Survey of Bacteria in the Farm of Faculty of Agriculture ,Al-Azahr University, Assiut Governorate,Egypt

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ABSTRACT

Two hundred and fifteen soil bacteria isolates able to reduce NO^-_3, 210 produced nitrous oxide (N_2O), even only 47 were respiratory denitrifiers . Nitrite or NH^+_4 was the major product of NO^-_3 reduction by the non-denitrifying organisms, but typically about 5 to 10% and up to 34% of the NO^-_3 reduced by them was released as N_2O during a 2-week incubation period. *Bacillus* and *Enterobacter* were the most commonly observed genera of non-denitrifying N_2O producers. Fermentative NO^-_2reduction and N_2O production by a *Bacillus* sp. and a *Citrobacter* sp. were characterized in pure culture studies. Dinitrogen (N_2) was not produced in detectable quantities by these organisms. When added to autoclaved soil, they accumulated more N_2O than two denitrifying *pseudomonads*, since the latter consume and produce N_2O. In tryptic soy broth (TSB), which allows active fermentative growth, NH^+_4 was apparently the major product of NO^-_3 reduction. Added NH^+_4 did not inhibit N_2O production or apparent reduction to NH^+_4, indicating that these processes are not essimilatory . The effect added glucose on N_2O production varied with the organism and media composition. Nitrous oxide production from NO^-_2 by these organisms was shown to be at least partially a biochemical reaction. The N_2O evolved slowly in bath cultures and mostly after apparent growth ceased. This is apparently a novel mechanism of N_2O generation which differs significantly from respiratory denitrification .

Key words: Nutrient broth (NB), tryptie soy broth (TSB), nitrite reduction, denitrification, dissimilatory ammonium production.

INTRODUCTION

There is much current interest in soil denitrification, promoted by the need to utilize nitrogen (N) fertilizer more efficiently, and in soil evolution of nitrous oxide (N_2O), promoted by the hypothesized role of this gas in the destruction of atmospheric ozone (C.A.S.T,1979; Caskey and Tiedje, 1976; Crutzen and Ehhalt, 1977; Mc Elroy *et al*., 1977).This has led to reevaluation of the mechanisms and organisms responsible for the production of gaseous N and the reduction of N oxides. Recent evidence suggests that NH^+_4 – oxidizing bacteria like *Nitrosomonas* are important sources of N_2O (Bremner and Blackmer, 1978; Breitenbeck *et al* ., 1980), and that production of this gas is not the exclusive province of denitrifying bacteria as previously believed.The term denitrification has been used to refer to any conversion of NO^-_3 or NO^-_2 to N gas. It is now commonly used by microbiologists; however, to describe only the reduction of NO^-_3 and NO^-_2 to NO, N_2O or N_2 in bacterial respiration, and the term will be used in this sense here. Other known mechanism of N_2O production include a variety of chemical reactions (Nelson and Bremner, 1970), production by nitrifiers, and by a miscellany of non-denitrifying fungi and bacteria (Yoshida and Alexander, 1970). The nature and relative significance of these various mechanisms are only vaguely understood at present.

Dissimilatory NO^-_3 –reducing bacteria have conventionally been considered to be of two types: (i) respiratory denitrifiers which can reduce NO^-_3 completely to N gasses, and (ii) NO^-_3 respires or NO^-_2 accumulators which are able to respire NO^-_3 only as far as NO^-_2 (Payne, 1973 and EL-Sayed , 2002(a and b)). Though it has been known that at least a few of the latter type of organism growing fermentatively can further dissimilate NO^-_2 to NH^+_4, the significance of this process has been suggested only in publications (Caskey and Tiedje, 1979; Sorenson, 1978 ; EL-Sayed , 2003 a).These fermentative organisms presumably attain greater ATP yields , by recycling reduced nucleotides via NO^-_2 reduction to NH^+_4 (Cole and Brown, 1980 ; EL-Sayed , 2003 b), from substrate level phosphorylation. In contrast, respiratory denitrifiers reduce NO^-_2 to N gases and generate ATP by electron transport phosphorylation. Ammonium is the only product of fermentative, dissimilatory NO^-_2 reduction which has been considered in the literature.In earlier work, however, it was noted that N_2O was evolved from NO^-_3 by a variety of organisms presumed to be non-denitrifying NO^-_3 reduces (Caskey and Tiedje , 1979 ; EL-Sayed , 2013). The objectives of this study were to characterize the mechanism of N_2O production, examine its significance as a source of N_2O in soil, and determine the end products of NO^-_3 reduction for a large sample of soil isolates.

MATERIALS AND METHODS

Isolation of soil NO^-_3 Reducers:-

Samples of silt loam soil (mixed mesic Typic), pH 7.1, and 1% organic matter) and silty clay loam soil

(mixed thermic cumulic of pH 7.1 and 1.3% organic matter) were collected from the surface (0-15cm) of

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corn fields. These samples were stored at field moisture contents at 2 to 4^{o} C. In some experiments 20.0 gm, soil samples were pre incubated anaerobically for 78 hours prior to isolation of NO^{-}_{3} reducers. Twenty grams of soil were blended for 60 seconds in 190 ml 0.85% Nacl solution with 1 drop of Tween 80. A tenfold dilution series was prepared in sterile Nacl solution. Dilutions were spread on plates of either tryptic soy or nutrient agar (Difco) with 5 m *M* KNO_3. Results for the two media were pooled since no significant differences were observed among the NO^{-}_{3} reducing organisms . Plates were incubated 4 days in anaerobic chamber at room temperature. Isolated colonies were picked at random and in some experiments were further purified by streaking on NO^{-}_{3} nutrient agar and incubating anaerobically. In other experiments, isolated colonies were assayed directly without further purification(EL-Sayed *et al*.,2001).

