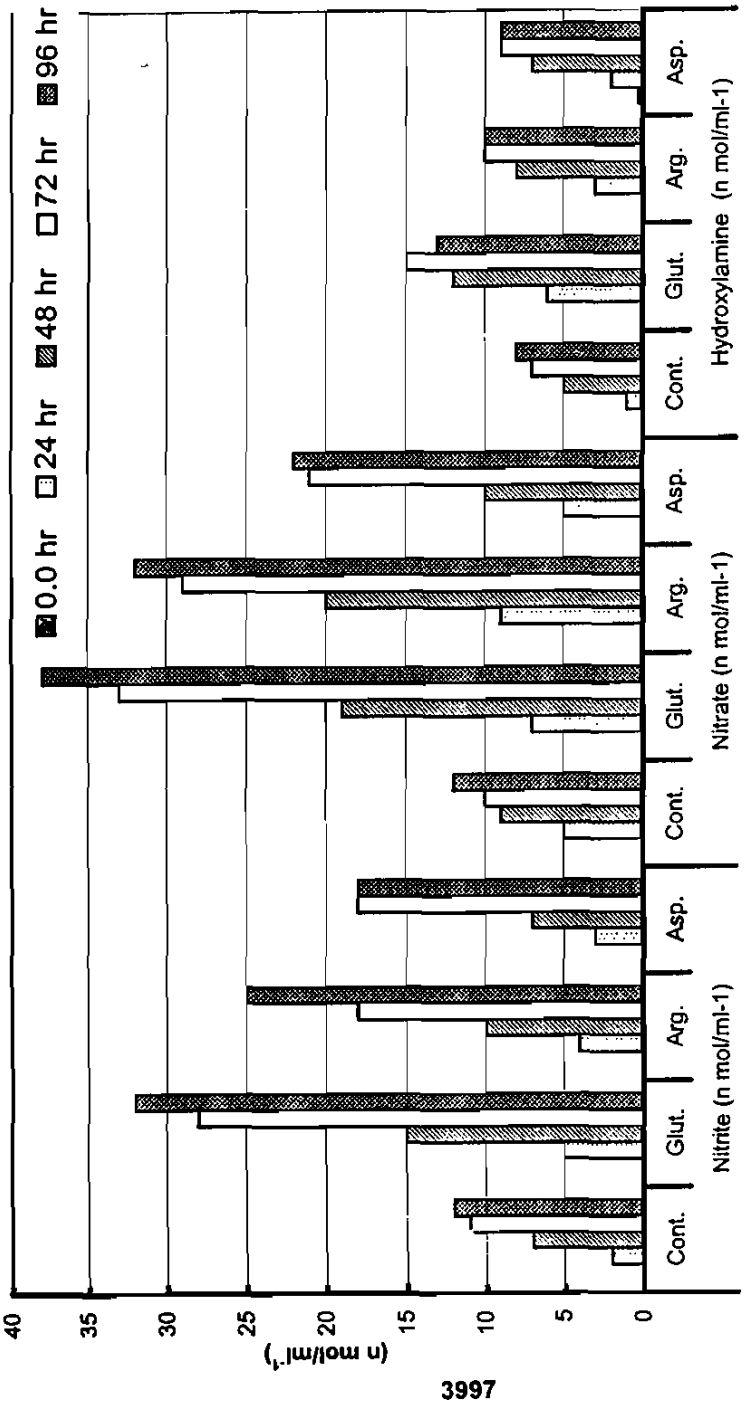

Fig. 2: Effect of glutamine, arginine and asparagine on the production of nitrite nitrate and hydroxylamine

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 flavous*-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 obvserved, when L-asprtate 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.

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دراسات على بكتريا التأزت غير ذاتية التغذية عن طريق سيدوموناس فلوراسنس

سامية فرحات محمد أحمد

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تلعب ميكروبات التأزت غيرذاتية التغذية دورا هاما فى دورة النتروجين فـــى التربـــة ، ولذلك فهى تشكل أهمية كبيرة فى التربة الزراعية. يهدف البحث الحالى الى دراسة تأثيرالـــpH وكذلك نوع الحامض الأميني المستخدم كمصدر للنيتروجين في البيئة علي معدل النمـــو وكذلـــك إنتاج النيتريت والنترات والهيدروكسيل أمين على واحدة من البكتريا غير ذاتيـــة التغذيـــة وهـــى سيدوموناس فلوراسنس وأظهرت النتائج ما يلي:

١-زيادة معدل نمول البكتريا علي p H ٦,٥ بالمقارنة بالـ pH ٦ و ٧ وكان ذلـــك ايضـــا مصحوبا بزيادة في إنتاج النواتج الأولية الأساسية وهى النتريت ، النترات و هيدروكسيل أميـن أثناء عملية التأزت عند تنمية البكتريا على p H ٦,٥

٢- كان الجلوتامين (ذو نسبة ك/ن ١:٣) أفضل مصادر النتروجين لعملية التأزت الهتروتروفيـــة بالمقارنة بالأرجنين و الأسبارجين من حيث تأثيره علي إنتاج النتريت ، النترات و الهيدروكسيل أمين وايضا معدل النمو.