Characterization of NO^{-}_{3} Reducing Isolates

Isolates were used to inoculate 5 ml of nutrient broth plus 5 m *M* KNO_3, contained in Hungate tubes (Bellco). Oxygen was removed from the tubes by evacuating and flushing with N_2 gas passed through 0.45- u Gelman filters.

To fervent the reduction of N_2O to N_2, acetylene (C_2H_2) was injected aseptically to a partial pressure of 8 KPa. In the same experiments , the isolates were also cultured in tubes without C_2H_2 or with trypic soy broth (with dextrose) in place of nutrient broth. Nitrous oxide was sampled after through mixing by withdrawing 0.5 ml from the headspace with a tuberculin syringe. Following gas analysis, cultures were centrifuged, and the clear supernatants were frozen for later NO^{-}_{3} and NO^{-}_{2} determinations(EL-Sayed,2005).

Pure Culture Studies

Cultures from selected tubes were checked for purity and maintained on NO^{-}_{3} agar (Difco) for further taxonomic or physiological characterization. Gram stain, sporulation, motility, and standard tests for reaction with sugars, litmus milk, citrate, and indole were observed for these isolates to identify them at the genus level.

All pure cultures were grown at 24^{o}C with 10 ml of the appropriate media in Hungate tubes. Tubes were inoculated with 0.1 ml of an (18 to 24) , hour culture (early stationary phase) grown anaerobically in NO^{-}_{3} broth, then immediately evacuated and flushed with N_2 gas aseptically. The time course of NO^{-}_{3} reduction and NO^{-}_{2} and N_2O production during growth in nutrient broth with 5 m *M* KNO_3 was determined by periodical analyzing three replicate tubes for N_2O an absorbance and by sacrificing, at frequent intervals, three additional replicates for NO^{-}_{3} and NO^{-}_{2} analysis. Growth yield responses to NO^{-}_{3} and NO^{-}_{2} were measured gravimetrically after washing cells in distilled water and drying at 85^{o} C. Filter- sterilized NO^{-}_{2} was added to media after autoclaving, but NO^{-}_{3} was autoclaved with the broth. The effect of pH on N_2O production was observed by adding HC l or NaoH to nutrient broth before autoclaving. The pH of replicate tubes was measured after autoclaving and was either unchanged or increased by only 0.1 unit. The appropriate quantities of $(NH_4)_2$ SO_4, KNO_3, or glucose were added to either tryptic soy broth (without dextrose) or nutrient broth to observe the effects of media composition on N_2O production. To assay for N_2 production, tubes were initially flushed with helium and incubated under water to minimize atmospheric contamination.

Resting cell suspensions were prepared from early stationary phase cultures harvested by centrifugation and repeated washing in 50 *mM* pH 7.0 phosphate buffer plus 200 ug. M /L chloramphenicol to inhibit protein synthesis. In separate assays this chloramphenicol concentration was sufficient to totally inhibit growth of the organisms studied. Cell density in the reaction mixture was approximately 2 times the maximum cell density attained in culture. Complete reaction mixtures consisted of 10 ml of 50 m *M* phosphate buffer (pH 7.0) , 200 ug M/L chloramphenicol ,2.8 m *M* glucose, 5 m*M* $NaNO_2$, N_2 atmospheres, and cells in Hungate tubes. Resting cell suspensions were continuously shaken during the 2-hour incubation at room temperature. Gas samples were removed periodically by syringe (EL-Soury *et al*.,2015).

N_2O production in Inoculated Axenic Soils

Twenty- gram samples of the soil were autoclaved for 1 hour on 2 consecutive days. Twenty- four hours anaerobic cultures of two NO^{-}_{2} accumulators and two denitrifiers in nitrate broth were harvested and washed in 10m *M* $CaCl_2$ plus 1.7m *M* KNO_3. The two denitrifiers had been isolated from soil and characterized in previous studies (Gamble *et al*, 1977; and EL-Sayed , 2005). Suspensions were diluted in $CaCl_2$, KNO_3 solution to give equal optical densities for all the organisms, and 10ml was added to 20 g of autoclaved soil, given approximately $5x10^{8}$ cells/g of soil. Control soils not inoculated received 10ml of $CaCl_2$, KNO_3 solution with no cells. The flask containing the slurries were made anaerobic and incubated on a rotary shaker (150 rpm) at room temperature. Frequent headspace samples were removed by syringe for N_2O analysis.

Chemical Analysis

Nitrous oxide (N_2O) was measured with a Varian 3700 gas chromatograph equipped with Porapak Q columns and operated isothermally at 50^{o}C. Samples

containing 0.05 to 30 ppm (v/v) N_2O were measured with a ^{63}Ni electron capture detector at 340°C with 10% CH_4 in argon carrier gas. Separation between CO_2 and N_2O was sufficient to prevent CO_2 interaction with N_2O response. A four-port in-oven venting value was used to prevent other gases from reaching the detector. Samples with N_2O concentrations exceeding 30 ppm were measured by thermal conductivity detector with helium as the carrier gas, again using Porapak Q columns at 50°C. Quantities of N_2O in solution were calculated using published values of the Bunsen absorption coefficient. Dinitrogen was separated on a molecular sieve 5 Ation column and analyzed by thermal conductivity detector.

The presence of NO^-_3 was determined qualitatively in the characterization of soil isolates by treating a small subsample, about 0.1ml, with 2 drops of 5% sulfamic acid to remove NO^-_2, then adding 3 drops of diphenylamine HCl in concentrated H_2SO_4. A strong-to- moderate blue color was developed with concentrations greater than about 0.5m *M* NO^-_3,. Quantitative NO^-_3 determinations were made with an Orion NO^-_3 electrode after removing NO^-_2 with sulfamic acid, then mixing the sample with an equal volume of 0.052 *M* $AL_2 (SO_4)_3$.The validity of this technique was verified by analysis of NO^-_3 an NO^-_2 . Nitrite was measured by autoanalyzer using the reaction with N-1-naphthyleth-ylenediamine dihydrochloride and sulfanilamide (Lowe and Hamilton, 1967 ; and EL-Sayed , 2004).

RERSULTS AND DISCUSSION

Isolation and classification of soil NO^-_3 reducers:-

Soil isolates classified into four distinct categories with regard to the products of NO^-_3- reduction (Table 1).Those for which> 50 %(usually 75 to 100%) of the NO^-_3-N added in nutrient broth (N B) was recovered as NO^-_2 after 2 weeks and called NO^-_2 accumulators.Those for which <50% of the NO^-_3-N in nutrient broth was recovered as N_2O plus NO^-_2, called NH^+_4 producers, though NH^+_4 accumulation and was not directly measured.Those organisms which reduced >50 % (usually 75 to 100%) of the NO^-_3 to N_2O in nutrient broth with 8 KP_a C_2 H_2, presumed to be respiratory denitrifiers.Those organisms which did not reduce NO^-_3 or which grew too slowly to reliably determine the products of NO^-_3 reduction during a 2- week incubation (Abdel-Aziz *et al* , 2003 ; Christopher *et al*.,2017).

Table (1) gives average recoveries of N_2O and NO^-_2 for the various categories. These indicate that recovery of N was good (except for the NH^+_4 producers) and that the categories are distinctly different with regard to end products. Summing the results for all soils, there were 155 NO^-_2 –accumulating isolates, 46 denitrifiers, 13 NH^+_4 producers, and 136 inactive isolates.

Table 1. Characterization of soil isolates with regard to products of NO^-_3 reduction after a 2- week incubation in anaerobic nutrient broth (NB) or tryptic soy broth (TSB) with 5m *M* KNO_3 and 8K Pa C_2 H_2 added

Soil treatment and type of isolate	Number of isolates	Number producing N_2O	Number which depleted NO^-_3 and NO^-_2	%Recovery of added NO^-_3 –as N	
				N_2O	NO^-_2
no incubation:-					
NO^-_2 accumulators	59	59	$(49)^+$	8.8	87.8
Presumptive NH^+_4 producers.	13	13	8(10)	16.2	15.5
Denitrifiers.	19	19	18(17)	79.9	3.6
Poor growth or in active NO^-_3 reduction.	13	nd #	1 (1)	nd #	nd #
Anaerobic incubation:-					
NO^-_2 accumulators.	61	60	1	8.1	85.2
Presumptive NH^+_4 producers.	2	2	1	12.4	37.1
Denitrifiers.	17	17	17	89.4	0.9
Poor growth, inactive NO^-_3 reduction.	74	nd #	1	nd #	nd #
soil, no pre incubation:-					
NO^-_2 accumulators.	38	34	1	11.3	82.7
Presumptive NH^+_4 producers	1	1	1	1	1
Denitrifiers.	13	13	13	89.9	1
Poor growth or inactive NO^-_3 reduction.	52	nd #	1	nd #	nd #

(+) Number which completely reduced NO^-_3 and NO^-_2 in TSB with dextrose given in parenthese; all other results are for incubation in NB.
(++) nd = not determined.

Anaerobic preincubation of soil did not appear to have a large effect on the results, but the survey is too limited to be conclusive on this point.

A most significant observation is that 150 of 155 NO^-_2 accumulators and all of the presumptive NH^+_4 producers evolved significant quantities of N_2O, though the gaseous products were always less than the apparent ionic products (NO^-_2 and NH^+_4) (EL-Sayed , 1999 ; Faith *et al* ., 2017). From 2 to 24 %(mean 9.0%) of the NO^-_3- N added was converted to N_2O by isolates in the NO^-_2- accumulating category (EL-Sayed , 2013) . Nitrous oxide production by NO^-_2- accumulating isolates from the soil with no anaerobic soil incubation was measured in NB with C_2H_2, average 8.7% N_2O; in NB without C_2H_2, 6.9% N_2O; and in tryptic soy broth with dextrose (T S B) with C_2H_2, 6.3% N_2O.On the average, C_2H_2 and T S B had minimal effects on N_2O production through media composition by individual organisms as shown below.It is interesting that most of the organisms which accumulated NO^-_2 in NB apparently produced NH^+_4 in TSB; 48 of 58 isolates completely removed

NO^-_3, and NO^-_2 from TSB, but no one of them did so in NB.

Table (2) indicates that these categories of NO^-_3 reducers are taxonomically as well as functionally different. *Pseudomonas*, *Flavobacterium*, and *Alcaligenes* were the only denitrifying genera observed genera. This is in accord once with work by Gamble *et. al*. (1977) and (EL-Sayed , 2013) whom found that are these three genera numerically dominant in a large-Scale *Survey* of many soils. One NO^-_2 accumulator was also classified as a *Flavobacterium, but Bacillus* and *Enterobacter* were more frequently encountered, with one *Citrobacter* isolate.

Pure Culture Characterization Fermentative N_2O producers

There is an attempt to determine whether or not N_2 was evolved by that two N_2O^- producing , NO^-_2 accumulators, (*Bacillus* sp. B37 and *Citrobacter* sp. C48). In NB and in TSB with 5 m *M* KNO_3 and He atmosphere, no N_2 was detected after 14 days of incubation. All of the NO^-_3 and NO^-_2 was reduced in TSB, and all of the NO^-_3 was reduced in NB, during this time. The N_2could have reliable detection of N_2, in this experiment only if 1% or more of the N added were released in this from. This can be due to slight atmospheric contamination during syringe sampling and injection. In some of these sampled, very small peaks with retention time corresponding to nitric oxide (NO) were observed. It was apparent that NO was not a major end product of NO^-_3 reduction, and further work is needed to verify NO produced by these organisms (EL-Sayed,2005 ; and EL-Sayed 1995 a and b).

The inability of these isolates to produce N_2 is further indicated by the observation that C_2H_2 did not increase N_2O accumulation (Table 3) (EL-Sayed & Abo-ELwafa , 2001 ; Khalafalla and Hamed , 2015). Reduction of N_2O to N_2 is inhibited by C_2H_2. It appeared, in fact, that C_2H_2 slightly inhibited N_2O production. The effect of C_2H_2 shown in Table (3) was not statistically significant in some cases, but when this experiment was repeated with C48 in NB and in TSB,

Table 2. Taxonomic characterization of selected NO^-_3 reducing soil isolates

Type	Genus	Number of isolates
Denitrifiers.	*Pseudomonas*	7
	Flavobacterium	4
	Alcaligenes	3
NO^-_2 accumulators	*Bacillus*	10
	Enterobacter	4
	Flavobacterium	2
	Citrobacter	2

Table 3. Nitrous oxide production by *Bacillus* sp (B37) and *citrobacter* sp. (C48) with and without 8 KPa acetylene$^+$

Isolate	C_2H_2	Percent NO^-_3-N converted to N_2O		
		Day 2	Day 6	Day 13
B 37	-	2.53	3.45	4.76
B 37	+	1.75++	2.90 n.s	4.20 n.s
C 48	-	0.41	1.04	1.18
C 48	+	0.40 n.s	0.81++	0.92 n.s

+ Anaerobic incubaition in nutrient broth with 9.8m*M* KNO^-_3.

++ Comparisons followed by ++ are significantly different at 0.95 level by two-tailed T-test. N.s. = No significant differences. Values are means of two replicates.

Table 4. Effect of media composition on products of NO^-_3 reduction by *Bacillus* sp. (B37) and *Citrobacter* sp. (C48), measured after 14 days of anaerobic incubation

Isolate	Media	Added glucose g* $liter^{-1}$	Added $(NH_4)_2SO_4$g*$liter^{-1}$	% of NO^-_3-N added converted to (1/ v)		
				N_2O	NO^-_2	(NH^+_4) ++
(C 48)	NB	1	1	1.4	90.5	8.4
	NB	1	0.48	1.3	89.7	9.3
	NB	0.6	1	7.0	53.0	40.3
	NB	0.6	0.48	12.5	41.5	46.3
	NB	2.6	1	6.9	50.1	43.03
	NB	2.6	0.48	11.7	40.5	48.1
	TSBND	1	1	11.7	6.7	81.9
	TSBND	1	0.48	12.1	5.3	82.9
	TSBND	2.6	1	3.0	1	97.2
	TSBND	2.6	0.48	3.0	1	97.2
B37	NB	1	1	9.7	70.0	21.5
	NB	1	0.48	6.6	79.7	14.0
	NB	0.6	1	7.2	71.5	21.6
	NB	0.6	0.48	9.1	71.5	19.7
	NB	2.6	1	5.8	68.7	26.8
	NB	2.6	0.48	7.4	68.2	24.7
	TSBND	1	1	3.5	0	96.7
	TSBND	1	0.48	3.9	0	96.3
	TSBND	2.6	1	1.3	0	98.9
	TSBND	2.6	0.47	1.3	0	98.9

+ NB= nutrient broth, TSBND= Tryptic Soy broth without dextrose, all with 5m*M* KNO_3. ++ NH^+_4 not measured directly, estimated from $[NO^-_3-(N_2O+NO^-_2)]$.
All observation means of three replicats.

consistent inhibition, averaging 18%, was observed. The extent of inhibition was not related to C_2H_2 concentration in the range of 2 to 32 KPa.

The effects of media composition on the products of NO^-_3 reduction by C48 and B37 are shown in Table (4). Nitrous oxide was produced under all conditions , but was never the major product (EL-Sayed , 2002 (a and b) ;and Rajesh *et al* ., 2017). In complex media it is not feasible to measure directly NH^+_4 production from NO^-_3 without an ^{15}N label. This is due to confounding reactions such as amino acid degradation and NH^+_4 assimilon.

Since, showing that N_2 and NO are not major products, it is highly probable that the unaccounted - for N was reduced to NH^+_4, the observation of NH^+_4accumulation by these organisms, which relates well to the results in Table (4). In parallel experiments. Therefore, NH^+_4 production was estimated by subtracting the NO^-_2- N and N_2O- N from the NO^-_3- N added (no NO^-_3 remained after 13 days).The addition of NH^+_4 did not have a significant effect on the apparent reduction of NO^-_2 A NH^+_4, causing slight increases in NH^+_4 production with C48 and slight decreases with B37. Ammonium additions did not consistently alter N_2O production either. Assimilatory N_2O - reduction is repressed by NH^+_4 (Payne, 1973 ; EL-Sayed , 2013). It is concluded that reduction of NO^-_2 to NH^+_4 and N_2O by these organisms is dissimilatory (EL-Sayed,2016).

Glucose additions consistently increased the apparent production of NH^+_4 Table (4), presumably by permitting more fermentative growth. In NB, NO^-_2 was the major product; but in TSB, with or without dextrose, NH^+_4 was.This is consistent with results presented in Table (1).Glucose did not affect N_2O production consistently. *Citrobacter* C48 in NB produced significantly more N_2O when glucose was added, but B37 in NB tended to produce slightly but not significantly less. Glucose significantly depressed N_2O evolution by both organisms in TSB.

Nitrous oxide production and growth of C48 and B37 with various NO^-_3 concentrations are shown in Table (5). Growth responses in NB were observed up to 15 m *M* NO^-_3, AT 5 m *M* NO^-_3 in NB, therefore, the supply of electron acceptor is growth- limiting. Under these conditions, rapid reduction of both NO^-_3 and NO^-_2 would be expected for respiratory denitrifires, yet C48 and B37 do not rapidly reduce NO^-_2 as shown in Fig ,(1) and in Table (4).

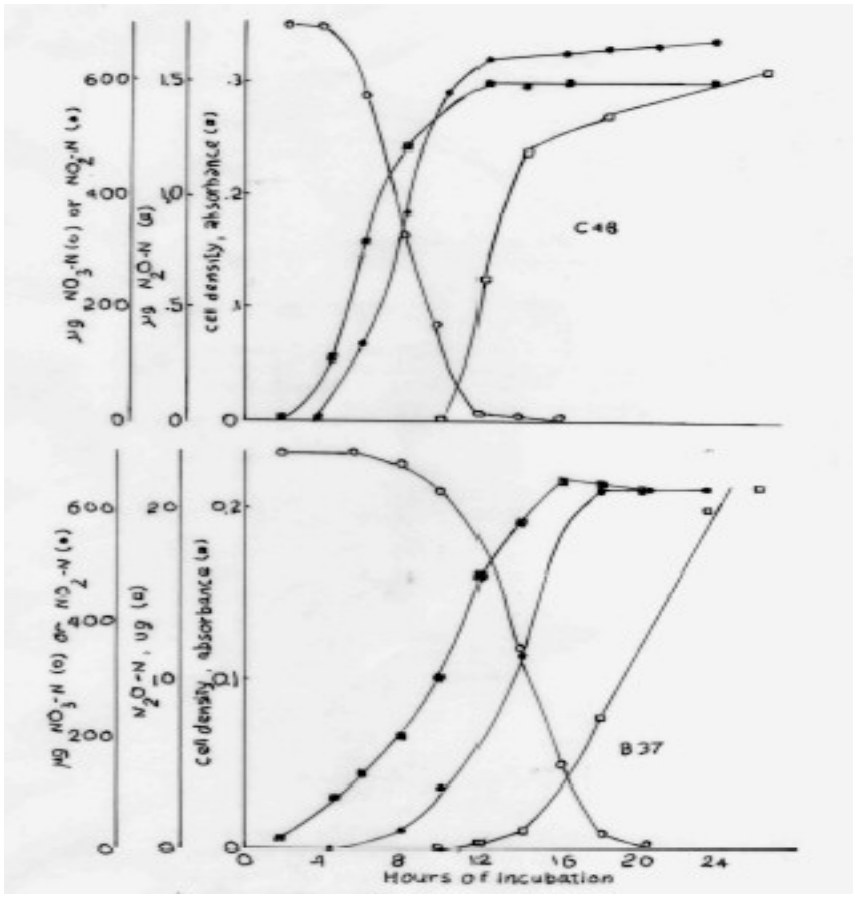

Fig. 1. reduction of NO_3^- (o), production of NO_2^- (●) and N_2O (□),and increase in turbidity (■) by cultures of Bacillus sp. B37 (1B) and Citrobacter sp. C48 (1A in anaerobic nutrient broth initially containing 5mM KNO_3. All observations are means of 3 replicates

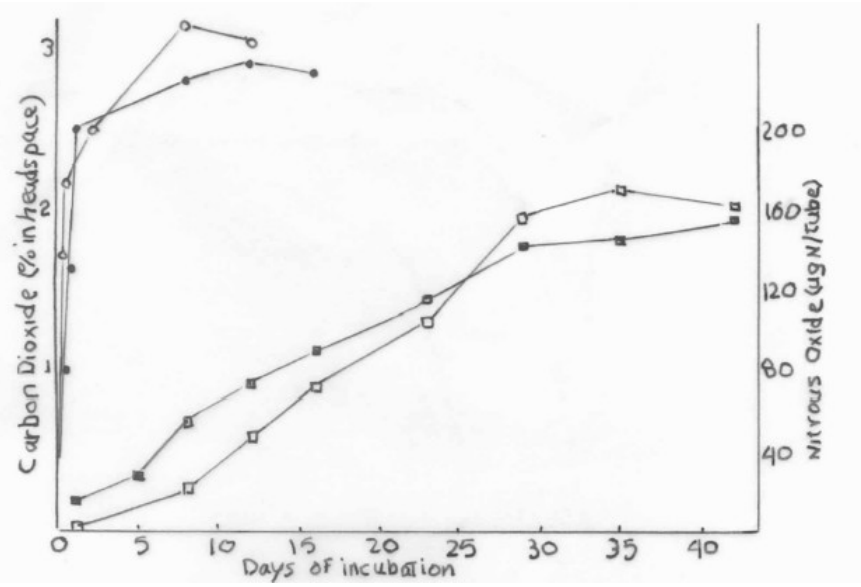

Fig. 2. Long-term production of N2O (Squares) and CO2 (circles) by Bacillus Sp. B37 (solid symbols) and Citrobacter sp. C48 (open symbols) in anaerobic nutrient broth initially conraining 5 mM KNO3. All observations are means of 3 replicates

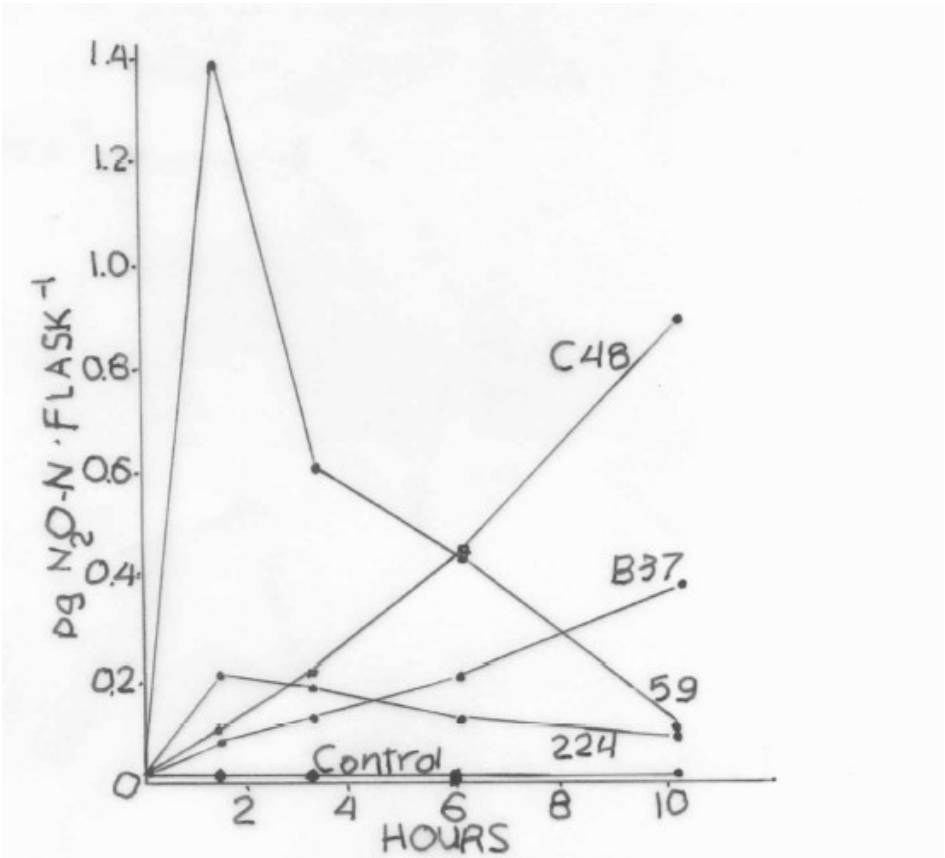

Fig. 3.N2O production by two denitrifiers (59 and 224) and Two Fermentative NO3- reducers (C48 and 837) following addition of cells to anaerobic soil slurries. All observations are means of 3 replicates. All comparisons at a given Time are significantly different at the 0.95 level except 59 vs. C48 at 6 hours and 59 vs.224 at 10 hours

Table 5.Effects of NO^-_3 concentration on growth and N_2O production by *Bacillus* sp.(B37) and *Citrobacter* sp. (C48) +

Isolate	NO^-_3 added (m*M*)	Maximum cell density (abs.)	N_2O produced (ug N)	N_2O-N as% of NO^-_3-N added
B 37	0	0.06	0	-
	2	0.12	9.2	13.1
	6	0.18	15.3	4.4
	16	0.29	43.1	4.2
	46	0.32	172.7	5.6
C 48	0	0.16	0	-
	2	0.20	7.9	11.2
	6	0.31	8.8	2.6
	16	0.36	11.9	1.2
	46	0.35	35.8	1.2

+ N_2O measured at 8 days. Values are means of three replicates in anaerobic nutrient broth.

Increased N_2O production with increased NO^-_3 was observed upto the highest concentration tested. The percentage of NO^-_3 – N converted to N_2O, however, generally decreased with increasing NO^-_3 concentrations (EL-Sayed and Ahmad , 2003).

Table (6) illustrates N_2O production at various pH values. Neither organism grew at pH 4.0, and there was no significant accumulation of N_2O in these tubes. Both organisms produced N_2O between pH 5.0 and 8.0, and

both accumulated maximum amounts at pH 8.0, the highest value tested.

In an attempt to elucidate the physiological function of N_2O production, the observation of growth yields with and without added NO^-_3 or NO^-_2 are shown in Table(7).The results are confounded by NO^-_2 toxicity which was observed in all cases at 10 m *M*. The greatest growth response occurred with NO^-_3 addition, presumably due to respiratory reduction to NO^-_2. No growth response to added NO^-_2 increased cell yield. Using the data in both Tables (7 and 4) TSB in Table(7) is chemically equivalent to TSBND plus 2.5 g. liter^{-1} glucose in Table(4); and NB in Table (7) is equivalent to unamended NB in (Table 4), the following observations are: (i) in TSB, growth responses to NO^-_2 occur, NO^-_2 is reduced mostly to NH^+_4, and small amounts of NO^-_2 are reduced to N_2O; (ii) in NB, there were no growth responses to NO^-_2, little of the NO^-_2 is reduced to NH^+_4 and small- to- moderate amounts are reduced to N_2O (EL-Sayed and Abo-EL-Wafa , 2001). The results are not conclusive but it appears likely that the observed growth responses to NO^-_2 are associated with reduction to NH^+_4 and not to N_2O (EL-Sayed,2013 ; EL-Soury *et al*,2015).

Figure (1) illustrates the temporal relationships among growth, NO^-_3 reduction, and N_2O production for B37 and C48 in NB. Nitrate is essentially reduced completely to NO^-_2 (90% recovery for B37, 96% for C48) at which time growth, as indicated by optical density, ceases. Only at this time does significant N_2O production occur.

Table 6. Effect of pH on N_2O production by *Citrobacter* sp. C48 and *Bacillus sp*. B37.+

Isolate	pH	Percent NO^-_3-N converted to N_2O		
		Day 2	Day 8	Day 15
B37	7.0	0	0	0
C48	7.0	0	0	0.09
B37	7.1	0.6	0.9	0.91
C48	7.1	6.9	7.3	6.7
B37	7.3	3.6	5.1	5.5
C48	7.3	2.0	2.4	2.5
B37	7.5	7.2	9.9	16.3
C48	7.5	2.9	6.7	9.1
B37	8.1	17.0	32.1	34.5
C48	8.1	4.1	12.5	20.8

+ In nutrient broth with 5m*M* KNO_3, anaerobic incubation. All observations are means of three replicates.

Table 7. Growth response of *Bacillus* sp. (B37) and *Citrobacter* sp. (C48) to added KNO_3 or Na NO_2

N amendment	Cell yield in NB# (ug cell dry wt. ml^{-1}) (+)		Cell yield in TSB# (ug cell dry wt. ml^{-1})	
	C48	B37	C48	B37
None	77	73	514	474
1m*M* NO^-_2	82	73	521	501
5m*M* NO^-_2	65#	70	577#	628#
10m*M* NO^-_2	45#	49#	497	271#
5m*M* NO^-_3	176#	162#	698#	698#

+ Cells harvested in distilled H_2O at early stationary phase when maximum optical density was attained, weight determined gravimetrically after drying at 85^{o}c.

++ NB is nutrient broth; TSB is tryptic soy broth with dextrose.

#Significantly different from broth with no NO^-_3 or NO^-_2 at 0.95 level by two-tailed T- test. Values are means of three replicates.

Table8.Production of N_2O by resting cell suspensions

Treatment	N_2O production P g N_2O-N.(min^{-1})	
	Citrobacter sp.	*Bacillus* sp.
No cells	0	0
Boiled cells	0	0
Aerobic atmosphere	87	0
Glucose omitted	356	50
Complete anaerobic reaction mixture+	1153	386

+ Reaction mixture consists of 50 m*M* pH 7.1 phosphate buffer, 200 ug*ml^{-1} chloramphenicol, 5m*M* Na NO_2, 2.8 m*M* glucose, washed, late log phase cells, and N_2 atmosphere in 10 ml H_2O. Values are means of two replicates.

Figure(2) presents a surprising aspect of N_2O production by these organisms . Nitrous oxide production occurs at a more or less linear rate for up to 35 days, long after growth steps(EL-Sayed, 2003 (a&b)). Most of the CO_2 in the headspace of these tubes accumulated within 24 hours, and no significant increases were observed after 8 days. The results suggest that N_2O production is not directly associated with growth of the organism (Fathi ,2014).

These results indicate that N_2O production by these organisms is a biological and not a chemical process. In one experiment, Hg Cl_2 was added to early stationary phase cultures which had accumulated NO^-_2 and were producing N_2O. This abolished N_2O production; distilled H_2O did not. Further evidence of enzymatic involvement ; in N_2O production was provided by resting cell suspensions prepared from washed, early stationary phase cells (Table 8). Boiling cells for 5 min abolished activity. These experiments also show that O_2 is an effective inhibitor of NO^-_2 reduction to N_2O. Furthermore, it was observed that glucose caused a several- fold increase in N_2O production. Boiled and live C48 cells from a 28-day-old culture were also assayed with results consistent with those in Table (8).

An additional resting cell experiment was performed to determine the effect of growth conditions on N_2O producing activity from NO^-_2, *Citrobacter* C48 was grown in TSB aerobically with no NO^-_3 or NO^-_2, anaerobically with neither NO^-_3 nor NO^-_2, and anaerobically with 5 m *M* KNO_3 (EL-Sayed , 2005). The relative activities were 1.4, 7.5, and 100, respectively. It appears that N_2O producing activity is inducible.

N_2O Production by Fermentative NO^-_2 Reducers in Soil

The results presented in Fig (3) showed that these organisms produce immediately N_2O when added to anaerobic autoclaved soils. Two denitrifying *pseudomonads* (isolates 59 and 224) were included in this experiment for purposes of comparison. The denitrifiers initially produced N_2O at a greater rate than the fermentative NO^-_2 reducers, but N_2O did not accumulate in the soils with denitrifiers due to reduction to N_2. After 10 hours of incubation, C48 and B37 had accumulated significantly more N_2O than the denitrifiers (EL-Sayed , 1999 and 2016).

CONCLUSIONS

Most soil isolates capable of dissimilatory NO^-_3 reduction to NO^-_2 also produced N_2O, though most of these isolates were not true respiratory denitrifiers. Nitrous oxide production by nondenitrifiers differed from denitrification in several ways. Ionic forms of nitrogen (NH^+_4 or NO^-_2) were the predominant products in the former process, with lesser amounts of N_2O, whereas denitrifiers have the potential for complete conversion of NO^-_3 sto nitrogen gas. The bacteria characterized in this study apparently cannot reduce N_2O to N_2, but most denitrifiers can.Production of N_2O by denitrifiers is directly linked to growth and respiration and so is a relatively rapid process when conditions are favorable.Production of N_2O by nondenitrifiers is slower and occurs mostly after apparent growth is completed.

The results suggest that N_2O production by nondenitrifying NO^-_3 reducers is enzymatic, though the involvement of a nonenzymatic step cannot be definitely ruled out. Boiling cells and treatment with $HgCl_2$ abolished activity. Nitrous oxide producing activity apparently was induced by anaerobic growth with NO^-_3. Activity was favored by high pH, suggesting that N_2O production is not due to chemical decomposition of HNO_2. The physiological function, if any exists, of N_2O production by these organisms is not clear. The results do not indicate that this process is directly linked to growth or energy generation, as is the case for respiratory denitrification and for fermentative reduction of NO^-_2 to NH^+_4. IF N_2O production serves as a means of detoxifying NO^-_2, it is a rather inefficient mechanism since NO^-_2 conversion is slow and incomplete. Since added NH^+_4 did not inhibit N_2O production; NO^-_2 reduction to N_2O is not associated with assimilatory NO^-_2 reduction.

The supply of energy substrate had variable effects on N_2O production. Glucose addition enhanced N_2O production in resting cell suspensions, but in Batch cultures it sometimes increased and sometimes decreased evolution of N_2O. Growth in TSB often resulted in less N_2O release than in nutrient broth, but in a few cases ; more N_2O was produced in TSB. No facile explanation for these varying effects is at hand.

Most of the nondenitrifying N_2O producers were apparently capable of fermentative dissimilatory reduction of NO^-_2 to NH^+_4 under the appropriate conditions. The *Bacillus* and *Citrobacter* isolates were NO^-_2 accumulators in NB, in TSB they were NH^+_4 producers. This was also true for 48 of 58 soil isolates which were initially classified as NO^-_2 accumulators in NB .This suggests that, for many bacteria, reduction beyond the initial NO^-_3 to NO^-_2 step is limited more by the environment than by the genetic potential of the organisms. In any case, more ionic N was produced than gaseous N, so fermentative NO^-_3 reducers might be less likely than denitrifiers to cause significant volatile

loss of fixed soil N. If NO^-_2 reduction to NH^+_4 were competitive with denitrification, gaseous N loss could actually by reduced by these organism.

It is difficult to evaluate the significance of nondenitrifying NO^-_3 reducers as a source of soil N_2O. These organisms did produce N_2O under a wide variety of conditions. From the survey it appears that they are more numerous than denitrifiers in soil. When added to autoclaved soil, fermentative NO^-_2 reducers initially produced N_2O at a lower rate than denitrifiers but, because they also consumed N_2O,denitrifiers accumulated less N_2O as the incubation proceeded. Nondenitrifying NO^-_3 reducers may thus contribute to N_2O evolution from soil .

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الملخص العربي

حصر للبكتيريا الموجودة بمزرعة كلية الزراعة – جامعة الأزهر بمحافظة أسيوط

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تم عزل ٢١٥ من البكتريا القادرة على اختزال النترات (NO^-_3) و كذلك ٢١٠ من البكتيريا المنتجة لأكسيد النيتروز (N_2O) وكذلك توجد ٤٧ من البكتيريا التنفسية كنتيجة لاختزال النترات الى غاز النيتروجين .

أوضحت النتائج أن النتريت أو الامنيوم كان الناتج الرئيسى نتيجة لاختزال النترات بواسطة الكائنات الحية الدقيقة نتيجة لبكتيريا عكس التأزت .

أشارت النتائج أن حوالى ٥ –١٠% وحتى ٣٤% من النترات يتم ا ختزالها وتتحررالى مادة N_2O نتيجه تحضينها لمدة اسبوعين ببكتيريا عكس التأزت، نتيجة لاستهلاك وانتاج N_2O.

أوضحت النتائج أن بكتيريا *Bacillus* و بكتيريا *Enterobacter* تكون ملحوظه نتيجة لوجود أجناس بكتيريا عكس التأزت والتى تنتج مادة N_2O وأن تخمر مادة N_2O تختزل وتنتج مادة بواسطة جنس بكتيريا *Bacillus* و كذلك جنس *Citrobacter* تكون مميزة لانتاج مزارع نقية، كذلك عند وضع التربة فى جهاز التعقيم (الاتوكلاف) تتجمع كميات كبيرة من N_2O نتيجة بكتيريا *Pseudomonads* نتيجة لاستهلاك وانتاج N_2O.

أوضح البحث أن النمو يكون نشطا عندما يحدث التخمر، لذلك فان الناتج الأعظم يكون الأمونيوم نتيجة لاختزال النترات، فى بيئة المرق المغذى (NB) و يحدث تجمع لمادة NO^-_2 . وعند اضافة N_2O لا يحدث تثبيط للناتج، أوحدوث اختزال للامونيوم كدليل لعدم التمثيل أو الامتصاص.

تأثير اضافة الجلوكوز الى الناتج يختلف باختلاف الكائنات الحية الدقيقة فى الوسط البيئى، اكسيد النيتروز الناتج بواسطة الكائنات الحية الدقيقة يكون واضحا فى هذا التفاعل الحيوى .و ينتج N_2O ببطء فى بيئة النمو و يكون واضحا ومعنويا وكنتيجة لتنفس البكتيريا ينطلق الازوت .